The Corticotropin-Releasing Factor System in Lactating Rats: Implications in the Regulation of Maternal Behavior with Special Focus on the BNST

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"Around here, however,
we don't look backwards for very long.
We keep moving forward, opening new doors
and doing new things, because we're curious…
And curiosity keeps leading us down new paths."

Walt Disney

Declaration of Included Manuscripts

Chapter 2: Reduced brain CRF receptor activation is required for adequate maternal care and maternal aggression in lactating rats.

Authors‘ contribution:

Stefanie Klampfl: experimental design, performance of experiments, data analysis, first draft of manuscript
Inga Neumann: revision of manuscript
Oliver Bosch: experimental design, performance of experiments, revision of manuscript

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Chapter 3: Hypo-activation of CRF receptors, predominantly type 2, in the medial-posterior BNST is vital for adequate maternal behavior in lactating rats.

Authors‘ contribution:

Stefanie Klampfl: experimental design, performance of experiments, data analysis, first draft of manuscript
Paula Brunton: performance of in situ hybridization, revision of manuscript
Doris Bayerl: performance of experiments, revision of manuscript
Oliver Bosch: experimental design, performance of experiments, revision of manuscript

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Chapter 4: Opposing effects of subtype-specific CRF receptor activation in the adBNST on maternal care and the stress axis in lactating rats

Authors‘ contribution:

Stefanie Klampfl: experimental design, performance of experiments, data analysis, first draft of manuscript

Doris Bayerl: performance of experiments

Oliver Bosch: experimental design, performance of experiments, revision of manuscript

Chapter 5: The CRF binding protein: an underestimated brain neuropeptide indispensable for the expression of maternal behavior in lactating rats

Authors‘ contribution:

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Doris Bayerl: performance of experiments

Oliver Bosch: experimental design, performance of experiments, revision of manuscript

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Abstract

The survival of the offspring strongly depends on the adequate expression of maternal behavior. Any dysregulations during the peripartum period can easily result in behavioral or physiological maladaptations that are detrimental to the offspring as well as to the mother herself. One factor, potentially contributing to dysregulations peripartum, is the corticotropin-releasing factor (CRF) system, which triggers nearly every behavioral and physiological response to stress. Given that the behavioral role of the CRF system during lactation is still under-investigated I aimed to characterize its implications in the regulation of maternal behavior in lactating rats. Therefore, I used a variety of methods including in situ hybridization, ELISA, repeated intravenous blood sampling, and central or local acute pharmacological manipulation of CRF receptors (CRF-R) or the CRF binding protein (CRF-BP) followed by various behavioral tests. For local manipulation, I focused on the bed nucleus of the stria terminalis (BNST) given its role in maternal behavior and its abundant expression of most members of the CRF family.

In the present thesis, I showed that the CRF system needs to be down-regulated postpartum given that central and local activation of CRF-R in the BNST impaired maternal care and maternal aggression, increased anxiety-related behavior but had no effect on maternal motivation. Importantly, the effects of CRF-R activation differed specifically in the various subdivisions of the BNST depending on the CRF-R subtype. In the mpBNST, predominantly CRF-R2 activation reduced maternal care and maternal aggression while both CRF-R1 and CRF-R2 mediated anxiety-related behavior. In the adBNST, CRF-R manipulation solely affected maternal care; CRF-R1 activation reduced maternal care while CRF-R2 stimulation reduced nursing but increased arched back nursing in a time-dependent manner. These behavioral

changes following specific CRF-R manipulation in the adBNST might be indirectly mediated via activation of the hypothalamo-pituitary-adrenal axis. Interestingly, I could demonstrate that the CRF-BP, which appears to sequester extracellular CRF / urocortin 1 and inhibit their binding to the CRF-R, has emerged as potent regulator of the required down-regulation of CRF-R activity as well as of maternal aggression, especially in the mpBNST.

In conclusion, both central and local activation of CRF-R in the BNST are detrimental to the appearance of maternal behavior in lactating rats. Therefore, CRF-R activation needs to be down-regulated postpartum, which is partly achieved and supported by altered expression patterns of CRF-R and its ligands as well as by the CRF-BP. This well balanced interplay of the CRF family members is essential to enable the mother showing adequate maternal behavior and, thus, to increase her fitness.

- Chapter 1 -

General Introduction

Across all species reproduction and, hence, successful rearing of the young represents the major aim in life. In mammals, this aim is achieved by the recruitment of a wide array of behavioral and physiological adaptations of the parents. Parental behavior is defined as “any behavior of a member of a species toward a reproductively immature conspecific that increases the probability that the recipient will survive to maturity” (Numan and Insel, 2003). Approximately 90 % of all mammalian species have established a promiscuous, uniparental care system with the mother as primary caregiver due to her ability to lactate (Numan and Insel, 2003). Thus, maternal behavior has emerged as the most important pro-social female behavior and guarantees the survival and development of the offspring.

1.1 Maternal behavior

1.1.1 Hormonal basis of maternal behavior

A variety of mammals show a basic level of responsiveness to the young that is activated by stimulation with the infant. However, a fundamental change in the (neuro-)endocrine system is indispensable in many species to prepare for motherhood (Numan and Insel, 2003). In rats, the onset of maternal behavior is induced by steroid hormones that are secreted in gestation-specific patterns throughout pregnancy (Bridges, 1996). Plasma estradiol concentrations are low during the first part of pregnancy and rise around day 15, after which peak levels are maintained until parturition. In contrast, progesterone levels are high throughout the first part of pregnancy, peaking at day 15 followed by a decline that becomes abrupt after day 20. Hence, shortly before parturition, a reversal of the estrogen-to-progesterone ratio can be observed (Figure 1). In primates and humans, pregnant females show similar changes in their hormonal system; however, the drastic drop in

progesterone is not required for the onset of labor and maternal behavior (Bahr et al., 2001).

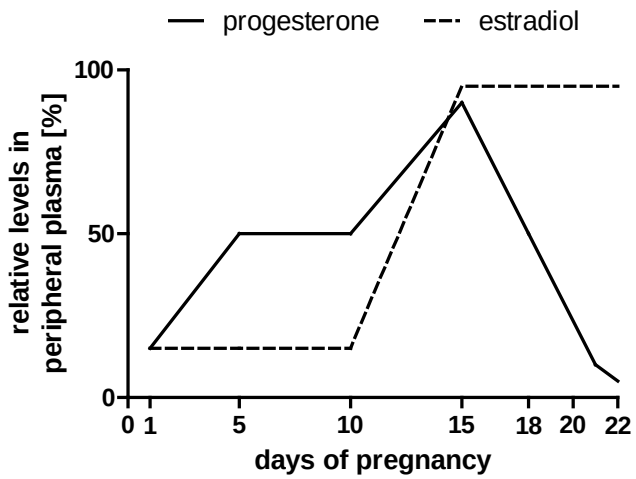

Figure 1. Relative blood plasma levels of estradiol and progesterone throughout pregnancy in rats. Data are depicted as the percentage of the maximum value detected during pregnancy. Adapted from Bridges, 1996.

These hormonal changes are accompanied by increased production and secretion of pituitary prolactin (PRL) and placental lactogens throughout most of pregnancy (Grattan, 2001). During the first half of pregnancy, PRL is released from the pituitary in two daily surges, while during most of the second half of pregnancy PRL secretion is low except for an increase on the final day of pregnancy. The low PRL release in the second half of pregnancy is accompanied by rising plasma levels of lactogens. Thus, pregnant rats are most of the time exposed to chronic lactogenic and progestin stimulation. The high levels of estradiol, PRL and lactogens act on neurons in the medial preoptic area (MPOA) and prime them for a successful onset of maternal behavior (Numan et al., 1977; Bridges et al., 1990; Bridges et al., 1997). This event is finally initiated by central neurotransmitter systems; in addition to PRL, the neuropeptides oxytocin (OXT) and arginine-vasopressin (AVP) are released to promote an immediate onset of maternal behavior (Pedersen and Prange, 1979; Pedersen et al., 1982; Pedersen et al., 1994).

1.1.2 Neuropeptidergic basis of maternal behavior

The nonapeptides OXT (van Leengoed et al., 1987; Numan and Insel, 2003; Bosch et al., 2005; Bosch and Neumann, 2012) and AVP (Bosch and Neumann, 2008, 2010; Bosch et al., 2010; Bosch, 2011; Bosch and Neumann, 2012) are not only fundamentally involved in the onset of maternal behavior but play also a crucial role in maintaining appropriate levels of maternal behavior (for further details see below). Before going into detail with the neuropeptidergic regulation, I will introduce the behavioral variety of maternal behavior, which can be divided into maternal care, maternal motivation, and maternal aggression and is accompanied by adaptations in maternal anxiety.

1.1.2.1 Maternal care

During motherhood, a rat dam shows a variety of pup-directed behaviors, which serves to ensure the well-being and development of the offspring. The dam licks and grooms the pups, which helps them to urinate and defecate, having a high impact on their social and emotional development (Caldji et al., 1998; Champagne, 2008). Additionally, the dam employs various nursing positions including blanket posture, hovering over the pups, and arched back nursing (ABN; Figure 2) in order to provide the young with sufficient nourishment.

Figure 2. Lactating rat fully engaged in ABN. ABN is characterized by a quiescent kyphotic posture, which enables the pups a perfect access to the teats in a protective environment (Stern and Johnson, 1990).

ABN is the most characteristic nursing behavior of rats and is classified as the only active nursing position with the dam being fully engaged in a quiescent kyphotic posture (Stern and Johnson, 1990). The quiescence of the dam is important not only to enable the pups to become and remain attached to the teats, but also for milk ejection to occur in response to the suckling stimuli. Sufficient offspring suckling is required to induce a behavioral quiescence, which is accompanied by slow-wave sleep and immobility (Voloschin and Tramezzani, 1979; Lincoln et al., 1980). The kyphotic posture is critical to prevent pups from being smothered and makes the 12 teats more readily available (Stern and Johnson, 1990). In order to induce kyphosis, which can be viewed as a reflex, ventral somatic sensory stimulation is required (Numan and Insel, 2003). Moreover, suckling as ventral tactile stimulus is able to subsequently maintain the ventroflexion for relatively long durations. Additional stimulation comes from the muzzle pushing against the teats surrounding region and from the paws treading on a wide area of the ventrum, including adjacent nipples (Stern and Johnson, 1990). This ventral stimulation is forwarded via the spinal cord to the caudal part of the periaqueductal gray (cPAG) in the midbrain (Yeziersky, 1991; Bandler and Shipley, 1994). Descending PAG projections regulate further the occurrence of motoric quiescence and immobility (Bandler and Shipley, 1994;

Cameron et al., 1995; Numan and Insel, 2003). Intriguingly, the PAG is interconnected with the bed nucleus of the stria terminalis (BNST) and the MPOA, two brain regions that are heavily involved in the promotion of maternal behavior. It was recently hypothesized that the BNST/MPOA primarily regulate active maternal behaviors like licking / grooming and pup retrieval, which are inhibited by descending projections from the PAG during kyphotic nursing (Numan and Insel, 2003). In order to terminate ABN, the BNST/MPOA send inhibiting projections to the PAG, thereby promoting the occurrence of active maternal behaviors.

The BNST and MPOA are part of the limbic system and are highly implicated in the regulation of maternal behavior (Numan and Insel, 2003; Bosch, 2011). Especially the ventral BNST (vBNST) and the dorsal MPOA (dMPOA) act in concert to form a maternal “super-region” (Numan and Insel, 2003). This neural complex receives input from the medial amygdala, which is the crucial relay site to forward olfactory stimulation from the pups either to a pup fear/avoidance circuit in virgins or to a pup attraction/approach circuit in lactating rats (Numan and Insel, 2003; Numan and Woodside, 2010). Forwarding the signal via the latter circuit includes further projections from the vBNST/dMPOA to hypothalamic nuclei such as the paraventricular nucleus (PVN) (Simerly and Swanson, 1988) thereby most likely stimulating OXT release (Numan and Woodside, 2010) or to extrahypothalamic sites such as the ventral tegmental area thereby mediating maternal motivation (Numan and Stolzenberg, 2009) (see 1.1.2.2). Interestingly, the state of the art indicates that maternal care is primarily mediated by the cPAG and the vBNST/dMPOA while other important limbic brain regions like the dorsal or posterior BNST (pBNST) have been largely neglected so far. To my knowledge, only one study investigated the implications of the pBNST in the occurrence of maternal care and assessed OXT and AVP release in the pBNST and their behavioral implications in lactating rats (Bosch

et al., 2010). They showed that both neuropeptide systems in the pBNST are apparently not involved in mediating maternal care but are rather vital for the regulation of maternal aggression (see 1.1.2.3).

1.1.2.2 Maternal motivation

The dam's brain needs to undergo further adaptations in order to be responsive to their young and to seek and maintain contact with them. In rats, retrieval behavior occurs when a mother carries pups in her mouth one at a time to transport displaced pups back to the nest or to move them to a new nest site. This process is referred to as maternal motivation and is described as an appetitive response that is voluntary, proactive and goal-directed (Numan and Stolzenberg, 2009; Numan and Woodside, 2010; Pereira and Morrell, 2011). Appetitive maternal motivation is regulated by the MPOA and its interactions with the telencephalon via the mesolimbic dopamine system appear to be the route through which MPOA neurons mediate such goal-directed behavior (Numan and Woodside, 2010). It is currently believed that OXT in the MPOA potentiates presumably glutamatergic input to the ventral tegmental area (Numan and Smith, 1984; Pedersen et al., 1994), thus stimulating the release of dopamine into the nucleus accumbens, which in turn projects to the ventral pallidum (Numan and Stolzenberg, 2009; Numan and Woodside, 2010). However, it is important to note that, even though this model has been established upon logical conclusions, it is still based on speculations and has not been causally proven yet.

Intriguingly and similar to maternal care, the BNST was not shown so far to play a role in the regulation of maternal motivation, which is supported by studies showing that manipulating OXT receptors in the anterior dorsal BNST (adBNST) (Consiglio et

al., 2005) or V1a receptors in the pBNST (Bosch et al., 2010) does not alter retrieval behavior in lactating rats.

1.1.2.3 Maternal aggression

Along with the establishment of maternal care and maternal motivation, lactating females exhibit a dramatic increase in aggressive behavior, which is specifically termed maternal aggression. The term was introduced 1968 (Moyer, 1968) to describe agonistic behavior displayed by females defending their young, and was distinguished from irritable, territorial, sex-related, fear-induced, predatory and intermale aggression. Thus, maternal aggression is not directed against the young but is aimed to protect the offspring from potential external threats, e.g. infanticidal conspecifics. Lactating females have an innate drive to fight against an intruder, which is unique to the dam, as in rodents a virgin female barely ever attacks an intruder (Bosch, 2013). This intruder-targeted maternal aggression is an adaptive and highly conserved behavior that likely increases the fitness of the offspring (Wolff, 1985). Interestingly, a pregnant rat shows first signs of maternal aggression already at the end of pregnancy, which transiently decreases at parturition, and peaks during the first week postpartum (Figure 3; Caughey et al., 2011).

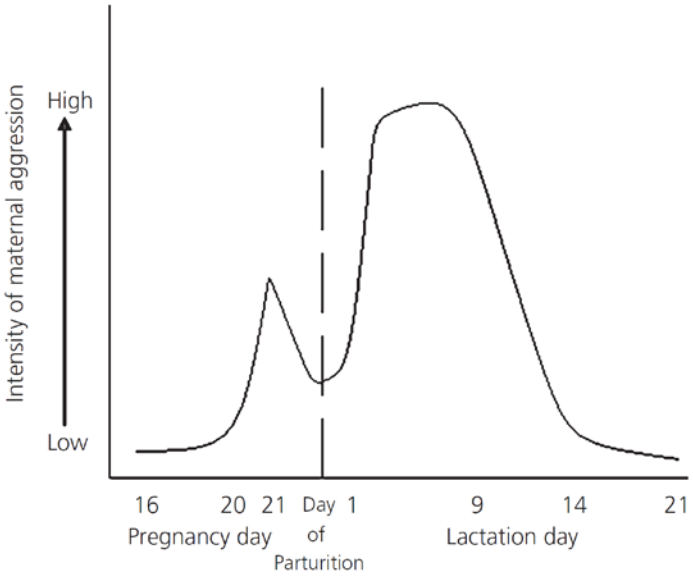

Figure 3. Changes in the intensity of maternal aggression from the third trimester of pregnancy to lactation in the rat. Maternal aggression is first observed the day before parturition (i.e. pregnancy day 21). Maximum levels of maternal aggression are apparent during the first week of lactation, declining afterwards until weaning. From Caughey et al., 2011.

Maternal aggression is assessed in the maternal defense test (Neumann et al., 2001) during which the dam, i.e. the resident, shows a variety of offensive and defensive behaviors toward the conspecific intruder (Erskine et al., 1978b; Lonstein and Gammie, 2002; Bosch et al., 2005). The dam's attacks are usually directed toward the neck or back of the intruder and can be paired with bites. Additionally, the mother is frequently engaged in threat behaviors such as lateral threat, where the dam approaches the intruder sideways to force the intruder aside, as well as pinning/keeping down and aggressive grooming of the intruder. Furthermore, defensive 'boxing' by the dam in an upright posture in front of the intruder can occur and is termed 'offensive upright'.

In rodents, maternal aggression is modulated by extrinsic and intrinsic factors (Bosch, 2013). On one hand, the presence of the pups, which depend on the dam's motivation to protect them, immediately after birth is crucial for the onset of maternal aggression (Gandelman and Simon, 1980). Interestingly, the suckling stimulus *per se* is not sufficient (Moltz et al., 1967; Bridges, 1975) but rather tactile and especially olfactory cues emanating from the pups are essential for stimulating and maintaining

maternal aggressive behavior (Lonstein and Gammie, 2002). On the other hand, the intruder itself which embodies a potential threat to the young's survival is highly responsible for the occurrence and intensity of maternal aggression (Bosch, 2013). For instance, the age and/or size (Erskine et al., 1978a; Flannelly and Flannelly, 1985), which is ideally 10 % lower than the resident's (Bosch, 2013), and the behavior of the intruder, which is usually defensive, directly impacts on the aggressive behavior of lactating rats. Furthermore, the sex of the intruder seems to influence maternal aggression in rats as male intruders receive less aggression from the lactating resident when compared with female intruders (Haney et al., 1989) independent of the intruders' reproductive status (Neumann et al., 2001).

Besides extrinsic factors, a variety of intrinsic factors modulates the onset and maintenance of maternal aggression. Increasing levels of estrogen, but not dropping progesterone, at the end of pregnancy prime for and stimulate a first onset of maternal aggression (Lonstein and Gammie, 2002). After birth, adaptations of neuropeptidergic systems and the mother's decreased innate anxiety (see 1.1.2.4) are crucially involved in the appearance of maternal aggression. OXT release in the BNST, PVN, central amygdala (CeA) and lateral septum (LS), and OXT receptor binding as well as general neuronal activity in the BNST and LS of lactating rats is increased during the maternal defense test (Bosch, 2013). Similarly to the adaptations in the OXT system, AVP release in the BNST, CeA and LS, and V1a receptor binding in the BNST and LS is elevated during a maternal aggressive encounter in lactating rats (Bosch, 2013). Intriguingly, the BNST in particular has emerged as a key region in the limbic system modulating maternal aggression.

1.1.2.4 Maternal anxiety

Given the importance of infant contact on almost all aspects of postpartum physiology and behavior, it seems intuitive that infants would be intimately involved in regulating the reduced anxiety seen in lactating mothers (Lonstein, 2007). In humans, breast-feeding is anxiolytic and increases positive mood in recently parturient women compared to bottle-feeding mothers (Fleming et al., 1990; Altshuler et al., 2000; Groer, 2005; Breitkopf et al., 2006). Similarly in rats, late pregnancy and lactation are accompanied by decreased anxiety compared to virgin females (Neumann et al., 2000; Lonstein, 2007). Here, physical contact with the pups, but not suckling, is necessary to reduce anxiety postpartum (Lonstein, 2005). Importantly, this effect appears to be transient as separation from the pups for as little as two hours before testing increases dam's anxiety (Neumann, 2003). However, not only extrinsic factors but also many neurochemical systems are involved in adapting anxiety-related behavior postpartum. For example, elevated PRL (Torner et al., 2001) and OXT levels peripartum act highly anxiolytic (Neumann et al., 2000; Bosch, 2011). Interestingly, the brain AVP system is also up-regulated in lactation but, in contrast to OXT, AVP has anxiogenic properties (Bosch and Neumann, 2008). Thus, OXT and AVP affect anxiety in an opposing manner but promote maternal behavior in the same way. Still, it needs to be considered that low anxiety levels do not necessarily entail a good performance in maternal behavior (also see 1.1.3) (Bosch, 2011). The behavioral output is generated by the integration of various signals from numerous brain sites, which most likely do not forward the anxiogenic or anxiolytic properties of neurotransmitters equally. Surprisingly, little evidence exists about specific brain regions mediating anxiety-related behavior in lactating rats. The ventrocaudal part of the midbrain PAG seems to be important as bilateral lesions further decrease anxiety-related behavior in lactating rats (Lonstein et al., 1998) while antagonizing

the OXT receptor in the ventrocaudal PAG increases anxiety to levels typically found in virgins (Lonstein, 2007). Interestingly, the AVP system in the MPOA (Bosch and Neumann, 2008) and the OXT system in the pBNST (Klampfl, Wöster, Bosch, unpublished data) are apparently not involved in the regulation of anxiety postpartum. However, an interesting model was proposed by Lonstein (2007) involving the vBNST; physical interaction with pups may decrease noradrenaline (NA) release in the vBNST when dams are exposed to an anxiogenic situation (Figure 4). This decrease disinhibits γ-aminobutyric acid-(GABA)ergic projections from the vBNST to various brain regions including the PVN, amygdala, and the ventrocaudal PAG. Potentially excitatory projections from the PVN and amygdala to the ventrocaudal PAG are also inhibited. Intriguingly, OXT and PRL release within and from the PVN may also be induced by vBNST disinhibition to exert anxiolytic actions postpartum.

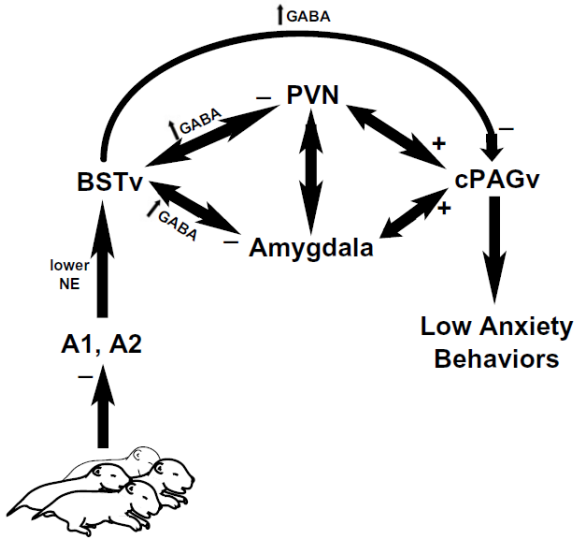

Figure 4. Proposed neural network of the anxiolytic effects of pup contact. Physical contact with pups reduces NA release from the locus coeruleus A1 and A2 region into the ventral BNST (BSTv) and promotes a disinhibition of GABAergic projections to the paraventricular nucleus (PVN), amygdala and ventrocaudal PAG (cPAGv). Excitatory projections from PVN and amygdala to cPAGv are inhibited. Decreased cPAGv activation promotes low anxiety behaviors. From Lonstein, 2007.

1.1.3 Rat animal model for extremes in anxiety and maternal behavior

The understanding of anxiety-related behavior has advanced in the last years and has been greatly promoted by the use of specific animal models. Starting in 1993, Wistar rats were selectively bred for extremes in anxiety-related behavior as

measured on the elevated plus-maze (EPM) (Liebsch et al., 1998). Two breeding lines have emerged termed HAB (high anxiety-related behavior) and LAB (low anxiety-related behavior). Rats are selected for experimental purposes and further breeding, when HAB and LAB rats spend less than 10 % and more than 35 % of time on the open arm of the EPM, respectively. The hyper-anxious phenotype of HAB rats, found in both males (Murgatroyd et al., 2004) and females (Bosch et al., 2006; Bosch and Neumann, 2008), derives from a single nucleotide polymorphism in the AVP promoter region, which leads to a disinhibition of the AVP gene transcription, and to increased AVP synthesis and release (Keck et al., 2002; Wigger et al., 2004). Furthermore, HAB rats have increased corticotropin-releasing factor (CRF) mRNA levels within the PVN compared to LABs (Bosch et al., 2006), which likely contribute to their hyper-anxious phenotype (see 1.2.3). In addition to the extremes in anxiety-related behavior, HAB and LAB rats are characterized by robust differences in various physiological and behavioral parameters. HAB rats show hyperresponsiveness of the hypothalamo-pituitary-adrenal (HPA) axis (see 1.2.2) (Landgraf et al., 1999; Keck et al., 2003). Additionally, in a behavioral test for depression HAB rats spend more time floating in the forced swim test (FST) indicating increased passive stress coping and, thus, increased depressive-like behavior compared to LABs (Liebsch et al., 1998; Neumann et al., 1998a; Keck et al., 2005; Slattery and Neumann, 2010). Profound differences are also found in the display of social behaviors; male LAB rats show a lack of social preference compared to HAB or non-selected (NAB) Wistar rats (Lukas et al., 2008). Furthermore, LAB rats are prone to show abnormal aggressive behaviors toward the intruder (Beiderbeck et al., 2012) while HAB rats show low intermale aggression in the resident-intruder test (Beiderbeck et al., 2007; Veenema et al., 2007; Neumann et al., 2010). Importantly, profound differences in social and aggressive behaviors are also observed in female

HAB and LAB rats, especially during lactation (Bosch et al., 2005; Neumann et al., 2005a; Bosch et al., 2006; Bosch and Neumann, 2008, 2010). The hyper-anxious phenotype of HAB dams is linked to a protective mothering style combined with high maternal care and motivation (Bosch, 2011). They retrieve faster and even more pups in the pup retrieval test (PRT). Moreover, HAB dams leave their nest less often and spend more time on ABN as well as on nursing or on pup-directed behavior in general (Neumann et al., 2005a; Bosch et al., 2006; Bosch and Neumann, 2008). In addition to the increased display of maternal care, HAB dams are more aggressive against a virgin female intruder in the maternal defense test (Bosch et al., 2005; Bosch, 2013). This is reflected by an increased number of attacks, reduced attack latency as well as by overall aggression compared to LAB dams. Interestingly, the behavioral performances in maternal care and maternal aggression positively correlate with their innate levels of anxiety (Bosch, 2011). A similar correlation was found for the number of attacks and the overall aggressive behavior during the maternal defense test. These results reveal that the intensity of maternal behavior depends on the dam's innate anxiety in the HAB/LAB animal model.

Investigating maternal behavior using lactating HAB and LAB rats has advanced our understanding of the neurochemical basis of this vital female social behavior. These studies support the role of AVP and OXT in the regulation of maternal behavior during lactation and demonstrate that high levels of both neuropeptide systems underlie high levels of maternal care and maternal aggression (Bosch, 2011). Furthermore, a vital implication of other neurotransmitter systems, e.g. the CRF system (see 1.2), is feasible.

1.2 The CRF system

The CRF system plays a key role in a diversity of behaviors accompanying stress, anxiety, and depression and in their underlying physiology. Additionally, substantial research has focused on relationships between social behaviors and the CRF system in a variety of taxa including fish, birds, rodents, and primates (Hostetler and Ryabinin, 2013).

1.2.1 CRF family members

In the last three decades, Wylie Vale's group has identified four peptides of the CRF family in mammals: CRF (Vale et al., 1981), Urocortin (Ucn) 1 (Vaughan et al., 1995; Donaldson et al., 1996), Ucn 2 (Reyes et al., 2001) and Ucn 3 (Lewis et al., 2001). These four peptides bind with different affinities to the two known receptors named CRF receptor 1 (CRF-R1) and CRF-R2 (De Souza et al., 1984; De Souza et al., 1985) as well as to the CRF binding protein (CRF-BP) (Behan et al., 1989; Potter et al., 1991; Behan et al., 1995b). In addition, two further CRF family members were discovered in non-mammalian species, i.e. frog sauvagine (Montecucchi et al., 1980; Montecucchi and Henschen, 1981) and fish urotensin 1 (Ichikawa et al., 1982).

1.2.1.1 CRF

CRF is a 41 amino acid neuropeptide and was first sequenced from ovine hypothalamus in 1980 (Vale et al., 1981). CRF was finally characterized as the ACTH releasing hormone and has been established as one of the major regulators of behavioral, neuroendocrine and autonomic responses to fear or stress (Behan et al., 1996b). The expression of CRF is encoded by only one gene, which is located on the

long arm of chromosome 8 (8q13). This gene basically codes for a 196 amino acid inactive pro-hormone termed ‘prepro-CRF’, which is transcribed and translated into the functionally active CRF peptide. The CRF gene consists of one functional promoter sequence, one intron of 800 base pairs (bps) and two exons of 582 bps. Interestingly, 97 % DNA sequence homology is found in the first 270 bps of the promoter in humans, sheep, mice and rats demonstrating a high conservation of the gene among mammals (King and Nicholson, 2007). This suggests that not only the signals, leading to CRF gene expression, are similar across animal species but also that the mechanisms, regulating the response to stress, are conserved. Importantly, this high conservation validates the use of animal models in the investigation of CRF’s functionality and increases construct and face validity.

Given that the CRF gene has only one functional promoter, differences in the regulation of gene expression are partly achieved by the variation in transcription factors that act on response elements (RE) within the promoter (King and Nicholson, 2007). Indeed, a substantial number of RE was identified for the transcription regulatory factors MTF1 (MTF1RE), nuclear hormone receptors (HRE), ecdysone (EcRE), glucocorticoid receptor (nGRE), YY1 (YY1RE), CREB (CRE), and CDXA (CDXARE). The elements MTF1RE, HRE and EcRE activate gene transcription by inhibiting the repressive elements nGRE and YY1RE. This allows cyclic adenosinmonophosphate (cAMP) to stimulate the binding of CREB to CRE and, thus, CRF gene expression. In addition to CREB, CRF gene transcription is under the control of the CREB co-activator CRTC2. The activation, i.e. dephosphorylation, of CRTC2 and its subsequent translocation to the nucleus is essential for a full transcriptional response of the CRF gene (Liu et al., 2008; Liu et al., 2010; Liu et al., 2011). Along with cAMP a variety of molecules can stimulate CRF gene expression. For instance, biogenic amines such as NA, acetylcholine, serotonin, cytokines and

gaseous neurotransmitters like NO and CO were shown to activate the CRF promoter (King and Nicholson, 2007). In contrast, glucocorticoids (GC) and GABA, among others, are able to inhibit CRF gene expression. The best studied inhibitory mechanism of CRF transcription is the negative feedback regulation by GC. Activation of the HPA axis induces the production and release of GC, which exert a central negative feedback in the brain hippocampus and PVN (see 1.2.2); the GC-GC receptor complex translocates to the nucleus of CRF neurons and binds the nGRE in the CRF promoter. This inhibits the binding of the transcription machinery to the CRE region, hence reducing CRF gene transcription (Jeanneteau et al., 2012).

CRF is expressed both peripherally and centrally. In the periphery, CRF can be found in blood vessels, skin, lung, testes, ovaries, and placenta (Boorse and Denver, 2006). Main functions for peripheral CRF can be assigned within the immune and the reproductive systems. ‘Immune CRF’ is secreted from spleen, thymus, and inflamed tissues and plays a direct immunomodulatory role as an autocrine or paracrine mediator of inflammation (Karalis et al., 1991; Baigent, 2001). Stimulated by inflammation, CRF is produced in peripheral inflammatory sites acutely from peripheral nerves, i.e. postganglionic sympathetic neurons and primary somatosensory fibers, and chronically from immune cells (Webster et al., 1998). Even though the exact mechanisms are still virtually unknown, some studies present evidence that CRF leads to the degranulation of mast cells (Theoharides et al., 1995), stimulates the secretion of interleukin 1, 2, and 6 (Singh and Leu, 1990; Angioni et al., 1993), promotes lymphocyte proliferation (Singh, 1989), and enhances the production of oxygen radicals by macrophages (Koshida and Kotake, 1994). ‘Reproductive CRF’ participates in various reproductive functions (Kalantaridou et al., 2007). Ovarial and endometrial CRF may participate in the regulation of steroidogenesis and the inflammatory processes of the ovary (ovulation and

luteolysis) and endometrium (decidualization and blastocyst implantation). Placental CRF is increasingly secreted mainly during the latter half of pregnancy and is responsible for the onset of labor (McLean et al., 1995; Magiakou et al., 1996; Chrousos et al., 1998). It has been proposed that there is a 'CRF placental clock', which determines the length of gestation and the timing of parturition (McLean et al., 1995).

Besides peripheral actions as a hormone, CRF has strong central effects acting as neurotransmitter or neuromodulator. It is expressed in the hypothalamus, BNST, amygdala, hippocampus, the septum, cerebrocortical areas, and some brain stem regions (Figure 5) (Swanson et al., 1983; Sawchenko and Swanson, 1985).

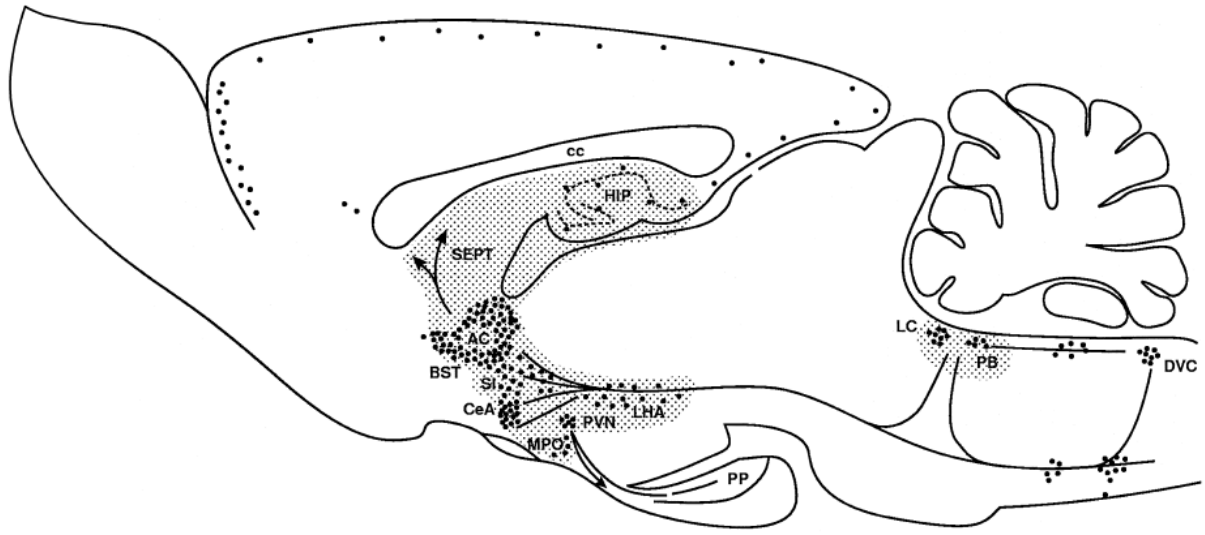

Figure 5. Sagittal section through a rat brain depicting the location of CRF cell bodies and pathways. Shaded areas represent sites identified as mediating the effects of CRF in behavioral responses to stressors. AC, anterior commissure; BST, bed nucleus of the stria terminalis; cc, corpus callosum; CeA, central amygdala; DVC, dorsal vagal complex; HIP, hippocampus, LC, locus coeruleus; LHA, lateral hypothalamus; MPO, medial preoptic area; PB, parabrachial nucleus; PP, pituitary; PVN, paraventricular nucleus; SEPT, septum; SI, substantia innominata. From Koob and Heinrichs, 1999.

Central CRF is involved in triggering nearly every physiological and behavioral response to stress. Expressed centrally in the PVN but secreted into the periphery, CRF is primarily known as hypophysiotropic factor stimulating the HPA axis (see 1.2.2). Within the central nervous system, application of CRF either intracerebroventricularly (icv) or locally at specific brain sites produces a wide variety of behavioral effects. The behavioral pharmacological profile resulting from exogenous administration of this neuropeptide depends on the baseline state of arousal and stress of the animal (Koob and Heinrichs, 1999). In non-stressed animals under low arousal conditions, icv CRF produces a dose-dependent behavioral activation that includes increased locomotor activity, rearing, and grooming when rats are tested in a familiar environment (Sutton et al., 1982; Koob et al., 1984; Sherman and Kalin, 1987; Dunn and Berridge, 1990). Electrophysiologically, CRF has excitatory properties as icv CRF infusion produces electroencephalographic activation characteristic of arousal at low doses (Ehlers et al., 1983) and seizure-like activity at higher doses (Ehlers et al., 1983; Marrosu et al., 1987). Furthermore, CRF can facilitate learning and memory, enhancing retention at low doses and impairing performance at higher doses (Koob and Bloom, 1985). In a more stressful environment, the profile of the behavioral effects of exogenously administered CRF changes to reflect an enhanced behavioral response to stress. The same icv doses that produce marked behavioral activation in a familiar environment induce behavioral suppression in a novel, stressful environment (Koob and Heinrichs, 1999). Rodents pretreated with CRF show decreases in locomotion in an open field (Takahashi et al., 1989), decreased exploration in a multi-compartment chamber (Berridge and Dunn, 1986), and decreased exploration in an EPM (Baldwin et al., 1991). Furthermore, CRF increases defensive burying (Diamant et al., 1992) and reduces sexual behavior (Sirinathsinghji et al., 1983), food intake (Arase et al.,

1988) and alcohol intake (Bell et al., 1998), though drug-induced craving is reinstated by CRF (Zorrilla et al., 2014).

Interestingly, the most studied aspect of CRF's impact on behavioral parameters is its anxiogenic property (Reul and Holsboer, 2002b). CRF increases emotionality in the open field test (Koob and Thatcher-Britton, 1985), enhances the acoustic startle response (Swerdlow et al., 1986), increases conditioned fear in a conditioned suppression test (Cole and Koob, 1988), and enhances stress-induced freezing behavior (Sherman and Kalin, 1988). The anxiogenic effects of CRF are mediated by various brain regions, including the amygdala (Bruchas et al., 2009), LS (Radulovic et al., 1999), and PAG (Miguel and Nunes-de-Souza, 2011). Another brain region which is of particular interest for CRF's effect on anxiety-related behavior is the BNST (Davis et al., 2010). This part of the extended amygdala contains numerous CRF synthesizing cell bodies (Cummings et al., 1983; Moga et al., 1989; Morin et al., 1999) and CRF-immunopositive fibers and terminals originating from the CeA (Figure 5) (Olschowka et al., 1982; Cummings et al., 1983; Swanson et al., 1983; Morin et al., 1999). Furthermore, various studies revealed stress- or anxiety-like behavioral and autonomic effects of CRF infusions into the BNST of rodents (Lee and Davis, 1997; Liang et al., 2001; Ciccocioppo et al., 2003), whereas infusions of non-selective CRF-R antagonists into the BNST conversely produce anxiolytic-like and anti-stress effects (Greenwell et al., 2004; Jasnow et al., 2004). Intriguingly, microinfusion of CRF into the BNST increases anxiety-related behaviors dose-dependently on the EPM in male rats (Sahuque et al., 2006).

1.2.1.2 Ucn 1

Shortly after the discovery of CRF, it emerged that other CRF-related peptides must exist given that, in addition to the CRF ortholog, fish and amphibians also express a paralog, i.e. urotensin 1 and sauvagine, respectively (Bale and Chen, 2012). Based on the similarities between urotensin 1 and sauvagine, Vale's group identified a new member of the mammalian CRF family, which was termed Ucn by that time and is now known as Ucn 1 (Vaughan et al., 1995).

The structure of the Ucn 1 gene is very similar to CRF; it contains one intron and two exons with the second exon encoding for the mature protein (Zhao et al., 1998). The promoter region contains transcription factor binding sites such as a TATA-like sequence, several GATA-binding sites, a C/EBP- and a Brn-2-binding site, a CRE sequence, and a NRSE/RE1. The latter two represent activating and inhibiting regulatory sequences, respectively.

Similar to CRF, Ucn 1 is expressed peripherally and centrally. In the periphery, Ucn 1 mRNA is found in lymphocytes, gastrointestinal tract, testes, thymus, spleen, kidney, and cardiomyocytes (Perrin and Vale, 1999; Boorse and Denver, 2006). Although the role of endogenous Ucn 1 in immune function is not completely clear, it is able to mimic some of the actions of CRF. For instance, Ucn 1 suppresses inflammation and cytokine release even more effectively than CRF, independently of endogenous GC (Agnello et al., 1998). Furthermore, the peptide protects myocytes from hypoxia-induced cell death (Okosi et al., 1998), reduces mean arterial blood pressure (Vaughan et al., 1995), and causes inhibition of gastric emptying (Nozu et al., 1999).

In the adult rat brain, Ucn 1 mRNA accumulates in the Edinger-Westphal nucleus and the lateral superior olive (Figure 6). Weaker signals are detected in the supraoptic nucleus (SON) and caudal lateral hypothalamic area as well as in neurons

of the facial, hypoglossal and ambiguual motor nuclei (Vaughan et al., 1995). Some, but not all, localization studies report further Ucn 1 mRNA in the hippocampus, amygdala, PVN, ventromedial hypothalamus, neocortex, olfactory system and cerebellum (Wong et al., 1996; Bittencourt et al., 1999). The highest density of Ucn 1-immunoreactive fibers is found in the LS, with more moderate Ucn 1 innervation in the SON, PVN, PAG, Edinger-Westphal nucleus, several brainstem nuclei, and the spinal cord (Vaughan et al., 1995; Kozicz et al., 1998; Bittencourt et al., 1999).

Many of the behavioral and physiological effects of central Ucn 1 administration are qualitatively similar to those of CRF. Many data indicate a prominent role for Ucn 1 in regulating appetite and feeding behavior. Icv Ucn 1 decreases food intake with an equal or greater potency than CRF (Spina et al., 1996). Interestingly, there is conflicting evidence with respect to a role for Ucn in anxiogenic behavioral responses. Icv Ucn 1 increases anxiety in the EPM in rats as well as in the light-dark box (LDB) and open field in mice (Moreau et al., 1997; Jones et al., 1998). However, in another study, Ucn 1 was reported to be far less potent in producing anxiety in the EPM than CRF, with no significant anxiogenesis at doses up to 1000 times that are required to suppress food intake (Spina et al., 1996). Furthermore, Ucn 1 has no effect on the amplitude of the acoustic startle response while CRF doubles the response (Jones et al., 1998). In one of the few studies of site-specific Ucn 1 administration, Ucn 1 injected into the basolateral amygdala is equipotent with CRF in producing anxiety in the social interaction test (Sajdyk et al., 1999). Similar to CRF, Ucn 1 produces a general increase in activity, i.e. grooming, alertness, locomotion (Spina et al., 1996; Jones et al., 1998), and can induce limbic seizures (Baram et al., 1997).

1.2.1.3 Ucn 2 and Ucn 3

Six years after the discovery of Ucn 1, two further family members were discovered, namely Ucn 2 (also called Stresscopin-related peptide) and Ucn 3 (also called Stresscopin) (Lewis et al., 2001; Reyes et al., 2001). The Ucn 2 gene encodes a 112 amino acid precursor protein where the C-terminus includes the coding region for the putative 38 amino acid mature peptide (Reyes et al., 2001). The Ucn 3 gene encodes a prepro-protein of 161 amino acids and a putative mature protein of 40 amino acids (Hsu and Hsueh, 2001). In contrast to CRF and Ucn 1, the genetic regulation of Ucn 2 and 3 is poorly understood and investigated to date. It was only shown that mouse Ucn 2 gene expression is up-regulated by GC acting on GRE in the promoter sequence (Chen et al., 2003).

Like CRF and Ucn 1, Ucn 2 and Ucn 3 are expressed both in the periphery and the brain. Peripherally, Ucn 2 mRNA is found in the heart, adrenal gland, human pregnant myometrial cells, and peripheral blood cells (Hsu and Hsueh, 2001; Reyes et al., 2001; Karteris et al., 2004). Ucn 2 produces vasodilatation via the adenylate cyclase - protein kinase A (PKA) pathway, inhibits cell death due to hypoxic stress in cardiomyocytes (Suda et al., 2004), reduces skeletal muscle mass and function loss during atrophy, and increases non-atrophying skeletal muscle mass and function (Hinkle et al., 2003). Ucn 3 mRNA is expressed in pancreas, the gastrointestinal tract, muscle, adrenal gland, and skin, though less abundantly than Ucn 2 mRNA (Hsu and Hsueh, 2001; Lewis et al., 2001). Similar to Ucn 1 and Ucn 2, Ucn 3 is able to inhibit cell death due to hypoxic stress in cardiomyocytes (Suda et al., 2004). Furthermore, Ucn 3 is involved in the local regulation of glucagon and insulin secretions (Li et al., 2003).

Within the central nervous system, Ucn 2 mRNA is expressed within the magnocellular division of the PVN, SON, arcuate nucleus of the hypothalamus, locus coeruleus (LC), and motor nuclei of the brain stem and spinal cord (Figure 6) (Hsu and Hsueh, 2001; Lewis et al., 2001). Ucn 3 mRNA is expressed in discrete areas including the pBNST, the medial amygdala, the hypothalamus, and brainstem (Figure 6) (Hsu and Hsueh, 2001; Reyes et al., 2001). In the hypothalamus, Ucn 3 mRNA is detected in the median preoptic nucleus, in the rostral perifornical region and in a region lateral to the PVN.

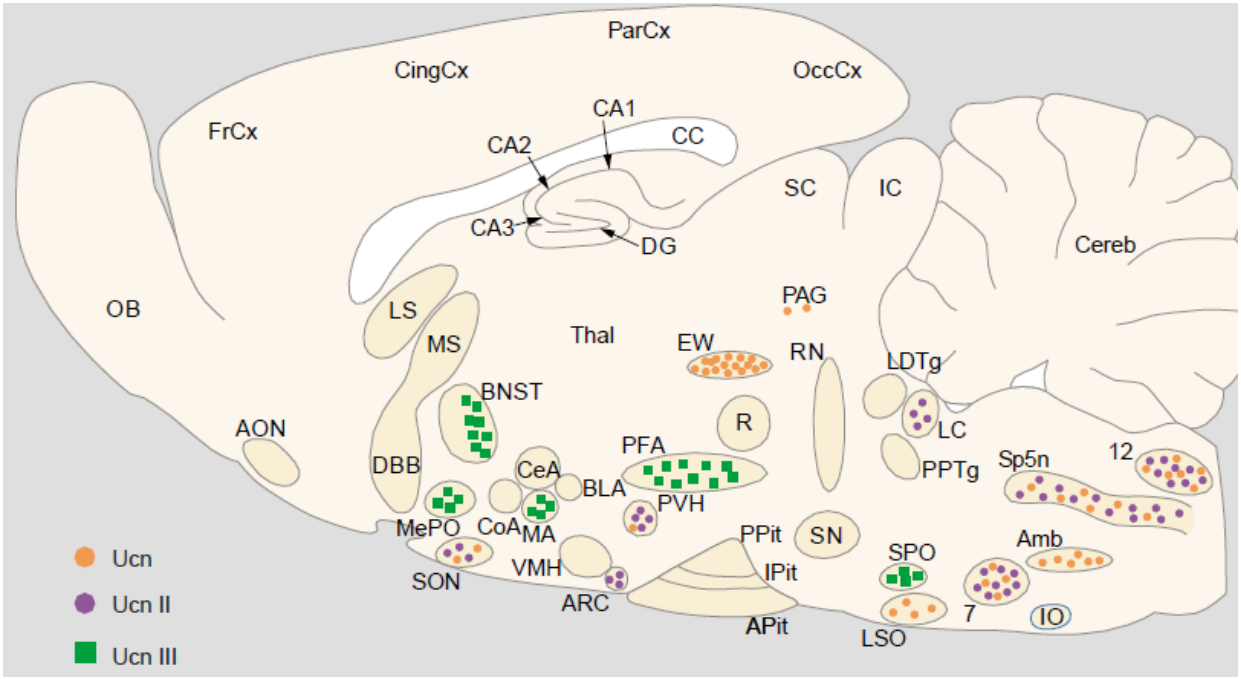

Figure 6. Distribution of Ucn 1, Ucn 2, and Ucn 3 mRNA in a sagittal section of the rat brain. 7: facial nucleus; 12, hypoglossal nucleus; Amb, amgibuus nucleus; AON, anterior olfactory nucleus; APit, anterior pituitary; ARC, arcuate nucleus; BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis; CA1-3, fields CA1-3 of Ammon horn; CC, corpus callosum; CeA, central amygdala; Cereb, cerebellum; CingCx, cingulate cortex; CoA, cortical amygdala; DG, dentate gyrus; DBB, diagonal band of Broca; EW, Edinger-Westphal nucleus; FrCx, frontal cortex; IC, inferior colliculi; IO, inferior olive; IPit, intermediate lobe of pituitary; LC, locus coeruleus; LDTg, laterodorsal tegmental nucleus; LS, lateral septum; LSO, lateral superior olive; MA, medial amygdala; MePO, median preoptic area; MS, medial septum; OB, olfactory bulb; OccCx, occipital cortex; PAG, periaqueductal gray; ParCx, parietal cortex; PFA, perifornical area; PPit, posterior pituitary; PPTg, pedunculopontine tegmental nucleus; PVH, paraventricular hypothalamus; R, red nucleus; RN, raphe nuclei; SC, superior colliculi; SN, substantia nigra; SON, supraoptic nucleus; SP5N: spinal trigeminal nucleus;

SPO, superior paraolivary nucleus; Thal, thalamus; VMH, ventromedial hypothalamic nucleus. From Reul and Holsboer, 2002b.

Central administration of Ucn 2 reveals mild motor suppressive effects (Valdez et al., 2002; Skorzewska et al., 2011), enhances a conditioned freezing fear response (Skorzewska et al., 2011), and is anorexic (Pelleymounter et al., 2004). Similarly to Ucn 2, central infusion of Ucn 3 shows acute locomotor suppressive effects, decreases stress-like behaviors (Valdez et al., 2003), and reduces food intake (Pelleymounter et al., 2004). However, the roles of Ucn 2 and Ucn 3 in the regulation of anxiety-related behavior are not clear yet. Valdez and coworkers report anxiolytic behavior 4 h after Ucn 2 infusion and 10 min after Ucn 3 infusion on the EPM (Valdez et al., 2002; Valdez et al., 2003) while Pelleymounter and colleagues show an anxiogenic effect of Ucn 2 and none of Ucn 3 on anxiety-related behavior (Pelleymounter et al., 2004). In another study, Ucn 3 did not affect anxiety in the shock probe test but was anxiolytic in the defensive withdrawal test (Zhao et al., 2007).

1.2.1.4 CRF receptors

CRF and its related peptides exert their physiological and behavioral effects via two receptors, termed CRF-R1 and CRF-R2 (Hauger et al., 2003). In general, CRF-R are G-protein coupled receptors (GPCR) belonging to the class B GPCR family of neuropeptide receptors and exhibit 70 % sequence homology to each other (Perrin and Vale, 1999). These receptors are encoded by distinct genes and have several splice variants expressed in various central and peripheral tissues. CRF-R1 has α and β isoforms in addition to subtypes designated c - h, which have been detected in human and rodent tissues. Most of these isoforms have been shown to be

nonfunctional (for detailed review see Grammatopoulos and Chrousos, 2002). CRF-R2 is expressed in three functional subtypes, i.e. α, β, and γ (Dautzenberg and Hauger, 2002). These isoforms differ in their N-terminus as well as in their distribution in both tissues and species. CRF-R2α and CRF-R2β have been detected in humans and rodents (Lovenberg et al., 1995; Liaw et al., 1996), while CRF-R2γ has only been reported in humans so far (Kostich et al., 1998). Peripherally, CRF-R1 is found in skin, ovaries, testes, and adrenals (Potter et al., 1994; Chalmers et al., 1995) while CRF-R2 is detected in heart, lungs, ovaries, testes, and adrenals (Potter et al., 1994; Chalmers et al., 1995; Lovenberg et al., 1995; Sanchez et al., 1999). Within the central nervous system, CRF-R1 and CRF-R2 mRNA show a distinct, but overlapping, distribution (Figure 7) (Potter et al., 1994; Chalmers et al., 1995; Lovenberg et al., 1995; Van Pett et al., 2000). CRF-R1 is widely distributed in regions involved in sensory information processing and motor control, in the BNST, hippocampus, amygdala, thalamic nuclei, and anterior pituitary. In contrast, CRF-R2 is virtually restricted to subcortical structures such as the BNST, LS, the ventromedial hypothalamic nucleus, SON and certain amygdaloid nuclei.

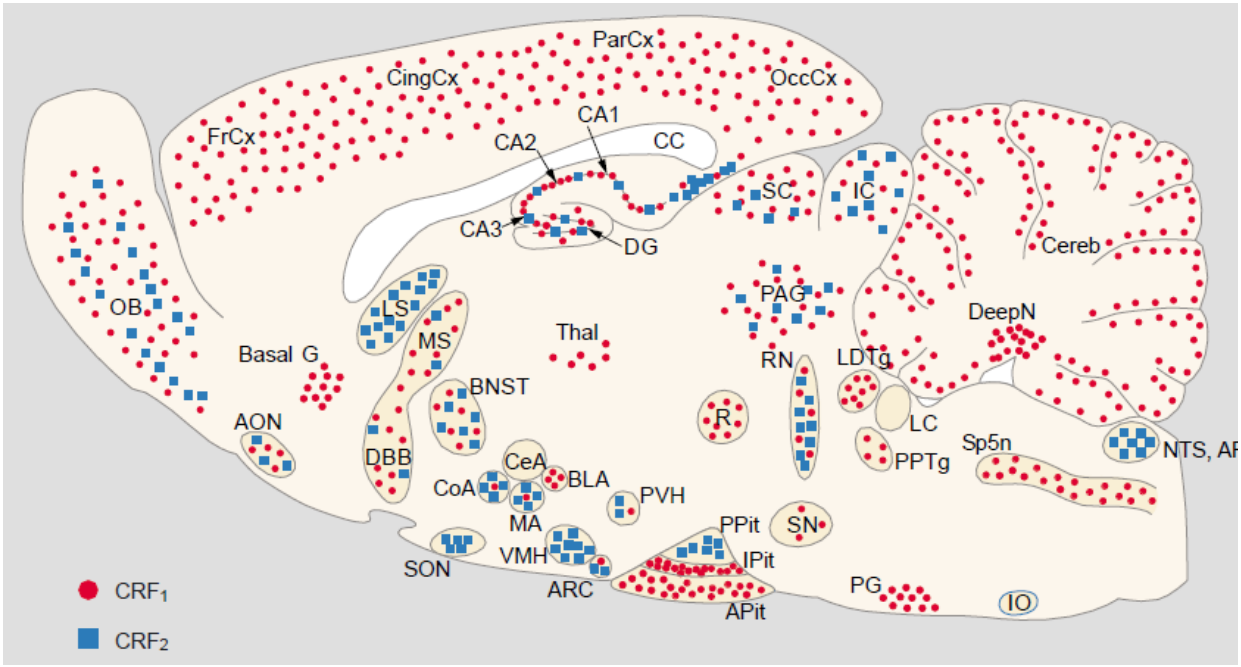

Figure 7. Distribution of CRF-R1 and CRF-R2 mRNA in a sagittal section of the rat brain. AON, anterior olfactory nucleus; AP, area postrema; APit, anterior pituitary; ARC, arcuate nucleus; Basal G, basal ganglia; BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis; CA1-3, fields CA1-3 of Ammon horn; CC, corpus callosum; CeA, central amygdala; Cereb, cerebellum; CingCx, cingulate cortex; CoA, cortical amygdala; Deep N, deep nuclei; DG, dentate gyrus; DBB, diagonal band of Broca; FrCx, frontal cortex; IC, inferior colliculi; IO, inferior olive; IPit, intermediate lobe of pituitary; LC, locus coeruleus; LDTg, laterodorsal tegmental nucleus; LS, lateral septum; MA, medial amygdala; MS, medial septum; NTS, nucleus tractus solitarius; OB, olfactory bulb; OccCx, occipital cortex; PAG, periaqueductal gray; ParCx, parietal cortex; PG, pontine gray, PPit, posterior pituitary; PPTg, pedunculopontine tegmental nucleus; PVH, paraventricular hypothalamus; R, red nucleus; RN, raphe nuclei; SC, superior colliculi; SN, substantia nigra; SON, supraoptic nucleus; SP5N, spinal trigeminal nucleus; Thal, thalamus; VMH, ventromedial hypothalamic nucleus. From Reul and Holsboer, 2002b.

CRF-R bind CRF and its related peptides with different affinities (Figure 8). CRF binds with 10-fold higher affinity to CRF-R1 than to CRF-R2 whereas Ucn 1 shows similar high affinity for both subtypes (Perrin et al., 1999; Hauger et al., 2003). Ucn 2 and Ucn 3 bind exclusively to CRF-R2 (Hsu and Hsueh, 2001; Lewis et al., 2001; Reyes et al., 2001).

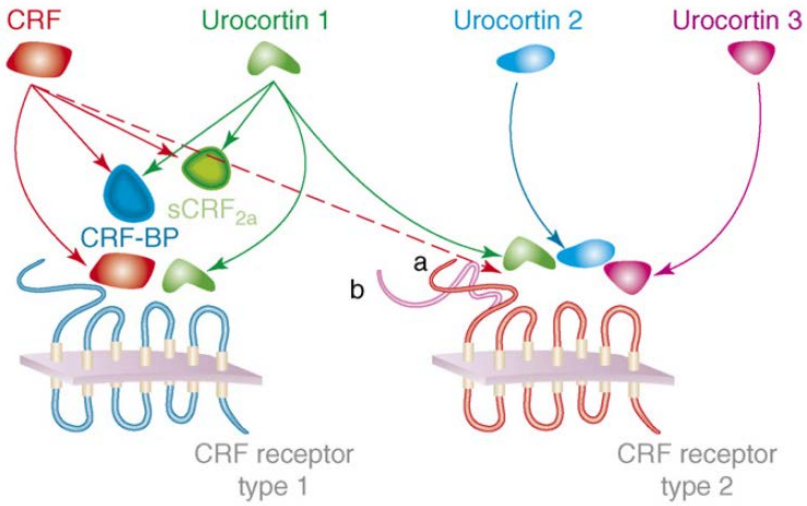

Figure 8. Schematic representation of the CRF system and the binding of the ligands to the receptors or the binding protein. Colored arrows indicate the CRF-R and CRF-BP with which each ligand interacts. Dotted arrow indicates relatively lower affinity compared with unbroken arrows. Adapted from Kuperman and Chen, 2008.

By means of selective agonists / antagonists, antisense oligonucleotides, and transgenic mice, physiological and behavioral effects of the ligands CRF and Ucn 1 – 3 could have been attributed to either CRF-R1 or CRF-R2 (Bale and Vale, 2004). Most findings of CRF and Ucn 1 can be assigned to signaling via CRF-R1 even though some aspects of Ucn 1 action are mediated via CRF-R2. The effects observed following Ucn 2 or Ucn 3 manipulation are exclusively mediated by CRF-R2. For instance, CRF's (and Ucn 1's) prominent role in anxiety-related and depressive-like behavior is mediated by CRF-R1 (Reul and Holsboer, 2002b) while the strong anorexic effects of Ucn 2 and Ucn 3 are mediated via CRF-R2 (Kuperman and Chen, 2008). Due to their specific binding profiles, the potential behavioral effects of Ucn 2 and Ucn 3 on anxiety (Valdez et al., 2002; Valdez et al., 2003; Pelleymounter et al., 2004; Zhao et al., 2007) might also be mediated via CRF-R2. However, CRF-R2 play a rather complicated role in anxiety and depressive pathophysiology, which entails the partly contradictory results of the different studies (see 1.2.1.3). One line of research supports the concept that CRF-R2 reestablish homeostasis by counteracting stress response-initiating effects and anxiety-like defensive behavior triggered by CRF-R1 signaling (Coste et al., 2001; Bale and Vale,

2004; Heinrichs and Koob, 2004; Muller and Holsboer, 2006). An alternative hypothesis proposes that CRF-R1 and CRF-R2 contribute to opposite defensive modes, with CRF-R1 mediating active defensive responses triggered by escapable stressors, and CRF-R2 mediating passive anxiety- and depression-like responses induced by inescapable, uncontrollable stressors (Maier and Watkins, 2004; Valentino and Commons, 2005).

CRF and its related peptides bind to the extracellular domains of CRF-R1 and CRF-R2, which transforms the membrane conformation of the receptors into an active state. CRF-R can signal via three distinct pathways, i.e. the adenylyl cyclase - PKA pathway, the phospholipase C (PLC) - protein kinase C (PKC) pathway, and the extracellular-signal related kinase (ERK) - mitogen-activated protein kinase (MAPK) pathway. In many endogenous and recombinant cell lines, CRF-R1 and CRF-R2 preferentially signal by $G_{s\alpha}$ coupling to the third intracellular loop, leading to the activation of adenylyl cyclase and generation of the second messenger cAMP. Increasing cAMP formation, in turn, stimulates PKA to phosphorylate downstream targets in the cytosol and CREB in the nucleus inducing transcription of certain genes (Dautzenberg et al., 2000; Dautzenberg and Hauger, 2002; Hillhouse and Grammatopoulos, 2006; Hauger et al., 2009). Interestingly, abnormalities in adenylyl cyclase – PKA signaling have been implicated in stress maladaptation, anxiety, and depression (Hauger et al., 2009). Signaling via the PLC - PKC pathway is presumably initiated by coupling to $G_{q\alpha}$, which in turn stimulates the formation of inositol(1,4,5)-triphosphate and contributes to intracellular calcium mobilization (Gutknecht et al., 2009; Hauger et al., 2009). Dysregulations of PKC signal transduction have been associated with severe anxiety and suicide (Hauger et al., 2009). It is still unknown what factors are responsible for coupling of $G_{s\alpha}$ or $G_{q\alpha}$ and thus, the activation of the different signaling pathways. However, it was recently

shown that Ucn 2 and Ucn 3, but not CRF, binding to CRF-R2 induce ERK – MAPK signaling via a PLC-dependent mechanism (Arzt and Holsboer, 2006) whereas activation of CRF-R1 by CRF or Ucn 1 on corticotropes induces a MAPK signal transduction pathway that is downstream of PKA (Kovalovsky et al., 2002). These studies indicate that the activation of the distinct signaling pathways is dependent on ligand specificity and the induction conditions of the intracellular pathways in each cell type (Arzt and Holsboer, 2006).

The magnitude, duration and specificity of cellular signaling by GPCRs depend upon rapid and stringent regulation to prevent deleterious effects of unrestrained, excessive receptor signal transduction. While agonist binding induces conformation changes to activate the receptor and initiate signaling, specific G-protein receptor kinases (GRK) are rapidly recruited to commence homologous desensitization by phosphorylating a specific pattern of serines and threonines in the third intracellular loop and the C-terminus of the receptor (Kohout and Lefkowitz, 2003; Krasel et al., 2005; Moore et al., 2007). This GRK-mediated phosphorylation of the receptor immediately increases its affinity for β-arrestins by approximately 30-fold, triggering their translocation from the cytosol to the cell surface (Kohout and Lefkowitz, 2003; Krasel et al., 2005; DeWire et al., 2007; Moore et al., 2007). Rapid, high-affinity binding of a single arrestin sterically uncouples the receptor from its cognate G_{α} protein, thereby terminating signal transduction. Following binding, class B GPCRs form stable complexes with arrestins that internalize as a unit into endocytic vesicles (Oakley et al., 1999; Oakley et al., 2000). Internalized receptors are dephosphorylated in endosomes and recycled as resensitized GPCRs back to the plasma membrane. During very prolonged exposure to high agonist concentrations, internalized GPCRs do not recycle to the membrane but move into lysosomes, where they undergo proteolytic degradation resulting in down-regulation to decrease the

total number of cellular receptors (Kohout and Lefkowitz, 2003; Moore et al., 2007). Indeed, cell stimulation with CRF for 30 min leads to a prominent internalization of CRF-R1 (Oakley et al., 2007), which resensitize within 1 – 2 h (Hoffman et al., 1985; Teli et al., 2005; Holmes et al., 2006). However, stimulation with CRF for 24 h decreases CRF-R1 by 70 – 80 %, presumably due to proteolytic degradation of receptors in lysosomes (Hauger et al., 1997; Perry et al., 2005). Intriguingly, following binding and activation by Ucn 2, CRF-R2 desensitize more rapidly and to a greater extent than the rate and magnitude of homologous desensitization of CRF-R1 by CRF (Hauger et al., 1997; Teli et al., 2005; Gutknecht et al., 2008; Markovic et al., 2008).

1.2.1.5 CRF-BP

In addition to the two CRF-R, the activity of CRF and CRF-related peptides is modulated by the CRF-BP, a 37 kDa secreted glycoprotein (Orth and Mount, 1987; Behan et al., 1989). The CRF-BP gene encodes a 322 amino acid protein, of which the mature protein contains one N-linked glycosylation site (Potter et al., 1991; Cortright et al., 1995; Jahn et al., 2001). Additionally, the CRF-BP lacks transmembrane domains or a phosphatidyl inositol anchor signal motif, suggesting that the CRF-BP does not have a direct association with the membrane (Potter et al., 1991; Eckart et al., 2001). The gene organization as well as nucleotide and amino acid sequence of the CRF-BP is highly conserved across evolution, suggesting the maintenance of a structural conformation necessary for biological activity. CRF-BP gene expression is positively regulated by stress and GC, providing an important negative feedback mechanism of the HPA axis (McClennen et al., 1998; Lombardo et al., 2001). Interestingly, the stress-induced increase in CRF-BP mRNA can persist up

to 21 h post-stress and has important implications for the effects of future stressors (Herringa et al., 2004). Furthermore, it was shown that CRF, cAMP, and interleukin-6 positively regulate CRF-BP promoter activity (Cortright et al., 1997; Mulchahey et al., 1999; Seasholtz et al., 2001). However, the CRF-BP is not only regulated by stress-related factors but is strongly influenced by reproductive hormones (Westphal and Seasholtz, 2006). For instance, the expression of CRF-BP mRNA varies over the estrous cycle, with nearly 3-fold higher levels at proestrous, when estrogen levels peak (Speert et al., 2002). This is potentially mediated by three estrogen RE half-sites in the CRF-BP promoter (van de Stolpe et al., 2004).

The CRF-BP is expressed in a highly tissue specific pattern (for review see Kemp et al., 1998). Human CRF-BP is found in plasma, amniotic fluid, synovial fluid, placenta, pituitary, and brain. In contrast, CRF-BP is not detected in rodent or ovine plasma and rat, mouse, and ovine CRF-BP mRNA and protein have been found only in brain and pituitary. In the rat brain, the CRF-BP is expressed in many specific regions including the BNST, amygdala, PVN, hippocampus, cerebral cortex, olfactory bulb, and sensory relays associated with the auditory, olfactory, vestibular, and trigeminal systems (Potter et al., 1992). The CRF-BP is expressed in both neurons and astrocytes (Behan et al., 1995a) and overlaps with CRF and CRF-R expression in several areas such as the BNST, amygdala, and pituitary (Kemp et al., 1998).

The CRF-BP is distinct from the CRF-R and binds CRF and Ucn 1, but not Ucn 2 and Ucn 3, with an equal or greater affinity than the CRF-R (Figure 8) (Sutton et al., 1995; Vaughan et al., 1995). The CRF-BP has important modulatory roles with regards to CRF activity and may have different functions depending on the specific cell-type or context in which it is expressed (Westphal and Seasholtz, 2006). Three major hypotheses exist with regard to the central and pituitary function of CRF-BP. The

glycoprotein could act in an inhibitory fashion to sequester CRF and/or target it for degradation, which would reduce CRF-R activity. Most studies support a role for CRF-BP in limiting the availability of endogenous CRF (Potter et al., 1991; Woods et al., 1994; Cortright et al., 1995; Peto et al., 1999). In contrast to its inhibitory role, the CRF-BP could bind CRF and potentiate CRF signaling by modulating its interaction with the receptor, delivering CRF to the receptor, or extending the half-life of CRF. Finally, CRF-BP could exert ligand and/or receptor-independent activity given that the CRF-BP is expressed at numerous sites where CRF and Ucn 1 are not detected (Potter et al., 1992).

Intriguingly, very little is known about the functional roles of the CRF-BP. Genetic mouse models have been created to elucidate physiological, neuroendocrine, and behavioral effects of CRF-BP overexpression (Burrows et al., 1998; Lovejoy et al., 1998) or deficiency (Karolyi et al., 1999) (for review see Seasholtz et al., 2001). For example, deletion of the CRF-BP gene results in increased anxiety-related behavior in males (Karolyi et al., 1999) without affecting the HPA axis (Seasholtz et al., 2001). In order to manipulate the CRF-BP in rats, the peptide can be inhibited by a truncated version of CRF, i.e. $CRF_{(6\text{-}33)}$, which binds with high affinity exclusively to the CRF-BP, displaces bound CRF/Ucn 1 and, thus, increases 'free' extracellular CRF levels (Behan et al., 1995c). Via this approach, it was shown that inhibition of the CRF-BP leads to cognition-enhancing properties in models of learning and memory. However, barely any other behavior has been investigated using this elegant technique.

1.2.2 CRF as regulator of the HPA axis

One of CRF's major functions is the activation of the HPA axis. Following stressor exposure, CRF and the co-secretagogue AVP are released from the parvocellular

part of the PVN (pPVN) at the level of the median eminence (Vale et al., 1981; Gillies et al., 1982). Both peptides reach the anterior pituitary corticotropes through the hypophyseal portal system where they stimulate ACTH production and release. ACTH is released into the circulatory system and stimulates GC production and secretion from the adrenal cortex (Aguilera, 1998). Via a negative feedback mechanism, GC reduce HPA axis activation on the level of the hippocampus, hypothalamus, and pituitary. For example, GC are able to directly inhibit corticotropic function by inhibiting hypothalamic CRF and AVP expression (Ma et al., 1997) and release from the median eminence (Spinedi et al., 1991), and by direct inhibition of proopiomelanocortin transcription and ACTH secretion by corticotropes (Levin and Roberts, 1991).

Another important relay site for signal integration and regulation of the HPA axis is the BNST. It receives rich innervations from limbic forebrain structures such as the ventral subiculum, medial amygdala and CeA (Herman et al., 2003), as well as more limited input from the medial prefrontal cortex (Spencer et al., 2005; Radley et al., 2009). Furthermore, the PVN receives abundant projections from the BNST (Sawchenko and Swanson, 1983; Cullinan et al., 1993; Dong et al., 2001b; Gu et al., 2003; Dong and Swanson, 2004a, 2006). The BNST can be subdivided into many cyto- and chemoarchitecturally distinct subregions; within the anterior BNST, the dorsomedial and fusiform nuclei express CRF (Phelix and Paull, 1990), and send the heaviest BNST projections to the pPVN (Dong et al., 2001b; Dong and Swanson, 2006). Within the pBNST, the principal nucleus contains a GABAergic neuronal population (Ju et al., 1989; Cullinan et al., 1993) that also heavily innvervates the pPVN (Gu et al., 2003; Dong and Swanson, 2004a). Electrical stimulation of the BNST can either excite or inhibit HPA axis activity (Dunn, 1987; Feldman et al., 1990; Casada and Dafny, 1991; Zhu et al., 2001), depending on the region targeted.

Additionally, anterior BNST lesions targeting the dorsomedial/fusiform nucleus attenuate HPA axis activation, as noted by decreased corticosterone (CORT) and PVN c-fos mRNA responses to acute stress (Choi et al., 2007). In contrast, lesions of the pBNST increase CRF and AVP mRNA expression in the pPVN, enhance stress-induced secretion of ACTH and CORT, as well as stress-induced c-fos mRNA (Gray et al., 1993; Herman et al., 1994; Choi et al., 2007). This positions the BNST in a central role integrating forebrain and limbic information and modulating the HPA stress responses. Indeed, a balanced regulation is vital for the maintenance of body homeostasis. Dysfunctions of the HPA axis have been often implicated in the appearance of mood disorders like anxiety and depression. Our animal model for extremes in anxiety, i.e. the HAB and LAB rats, connects differences in the rats' HPA axis reactivity with a depressive-like phenotype. Male and female HAB rats display a hyper-responsiveness of the HPA axis to a mild emotional stressor (Liebsch et al., 1998; Neumann et al., 1998a; Landgraf et al., 1999; Keck et al., 2002) and a pathological ACTH response during the combined dexamethasone / CRF challenge test in males (Keck et al., 2003), which is a diagnostic marker for major depression (Holsboer et al., 1994). These physiological abnormalities are based on differences in CRF and AVP expression within the PVN. Male HAB rats do not only have higher AVP levels, they also express elevated levels of CRF mRNA under basal conditions (Bosch et al., 2006), which is most likely responsible for the increased drive of the HPA axis following stressor exposure. Hence, especially CRF appears to be highly implicated in the regulation of mood disorders.

1.2.3 The CRF system and mood disorders – focus on the peripartum period

Mood disorders like anxiety and depression are among the most common neuropsychiatric disorders worldwide. In fact, depression affects approximately 20 % of the population with the incidence being up to three times higher in women than in men (Gutierrez-Lobos et al., 2002; Kornstein et al., 2002). Accumulating evidence suggests that an increased central CRF drive is a key feature often seen in major depression and anxiety disorders (Reul and Holsboer, 2002a). For example, increased CRF concentrations in the cerebrospinal fluid have been repeatedly observed in major depression (Nemeroff et al., 1984; Banki et al., 1987; Hartline et al., 1996), posttraumatic stress disorder patients (Bremner et al., 1997), and suicide victims (Arato et al., 1989). Furthermore, CRF mRNA and peptide expression is increased in the hypothalamus, cortical areas, pontine nuclei and the LC postmortem (Raadsheer et al., 1994; Austin et al., 2003; Bissette et al., 2003; Merali et al., 2006). This is paralleled by a down-regulation of CRF-R1, but not CRF-R2, in cortical areas of suicide victims, all pointing to an hyperactive CRF/CRF-R1 system in depression (Nemeroff et al., 1988; Merali et al., 2004). Furthermore, the CRF-BP has been found to be down-regulated in the amygdala of patients with bipolar disorder (Herringa et al., 2006) and Ucn 1 expression is upregulated in the Edinger-Westphal nuclei of suicide victims (Kozicz et al., 2008). The relevance of overactive limbic CRF-R1 transmission in depression is underlined by the fact that selective CRF-R1 antagonists exert antidepressant effects at doses that do not influence baseline or stimulated HPA axis activation (Zobel et al., 2000; Kunzel et al., 2003). Along with CRF's hyperactivity in mood disorders, evidence has been accumulating that disturbances in the regulatory control of the HPA axis play a pivotal role in the etiology of mood disorders, particularly major depression. (Steckler et al., 1999;

Holsboer, 2000). The reported hyperactivity of the HPA axis is most likely caused by the hyperactivity of CRF/CRF-R1 signaling (Reul and Holsboer, 2002b).

As briefly mentioned above, women are more likely to be affected by mood disorders than men. The time of the highest risk for women to develop anxiety and depressive disorders is during their childbearing years (Kessler, 2003; Marcus, 2009). Hormonal fluctuations during pregnancy and postpartum are proposed to play an essential role in the establishment of depressive symptoms. Particularly women who have a history of high anxiety, experienced pregnancy complications, gave birth prematurely, delivered a low birthweight infant or are caring for an infant with a birth defect are particularly prone to suffer from a (new) episode of high anxiety during this greatly susceptible period of life. Mothers with postpartum anxiety exhibit bidirectional parenting styles; one subset shows reduced coping and reactivity to the infant while the second group displays a highly protective mothering style, often termed ‘helicopter parenting’. The latter was proposed to be found in lactating HAB rats, displaying high anxiety-related behavior on the one hand and very elaborate and protective maternal behavior on the other hand (Bosch, 2011). Given that the peripartum period is associated with dramatic hormonal changes, it is often speculated that these fluctuations are responsible for the high susceptibility to develop mood disorders. However, dysfunctions in neuropeptidergic systems regulating anxiety-related behavior peripartum are highly feasible as well. Unfortunately, postpartum anxiety disorders are still under-investigated because the likelihood of detecting elevated anxiety in postpartum women is very low (Coates et al., 2004) and suitable animal models that allow the investigation of peripartum dysregulations of anxiety are rare. The need for a better understanding is even increased by the fact that women are more likely to be anxious than depressed and that peripartum anxiety is a very strong predictor of later postpartum depression

(Stuart et al., 1998; Bergant et al., 1999; Heron et al., 2004; Ross et al., 2004). Thus, a better understanding of postpartum anxiety could help prevent a trajectory toward postpartum depression for some women (Lonstein, 2007).

Similar to anxiety, depressive symptoms are also influenced by reproductive events in women. After parturition, women can suffer from three different forms of depressive disorders: postpartum blues, postpartum depression, and postpartum psychosis (Hillerer et al., 2014). Postpartum blues, which is a transient and mild condition characterized by mood disturbances beginning a few days after parturition and lasting less than two weeks, is extremely common with prevalence estimates of up to 84 % of parturient women (Henshaw et al., 2004). Postpartum blues often resolves spontaneously within two weeks without bearing negative consequences for the mother or child (Seyfried and Marcus, 2003). In contrast, postpartum depression is more serious for both the mother and the infant (O'Connor et al., 2002; Deave et al., 2008) but is not as prevalent as postpartum blues with estimates of 15 % (Goodman, 2007). However, the actual number is likely higher due to the reluctance of mothers to admit their depressive state during a time of expected happiness (Marcus, 2009). Postpartum depression represents an episode of major depression with a specific temporal manifestation which is still under debate. A crucial feature of maternal depression, which distinguishes postpartum depression from other depressive episodes, is the loss of interest in the infant (Atkinson et al., 2000; Lovejoy et al., 2000), which may lead to infant neglect due to the aversive perception of the child (Adamakos et al., 1986; Bifulco et al., 2004). Finally, postpartum psychosis is probably the most serious postpartum disorder and has a prevalence of 0.1 - 0.5 % in parturient women. It has been hypothesized to be a feature of bipolar or schizoaffective disorder and it may culminate with suicide and/or filicide (Appleby et al., 1998).

1.3 The CRF system in the peripartum period

In order to protect a mother from the detrimental consequences of postpartum mood disorders, the maternal brain undergoes tremendous changes, thereby, adapting to the changing demands of maternity. Besides behavioral and neuropeptidergic adaptations (see 1.1), pregnant and lactating females show remarkable alterations in their stress reactivity. During pregnancy, the basal and stress-induced HPA axis activity is markedly reduced in rats, mice (Johnstone et al., 2000; Douglas et al., 2003) and humans (Schulte et al., 1990; Hartikainen-Sorri et al., 1991). In rats, this hyporesponsiveness is evident from day 15 of gestation and persists through pregnancy (Neumann et al., 1998b), parturition (Wigger et al., 1999) and lactation, until weaning (Windle et al., 1997). It is reflected by reduced ACTH and CORT secretion following stress and involves adaptations at the level of both the anterior pituitary and the hypothalamus, as well as at higher brain areas (da Costa et al., 1996). In the pituitary of pregnant and lactating rats, corticotropes are less reactive to administered CRF (Neumann et al., 1998b; Toufexis et al., 1999) and AVP (Ma et al., 2005) than in virgins. At the level of the hypothalamus, CRF and AVP neurons in the pPVN are less stimulated by stressors compared to virgin rats, which is evident by reduced CRF and AVP synthesis (da Costa et al., 2001; Brunton et al., 2006). In addition, CRF's ability to activate neurons within the PVN is reduced from early lactation onward (da Costa et al., 1997; da Costa et al., 2001). Interestingly, the excitatory drive to PVN neurons from both the limbic forebrain (da Costa et al., 1996) and the brainstem nuclei (Brunton et al., 2005) is reduced in response to emotional and physical stressors, respectively. At the level of higher brain regions, expression of CRF mRNA is reduced in the CeA, whereas it is increased in the dorsolateral portion of the BNST during lactation (Walker et al., 2001). Furthermore, immediate early gene expression in the BNST, arcuate nucleus, LS, and medial amygdala is

elevated in response to icv CRF (da Costa et al., 1996; da Costa et al., 1997). In general, the central nervous system of lactating rats seems to be less responsive to CRF compared to virgin females (da Costa et al., 1996; da Costa et al., 1997). Thus, decreased synthesis of CRF, reduced stimulation of CRF release, or decreased responsiveness of the brain to CRF could contribute to the reduced anxiety and stress reactivity occurring during lactation.

In addition to CRF's prominent role in the dam's stress reactivity postpartum, the neuropeptide system appears to be also involved in the behavioral adaptations associated with the peripartum period. A few studies have investigated potential effects of CRF and its related peptides on maternal behavior. For instance, icv CRF inhibits maternal-like care in nulliparous ovariectomized, steroid-primed virgin rats that had three days of mothering experience and induces pup-killing in rats that are naïve to the pups (Pedersen et al., 1991). Furthermore, in lactating marmosets, icv CRF impaired the occurrence of maternal care (Saltzman et al., 2011). In lactating mice, the effects of various CRF family members on maternal aggression were investigated either by pharmacological manipulation or by genetic approaches. Gammie and co-workers revealed that icv administration of CRF (Gammie et al., 2004), Ucn 1, or Ucn 3 (D'Anna et al., 2005) impairs the occurrence of maternal aggression in lactating mice. C-fos studies examining neuronal activation revealed increased activation in various regions like the BNST, medial and LS, medial and CeA, and the PAG after icv CRF, Ucn 1 and Ucn 3 administration (Gammie et al., 2004; D'Anna et al., 2005). Assigning impairing effects of CRF and its related peptides to one of the CRF-R subtypes and to distinct brain regions, activation of CRF-R2 in the LS impairs maternal aggression, which apparently further activates the ventromedial hypothalamus, lateral hypothalamus and parabrachial nucleus as downstream mediators (D'Anna and Gammie, 2009). By means of transgenic mice,

the CRF-R2 subtype was further highlighted as a main mediator of maternal aggression (Gammie et al., 2005). However, also the CRF-R1 subtype might play a role as lactating CRF-R1 knockout mice display impaired maternal care and maternal aggression while maternal anxiety is not altered (Gammie et al., 2007a). In addition to the ligands and receptors of the CRF system, the CRF-BP seems to be another important mediator of maternal behavior. Lactating mice that were selected for high maternal aggression express elevated levels of CRF-BP compared to low aggressive females (Gammie et al., 2007b). Furthermore, knocking out the CRF-BP gene leads to impaired maternal aggression but no changes in maternal care and anxiety in lactating mice (Gammie et al., 2008). These studies provide a first insight in the behavioral role postpartum and reveal the CRF family as an interesting candidate system for the fine-tuned regulation of maternal behavior.

1.4 Aim of the thesis

The maternal brain is well known for the numerous physiological, neuroendocrine and behavioral adaptations occurring around parturition. Neuropeptide systems like the OXT or AVP system have been extensively studied and implicated in mediating these changes in a variety of species. To date, only little attention has been paid to potential implications of the CRF system, particularly in terms of behavioral adaptations peripartum. The existing studies are limited by either investigating an artificial animal model for maternal behavior, i.e. sensitized virgin rats, or by focusing predominantly on one behavioral parameter, i.e. maternal aggression, in lactating mice. Therefore, in the present thesis, I aimed to characterize the CRF system in the regulation of maternal care, maternal motivation, maternal aggression, and maternal anxiety using a fully authentic model for maternal behavior, i.e. early lactating rats.

In the first part of my thesis, I used lactating HAB and LAB rats, an animal model for anxiety and maternal behavior, to investigate potential differences in the CRF system during lactation compared to virginity. Furthermore, I aimed to elucidate the effects of acute central administration of CRF or a non-selective CRF-R antagonist on the full repertoire of maternal behavior. In the second and third part, I tried to assign the behavioral effects of CRF-R activation on maternal behavior to the BNST, a crucial region for maternal behavior and an abundant expression site for the CRF system. Thereby, I distinguished between the medial-posterior BNST (mpBNST) and the adBNST and aimed to include potential neuroendocrine mechanisms in the behavioral regulation by the CRF system. In the last part, I intended to assess the role of the CRF-BP in the regulation of maternal behavior in lactating rats using a pharmacological approach. Therefore, the CRF-BP was acutely inhibited to increase free CRF/Ucn 1 concentrations either centrally or within the mpBNST or adBNST.

These studies were designed to contribute evidently to the understanding of the CRF system's role in the maternal brain. Advancing our understanding may be helpful unraveling a vital candidate system in mediating some of the dysregulations that might occur during the most prevalent time to develop mood disorders, the peripartum period.

- Chapter 2 -

Reduced brain CRF receptor activation is required for adequate maternal care and maternal aggression in lactating rats

Authors‘ contribution:

Stefanie Klampfl: experimental design, performance of experiments, data analysis, first draft of manuscript

Inga Neumann: revision of manuscript

Oliver Bosch: experimental design, performance of experiments, revision of manuscript

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2.1 Abstract

The brain CRF system triggers a variety of neuroendocrine and behavioral responses to stress. Whether maternal behavior and emotionality in lactation are modulated by CRF has rarely been investigated.

In the present study, we measured CRF mRNA expression within the pPVN in virgin and lactating Wistar rats bred for high (HAB) and low (LAB) anxiety-related behavior or non-selected for anxiety (NAB). Further, we infused icv synthetic CRF or the CRF-R antagonist D-Phe to manipulate CRF-R1/2 non-specifically in lactating HAB, LAB, and NAB dams and monitored maternal care, maternal motivation, maternal aggression, and anxiety.

The CRF mRNA expression in the pPVN was higher in HAB versus LAB rats independent of reproductive status. The lactation-specific decrease of CRF mRNA was confirmed in LAB and NAB dams but was absent in HABs. Icv CRF decreased maternal care under basal conditions in the home cage in all breeding lines and reduced attack behavior in HAB and LAB dams during maternal defense. In contrast, D-Phe rescued maternal care after exposure to maternal defense in the home cage without influencing maternal aggression. Furthermore, D-Phe decreased and CRF tended to increase anxiety in HAB/NAB and in LAB dams, respectively, suggesting an anxiogenic effect of CRF in lactating females.

In conclusion, low CRF-R activation during lactation is an essential prerequisite for the adequate occurrence of maternal behavior, at least in LAB and NAB rats.

2.2 Introduction

In mammals, CRF is the primary activator of the HPA axis and is also strongly involved in the central stress response (Fisher and Brown, 1991). CRF is expressed in different brain regions like the pPVN, the extended amygdala, or the LS (Potter et al., 1994; Boorse and Denver, 2006). Via CRF-R1 and -R2, CRF has anxiogenic effects and increases passive stress coping in males (Reul and Holsboer, 2002b; Hauger et al., 2009). Additionally, CRF modulates social behaviors, e.g. icv infused CRF reduces aggression in male mice (Mele et al., 1987), while central application of a CRF-R antagonist impairs social recognition in female rats (Heinrichs, 2003). Furthermore, there are first indications for CRF playing a significant role in maternal behavior; icv CRF reduces maternal-like behavior and induces pup-killing in ovariectomized, steroid-primed virgin rats (Pedersen et al., 1991) as well as decreases maternal aggression against a male intruder in lactating mice (Gammie et al., 2004).

To further investigate the role of brain CRF in social behavior, the use of animal models with differences in the CRF system is advantageous. Here, Wistar rats bred for high (HAB) and low (LAB) anxiety-related behavior are particularly suitable (Liebsch et al., 1998; Bosch, 2011) as male HAB rats have higher CRF mRNA levels in the pPVN in comparison to male LAB rats (Bosch et al., 2006). This difference exists in addition to the well-characterized elevated expression and release of AVP in both male and female HAB compared to LAB rats (Keck et al., 2002; Murgatroyd et al., 2004; Bosch and Neumann, 2008). Moreover, profound differences in social behaviors are observed in male (Henniger et al., 2000; Beiderbeck et al., 2007; Veenema et al., 2007) as well as in lactating HAB and LAB rats (Bosch et al., 2005; Neumann et al., 2005a; Bosch and Neumann, 2008). With respect to maternal behavior, the high anxiety level of HAB dams is linked to a more protective mothering

style. Likely due to their increased brain AVP activity, lactating HAB dams retrieve their pups faster, leave their nest less often, spend more time on ABN and display more maternal aggression against an intruder (Bosch, 2011).

In order to study the contribution of the brain CRF system to the differences in maternal behavior, we compared the CRF mRNA expression in the pPVN of virgin and lactating HAB, LAB, and NAB Wistar rats. Furthermore, to elucidate the involvement of central CRF-R1/2 in the regulation of maternal care, motivation, and aggression as well as anxiety in lactation either a CRF-R agonist (synthetic CRF) or a CRF-R1/2 antagonist (D-Phe) was applied.

2.3 Materials & Methods

2.3.1 Animals

The studies were conducted in accordance with the European Communities Council Directive (86/609/EEC), and received approval by the local government of the Oberpfalz (Bavaria). All efforts were made to minimize the number of animals used and their suffering.

Virgin female HAB and LAB (220 – 250 g; local breeding colony; for selection procedures see Neumann et al., 2005b) as well as NAB rats (220 – 250 g; Charles River Laboratories, Sulzfeld, Germany) were kept under standard laboratory conditions (change of bedding once per week; 12 : 12 h light / dark cycle, lights on at 6 a.m.; RT 22 ± 2 °C, 55 ± 5 % relative humidity) with access to water and standard rat chow *ad libitum*. Female rats were mated with sexually experienced male rats and pregnancy was confirmed by the presence of sperm in vaginal smears (pregnancy day (PD) 1). Afterwards, the females were group-housed in standard polycarbonate rat cages (40 x 60 x 20 cm, 3 - 4 animals per cage) until PD 18, when they underwent surgery (behavioral experiment, only) and were single-housed in plexiglas

observation cages (38 x 22 x 35 cm) for undisturbed delivery (6 - 15 pups on PD 22 - 23). Litters were culled to 8 pups of mixed sexes. For comparison of virgin female rats with lactating dams, both groups were treated identically, i.e. virgins were single-housed 7 days prior to brain removal, which complies with the single-housing period of the lactating rats. During this period, all rats were handled carefully twice a day to familiarize them with the experimental procedures and to reduce non-specific stress responses during the experiment.

Naïve virgin female Wistar rats (200 – 220 g, Charles River Laboratories) at random stages of the estrous cycle served as intruders in the maternal defense test (Neumann et al., 2001; Bosch et al., 2005). The intruder rats were kept in a separate room to avoid olfactory recognition possibly influencing the aggressive behavior of the lactating residents during the maternal defense test.

2.3.2 In situ hybridisation for CRF mRNA expression

In order to compare CRF mRNA expression within the pPVN of virgin and lactating HAB, LAB, and NAB rats on lactation day (LD) 4 or equivalent in virgin rats, the animals were briefly anesthetized with CO_2, their brains removed rapidly and flash frozen on dry ice. Brains were sectioned at 16 µm using a cryostat (CM3050S, Leica Microsystems GmbH, Nussloch, Germany), slide mounted, and stored at -20°C until further processing. Slides from all groups were processed simultaneously and autoradiograms were coded to obscure the identity of the tissue.

CRF mRNA *in situ* hybridisation was performed using a highly specific single, 48 base, ^{35}S-labeled oligonucleotide probe (5´ ggc ccg cgg cgc tcc aga gac gga tcc cct gct cag cag ggc cct gca) using an established protocol (De Vries et al., 1994; Wang et al., 1994). Afterwards, the air-dried slides were exposed to a BioMax MR film (Kodak Bio Max MR Film, Rochester, NY, USA) for 12 days. For analysis, the film

was digitised and slices containing comparable sections of pPVN were analysed using ImageJ 1.32j (National Institutes of Health, Bethesda, MD, USA). The gray density within each brain region was measured and background was subtracted to reveal differences in radioactivity binding. Measurements were made bilaterally over six sections per rat by an experienced experimenter blind to the treatments.

2.3.3 Implantation of icv guide cannula in lactating rats

On PD 18, a different set of females were deeply anaesthetised using the inhalation anaesthesia isoflurane (Baxter Germany GmbH, Unterschleißheim, Germany). Under semi-sterile conditions, a 21 G stainless steel guide cannula (length: 12 mm) was implanted stereotaxically targeting the right lateral ventricle (1.0 mm posterior to bregma, 1.6 mm lateral, 1.8 mm ventral (Paxinos and Watson, 1998)) and secured in place with dental cement to two stainless steel screws inserted into the skull (Neumann et al., 2000). The guide cannula was closed using a 25 G stainless steel stylet of the same length as the guide cannula. After surgery, all animals received a subcutaneous injection of antibiotic (120 µl, Baytril; Bayer, Leverkusen, Germany).

2.3.4 Acute icv infusion of CRF or D-Phe in lactating rats

For acute icv infusion, a 25 G stainless steel infusion cannula (length: 14 mm) was connected to a 10 µl microsyringe via a PE-50 tubing (50 cm) and lowered into the guide cannula, where it was kept in place by a piece of silicon tubing during slow substance infusion for approximately 30 s (Neumann et al., 2000). Lactating HAB, LAB, and NAB rats received an acute icv infusion of either vehicle (VEH; 5 µl sterile Ringer´s solution; pH adjusted to 7.4; Braun, Melsungen, Germany), synthetic human / rat CRF (1 µg / 5 µl; Tocris Bioscience, Ellisville, Missouri, USA), which binds to both receptors CRF-R1 and CRF-R2, or the non-specific CRF-R1/2 antagonist D-Phe

((D-Phe^{12}, Nle^{21,38}, α-Me-Leu^{37})-CRF (12-41); human / rat; 10 µg / 5 µl; Bachem, Bubendorf, Switzerland). Doses were based on previous studies (Pedersen et al., 1991; Gammie et al., 2004).

2.3.5 Experimental design

On LD 1, maternal care was observed for 60 min under basal conditions in the home cage starting at 8 a.m. The dams were icv infused with VEH, CRF, or D-Phe at 9 a.m. and immediately observed after injection for 120 min and at 2 p.m. for another 60 min. The same treatment was injected on the subsequent days at 10 a.m. Ten min later the PRT (LD 2), the test for anxiety-related behavior on the EPM (LD 3) or the maternal defense test against a virgin intruder (LD 4) were performed. In addition, on LD 4, maternal care was observed for 60 min before and after the maternal defense test.

2.3.6 Behavioral tests

Maternal care. Maternal care was monitored in the home cage according to an established protocol (Bosch and Neumann, 2008). In more detail, the behavior of the mother was scored for 10 s every 2nd min in 30-min blocks resulting in a maximum score of 15 counts per block. The behaviors of the lactating dams were monitored on LD 1 (under basal conditions) and LD 4 (before and after psychosocial stress) according to the experimental design. The main parameter for the quality of maternal care was the occurrence of ABN (Bosch, 2011), the only active nursing posture, where the mother is engaged in a quiescent kyphosis (Stern and Johnson, 1990). Other behavioral parameters scored were hovering over the pups and blanket nursing posture, which together with ABN were counted as the sum of nursing. Pup retrieval/mouthing and licking/grooming were assessed as ‘other maternal behaviors’.

Additionally, non-maternal behaviors were scored, i.e. locomotion (including digging/burrowing and cage exploration), self-grooming, and sleeping/resting, which were summed up and are presented as off-nest behavior.

Maternal motivation. In order to test the dams' maternal motivation, the PRT was performed on LD 2 (van Leengoed et al., 1987; Neumann et al., 2005a). Here, 60 min prior to the test, the dams were separated from their litter and moved to a separate room. Ten min prior to the PRT, the dams received their respective icv treatment. All pups of the litter were then distributed in a plastic box (54 x 34 x 31 cm) covered with bedding from their home cage, the mother was placed in the box, and the time of collection of pups was measured for a maximum of 900 s.

Maternal aggression. To assess maternal aggression, the maternal defense test was performed on LD 4 in a separate room, to which the animals were transported 60 min prior to the test. Ten min after treatment infusion, the lactating residents were confronted with an unknown virgin female intruder in their home cage in the presence of the litter for 10 min as described previously (Neumann et al., 2001; Bosch et al., 2005). The dams' behavior was videotaped for later analysis by an experienced observer blind to the treatment. The following behavioral parameters were scored: total number of attacks, latency to first attack, keep down, lateral threat, and offensive upright as well as non-aggressive behaviors. After termination of the maternal defense test, the intruder was removed, the cage was transported back to the observation room, and maternal care of the lactating mother was monitored for 60 min as described above.

Anxiety-related behavior. Anxiety-related behavior was tested on the EPM on LD 3 as described earlier (Pellow et al., 1985; Neumann et al., 2000; Bosch and Neumann, 2008). Briefly, the plus-shaped maze consists of two open (50 x 10 cm, 80 lux) and two closed arms (50 x 10 x 30 cm, 10 lux) surrounding a neutral square-shaped central zone (10 x 10 cm, 65 lux) and is elevated 80 cm over the floor. During the 5-min testing period, the percentage of time spent on the open versus all arms was indicative of anxiety-related behavior and the number of closed arm entries was used to assess locomotion (Neumann et al., 2000).

2.3.7 Histology

At the end of the behavioral experiments, animals were decapitated and blue ink was infused via an infusion system through the icv cannula to verify the correct infusion site indicated by the presence of ink in the ventricles. No subjects of the HAB and LAB groups, but three NAB dams, had to be excluded.

2.3.8 Statistical analysis

In situ hybridisation was analysed using two-way analysis of variance (ANOVA; factors: breeding line x reproductive status) and Fisher's LSD test has been applied as *post hoc* test. Behavioral data were analysed using independent t-test, one-way ANOVA or ANOVA for repeated measures (factors: time x treatment). One-way ANOVA was followed by Tukey-Kramer HSD *post hoc* test, and ANOVA for repeated measures was corrected with Fisher's LSD *post hoc* test. For all tests, the software package IBM SPSS (Version 19.0) was used. Data are presented as means + S.E.M. and significance was accepted at $p \leq 0.05$.

2.4 Results

2.4.1 Experiment 1: CRF mRNA expression in the pPVN of virgin versus lactating rats

CRF mRNA expression within the pPVN differed between HAB, LAB, and NAB rats depending on the reproductive status (two-way ANOVA; $F_{2,28} = 3.57$, $p = 0.04$; Figure 9). In virgin rats, CRF mRNA expression in HABs was significantly higher and tended to be higher compared with LAB ($p = 0.02$) and NAB rats ($p = 0.08$), respectively. Similarly, in lactating rats, CRF mRNA expression in HABs was higher compared to LAB and NAB dams ($p < 0.01$, in each case). Within group comparison revealed reduced CRF mRNA expression in lactating versus virgin LAB ($p = 0.02$) and NAB rats ($p = 0.01$), while both breeding lines did not differ from each other. In contrast, in lactating HAB dams, CRF mRNA expression was not reduced compared to the virgins.

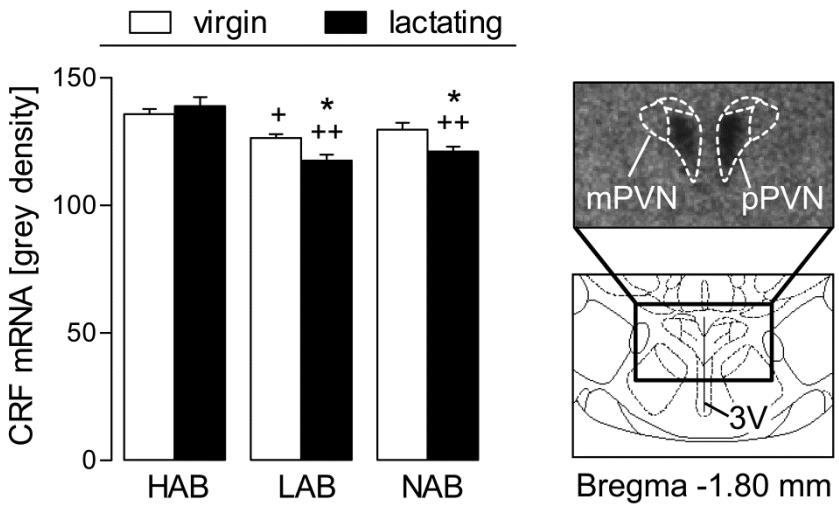

Figure 9. Expression of CRF mRNA within the parvocellular part of the paraventricular nucleus of virgin and lactating (LD 4) HAB, LAB, and NAB rats under basal, non-stress conditions (left). A representative photomicrograph is shown on the right side (3V: third ventricle; mPVN: magnocellular paraventricular nucleus; pPVN: parvocellular paraventricular nucleus). Hybridization is evident as gray density. Data is presented as mean + SEM. * $p \leq 0.05$ versus corresponding virgins; ++ $p \leq 0.01$, + $p \leq 0.05$ versus corresponding HAB.

2.4.2 Experiment 2: Behavioral effects of non-specific icv CRF-R1/2 manipulation on maternal behavior in lactating rats

Effects of CRF-R manipulation on maternal care in the home cage

ABN: On LD 1, ABN was observed before and after treatment infusion. ANOVA for repeated measures revealed a main effect in HAB (factor: time x treatment: $F_{10,20}$ = 1.95, p = 0.04; Figure 10) and LAB (factor: treatment: $F_{2,23}$ = 10.34, p < 0.01), but not in NAB, dams.

In detail, before icv infusion the HAB / LAB specific difference in ABN (Bosch and Neumann, 2008) was confirmed in the prospective VEH groups with HABs showing more ABN than LABs (independent t-test; t -60 min: t_{12} = -2.38, p = 0.03; t -30 min: t_{12} = -3.86, p = 0.002). Comparing the groups within each line, no differences were seen in ABN within any breeding line. The infusion procedure *per se* resulted in a drop in ABN in all treatment groups of the HAB line (p < 0.01, in each case), only. After treatment infusion, HAB dams treated with CRF showed reduced ABN at t +30 min (p = 0.03), t +60 min (p = 0.05), and t +90 min (p < 0.01) compared to VEH-treated mothers. Similarly, in LAB dams, CRF reduced ABN at t +30 min by trend (p = 0.06) and t +90 min (p = 0.01). Icv D-Phe did not change ABN in any breeding line. The display of maternal care in the afternoon did not differ in any breeding line (data not shown).

Nursing: With respect to nursing, which comprises all nursing positions, differences were found in all breeding lines depending on time (ANOVA for repeated measures; factor: time; HAB: $F_{5,21}$ = 7.92, p < 0.01; LAB: $F_{5,23}$ = 2.22, p = 0.05; NAB: $F_{5,22}$ = 4.23, p < 0.01; Figure 10) and on treatment (factor: treatment; HAB: $F_{2,21}$ = 5.24, p = 0.01; LAB: $F_{2,23}$ = 10.40, p < 0.01; NAB: $F_{2,22}$ = 3.56, p = 0.04).

In detail, before treatment infusion no differences were seen in nursing within any breeding line. The infusion procedure *per se* led to a drop in nursing in all treatment

groups of HAB dams (VEH: p = 0.04; CRF: p < 0.01; D-Phe: p = 0.01) and in CRF-treated LAB (p = 0.01) and NAB dams (p < 0.01). After treatment infusion, CRF-treated HAB dams showed less nursing at t 0 min (p = 0.01), t +30 min by trend (p = 0.08), and t +90 min (p = 0.05) compared to VEH-treated mothers. In LAB dams, CRF treatment reduced nursing at t +30 min (p < 0.01), t +60 min (p = 0.01), and t +90 min by trend (p = 0.08). In NAB dams, CRF treatment reduced nursing at t 0 min (p = 0.02) and t +30 min (p < 0.01) compared to VEH. Icv D-Phe did not change nursing in any breeding line. Observation of maternal care in the afternoon showed no differences in nursing in any breeding line (data not shown).

In summary, CRF treatment reduced whereas D-Phe did not alter maternal care in all breeding lines under basal conditions.

Other maternal behaviors: No significant differences or interactions depending on time and/or treatment were found in licking/grooming and pup retrieval/mouthing (data not shown). We did not observe any pup-killing following any of the treatments.

Non-maternal behaviors: Significant differences in off-nest behavior were found in HAB and LAB dams depending on time (ANOVA for repeated measures; factor: time; HAB: $F_{5,105} = 10.17$, $p < 0.001$; LAB: $F_{5,115} = 2.48$, $p = 0.03$; Table 1) and treatment (factor: treatment; HAB: $F_{2,21} = 4.04$, $p = 0.03$; LAB: $F_{2,23} = 6.78$, $p = 0.005$). No significant differences were detected in NAB dams. In detail, HAB dams showed off-nest behavior more frequently at t 0 min compared to the previous time-point independent of treatment (VEH: p = 0.03, CRF: p < 0.001, D-Phe: p 0.002). Furthermore, CRF-treated HAB dams showed more off-nest behavior at t 0 min (p = 0.009) and at t +30 min by trend (p = 0.06) compared to VEH. LAB dams treated with CRF showed off-nest behavior more frequently at t 0 min compared to the previous time-point (p = 0.005) as well as at t +30 min (p = 0.004) and at t +60 min by trend (p = 0.06) compared to VEH.

Locomotion was significantly altered in HAB and LAB dams depending on time (factor: time; HAB: $F_{5,105} = 19.89$, $p < 0.001$; LAB: $F_{5,10} = 4.03$, $p = 0.002$; Table 1) and treatment (factor: treatment; HAB: $F_{2,21} = 6.49$, $p = 0.006$; LAB: $F_{2,23} = 8.20$, $p = 0.002$). No significant differences were found in NAB dams. In detail, CRF- ($p < 0.001$) and D-Phe-treated HAB dams ($p = 0.02$) showed more locomotion at t 0 min compared to the previous time-point. Furthermore, CRF-treated HAB dams showed more locomotion at t 0 min ($p = 0.004$) and t +90 min ($p = 0.04$) as well as at t +30 min and t +60 min by trend ($p = 0.06$, in each case). LAB dams treated with VEH ($p = 0.05$) or CRF ($p = 0.004$) showed more locomotion at t 0 min compared to the previous time-point. Moreover, CRF-treated LAB dams displayed more locomotion at t +30 min ($p = 0.007$) and t +60 min ($p = 0.009$).

Significant interactions were detected in the occurrence of self-grooming in HAB (factor: time x treatment; $F_{10,105} = 2.52$, $p = 0.009$; Table 1) and LAB dams ($F_{10,115} = 3.46$, $p = 0.001$) while no differences were found in NAB dams. In detail, CRF-treated HAB dams showed more self-grooming at t 0 min compared to the previous time-point ($p = 0.01$) and at t +30 min compared to VEH ($p = 0.03$). CRF-treated LAB dams displayed more self-grooming at t 0 min compared to the previous time-point ($p < 0.001$) as well as at t 0 min ($p = 0.001$), t +30 min ($p = 0.004$) and t +60 min by trend ($p = 0.06$) compared to VEH.

No significant differences were found in the occurrence of sleeping in either breeding line.

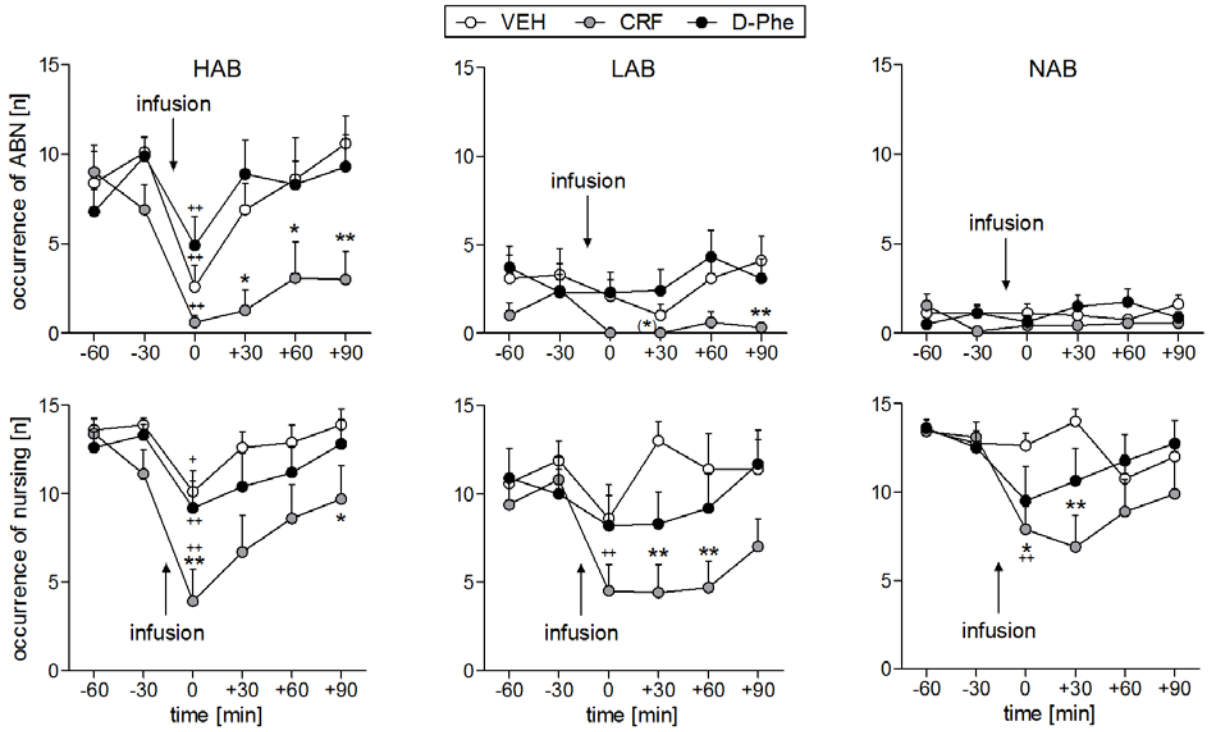

Figure 10. Effect of CRF-R manipulation on maternal care of lactating HAB, LAB, and NAB dams in the home cage. Dams received an acute icv infusion of VEH (5 µl sterile Ringer's solution; pH 7.4), CRF (1 µg / 5 µl), or D-Phe (10 µg / 5 µl). Arched back nursing (ABN, top) and sum of nursing (bottom) were scored for 60 min before and for 120 min after infusion. Data is presented as mean + SEM. n = 7 - 10 per group. * $p \leq 0.05$, (*) $p = 0.06$ versus corresponding VEH; + $p \leq 0.05$ versus t -30 min, ++ $p \leq 0.01$ versus t -30 min.

Effects of CRF-R manipulation on stress-induced reduction of maternal care

ABN: On LD 4, maternal care was observed before and after the maternal defense test. Differences in ABN depending on time were detected in lactating HAB (ANOVA for repeated measures; $F_{3,16} = 20.20$, $p < 0.01$; Figure 11) and LAB dams ($F_{3,22} = 21.08$, $p < 0.01$). A treatment effect on ABN was found in HAB dams ($F_{2,16} = 5.16$, $p = 0.01$), only.

In detail, before treatment infusion and maternal defense, no alterations in ABN were detected within any breeding line. The infusion procedure combined with the following maternal defense test led to a drop in ABN in all treatment groups of HAB

(VEH: $p < 0.01$; CRF, D-Phe: $p = 0.01$, in each case) and LAB dams (VEH by trend: $p = 0.06$; CRF, D-Phe: $p < 0.01$, in each case).

After icv treatment and maternal defense, only D-Phe-treated HAB dams showed more ABN at t 0 min compared to VEH-treated dams ($p = 0.01$). Icv CRF did not change ABN in any breeding line. No effect of time or treatment was found in NAB dams, which might be attributed to a floor effect.

Nursing: With respect to nursing, differences were found in HAB and LAB dams depending on time (ANOVA for repeated measures; factor: time; HAB: $F_{3,17} = 6.71$, $p < 0.01$; LAB: $F_{3,22} = 2.85$, $p = 0.04$; Figure 11). Additionally, a treatment effect was detected in HAB dams (factor: treatment; $F_{2,17} = 3.95$, $p = 0.03$) and a time x treatment interaction was found in NAB dams ($F_{6,20} = 2.21$, $p = 0.05$).

In detail, no differences were observed in any breeding line before treatment and maternal defense. The infusion procedure and the following maternal defense test led to a drop in nursing behavior in VEH-treated HAB dams ($p = 0.01$), in CRF-treated LAB dams ($p = 0.04$), and in CRF- ($p < 0.01$) and D-Phe-treated NAB dams ($p = 0.03$).

After treatment infusion and consecutive exposure to the maternal defense test, D-Phe treatment increased nursing at t 0 min in HAB dams ($p = 0.03$) and at t +30 min in LAB dams ($p = 0.05$) compared to VEH treatment. In NAB dams, icv D-Phe showed no effect whereas CRF decreased nursing at t +30 min ($p = 0.02$).

In summary, D-Phe restored maternal care back to pre-stress levels in HAB and LAB dams while CRF even prolonged the impairing effect of maternal defense exposure in lactating NAB dams.

Other maternal behaviors: No significant differences or interactions depending on time and/or treatment were found in licking/grooming and pup retrieval/mouthing (data not shown). We did not observe any pup-killing following any of the treatments.

Non-maternal behaviors: A significant interaction of time and treatment was detected in the occurrence of locomotion in NAB (Table 2; ANOVA for repeated measures: factor: time x treatment; $F_{6,66} = 3.68$, $p = 0.003$) but not in HAB or LAB dams. In detail, CRF- ($p < 0.001$) and D-Phe-treated NAB dams ($p = 0.003$) showed more locomotion at t 0 min compared to the previous time-point. Furthermore, CRF-treated NAB dams displayed more locomotion at t 0 min ($p = 0.008$) and t +30 min ($p = 0.01$) compared to VEH. A significant interaction of time and treatment was detected in the occurrence of self-grooming in LAB dams (factor: time x treatment; $F_{6,66} = 2.20$, $p = 0.05$) but not in HAB or NAB dams. In detail, CRF-treated LAB dams showed more self-grooming at t 0 min compared to the previous time-point ($p = 0.02$). No significant differences or interactions depending on time and/or treatment were found in the occurrence of off-nest behavior and sleeping / resting in all breeding lines.

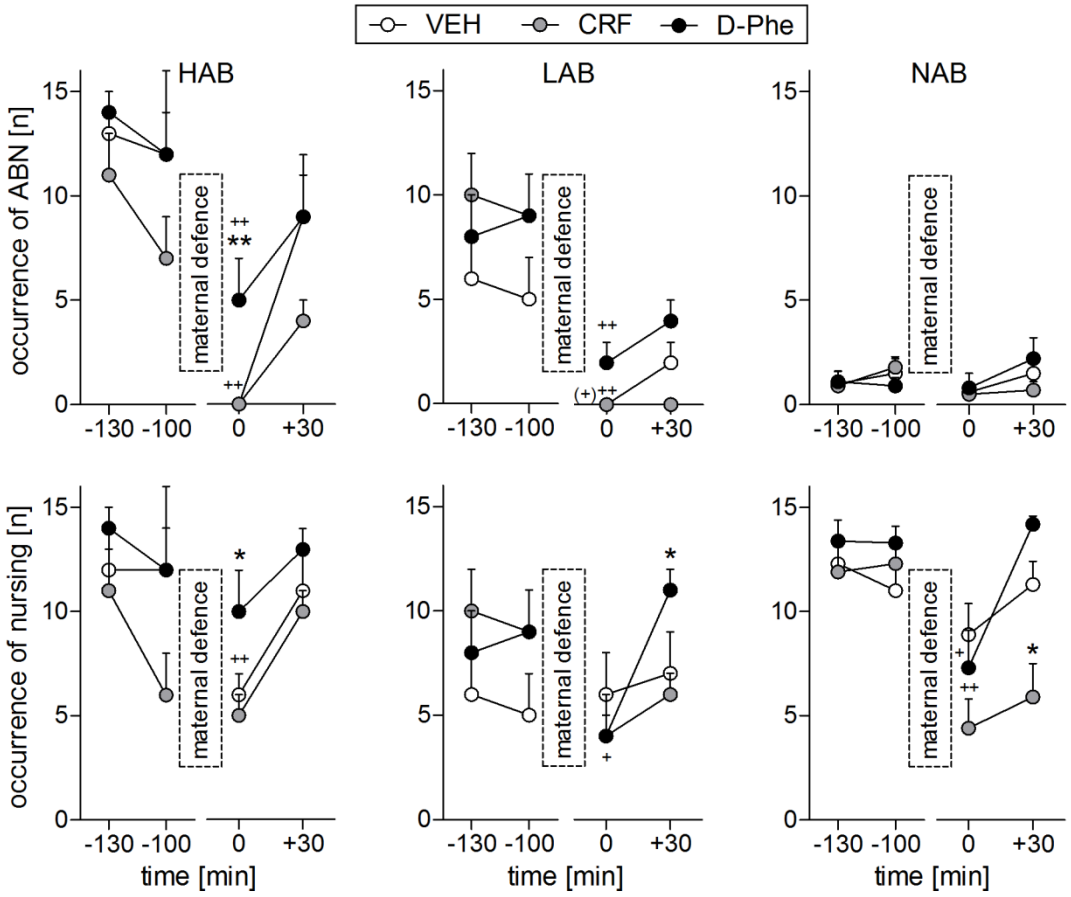

Figure 11. Effect of CRF-R manipulation followed by the maternal defense test on maternal care of lactating HAB, LAB, and NAB dams. Dams received an icv infusion of VEH (5 µl sterile Ringer's solution; pH 7.4), CRF (1 µg / 5 µl), or D-Phe (10 µg / 5 µl) 10 min prior to the maternal defense test. Arched back nursing (ABN, top) and sum of nursing (bottom) were scored for 60 min before and 60 min immediately after the maternal defense test. Data is presented as mean + SEM. n = 4 - 9 per group. * $p \leq 0.05$ versus corresponding VEH; + $p \leq 0.05$, ++ $p \leq 0.01$, (+) $p = 0.06$ versus t -100 min.

Effects of CRF-R manipulation on maternal motivation

In the PRT on LD 2, an interaction effect of number of retrieved pups over the 15-min trial comparing VEH groups of all breeding lines was found (two-way ANOVA for repeated measures; factor: time x treatment: $F_{90,19} = 1.54$, $p < 0.01$; data not shown) but *post hoc* correction revealed no significant effect. Moreover, neither treatment affected pup retrieval within any breeding line.

Effects of CRF-R manipulation on maternal aggression

On LD 4, maternal aggression as reflected by the number of attacks against an intruder displayed during the maternal defense test depended on the treatment in HAB (one-way ANOVA; $F_{2,18} = 3.59$, $p = 0.04$; Figure 12) and LAB ($F_{2,23} = 4.45$, $p = 0.02$), but not in NAB, dams with CRF decreasing this behavior (HAB: $p = 0.04$; LAB: $p = 0.03$). Other differences in aggressive or non-aggressive behaviors between treatment groups were not observed (data not shown). Icv D-Phe had no effect in any breeding line.

The line-specific differences in maternal aggression (Bosch et al., 2005) were not present in the VEH-treated HAB and LAB dams, likely due to a high behavioral variation in the HAB residents.

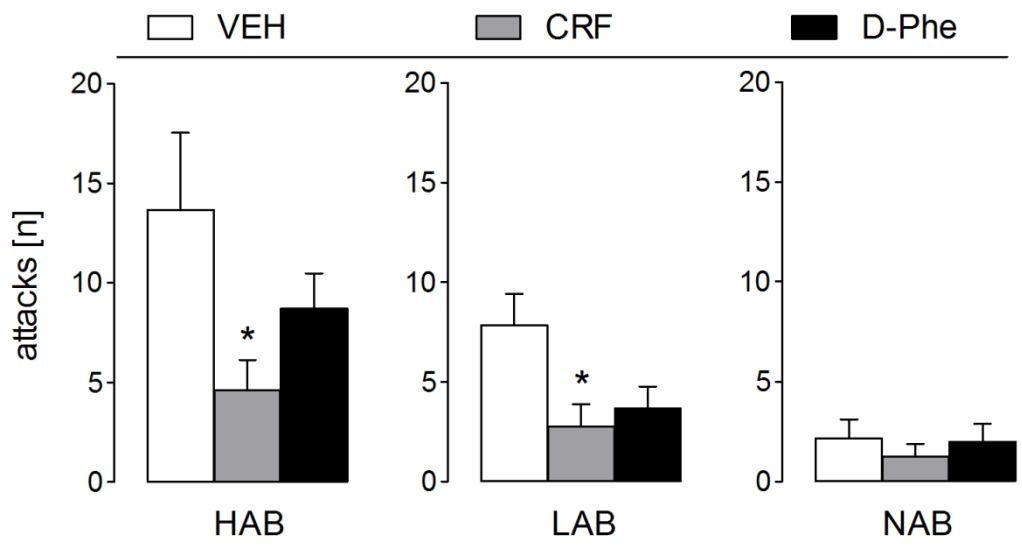

Figure 12. Effect of CRF-R manipulation on maternal aggression of lactating HAB, LAB, and NAB dams measured in the maternal defense test. Dams received an acute icv infusion of VEH (5 µl sterile Ringer's solution; pH 7.4), CRF (1 µg / 5 µl), or D-Phe (10 µg / 5 µl) 10 min prior to the test. Maternal aggression against a virgin female intruder was scored during the 10-min trial. Number of attacks by the resident is shown. Data is presented as mean + SEM. n = 6 – 9 per group. * $p \leq 0.05$ versus corresponding VEH.

Effects of CRF-R manipulation on anxiety-related behavior

On LD 3, anxiety-related behavior was assessed on the EPM. VEH-treated HAB dams spent significantly less percentage of time on the open arms of the EPM than the respective LABs (independent t-test; t_{11} = -8.46, $p < 0.01$; Figure 13) thus confirming their high level of innate anxiety also in lactation (Neumann et al., 2005a). With respect to manipulations of the CRF system, a treatment effect was present in HAB (one-way ANOVA; $F_{2,18}$ = 12.48, $p < 0.01$) and NAB dams ($F_{2,18}$ = 11.83, $p < 0.01$), while a trend was found in LABs ($F_{2,23}$ = 3.04, $p = 0.06$). In detail, D-Phe increased the percentage of time spent on the open arms in HAB ($p < 0.01$) and NAB dams ($p = 0.01$) while CRF reduced it in LABs by trend ($p = 0.06$). With respect to locomotion, a treatment effect was found in the number of closed arm entries in HAB ($F_{2,18}$ = 3.88, $p = 0.04$) and NAB ($F_{2,18}$ = 4.32, $p = 0.03$), but not in LAB, dams. *Post hoc* corrections revealed no differences in HAB dams while it showed a decreased number of closed arm entries in CRF-treated NABs ($p = 0.03$).

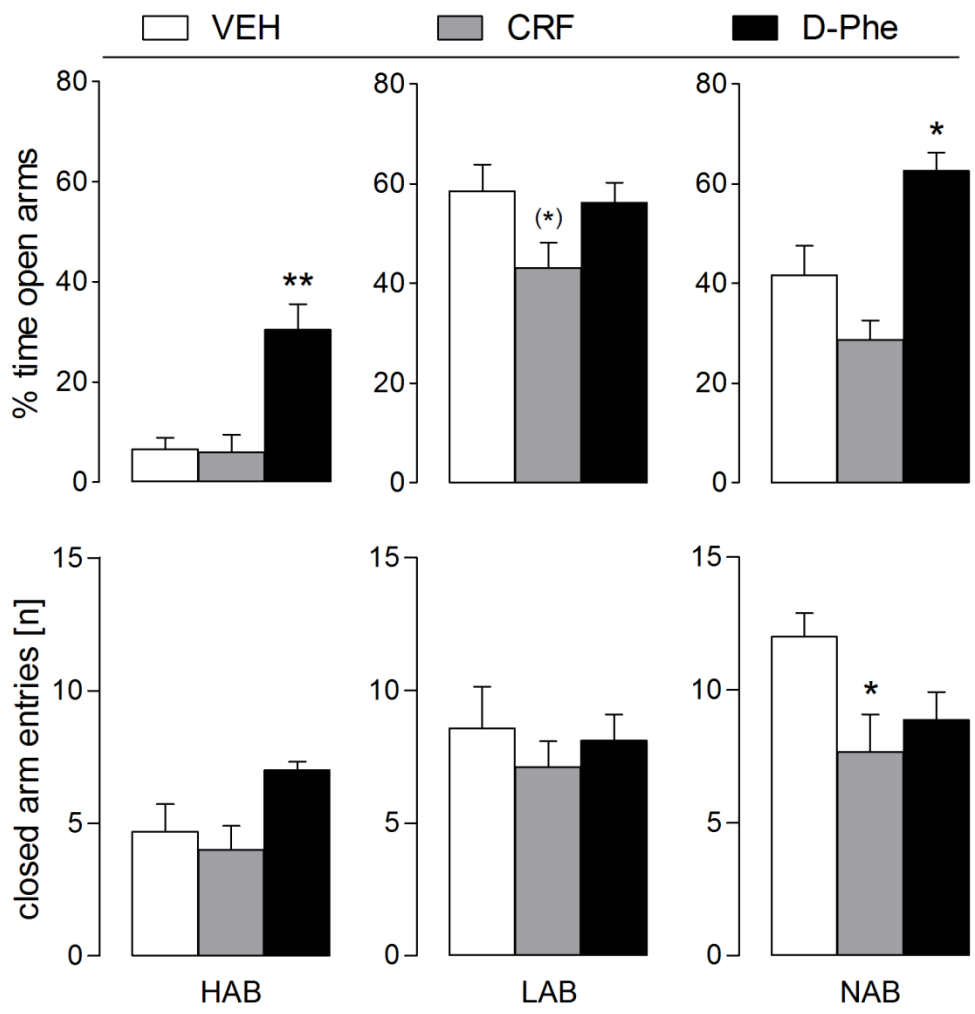

Figure 13. Effect of CRF-R manipulation on anxiety-related behavior of lactating HAB, LAB, and NAB dams measured on the elevated plus-maze. Dams received an acute icv infusion of VEH (5 µl sterile Ringer's solution; pH 7.4), CRF (1 µg / 5 µl), or D-Phe (10 µg / 5 µl) 10 min prior to the test. The percentage of time spent on the open arms (top) and the number of entries into the closed arms (bottom) during the 5-min test are shown. Data is presented as mean + SEM. n = 6 – 10 per group. * $p \leq 0.05$, ** $p < 0.01$, (*) $p = 0.06$ versus corresponding VEH.

2.5 Discussion

The brain neuropeptide CRF appears to be an important modulator of maternal care and maternal aggression as well as of emotionality in lactating rats. We could demonstrate a reduction in the expression of CRF mRNA within the pPVN of lactating LAB and NAB rats on LD 4 compared with virgin rats. However, this lactation-specific adaptation was absent in HAB dams, which generally showed the

highest hypothalamic CRF mRNA expression level independent of the reproductive status. Central activation of the CRF system via icv infusion of a CRF-R agonist, thus mimicking a central stress response, impaired maternal care under basal conditions in the home cage in HAB, LAB, and NAB dams. In contrast, after exposure to an acute stressor, i.e. the maternal defense test, and subsequent impairment of maternal care, blockade of central CRF-R restored this behavior in HAB and LAB dams, while activation of CRF-R resulted in prolonged decrease of maternal care in NABs. Furthermore, icv infusion of synthetic CRF reduced maternal aggression during the maternal defense test in HAB and LAB dams. These findings imply that the lactation-related reduction in central CRF expression is an essential prerequisite for the mother to show appropriate maternal behavior independent of her innate anxiety. Moreover, icv CRF increased anxiety-related behavior in LAB dams, whereas D-Phe decreased the level of anxiety in HAB and NAB dams demonstrating a role for CRF as an anxiogenic neuropeptide also in females, at least during lactation.

Across all mammalian species, successful rearing of the young requires the recruitment of a wide array of behavioral and physiological adaptations. Postpartum female rats are highly motivated to care for, nurse, and defend the pups. Both maternal care and maternal aggression are well known to be mediated by brain neuropeptides such as OXT and AVP, whose expression, release, and/or receptor binding are up-regulated in lactation (Bosch and Neumann, 2012). Initial studies on the role of CRF in maternal-like behavior in ovariectomized, primed virgin rats (Pedersen et al., 1991) and maternal aggression in lactating mice (Gammie et al., 2004; D'Anna and Gammie, 2009) point toward brain CRF as an additional neuropeptidergic candidate in the regulation of maternal behavior.

Indeed, we were able to confirm, at least in NAB and LAB rats, that CRF mRNA expression is reduced within the pPVN during lactation (Johnstone et al., 2000; Lightman et al., 2001; Walker et al., 2001) (but see da Costa et al., 2001; Deschamps et al., 2003). However, in HAB females, CRF mRNA expression was not lowered in lactation and they had generally the highest CRF mRNA expression compared to LAB and NAB rats independent of the reproductive status, thereby expanding our previous findings from males to females (Bosch et al., 2006). Importantly, the high CRF mRNA expression in the pPVN accounts for the described stress hyper-responsiveness of the HPA axis of HAB rats independent of sex or reproductive status (Landgraf et al., 1999; Douglas et al., 2007). In this context it is of interest to note that, despite their elevated level of CRF mRNA, lactating HAB dams still show the highest level of ABN and maternal aggression (Bosch and Neumann, 2010). While we cannot prove a causal relationship at this point, we assume that the higher activity of the brain AVP system in HAB dams contributes to the behavioral differences (Bosch and Neumann, 2008). Brain AVP is an important neuropeptide promoting maternal care and maternal aggression and, therefore, probably capable of compensating the negative effects of increased CRF activity on maternal behavior in HAB dams. However, future studies need to show whether changes on CRF mRNA levels also reflect altered levels of CRF protein. Moreover, the lactation-associated changes in CRF system activity might be region-dependent. For example, in the BNST (Walker et al., 2001), the medial and CeA, and the MPOA (da Costa et al., 2001) CRF mRNA expression was found to be elevated in lactating rats indicating region-dependent functions of the CRF system.

While HAB, LAB, and NAB rats differed in the CRF mRNA expression, the behavioral effects of central manipulations of the CRF-R1/2 were similar between the strains. Under basal conditions, icv CRF decreased maternal care in all breeding lines further

supporting the impairing effect of CRF on maternal care as described for ovariectomized, primed virgin rats (Pedersen et al., 1991). Interestingly, the same study describes increased pup-killing behavior after icv CRF infusion in these virgins, which we could not observe in our lactating rats. Here, blocking the CRF-R 1/2 with the unspecific antagonist D-Phe did not further increase maternal care under basal conditions in the home cage pointing toward an already attenuated activity of the endogenous brain CRF system in lactation. In contrast, stimulation of CRF-R with synthetic CRF mimicked the activation of the brain CRF system following stressor exposure, which in turn reduced maternal care (Vilela and Giusti-Paiva, 2011). In support, exposure to the maternal defense test and, consequently, an acute activation of the brain CRF system impaired maternal behavior in HAB, LAB, and NAB dams, which could be improved by D-Phe in HABs and LABs. This provides direct evidence for the involvement of the brain CRF system as inhibitor of maternal behavior.

Generally, we cannot exclude the possibility that CRF exerts this behavioral effect by acting on other neurotransmitter / neuromodulator systems, e.g. the brain OXT and AVP systems, which both promote social and specifically maternal behaviors (Bosch and Neumann, 2012; Neumann and Landgraf, 2012). Importantly, it is known that the release of OXT and AVP into the periphery is inhibited by icv CRF in male rats. Since both neuropeptides promote maternal behavior (Bosch and Neumann, 2012), inhibition of either of these neuropeptide systems within the brain is likely to cause the impairment of maternal behavior. However, CRF-induced alterations in central OXT/AVP release have not been demonstrated yet.

In order to investigate the role of the CRF system in maternal care after a natural psychosocial stressor, i.e. defending the offspring against a potential threat, maternal care was also observed immediately after the maternal defense test. The latter has

been validated as an acute psychosocial stressor for lactating dams indicated by elevated adrenocorticotropic hormone (ACTH) and CORT levels in NAB (Neumann et al., 2001) as well as in HAB and LAB dams (Douglas et al., 2007). In confirmation, exposure to a virgin intruder rat resulted in decreased ABN in HAB and LAB dams within 30 min after the intruder rat has been removed. Nursing was also decreased in VEH-treated HAB dams, in CRF-treated LABs, and in CRF- and D-Phe-treated NAB dams. Importantly, blocking CRF-R1/2 by D-Phe prior to the maternal defense test improved maternal care thereafter; specifically, it increased the occurrence of ABN (HAB) and nursing (HAB, LAB) compared to VEH-treated dams. Therefore, we conclude that exposure to this stressor elevates centrally released CRF in lactating rats (for further discussion see below) resulting in impaired maternal care, which can be rescued by blocking CRF-R.

Maternal motivation of lactating HAB, LAB, and NAB dams was assessed in the PRT (van Leengoed et al., 1987; Neumann et al., 2005a). Manipulation of the CRF system did not alter retrieval behavior, which is in line with studies revealing that mice deficient for one of the two CRF-R (Gammie et al., 2007a; D'Anna et al., 2008) or for the CRF-BP (Gammie et al., 2008) do not show differences in retrieval behavior compared to wildtype mice. Hence, maternal motivation seems to be unaffected by the CRF system.

We further studied the involvement of the central CRF system in maternal aggression in detail. While icv D-Phe had no effect on maternal aggression in any of the rat lines, CRF decreased the number of attacks in both HAB and LAB residents, thus confirming results described in lactating mice (Gammie et al., 2004). However, we could not reveal a comparable treatment effect in lactating NAB females, which might be due to a floor effect as NAB dams displayed only low levels of aggression throughout the different treatment groups. Our results demonstrate that - at least in

lactation - activating but not blocking CRF-R does affect maternal behavior during exposure to psychosocial stress. However, since D-Phe did not affect maternal aggression but was capable of improving maternal care after the maternal defense test, it is conceivable that the activation of the brain CRF system is not immediate. Indeed, an activation of the CRF system is required for an adequate behavioral response to stress (Hemley et al., 2007) but in lactating animals probably only to a certain extent possible to keep the dam alert. Beyond this level, further activation seems to be counterproductive since we found that CRF infusion impaired maternal aggression in lactating rats. Additionally, maternal care after maternal defense was altered in both directions; CRF prolonged the stress-induced decrease of maternal care whereas D-Phe improved it in NAB and HAB / LAB rats, respectively. This accounts for a counterbalanced activation of the CRF system upon stressor exposure during lactation depending on dam's stress reactivity.

In order to assess the involvement of CRF in anxiety-related behavior in lactating HAB, LAB, and NAB dams, they were tested on the EPM. In NAB and the more anxious HAB dams, D-Phe induced an anxiolytic effect likely by blocking CRF-R-mediated signal transmission and, thus, inhibition of the anxiogenic-like action of endogenous CRF. The latter might be released upon exposure to the EPM as a mild emotional stressor (Neumann et al., 1998b) and/or the separation from the pups being important for low anxiety levels during lactation (Neumann, 2003; Lonstein, 2005). The anxiogenic property of synthetic CRF (Reul and Holsboer, 2002b) was indicated by trend in LAB dams ($p = 0.06$), which did not reach statistical significance probably due to limited power in that group. On the other hand, the high innate level of anxiety of HABs could not be further enhanced by CRF. Since we did not find changes in emotionality after treatment with CRF in HAB dams and with D-Phe in LAB dams, we believe that this was due to a ceiling (HAB) or floor (LAB) effect of

their respective innate anxiety levels (Bosch, 2011). However, we could not find an effect of icv CRF on anxiety-related behavior in NAB dams either what might be a result of the unspecific, central activation of CRF-R. This is supported by the fact that CRF increased anxiety in LAB dams only by trend accounting for an insufficient evolvement of its anxiogenic property after icv infusion. In support, icv CRF is known to have an attenuated effect on activating central neural pathways during lactation (da Costa et al., 1997). Additionally, it is conceivable that the CRF-BP is involved in the regulation of anxiety during lactation. The CRF-BP is supposed to be important during lactation (Gammie et al., 2008) and might partly bind the infused CRF, thereby reducing CRF-R activation and the anxiogenic effect.

Besides the anxiogenic effect of CRF it is well known that locomotion is increased after central CRF-R manipulation (Eckart et al., 2002). However, in the present study we found that CRF decreases locomotion on the EPM when compared to the VEH-treated control groups, at least in NAB dams. We speculate that this might be due to the relatively high number of closed arm entries in the VEH group. But further investigation is needed to address this potential controversy.

In the present study, we could show an involvement of the CRF system in maternal behavior in lactating rats. However, further studies are required to assign the behavioral consequences to region-dependent CRF-R1 or CRF-R2 activation/inhibition. Candidate regions mediating these behavioral effects include the PVN, the BNST and the LS since they contain most members of the CRF family (Potter et al., 1992; Potter et al., 1994; Li et al., 2002) and are relevant for regulating maternal behavior (Numan and Insel, 2003; Bosch, 2011; Caughey et al., 2011). A single study points to the LS as important region mediating murine maternal aggression by CRF-R2 (D'Anna and Gammie, 2009). However, region- and receptor subtype-specific experiments in lactating rats are missing.

In conclusion, our finding of a lactation-specific reduction in hypothalamic CRF mRNA expression in LAB and NAB rats further supports the hypothesis of a stress hypo-responsiveness postpartum, which seems to be absent in HAB dams. Furthermore, pharmacological manipulation of brain CRF-R revealed that the brain CRF system (i) inhibits both maternal care and aggression, (ii) does not influence maternal motivation, (iii) mediates the acute stress-induced impairment of maternal care, and (iv) influences anxiety also in females independent of their innate anxiety. Therefore, a reduced activity of the brain CRF system is a necessary prerequisite for the expression of appropriate maternal care and maternal aggression as well as for the attenuation of anxiety-related behavior during lactation.

- Chapter 3 -

Hypo-activation of CRF receptors, predominantly type 2, in the medial-posterior BNST is vital for adequate maternal behavior in lactating rats

Authors‘ contribution:

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3.1 Abstract

Maternal behavior ensures the proper development of the offspring. In lactating mammals, maternal behavior is impaired by stress, the physiological consequence of central CRF-R activation. However, which CRF-R subtype in which specific brain area(s) mediates these effects is unknown.

Here, we confirmed that an icv injected non-selective CRF-R antagonist enhances, whereas an agonist impairs maternal care. The agonist also prolonged the stress-induced decrease in nursing and, furthermore, reduced maternal aggression and increased anxiety. Focusing on the BNST, CRF-R1 and -R2 mRNA expression did not differ in virgin versus lactating rats. However, CRF-R2 mRNA was more abundant in the posterior than the medial BNST. Pharmacological manipulations within the mpBNST showed that both CRF-R1 and -R2 agonists reduced ABN rapidly or delayed, respectively. Following stress, both antagonists prevented the stress-induced decrease in nursing while the CRF-R2 antagonist even increased ABN. During the maternal defense test, maternal aggression was abolished by the CRF-R2, but not the -R1, agonist. Anxiety-related behavior was increased by the CRF-R1 agonist and reduced by both antagonists. Both antagonists were also effective in virgin females but not in males, revealing a sexual dimorphism in the regulation of anxiety within the mpBNST.

In conclusion, the detrimental effects of increased CRF-R activation on maternal behavior are mediated via CRF-R2 and, to a lesser extent, via CRF-R1 in the mpBNST in lactating rats. Moreover, both CRF-R1 and -R2 regulate anxiety in females independent of their reproductive status.

3.2 Introduction

The maternal brain is a complex and perfectly organized system, which undergoes vital adaptations peripartum to ensure the onset and maintenance of maternal behavior (Bosch, 2011). Consequently, maladaptive alterations can cause severe problems like increased vulnerability to mood disorders affecting 20 - 30 % of mothers (Brummelte and Galea, 2010a). One factor that evidently contributes to such maladaptations is CRF (Magiakou et al., 1996; O'Keane et al., 2011).

CRF is a 41-amino acid neuropeptide binding to CRF-R1, and with 40-fold lower affinity to CRF-R2 (Hauger et al., 2003), which is primarily activated by Ucn 2 and 3 (Hsu and Hsueh, 2001; Lewis et al., 2001; Reyes et al., 2001). CRF is the primary initiator of the HPA axis (Vale et al., 1981). Furthermore, CRF exerts anxiogenic actions via CRF-R1 when centrally (Koob and Thatcher-Britton, 1985; Bruchas et al., 2009) or locally injected, e.g. into the BNST of male rats (Lee and Davis, 1997; Liang et al., 2001; Sahuque et al., 2006). The CRF system also modulates male social behaviors including aggression (Mele et al., 1987; Tazi et al., 1987) and social recognition (Heinrichs, 2003). In females, activated CRF-R impair maternal behavior as reported in a few studies in rodents (Pedersen et al., 1991; Gammie et al., 2004; D'Anna et al., 2005; D'Anna and Gammie, 2009; Klampfl et al., 2013) and primates (Saltzman et al., 2011). In lactating mice, icv administration of CRF or Ucn 3 decreases maternal aggression (Gammie et al., 2004; D'Anna et al., 2005), which has been linked to CRF-R2 in the LS (D'Anna and Gammie, 2009). In ovariectomized, steroid-primed virgin rats, icv CRF decreases maternal-like behavior and induces pup-killing (Pedersen et al., 1991), which is not observed in lactating rats (Klampfl et al., 2013). In the latter, icv CRF-R1/2 activation decreases maternal care and aggression and increases anxiety-related behavior while CRF-R1/2 inhibition restores maternal care after stress and is anxiolytic (Klampfl et al., 2013).

However, the potential brain sites of action and the specific role of the different CRF-R subtypes in maternal behavior and anxiety in lactating rats are not known.

Here, we first aimed to confirm our finding of impaired maternal behavior after central manipulation of CRF-R1/2 (Klampfl et al., 2013) using a different, more non-specific receptor agonist. Thereafter, we focused on the BNST, a key brain region for maternal behavior (Terkel et al., 1979; Numan et al., 1985) and anxiety (Lee and Davis, 1997), which expresses most members of the CRF family (Potter et al., 1992; Potter et al., 1994; Li et al., 2002). We assessed CRF-R1 and -R2 mRNA expression in the medial BNST (mBNST) and pBNST of virgin and lactating rats. Based on these results, we studied maternal care, motivation, aggression, and emotionality in lactating rats after local pharmacological manipulation with CRF-R1 and -R2 specific (ant-)agonists in the mpBNST. Additionally, we investigated a potential sexual dimorphism in the regulation of anxiety within the mpBNST in rats.

3.3 Materials & Methods

3.3.1 Animals

Virgin female or male Wistar rats (220 – 250 g; Charles River Laboratories, Sulzfeld, Germany) were kept under standard laboratory conditions (change of bedding once per week, 12 : 12 h light / dark cycle, lights on at 6 a.m., RT 22 ± 2 °C, 55 % relative humidity) with access to water and standard rat chow *ad libitum*. For experiments 1 - 3, females were mated and housed until delivery as described elsewhere (Klampfl et al., 2013). Litters were culled to eight pups of mixed sexes. For comparison of virgin females versus lactating rats in experiment 2, both groups were treated identically, i.e. virgins were single-housed 7 days prior to brain removal, consistent with the single-housing period of the lactating rats. For experiments 4 and 5, virgin female and male rats were kept in groups of 3 – 4 rats until surgery whereafter they were

single-housed as described earlier (Klampfl et al., 2013). During the single-housing period, all rats were handled twice a day to reduce non-specific stress responses during the experiments (Neumann et al., 1998b).

For the maternal defense test, naïve virgin female rats (200 – 220 g, Charles River Laboratories) were used as intruders at random stages of their estrous cycle. Intruder rats were kept in a separate room to avoid olfactory recognition.

The experiments were approven by the Committee on Animal Health and Care of the local government and conformed to international guidelines on the ethical use of animals. All efforts were made to minimize the number of rats used and their suffering.

3.3.2 Behavioral tests

Maternal care. Maternal care was monitored on LD 1 before and after substance infusion under 'non-stress conditions' as well as on LD 5 before and after substance infusion, which was combined with a psychosocial stressor (i.e. maternal defense test (Neumann et al., 2005a); 'stress conditions'). The authors acknowledge that there is a limited amount of stress associated with the infusion procedure, though the 'non-stress' term is used to distinguish between the observations made on LD 1 which did not involve the maternal defense test from those conducted on LD 5 which did. Observations were conducted for 10 s every 2nd min in 30 min blocks according to an established protocol (Bosch and Neumann, 2008). In detail, on LD 1, dams were observed from 8 – 9 a.m., infused at 9 a.m. and observation continued from 9.30 – 11 a.m. Additionally, dams were observed from 2 – 3 p.m. to assess potential long-lasting effects of drug treatment. On LD 5, dams were observed from 8 – 9 a.m., transported to another room, and infused at 10 a.m. Dams were tested 30 min after infusion in the maternal defense test, immediately afterwards transported back to the

observation room, and maternal care was observed for another 60 min in order to assess potential effects of the stressor on maternal care. The main parameter for the quality of maternal care was the occurrence of ABN (Bosch, 2011; Bosch and Neumann, 2012), the only active nursing posture where the dam is engaged in a quiescent kyphosis (Stern and Johnson, 1990). Other nursing parameters scored were hovering over the pups and blanket nursing posture, which together with ABN were counted as the sum of nursing indicating the quantity of maternal care as both active and passive nursing postures were included. Pup retrieval/mouthing and licking/grooming were assessed as 'other maternal behaviors'. Additionally, non-maternal behaviors were scored, i.e. locomotion (including digging/burrowing and cage exploration), self-grooming, and sleeping/resting, which were summed up and are presented as off-nest behavior. Data is shown in 30 min blocks before and after treatment infusion with a maximal count of 15 observations per block.

Maternal motivation. The dams' maternal motivation was tested in the PRT on LD 2 (van Leengoed et al., 1987; Neumann et al., 2005a). The dams were separated from their litter 60 min prior to the test and moved to a separate room. Thirty minutes prior to the test, dams received their respective treatment. All 8 pups of the litter were then distributed in a plastic box (54 x 34 x 31 cm) covered with bedding from their home cage, the mother was placed in the box, and the number of retrieved pups within the 15-min testing period was counted.

Maternal aggression. To assess maternal aggression, the maternal defense test was performed on LD 5 in a separate room, to which the dams were transported 60 min prior to the test (see above). Thirty minutes after treatment infusion, the lactating residents were confronted with an unknown virgin female intruder in their home cage

in the presence of the litter for 10 min as described previously (Neumann et al., 2001; Bosch et al., 2005). The dam's behavior was videotaped for subsequent analysis by an experienced observer blind to the treatment. The following behavioral parameters were scored: total number of attacks, latency to first attack, keep down, lateral threat, and offensive upright as well as non-aggressive behaviors (for detailed description see Bosch, 2013).

Anxiety-related behavior. Anxiety-related behavior was tested on the EPM on LD 3 in lactating rats and additionally in virgin female and male rats as described earlier (Pellow et al., 1985; Neumann et al., 2000). Male rats were also tested in the LDB (adapted from Waldherr and Neumann, 2007; Slattery and Neumann, 2010).

Elevated plus-maze: The plus shaped maze consists of two open arms (50 x 10 cm, 80 lux) and two closed arms (50 x 10 x 30 cm, 10 lux) connected by a square shaped neutral zone (10 x 10 cm, 65 lux) and is elevated 82 cm over the floor. The rats were placed in the neutral zone of the maze and were allowed to freely explore the maze for 5 min. The percentage of time spent on the open arms (ratio of time spent on open arms to total time spent on all arms) and the percentage of open arm entries (ratio of entries into open arms to total number of entries into all arms) were taken as indicator of anxiety-related behavior. An entry was recorded when both front legs and shoulders of the rat crossed into an arm or the neutral zone. Since the rat always had to cross the neutral zone, every open/closed arm entry was considered as a new entry. The number of closed arm entries was used to measure locomotion (Neumann et al., 2000).

Light-dark box: The LDB consists of a light (40 x 50 cm, 400 lux) and a dark compartment (40 x 30 cm, 50 lux). A small opening (7.5 x 7.5 cm) connecting both compartments enables transition between the light and dark box. The floor in each

compartment is divided into squares (10 x 10 cm) to assess locomotor activity via line-crosses. The rats were placed in the light box and time spent in each box, latency to enter the dark box as well as to re-enter the light box, line-crosses, and rearing were assessed during the 5-min test.

3.3.3 Experimental design

Experiment 1: Non-specific icv manipulation of CRF-R1/2 in lactating rats

On PD 18, females were implanted with a 21 G guide cannula targeting the right lateral ventricle (1.0 mm caudal, 1.6 mm lateral, 1.8 mm ventral to bregma (Paxinos and Watson, 1998)) under inhalation anesthesia (Isoflurane; Baxter Germany GmbH, Unterschleißheim, Germany) and semi-sterile conditions as described earlier (Bosch et al., 2010). On the experimental days, a 23 G infusion cannula, prepared as previously described (Neumann et al., 2000), was used to infuse either (i) VEH (5 µl sterile Ringer´s solution + 4 % DMSO; pH 7.4; Braun, Melsungen, Germany), (ii) the non-specific CRF-R1/2 agonist Ucn 1 (1 µg / 5 µl; Bachem, Bubendorf, Switzerland), or (iii) the non-specific CRF-R1/2 antagonist D-Phe (D-Phe^{12}, Nle^{21,38}, α-Me-Leu^{37})-CRF (12-41, human/rat; 10 µg / 5 µl; Bachem). Doses were chosen based on previous studies (Jones et al., 1998; Gammie et al., 2004).

On LD 1, 2, 3, and 5, lactating dams received a single acute icv infusion 30 min prior to the tests. Each animal received the same treatment on every testing day as assigned on the first testing day. In each case, dams were immediately returned to their home cage after infusion. Maternal care was observed under non-stress conditions (LD 1) and stress conditions (LD 5) in the home cage as described above. Additionally, maternal motivation (LD 2), anxiety-related behavior (LD 3), and maternal aggression (LD 5) were tested as described above. All tests were performed between 8 a.m. and 3 p.m. in the light phase of the cycle.

Experiment 2: Expression of CRF-R1 and -R2 mRNA within the BNST of virgin versus lactating rats

In order to compare mRNA expression of CRF-R1 and CRF-R2 between virgin and lactating rats, two separate groups of untreated rats were killed by conscious decapitation under basal conditions on LD 4 or equivalent in virgin rats. The brains were rapidly removed, flash frozen on dry ice and stored at -20 °C until subsequent processing by *in situ* hybridization as described below.

Experiment 3: Intra-mpBNST manipulation of CRF-R1 or -R2 in lactating rats

On PD 18, females were implanted bilaterally with 23 G guide cannula targeting the pBNST (0.7 mm caudal, 1.5 mm lateral, 4.5 mm ventral to bregma (Paxinos and Watson, 1998)) as described above. Substances were infused using a 27 G infusion cannula (Neumann et al., 2000). Lactating rats received either (i) VEH (0.5 µl sterile Ringer's solution + 4 % DMSO; pH 7.4; Braun), (ii) CRF-R1 agonist human/rat CRF (1 µg / 0.5 µl; Tocris Bioscience, Ellisville, Missouri, USA), (iii) CRF-R1 specific antagonist CP-154,526 (12 µg / 0.5 µl; Tocris Bioscience), (iv) CRF-R2 specific agonist hUcn 3 (Stresscopin; 3 µg / 0.5 µl; Phoenix Pharmaceuticals, Karlsruhe, Germany), or (v) CRF-R2 specific antagonist Astressin-2B (4 µg / 0.5 µl; Sigma-Aldrich, Steinheim, Germany). Doses were chosen based on previous studies (Gammie et al., 2004; D'Anna et al., 2005; Sahuque et al., 2006; D'Anna and Gammie, 2009). To assess how far the drug diffused from the injection site we infused ink (Pelikan Ink 4001, Hanover, Germany; diluted 1:20 in Ringer's solution) into the pBNST of three rats and collected the brains for histological analysis. Infusion of treatment and assessment of behaviors on LD 1, 3 and 5 were conducted as described above. Importantly, the repeated acute infusion of the CRF-R1 or -R2 (ant-)agonist separated by 48 h intervals and at the doses used here is not expected

to result in receptor (de)sensitization (personal communication with Prof. J. Radulovic, Northwestern University, USA; see also Spiess et al., 1998; Hauger et al., 2009). Moreover, *in vitro* studies have demonstrated rapid (within 1 - 2 hours) resensitization of the CRF-R following CRF-induced receptor internalization (Hauger et al., 2009). A different set of lactating dams was used for the PRT on LD 2. All tests were performed between 8 a.m. and 3 p.m.

Experiment 4: Intra-mpBNST manipulation of CRF-R1 or -R2 in virgin rats

Virgin rats underwent the same surgery as females in experiment 3 approximately two weeks after arrival. Starting three days after surgery vaginal smears were taken to assess estrous cycle stage. Females in metestrous were tested the following day, i.e. presumed to be diestrous, on the EPM between 8 a.m. and 12 p.m. The females were placed on the EPM 10 min after intra-mpBNST manipulation with VEH, CP-154,526, or Astressin-2B (for details see above) to assess whether the effects of CRF-R manipulation on anxiety in lactating rats are sex- or lactation-specific as anxiety-related behavior of male rats is seemingly modulated only by CRF-R1 manipulation (Sahuque et al., 2006). After the test, estrous cycle stage was verified via a vaginal smear and virgin rats not in diestrous were omitted from the data analysis.

Experiment 5: Intra-mpBNST manipulation of CRF-R1 or -R2 in male rats

Recently, the effects of CRF-R manipulation in the BNST on male anxiety were reported (Sahuque et al., 2006). As numerous subdivisions of the BNST were manipulated at the same time in that study, we focused exclusively on the mpBNST to clearly assess a potential sexual dimorphic effect of CRF in regulating anxiety in this subdivision of the BNST. Therefore, male rats were implanted bilaterally with

cannula targeting the pBNST approximately two weeks after arrival at our animal facility (for details see experiment 3). Six days after surgery, the males were administered VEH, CRF, or Stresscopin into the mpBNST (for details see experiment 3) and were placed 10 min (VEH, CRF) or 25 min (Stresscopin) after infusion on the EPM. Two days later, the males were placed in the LDB after infusion with the same treatments as assigned for the EPM. In a different set of animals, we tested whether the application of CRF-R antagonists *per se* has anxiolytic effects as shown for females (see experiment 3 and 4). Therefore, males were placed on the EPM 10 min after intra-mpBNST manipulation with VEH, CP-154,526, or Astressin-2B (for details see experiment 3) using the same doses as for virgin and lactating females.

3.3.4 Histology

At the end of the behavioral experiments, rats were decapitated. For icv cannula verification, brains were infused with blue ink, removed, and cut with a razor blade at the infusion site. Blue colored ventricles indicated correct placement of the icv cannula (experiment 1). To verify the correct placements of local cannula within the mpBNST brains were removed, flash frozen, cut in 40 µm coronal sections, slide mounted and stained via quick Nissl staining (experiments 3 - 5).

3.3.5 *In situ* hybridization for CRF-R mRNA expression

Brains were sectioned at 16 µm using a cryostat (Model CM3050S Leica Microsystems GmbH, Nussloch, Germany), slide mounted, and stored at -20°C until further processing.

CRF-R1/2 mRNA *in situ* hybridization was conducted following an established protocol and using previously described plasmids for CRF-R1 or -R2 (Brunton et al., 2009; Brunton et al., 2011). Some extra slides were also hybridized with ^{35}S-UTP-

labeled cRNA sense probes to serve as negative controls. Autoradiograms of the mBNST (bregma -0.2 mm – -0.4 mm) and the pBNST (bregma -0.4 mm – -0.9 mm) (Paxinos and Watson, 1998) were examined with Image J (V1.46, NIH image software) by an experienced observer blind to the groups as described recently (Brunton et al., 2011). Additionally, all pictures were converted to 8 bit and their contrast was enhanced to the same extent. Measurements were made bilaterally over six sections per rat. Brain sections hybridized with ^{35}S-UTP-labeled cRNA sense probes showed no signal above background.

3.3.6 Statistical analysis

In situ hybridization data was analyzed using a two-way ANOVA (factors: reproductive status x brain site). For the behavioral studies, only animals that had been fitted correctly with the local cannula were included in the analysis. Behavioral data was analyzed using either one-way ANOVA (factor: treatment) or ANOVA for repeated measures (factors: time x treatment). One-way ANOVA was followed by SIDAK and ANOVA for repeated measures by Fisher's LSD *post hoc* test. For all tests, the software package SPSS 19.0 (IBM) was used. Data are presented as means + S.E.M. and significance was accepted at $p \leq 0.05$.

3.4 Results

3.4.1 Experiment 1: Behavioral effects of non-specific icv manipulation of CRF-R1/2 in lactating rats

Maternal care under non-stress conditions on LD 1

ABN: Neither significant differences depending on time and/or treatment nor an interaction were revealed by two-way ANOVA for repeated measures (Figure 14A, top).

Nursing: Differences in nursing, which comprises all nursing positions, were found depending on treatment (two-way ANOVA for repeated measures; $F_{2,16} = 4.17$, $p = 0.03$) but not on time. However, there was a significant time x treatment interaction ($F_{8,64} = 2.20$, $p = 0.03$; Figure 14A, bottom). In detail, no group differences were detected before treatment infusion. The infusion procedure decreased the occurrence of nursing in VEH-treated dams significantly ($p = 0.01$) and there was a tendency for a reduction in the CRF-R1/2 agonist-treated dams ($p = 0.07$) at t +30 min. The CRF-R1/2 antagonist prevented this infusion-induced decrease at t +30 min ($p = 0.01$) while the agonist even prolonged the impairing effect on nursing at t +90 min ($p = 0.01$) compared to VEH. In the afternoon, the occurrence of nursing did not differ between the groups (data not shown).

Other maternal behaviors: No significant differences or interactions depending on time and/or treatment were found in licking/grooming and pup retrieval/mouthing (data not shown). We did not observe pup-killing following any of the treatments.

Non-maternal behaviors: A significant interaction was found in the occurrence of off-nest behavior (two-way ANOVA for repeated measures; $F_{8,68} = 2.20$, $p = 0.03$; Table 3). No differences were found depending on time or treatment. In detail, VEH-treated dams showed off-nest behavior more frequently at t +30 min compared to before infusion ($p = 0.01$) and to CRF-R1/2 antagonist-treated dams ($p = 0.04$). CRF-R1/2 agonist-treated dams showed differences in self-grooming depending on treatment ($F_{2,17} = 10.27$, $p < 0.01$) but not on time. No interaction was detected between the two factors. In detail, these dams showed significantly more self-grooming than VEH-treated dams ($p < 0.01$). No differences were detected for locomotion and sleeping/resting.

Maternal care under stress conditions on LD 5

ABN: Differences in ABN were found depending on time (ANOVA for repeated measures; $F_{3,48} = 6.32$, $p < 0.01$; Figure 14B, top), but not on treatment. No interaction was found between the two factors.

Nursing: Differences in nursing were found depending on time (two-way ANOVA for repeated measures; $F_{3,48} = 46.09$, $p < 0.01$; Figure 14B, bottom), but not on treatment. No interaction effect was found between the two factors. However, analysis of the within-subject contrasts revealed a significant linear interaction between the treatment groups at various time-points (two-way ANOVA for repeated measures, $F_{2,16} = 4.09$, $p = 0.03$). In all three groups, nursing was decreased at t 0 min compared to all other intervals (VEH: $p < 0.01$, in each case; CRF-R1/2 agonist: t -130 min / t -100 min: $p < 0.01$, t +30 min: $p = 0.04$; CRF-R1/2 antagonist: $p < 0.01$, in each case). Furthermore, CRF-R1/2 ago-treated dams showed less nursing at t +30 min compared to VEH-treated mothers ($p = 0.05$).

Other maternal behaviors: No significant differences or interactions depending on time and/or treatment were found in pup retrieval/mouthing and licking/grooming (data not shown). We did not observe pup-killing following any of the treatments.

Non-maternal behaviors: All dams showed differences in the occurrence of off-nest behavior (two-way ANOVA for repeated measures; $F_{3,51} = 30.92$, $p < 0.01$; Table 4), in locomotion ($F_{3,51} = 50.14$, $p < 0.01$), and in sleeping/resting ($F_{3,51} = 4.54$, $p < 0.01$) depending on time but not on treatment. No interactions between the two factors were detected. Significant interactions were found in self-grooming ($F_{6,51} = 4.48$, $p < 0.01$), which also differed depending on time ($F_{3,51} = 9.91$, $p < 0.01$) and treatment ($F_{2,17} = 3.64$, $p = 0.04$). In detail, CRF-R1/2 agonist-treated dams showed more self-grooming at t 0 min compared to the previous time-point and compared to VEH-treated dams ($p < 0.01$, in each case).

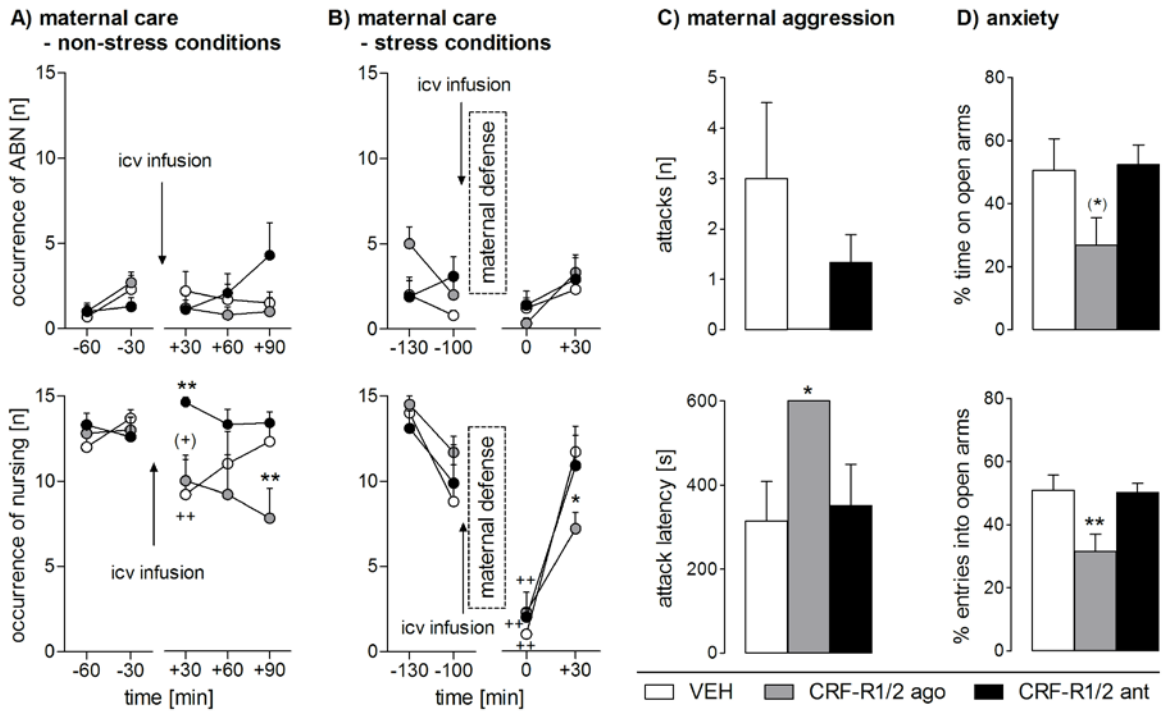

Figure 14. Effect of non-specific icv CRF-R1/2 manipulation on maternal care under (A) non-stress conditions on LD 1, and (B) stress conditions on LD 5, (C) on maternal aggression during the maternal defense test on LD 5, and (D) on anxiety-related behavior of lactating rats on LD 3. Arched back nursing (ABN; A, B top) and sum of nursing (A, B bottom) were scored for 60 min before and for 90 min after infusion (A) or 60 min after maternal defense (B). Maternal aggression was scored as number of attacks (C top) and attack latency (C bottom) by the resident. Anxiety-related behavior was measured as percentage of time spent on the open arms (D top) and the percentage of entries into the open arms (D bottom) on the EPM. Dams received an acute icv infusion of vehicle (VEH; 5 µl sterile Ringer's solution; pH 7.4), CRF-R1/2 agonist Ucn 1 (CRF-R1/2 ago; 1 µg / 5 µl), or CRF-R1/2 antagonist D-Phe (CRF-R1/2 ant; 10 µg / 5 µl). Data is presented as mean + SEM. n = 6 - 7 per group. * $p \leq 0.05$, ** $p \leq 0.01$ versus VEH; ++ $p \leq 0.01$, (+) $p = 0.07$ versus previous time-point (two-way ANOVA for repeated measures; factors: time x treatment).

Maternal motivation on LD 2

In the PRT, a strong trend toward reduced pup retrieval in CRF-R1/2 agonist-treated dams was observed (VEH: 3.3 ± 1.5; Ucn: 0.4 ± 0.3; D-Phe: 3.5 ± 0.8 number of pups collected in 15 min; one-way ANOVA; $F_{2,18} = 3.23$, $p = 0.06$). No differences were observed in CRF-R1/2 antagonist-treated dams.

Maternal aggression on LD 5

The number of attacks did not differ between the groups even though CRF-R1/2 agonist injection completely abolished maternal aggression (Figure 14C, top). However, the latency to the first attack was significantly affected by the treatment (one-way ANOVA; $F_{2,15} = 3.93$, $p = 0.04$; Figure 14C, bottom). The CRF-R1/2 agonist significantly increased the attack latency compared to VEH ($p = 0.05$). No other behavioral parameter (e.g. keep down, lateral threat, offensive upright) measured during maternal defense test differed between the groups.

Anxiety-related behavior on LD 3

The treatment tended to alter the percentage of time spent on the open arms of the EPM (one-way ANOVA; $F_{2,16} = 3.23$, $p = 0.06$; Figure 14D, top), while the percentage of open arm entries was significantly altered by the treatment ($F_{2,16} = 6.43$, $p < 0.01$; Figure 14D, bottom). CRF-R1/2 agonist-infused dams made significantly fewer entries into the open arms compared to VEH ($p = 0.02$). Importantly, entries into closed arms did not differ between the groups, indicating the icv infusion did not affect locomotor activity (data not shown).

3.4.2 Experiment 2: Expression of CRF-R1 and CRF-R2 mRNA in the BNST of virgin versus lactating rats

CRF-R1 mRNA expression did not differ between virgin and lactating rats in either the mBNST or the pBNST (Figure 15). CRF-R2 mRNA expression was higher in the pBNST compared to the mBNST (two-way ANOVA; factor: brain site; $F_{1,19} = 12.05$, $p < 0.01$; Figure 15) but was not altered by the reproductive status nor an interaction was found between the two factors. There was no significant difference in the ratio of CRF-R1:R2 mRNA expression in either the mBNST (virgin = 9.5 +/- 3.1; lactating =

8.9 +/- 1.5) or the pBNST (virgin = 2.7 +/- 0.5; lactating = 2.8 +/- 0.1) between virgin and lactating rats.

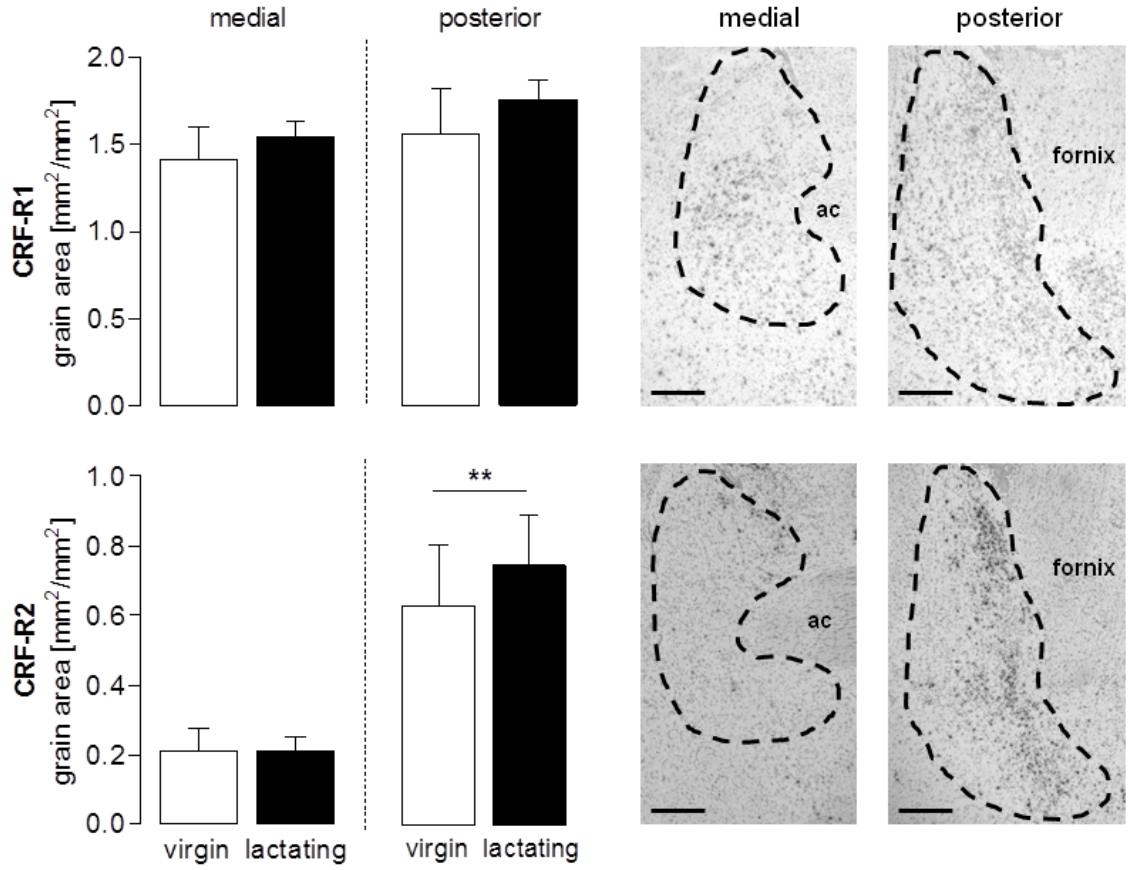

Figure 15. CRF-R1 (top) and CRF-R2 (bottom) mRNA expression in the medial and posterior part of the BNST comparing virgin and lactating rats. Data is presented as mean grain area + SEM. n = 4 – 7 per group. ** $p \leq 0.01$ versus medial part (two-way ANOVA; factors: reproductive status x brain site). Representative photomicrographs are shown on the right side (x 4 objective, scale bar = 500 µm). Hybridization is evident as localized clumps of silver grains. ac: anterior commissure.

3.4.3 Experiment 3: Behavioral effects of intra-mpBNST CRF-R1 or -R2 manipulation in lactating rats

The precise cannula placement sites within the mpBNST are illustrated in Figure 16A. The slow infusion of 0.5 µl spreads out over an area of 1 mm³ thus mainly affecting the pBNST but also the mpBNST (Figure 16B), consistent with previous findings (Engelmann et al., 1999).

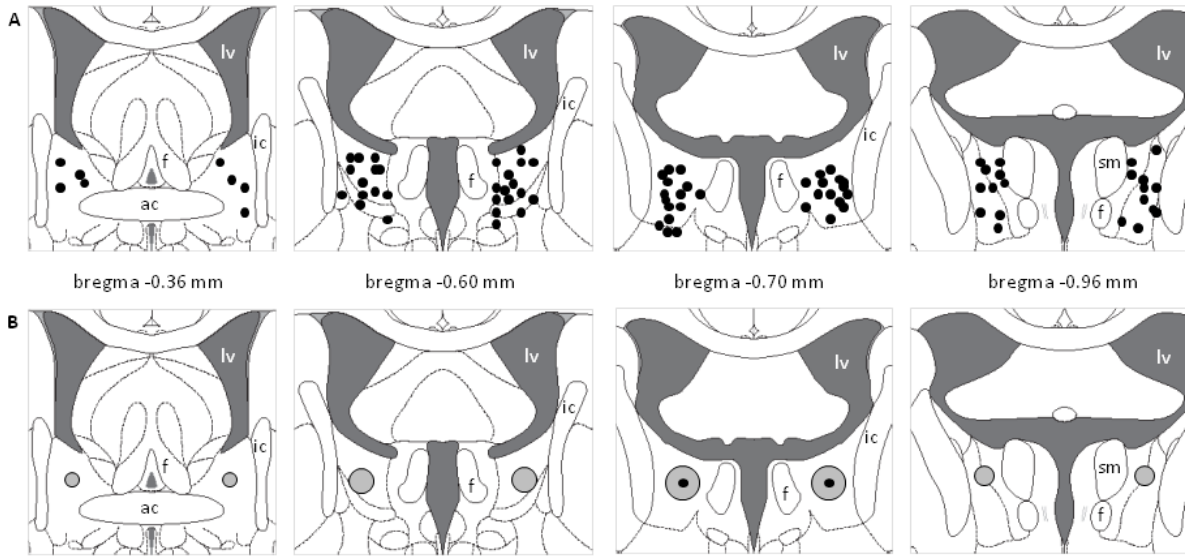

Figure 16. Histological localization of infusion cannula within the medial-posterior BNST (mpBNST). (A) The cannula placement sites for subsequent drug infusion are shown as black dots within the mpBNST on schematic plates from the Paxinos & Watson (1998) stereotaxic atlas. (B) The extent of substance spreading after ink infusion (black dot) in 3 rats is schematically demonstrated as gray circles within the mpBNST. ac: anterior commissure, f: fornix, ic: internal capsule, lv: lateral ventricle, sm: stria medullaris of the thalamus.

Maternal care under non-stress conditions on LD 1

ABN: Differences in ABN were found depending on treatment (two-way ANOVA for repeated measures; $F_{4,48} = 3.33$, $p = 0.01$) but not on time. However, there was a significant time x treatment interaction ($F_{24,288} = 1.57$, $p = 0.04$; Figure 17A, top). In detail, before the infusion, no differences were detected between the groups. Shortly afterwards, dams treated with the CRF-R1 agonist showed less ABN at t +30 min and t +60 min ($p = 0.03$, in each case) compared to VEH-treated dams. During the observation in the afternoon, less ABN was observed in CRF-R1 antagonist-treated dams at t +300 min and in CRF-R2 agonist-treated dams at t +300 min and t +330 min compared to VEH ($p < 0.01$, in each case).

Nursing: Differences in nursing were found depending on time (two-way ANOVA for repeated measures; $F_{6,24} = 2.85$, $p = 0.01$) and on treatment ($F_{4,48} = 4.24$, $p < 0.01$).

Moreover, a significant time x treatment interaction was revealed ($F_{24,288}$ = 1.69, p = 0.02; Figure 17A, bottom). In detail, before the infusion, no differences were found. Shortly afterwards, the CRF-R1 agonist-treated dams showed less nursing at t +30 min (p < 0.01) and t +60 min (p = 0.05) compared to VEH. During the observation in the afternoon, the CRF-R2 agonist resulted in significantly less nursing at t +300 min (p = 0.03) and t +330 min (p < 0.01) compared to VEH.

Other maternal behaviors: No significant differences or interactions depending on time and/or treatment were found in pup retrieval/mouthing and licking/grooming (data not shown). We did not observe any pup-killing following any of the treatments.

Non-maternal behaviors: Significant interactions were found in the occurrence of off-nest behavior (two-way ANOVA for repeated measures; $F_{24,288}$ = 1.62, p = 0.03; Table 5), which also differed depending on time ($F_{6,288}$ = 3.14, p < 0.01) and treatment ($F_{4,48}$ = 3.20, p = 0.02). In detail, CRF-R1 and CRF-R2 agonist-treated dams showed off-nest behavior more frequently compared to the respective previous time-point (p < 0.01, in each case) and compared to VEH at t +30 min (CRF-R1 agonist: p < 0.01, CRF-R2 agonist: p = 0.01). Additionally, CRF-R2 agonist-treated dams displayed more off-nest behaviors at t +330 min (p = 0.01). Differences in locomotion were found depending on time ($F_{6,288}$ = 8.05, p < 0.01) but not on treatment. However, a significant interaction was detected ($F_{24,288}$ = 2.78, p < 0.01). In detail, CRF-R1 agonist-treated dams showed more locomotion compared to the previous time-point and compared to VEH at t +30 min (p < 0.01, in each case). CRF-R2 agonist-treated dams showed more locomotion at t +330 min compared to VEH (p = 0.05). Differences in self-grooming were found depending on time ($F_{6,288}$ = 8.56, p < 0.01) but not on treatment. However, a significant interaction was detected ($F_{24,288}$ = 3.15, p < 0.01). In detail, CRF-R1 (p < 0.01) and CRF-R2 agonist-treated dams (p = 0.02) showed more self-grooming at t +30 min compared to the respective

previous time-point. Additionally, CRF-R1 agonist-treated dams showed more self-grooming at t +30 min ($p < 0.01$) as did CRF-R1 antagonist-treated dams at t +330 min compared to VEH. CRF-R2 antagonist-treated dams showed less self-grooming at t +90 min ($p = 0.05$). Differences in sleeping/resting were found depending on time ($F_{6,288} = 4.72$, $p < 0.01$) and on treatment ($F_{4,48} = 3.89$, $p < 0.01$). However, no significant interaction was detected. In detail, CRF-R2 agonist-treated dams showed more sleeping/resting at +30 min compared to the previous time-point ($p < 0.01$) and at t +300 min ($p = 0.01$) and t +330 min ($p < 0.01$) compared to VEH.

Maternal care under stressor conditions on LD 5

ABN: Differences in ABN depending on time (two-way ANOVA for repeated measures; factor: time; $F_{3,120} = 4.64$, $p < 0.01$; Figure 17B top) and treatment (factor: treatment; $F_{4,40} = 4.17$, $p < 0.01$) were detected; however, no interaction effect was found. While no differences in ABN were found before any manipulation the infusion paired with the maternal defense test led to a significant reduction in the occurrence of ABN in the VEH group (t -100 min versus t 0 min; $p = 0.01$). Only the CRF-R2 antagonist-treated dams showed more ABN at t +30 min ($p = 0.04$) compared to the VEH-treated dams.

Nursing: Differences in nursing were found depending on time (ANOVA for repeated measures; $F_{3,120} = 9.57$, $p < 0.01$; Figure 17B, bottom), but not on treatment nor was an interaction effect detected. However, single analysis of the time courses within the different treatment groups revealed main effects in VEH- (one-way ANOVA for repeated measures; $F_{3,33} = 4.29$, $p = 0.01$) and CRF-R1 ago-treated dams ($F_{3,21} = 3.6$, $p = 0.03$) as well as a trend in CRF-R2 ago-treated dams ($F_{3,18} = 2.53$, $p = 0.08$). The occurrence of nursing was decreased at t +30 min compared to pre-stress levels (VEH: t 0 min vs t -130 min: $p = 0.01$, t 0 min vs t -100 min: $p = 0.06$; CRF: t 0 min vs

t -130 min: p = 0.03, t 0 min vs t -100 min: p = 0.05). Treatment with either the CRF-R1 or -R2 antagonist prevented this stress-induced drop in nursing.

Other maternal behaviors: No significant differences or interactions depending on time and/or treatment were found in pup retrieval/mouthing and licking/grooming (data not shown). We did not observe any pup-killing following any of the treatments.

Non-maternal behaviors: Dams differed in the occurrence of off-nest behavior (two-way ANOVA for repeated measures; $F_{3,126} = 8.59$, $p < 0.01$; Table 6), self-grooming ($F_{3,126} = 7.33$, $p < 0.01$), and locomotion ($F_{3,126} = 16.52$, $p < 0.01$) depending on time. However, no treatment or interaction effects were observed. For locomotion, a strong trend for an interaction was detected ($F_{12,126} = 1.76$, $p = 0.06$). No differences were found in sleeping/resting.

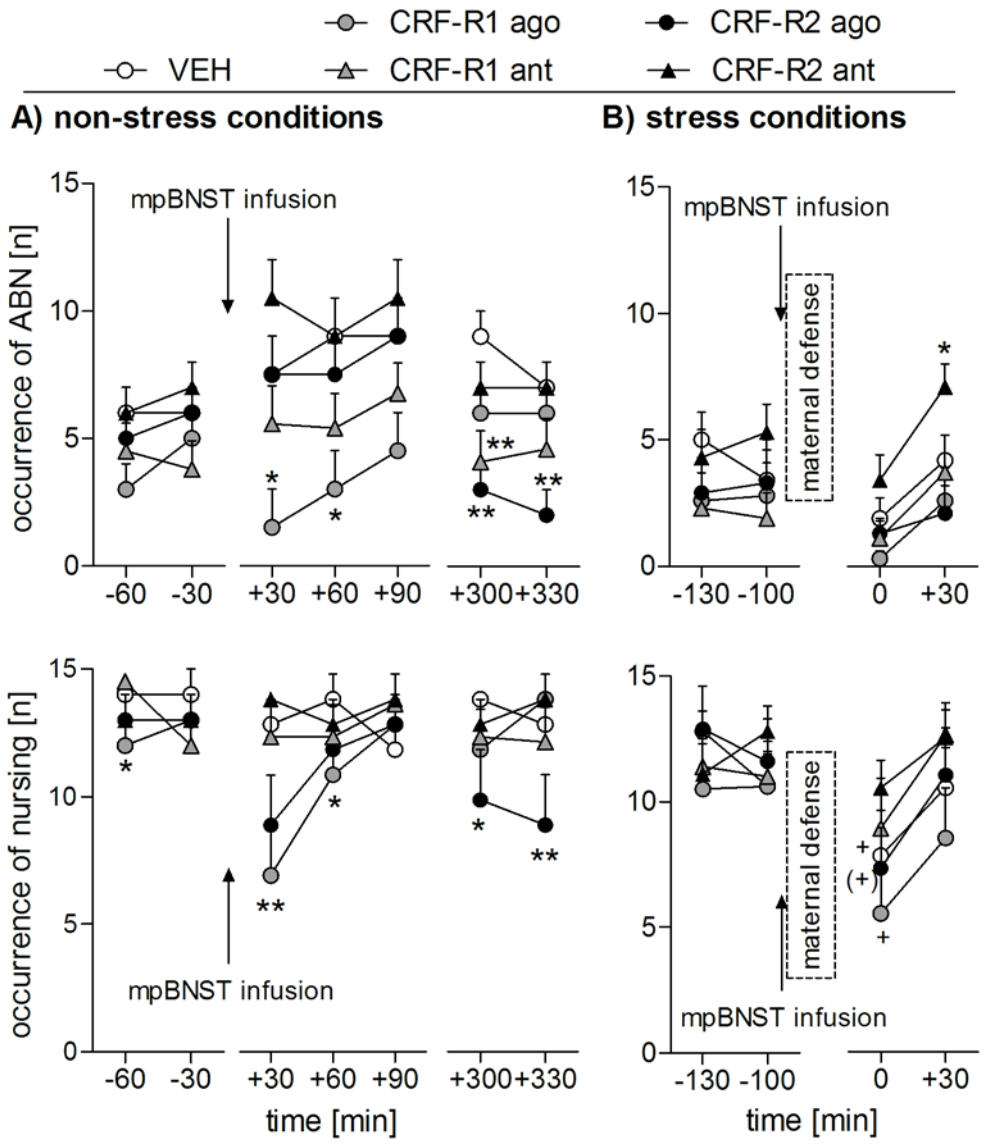

Figure 17. Effect of intra-mpBNST CRF-R1 or -R2 specific agonist (ago) or antagonist (ant) treatment on maternal care of lactating dams under (A) non-stress conditions on LD 1, and (B) stress conditions on LD 5. Arched back nursing (ABN, top) and sum of nursing (bottom) were scored for 60 min before and for 90 min after infusion (A) or 60 min after maternal defense (B). Under non-stress conditions, ABN and nursing were also observed for additional 60 min in the afternoon (A). Dams received an acute bilateral infusion of vehicle (VEH; 0.5 µl sterile Ringer's solution; pH 7.4), CRF-R1 agonist human/rat CRF (CRF-R1 ago; 1 µg / 0.5 µl), CRF-R1 antagonist CP-154,526 (CRF-R1 ant; 12 µg / 0.5 µl), CRF-R2 agonist Stresscopin (CRF-R2 ago; 3 µg / 0.5 µl), or CRF-R2 antagonist Astressin-2B (CRF-R2 ant; 4 µg / 0.5 µl) into the mpBNST. Data is presented as mean + SEM. n = 8 – 14 per group. ** $p \leq 0.01$, * $p \leq 0.05$ versus VEH (two-way ANOVA for repeated measures; factors: time x treatment); ++ $p \leq 0.01$, + $p \leq 0.05$, (+) $p = 0.08$ versus t -100 min (one-way ANOVA for repeated measures; factor: time).

Maternal motivation on LD 2

None of the treatments affected pup retrieval behavior (pups retrieved within 15 min: VEH: 7.3 ± 0.5; CRF-R1 agonist: 7.0 ± 1.0; CRF-R1 antagonist: 8.0 ± 0.0; CRF-R2 agonist: 5.3 ± 2.7; CRF-R2 antagonist: 2.8 ± 1.5).

Maternal aggression on LD 5

The number of attacks (one-way ANOVA; $F_{4,39}$ = 5.53, p = 0.01; Figure 18 left) and the attack latency ($F_{4,39}$ = 10.16, p < 0.01; Figure 18 right) differed between the groups. In detail, the CRF-R2 agonist completely blocked attacks (p = 0.02) and, thus, increased the attack latency (p < 0.01) compared to the VEH-treated dams. Accordingly, the CRF-R2 antagonist increased the number of attacks (p = 0.02) and decreased the attack latency (p = 0.03). Neither the CRF-R1 agonist nor antagonist had any significant effect on maternal aggression. No other behavioral parameters (e.g. keep down, lateral threat, offensive upright) measured during maternal defense differed between the groups.

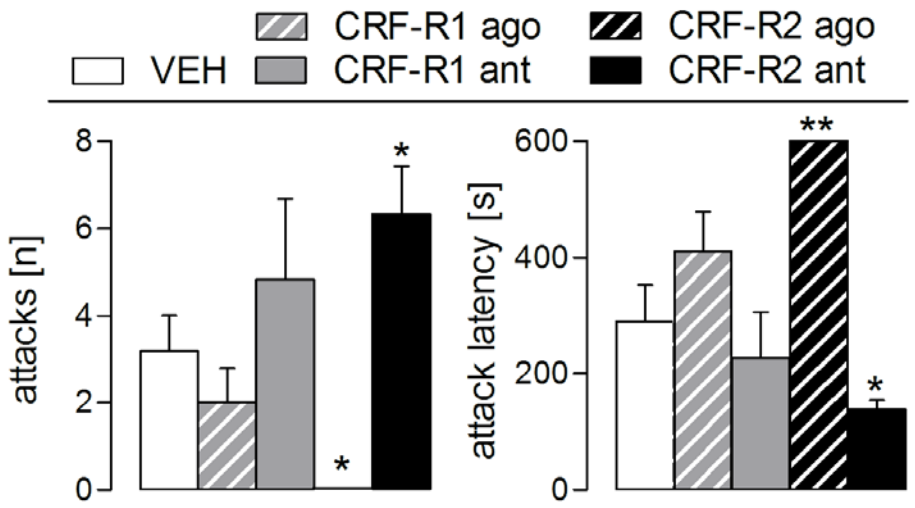

Figure 18. Effect of intra-mpBNST CRF-R1 or -R2 specific agonist (ago) or antagonist (ant)

treatment on maternal aggression of lactating rats measured in the maternal defense test. Maternal aggression against a virgin female intruder was scored during the 10-min trial. Number of attacks (left) and attack latency (right) by the resident is shown. For details on treatments see legend to Figure 17. Data is presented as mean + SEM. n = 7 – 11 per group. ** p ≤ 0.01, * p ≤ 0.05 versus VEH (one-way ANOVA; factor: treatment).

Anxiety-related behavior on LD 3

Treatment significantly altered the percentage of time spent on the open arms of the EPM in the lactating dams (one-way ANOVA; $F_{4,43}$ = 16.21, p < 0.01; Figure 19A). Mothers treated with the CRF-R1 agonist spent significantly less (p = 0.04) whereas dams injected with the antagonist for CRF-R1 (p < 0.01) or CRF-R2 (p < 0.01) spent significantly more time on the open arms compared to the VEH-treated dams. CRF-R2 agonist-infused mothers did not significantly differ from VEH-treated mothers. Regarding locomotor activity, no group differences were detected in number of entries into the closed arms (data not shown).

3.4.4 Experiment 4 and 5: Behavioral effects of intra-mpBNST CRF-R1 or -R2 blockade on anxiety in virgin and male rats

Virgin rats: The percentage of time spent on the open arms of the EPM significantly differed depending on the treatment (one-way ANOVA; $F_{2,11}$ = 7.03, p = 0.01; Figure 19A). Females treated with the CRF-R1 antagonist (p = 0.03) or the CRF-R2 antagonist (p < 0.01) spent significantly more time on the open arms compared to the VEH-treated females. No difference was found in the number of closed arm entries between any of the groups (data not shown).

Male rats: No statistically significant differences were found in any of the parameters tested in male rats, either on the EPM or in the LDB when infused with subtype-specific CRF-R agonists or antagonists (Figure 19B).

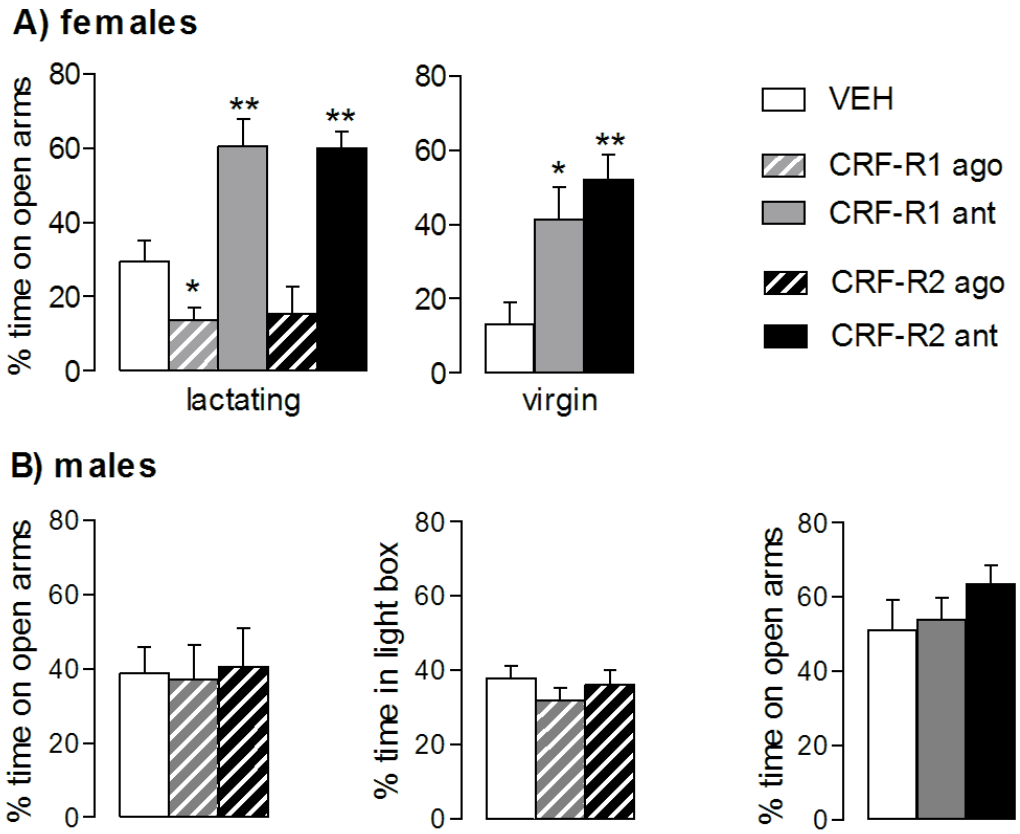

Figure 19. Effect of intra-mpBNST CRF-R1 or -R2 specific agonist (ago) or antagonist (ant) treatment on anxiety-related behavior of lactating and virgin female (A) and male rats (B) on the elevated plus-maze or the light-dark box (males only). Virgin females were treated with antagonists, only. The percentage of time spent on the open arms or the percentage of time spent in the light box during the 5-min tests is shown. For details on treatments see legend to Figure 17. Data is presented as mean + SEM. n = 7 – 12 per group. ** $p < 0.01$, * $p \leq 0.05$ versus VEH (one-way ANOVA; factor: treatment).

3.5 Discussion

This is the first study to provide evidence that CRF-R2, and to a lesser extent CRF-R1, in the mpBNST are importantly involved in regulating maternal behavior in lactating rats. CRF-R2, but not CRF-R1, mRNA expression was higher in the pBNST versus mBNST independent of reproductive status. The behavioral experiments revealed that ABN and total nursing were rapidly impaired by intra-mpBNST CRF-R1 agonist and after a delay by the CRF-R2 agonist. However, under stress conditions,

ABN was increased only by the CRF-R2 antagonist, whereas both antagonists prevented the typical decrease in nursing after stress. During the maternal defense test, the CRF-R2 agonist abolished maternal aggression while the CRF-R2 antagonist increased aggression; however, CRF-R1 manipulation had no significant effect. Furthermore, the CRF-R1 agonist increased while both antagonists decreased anxiety-related behavior in virgin and lactating rats but not in male rats.

Several studies suggest a crucial contribution of CRF in regulating cellular (da Costa et al., 2001; Lightman et al., 2001; Walker et al., 2001; Deschamps et al., 2003) and behavioral adaptations (Pedersen et al., 1991; Gammie et al., 2004; Klampfl et al., 2013) in lactating females. We recently demonstrated that activation of central CRF-R1/2 reduces, and their blockade increases maternal behavior while anxiety was altered conversely (Klampfl et al., 2013). As the previously infused agonist CRF binds with 40-fold higher affinity to CRF-R1 (Hauger et al., 2003) it is often used as a CRF-R1 specific agonist (e.g. Magalhaes et al., 2010). Therefore, we aimed to confirm our earlier results using the non-specific CRF-R1/2 agonist Ucn 1 (Figure 14). Importantly, the behavioral effects were similar to our recent data (Klampfl et al., 2013) and additionally support previous studies demonstrating a detrimental effect of CRF-R activation on maternal behavior (Pedersen et al., 1991; Gammie et al., 2004; Klampfl et al., 2013). Consistent with our findings in rats, icv Ucn 1 impairs maternal aggressive behavior in lactating mice (D'Anna et al., 2005). Furthermore, Ucn 1 is known to be anxiogenic in male rodents (Moreau et al., 1997; Spina et al., 2002), which we now extend to lactating rats (Figure 14). These results confirm the impairing effects of central CRF-R activation on maternal behavior and postpartum anxiety. We further investigated the effects of specific CRF-R1 and -R2 manipulation in the mpBNST on maternal behavior and anxiety in lactating rats.

We focused on the BNST due to its importance in mediating maternal care (Numan and Insel, 2003), maternal aggression (Bosch et al., 2010; Bosch, 2011; Caughey et al., 2011), and anxiety (Sahuque et al., 2006; Walker et al., 2009). Importantly, the BNST contains most members of the CRF family, i.e. CRF (Potter et al., 1994), Ucn 2 (Reyes et al., 2001), and Ucn 3 (Lewis et al., 2001) as well as CRF-R1 and -R2 (Potter et al., 1994; Chalmers et al., 1995). We found no differences in either CRF-R1 or -R2 mRNA within the mBNST or the pBNST between virgin and lactating rats (Figure 15). However, CRF-R2 mRNA expression was higher in the pBNST compared to the mBNST. Thus, we hypothesized that CRF-R2 might play a special role in the pBNST but that CRF-R1 could equally contribute to possible behavioral changes.

Intra-mpBNST application of either the CRF-R1 or -R2 antagonist had no effect on nursing behavior under non-stress conditions. This indicates a minimal activation of the CRF-R under basal conditions during lactation and, thus, strengthens the hypothesis that down-regulation of the CRF system in the maternal brain is vital. However, infusion of either agonist reduced nursing behavior in a time-dependent manner: the effects of the CRF-R1 agonist were rapid, while those of the CRF-R2 agonist were delayed. At the same time, the dams displayed more off-nest behaviors; CRF-R1 agonist-treated dams showed more locomotion and self-grooming while CRF-R2 agonist-treated dams showed more sleeping/resting (Table 5). The time-delayed effect of the CRF-R2 agonist might be due to a longer latency until the agonist exerts its actions under basal conditions (Pelleymounter et al., 2004; D'Anna et al., 2005; D'Anna and Gammie, 2009). Thus, the relative quiescence of both receptor subtypes in the mpBNST is necessary for the expression of appropriate maternal care under basal conditions. As CRF-R mRNA expression was not different (Figure 15) and CRF mRNA levels are elevated in the pBNST compared to virgin

females (Walker et al., 2001) the proposed quiescence could result from either reduced CRF-R protein expression or reduced CRF/Ucn release. This might result from reduced NAergic input to (Forray and Gysling, 2004) or reduced NAergic activity within the BNST (Smith et al., 2012) postpartum. Additionally, increased OXT receptor binding within the BNST postpartum (Bosch et al., 2010) might attenuate the activity of CRF neurons as has been shown for the PVN (Windle et al., 2004) and, moreover, has been recently proposed for the BNST (Dabrowska et al., 2013). Furthermore, CRF neurons projecting to the BNST from the CeA express reduced levels of CRF mRNA during lactation (Walker et al., 2001), thus supporting reduced CRF-R activation also within the mpBNST.

However, following stressor exposure, distinct roles for CRF-R1 and -R2 emerged during subsequent maternal care observation. ABN reflecting the quality of maternal care returned rapidly to pre-stress levels only in the CRF-R2 antagonist-treated group. The occurrence of nursing reflecting the quantity of maternal care was affected by both receptor antagonists as a small but significant decrease in nursing after the maternal defense test was prevented by intra-mpBNST administration of both the CRF-R1 and -R2 antagonist.

In contrast to maternal care, we did not find any changes in maternal motivation after subtype-specific CRF-R manipulations within the mpBNST. This seems to be contrary to our own results as we show a trend for reduced maternal motivation in the CRF-R1/2 agonist-treated rats (experiment 1). However, one has to distinguish between central and, hence, very broad receptor manipulation versus local (ant-)agonism of CRF-R within the mpBNST (Bosch, 2011). Since the BNST has not been reported to mediate maternal motivation the lack of an effect after intra-mpBNST manipulation was anticipated.

With respect to maternal aggression, activation and blockade of CRF-R2 (but not CRF-R1) abolished and increased maternal aggression, respectively (Figure 18). Interestingly, the CRF-R2 agonist elicited an immediate behavioral effect in a stressful situation in contrast to basal conditions. These findings support our hypothesis that CRF-R activation in the mpBNST needs to be low during lactation for appropriate maternal behavior to occur. Additionally, this further highlights the importance of the CRF-R2 subtype in the regulation of maternal behavior. Consistent with this, CRF-R2, but not CRF-R1, within the LS mediate maternal aggression in lactating mice (Gammie et al., 2005; D'Anna and Gammie, 2009). Hence, signal transmission via CRF-R2 modulates maternal aggressive behavior, at least within the mpBNST and the LS.

Besides maternal behavior, manipulation of CRF-R activity within the mpBNST also affected anxiety-related behavior (Figure 19). Activation of CRF-R1 was anxiogenic while blockade of CRF-R1 or -R2 was anxiolytic in dams. Interestingly, CRF-R1 activation in lactating rats was anxiogenic at a dose that was not effective in male rats. However, a 2.5-fold higher dose elicits an anxiogenic effect in males (Klampfl and Bosch, unpublished), thereby confirming a previous study showing a similar dose-dependent effect on anxiety (Sahuque et al., 2006). This suggests a higher activation threshold in males. Furthermore, the CRF-R1 or -R2 antagonists reduced anxiety-related behavior in the lactating females but not in males, which indicate these antagonists have anxiolytic actions only in combination with previous activation of the receptor in males (Sahuque et al., 2006). Interestingly, the anxiolytic effects of both antagonists were also found in virgin rats suggesting a higher basal activity of intra-mpBNST CRF-R in females. Thus, the higher basal activity and the lower activation threshold in females render the system more sensitive and potentially vulnerable as shown for the LC (Valentino et al., 2013). Interestingly, a recent study

demonstrated that CRF infusions into the dorsal raphe nucleus of female mice have no effect on anxiety whereas the same treatment is anxiogenic in males (Howerton et al., 2014). However, our study is the first to prove evidence that the regulation of emotionality within the BNST by the CRF system is sexually dimorphic and independent of reproductive status.

Regarding the lack of effect on anxiety-related behavior after CRF-R2 activation with Stresscopin in lactating rats, it is possible that the dose used was sub-threshold or that the agonist requires more time to elicit an anxiogenic-like response. Indeed, the CRF-R2 ligand Ucn 2, which shares high homology with Stresscopin and binds with similar high affinity to CRF-R2 (Reyes et al., 2001), exerts its anxiogenic effect only four hours after central infusion in male rats (Pelleymounter et al., 2002; Valdez et al., 2002; Pelleymounter et al., 2004). The role of CRF-R2 in anxiety-related behavior appears to be complex - especially with respect to CRF-R2 knock-out mice (Reul and Holsboer, 2002b). The region- and neuron-specific location of receptors and, thus, the differential modulation of neurotransmitter systems is likely responsible for an anxiogenic or anxiolytic behavioral outcome following CRF-R2 activation.

In conclusion, low CRF-R activation within the mpBNST postpartum is an indispensable prerequisite for the adequate rearing and defense of the offspring. As dysregulation of the mother's CRF system is evident in postpartum mood disorders, our findings serve to better understand the fine-tuned regulation of the maternal brain especially under stressful conditions.

- Chapter 4 -

Opposing effects of subtype-specific CRF receptor activation in the adBNST on maternal care and the stress axis in lactating rats

Authors‘ contribution:

Stefanie Klampfl: experimental design, performance of experiments, data analysis, first draft of manuscript

Doris Bayerl: performance of experiments

Oliver Bosch: experimental design, performance of experiments, revision of manuscript

4.1 Abstract

In lactation, maternal neglect and impaired defense of the offspring are mediated by activation of CRF-R in the mpBNST. Based on the cyto- and chemoarchitectural heterogeneity of the BNST, we hypothesized that manipulation of CRF-R in another functional part of this brain area, i.e. the adBNST, modulates maternal behavior differently from the mpBNST in lactating rats. Furthermore, these behavioral effects might be indirectly mediated by altered activity of the HPA axis.

Indeed, maternal care was differentially changed by CRF-R subtype-specific manipulation; ABN was impaired by the CRF-R1 agonist but improved by the CRF-R2 agonist with a delay. Total nursing was reduced by both agonists. After the stressful maternal defense test, which is generally detrimental to maternal care, ABN tended to be improved by the CRF-R1 antagonist. Maternal motivation, maternal aggression, and anxiety were not affected by any manipulation. Assessing HPA axis activity following CRF-R manipulation, solely the CRF-R1 agonist increased basal ACTH release which was not further elevated by exposure to a stressor. CORT release was stimulated by both agonists under basal conditions. Under stressful conditions, CORT levels remained high in CRF-R1 agonist-treated dams while a switch to decreased release was observed in the CRF-R2 agonist group. Both antagonists prevented the stress-induced increase in CORT.

In conclusion, maternal behavior is differentially regulated by CRF-R1 and CRF-R2 in the adBNST, which is clearly distinct to the mpBNST. Interestingly, altered HPA axis activity might be involved in or even responsible for the observed changes in behavioral patterns. These data extend our basic understanding of the maternal brain and provide new insights into potential dysregulations postpartum.

4.2 Introduction

The peripartum period is accompanied by numerous physiological and behavioral adaptations of the maternal brain. These changes are essential for the mothers' mental health and an adequate expression of maternal behavior, which ensures the proper development of the offspring (Bosch, 2011). However, 20 – 30 % of mothers develop postpartum mood disorders, show infant neglect or even infanticide (Friedman and Resnick, 2009; Brummelte and Galea, 2010a). One peptidergic system that contributes evidently to such maladaptations during this highly sensitive period is the central CRF system (Magiakou et al., 1996; Klampfl et al., 2013; Klampfl et al., 2014b).

The CRF system consists of CRF and its related peptides Ucn 1 - 3, which bind to CRF-R1, CRF-R2, and the CRF-BP with different affinities (Reul and Holsboer, 2002b). CRF was first discovered as the main initiator of the HPA axis and, thus, is the major secretagogue of ACTH from the anterior pituitary into the portal blood system. ACTH leads to the release of CORT from the adrenal glands causing a negative feedback on the level of the hippocampus, PVN, and pituitary (Vale et al., 1981). However, CRF and its related peptides also exert central functions and influence a variety of non-social and social behaviors, e.g. increased anxiety-related behavior (Britton et al., 1986; Sahuque et al., 2006; Klampfl et al., 2013; Klampfl et al., 2014b), induced pup-killing (Pedersen et al., 1991) and reduced maternal behavior (Pedersen et al., 1991; Gammie et al., 2004; Klampfl et al., 2013; Klampfl et al., 2014b). Intriguingly, some of these effects could be assigned to the CRF system of the BNST.

The BNST is a complex and particularly heterogeneous structure within the limbic system. Initially, it was divided into lateral and medial divisions due to neuronal innervations from the amygdala (Krettek and Price, 1978). However, developmental

and cyto- and chemo-architectonic studies led to an alternative designation along an anterior-posterior gradient (Bayer, 1987; Ju and Swanson, 1989; Ju et al., 1989). The BNST acts as central relay site for the integration of a variety of neuronal signals, thus mediating behavioral and physiological responses. Interestingly, the anterior part of the BNST is mainly connected with hypothalamic and lower brainstem regions associated with autonomic activity (Dong et al., 2001a) while the posterior division is involved in controlling neuroendocrine and social behaviors (Dong et al., 2001a; Dong and Swanson, 2004a; Choi et al., 2007). For example, anterolateral and posteriomedial parts send direct projections to CRF containing cells of the pPVN (Dong et al., 2001b; Dong and Swanson, 2004a, 2006), thereby providing a mechanism for direct actions on the HPA axis (Crestani et al., 2013).

Such a tremendous heterogeneity within one single brain site raises the question whether social and non-social behaviors regulated by the BNST might be influenced differently depending on the specific site of manipulation. We recently demonstrated that within the mpBNST CRF-R activation, predominantly subtype 2, impairs maternal care and maternal aggression in lactating rats (Klampfl et al., 2014b). Furthermore, anxiety-related behavior is increased by activation of both receptor subtypes in lactating and virgin females. In the present study, we focused on the adBNST, which contains the anterodorsal area and the oval nucleus (Dong et al., 2001b). We compared CRF-R mRNA expression patterns in virgin versus lactating rats. Furthermore, we manipulated CRF-R1 and CRF-R2 with selective agonists and antagonists in the adBNST and assessed possible effects on maternal care, maternal motivation, maternal aggression, and anxiety-related behavior during early lactation. Finally, we investigated a potential impact of selective intra-adBNST CRF-R manipulation under basal and stressful conditions on the HPA axis in lactating rats.

4.3 Materials & Methods

4.3.1 Animals

Virgin female Sprague-Dawley rats (220 – 250 g; Charles River Laboratories, Sulzfeld, Germany) were kept under standard laboratory conditions (change of bedding once per week, RT 22 ± 2 °C, 55 % relative humidity, 12 : 12 h light / dark cycle, lights on at 6 a.m.) with access to water and standard rat chow *ad libitum*. Females were mated and housed in groups of 3 to 4 animals until PD 18. For experiment 1, females underwent surgery whereafter they were single-housed to guarantee recovery and undisturbed delivery; for experiment 2, females were single-housed at PD 18 to assure undisturbed delivery as described recently (Klampfl et al., 2013). On the day of birth, litters were culled to eight pups of mixed sexes. During the single-housing period (except the day before and the day of delivery), all rats were handled twice a day to reduce non-specific stress responses during the experiments (Neumann et al., 1998b).

For the maternal defense test, naïve virgin female Wistar rats (200 – 220 g, Charles River Laboratories) were used as intruders at random stages of their estrous cycle. Intruder rats were kept in a separate room to avoid olfactory recognition.

The experiments were approved by the Committee on Animal Health and Care of the local government and conformed to international guidelines on the ethical use of animals. All efforts were made to minimize the number of rats used and their suffering.

4.3.2 Behavioral tests

Maternal care. Maternal care was monitored on LD 1 before and after substance infusion (non-stress condition) as well as on LD 7 before and after substance infusion, which was combined with a psychosocial stressor (i.e. maternal defense test

(Neumann et al., 2005a); stress condition). The authors acknowledge that there is a limited amount of stress associated with the infusion procedure, though the 'non-stress' term is used to distinguish between the maternal care observations on LD1 which did not involve the maternal defense test from those conducted on LD 7 which did. Observations were conducted for 10 s every 2nd min in 30 min blocks according to an established protocol (Bosch and Neumann, 2008). In detail, on LD 1, dams were observed under non-stress conditions from 8 – 9 a.m., infused at 9 a.m. and observation continued afterwards from 9.30 – 11 a.m. Additionally, dams were observed 5 h after infusion, i.e. from 2 – 3 p.m. to assess potential long-lasting effects of drug treatment. On LD 7, dams were observed from 8 – 9 a.m., transported to another room, and infused at 10 a.m. Dams were tested either 10 or 25 min after infusion (depending on the treatment, see below) in the maternal defense test, immediately afterwards transported back to the observation room, and maternal care was observed for another 60 min in order to assess effects of the stressor on maternal care. The main parameter for the quality of maternal care was the occurrence of ABN (Bosch, 2011), the only active nursing posture where the dam is engaged in a quiescent kyphosis (Stern and Johnson, 1990). Other behavioral parameters scored were hovering over the pups and blanket nursing posture, which together with ABN were counted as total nursing, thereby indicating the quantity of maternal care (Klampfl et al., 2014b). Pup retrieval/mouthing and licking/grooming were assessed as 'other maternal behaviors'. Additionally, non-maternal behaviors were scored, i.e. locomotion (including digging/burrowing and cage exploration), self-grooming, and sleeping/resting, which were summed up and are presented as off-nest behavior. Data is shown in 30 min blocks before and after treatment infusion with a maximal count of 15 observations per block.

Maternal motivation. The dams' maternal motivation was tested in the PRT on LD 3 (van Leengoed et al., 1987; Neumann et al., 2005a). The dams were separated from their litter 60 min prior to the test and moved to a separate room. Ten or 25 min prior to the test (depending on treatment, see below), dams received their respective treatment. All 8 pups of the litter were then distributed in a plastic box (54 x 34 x 31 cm) covered with bedding from their home cage, the mother was placed in the box, and the number of retrieved pups within the 15-min testing period was counted.

Maternal aggression. To assess maternal aggression, the maternal defense test was performed on LD 5 in a separate room, to which the dams were transported 60 min prior to the test (see above). Ten or 25 min after treatment infusion (depending on treatment, see below), the lactating residents were confronted with an unknown virgin female intruder in their home cage in the presence of the litter for 10 min as described previously (Neumann et al., 2001; Bosch et al., 2005). The dam's behavior was videotaped for subsequent analysis by an experienced observer blind to the treatment. The following behavioral parameters were scored: total number of attacks, latency to first attack, lateral threat, keep down, and offensive upright as well as non-aggressive behaviors (for detailed description see Bosch, 2013).

Anxiety-related behavior. Anxiety-related behavior was tested on the EPM on LD 3 as described earlier (Pellow et al., 1985; Neumann et al., 2000). Briefly, the plus shaped maze consists of two open arms (50 x 10 cm^2, 80 lux) and two closed arms (50 x 10 x 30 cm^3, 10 lux) surrounding a neutral square shaped central zone (10 x 10 cm^2, 65 lux) and is elevated 82 cm over the floor. Ten or 25 min after infusion (depending on treatment, see below), the rats were placed in the neutral zone of the maze and were allowed to freely explore the maze for 5 min. The percentage of time

spent on the open arms versus all arms/zones (open arm, closed arm, and neutral zone) and the percentage of open arm entries versus all entries (open and closed arms) were taken as indicator of anxiety-related behavior. The number of closed arm entries was used to measure locomotion (Neumann et al., 2000).

4.3.3 Experimental design

Experiment 1: Intra-adBNST manipulation of CRF-R1 or -R2 in lactating rats

On PD 18, females were implanted bilaterally with 23 G guide cannula targeting the adBNST (-0.2 mm caudal, 3.0 mm lateral, 4.9 mm ventral to bregma (Paxinos and Watson, 1998) with an angle of 12.5°) under inhalation anesthesia (Isoflurane; Baxter Germany GmbH, Unterschleißheim, Germany) and semi-sterile conditions as described earlier (Bosch et al., 2010). Substances were infused using a 27 G infusion cannula (Neumann et al., 2000). Lactating rats received either (i) VEH (0.5 µl of sterile Ringer's solution + 4 % DMSO; pH 7.4; Braun), (ii) CRF-R1 agonist human/rat CRF (1 µg / 0.5 µl; Tocris Bioscience, Ellisville, Missouri, USA), (iii) CRF-R1 specific antagonist CP-154,526 (12 µg / 0.5 µl; Tocris Bioscience), (iv) CRF-R2 specific agonist hUcn 3 (Stresscopin; 3 µg / 0.5 µl; Phoenix Pharmaceuticals, Karlsruhe, Germany), or (v) CRF-R2 specific antagonist Astressin-2B (4 µg / 0.5 µl; Sigma-Aldrich, Steinheim, Germany). Doses were chosen based on our previous study (Klampfl et al., 2014b). On LD 1, 3, 5, and 7, lactating dams received a single acute bilateral infusion either 10 (VEH, CRF-R1 ago, CRF-R1 ant, CRF-R2 ant) or 25 min (CRF-R2 ago) prior to the tests. Each animal received the same treatment on every testing day as assigned on LD 1. Importantly, the repeated infusions of the CRF-R1 or –R2 (ant)agonists separated by 48 h intervals is not expected to result in receptor (de)sensitization (personal communication with Prof. J Radulovic, Northwestern University, USA; see also Spiess et al., 1998; Hauger et al., 2009). Between infusion

and the respective tests, dams were placed back to their home cages. Maternal care was observed under non-stress conditions (LD 1) and stress conditions (LD 7) in the home cage as described above. Additionally, maternal motivation (LD 3), anxiety-related behavior (LD 5), and maternal aggression (LD 7) were tested as described above. All tests were performed between 8 a.m. and 3 p.m. in the light phase of the cycle.

Experiment 2: Effects of intra-adBNST manipulation of CRF-R1 or -R2 on HPA axis activity

On LD 1, females were bilaterally implanted with local guide cannula targeting the adBNST as described in experiment 1. In order to determine HPA axis activity following intra-adBNBST CRF-R manipulations, dams were also fitted with a jugular vein catheter for repeated blood sampling in the conscious, freely moving rat as described earlier (Bosch et al., 2007).

On LD 6 at 8 a.m., the catheters were connected to a sampling syringe filled with heparinized saline (0.6 %; Ratiopharm, Ulm, Germany). After 1.5 hours of habituation, blood samples (0.25 ml) were collected in EDTA-coated tubes and stored on ice. The first two samples were drawn 30 min apart under basal conditions. Immediately afterwards, the dams were infused bilaterally into the adBNST with VEH, CRF-R1 agonist, CRF-R2 agonist, CRF-R1 antagonist, or CRF-R2 antagonist according to experiment 1. Further blood samples were taken 10 and 30 min after treatment to assess respective effects of specific CRF-R activation/inhibition on the HPA axis. Afterwards, dams were exposed to strong stressor, i.e. a virgin intruder in the maternal defense test, for 10 min (see above). Additional blood samples were collected 5, 15, and 60 min after termination of the aggression test. All blood samples were immediately replaced by sterile saline. Samples were centrifuged for 5 min at

4°C (5000 rpm) and stored at -20°C until further processing. Plasma ACTH and CORT concentrations were measured using ELISA (IBL International GmbH, Hamburg, Germany).

4.3.4 Histology

At the end of the experiments, rats were decapitated. To verify the correct placements of local cannula within the adBNST brains were infused with 0.5 µl of ink (Pelikan Ink 4001, Hanover, Germany; diluted 1:20 in Ringer's solution), removed, flash frozen, cut in 40 µm coronal sections, and slide mounted. Ink diffusion was assessed to exclude any diffusion to the mpBNST. Furthermore, slides were stained via quick Nissl staining to locate the tip of the infusion cannula. Only rats with correctly fitted cannula and properly targeted ink diffusion were included in the statistical analysis. The precise cannula placement sites within the adBNST are illustrated in Figure 20.

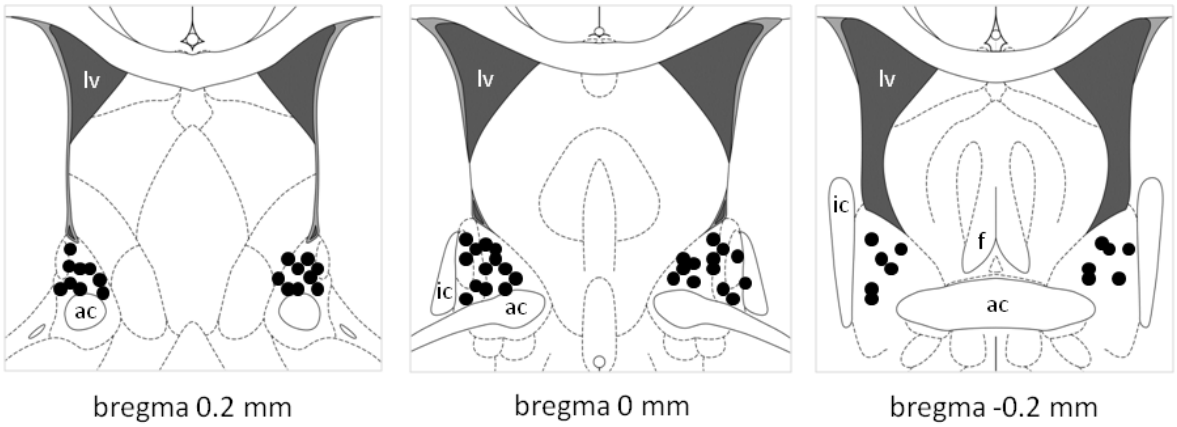

Figure 20. Histological localization of infusion cannula within the adBNST. Cannula placement sites for subsequent drug infusion are shown as black dots within the adBNST on schematic plates from the Paxinos and Watson (1998) stereotactic atlas. ac: anterior commissure, f: fornix, ic: internal capsule, lv: lateral ventricle.

4.3.5 Statistical analysis

In situ hybridization data was analyzed using two-way ANOVA (factors: reproductive status x brain site). Behavioral and physiological data were analyzed using either one-way ANOVA (factor: treatment) or two-way ANOVA for repeated measures (factors: time x treatment) followed by Fisher's LSD *post hoc* test. An independent t-test was performed where appropriate. For all tests, the software package SPSS 19.0 (IBM) was used. Data are presented as means ± S.E.M. and significance was accepted at $p \leq 0.05$.

4.4 Results

4.4.1 Experiment 1: Intra-adBNST manipulation of CRF-R1 or -R2 in lactating rats

Maternal care under non-stress conditions on LD 1

ABN: Differences in ABN were found depending on time (two-way ANOVA for repeated measures; $F_{6,192} = 3.14$, $p = 0.006$; Figure 21, time course top) and treatment ($F_{4,32} = 6.62$, $p = 0.001$), which also interacted significantly ($F_{24,192} = 1.89$, $p = 0.01$). Before infusion, CRF-R1 antagonist ($p = 0.03$) and CRF-R2 agonist-treated dams ($p = 0.005$) showed more ABN at t -30 min compared to VEH. Immediately after infusion, CRF-R1 agonist infusion decreased ABN by trend compared to t -30 min ($p = 0.07$). Furthermore, ABN was increased at t +60 min by the CRF-R1 antagonist ($p = 0.006$) and CRF-R2 agonist ($p = 0.02$) compared to VEH. Five hours after infusion, the CRF-R2 agonist ($p < 0.001$) and the CRF-R2 antagonist ($p = 0.04$) increased ABN compared to VEH.

Summing up the occurrence of ABN after infusion, i.e. from t +30 min until t +90 min, significant differences were found (one-way ANOVA, $F_{4,32} = 5.31$, $p = 0.02$; Figure

21, sum top). Both the CRF-R1 antagonist and the CRF-R2 agonist increased ABN significantly ($p = 0.02$) and by trend ($p = 0.06$), respectively. In addition, the CRF-R1 agonist decreased ABN after infusion versus VEH (independent t-test; $t_{14} = 2.95$, $p = 0.01$).

Total nursing: Differences in nursing were found depending on treatment (two-way ANOVA for repeated measures; $F_{4,32} = 12.18$, $p < 0.001$; Figure 21, time course bottom) but not on time. However, a significant interaction was revealed ($F_{24,192} = 2.20$, $p = 0.002$). Before infusion, no differences were found. After infusion, CRF-R1 and -R2 agonist-treated dams showed less nursing at t +30 min compared to the previous time-point (CRF-R1 agonist: $p = 0.02$, CRF-R2 agonist: $p = 0.03$) and compared to VEH (CRF-R1 agonist: $p = 0.004$, CRF-R2 agonist: $p = 0.03$). This decrease persisted in CRF-R1 agonist-treated dams until t +300 min ($p \leq 0.001$, in each case) and was similarly found in CRF-R2 agonist-treated dams (t +90 min: $p = 0.05$).

Summing up the occurrence of total nursing after the infusion, i.e. from t +30 min until t +90 min, significant differences were found (one-way ANOVA, $F_{4,32} = 15.83$, $p < 0.001$; Figure 21 sum bottom). The CRF-R1 ($p < 0.001$) and -R2 agonist ($p = 0.002$) reduced nursing compared to VEH.

Other maternal behaviors: No significant differences in pup retrieval/mouthing and licking/grooming were found.

Non-maternal behaviors: Significant interactions were found in the occurrence of off-nest behavior (two-way ANOVA for repeated measures; $F_{24,192} = 2.51$, $p < 0.001$; Table 7), which also differed depending on time ($F_{6,192} = 3.65$, $p = 0.002$) and treatment ($F_{4,32} = 9.82$, $p < 0.001$). CRF-R1 agonist- and CRF-R2 agonist-treated dams showed more off-nest behavior at t +30 min compared to the previous time-point (CRF-R1 agonist: $p = 0.001$; CRF-R2 agonist: $p = 0.005$) and to VEH (CRF-R1

agonist: p = 0.01; CRF-R2 agonist: p = 0.02). Furthermore, CRF-R1 agonist-treated dams showed more off-nest behavior at t +60 min (p < 0.001), t +90 min (p = 0.001), and t +300 min (p = 0.002).

Locomotion differed depending on time ($F_{6,192}$ = 2.47, p = 0.02) and treatment ($F_{4,32}$ = 17.44, p < 0.001), which also interacted ($F_{24,192}$ = 3.41, p < 0.001). CRF-R1 agonist-treated dams showed more locomotion at t +30 min compared to the previous time-point and to VEH (p < 0.001, in each case) as well as at t +60 min and t +90 min (p < 0.001, in each case) and t +300 min (p = 0.002) compared to VEH. The CRF-R2 and the CRF-R1 antagonist-treated dams showed less locomotion at t -60 min (p = 0.05) and t -30 min (p = 0.03), respectively.

Self-grooming differed depending on time ($F_{6,186}$ = 4.61, p < 0.001) and treatment ($F_{4,31}$ = 10.13, p < 0.001), which also interacted ($F_{24,186}$ = 3.18, p < 0.001). CRF-R1 (p = 0.001) and CRF-R2 agonist-treated dams (p = 0.02) showed more self-grooming at t +30 min compared to the previous time-point. Additionally, CRF-R1 agonist-treated dams showed more self-grooming at t +30 min (p = 0.03), t +60 min (p < 0.001) and t +90 min (p = 0.005) compared to VEH.

Sleeping/resting differed depending on treatment ($F_{4,32}$ = 2.92, p = 0.03), but not on time, which interacted significantly ($F_{24,192}$ = 1.67, p = 0.02). CRF-R2 agonist-treated dams showed more sleeping/resting at t +30 min compared to the previous time-point (p < 0.001) and to VEH (p = 0.006) as well as at t +60 min by trend (p = 0.06), and t+90 min (p = 0.03) compared to VEH. CRF-R1 agonist-treated dams showed more sleeping/resting at t -60 min (p = 0.05).

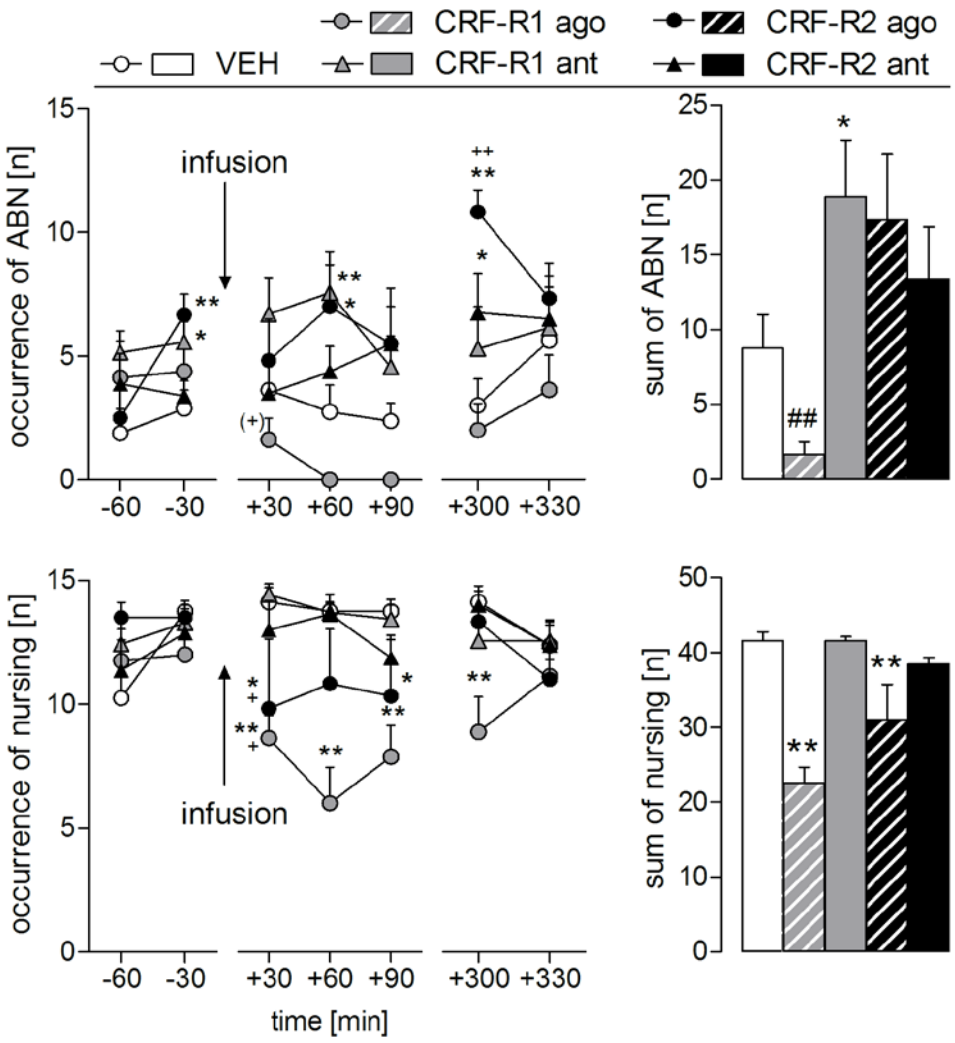

Figure 21. Effect of intra-adBNST CRF-R1 or -R2 specific agonist (ago) or antagonist (ant) treatment on maternal care of lactating dams under non-stress conditions on LD 1. Arched back nursing (ABN, top) and sum of nursing (bottom) were scored for 60 min before, for 90 min after infusion and for additional 60 min with a 5 h delay. The 90 min after infusion are summed up and shown on the right side. Dams received an acute bilateral infusion of vehicle (VEH; 0.5 µl sterile Ringer's solution; pH 7.4), CRF-R1 agonist human/rat CRF (CRF-R1 ago; 1 µg / 0.5 µl), CRF-R1 antagonist CP-154,526 (CRF-R1 ant; 12 µg / 0.5 µl), CRF-R2 agonist Stresscopin (CRF-R2 ago; 3 µg / 0.5 µl), or CRF-R2 antagonist Astressin-2B (CRF-R2 ant; 4 µg / 0.5 µl) into the adBNST. Data is presented as mean + SEM. n = 6 - 8 per group. ** $p \leq 0.01$, * $p \leq 0.05$ versus VEH; ++ $p \leq 0.01$, + $p \leq 0.05$, (+) $p = 0.07$ versus t -30 min (time course: two-way ANOVA for repeated measures; sum: one-way ANOVA), ## $p \leq 0.01$ versus VEH (independent t-test).

Maternal care under stress conditions on LD 7

ABN: Differences in ABN were found depending on time (two-way ANOVA for repeated measures; $F_{3,93} = 6.56$, $p < 0.001$; Figure 22, time course top) but not on treatment. No significant interaction was detected.

Summing up ABN after infusion and the maternal defense test, no significant differences were found. However, an independent t-test revealed a strong trend toward increased ABN in CRF-R1 antagonist-treated dams compared to VEH ($t_{13} = -2.00$, $p = 0.06$; Figure 22, sum top).

Total nursing: Differences in nursing were found depending on time (two-way ANOVA for repeated measures; $F_{3,93} = 10.05$, $p < 0.001$) but not on treatment. No significant interaction was detected (Figure 22, time course bottom). Summing up nursing did not reveal any differences (Figure 22, sum bottom).

Other maternal behaviors: No significant differences in pup retrieval/mouthing and licking/grooming were found.

Non-maternal behaviors: Off-nest behavior ($F_{3,93} = 15.47$, $p < 0.001$), locomotion ($F_{3,93} = 21.33$, $p < 0.001$), and self-grooming ($F_{3,93} = 6.09$, $p = 0.001$) differed depending on time but not on treatment (Table 8). No significant interactions were detected. No differences in sleeping/resting were found.

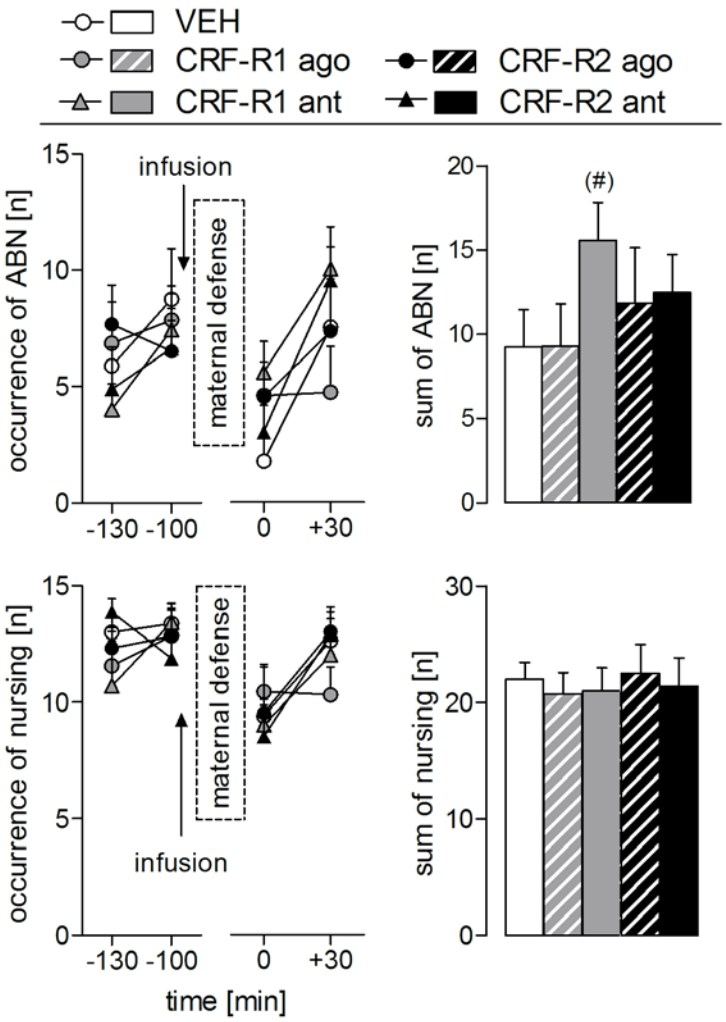

Figure 22. Effect of intra-adBNST CRF-R1 or -R2 specific agonist (ago) or antagonist (ant) treatment on maternal care of lactating dams under stress conditions on LD 7. Arched back nursing (ABN, top) and total nursing (bottom) were scored for 60 min before and after infusion combined with the maternal defense test. The 60 min afterwards are summed up and shown on the right side. For details on treatment see Figure 21. Data is presented as mean + SEM. n = 6 - 8 per group. (#) $p \leq 0.06$ versus VEH (independent t-test).

Maternal motivation on LD 3

None of the treatments affected pup retrieval behavior (pups retrieved within 15 min: VEH: 3.8 ± 1.2, CRF-R1 agonist: 3.3 ± 1.3, CRF-R1 antagonist: 3.4 ± 1.3, CRF-R2 agonist: 1.0 ± 0.8, CRF-R2 antagonist: 2.1 ± 1.1).

Maternal aggression on LD 7

In the maternal defense test, no significant differences were found comparing both aggressive and non-aggressive behaviors between the treatment groups.

Anxiety-related behavior on LD 5

On the EPM, no significant differences were found comparing both the percentage of time on and percentage of entries into the open arms between the treatment groups. Locomotor activity as measured by closed arm entries did not differ.

4.4.2 Experiment 2: Effects of intra-adBNST manipulation of CRF-R1 or -R2 on the HPA axis under basal and stressful conditions

ACTH

ACTH levels in the blood plasma differed depending on time (two-way ANOVA for repeated measures; $F_{1.94,156} = 24.04$, $p < 0.001$; Figure 23, top) and treatment ($F_{4,26} = 16.11$, $p < 0.001$), which also interacted ($F_{7.8,156} = 9.26$, $p < 0.001$; corrected after Greenhouse-Geisser as Mauchly-Test for sphericity revealed $p < 0.001$).

In detail, dams treated with VEH or one of both agonists showed significant changes in ACTH levels over time. In VEH-treated dams, no effects were found immediately after infusion. However, ACTH levels significantly increased at MD+5 compared to basal levels (MD+5 versus basal 1: $p = 0.01$, MD+5 versus basal 2: $p = 0.02$), which again decreased at MD+15 ($p = 0.05$) compared to the previous sample. In CRF-R1 agonist-treated dams, ACTH levels increased immediately after infusion (I+10: $p < 0.001$), decreased at MD+5 ($p = 0.005$) to increase again at MD+15 ($p = 0.02$), and finally decreased at MD+60 ($p < 0.001$) compared to the respective previous samples. In CRF-R2 agonist-treated dams, ACTH levels increased at I+30 compared

to basal levels (I+30 versus basal 1: p = 0.04, I+30 versus basal 2: p = 0.05). No changes over time were observed in the antagonist-treated dams.

Comparing the different treatment groups at various time-points, CRF-R1 agonist infusion resulted in a persistent increase of ACTH levels at all time-points after infusion (p < 0.001, in each case) compared to VEH. No differences were observed in all other treatment groups.

CORT

CORT levels in the blood plasma differed depending on time (two-way ANOVA for repeated measures; $F_{3.8,186}$ = 21.83, p < 0.001; Figure 23, bottom) and treatment ($F_{4,31}$ = 8.46, p < 0.001), which also interacted ($F_{15.3,186}$ = 2.71, p = 0.001; corrected after Greenhouse-Geisser as Mauchly-Test for sphericity revealed p < 0.001).

In detail, except the CRF-R1 antagonist-treated dams all mothers showed significant changes in CORT levels over time. In VEH-treated dams, CORT levels rose at I+10 (p = 0.001, in each case) and at MD+5 (p < 0.001, in each case) compared to both basal levels. Additionally, CORT levels decreased at MD+60 back to basal levels compared to the previous sample (p = 0.001). In CRF-R1 agonist-treated dams, CORT concentrations increased after infusion compared to the previous sample (p < 0.001). In CRF-R2 agonist-treated dams, CORT levels increased after infusion at I+10 (p < 0.001) and I+30 (p = 0.01) compared to the previous sample. After stressor exposure, CORT concentrations decreased at MD+5 (p = 0.04) and MD+15 (p = 0.001) but increased again at MD+60 (p = 0.02) compared to the respective previous samples. In CRF-R1 antagonist-treated dams, a decrease in CORT concentrations was observed at MD+60 (p = 0.02) compared to the previous sample.

Comparing the different treatment groups at various time-points, all dams showed differences in CORT concentrations after infusion compared to VEH-treated rats.

CRF-R1 agonist infusion resulted in an increase of CORT levels at I+30 ($p = 0.002$), MD+15 ($p = 0.02$), and MD+60 ($p = 0.001$). CRF-R1 antagonist treatment significantly prevented a stress-induced increase at MD+15 ($p = 0.008$). Infusion of the CRF-R2 agonist increased CORT concentrations at I+30 ($p = 0.02$). However, in combination with the maternal defense test, CORT concentrations decreased at MD+15 ($p = 0.01$) to finally increase again at MD+60 ($p = 0.01$). Infusion of the CRF-R2 antagonist prevented an infusion- (I+10: $p = 0.02$) and stress-induced (I+5: $p = 0.02$, I+15: $p = 0.03$) increase in CORT levels.

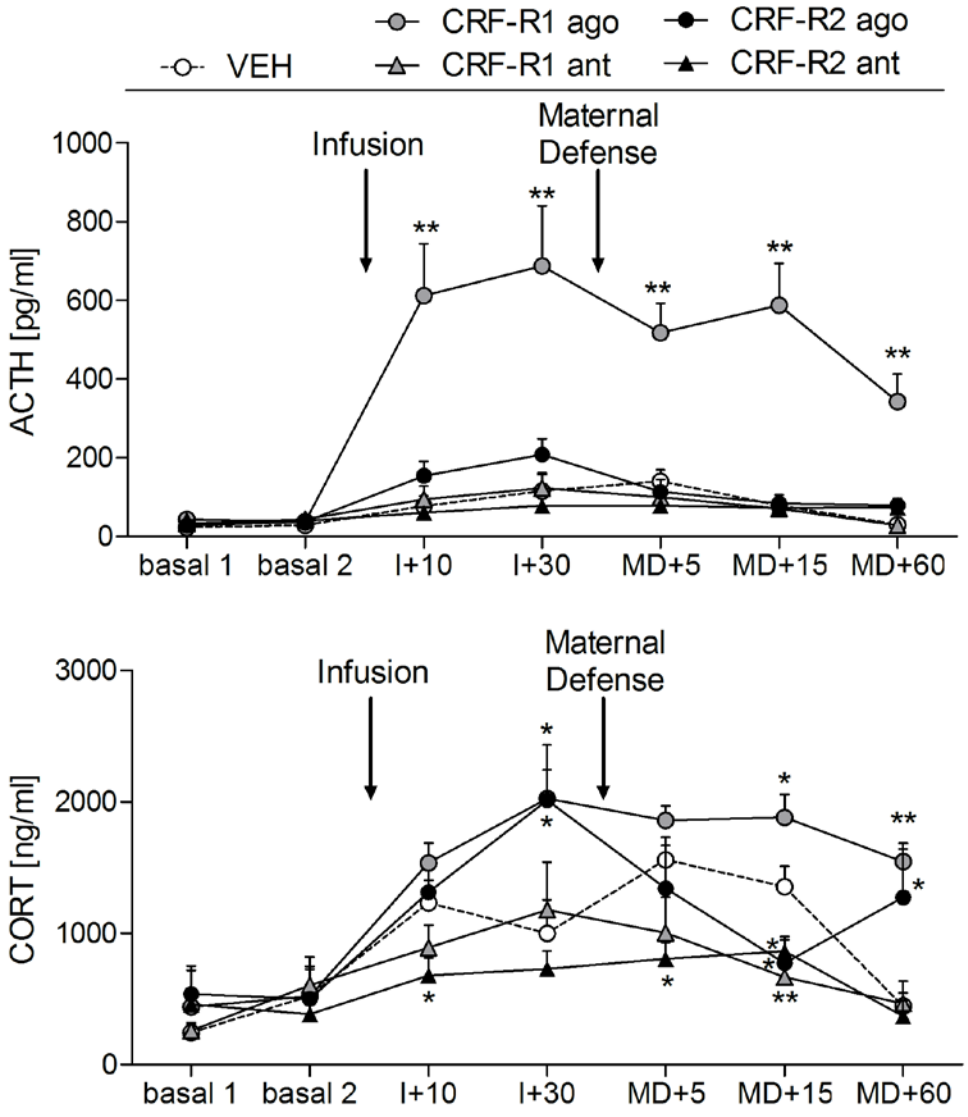

Figure 23. Effect of intra-adBNST CRF-R1 or -R2 specific agonist (ago) or antagonist (ant)

treatment on plasma adrenocorticotropic-hormone (ACTH) and corticosterone (CORT) concentrations. ACTH and CORT were measured under basal conditions (basal 1, basal 2), 10 (I+10) and 30 min (I+30) after infusion, and 5 (MD+5), 15 (MD+15), and 60 min (MD+60) after the maternal defense test. For details on treatment see Figure 21. Data is presented as mean + SEM. n = 5 - 8 per group. For simplification only treatment effects are shown. ** $p \leq 0.01$, * $p \leq 0.05$ versus VEH (two-way ANOVA for repeated measures).

4.5 Discussion

In order to enable the expression of appropriate maternal behavior, the CRF system needs to be down-regulated postpartum (Pedersen et al., 1991; Gammie et al., 2004; Klampfl et al., 2013; Klampfl et al., 2014b). We recently demonstrated that activation of CRF-R, predominantly CRF-R2, in the mpBNST impairs maternal care and aggression while maternal anxiety is decreased by independent inhibition of both receptor types in a sex-specific manner (Klampfl et al., 2014b). Investigating the adBNST we are the first to provide evidence that the CRF system in another functional part of the BNST exerts different roles in mediating maternal behavior. Under basal conditions, activation of CRF-R1 or -R2 resulted either in opposing or similar effects on nursing depending on whether the quality (occurrence of ABN) or quantity (occurrence of total nursing) of maternal care was investigated, respectively. We found that activation of CRF-R1 in the adBNST impaired ABN and total nursing. This data is consistent with our recent study showing a similar impairing effect of CRF-R1 activation in the mpBNST (Klampfl et al., 2014b). The fact that both the quality and quantity of nursing were reduced by CRF-R1 activation in the adBNST and mpBNST (Klampfl et al., 2014b) shows that stimulation of this receptor subtype leads to a general neglect of the offspring during lactation. Importantly, even though inhibition of CRF-R1 revealed increased ABN compared to VEH, this effect can be disregarded as these dams showed similar high levels of ABN already before treatment infusion. Furthermore, activation of CRF-R2 improved ABN, especially 5

hours after infusion. As the CRF-R2 agonist-treated dams showed more ABN before infusion (similar to the CRF-R1 antagonist), the elevated levels at t +60 min can be neglected. However, a strong increase was detected with a delay, i.e. 5 hours after infusion, which most likely reflects a drug-induced change in behavior. This time course of treatment-effectiveness on ABN is similar to that in the mpBNST (Klampfl et al., 2014b) supporting the finding of the drug-induced increase of ABN after 5 hours. However, CRF-R2 activation in the mpBNST is detrimental to ABN, which is in sharp contrast to the current study. This indicates that a potential diffusion to the respective other part of the BNST can be excluded and, more importantly, that the CRF-R2 subtype takes on different functional roles in the regulation of nursing depending on the site of location in the BNST. Moreover, we found that CRF-R2 activation decreased the occurrence of total nursing showing that stimulation of CRF-R2 leads to reduced quantity but improved quality of nursing in a time-dependent context. This implies that CRF-R2 agonist-treated dams nursed less promptly after infusion, which is later even reversed. At that time-point, the dams showed elevated levels of high quality nursing, i.e. ABN. CRF-R2 are generally described as receptors mediating beneficial and stress recovering effects upon activation (Reul and Holsboer, 2002b; Bale and Vale, 2004), which is broadly supported by our study. For example, genetic deletion of CRF-R2 results in stress hypersensitivity and anxiogenesis in male mice (Bale et al., 2002) and impaired maternal aggression in addition to stress hypersensitivity in lactating mice (D'Anna et al., 2008). However, it is important to not generalize the role of the CRF-R2 as beneficial mediator of the stress response but to scrutinize expression sites and temporal context.

After exposure to a strong, psycho-social stressor, i.e. the maternal defense test, only inhibition of CRF-R1 increased ABN by trend compared to VEH. This indicates that a hyperactivation of CRF-R1 in the adBNST might be detrimental to the

expression of appropriate maternal care, even under stressful conditions. However, the receptors need to be activated to a certain extent upon stressor exposure as being aroused and vigilant is indeed vital for the dam to defend her litter (Bosch, 2013). This provides an adequate coping with a potentially dangerous situation to the offspring. Interestingly, our finding is in contrast to the regulation in the mpBNST (Klampfl et al., 2014b) where inhibition of CRF-R2 has similar improving behavioral effects as inhibition of CRF-R1 in the adBNST. These data strongly indicate a different regulatory mechanism of maternal care by CRF-R depending on the temporal context and the site of manipulation within the BNST with the anterior part being dominated by CRF-R1 and the posterior part by CRF-R2.

Assessing maternal motivation in the PRT did not reveal any involvement of the CRF system in this motivated maternal behavior. This result was anticipated as we manipulated receptors in the dorsal but not ventral part of the anterior BNST. The ventral part strongly interconnects with the MPOA, which together form a “maternal super-region” that was shown to regulate maternal motivation (Numan and Insel, 2003). Even though the dorsal division projects to the ventral nuclei (see below) a functional connection important for the control of maternal motivation can likely be excluded. Furthermore, our results are in concordance with our previous study (Klampfl et al., 2014b), hence, further supporting that the CRF system in the BNST, at least in the ad- and mpBNST, is not involved in the regulation of maternal motivation in lactating rats.

Similar to maternal motivation, maternal aggression was not affected by manipulation of CRF-R in the adBNST. In contrast, in the mpBNST, CRF-R2 manipulation has strong effects on maternal aggressive behavior (Klampfl et al., 2014b). The mpBNST, especially the principal and interfascicular nuclei, contains large amounts of CRF-R2 (Klampfl et al., 2014b) and has been implicated in defensive behaviors (Shaikh et al.,

1986; Dong and Swanson, 2004a; Bosch, 2013). Additionally, the pBNST strongly projects to the LS (Dong and Swanson, 2004a), which is known for regulating maternal aggression (D'Anna and Gammie, 2009; Bosch, 2013) whereas the adBNST is lacking such projections (Dong et al., 2001b). This might contribute to the different regulation patterns in the BNST.

Furthermore, we did not find an effect of CRF-R manipulation in the adBNST on anxiety-related behavior in lactating rats. This lack of effect was unexpected as the CRF system in the BNST is well known for regulating anxiety (Walker et al., 2003; Sahuque et al., 2006; Davis et al., 2010; Klampfl et al., 2014b; Tran et al., 2014). Additionally, it was shown that inhibition of CRF-R2 with Astressin-2B in the anterolateral BNST (part of the adBNST) is anxiogenic (Tran et al., 2014). However, it needs to be mentioned that this study was performed in male Fisher-344 rats, which have an abnormally functioning HPA axis and also exhibited extreme low levels of anxiety (80 % time on the open arms), whereas we used female, lactating Sprague-Dawley rats. Due to unusual low anxiety levels in the VEH group (Tran et al., 2014) and sex differences in the regulation of anxiety (Toufexis, 2007; Klampfl et al., 2014b), these studies are hardly comparable. Given the lack of effect on the adBNST in the present study and a strong anxiolytic effect when inhibiting CRF-R in the mpBNST (Klampfl et al., 2014b), we suggest that the CRF system in the BNST differentially regulates anxiety-related behavior; the adBNST appears to be not involved while the mpBNST is an important mediator postpartum.

The BNST is a vital limbic region for signal integration and plays an essential role in the regulation of the HPA axis (Crestani et al., 2013). During lactation, the HPA axis shows blunted responses to stressors and hypercorticism (Stern et al., 1973; Brunton et al., 2008). However, when CORT levels are abnormally elevated, maternal care is affected detrimentally (Brummelte and Galea, 2010b). Therefore, we aimed to

investigate whether manipulation of CRF-R in the adBNST might influence HPA axis activity and, thus, indirectly impact on maternal care during lactation. We found that CRF-R1 activation increased ACTH and CORT levels while CRF-R2 activation only affected the latter. This implicates a different regulation of the HPA axis by the two receptor subtypes. It can be speculated that CRF-R1 and -R2 are located on different neurons with unlike projection sites. An intrinsic connection study revealed no direct projections from the adBNST to the PVN but direct GABAergic innervation of the anterior ventral BNST, i.e. the fusiform nucleus (Turesson et al., 2013). This site, in turn, sends GABAergic projections to the PVN, thereby inhibiting HPA axis-stimulating CRF neurons in the hypothalamus (Crestani et al., 2013). Thus, activation of CRF-R1 could activate the HPA axis via a disinhibition of the GABAergic connection between the BNST and PVN. However, the fusiform nucleus also sends excitatory CRFergic projections to the PVN (Choi et al., 2007), which might be inhibited by stimulation of GABAergic neurons in the adBNST. As this would result in an inhibition of the HPA axis, we assume that activation of CRF-R1 in the adBNST leads to a disinhibition via a GABAergic-mediated mechanism passing the vBNST. Given the increased CORT release without significantly augmenting ACTH after CRF-R2 stimulation, it can be suggested that CORT release is not induced by changes at the hypothalamic and pituitary level but might be stimulated via activation of the sympathetic nervous system. This is supported by studies showing heavy projections from the adBNST to brainstem nuclei (Dong et al., 2001b; Herman et al., 2003; Spencer et al., 2005). These neuronal interconnections are important for the regulation of autonomic functions and might induce sympathetic activation of CORT release from the adrenals via a CRF-R2-mediated mechanism. However, as both agonists lead to a strong activation of the HPA axis on the adrenal level, a ceiling effect in terms of CORT release is also feasible, which would not allow detecting

differences as seen on the pituitary level with ACTH. Interestingly, CRF-R2 activation-induced CORT release is drastically decreased when a strong stressor is added. It can be suggested that CRF-R2 are located on neuronal populations which change their influencing activity on the HPA axis by stress experience; for example, NAergic neurons inhibit HPA axis responses to acute stress (Forray and Gysling, 2004). This might lead to a switch in CORT release during the endogenous stress response. Our data demonstrates that CRF-R manipulation in the adBNST strongly influences HPA axis activity depending on the stress context. Therefore, an indirect regulation of maternal care via a hyper-activation of the HPA axis is conceivable. In order to demonstrate such a relation, further studies are needed that investigate potential effects on maternal care after acute CORT treatment as chronic CORT administration decrease maternal care (Brummelte and Galea, 2010b). Additionally, effects of intra-adBNST CRF-R activation with a concomitant disabled HPA axis on maternal behavior needs to be studied.

The present data on the adBNST together with data from our previous study on the mpBNST (Klampfl et al., 2014b) demonstrate the tremendous heterogeneity of the BNST in terms of behavioral variability regulated by one neuropeptidergic system within various subdivisions of the BNST. The different behavioral outcomes of CRF-R manipulation in the anterior or posterior part of the BNST can likely be explained by the different input and projection sites in the respective division (Dong et al., 2001a; Dong et al., 2001b; Dong and Swanson, 2004a, b, 2006). Importantly, the two divisions of the BNST also vary in the distribution of CRF system components. CRF-ir cell bodies and moderate to dense accumulations of CRF-ir fibres with appearance of terminal fields are concentrated within the anterior division (Ju et al., 1989) whereas a natural ligand for CRF-R2, i.e. Ucn 3, is primarily found in the posterior part (Li et al., 2002). These different neuroanatomical and neurochemical profiles can certainly

result in the distinct mediation of maternal behavior depending on the site of neuronal activation.

In conclusion, we showed that manipulation of the CRF system in the BNST exerts different effects on maternal and anxiety-related behavior depending on the manipulated subdivision. Furthermore, an indirect and stress-dependent regulation of maternal care via the HPA axis is feasible. These results demonstrate the huge complexity of the maternal brain and physiology, which, in cases of dysregulations, requires specificity on the one hand and systemic approaches on the other. Thus, systemic treatments need to be highly individualized in order to reach a perfect balance that is vital for the health of both the mother and the offspring.

- Chapter 5 -

The CRF binding protein:

an underestimated brain neuropeptide indispensable for the

expression of maternal behavior in lactating rats

Authors‘ contribution:

Stefanie Klampfl:	experimental design, performance of experiments, data analysis, first draft of manuscript
Doris Bayerl:	performance of experiments
Oliver Bosch:	experimental design, performance of experiments, revision of manuscript

5.1 Abstract

Stimulation of CRF-R postpartum revealed detrimental effects on maternal behavior, which renders a down-regulation of receptor activity essential during lactation. This down-regulation might be partly achieved and supported by the CRF-BP. The CRF-BP is an important regulator of CRF/Ucn 1 as it most likely sequesters these ligands away from CRF-R. Thus, we investigated potential effects of acute CRF-BP inhibition with $CRF_{(6-33)}$, thereby increasing the endogenously available CRF/Ucn 1, either centrally or locally within the mpBNST or the adBNST on maternal behavior as well as anxiety-related and depressive-like behavior in lactating rats.

We found that CRF-BP inhibition had no effect on maternal care under non-stress conditions. However, following stressor exposure, i.e. the maternal defense test, dams showed impaired recovery from the stress-induced drop in nursing after infusion of the inhibitor only into the mpBNST. During the maternal defense test, icv and intra-mpBNST CRF-BP inhibition increased maternal threat behavior against a virgin intruder. Anxiety-related behavior on the EPM was only increased by CRF-BP inhibition in the mpBNST. No differences were found in the FST after icv inhibition. Moreover, no behavioral effects were detected following CRF-BP inhibition in the adBNST in lactating rats.

The present study demonstrates an indispensable involvement of the CRF-BP, especially within the mpBNST, in the regulation of behavioral adaptations on the CRF level postpartum. It is important for ending the stress response, for modulating threat behavior as part of maternal aggression and for maintaining a hypo-anxious state in lactating rats. Thus, the CRF-BP contributes to the postpartum-associated down-regulation of the CRF system.

5.2 Introduction

The CRF-BP is a 37 kDa secreted glycoprotein and binds CRF and Ucn 1 with similarly high or even greater affinity than the CRF-R (Sutton et al., 1995; Westphal and Seasholtz, 2006). Despite sharing ligands, CRF-R are structurally unrelated to the the CRF-BP (Orth and Mount, 1987; Behan et al., 1989). It binds CRF at a different amino acid sequence than the receptors (Behan et al., 1995b) and an association to the cell membrane is questionable due to the lack of transmembrane domains or a phosphatidyl inositol anchor signal motif (Potter et al., 1991; Cortright et al., 1995). The CRF-BP is found in various peripheral structures like placenta, amniotic fluid and plasma, where it is implicated in human parturition (Fadalti et al., 2000). However, it is also expressed abundantly in cerebral neurons and astrocytes (Behan et al., 1995a), including cortical and subcortical limbic structures like the BNST, amygdala, hippocampus and various hypothalamic nuclei (Potter et al., 1992). Histological studies provide evidence for a large co-localization of CRF-BP with CRF and CRF-R expression sites in the BNST, LS, CeA and MPOA (Potter et al., 1992). This co-localization places the CRF-BP in an important regulatory position between CRF and its receptors in these limbic brain regions. However, the CRF-BP is also expressed in numerous brain areas where its ligands are missing, thus, suggesting additional roles for this peptide (Potter et al., 1992).

Intriguingly, very little is known about the functional roles of the CRF-BP. Genetic mouse models have been created to elucidate physiological, neuroendocrine, and behavioral effects of CRF-BP overexpression (Burrows et al., 1998; Lovejoy et al., 1998) or deficiency (Karolyi et al., 1999)(for review see Seasholtz et al., 2001). For example, deletion of the CRF-BP gene results in increased anxiety-related behavior in males (Karolyi et al., 1999) and impaired maternal aggression in lactating female mice (Gammie et al., 2008) without affecting the stress axis (Seasholtz et al., 2001).

In order to manipulate the CRF-BP in rats, the peptide can be inhibited by a truncated version of CRF, i.e. $CRF_{(6-33)}$, which binds with high affinity exclusively to the CRF-BP, displaces bound CRF/Ucn 1 and, thus, increases 'free' extracellular CRF concentrations (Behan et al., 1995c). In this context, it was shown that CRF-BP inhibition leads to cognition-enhancing properties in models of learning and memory. However, barely any other behavior has been investigated using this elegant technique.

Given that the CRF-BP is apparently an important regulator of CRF, its related peptides, and CRF-R and that the CRF-BP influences behaviors like cognition, anxiety, and murine maternal aggression, similar regulatory activities might also impact other behaviors mediated by the CRF system. We have recently demonstrated that hypoactivation of CRF-R (Klampfl et al., 2013), predominantly type 2 in the mpBNST (Klampfl et al., 2014b) and type 1 in the adBNST (Klampfl et al., 2014a), is essential for the adequate expression of maternal behavior in lactating rats. Given that maternal behavior is the most important pro-social behavior in females, being vital for the survival of the offspring (Bosch, 2011), potential dysregulations of its regulators like the CRF system would be detrimental to the occurrence of maternal behavior. Thus, it is important to know the underlying mechanisms for the essential hypoactivation of CRF-R postpartum but these are still largely unclear. The CRF-BP could represent an interesting target and might be involved in the down-regulation of the CRF system during lactation and, hence, in the regulation of maternal behavior.

Therefore, in the present study, we investigated behavioral effects of CRF-BP inhibition either centrally or locally in the adBNST or mpBNST. We focused on the display of maternal care both under non-stress and stress conditions and on maternal aggression. Given the important role of the CRF system in the expression of

anxiety and depression (Reul and Holsboer, 2002b, a), we also assessed behavioral effects of CRF-BP inhibition on anxiety-related and depressive-like behavior in early lactating rats.

5.3 Materials & Methods

5.3.1 Animals

Virgin female Sprague-Dawley rats (220 – 250 g; Charles River Laboratories, Sulzfeld, Germany) were kept under standard laboratory conditions (change of bedding once per week, RT 22 ± 2 °C, 55 % relative humidity, 12 : 12 h light / dark cycle, lights on at 6 a.m.) with access to water and standard rat chow *ad libitum*. Females were mated and housed in groups of 3 to 4 rats until PD 18 where they underwent surgery. Afterwards, they were single-housed to guarantee recovery and undisturbed delivery (Klampfl et al., 2013). During the prepartum single-housing period (except the day before and the day of delivery), all rats were handled twice daily to reduce non-specific stress responses during the experiments (Neumann et al., 1998b). On the day of birth, litters were culled to eight pups of mixed sexes.

For the maternal defense test, naïve virgin female Wistar rats (200 – 220 g, Charles River Laboratories) were used as intruders at random stages of their estrous cycle. Intruder rats were kept in a separate room to avoid olfactory recognition.

The experiments were approven by the Committee on Animal Health and Care of the local government and conformed to international guidelines on the ethical use of animals. All efforts were made to minimize the number of rats used and their suffering.

5.3.2 Behavioral tests

Maternal care. Maternal care was monitored on LD 1 before and after substance infusion (non-stress condition) as well as on LD 5 before and after substance infusion, which was combined with a psychosocial stressor (i.e. the maternal defense test (Neumann et al., 2005a); stress condition). We acknowledge that there is a limited amount of stress associated with the infusion procedure, though the 'non-stress' term is used to distinguish between the maternal care observations on LD 1 which did not involve the maternal defense test from those conducted on LD 5 which did. Observations were conducted for 10 s every 2nd min in 30 min blocks according to an established protocol (Bosch and Neumann, 2008). In detail, on LD 1, dams were observed under non-stress conditions from 8 – 9 a.m., infused at 9 a.m. and observation continued 30 min after infusion from 9.30 – 11 a.m. Additionally, dams were observed from 2 – 3 p.m. to assess potential long-lasting effects of drug treatment. On LD 5, dams were observed from 8 – 9 a.m., transported to another room, and infused at 10 a.m. Dams were tested 20 min after infusion in the maternal defense test, immediately afterwards transported back to the observation room, and maternal care was observed for another 60 min in order to assess effects of the stressor on maternal care. The main parameter for the quality of maternal care was the occurrence of ABN (Bosch, 2011), the only active nursing posture where the dam is engaged in a quiescent kyphosis (Stern and Johnson, 1990). Other behavioral parameters scored were hovering over the pups and blanket nursing posture, which together with ABN were counted as total nursing, thereby indicating the quantity of maternal care (Klampfl et al., 2014b). Pup retrieval/mouthing and licking/grooming were also scored. Additionally, non-maternal behaviors were scored, i.e. locomotion (including digging/burrowing and cage exploration), self-grooming, and sleeping/resting, which were summed up and are presented as off-nest behavior.

Data is shown in 30 min blocks before and after treatment infusion with a maximal count of 15 observations per block.

Maternal aggression. To assess maternal aggression, the maternal defense test was performed on LD 5 in a separate room, to which the dams were transported 60 min prior to the test (see above). Twenty min after treatment infusion, the lactating residents were confronted with an unknown virgin female intruder in their home cage in the presence of the litter for 10 min as described previously (Neumann et al., 2001; Bosch et al., 2005). The dam's behavior was videotaped for subsequent analysis by an experienced observer blind to the treatment. The following behavioral parameters were scored: total number of attacks, latency to first attack, lateral threat, keep down, and offensive upright as well as non-aggressive behaviors (for detailed description see Bosch, 2013).

Anxiety-related behavior. Anxiety-related behavior was tested on the EPM on LD 3 as described earlier (Pellow et al., 1985; Neumann et al., 2000). Briefly, the plus shaped maze consists of two open arms (50 x 10 cm^2, 80 lux) and two closed arms (50 x 10 x 30 cm^3, 10 lux) surrounding a neutral square shaped central zone (10 x 10 cm^2, 65 lux) and is elevated 82 cm over the floor. Twenty min after infusion, the rats were placed in the neutral zone of the maze and were allowed to freely explore the maze for 5 min. The percentage of time spent on the open arm versus all areas (open arm, closed arm, and neutral zone) and the percentage of open arm entries versus all entries (open and closed arms) are indicators for anxiety-related behavior. The number of closed arm entries was used to measure locomotion (Neumann et al., 2000).

Depressive-like behavior. The FST (Porsolt et al., 1977) was used to assess depressive-like behavior on LD 7 in a single test session (Cryan and Mombereau, 2004; Cryan et al., 2005). Twenty min after infusion, the rats were placed in the FST container (49 cm high, 30 cm diameter, filled up to 37 cm with tap water, 22 - 24°C, changed for each rat) for 10 min. The dams' behavior was videotaped for subsequent analysis by an experienced observer blind to the treatment. The following behavioral parameters were scored: (1) struggling, defined as movements during which the forepaws break the water's surface, (2) swimming, defined as movements of all four paws resulting in forward motion without breaking the water surface, and (3) floating, defined as movements that are necessary to prevent drowning as an indicator of passive stress-coping.

5.3.3 Experimental design

Experiment 1: Icv inhibition of the CRF-BP in lactating rats

On PD 18, females were implanted with a 21 G guide cannula targeting the right lateral ventricle (1.0 mm caudal, 1.6 mm lateral, 2.1 mm ventral to bregma (Paxinos and Watson, 1998)) under inhalation anesthesia (Isoflurane; Baxter Germany GmbH, Unterschleißheim, Germany) and semi-sterile conditions as described earlier (Bosch et al., 2010). On the experimental days, a 25 G infusion cannula, prepared as previously described (Neumann et al., 2000), was used to infuse either (i) VEH (5 µl sterile Ringer´s solution; pH 7.4; Braun, Melsungen, Germany) or the CRF-BP inhibitor $CRF_{(6\text{-}33)}$ (5 µg / 5 µl; Bachem, Bubendorf, Switzerland). The dose was chosen based on a previous study (Zorrilla et al., 2001).

On LD 1, 3, 5, and 7, lactating dams received a single acute icv infusion 20 min prior to the tests. Each animal received the same treatment on every testing day as assigned on the first testing day. In each case, dams were immediately returned to

their home cage after infusion. Maternal care was observed under non-stress conditions (LD 1) and stress conditions (LD 5) in the home cage. Additionally, anxiety-related behavior (LD 3), maternal aggression (LD 5), and depressive-like behavior (LD 7) were tested. All tests were performed between 8 a.m. and 3 p.m. in the light phase of the cycle.

Experiments 2 and 3: Intra-BNST inhibition of the CRF-BP in lactating rats

On PD 18, females were implanted bilaterally with 23 G guide cannula targeting either the mpBNST (experiment 2: 0.7 mm caudal, 1.5 mm lateral, 4.5 mm ventral to bregma) or the adBNST (experiment 3: -0.2 mm caudal, 3.0 mm lateral, 4.9 mm ventral to bregma (Paxinos and Watson, 1998) with an angle of 12.5°) under inhalation anesthesia (Isoflurane; Baxter Germany GmbH, Unterschleißheim, Germany) and semi-sterile conditions as described earlier (Bosch et al., 2010). Substances were infused using a 30 G infusion cannula (Neumann et al., 2000). Lactating rats received either (i) VEH (0.5 µl of sterile Ringer's solution; pH 7.4; Braun) or the CRF-BP inhibitor $CRF_{(6\text{-}33)}$ (5 µg / 0.5 µl; Bachem, Bubendorf, Switzerland). The dose was adapted from experiment 1.

On LD 1, 3, and 5, lactating dams received a single acute bilateral infusion 20 min prior to the tests. Each animal received the same treatment on every testing day as assigned on LD 1. After infusion, dams were immediately placed back to their home cages. Maternal care was observed under non-stress conditions (LD 1) and stress conditions (LD 5) in the home cage as described above. Additionally, anxiety-related behavior (LD 3), and maternal aggression (LD 5) were tested. The FST was not performed as we found no effects after icv manipulation. All tests were performed between 8 a.m. and 3 p.m. in the light phase of the cycle.

5.3.4 Histology

At the end of the behavioral experiments, rats were decapitated. To verify the correct placements of the icv cannula brains were infused with 5 µl of ink (experiment 1; Pelikan Ink 4001, Hanover, Germany), removed and cut at the site of implantation with a razor blade. A bluish colored ventricle indicated the correct placement of the cannula. To verify the correct placements of the local cannula brains were infused with 0.5 µl of ink (experiment 2 and 3; Pelikan Ink 4001; diluted 1:20 in Ringer's solution), removed, flash frozen, cut in 40 µm coronal sections, and slide mounted. Ink diffusion was assessed to exclude any diffusion to other parts of the BNST. Afterwards, slides were stained via quick Nissl staining to locate the tip of the infusion cannula. Only rats with correctly fitted cannula were included in the statistical analysis.

5.3.5 Statistical analysis

Data were analyzed using either independent t-test (factor: treatment) or two-way ANOVA for repeated measures (factors: time x treatment) followed by Fisher's LSD *post hoc* test. For all tests, the software package SPSS 19.0 (IBM) was used. Data are presented as means ± S.E.M. and significance was accepted at $p \leq 0.05$.

5.4 Results

5.4.1 Experiment 1: Behavioral effects of icv inhibition of the CRF-BP

Maternal care under non-stress conditions on LD 1

ABN: Differences in ABN were found depending on time (two-way ANOVA for repeated measures; $F_{6,114} = 3.51$, $p = 0.003$; Figure 24A) but not on treatment. No interaction was detected.

Nursing: Differences in nursing were found depending on time (two-way ANOVA for repeated measures; $F_{6,114} = 3.26$, $p = 0.005$; Figure 24A) but not on treatment. No interaction was detected.

Other maternal behaviors: Licking/grooming differed depending on time (two-way ANOVA for repeated measures; $F_{6,114} = 2.26$, $p = 0.04$) but not on treatment neither was an interaction detected. No differences were found for pup retrieval/mouthing. We did not observe any pup-killing following any treatment.

Non-maternal behaviors: Differences in off-nest behavior and locomotion were found depending on time (two-way ANOVA for repeated measures; off-nest: $F_{6,114} = 2.99$, $p = 0.009$; locomotion: $F_{6,114} = 2.90$, $p = 0.01$; Table 9) but not on treatment neither was an interaction detected. No differences were found for self-grooming and sleeping/resting.

Maternal care under stress conditions on LD 5

ABN: Differences in ABN were found depending on time (two-way ANOVA for repeated measures; $F_{3,57} = 5.82$, $p = 0.002$; Figure 24B) but not on treatment neither was an interaction detected.

Nursing: Differences in nursing were found depending on time (two-way ANOVA for repeated measures; $F_{3,57} = 4.63$, $p = 0.006$; Figure 24B) but not on treatment neither was an interaction detected.

Other maternal behaviors: No significant differences or interactions depending on time and/or treatment were found in licking/grooming and pup retrieval/mouthing (data not shown). We did not observe any pup-killing following any treatment.

Non-maternal behaviors: Differences depending on time were found for off-nest behavior (two-way ANOVA for repeated measures; $F_{3,57} = 4.51$, $p = 0.007$; Table 10), for locomotion ($F_{3,57} = 8.57$, $p < 0.001$), and for self-grooming ($F_{3,57} = 3.32$, $p = 0.03$)

but not for sleeping/resting. No differences were found depending on treatment neither were interactions detected.

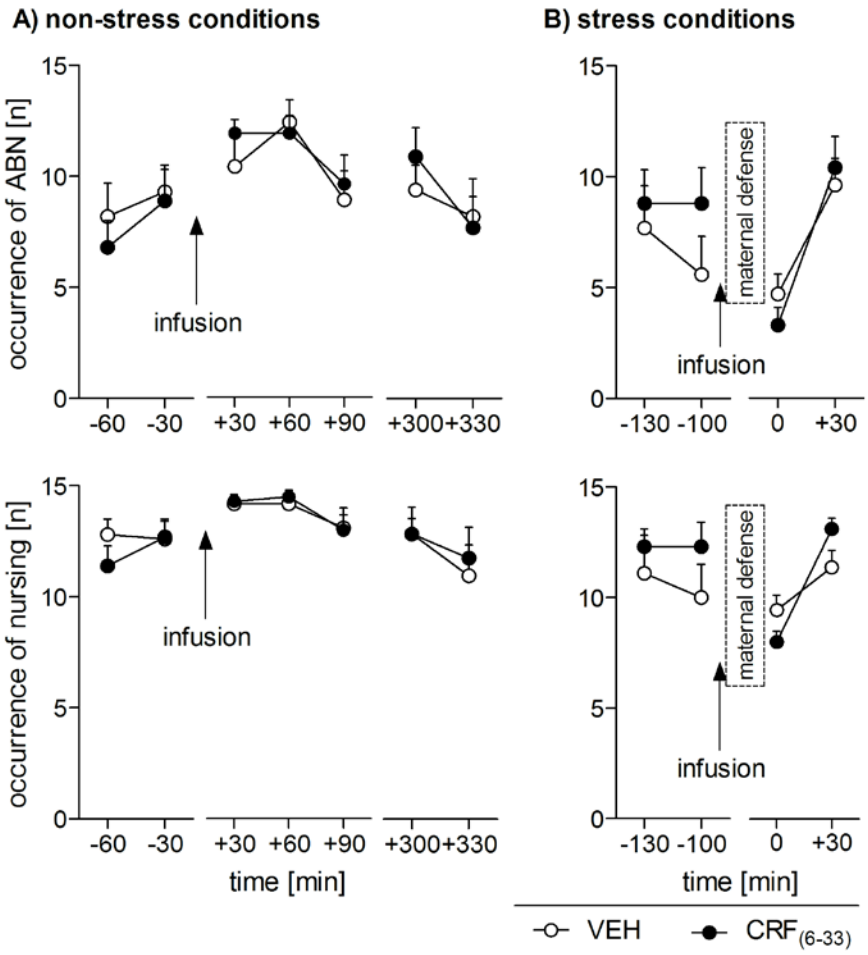

Figure 24. Effects of icv CRF-BP inhibition on maternal care under non-stress conditions on LD 1 (A) and stress conditions on LD 5 (B). ABN (top) and nursing (bottom) were scored for 60 min before and for 90 min after infusion (A) or 60 min after maternal defense (B). Under non-stress conditions, ABN and nursing were also observed for additional 60 min in the afternoon (A). Dams received an acute icv infusion of VEH (5 µl of sterile Ringer's solution) or the CRF-BP inhibitor $CRF_{(6-33)}$ (5 µg / 5 µl). Data are presented as mean + SEM. n = 9 – 12 per group.

Maternal aggression on LD 5

In the maternal defense test, $CRF_{(6-33)}$-treated dams showed more keep down (independent t-test; $t_{19} = -2.49$, $p = 0.02$; Figure 25) and spent more time displaying aggressive behavior ($t_{19} = -2.75$, $p = 0.01$) compared to VEH. At the same time, these

dams showed less exploration of the intruder (VEH: 70.9 ± 15.2 s, $CRF_{(6-33)}$: 36.0 ± 5.4 s; $t_{10.01}$ = 2.17, p = 0.05). No differences were found in the number of attacks, attack latency, offensive upright or in any other behavior.

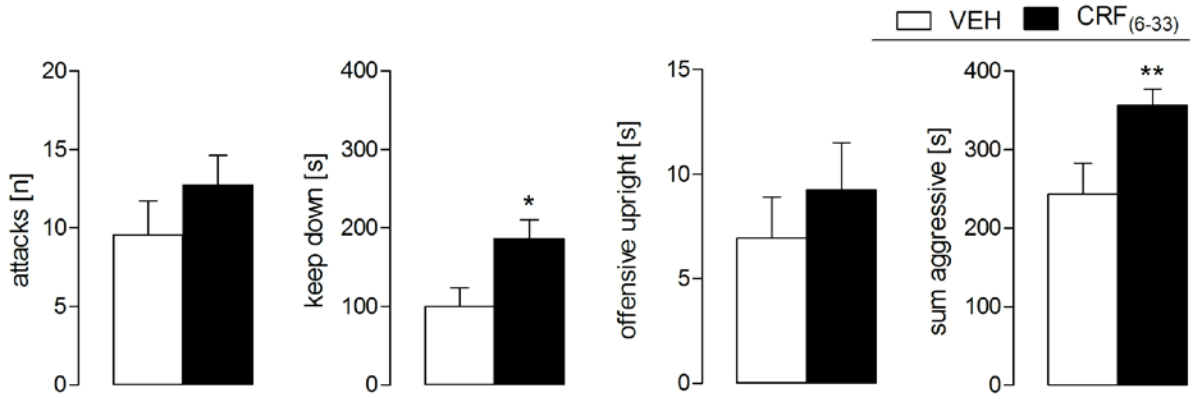

Figure 25. Effect of icv CRF-BP inhibition on maternal aggression of lactating rats measured in the maternal defense test. Maternal aggression against a virgin female intruder was scored during the 10 min trial. Number of attacks, keep down, offensive upright and the sum of aggressive behaviors by the resident is shown. For details on treatment, see legend to Figure 24. Data are presented as mean + SEM. n = 9 – 12 per group. ** p ≤ 0.01, * p ≤ 0.05 versus VEH (independent t-test).

Anxiety-related behavior on LD 3

On the EPM, no significant differences were found in the percentage of time spent on the open arms or of entries into the open arms between the groups. However, CRF-BP inhibitor-treated dams showed less entries into the closed arms compared to VEH (independent t-test; t_{16} = 2.36, p = 0.03; Figure 26) indicative of decreased locomotion.

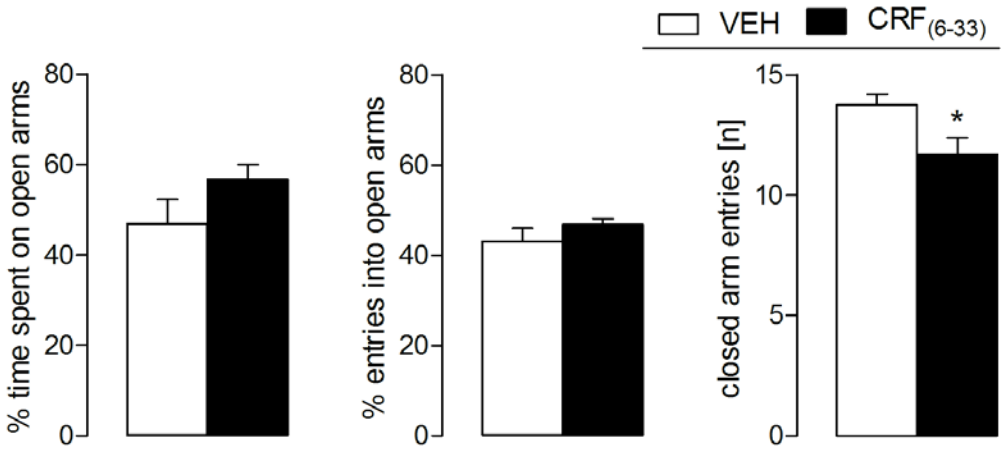

Figure 26. Effect of icv CRF-BP inhibition on anxiety-related behavior of lactating rats measured on the EPM. The percentage of time spent on the open arms (left) or the percentage of entries into the open arms (middle) were assessed to measure anxiety-related behavior. Closed arm entries (right) reflect locomotion on the EPM. For details on treatment see legend to Figure 24. Data are presented as mean + SEM. n = 9 – 12 per group. * $p \leq 0.05$ versus VEH (independent t-test).

Depressive-like behavior on LD 7

In the FST, no significant differences were detected with respect to struggling (VEH: 109.0 ± 11.1 s, $CRF_{(6-33)}$: 119.0 ± 9.9 s), swimming (VEH: 0.5 ± 0.3 s, $CRF_{(6-33)}$: 0.9 ± 0.4 s), or floating (VEH: 483.5 ± 10.3 s, $CRF_{(6-33)}$: 464.1 ± 14.6 s).

5.4.2 Experiment 2: Behavioral effects of local inhibition of the CRF-BP in the mpBNST

Maternal care under non-stress conditions on LD 1

ABN: No significant differences or interactions in ABN were observed depending on time and/or treatment (Figure 27A).

Nursing: No significant differences or interactions in nursing were detected depending on time and/or treatment (Figure 27A).

Other maternal behaviors: No significant differences or interactions in licking/grooming and retrieval/mouthing were observed depending on time and/or

treatment (data not shown). We did not observe any pup-killing following any treatment.

Non-maternal behaviors: No significant differences or interactions in off-nest behavior, self-grooming, and locomotion were found depending on time and/or treatment (Table 11). No differences in sleeping/resting were detected depending on time or treatment, but a significant interaction was found (two-way ANOVA for repeated measures; $F_{6,108} = 2.29$, $p = 0.04$). Post hoc testing revealed no relevant differences.

Maternal care under stress conditions on LD 5

ABN: Differences in ABN were found depending on time (two-way ANOVA for repeated measures; $F_{3,51} = 3.76$, $p = 0.01$; Figure 27B) but not on treatment. No significant interaction was detected.

Nursing: Differences in nursing were found depending on time ($F_{3,51} = 3.97$, $p = 0.01$; Figure 27B) but not on treatment. No significant interaction was detected. However, an independent t-test revealed significantly less nursing in $CRF_{(6\text{-}33)}$-treated dams at t +30 min compared to VEH ($t_{17} = 2.42$, $p = 0.02$).

Other maternal behaviors: No significant differences or interactions in licking/grooming and retrieval/mouthing were observed depending on time and/or treatment (data not shown). We did not observe any pup-killing following the treatments.

Non-maternal behaviors: Differences depending on time were found for off-nest behavior (two-way ANOVA for repeated measures; $F_{3,51} = 4.74$, $p = 0.005$; Table 12), self-grooming ($F_{3,51} = 5.44$, $p = 0.003$) and locomotion ($F_{3,51} = 16.5$, $p < 0.001$). No treatment effects or interactions were detected. No significant differences or interactions in sleeping were observed depending on time and/or treatment.

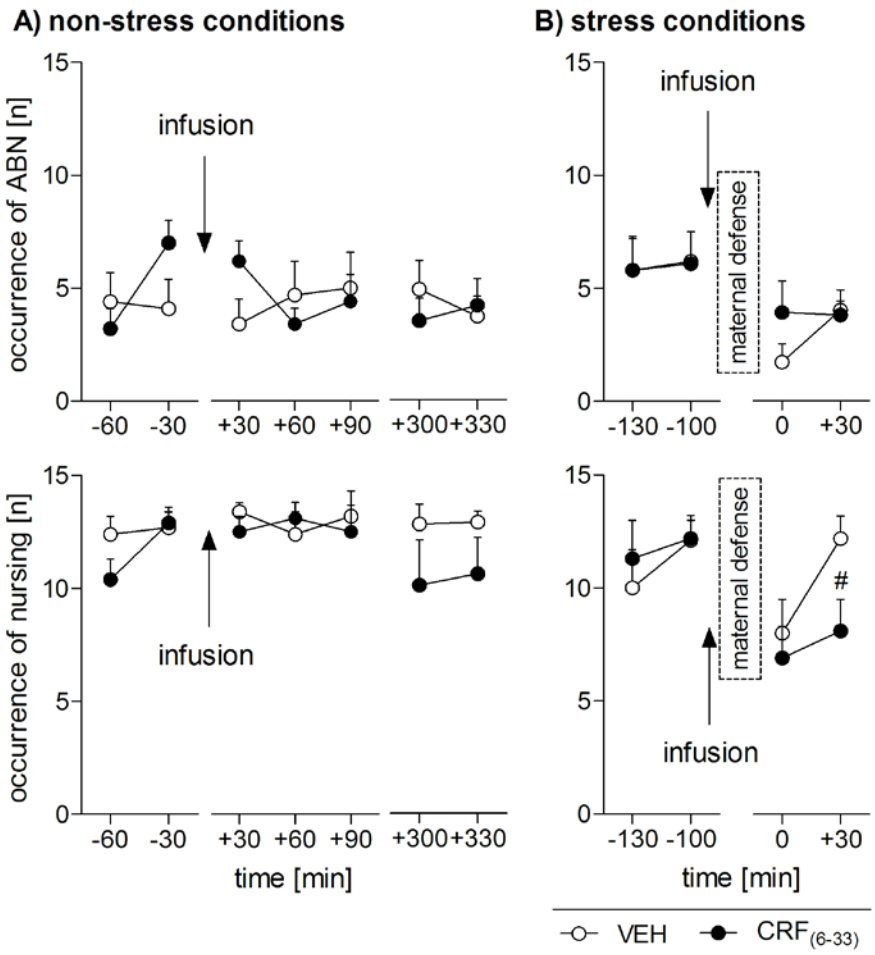

Figure 27. Effects of intra-mpBNST CRF-BP inhibition on maternal care under non-stress conditions on LD 1 (A) and stress conditions on LD 5 (B). ABN (top) and nursing (bottom) were scored for 60 min before and for 90 min after infusion (A) or 60 min after maternal defense (B). Under non-stress conditions, ABN and nursing were also observed for additional 60 min in the afternoon (A). Dams received an acute intra-mpBNST infusion of VEH (0.5 µl of sterile Ringer's solution) or the CRF-BP inhibitor $CRF_{(6-33)}$ (5 µg / 0.5 µl). Data are presented as mean + SEM. n = 9 per group. # $p \leq 0.05$ versus VEH (independent t-test).

Maternal aggression on LD 5

In the maternal defense test, $CRF_{(6-33)}$-treated dams showed more offensive upright compared to VEH (independent t-test; $t_{8.29} = -2.66$, $p = 0.02$; Figure 28). No significant differences were found in attack latency (data not shown), the number of attacks, keep down, or the sum of aggressive behaviors (Figure 28).

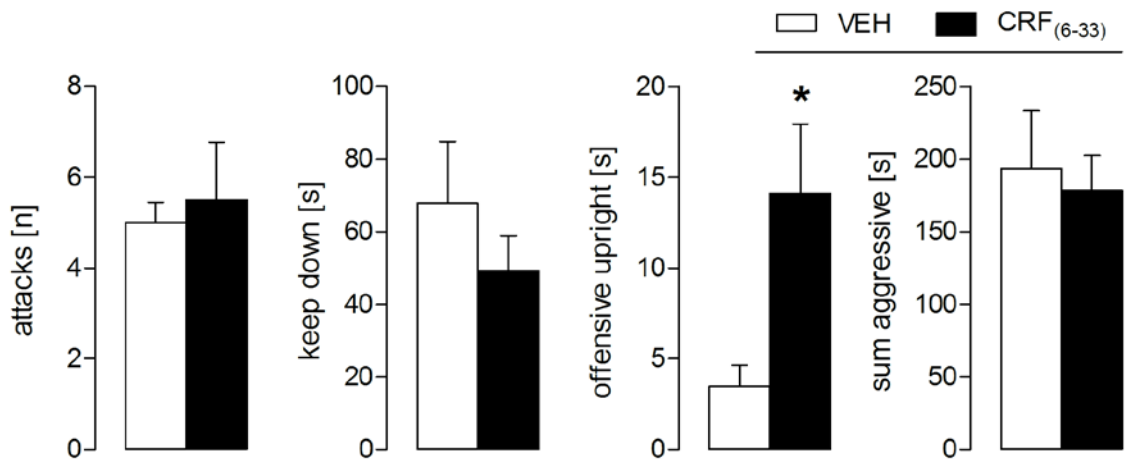

Figure 28. Effect of intra-mpBNST CRF-BP inhibition on maternal aggression of lactating rats measured in the maternal defense test. Maternal aggression against a virgin female intruder was scored during the 10-min trial. Number of attacks, keep down, offensive upright, and the sum of aggressive behaviors by the resident are shown. For details on treatment see legend to Figure 27. Data are presented as mean + SEM. n = 8 - 9 per group. * $p \leq 0.05$ versus VEH (independent t-test).

Anxiety-related behavior on LD 3

On the EPM, $CRF_{(6-33)}$-treated dams showed a reduced percentage of entries into the open arms (independent t-test; $t_{13} = 3.91$, $p = 0.002$; Figure 29) and increased entries into the closed arms ($t_{13} = -3.48$, $p = 0.004$) as indicator of locomotion. No significant difference was found in the percentage of time spent on the open arms.

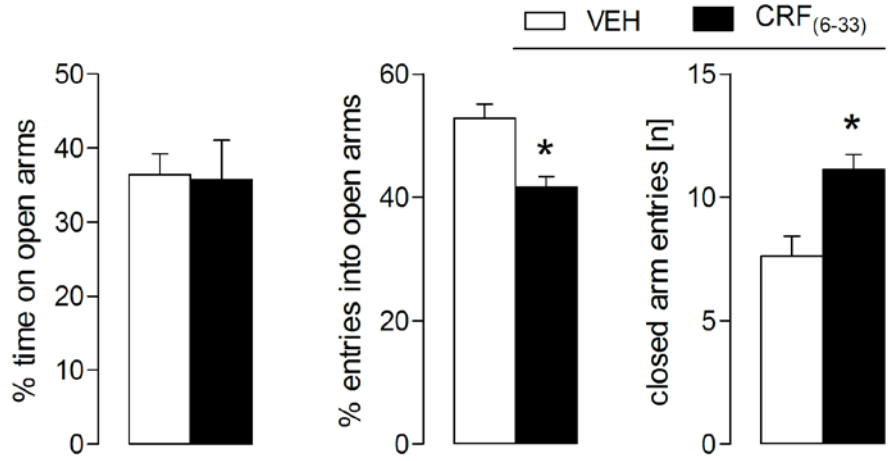

Figure 29. Effect of intra-mpBNST CRF-BP inhibition on anxiety-related behavior of lactating

rats measured on the EPM. The percentage of time spent on the open arms (left) or the percentage of entries into the open arms (middle) were assessed to measure anxiety-related behavior. Closed arm entries (right) reflect locomotion on the EPM. For details on treatment see legend to Figure 27. Data are presented as mean + SEM. n = 7 – 9 per group. * $p \leq 0.05$ versus VEH (independent t-test).

5.4.3 Experiment 3: Behavioral effects of local inhibition of the CRF-BP in the adBNST

Maternal care under non-stress conditions on LD 1

ABN: No significant differences or interactions in ABN were observed depending on time and/or treatment (Figure 30A).

Nursing: No significant differences or interactions in nursing were detected depending on time and/or treatment (Figure 30A).

Other maternal behaviors: No significant differences or interactions in licking/grooming and retrieval/mouthing were observed depending on time and/or treatment (data not shown). We did not observe pup-killing following any treatment.

Non-maternal behaviors: No significant differences or interactions were found depending on time and/or treatment (Table 13).

Maternal care under stress conditions on LD 5

ABN: Differences in ABN were found depending on time (two-way ANOVA for repeated measures; $F_{3,30} = 3.15$, $p = 0.04$; Figure 30B) but not on treatment. No significant interactions were detected.

Nursing: Differences in nursing were found depending on time (two-way ANOVA for repeated measures; $F_{3,30} = 8.21$, $p < 0.001$; Figure 30B) but not on treatment. A trend for a significant interaction was detected ($F_{3,30} = 2.59$, $p = 0.07$).

Other maternal behaviors: No significant differences or interactions in licking/grooming and retrieval/mouthing were observed depending on time and/or treatment (data not shown). We did not observe pup-killing following any treatment.

Non-maternal behaviors: Differences in off-nest behavior were found depending on time (two-way ANOVA for repeated measures; $F_{3,30} = 7.67$, $p = 0.001$; Table 14) but not on treatment. Additionally, a significant interaction was detected ($F_{3,30} = 4.15$, $p = 0.01$). In detail, off-nest behavior increased at t 0 min in $CRF_{(6-33)}$-treated dams ($p = 0.001$) and decreased again at t +30 min ($p = 0.004$) compared to the respective previous time-point.

Differences in self-grooming were found depending on time (two-way ANOVA for repeated measures; $F_{3,30} = 8.47$, $p < 0.001$) but not on treatment. Additionally, a significant interaction was detected ($F_{3,30} = 2.82$, $p = 0.05$). In detail, self-grooming increased at t 0 min in $CRF_{(6-33)}$-treated dams ($p = 0.003$) and decreased again at t +30 min ($p = 0.008$) compared to the respective previous time-point. Moreover, $CRF_{(6-33)}$-treated dams showed more self-grooming at t +30 min compared to VEH ($p = 0.04$).

Differences in locomotion were found depending on time (two-way ANOVA for repeated measures; $F_{3,30} = 8.26$, $p < 0.001$) but not on treatment. No significant interaction was detected.

No significant differences or interactions in sleeping/resting were found depending on time and/or treatment.

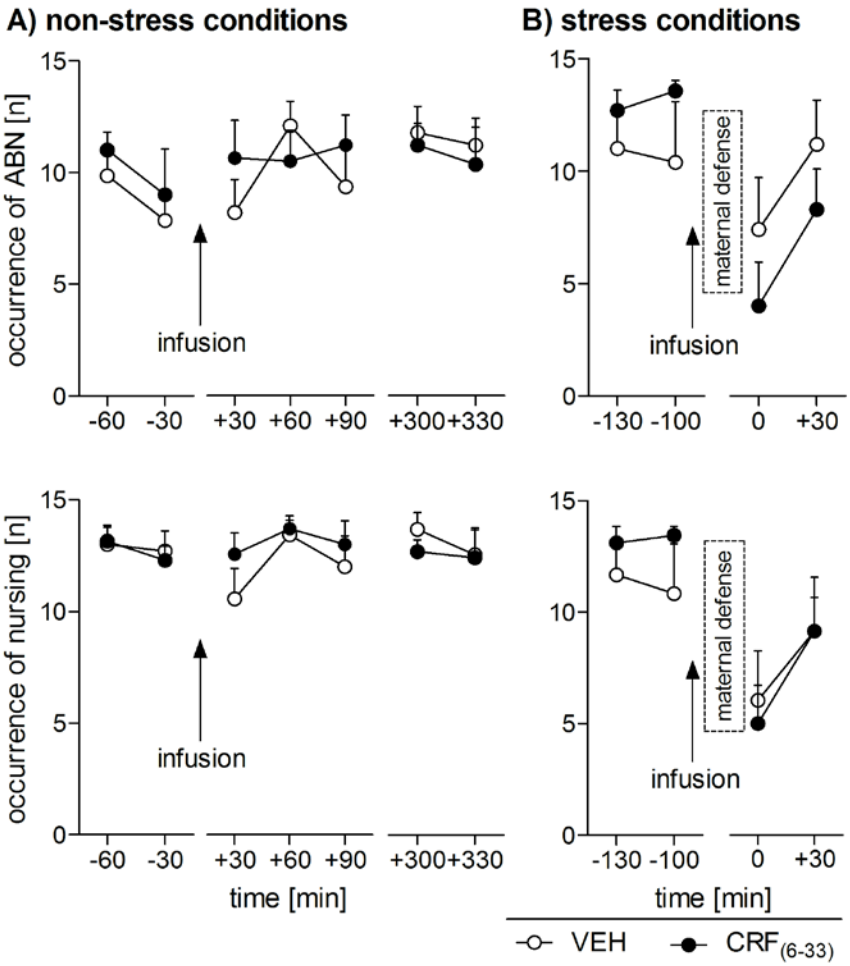

Figure 30. Effects of intra-adBNST CRF-BP inhibition on maternal care under non-stress conditions on LD 1 (A) and stress conditions on LD 5 (B). ABN (top) and nursing (bottom) were scored for 60 min before and for 90 min after infusion (A) or 60 min after maternal defense (B). Under non-stress conditions, ABN and nursing were also observed for additional 60 min in the afternoon (A). Dams received an acute intra-adBNST infusion of VEH (0.5 µl of sterile Ringer's solution) or the CRF-BP inhibitor $CRF_{(6-33)}$ (5 µg / 0.5 µl). Data are presented as mean + SEM. n = 5 - 7.

Maternal aggression on LD 5

No differences in maternal aggression were detected during the maternal defense test (number of attacks: VEH: 7.8 ± 1.4, $CRF_{(6-33)}$: 11.2 ± 4.5; attack latency: VEH: 100.4 ± 21.9 s, $CRF_{(6-33)}$: 71.0 ± 17.7 s; keep down: VEH: 46.7 ± 11.1 s, $CRF_{(6-33)}$: 74.8 ± 24.4 s; offensive upright: VEH: 5.1 ± 1.8 s, $CRF_{(6-33)}$: 4.2 ± 1.7 s; sum aggressive: VEH: 187.0 ± 27.8 s, $CRF_{(6-33)}$: 235.4 ± 64.6 s).

Anxiety-related behavior on LD 3

No differences were detected for anxiety-related behavior on the EPM (% time on the open arms: VEH: 26.0 ± 6.0, $CRF_{(6-33)}$: 33.2 ± 8.5; % entries into open arms: VEH: 45.1 ± 1.7, $CRF_{(6-33)}$: 41.2 ± 1.2; closed arm entries: VEH: 10.9 ± 1.6, $CRF_{(6-33)}$: 12.8 ± 1.5).

5.5 Discussion

Activation of CRF-R postpartum impairs the expression of maternal behavior (Pedersen et al., 1991; Gammie et al., 2004; D'Anna and Gammie, 2009; Klampfl et al., 2013; Klampfl et al., 2014a; Klampfl et al., 2014b). Hence, hypoactivation of CRF-R during this sensitive period is an indispensable prerequisite for the appearance of appropriate maternal behavior in lactating rats. In order to achieve such a hypoactivation, the CRF system has to adapt to this unique and demanding period on cellular (da Costa et al., 2001; Lightman et al., 2001; Walker et al., 2001; Deschamps et al., 2003; Klampfl et al., 2013; Klampfl et al., 2014b) and neuroendocrine levels (Brunton et al., 2008; Klampfl et al., 2014a). The CRF-BP, which is a potent regulator of CRF and Ucn 1, is an interesting yet poorly studied candidate for controlling such adaptations postpartum. In the present study, we demonstrated that inhibition of the CRF-BP had no effect on maternal care under basal conditions independent of the site of manipulation. Following stress, CRF-BP inhibition impaired the recovery from the stress-induced drop in nursing within the mpBNST, but not icv or in the adBNST. Maternal aggression, especially threat behavior, was increased by icv inhibition, an effect which was localized in the mpBNST. Furthermore, inhibition of the CRF-BP in the mpBNST was anxiogenic and decreased locomotion whereas icv inhibition did not affect anxiety but increased locomotion. No effects on maternal aggression and anxiety-related behavior were found in the adBNST. Additionally, no differences were

detected after icv inhibition in the FST, which was not performed following local manipulation.

The appearance of maternal care is not only regulated by OXT, AVP and PRL (Bosch, 2011) but is importantly mediated by CRF and its related peptides (Pedersen et al., 1991; Gammie et al., 2004; D'Anna and Gammie, 2009; Klampfl et al., 2013; Klampfl et al., 2014a; Klampfl et al., 2014b). We have recently shown that maternal care can be impaired by infusion of CRF or Stresscopin (i.e. human Ucn 3) either centrally (Klampfl et al., 2013) or locally into the mpBNST (Klampfl et al., 2014b) or the adBNST (Klampfl et al., 2014a). These findings support the vital requirement of a down-regulated CRF-R activation in mentally healthy, lactating individuals. However, inhibition of the most likely regulatory mechanism, i.e. the CRF-BP, either centrally or locally within the BNST revealed no behavioral changes under basal, stress-free conditions. This suggests no functional implication of the CRF-BP in the down-regulation of the CRF system under non-stress conditions and is supported by a previous study in lactating CRF-BP deficient mice showing no differences in nursing compared to wildtype mice (Gammie et al., 2008). Furthermore, this outcome was somewhat anticipated as CRF and its related peptides are not released until stimulated by a stressor (Reul and Holsboer, 2002b). Importantly, inhibition of CRF-BP after stressor exposure did affect maternal care but only when manipulated locally within the mpBNST; the recovery from a stress-induced drop in nursing was impaired. Thus, the CRF-BP in the mpBNST seems to be involved in ending the stress response, which guarantees a fast resumption of appropriate levels of maternal care. The CRF-BP is expressed in neurons and astrocytes (Behan et al., 1995a), and can be released upon stimulation from secretory vesicles (Blanco et al., 2011). Furthermore, the expression of the CRF-BP is up-regulated by acute stressor exposure as shown for the CeA (Herringa et al., 2004). These data together with our

findings suggest that in the mpBNST the CRF-BP expression is increased and its release is induced by exposure to the maternal defense test, which is a strong psychosocial stressor to the dam (Neumann et al., 2001). This is certainly important in order to protect the dam from exceeding levels of CRF, which are detrimental for the appearance of maternal care (Pedersen et al., 1991; Gammie et al., 2004; D'Anna and Gammie, 2009; Klampfl et al., 2013; Klampfl et al., 2014a; Klampfl et al., 2014b). The fact that inhibition of the CRF-BP in the mpBNST, but not in the adBNST or even centrally, impaired stress recovery suggests an exclusive function for the CRF-BP in the mpBNST in mediating maternal care in a stress context. This is supported by the local expression patterns of the CRF-BP, which are abundant in the mpBNST and barely seen in the adBNST (Potter et al., 1992).

Maternal aggression was increased by central and intra-mpBNST inhibition of the CRF-BP in lactating rats. After icv inhibition, dams displayed generally more aggressive behavior, which is reflected by increased levels of keeping down the intruder. This is supported by our previous studies showing a modulation of maternal aggression after manipulating CRF-R icv (Klampfl et al., 2013; Klampfl et al., 2014b). Interestingly, the increase in threat behavior following icv CRF-BP inhibition is similarly found in intra-mpBNST-treated dams, however, only affecting offensive upright, which is supported by our previous study (Klampfl et al., 2014b). Interestingly, no effects were detected in the adBNST being consistent with the lack of an effect of CRF-R manipulation on maternal aggression in the adBNST (Klampfl et al., 2014a). These data not only highlight the CRF system in the mpBNST as important regulator of maternal aggression but, additionally, reinforce that the CRF-BP is a particular modulator of threat behavior in lactating dams. However, CRF-BP deficient mice display less maternal aggression as reflected by reduced number of attacks and increased attack latency; no data on threat behavior was reported in this

study (Gammie et al., 2008). The diverging outcomes of the knockout mouse study and our present data might be based on the different species, on the use of mice selected for high maternal aggression expressing elevated CRF-BP levels, and moreover, on the method of inactivation of the CRF-BP. In these mice, the CRF-BP gene was chronically deleted which might have resulted in various compensatory mechanisms throughout development and adulthood (Nelson, 1997) whereas in the present study, the CRF-BP was acutely inhibited. Intriguingly, an increase of aggression following CRF-BP inhibition was unpredicted as the likely resulting elevated levels of CRF/Ucn 1 seem to improve maternal aggression whereas previous studies demonstrate impairing effects of increased CRF-R activation (Klampfl et al., 2013; Klampfl et al., 2014b). However, extracellular CRF concentrations are presumably different when administered exogenously compared to the slight increase of endogenous CRF after CRF-BP inhibition (Westphal and Seasholtz, 2006). We speculate that the appropriate display of maternal aggression requires perfectly balanced CRF release and, hence, CRF-R activation. A slight increase of extracellular CRF levels by CRF-BP inhibition seemingly promotes threat, but not attack, behavior whereas a stronger increase as evoked by exogenous administration of CRF can exert detrimental effects on the display of maternal aggression in lactating rats (Klampfl et al., 2013).

Anxiety-related behavior was differentially affected by inhibition of the CRF-BP depending on the site of manipulation. Icv inhibition had no effect on anxiety while inhibition in the mpBNST was anxiogenic. Furthermore, locomotor activity was decreased following icv inhibition while it was increased after intra-mpBNST inhibition. No effects on anxiety or locomotion were found in the adBNST. These diverging data might be explained by the different ways of administration. Icv infusion affects all centrally expressed CRF-BP, which may vary in function depending on the

expression site. The current knowledge about the CRF-BP proposes three hypotheses of how the CRF-BP might affect CRF and its related ligands, i.e. sequestering, potentiating, or signaling independent of the ligand (Seasholtz et al., 2002). As icv manipulation induces the integration of various single local effects resulting in a behavioral change (Bosch, 2013), it is possible that local anxiogenic effects as seen in the mpBNST disappear following icv manipulation. This is supported by lactating knock out mice which are globally deficient for the CRF-BP within the brain and do not display altered anxiety-related behavior compared to lactating wildtype mice (Gammie et al., 2008). Furthermore, icv CRF-BP inhibition in male rats reveales no anxiogenic phenotype (Behan et al., 1995c). Therefore, solely local CRF-BP inhibition in the mpBNST appears efficient and demonstrates that the CRF-BP in this brain region is important for maintaining a hypoanxious state.

The lack of an effect of CRF-BP inhibition in the adBNST on anxiety-related behavior is consistent with the lacking effect of intra-adBNST CRF-R manipulation on anxiety-related behavior (Klampfl et al., 2014a). This demonstrates that the CRF system in the adBNST is not involved in the regulation of anxiety, at least during lactation.

Along with anxiety-related behavior, changes in locomotion were also measured on the EPM following CRF-BP inhibition. Icv inhibition revealed decreased locomotion which is similarly found after icv CRF administration in lactating, non-selected Wistar rats (Klampfl et al., 2013). These data are consistent with previous studies showing reduced locomotor activity in a novel environment like the EPM following icv CRF infusion (Baldwin et al., 1991; Koob and Heinrichs, 1999) (but see Karolyi et al., 1999). However, increasing endogenous ‘free’ CRF by CRF-BP inhibition in the mpBNST led to higher locomotion in lactating rats. Therefore, it can be speculated that a local increase of endogenous CRF rather promotes arousal and, thus, increases locomotion whereas a global increase is locomotor suppressive.

Depressive-like behavior was investigated only after icv inhibition of the CRF-BP, which revealed no behavioral effects in the FST. This is supported by findings in lactating CRF-BP deficient mice, which did not differ from wildtype mice in the same test (Gammie et al., 2008). Even though icv CRF increases immobility in the FST in rats (Dunn and Swiergiel, 2008), the slight increase of endogenous, extracellular CRF concentrations by CRF-BP inhibition seems to be insufficient to induce a depressed phenotype. Moreover, the pro-depressive effects of CRF in rats cannot be generalized as this seems to be species-, reproductive status- and/or sex-specific (Dunn and Swiergiel, 2008; Gammie et al., 2008). For example, in contrast to rats icv CRF decreases immobility in male mice (Swiergiel et al., 2008), whereas increasing extracellular CRF levels by CRF-BP deficiency is not sufficient to change depressive-like behavior in lactating mice (Gammie et al., 2008).

The present study provides essential evidence on the behavioral implications of the CRF-BP during lactation and its important function as a fine-tuning regulator of other components of the CRF system, especially under stressful conditions. Demonstrating a vital role in ending the stress response, in modulating threat behavior as part of maternal aggression and in maintaining a hypo-anxious state, the CRF-BP contributes evidently to a postpartum-associated down-regulation of the CRF system particularly in the mpBNST. This protein might represent a potential target for future therapeutic approaches in order to treat CRF- and/or stress-induced maladaptations of the postpartum brain leading to postpartum mood disorders and infant neglect. Such restoration of the required balance in the CRF system would allow a beneficial and adequate expression of maternal behavior in lactating females.

- Chapter 6 -

General Discussion

6.1. Summary of results

The study presented in Chapter 2 was designed to get a first indication of CRF's action on maternal behavior in lactating rats. Along with NAB Wistar rats I used the HAB and LAB animal model, in which males are known to differ in their CRF system (Bosch et al., 2006), in order to detect possible effects of acute CRF-R manipulation on maternal behavior. I could show a lactation-specific reduction of CRF mRNA in LAB and NAB dams. This reduction was absent in lactating HAB rats, which had generally higher CRF mRNA levels than LAB and NAB rats. Furthermore, icv CRF infusion decreased maternal care under non-stress conditions in all breeding lines and reduced maternal aggression in HAB and LAB dams. Under stress conditions, the CRF-R antagonist prevented the stress-induced drop in maternal care. Furthermore, the antagonist decreased and CRF tended to increase anxiety in HAB/NAB and in LAB dams, respectively, suggesting an anxiogenic effect of CRF in lactating females.

In Chapter 3, I aimed to localize the detrimental effects of CRF-R manipulation on maternal behavior within the rat brain and focused specifically on CRF-R1 and CRF-R2 in the mpBNST. First, I confirmed the behavioral results obtained in Chapter 2 by using a more non-selective CRF-R agonist, i.e. Ucn 1, in lactating Wistar rats. Furthermore, I showed that CRF-R2, but not CRF-R1, mRNA expression was higher in the pBNST versus mBNST independently of reproductive status. Finally, acute pharmacological manipulation in the mpBNST revealed that ABN and total nursing were rapidly impaired by the CRF-R1 agonist and after a delay by the CRF-R2 agonist. Under stress conditions, ABN was increased only by the CRF-R2 antagonist, whereas both antagonists prevented the typical decrease in nursing after stress. During the maternal defense test, only the CRF-R2 agonist abolished maternal

aggression while the CRF-R2 antagonist increased it. Furthermore, the CRF-R1 agonist increased while both antagonists decreased anxiety-related behavior in virgin and lactating rats but not in male rats.

The study described in Chapter 4 was conducted to potentially extend the findings in maternal behavior from the mpBNST to another subdivision of the BNST, i.e. the adBNST. Acute manipulation of CRF-R1 and CRF-R2 in the adBNST revealed impaired ABN by the CRF-R1 agonist while the CRF-R2 agonist improved it with a delay. Total nursing was reduced by both agonists. A stress-induced decrease in ABN tended to be improved by the CRF-R1 antagonist. No other maternal behaviors were affected by subtype-selective CRF-R manipulation in the adBNST. In order to assess potentially indirect, behavioral effects via activation of the HPA axis, ACTH and CORT were measured following acute intra-adBNST CRF-R1 or CRF-R2 manipulation. Solely the CRF-R1 agonist increased basal ACTH release which was not further elevated by exposure to a stressor. CORT release was stimulated by both agonists under basal conditions. After stressor exposure, CORT levels remained high in CRF-R1-treated dams while a switch to decreased release was found in CRF-R2-treated dams. Both antagonists prevented the stress-induced increase in CORT.

In Chapter 5, I focused on the often neglected member of the CRF system, the CRF-BP. I aimed to characterize the role of the CRF-BP in the regulation of maternal behavior in lactating rats after acute inhibition of the CRF-BP both centrally as well as locally within the mpBNST or adBNST. Under non-stress conditions, central and local CRF-BP inhibition had no effect on maternal care. Following stressor exposure only intra-mpBNST inhibitor-treated dams showed impaired recovery from the stress-induced drop in nursing. During the maternal defense test, icv and intra-mpBNST inhibition increased maternal threat behavior. Anxiety-related behavior on the EPM

was only increased by CRF-BP inhibition in the mpBNST. No behavioral effects were detected following CRF-BP inhibition in the adBNST.

In summary, the results of the present thesis demonstrate an immense relevance for the CRF system in the regulation of maternal behavior in lactating rats. Various behavioral functions could be attributed to the members of the CRF family. Moreover, one brain region, the BNST, was particularly highlighted, appearing vital in the signal integration and mediation of behavioral effects induced by the CRF system. This promotes considerably our understanding of the complex adaptations of the maternal brain in the peripartum period.

6.2. The CRF system and its impact on maternal care

Maternal care represents probably the most important aspect of the wide behavioral repertoire displayed by lactating rats (Numan and Insel, 2003). It guarantees the all-encompassing well-being and proper development of the offspring. Interestingly, studies on the regulation of maternal care have almost exclusively focused on neurotransmitter systems which promote this behavior whereas other systems exerting negative effects like the CRF system have been virtually under-studied.

In the present thesis, acute effects of the CRF system on maternal care were investigated under non-stress and stressful conditions given that most family members are heavily involved in an individual's stress response (Bale and Vale, 2004) and that maternal care might be differentially affected under either condition. Under non-stress conditions, I provided evidence that both central and local activation of CRF-R by various ligands of the CRF family reduces the occurrence of maternal care, especially nursing. Following icv manipulation with CRF (Chapter 2) or Ucn 1 (Chapter 3), dams showed less ABN and nursing independent of their innate

anxiety. Similar effects were found in ovariectomized, steroid-primed virgin rats, which had three days of mothering experience and show reduced maternal-like care after icv CRF treatment (Pedersen et al., 1991). The same study also describes increased pup-killing behavior in primed virgin rats without pup contact, which I did not observe in lactating females. This indicates that icv CRF administration enhances the aversive responses elicit by pup presence in naïve, unsensitized females (Numan and Insel, 2003), which culminates in infanticide (Pedersen et al., 1991). As soon as the switch from aversive to approach behavior has occurred - as seen in sensitized or lactating females – the behavioral effects of CRF-R activation switches from infanticide to reduced maternal care, which represents infant neglect. Thus, it is not surprising that lactating females did not show any indices of pup-killing but displayed less maternal care as found in the present thesis. Intriguingly, the present results are similarly found in primates showing a reduction in maternal care after icv CRF infusion in lactating marmoset monkeys (Saltzman et al., 2011) and, thus, revealing a transspecies effect of CRF-R manipulation on maternal care.

Icv administration of a non-selective antagonist revealed no effects on maternal care under basal conditions (Chapters 2 and 3), implying that CRF-R activation is suppressed under basal conditions in lactating rats in order to assure adequate and sufficient levels of maternal care. This is supported by the lack of an effect after CRF-BP inhibition (Chapter 5), which increases free endogenous levels of CRF/Ucn 1 (Behan et al., 1995c; Westphal and Seasholtz, 2006). However, under non-stress conditions, the CRF-BP seemingly has bound too low levels of CRF/Ucn 1 to elicit a behavioral effect upon release.

Additionally contributing to the inevitable down-regulation of the CRF system postpartum, CRF mRNA expression is reduced within the CeA (Walker et al., 2001) and pPVN during lactation (Johnstone et al., 2000; Lightman et al., 2001; Walker et

al., 2001), which could be confirmed in the present thesis for the pPVN in NAB and LAB rats (Chapter 2). However, CRF mRNA expression is not reduced globally within the maternal brain. For instance, in the dorsolateral portion of the BNST (part of the mpBNST) CRF mRNA is increased in lactating versus virgin rats (Walker et al., 2001). Importantly, changes in CRF mRNA levels do not necessarily reflect altered expression levels of functional CRF protein, which is why further studies are needed to pursue the exact molecular underpinnings of a down-regulated CRF system postpartum.

Focusing on the BNST, subtype-specific manipulation of CRF-R1 and CRF-R2 confirmed the findings obtained in the icv approach and, in addition, allowed an assignment of behavioral effects of CRF-R manipulation to the BNST. Here, it is important to distinguish between the subdivisions of the BNST given their distinct cyto- and chemoarchitectonic differences (Bayer, 1987; Ju and Swanson, 1989; Ju et al., 1989). Acute activation of CRF-R in the mpBNST revealed reduced basal levels of ABN and nursing in a time-dependent manner; the effects of CRF-R1 activation were rapid, whereas those of CRF-R2 stimulation were delayed (Chapter 3). According to the decreases in maternal care, CRF-R1 activation with CRF revealed more locomotion and self-grooming, whereas CRF-R2 activation with Ucn 3 increased sleeping/resting. The increase in locomotion by CRF is supported by studies showing heightened locomotor activity and grooming behavior under basal conditions in the home cage (Sutton et al., 1982; Koob et al., 1984; Sherman and Kalin, 1987; Dunn and Berridge, 1990). The increase in sleeping/resting indicates sedative properties of Ucn 3 and concomitant CRF-R2 activation, which is supported by a study demonstrating motor suppressive effects upon CRF-R2 stimulation (Ohata and Shibasaki, 2004).

Interestingly, manipulation of CRF-R in the adBNST revealed a quite distinct picture; activation of CRF-R1 impaired ABN and total nursing whereas activation of CRF-R2 improved ABN with a delay but impaired nursing immediately (Chapter 4). The changes in maternal care following CRF-R1 stimulation are consistent with the alterations found after CRF-R1 activation in the mpBNST (Chapter 3). Thus, I propose that CRF-R1 activation in the BNST, independent of anterior or posterior portion, is detrimental for maternal care and requires hypo-activation during lactation. CRF, but not Ucn 1, is most likely the endogenous ligand for CRF-R1 in the BNST as CRF neuronal cell bodies and terminals were abundantly described (Olschowka et al., 1982; Cummings et al., 1983; Swanson et al., 1983; Moga et al., 1989; Morin et al., 1999) while Ucn 1 expression or projections have not been detected in this limbic brain region (Vaughan et al., 1995; Kozicz et al., 1998; Bittencourt et al., 1999).

In contrast to the effects of CRF-R1 on maternal care, CRF-R2 appears to play a more complex role within the BNST. With respect to ABN, the time course of treatment-effectiveness is similar to the mpBNST indicating a slow, delayed change of ABN. However, CRF-R2 activation in the adBNST improves ABN in contrast to its impairment in the mpBNST. This implies a positive role for CRF-R2 in the regulation of ABN in the adBNST and a negative role in the mpBNST. Interestingly, CRF-R2 activation in the adBNST decreased total nursing, which is again similar to its effects in the mpBNST. This demonstrates that CRF-R2 activation causes less nursing promptly after infusion, which is reversed at the time when these dams show increased ABN. Together, these data show that the quality and quantity of maternal care are affected equally by CRF-R2 activation in the mpBNST whereas they dissociate in the adBNST. Further research is needed to determine the relevance of this dissociation.

The behavioral changes following CRF-R activation in the adBNST might be mediated via an indirect pathway. Stimulation of CRF-R1 and CRF-R2 were shown to increase HPA axis activity (Chapter 4) and elevated CORT concentrations could potentially be detrimental to maternal behavior (Brummelte and Galea, 2010b). Inhibition of either CRF-R or of the CRF-BP as regulator of CRF-R activation in both the mpBNST and adBNST revealed no behavioral effects under non-stress conditions. This supports data from the icv studies (Chapters 2, 3 and 5) and strengthens the hypothesis of a down-regulated CRF system as an indispensable prerequisite for the occurrence of adequate maternal care.

While the effects of the CRF system on maternal behavior under non-stress conditions were discussed above, the effects under stress conditions will be discussed in the following. Under stressful conditions, maternal care was shown to be impaired by exposure to a psychosocial stressor, i.e. the maternal defense test (Neumann et al., 2001). Both central and local inhibition of CRF-R by unspecific or specific antagonists, respectively, can rescue maternal care depending on receptor subtype and brain region. Following icv manipulation, the non-selective inhibition of CRF-R prior to the maternal defense test improved ABN in HAB and nursing in HAB and LAB dams compared to the respective VEH group (Chapter 2). No effects were found in NAB Wistar dams (Chapters 2 and 3). However, this data indicates that exposure to a stressor elevates centrally released CRF in lactating rats, which in turn, results in impaired maternal care and can be rescued by blocking the CRF-R. Importantly, a balanced release of CRF is necessary for a proper stress response and, thus, for arousal and attention toward the intruder during the maternal defense test (Hemley et al., 2007). Icv inhibition of the CRF-BP prior to the maternal defense test revealed no significant changes in maternal care immediately after maternal

aggression. This might result from the global inhibition of the CRF-BP, which is apparently responsible for the fine-tuning of CRF-R activation in a region-specific fashion.

Following manipulation of CRF-R1 or CRF-R2 in the BNST and stressor exposure, different roles emerged for the two subtypes depending on behavior and division of the BNST. In the mpBNST, ABN returned rapidly to pre-stress levels only in the CRF-R2 antagonist-treated dams, whereas both antagonists for CRF-R1 and CRF-R2 prevented the stress-induced decrease. This shows that under stressful conditions CRF-R2 activation mediates all important parameters of maternal care while CRF-R1 activation affects only nursing behavior in the mpBNST. In contrast to the mpBNST, ABN was improved only by the CRF-R1 antagonist in the adBNST, which points to a crucial role of intra-adBNST CRF-R1 in mediating stress-induced changes of maternal care. Together, the CRF-R2 subtype appears to be more important in mediating maternal care in the mpBNST whereas the CRF-R1 subtype most likely is the crucial receptor in the adBNST, especially in a stress context. This region-specific pattern is supported by CRF-R mRNA expression levels; the more posterior in the BNST the more abundant CRF-R2 are expressed (Chapter 3). However, CRF-R mRNA expression for the adBNST is still under investigation (collaboration with Dr. Paula J Brunton, The Roslin Institute, Edinburgh, UK). Interestingly, the CRF-BP in the adBNST was shown to have no functional implication in the regulation of maternal care under stressful conditions while its inhibition prolonged the stress-induced decrease of nursing in the mpBNST. Given that the CRF-BP binds mostly the endogenous ligands for the CRF-R1, i.e. CRF and Ucn 1, it is surprising to see no effects in the adBNST, which is supposed to mediate its effect via CRF acting on CRF-R1. In contrast, the CRF-BP in the mpBNST appears to impact maternal care, which is probably mediated via CRF-R2 activation by Ucn 3. However, Ucn 3 does

not bind to the CRF-BP (Westphal and Seasholtz, 2006) which is why the mode of action of the CRF-BP in the mpBNST remains uncertain. Thus, further studies are needed to elucidate molecular actions of the CRF-BP, promoting our understanding of the CRF-BP during lactation with special focus on maternal care.

The current data demonstrate that the adequate appearance of maternal care requires hypo-activation of CRF-R postpartum, which appears to be supported by the CRF-BP. Furthermore, stress-induced impairment of maternal care was shown to be mediated predominantly by CRF-R2 in the mpBNST and by CRF-R1 in the adBNST, concomitantly assigning the main receptor subtype of signal transmission to either of the BNST subdivisions.

6.3. The CRF system and its impact on maternal motivation

Maternal motivation, which is represented by retrieving the litter into the nest, is an essential appetitive behavior and usually precedes maternal care (Numan and Woodside, 2010). In the present thesis, maternal motivation was investigated following central or intra-BNST CRF-R manipulation using the PRT. Upon acute icv manipulation, pup retrieval behavior was not changed after CRF infusion (Chapter 2), whereas central Ucn 1 administration impaired maternal motivation by trend (Chapter 3). On the one hand, it is feasible that CRF-R signaling is not involved in the regulation of maternal motivation and that Ucn 1's effects are artifacts of the global drug administration. On the other hand, Ucn 1, but not CRF, might generally mediate appetitive behavior in rats. The latter suggestion is supported by the suppressing effects of Ucn 1 on another appetitive behavior, i.e. feeding (Spina et al., 1996). However, a causal relationship remains to be proven.

Local and subtype-specific manipulation of CRF-R in the mpBNST and adBNST revealed no effects on pup retrieval behavior. As CRF and Ucn 3 were used for CRF-

R activation, either suggestion of the effects of the CRF system on maternal motivation (see above) could be supported. However, it has to be noted that the BNST has not been reported to mediate maternal motivation (see 1.1.2.2), which is why a lack of an effect after intra-BNST manipulation was anticipated. This lack was also the cause why no PRT was performed following CRF-BP inhibition (Chapter 5). In order to finally elucidate the role of the CRF system in the regulation of maternal motivation further experiments are required, going into greater detail of this complex aspect of maternal behavior.

6.4. The CRF system and its impact on maternal aggression

In contrast to maternal care and maternal motivation, which both represent pup-directed behaviors, maternal aggression is directed toward an intruder and is aimed to defend the litter in a potentially threatening situation. Especially the BNST has been implicated in mediating maternal aggression (Bosch, 2013). To date, this aggressive behavior has been demonstrated to be primarily promoted by OXT and AVP in lactating rats and mice. In the present thesis, I aimed to extend our knowledge to the CRF system, which is a promising candidate system involved in modulating maternal aggressive behavior, with special focus on the BNST.

To begin with, CRF-R were manipulated icv and non-selectively prior to the maternal defense test. Icv inhibition of CRF-R did not reveal any changes in maternal aggression (Chapters 2 and 3) while activation of CRF-R (CRF: Chapter 2; Ucn 1: Chapter 3) decreased maternal aggressive behavior. The detrimental effects of CRF (Gammie et al., 2004) and Ucn 1 (D'Anna et al., 2005) are similarly found in lactating mice, which supports a species-independent negative effect of CRF-R activation on maternal aggression during lactation.

Focusing on the BNST, differential results of CRF-R manipulation on aggression were detected depending on the targeted subdivision within the BNST. Acute CRF-R subtype-specific stimulation in the mpBNST (Chapter 3) revealed that inhibition and activation of CRF-R2 increased and decreased, respectively, maternal aggression. Interestingly, CRF-R1 manipulations had no behavioral consequences in the maternal defense test. These findings are supported by studies in lactating mice showing reduced maternal aggression after CRF-R2 stimulation and increased aggression after CRF-R2 inhibition in the LS (D'Anna and Gammie, 2009). Furthermore, lactating mice deficient for CRF-R2 show deficits in the display of maternal aggression (D'Anna et al., 2008). Together, these data indicate a potent role of CRF-R2 within the mpBNST and LS in regulating maternal aggression postpartum.

Interestingly, maternal aggression was completely abolished in Ucn 1- and Ucn 3-treated dams but was only reduced in CRF-treated rats. Thus, I propose that maternal aggression is mediated by Ucn-induced CRF-R2 activation with Ucn 3 being the preferred ligand in the mpBNST. This is supported by the endogenous expression patterns of the CRF-R2 ligands (see 1.2.1.3) (Vaughan et al., 1995; Hsu and Hsueh, 2001; Lewis et al., 2001; Reyes et al., 2001). It needs to be mentioned that Ucn 1 and Ucn 3 infusion in the LS of lactating mice only reduced maternal aggressive behavior but did not abolish it (D'Anna and Gammie, 2009). However, in the present mpBNST study in rats, a higher dosage was used than in the LS study in mice, suggesting an unsaturated CRF-R2 activation and, thus, incomplete reduction of maternal aggression in mice. With respect to CRF-R1, lactating mice deficient for this receptor subtype displayed impaired maternal aggression in the maternal defense test (Gammie et al., 2007a). However, these dams showed a high variance of aggressive behavior toward the intruder and did not display constantly lower levels

of maternal aggression over several experimental days compared to wildtype mice. Additionally, if CRF-R1 activation mediates maternal aggression along with CRF-R2, deletion of the CRF-R1 gene would result in an improvement but not impairment of maternal aggression. Therefore, the altered levels of aggressive behavior in lactating CRF-R1 deficient mice most likely result from compensatory mechanisms throughout development and adulthood (Nelson, 1997).

In contrast to the negative effects of CRF-R2 activation in the mpBNST, selective manipulation of CRF-R1 or CRF-R2 in the adBNST did not affect maternal aggression at all. Assuming that Ucn 3 as endogenous ligand for CRF-R2 is mediating maternal aggression (see above) and that Ucn 3 mRNA (Hsu and Hsueh, 2001; Reyes et al., 2001) and fibers (Li et al., 2002) are found in the mpBNST, but not adBNST, it was not surprising to detect no behavioral changes after CRF-R manipulation in the adBNST. Furthermore, it was shown that the mpBNST heavily projects to the LS (Dong and Swanson, 2004a), another vital regulating site for maternal aggression (D'Anna and Gammie, 2009; Bosch, 2013), while the adBNST is lacking projections to the LS (Dong et al., 2001b). Such projections to the LS might help regulating the display of maternal aggression during lactation.

In conclusion, the current findings strongly indicate that the mpBNST is the subdivision of the BNST in which CRF-R2 is the predominant receptor subtype regulating maternal aggression. The adBNST with CRF-R1 as the main receptor subtype seems to rather play a role in mediating maternal care (see 6.2).

6.5. The CRF system and its impact on maternal anxiety

Anxiety-related behavior is reduced in lactating rats compared to non-lactating females (Lonstein, 2007; Bosch, 2011), which appears to be necessary for the mother’s acceptance of and attraction to unfamiliar and anxiety-generating neonates

(Fleming and Luebke, 1981). Furthermore, reduced anxiety is proposed to be essential for dam's increased aggression toward potentially dangerous conspecifics (Hansen et al., 1985). Various neurotransmitter systems like OXT, AVP and PRL have been associated with the regulation of maternal anxiety (Torner et al., 2001; Bosch, 2011; Neumann and Landgraf, 2012). Additionally, the CRF system, which is well known for its implication in anxiety in males (Reul and Holsboer, 2002a, b), was proposed to eventually play a role in the regulation of maternal anxiety (Lonstein, 2007) but was only investigated under stress conditions (Gammie et al., 2008; D'Anna and Gammie, 2009). Therefore, I assessed anxiety-related behavior after central or intra-BNST manipulation of the CRF system in lactating rats without additional stressors.

Acute central and non-selective inhibition of CRF-R revealed differential effects in the two icv studies. In the first study, CRF-R inhibition was anxiolytic in lactating NAB, as well as HAB, Wistar rats (Chapter 2) whereas it did not alter anxiety-related behavior in the second study in lactating NAB Wistar rats (Chapter 3). Given that HAB rats generally show heightened levels of anxiety (Murgatroyd et al., 2004; Bosch et al., 2006; Bosch and Neumann, 2008) and have an up-regulated CRF system (Chapter 2) (Bosch et al., 2006), exposure to the EPM might act as a mild stressor (Neumann et al., 1998b) and elevate endogenous CRF/Ucn levels during lactation. These, in turn, can be antagonized by icv infusion of D-Phe, resulting in an anxiolytic effect. However, in the NAB Wistar dams of both studies, the EPM might have been perceived differently as stressor, explaining anxiolytic effects in higher stressed dams in the first study (Chapter 2) compared to no effect in potentially less stressed dams in the second study (Chapter 3).

With respect to central, non-selective activation of CRF-R, CRF tended to be anxiogenic in LAB dams only (Chapter 2). This weak or even absent anxiogenic

effect of CRF might be explained by the unspecific and global activation of CRF-R in the brain following icv infusion (Bosch, 2011). Indeed, CRF can exert both anxiogenic and anxiolytic actions depending on the brain site of receptor activation, like the BNST (Sahuque et al., 2006) / amygdala (Davis et al., 2010) and frontal cortex (Zieba et al., 2008; Ohata and Shibasaki, 2011) / globus pallidus (Sztainberg et al., 2011), respectively. These opposing effects in various brain regions might cancel each other's effect out (Bosch, 2011), resulting in no or just a slight change in maternal anxiety. Interestingly, the second study on central, non-selective activation of CRF-R with Ucn 1 revealed a significant increase in anxiety-related behavior (Chapter 3). Given that Ucn 1 has mostly been shown to be anxiogenic but never anxiolytic (Spina et al., 1996; Moreau et al., 1997; Jones et al., 1998), it is feasible that icv CRF-R activation with Ucn 1 was sufficient to induce an anxiogenic phenotype. Additionally, Ucn 1 is able to dissociate CRF from the CRF-BP, thus elevating endogenous CRF levels (Behan et al., 1996a), which could potentiate the anxiogenic effects of Ucn 1. Interestingly, inhibition of the CRF-BP was ineffective in altering maternal anxiety (Chapter 5) but supports the lack of effect following icv CRF. However, given that inhibition of the CRF-BP can also increase endogenous levels of Ucn 1 (Behan et al., 1996a) but was not anxiogenic as after icv Ucn 1 infusion (Chapter 3), it can be speculated that the slight elevation of 'free' Ucn 1 by CRF-BP inhibition is not sufficient to induce an anxiogenic phenotype. In support, central CRF-BP inhibition is not anxiogenic in male rats either (Behan et al., 1995c) demonstrating no functional implication of central CRF-BP in the regulation of anxiety-related behavior.

Manipulations of CRF-R and CRF-BP icv gave a first indication of the CRF system's role in the regulation of maternal anxiety. Interestingly, when focusing on the CRF system in the BNST, which is an important regulator of anxiety-related behavior in

males (Lee and Davis, 1997; Liang et al., 2001; Ciccocioppo et al., 2003; Greenwell et al., 2004; Jasnow et al., 2004; Sahuque et al., 2006), maternal anxiety was clearly affected by subtype-selective CRF-R manipulation. Microinfusion of CRF into the mpBNST revealed a significant reduction in anxiety (Chapter 3), which extends the knowledge of CRF's anxiogenic property in males to lactating females. Furthermore, intra-mpBNST administration of either a selective CRF-R1 or CRF-R2 antagonist was strongly anxiolytic in lactating rats. Interestingly, the anxiolytic effects of both antagonists were also found in virgin rats, suggesting that both receptor subtypes mediate anxiety-related behavior in females independent of reproductive status. In contrast, selective CRF-R inhibition has no effect in males (Sahuque et al., 2006). Only a combined infusion of CRF with a CRF-R1, but not CRF-R2, antagonist has anxiolytic properties in male rats. Thus, the current thesis is the first to provide evidence that both CRF-R subtypes in the mpBNST are involved in the regulation of anxiety-related behavior, and furthermore, that emotionality is regulated by the CRF system in a sexually dimorphic and reproductive status-independent way.

Continuing with the adBNST, maternal anxiety was not changed following any selective CRF-R1 and CRF-R2 manipulation in the adBNST (Chapter 4). In confirmation of this region-dependent regulation of maternal anxiety within the BNST, inhibition of the CRF-BP within the mpBNST was anxiogenic (Chapter 3) while no effects were found following CRF-BP inhibition in the adBNST (Chapter 4). All of these findings strongly support the the CRF system in the mpBNST, but not the adBNST, as a vital regulator of maternal anxiety during lactation.

In conclusion, maternal anxiety is similarly regulated by both CRF-R1 and CRF-R2 in the mpBNST, which need to by hypo-activated during lactation to guarantee the postpartum-associated anxiolysis in lactating rats. This typical hypoanxious state is likely supported and maintained by the CRF-BP.

Importantly, locomotor activity was also measured on the EPM in order to assess a potential influence on anxiety-related behavior via changes in locomotion. Following icv manipulation, only activation, but not inhibition, of CRF-R changed locomotor activity on the EPM. While Ucn 1 infusion had no effect (Chapter 3), icv CRF (Chapter 2) and CRF-BP inhibitor infusion (Chapter 5) decreased locomotor activity. Even though CRF effects were discussed to be generated by unusual high locomotion of VEH dams in Chapter 2, considering data from all studies rather indicates that CRF might be locomotor suppressive. This assumption is supported by studies showing decreased locomotor activity following CRF infusion in a novel environment such as the EPM (Baldwin et al., 1991; Koob and Heinrichs, 1999). This effect seems to be more prominent after icv manipulation as intra-BNST CRF-R manipulation did not change locomotion on the EPM (Chapters 3 and 4). However, inhibition of the CRF-BP in the mpBNST revealed increased locomotion (Chapter 5). This indicates that locomotion is not only affected in a stress context but is also sensitive to the amounts of endogenously available CRF. Apparently, slight elevation of free CRF in the mpBNST increases locomotion while CRF infusion into the mpBNST has no effect on locomotor activity. Thus, these inconsistent data on locomotion on the EPM following manipulation of the CRF system imply that a clear link between locomotion and anxiety cannot be made as both parameters were not always changed similarly.

6.6. The CRF system in the BNST and potential interactions with other neuropeptide systems

Complex behaviors, such as maternal behavior, are most likely mediated not only by single neurotransmitter systems but are triggered by a variety of systems belonging to intricate neuronal circuits. Indeed, important neuropeptide systems in the

postpartum period like OXT and AVP act in concert to mediate maternal behavior (Bosch and Neumann, 2012; Neumann and Landgraf, 2012). Thus, it is highly feasible that CRF and Ucn not only act as neurotransmitters but have rather neuromodulatory properties.

Central CRF neurons may not be regarded as a homogeneous cell population even though they share a common neuropeptide phenotype (Dabrowska et al., 2013). For example, CRF neurons co-localize serotonin in the raphe nuclei (Valentino et al., 2010), NA in the LC (Valentino et al., 1983; Valentino et al., 2010), and glutamate (Ziegler et al., 2002; Lin et al., 2003; Hrabovszky et al., 2005; Hrabovszky and Liposits, 2008) and OXT in the PVN (Dabrowska et al., 2013). Within the BNST, CRF neurons co-localize mainly GABA, which is the major neurotransmitter phenotype detected in the BNST (Dabrowska et al., 2013). Moreover, CRF-R are predominantly found on GABAergic neurons (Dabrowska et al., 2011). Due to this prominent co-expression, signaling via a GABA-mediated mechanism might be essentially involved in the regulation of maternal behavior. For instance, I presented evidence that the negative effects of CRF and Ucn 3 on maternal care might be indirectly mediated via an increased HPA axis activity (Chapter 4). This indirect effect is suggested to be triggered by a GABAergic-mediated disinhibition of the PVN. Moreover, it is reasonable that ligand-induced CRF-R activation in the BNST increases GABAergic signaling in other brain regions like the PAG, where enhanced GABA release was shown to reduce nursing behavior in lactating rats (Stern and Lonstein, 2001). Hence, the CRF system can certainly regulate maternal behavior via local interactions with GABA.

Besides expression profiles on GABAergic neurons, especially the CRF-R2 subtype is also found on OXT fibers in the BNST (Dabrowska et al., 2011; Dabrowska et al., 2013). This strongly suggests an interaction between CRF-R activation and OXT

release in the BNST, which might directly influence maternal behavior. As OXT in the mpBNST has been implicated in the regulation of maternal behavior, in particular maternal aggression (Bosch, 2013), it is feasible that the reduction in maternal aggression following CRF-R2 activation (Chapter 3) is triggered by concomitantly decreased OXT release in the mpBNST. In the adBNST, the CRF and OXT system might interact differently compared to the mpBNST; on the one hand, CRF-R manipulation did not affect maternal aggression (Chapter 4) whereas, on the other hand, OXT receptor activation impairs aspects of maternal aggressive behavior (Consiglio et al., 2005). In contrast to maternal aggression, an interactive regulation of maternal care by the CRF and OXT system is unlikely as OXT in the BNST was shown to have no effect on maternal care (Bosch, 2011). Thus, the influence of the CRF system on maternal care is most likely mediated by interactions with other neurotransmitter systems such as the GABAergic system (see above). With respect to anxiety-related behavior, the anxiogenic effects of CRF-R activation in the mpBNST seem to be regulated independently of the OXT system because antagonizing the OXT receptor in this portion of the BNST does not alter anxiety-related behavior in lactating rats (Klampfl, Wöster, Bosch, unpublished data). Thus, CRF-R signaling in the BNST most likely influences other downstream targets mediating anxiety-related behavior. Intriguingly, both the CRF and OXT system within the BNST (Dabrowska et al., 2011; Dabrowska et al., 2013) and PVN (Dabrowska et al., 2011) could presumably influence each other also reversely, i.e. OXT modulates CRF synthesis / release via OXT receptors expressed by CRF neurons. In the PVN, OXT release might have suppressing effects on CRF neurons given that icv OXT administration attenuates the stress-induced increase of CRF mRNA in the PVN (Windle et al., 2004), being important for a blunted stress response postpartum. Even

though such an interaction is also feasible in the BNST, no studies have been performed to date investigating physiological or behavioral effects.

In contrast to the feedback loop between the OXT and CRF system, interactions with the AVP system seem to be not as prominent. It was shown that CRF neurons in the BNST and PVN express the AVP V1b receptor (Dabrowska et al., 2013), which probably promotes HPA axis activity forming heterodimers with CRF-R1 (Young et al., 2007). In a behavioral context, AVP was shown to potentiate CRF's effects on stress-induced fighting in male rats (Elkabir et al., 1990), indicating a link of the two systems in the regulation of aggressive behavior. Therefore and due to AVP's essential role in the regulation of maternal behavior (see 1.1.2), it is intriguing to speculate about possible interactions with the CRF system also during lactation. However, maternal behavior is predominantly mediated by V1a (Bosch et al., 2010) but not V1b receptor in the BNST (Bayerl, Klampfl, Bosch, unpublished). V1a receptors are not expressed on CRF neurons in the BNST (Dabrowska et al., 2013), which is why an interaction appears unlikely. Still, V1a receptors might be expressed by Ucn 3 neurons in the BNST but this has not been investigated so far. Thus, to date, it needs to be assumed that the AVP and CRF systems in the BNST do not mediate maternal behavior via a common feedback loop.

Another candidate neurotransmitter system for an interaction with the CRF family postpartum is the NAergic system. Its signal transmission in the BNST strongly influences maternal behavior in lactating rats; NAergic α2 receptor activity in the vBNST needs to be down-regulated during lactation to guarantee adequate maternal behavior (Smith et al., 2012; Smith et al., 2013). Furthermore, the adBNST and mpBNST, which protect from the effects of the CRF system on maternal behavior, receive very dense NAergic innervations through the ventral NAergic bundle from the A1 and A2 cell groups in the nucleus of the solitary tract (Ricardo and Koh, 1978;

Woulfe et al., 1988; Forray and Gysling, 2004; Park et al., 2009). Even though the NAergic system in these subdivisions of the BNST has not been implicated in the regulation of maternal behavior, it is tempting to suggest a similar down-regulation of NAergic activity in the adBNST and mpBNST. Here, NA is speculated to induce the release of endogenous CRF through the activation of α1-adrenergic receptors (Forray and Gysling, 2004), which would be detrimental for maternal behavior (Chapters 2 - 4) and would be similar to the neuronal interactions in the vBNST (Smith et al., 2012; Smith et al., 2013). Thus, it is quite reasonable that interactions between the NAergic and CRF system could be responsible for changes in the occurrence of maternal behavior following manipulation of either receptor. Importantly, both the NAergic and CRF system need to be down-regulated during lactation to guarantee the appearance of adequate maternal behavior.

In conclusion, massive evidence points to potential interactions of the CRF system with various neurotransmitter systems. Given that several neurotransmitter systems in single brain regions like the BNST have been implicated in maternal behavior, it is highly reasonable that these systems do not act on their own. It is rather feasible that they interconnect to act in concert and to trigger such a complex behavior like maternal behavior. Unfortunately, virtually no studies have investigated such crucial interactions and signaling circuits, which would strongly promote our understanding of the regulation of maternal behavior.

6.7. Translational aspects

During the last decades, the CRF system has evolved as an important factor in the development of psychopathologies like depression and anxiety disorders. Importantly, women are twice as likely affected than men suggesting a sexual dimorphism in the underlying mechanisms (Kessler, 2003; Marcus, 2009). The time

of highest risk for women to develop psychopathologies is during their childbearing years. Dysregulations leading to postpartum mood disorders have mostly been associated with a disturbed hormonal balance and hyperactivity of the HPA axis (Slattery and Neumann, 2008; Brummelte and Galea, 2010a). However, recent studies and especially the present thesis strongly indicate that the CRF system also plays an important role in the pathogenesis of postpartum mood disorders. In healthy mothers, the reduced activity of the brain CRF system is essential for adequate physiological and behavioral adaptations and may represent a protective mechanism of the maternal brain to cope with the dramatic alterations in various hormonal systems during motherhood (Slattery and Neumann, 2008). In the present thesis, I could support this hypothesis and demonstrated the detrimental effects on maternal behavior during the unfavorable, maybe even pathological condition of CRF-R (hyper-)activation in lactating rats. Indeed, CRF-R activation, especially within the BNST, impaired not only pup-directed behaviors but also reduced pup-protective behaviors, i.e. maternal aggression. These outcomes could be assumed to represent infant neglect, which is often observed in depressed mothers (Adamakos et al., 1986; Bifulco et al., 2004; Friedman and Resnick, 2009). Such a development of aversive tendencies toward the child could at worst culminate in filicide, which is almost exclusively seen in psychotic mothers (Appleby et al., 1998; Porter and Gavin, 2010). Interestingly, pup-killing was found in virgin rats, which were naïve to pups, following icv CRF administration (Pedersen et al., 1991). As discussed in Chapter 6.2, these effects are most likely mediated by a potentiation of the aversive circuit stimulated by pups in non-lactating females. Even though lactating females do not show filicide following CRF application, it needs to be considered that all studies were conducted using acute pharmacological manipulations. Chronic CRF-R activation, in contrast,

might not only result in infant neglect but could affect dams and, consequently, the young even more severely.

Together, previous and current data certainly indicate a massive dysregulation of the CRF system postpartum, among others, leading to infant neglect and filicide (Appleby et al., 1998; Porter and Gavin, 2010). Unfortunately, the therapeutic possibilities are not well advanced in the treatment of postpartum mood disorders. Given that most current antidepressants or anxiolytics have been developed in males and that psychopathologies are differentially regulated in females (Solomon and Herman, 2009; Valentino et al., 2013), suitable treatment options are essential for lactating mothers but still missing. Therefore, a better understanding of the underlying mechanisms is absolutely required and would certainly advance the development of suitable medication for postpartum mood disorders.

6.8. Conclusion and Perspective

In my thesis, I presented evidence for the inevitable hypoactivation of CRF-R in early lactating rats in order to show the full repertoire of maternal behavior. (Hyper)Activation of CRF-R, especially in the BNST, revealed detrimental effects on the occurrence of maternal care, maternal aggression and maternal anxiety, but not on maternal motivation (Figure 31). Importantly, both CRF-R subtypes were differently implicated in the regulation of maternal behavior depending on the site of action within the BNST. In the mpBNST, maternal care is impaired by the activation of CRF-R1 (CRF) and CRF-R2 (Ucn 3) while the effect of CRF might also be mediated by CRF-R2, which seems to be the predominant receptor subtype in the mpBNST. Indeed, CRF-R2 mediate the stress-induced reduction of maternal care and regulate maternal aggression. However, both receptor subtypes are involved in the regulation of maternal anxiety within the mpBNST. The CRF-BP in the mpBNST

appears to be vital in ending the stress response to assure a fast resumption of maternal care and is implicated in the hypoanxious state of lactating females.

In the adBNST, the functions of the two receptor subtypes are different; CRF-R1 activation reduced maternal care whereas CRF-R2 stimulation increased ABN and reduced nursing in a time-dependent manner. After stressor exposure, the CRF-R1 subtype seems to mediate the stress-induced impairment of maternal care in the adBNST. Moreover, CRF-R activation in the adBNST has no influence on maternal aggression, maternal motivation and maternal anxiety attributing an essential role in the regulation of maternal care to the adBNST in lactating rats. Importantly, CRF-R activity in the adBNST does not appear to be regulated by the CRF-BP postpartum. Together, even though the CRF-R1 seems to be the predominant subtype in the adBNST, the CRF-R2 subtype is apparently also implicated in the regulation of maternal behavior, in particular maternal care. Interestingly, the CRF system in the adBNST appears to be exclusively involved in the regulation of maternal care but no other maternal behaviors.

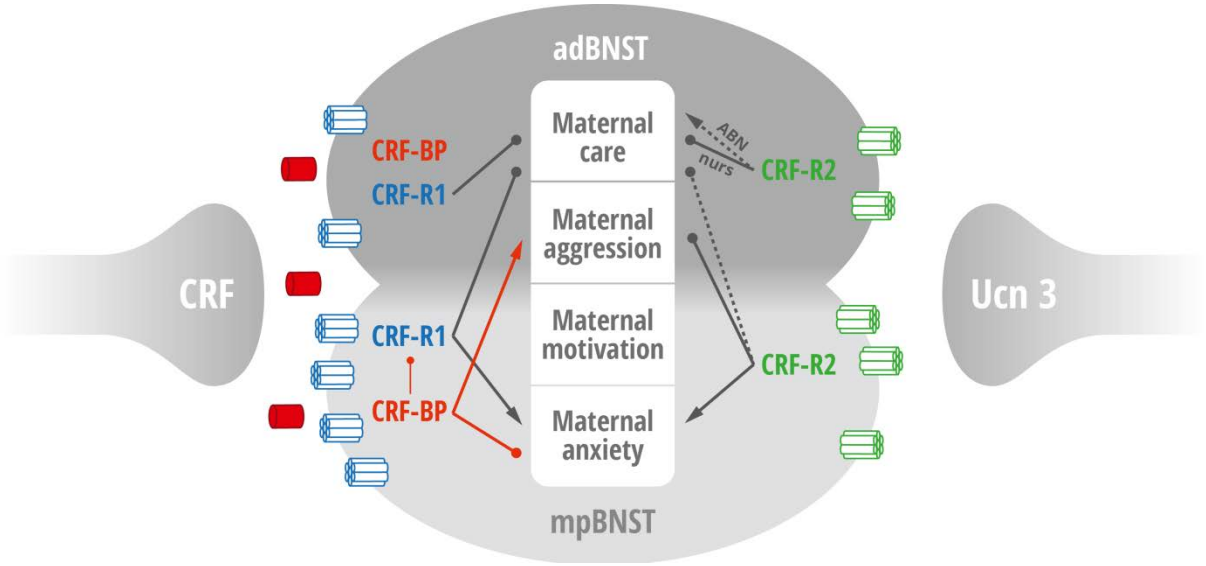

Figure 31. Schematic representation of the effects of the CRF system within the BNST on maternal behavior. The BNST is divided into the adBNST (dark gray) and mpBNST (light gray) of which each subdivision expresses CRF-R1 (blue), CRF-R2 (green) and the CRF-BP (red). CRF-R1 is

hypothesized to be stimulated by CRF while CRF-R2 is believed to be activated by Ucn 3. Arrows indicate stimulating effects while blunted ends represent inhibiting effects. Straight lines show immediate effects whereas dotted lines represent delayed effects. ABN, arched back nursing; nurs, nursing.

These data significantly advance our understanding of the role of the CRF system during the postpartum period. Considerable insight could be gained especially on a behavioral level. However, further experiments will be helpful to unravel particularly cellular and neuronal mechanisms underlying the down-regulation of the CRF system during lactation. For instance, CRF and CRF-R mRNA levels were shown to differ between virgin and lactating rats in some important brain regions for maternal behavior like the BNST, PVN (Chapters 2 and 3) (Walker et al., 2001), and CeA (Walker et al., 2001). Thus, assessing expression levels in other brain areas important for maternal behavior like the MPOA, LS or PAG could reveal further CRF system candidate regions. Importantly, the assessment of CRF-R1 and CRF-R2 mRNA in the adBNST is currently under examination in collaboration with Dr. Paula Brunton (The Roslin Institute, Edinburgh, UK) in order to improve our understanding of the behavioral results obtained following CRF-R manipulation in the adBNST. Furthermore, expression patterns of the remaining CRF family members, i.e. Ucn 1 – 3 and the CRF-BP, urgently need to be assessed. CRF-BP expression levels in the BNST, MPOA, LS, PVN, amygdala, and hippocampus of virgin and lactating rats are currently in process in collaboration with Prof. Dr. Audrey Seasholtz (University of Michigan, Ann Arbor, MI, USA).

The current thesis presents a thorough overview over the CRF system in the BNST during lactation; however, not all subdivisions were tested. In order to complete this overview the effects of the CRF system need to be investigated in the vBNST as well. The vBNST expresses CRF abundantly especially in the fusiform nucleus (Koob and

Heinrichs, 1999), is strongly interconnected with the adBNST (Turesson et al., 2013) and MPOA (Numan and Insel, 2003), and plays an important role in the regulation of maternal behavior (Numan and Insel, 2003; Smith et al., 2012; Smith et al., 2013). Thus, the vBNST might represent another candidate site in the regulation of maternal behavior by the CRF system. Moreover, it would be intriguing to investigate potential differences in receptor signaling between virgin and lactating rats; so far, CRF-R signaling has only been shown to be sexually dimorphic (Valentino et al., 2013) while reproductive status-specific studies are missing.

In order to find out more about potential interactions with other neurotransmitter systems, acute double-infusion approaches are feasible. Furthermore, once behavioral effects and signaling mechanisms following acute CRF-R manipulation are unraveled, the impact of chronic CRF-R activation on maternal behavior needs to be investigated. Such an approach might help to better understand the suggested pathological condition of CRF-R hyperactivity in mood disorders like depression and anxiety (Reul and Holsboer, 2002a, b).

Together, these experiments will help to elucidate further implications of the CRF system in the regulation of maternal behavior and to reveal potential mechanisms responsible for a dysbalanced activity of CRF-R in postpartum mood disorders. Such an advanced understanding is indispensable for the development of therapeutics particularly suitable for lactating females and, consequently, for the expression of adequate maternal behavior.

Abbreviations

ABN	arched back nursing
ACTH	adrenocorticotropic hormone
adBNST	anterior dorsal bed nucleus of the stria terminalis
ANOVA	analysis of variance
AVP	arginine-vasopressin
BNST	bed nucleus of the stria terminalis
bps	base pairs
cAMP	cyclic adenosinmonophosphate
CeA	central amygdala
CORT	corticosterone
cPAG	caudal part of periaqueductal gray
CRF	corticotropin-releasing factor
CRF-BP	corticotropin-releasing factor binding protein
CRF-R	corticotropin-releasing factor receptor
dMPOA	dorsal medial preoptic area
EPM	elevated plus-maze
ERK	extracellular-signal related kinase
FST	forced swim test
GABA	γ-amino butyric acid
GC	glucocorticoid
GPCR	G-protein coupled receptor
GRK	G-protein receptor kinase
HAB	high anxiety-related behavior
HPA axis	hypothalamo-pituitary-adrenal axis
icv	intracerebroventricular
LAB	low anxiety-related behavior
LC	locus coeruleus
LD	lactation day
LDB	light-dark box
LS	lateral septum
MAPK	mitogen-activated protein kinase

mBNST	medial bed nucleus of the stria terminalis
mpBNST	medial-posterior bed nucleus of the stria terminalis
MPOA	medial preoptic area
NA	noradrenaline
NAB	non-selected for anxiety-related behavior
OXT	oxytocin
PAG	periaqueductal gray
pBNST	posterior bed nucleus of the stria terminalis
PD	pregnancy day
PKA	protein kinase A
PLC	phospholipase C
pPVN	parvocellular part of the paraventricular nucleus
PRL	prolactin
PRT	pup retrieval test
PVN	paraventricular nucleus
RE	response element
SON	supraoptic nucleus
Ucn	urocortin
vBNST	ventral bed nucleus of the stria terminalis
VEH	vehicle

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Supplementary

Table 1. Effects of non-specific icv CRF-R manipulation on non-maternal behaviors under non-stress conditions on LD 1. The occurrence of all off-nest behaviors was scored for 60 min before and for 120 min after infusion (indicated by the dotted line). Off-nest behavior is further divided into locomotion (including digging/burrowing and any explorative behavior in the home cage), self-grooming, and sleeping/resting. Dams received an acute icv infusion of vehicle (VEH; 5 µl sterile Ringer's solution; pH 7.4), CRF (1 µg / 5 µl), or D-Phe (10 µg / 5 µl). Data is presented as mean ± SEM. n = 6 - 7 per group. ** $p \leq 0.01$, * $p \leq 0.05$, (*) $p = 0.06$ versus VEH; ++ $p \leq 0.01$, + $p \leq 0.05$ versus previous time-point (two-way ANOVA for repeated measures; factors: time x treatment).

Behavior	Strain	Group	Occurrence [n]					
			-60 min	-30 min	0 min	+30 min	+60 min	+90 min
Off-nest	HAB	VEH	0.7±0.6	0.6±0.4	4.0±1.4^{+}	1.9±0.8	1.7±1.0	0.9±0.9
		CRF	0.4±0.4	2.0±1.0	10.4±1.5**$^{++}$	6.8±1.9$^{(*)}$	5.0±1.8	3.9±1.8
		D-Phe	1.1±0.7	0.3±0.2	5.2±1.6^{++}	3.3±2.0	3.3±1.5	2.0±1.4
	LAB	VEH	3.6±1.9	2.6±1.0	5.7±1.2	1.4±1.0	2.4±2.1	3.3±2.3
		CRF	5.0±1.6	1.9±1.4	10.3±1.5^{++}	9.4±1.8**	8.1±2.1$^{(*)}$	6.4±1.8
		D-Phe	3.2±1.6	3.7±1.5	6.4±1.8	6.0±2.0	5.2±1.9	2.2±1.5
	NAB	VEH	0.6±0.4	1.0±0.5	2.0±0.8	0.5±0.5	3.0±1.5	2.1±0.8
		CRF	0.1±0.1	0.6±0.4	5.6±1.5	5.8±1.9	4.8±1.8	4.0±1.9
		D-Phe	0.4±0.2	1.1±0.9	4.4±1.9	3.4±1.7	2.4±1.5	1.3±1.3
Locomotion	HAB	VEH	0.3±0.2	0.4±0.3	2.4±0.7	0.9±0.3	0.6±0.4	0.1±0.1
		CRF	0.1±0.1	1.1±0.5	7.9±1.7**$^{++}$	2.8±1.0$^{(*)}$	2.6±1.1$^{(*)}$	1.6±0.7*
		D-Phe	0.4±0.2	0.2±0.1	2.6±0.8^{+}	0.7±0.4	0.6±0.4	0.6±0.3
	LAB	VEH	2.9±1.5	1.3±0.4	4.4±1.7^{+}	1.0±0.7	0.3±0.3	1.1±0.8
		CRF	2.6±1.0	1.5±0.7	5.6±0.8^{++}	5.0±1.1**	4.8±1.4**	3.2±1.0
		D-Phe	1.3±0.8	1.3±0.5	3.8±1.0	2.7±0.8	2.2±0.9	1.0±0.7
	NAB	VEH	0.3±0.2	0.1±0.1	1.0±0.5	0.3±0.3	0.5±0.3	0.6±0.3
		CRF	0.1±0.1	0.3±0.2	2.7±0.6	1.7±0.5	1.6±0.7	1.2±0.6
		D-Phe	0.1±0.1	0.4±0.3	2.3±0.9	1.4±0.6	1.3±1.0	0.6±0.6
Self-grooming	HAB	VEH	0.1±0.1	0.1±0.1	0.6±0.3	0.3±0.3	0.3±0.2	0.3±0.3
		CRF	0.0±0.0	0.3±0.3	1.0±0.3^{+}	2.3±1.0*	1.3±0.6	0.6±0.4
		D-Phe	0.0±0.0	0.0±0.0	0.3±0.2	0.1±0.1	0.3±0.2	0.4±0.2
	LAB	VEH	0.0±0.0	0.9±0.5	0.6±0.4	0.1±0.1	0.3±0.2	0.3±0.2
		CRF	0.4±0.3	0.3±0.2	3.9±0.8**$^{++}$	3.5±1.0**	1.9±0.8$^{(*)}$	1.1±0.5
		D-Phe	0.8±0.7	0.9±0.6	0.9±0.3	0.6±0.2	0.4±0.2	0.2±0.1
	NAB	VEH	0.1±0.1	0.1±0.1	0.4±0.3	0.0±0.0	0.6±0.3	0.5±0.2
		CRF	0.0±0.0	0.2±0.1	0.1±0.1	1.2±0.5	0.8±0.5	0.4±0.2
		D-Phe	0.1±0.1	0.1±0.1	0.3±0.3	0.6±0.4	0.1±0.1	0.1±0.1
Sleeping/ resting	HAB	VEH	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.1
		CRF	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
		D-Phe	0.0±0.0	0.0±0.0	0.1±0.1	1.7±1.7	1.4±1.4	0.0±0.0
	LAB	VEH	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
		CRF	0.9±0.9	0.1±0.1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
		D-Phe	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	NAB	VEH	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.3±0.3
		CRF	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
		D-Phe	0.0±0.0	0.0±0.0	0.1±0.1	0.0±0.0	0.0±0.0	0.0±0.0

Table 2. Effects of non-specific icv CRF-R manipulation on non-maternal behaviors under stress conditions on LD 4. The occurrence of all off-nest behaviors was scored for 60 min before and for 60 min after the combined infusion with the maternal defense test (indicated by the dotted line). Off-nest behavior is further divided into locomotion (including digging/burrowing and any explorative behavior in the home cage), self-grooming, and sleeping/resting. For details on treatments see legend to Table 1. Data is presented as mean ± SEM. n = 6 - 7 per group. ** $p \leq 0.01$ versus VEH; ++ $p \leq 0.01$ versus previous time-point (two-way ANOVA for repeated measures; factors: time x treatment).

Behavior	Strain		Occurrence [n]			
			-130 min	-100 min	0 min	+30 min
Off-nest	HAB	VEH	2.0±1.3	2.8±2.8	6.6±1.7	2.6±2.6
		CRF	4.0±1.8	8.5±2.4	8.1±1.2	2.8±0.9
		D-Phe	1.4±0.9	2.9±2.0	6.1±2.0	1.9±1.2
	LAB	VEH	8.6±2.4	9.9±2.4	8.7±1.8	7.0±2.0
		CRF	4.7±1.6	6.1±2.2	10.2±2.1	6.9±1.9
		D-Phe	6.6±2.3	5.7±2.2	9.2±1.5	3.0±1.3
	NAB	VEH	1.6±0.9	3.4±2.0	4.8±1.3	2.9±1.0
		CRF	2.1±1.1	1.8±1.3	9.1±1.6	7.8±2.0
		D-Phe	1.3±1.0	0.9±0.6	7.9±1.8	2.5±1.8
Locomotion	HAB	VEH	0.8±0.6	1.2±1.2	2.8±0.6	1.0±1.0
		CRF	1.0±0.4	1.1±0.4	2.9±0.6	1.6±0.5
		D-Phe	1.0±0.6	0.3±0.2	2.0±0.7	0.9±0.6
	LAB	VEH	4.1±1.5	4.6±1.4	5.7±1.1	2.4±0.8
		CRF	1.3±0.5	2.8±1.2	6.1±1.3	3.6±1.0
		D-Phe	1.8±1.0	2.3±1.2	3.4±0.8	1.4±0.5
	NAB	VEH	0.3±0.3	0.5±0.5	1.6±0.4	0.8±0.3
		CRF	0.3±0.2	0.0±0.0	4.4±0.9**$^{++}$	3.2±0.9**
		D-Phe	0.3±0.2	0.5±0.4	3.0±0.7^{++}	1.0±0.6
Self-grooming	HAB	VEH	0.6±0.4	0.0±0.0	1.4±0.6	0.0±0.0
		CRF	0.6±0.3	1.3±0.6	5.0±1.4	0.6±0.4
		D-Phe	0.3±0.2	0.4±0.4	3.3±1.5	0.3±0.2
	LAB	VEH	1.1±0.6	1.9±0.8	1.6±0.6	1.1±0.4
		CRF	0.3±0.2	0.9±0.5*	3.6±1.0	1.9±0.7
		D-Phe	1.8±0.8	1.4±0.6	2.9±0.9	0.1±0.1
	NAB	VEH	0.4±0.2	0.5±0.3	1.6±0.5	0.9±0.3
		CRF	0.2±0.2	0.1±0.1	3.2±0.7	2.8±1.0
		D-Phe	0.1±0.1	0.1±0.1	2.5±0.7	0.8±0.6
Sleeping/ resting	HAB	VEH	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
		CRF	1.1±1.1	5.0±2.0	0.0±0.0	0.0±0.0
		D-Phe	0.0±0.0	1.6±1.6	0.0±0.0	0.0±0.0
	LAB	VEH	0.1±0.1	1.0±0.7	0.0±0.0	1.7±1.7
		CRF	2.0±1.3	0.7±0.6	0.0±0.0	0.1±0.1
		D-Phe	0.0±0.0	0.0±0.0	0.1±0.1	0.0±0.0
	NAB	VEH	0.5±0.3	1.8±1.6	0.1±0.1	0.5±0.5
		CRF	0.2±0.1	0.0±0.0	0.1±0.1	0.3±0.3
		D-Phe	0.0±0.0	0.0±0.0	0.1±0.1	0.1±0.1

Table 3. Effects of non-specific icv CRF-R manipulation on non-maternal behaviors under non-stress conditions on LD 1. The occurrence of all off-nest behaviors was scored for 60 min before and for 90 min after infusion (indicated by the dotted line). Off-nest behavior is further divided into locomotion (including digging/burrowing and any explorative behavior in the home cage), self-

grooming, and sleeping/resting. Dams received an acute icv infusion of vehicle (VEH; 5 µl sterile Ringer's solution; pH 7.4), CRF-R1/2 agonist Ucn 1 (CRF-R1/2 ago; 1 µg / 5 µl), or CRF-R1/2 antagonist D-Phe (CRF-R1/2 ant; 10 µg / 5 µl). Data is presented as mean ± SEM. n = 6 - 7 per group. * p ≤ 0.05 versus VEH; ++ p ≤ 0.01 versus previous time-point (two-way ANOVA for repeated measures; factors: time x treatment), ## p ≤ 0.01 versus VEH (two-way ANOVA for repeated measures; factor: treatment).

Behavior	Group	Occurrence [n]				
		-60 min	-30 min	+30 min	+60 min	+90 min
Off-nest	VEH	0.5 ± 0.5	0.0 ± 0.0	4.0 ± 2.4^{++}	2.5 ± 1.8	2.8 ± 1.2
	CRF-R1/2 ago	0.2 ± 0.2	0.8 ± 0.3	1.5 ± 0.8	1.2 ± 1.0	3.5 ± 1.5
	CRF-R1/2 ant	0.4 ± 0.2	0.9 ± 0.6	0.0 ± 0.0*	1.1 ± 0.6	0.4 ± 0.4
Locomotion	VEH	0.5 ± 0.5	0.0 ± 0.0	1.5 ± 0.9	0.3 ± 0.2	0.0 ± 0.0
	CRF-R1/2 ago	0.0 ± 0.0	0.5 ± 0.2	0.5 ± 0.3	0.7 ± 0.5	1.3 ± 0.6
	CRF-R1/2 ant	0.3 ± 0.2	0.3 ± 0.2	0.0 ± 0.0	0.3 ± 0.2	0.1 ± 0.1
Self-grooming	VEH	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.2	0.0 ± 0.0	0.2 ± 0.2
	CRF-R1/2 ago $^{\#\#}$	0.2 ± 0.2	0.3 ± 0.2	1.0 ± 0.5	0.3 ± 0.3	0.8 ± 0.4
	CRF-R1/2 ant	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.3 ± 0.2	0.0 ± 0.0
Sleeping/ resting	VEH	0.0 ± 0.0	0.0 ± 0.0	2.3 ± 2.3	1.8 ± 1.8	0.8 ± 0.8
	CRF-R1/2 ago	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	CRF-R1/2 ant	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Table 4. Effects of non-specific icv CRF-R manipulation on non-maternal behaviors under stress conditions on LD 5. The occurrence of all off-nest behaviors was scored for 60 min before and for 60 min after the combined infusion with the maternal defense test (indicated by the dotted line). Off-nest behavior is further divided into locomotion (including digging/burrowing and any explorative behavior in the home cage), self-grooming, and sleeping/resting. For details on treatments see legend to Table 3. Data is presented as mean ± SEM. n = 6 - 7 per group. ** p ≤ 0.01 versus VEH; ++ p ≤ 0.01 versus previous time-point (two-way ANOVA for repeated measures; factors: time x treatment).

Behavior	Group	Occurrence [n]			
		-130 min	-100 min	0 min	+30 min
Off-nest	VEH	0.0 ± 0.0	1.7 ± 1.3	8.5 ± 1.7	2.2 ± 1.3
	CRF-R1/2 ago	0.3 ± 0.3	1.5 ± 0.6	10.0 ± 1.5	5.2 ± 1.8
	CRF-R1/2 ant	0.9 ± 0.4	2.8 ± 1.2	8.5 ± 1.3	4.6 ± 2.0
Locomotion	VEH	0.0 ± 0.0	1.0 ± 0.7	5.5 ± 1.3	0.2 ± 0.2
	CRF-R1/2 ago	0.0 ± 0.0	0.3 ± 0.2	4.3 ± 1.0	0.7 ± 0.2
	CRF-R1/2 ant	0.4 ± 0.3	0.9 ± 0.6	3.8 ± 0.6	0.8 ± 0.4
Self-grooming	VEH	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 0.4	0.3 ± 0.3
	CRF-R1/2 ago	0.2 ± 0.2	0.2 ± 0.2	2.8 ± 0.7**$^{++}$	0.5 ± 0.3
	CRF-R1/2 ant	0.4 ± 0.3	0.5 ± 0.3	0.5 ± 0.3	0.5 ± 0.3
Sleeping/ resting	VEH	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.6
	CRF-R1/2 ago	0.2 ± 0.2	0.3 ± 0.3	0.5 ± 0.3	2.7 ± 1.7
	CRF-R1/2 ant	0.0 ± 0.0	0.3 ± 0.3	1.3 ± 0.9	2.6 ± 1.7

Table 5. Effects of specific intra-mpBNST CRF-R manipulation on non-maternal behaviors under non-stress conditions on LD 1. The occurrence of all off-nest behaviors was scored for 60 min before and 90 min after infusion (indicated by the dotted line) as well as 60 min in the afternoon. Off-nest behavior is further divided into locomotion (including digging/burrowing and any explorative behavior in the home cage), self-grooming, and sleeping/resting. Dams received an acute bilateral infusion of vehicle (VEH; 0.5 µl sterile Ringer's solution; pH 7.4), CRF-R1 agonist human/rat CRF (CRF-R1 ago; 1 µg / 0.5 µl), CRF-R1 antagonist CP-154,526 (CRF-R1 ant; 12 µg / 0.5 µl), CRF-R2 agonist Stresscopin (CRF-R2 ago; 3 µg / 0.5 µl), or CRF-R2 antagonist Astressin-2B (CRF-R2 ant; 4 µg / 0.5 µl) into the mpBNST. Data is presented as mean ± SEM. n = 8 – 14 per group. ** $p \leq 0.01$, * $p \leq 0.05$ versus VEH; ++ $p \leq 0.01$ versus previous time-point (two-way ANOVA for repeated measures; factors: time x treatment).

Behavior	Group	Occurrence [n]						
		-60 min	-30 min	+30 min	+60 min	+90 min	+300 min	+330 min
Off-nest	VEH	0.4±0.1	1.0±0.6	1.3±1.0	0.9±0.7	2.3±1.2	0.3±0.2	0.5±0.4
	CRF-R1 ago	1.1±0.7	0.6±0.6	6.6±1.4**$^{++}$	1.4±0.5	0.5±0.3	1.9±1.7	0.6±0.6
	CRF-R1 ant	0.0±0.0	1.7±1.0	2.0±1.3	1.7±1.3	0.5±0.2	1.7±1.2	2.5±1.4
	CRF-R2 ago	1.4±0.6	1.2±0.6	5.3±1.8*$^{++}$	2.8±1.6	1.9±0.9	3.3±1.7	4.8±2.2*
	CRF-R2 ant	1.3±0.7	0.7±0.4	0.5±0.2	1.7±0.7	1.2±0.9	1.4±1.1	0.2±0.2
Locomotion	VEH	0.3±0.1	0.3±0.2	0.1±0.1	0.0±0.0	0.4±0.3	0.2±0.1	0.0±0.0
	CRF-R1 ago	0.5±0.4	0.1±0.1	3.9±1.0**$^{++}$	1.0±0.3	0.1±0.1	0.1±0.1	0.0±0.0
	CRF-R1 ant	0.0±0.0	1.1±0.7	1.6±1.2	1.0±0.7	0.2±0.2	0.3±0.1	0.2±0.1
	CRF-R2 ago	0.4±0.2	1.1±0.7	1.4±0.6	0.8±0.4	0.2±0.1	0.1±0.1	0.2±0.1*
	CRF-R2 ant	0.4±0.2	0.4±0.2	0.2±0.1	0.5±0.3	0.7±0.5	0.0±0.0	0.0±0.0
Self-grooming	VEH	0.1±0.1	0.3±0.3	0.3±0.3	0.3±0.2	0.4±0.2	0.2±0.2	0.0±0.0
	CRF-R1 ago	0.1±0.1	0.1±0.1	2.0±0.5**$^{++}$	0.4±0.3	0.1±0.1	0.1±0.1	0.0±0.0
	CRF-R1 ant	0.0±0.0	0.2±0.1	0.1±0.1	0.2±0.2	0.3±0.2	0.1±0.1	0.3±0.1*
	CRF-R2 ago	0.2±0.1	0.0±0.0	0.9±0.4^{+}	0.3±0.2	0.1±0.1	0.1±0.1	0.1±0.1
	CRF-R2 ant	0.2±0.2	0.1±0.1	0.2±0.1	0.2±0.2	0.0±0.0*	0.1±0.1	0.0±0.0
Sleeping/ resting	VEH	0.0±0.0	0.0±0.0	0.6±0.6	0.0±0.0	1.3±1.3	0.0±0.0	0.0±0.0
	CRF-R1 ago	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	1.6±1.6	0.6±0.6
	CRF-R1 ant	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	1.4±1.1	1.9±1.4
	CRF-R2 ago	0.0±0.0	0.0±0.0	2.1±1.4^{++}	1.4±1.4	1.0±0.7	4.7±2.1**	4.4±2.2**
	CRF-R2 ant	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	1.2±1.1	0.2±0.2

Table 6. Effects of specific intra-mpBNST CRF-R manipulation on non-maternal behaviors under stress conditions on LD 5. The occurrence of all off-nest behaviors was scored for 60 min before and 60 min after the combined infusion with the maternal defense test (indicated by the dotted line). Off-nest behavior is further divided into locomotion (including digging/burrowing and any explorative behavior in the home cage), self-grooming, and sleeping/resting. For details on treatments see legend to Table 5. Data is presented as mean ± SEM. n = 8 – 14 per group.

Behavior	Group	Occurrence [n]			
		-130 min	-100 min	0 min	+30 min
Off-nest	VEH	2.4 ± 1.1	4.1 ± 1.5	6.3 ± 1.7	4.0 ± 1.6
	CRF-R1 ago	3.9 ± 2.2	3.5 ± 1.8	8.3 ± 2.0	4.9 ± 2.0
	CRF-R1 ant	1.1 ± 0.6	3.1 ± 0.8	5.4 ± 2.0	1.7 ± 0.9
	CRF-R2 ago	1.6 ± 1.3	3.3 ± 1.5	5.4 ± 1.5	2.7 ± 1.6
	CRF-R2 ant	3.0 ± 1.1	1.7 ± 0.7	3.8 ± 1.2	2.2 ± 1.3
Locomotion	VEH	0.2 ± 0.1	0.5 ± 0.3	2.5 ± 0.8	0.7 ± 0.3
	CRF-R1 ago	1.5 ± 1.1	0.8 ± 0.5	4.6 ± 1.4	0.9 ± 0.5
	CRF-R1 ant	0.3 ± 0.2	1.3 ± 0.7	3.0 ± 1.2	1.1 ± 0.6
	CRF-R2 ago	0.2 ± 0.1	1.7 ± 0.9	1.7 ± 0.4	0.6 ± 0.3
	CRF-R2 ant	1.3 ± 0.5	0.5 ± 0.3	1.7 ± 0.6	0.5 ± 0.2
Self-grooming	VEH	0.6 ± 0.3	1.0 ± 0.6	1.7 ± 0.5	0.3 ± 0.2
	CRF-R1 ago	0.1 ± 0.1	0.4 ± 0.2	1.1 ± 0.5	1.0 ± 0.4
	CRF-R1 ant	0.1 ± 0.1	0.7 ± 0.4	2.0 ± 1.6	0.6 ± 0.4
	CRF-R2 ago	0.1 ± 0.1	0.3 ± 0.3	1.1 ± 0.4	0.0 ± 0.0
	CRF-R2 ant	0.4 ± 0.2	0.5 ± 0.5	0.7 ± 0.3	0.0 ± 0.0
Sleeping/ resting	VEH	0.3 ± 0.3	0.2 ± 0.1	0.8 ± 0.6	2.4 ± 1.6
	CRF-R1 ago	0.0 ± 0.0	1.6 ± 1.6	1.3 ± 1.1	2.8 ± 2.0
	CRF-R1 ant	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	CRF-R2 ago	1.0 ± 1.0	0.4 ± 0.4	1.4 ± 1.1	1.6 ± 1.3
	CRF-R2 ant	0.0 ± 0.0	0.0 ± 0.0	0.9 ± 0.7	1.2 ± 1.2

Table 7. Effects of specific intra-adBNST CRF-R manipulation on non-maternal behaviors under non-stress conditions on LD 1. The occurrence of all off-nest behaviors was scored for 60 min before and 90 min after infusion (indicated by the dotted line) as well as 60 min in the afternoon. Off-nest behavior is further divided into locomotion (including digging/burrowing and any explorative behavior in the home cage), self-grooming, and sleeping/resting. For details on treatments see legend to Table 5. Data is presented as mean ± SEM. n = 6 - 8 per group. ** $p \leq 0.01$, * $p \leq 0.05$, (*) $p = 0.06$ versus VEH; ++ $p \leq 0.01$ versus previous time-point (two-way ANOVA for repeated measures; factors: time x treatment).

Behavior	Group	Occurrence [n]						
		-60 min	-30 min	+30 min	+60 min	+90 min	+300 min	+330 min
Off-nest	VEH	1.9±1.2	0.0±0.0	0.4±0.4	0.4±0.4	0.1±0.1	0.0±0.0	0.3±0.2
	CRF-R1 ago	2.4±1.1	0.1±0.1	4.5±0.9**$^{++}$	4.9±1.0**	5.6±1.5**	3.6±1.6**	1.0±0.5
	CRF-R1 ant	0.1±0.1	0.4±0.3	2.9±1.2	0.0±0.0	0.6±0.4	0.3±0.3	0.9±0.4
	CRF-R2 ago	0.0±0.0	0.2±0.2	4.8±2.9*$^{++}$	2.8±2.5	3.0±2.4	0.0±0.0	1.3±1.0
	CRF-R2 ant	1.1±0.8	0.9±0.6	2.4±0.9	1.3±1.3	0.6±0.4	0.0±0.0	1.3±0.9
Locomotion	VEH	0.9±0.4	0.0±0.0	0.3±0.3	0.0±0.0	0.0±0.0	0.0±0.0	0.3±0.2
	CRF-R1 ago	0.8±0.5	0.0±0.0	2.9±0.6**$^{++}$	3.5±1.0**	2.8±0.8**	2.3±1.0**	0.9±0.5
	CRF-R1 ant	0.1±0.1	0.4±0.3*	0.0±0.0	0.0±0.0	0.4±0.3	0.0±0.0	0.3±0.2
	CRF-R2 ago	0.0±0.0	0.2±0.2	0.3±0.2	0.2±0.2	0.7±0.5	0.0±0.0	0.2±0.2
	CRF-R2 ant	0.0±0.0*	0.0±0.0	0.0±0.0	0.1±0.1	0.1±0.1	0.0±0.0	0.3±0.2
Self-grooming	VEH	0.0±0.0	0.0±0.0	0.1±0.1	0.0±0.0	0.1±0.1	0.0±0.0	0.0±0.0
	CRF-R1 ago	0.1±0.1	0.0±0.0	1.1±0.4*$^{++}$	2.9±1.0**	2.0±0.8**	0.1±0.1	0.1±0.1
	CRF-R1 ant	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.1	0.0±0.0	0.1±0.1
	CRF-R2 ago	0.0±0.0	0.0±0.0	0.8±0.7^{+}	0.2±0.2	0.2±0.2	0.0±0.0	0.0±0.0
	CRF-R2 ant	0.3±0.3	0.0±0.0	0.3±0.3	0.4±0.3	0.1±0.1	0.0±0.0	0.1±0.1
Sleeping/ resting	VEH	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	CRF-R1 ago	1.4±1.0*	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.6±0.6	0.0±0.0
	CRF-R1 ant	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	CRF-R2 ago	0.0±0.0	0.0±0.0	3.2±2.0**$^{++}$	2.5±2.5$^{(*)}$	1.3±1.1*	0.0±0.0	0.0±0.0
	CRF-R2 ant	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

Table 8. Effects of specific intra-adBNST CRF-R manipulation on non-maternal behavior under stress conditions on LD 7. The occurrence of all off-nest behaviors was scored for 60 min before and 60 min after the combined infusion with the maternal defense test (indicated by the dotted line). Off-nest behavior is further divided into locomotion (including digging/burrowing and any explorative behavior in the home cage), self-grooming, and sleeping/resting. For details on treatments see legend to Table 5. Data is presented as mean ± SEM. n = 6 – 8 per group.

Behavior	Group	Occurrence [n]			
		-130 min	-100 min	0 min	+30 min
Off-nest	VEH	0.5±0.3	0.1±0.1	3.8±1.0	0.6±0.3
	CRF-R1 ago	0.4±0.3	1.1±0.9	1.6±0.6	1.0±0.5
	CRF-R1 ant	1.3±0.6	0.1±0.1	3.9±1.5	0.7±0.4
	CRF-R2 ago	1.3±1.0	0.3±0.3	4.2±2.3	0.7±0.7
	CRF-R2 ant	0.3±0.3	0.6±0.4	3.6±1.7	0.6±0.6
Locomotion	VEH	0.3±0.2	0.1±0.1	2.0±0.6	0.1±0.1
	CRF-R1 ago	0.3±0.2	0.6±0.4	1.0±0.3	0.4±0.3
	CRF-R1 ant	0.3±0.2	0.1±0.1	1.9±0.6	0.4±0.2
	CRF-R2 ago	0.3±0.2	0.0±0.0	1.8±0.7	0.2±0.2
	CRF-R2 ant	0.0±0.0	0.6±0.4	0.9±0.3	0.1±0.1
Self-grooming	VEH	0.3±0.2	0.0±0.0	0.5±0.3	0.1±0.1
	CRF-R1 ago	0.1±0.1	0.1±0.1	0.3±0.3	0.1±0.1
	CRF-R1 ant	0.1±0.1	0.0±0.0	0.7±0.3	0.1±0.1
	CRF-R2 ago	0.0±0.0	0.2±0.2	0.8±0.5	0.2±0.2
	CRF-R2 ant	0.0±0.0	0.0±0.0	2.1±1.3	0.3±0.3
Sleeping/ resting	VEH	0.0±0.0	0.0±0.0	0.9±0.6	0.3±0.2
	CRF-R1 ago	0.0±0.0	0.1±0.1	0.0±0.0	0.0±0.0
	CRF-R1 ant	0.0±0.0	0.0±0.0	0.6±0.6	0.0±0.0
	CRF-R2 ago	1.0±1.0	0.0±0.0	0.2±0.2	0.3±0.3
	CRF-R2 ant	0.3±0.3	0.0±0.0	0.4±0.2	0.0±0.0

Table 9. Effects of icv CRF-BP inhibition on non-maternal behaviors under non-stress conditions on LD 1. The occurrence of all off-nest behaviors was scored for 60 min before and for 90 min after infusion (indicated by the dotted line) as well as 60 min in the afternoon. Off-nest behavior is further divided into locomotion (including digging/burrowing and any explorative behavior in the home cage), self-grooming, and sleeping/resting. Dams received an acute icv infusion of vehicle (VEH; 5 µl of sterile Ringer's solution) or the CRF-BP inhibitor $CRF_{(6-33)}$ (5 µg / 5 µl). Data are presented as mean ± SEM. n = 9 – 12 per group.

Behavior	Group	Occurrence [n]						
		-60 min	-30 min	+30 min	+60 min	+90 min	+300 min	+330 min
Off-nest	VEH	0.6±0.3	1.1±0.7	0.2±0.2	0.1±0.1	0.6±0.3	0.9±0.5	3.2±1.4
	$CRF_{(6-33)}$	1.5±0.8	1.3±0.6	0.0±0.0	0.0±0.0	0.6±0.3	1.3±1.2	2.1±1.3
Locomotion	VEH	0.4±0.2	0.8±0.5	0.2±0.2	0.0±0.0	0.3±0.2	0.4±0.2	1.2±0.7
	$CRF_{(6-33)}$	0.8±0.4	1.3±0.5	0.0±0.0	0.0±0.0	0.5±0.2	0.1±0.1	0.3±0.2
Self-grooming	VEH	0.1±0.1	0.1±0.1	0.0±0.0	0.1±0.1	0.1±0.1	0.0±0.0	0.2±0.2
	$CRF_{(6-33)}$	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.3±0.2
Sleeping/ resting	VEH	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.4±0.4	0.4±0.4
	$CRF_{(6-33)}$	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	1.3±1.3	1.3±1.3

Table 10. Effects of icv CRF-BP inhibition on non-maternal behaviors under stress conditions on LD 5. The occurrence of all off-nest behaviors was scored for 60 min before and for 60 min after the combined infusion with the maternal defense test (indicated by the dotted line). Off-nest behavior is further divided into locomotion (including digging/burrowing and any explorative behavior in the home cage), self-grooming, and sleeping/resting. For details on treatments see legend to Table 9. Data are presented as mean ± SEM. n = 9 – 12 per group.

Behavior	Group	Occurrence [n]			
		-130 min	-100 min	0 min	+30 min
Off-nest	VEH	3.0±1.7	3.7±1.4	3.4±0.6	1.0±0.5
	$CRF_{(6-33)}$	0.9±0.8	1.5±0.9	4.4±0.7	0.3±0.2
Locomotion	VEH	1.4±1.1	1.6±0.7	2.3±0.4	0.3±0.2
	$CRF_{(6-33)}$	0.0±0.0	0.4±0.2	2.3±0.5	0.2±0.1
Self-grooming	VEH	0.8±0.5	0.3±0.3	0.7±0.4	0.4±0.2
	$CRF_{(6-33)}$	0.0±0.0	0.1±0.1	1.3±0.3	0.1±0.1
Sleeping/ resting	VEH	0.7±0.7	0.7±0.4	0.0±0.0	0.0±0.0
	$CRF_{(6-33)}$	0.9±0.8	1.0±0.9	0.3±0.3	0.0±0.0

Table 11. Effects of intra-mpBNST CRF-BP inhibition on non-maternal behaviors under non-stress conditions on LD 1. The occurrence of all off-nest behaviors was scored for 60 min before and for 90 min after infusion (indicated by the dotted line) as well as 60 min in the afternoon. Off-nest behavior is further divided into locomotion (including digging/burrowing and any explorative behavior in the home cage), self-grooming, and sleeping/resting. Dams received an acute intra-mpBNST infusion of vehicle (VEH; 0.5 µl of sterile Ringer's solution) or the CRF-BP inhibitor $CRF_{(6-33)}$ (5 µg / 0.5 µl). Data are presented as mean ± SEM. n = 9 per group.

Behavior	Group	Occurrence [n]						
		-60 min	-30 min	+30 min	+60 min	+90 min	+300 min	+330 min
Off-nest	VEH	0.8±0.6	0.8±0.6	0.4±0.2	1.2±0.9	1.2±1.1	0.9±0.6	0.7±0.3
	$CRF_{(6-33)}$	1.5±0.8	0.8±0.5	0.2±0.2	1.0±0.7	1.3±1.2	4.0±2.1	3.0±1.6
Locomotion	VEH	0.2±0.2	0.2±0.2	0.3±0.2	0.4±0.3	0.3±0.2	0.5±0.4	0.6±0.2
	$CRF_{(6-33)}$	0.2±0.2	0.1±0.1	0.0±0.0	0.5±0.3	1.0±0.9	0.1±0.1	0.6±0-3
Self-grooming	VEH	0.2±0.2	0.0±0.0	0.1±0.1	0.0±0.0	0.0±0.0	0.1±0.1	0.1±0.1
	$CRF_{(6-33)}$	0.0±0.0	0.2±0.2	0.2±0.2	0.0±0.0	0.3±0.3	0.0±0.0	0.0±0.0
Sleeping/ resting	VEH	0.4±0.4	0.6±0.6	0.0±0.0	0.8±0.8	0.9±0.9	0.3±0.3	0.0±0.0
	$CRF_{(6-33)}$	0.8±0.8	0.2±0.2	0.0±0.0	0.1±0.1	0.0±0.0	3.8±2.0	1.6±1.3

Table 12. Effects of intra-mpBNST CRF-BP inhibition on non-maternal behaviors under stress conditions on LD 5. The occurrence of all off-nest behaviors was scored for 60 min before and for 60 min after the combined infusion with the maternal defense test (indicated by the dotted line). Off-nest behavior is further divided into locomotion (including digging/burrowing and any explorative behavior in the home cage), self-grooming, and sleeping/resting. For details on treatments see legend to Table 11. Data are presented as mean ± SEM. n = 9 per group.

Behavior	Group	Occurrence [n]			
		-130 min	-100 min	0 min	+30 min
Off-nest	VEH	1.9±1.5	1.6±0.6	4.6±1.4	1.1±0.4
	$CRF_{(6-33)}$	2.4±1.6	1.2±0.6	6.2±1.5	4.2±1.1
Locomotion	VEH	0.4±0.2	0.5±0.2	2.1±0.6	0.6±0.3
	$CRF_{(6-33)}$	0.2±0.1	0.4±0.2	3.3±0.8	1.2±0.4
Self-grooming	VEH	0.1±0.1	0.3±0.3	1.2±0.8	0.5±0.3
	$CRF_{(6-33)}$	0.0±0.0	0.7±0.4	1.0±0.5	1.7±0.6
Sleeping/ resting	VEH	1.4±1.4	0.5±0.4	0.4±0.3	0.0±0.0
	$CRF_{(6-33)}$	2.2±1.6	0.1±0.1	1.2±0.9	0.8±0.8

Table 13. Effects of intra-adBNST CRF-BP inhibition on non-maternal behaviors under non-stress conditions on LD 1. The occurrence of all off-nest behaviors was scored for 60 min before and for 90 min after infusion (indicated by the dotted line) as well as 60 min in the afternoon. Off-nest behavior is further divided into locomotion (including digging/burrowing and any explorative behavior in the home cage), self-grooming, and sleeping/resting. For details on treatments see legend to Table 11. Data are presented as mean ± SEM. n = 5 - 7 per group.

Behavior	Group	Occurrence [n]						
		-60 min	-30 min	+30 min	+60 min	+90 min	+300 min	+330 min
Off-nest	VEH	0.6±0.4	0.4±0.4	2.0±1.1	0.7±0.6	0.7±0.6	0.0±0.0	1.4±1.0
	$CRF_{(6-33)}$	0.3±0.3	1.1±0.6	1.3±0.7	0.0±0.0	0.0±0.0	0.1±0.1	1.0±0.7
Locomotion	VEH	0.0±0.0	0.3±0.3	1.7±0.8	0.3±0.3	1.4±1.3	0.0±0.0	0.6±0.4
	$CRF_{(6-33)}$	0.3±0.3	0.7±0.4	0.4±0.4	0.0±0.0	0.9±0.9	0.1±0.1	0.3±0.2
Self-grooming	VEH	0.4±0.3	0.1±0.1	0.0±0.0	0.3±0.2	0.1±0.1	0.0±0.0	0.3±0.2
	$CRF_{(6-33)}$	0.0±0.0	0.0±0.0	0.6±0.4	0.0±0.0	0.0±0.0	0.0±0.0	0.4±0.3
Sleeping/ resting	VEH	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	$CRF_{(6-33)}$	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

Table 14. Effects of intra-adBNST CRF-BP inhibition on non-maternal behaviors under stress conditions on LD 5. The occurrence of all off-nest behaviors was scored for 60 min before and for 60 min after the combined infusion with the maternal defense test (indicated by the dotted line). Off-nest behavior is further divided into locomotion (including digging/burrowing and any explorative behavior in the home cage), self-grooming, and sleeping/resting. For details on treatments see legend to Table 11. Data are presented as mean ± SEM. n = 5 - 7 per group.

Behavior	Group	Occurrence [n]			
		-130 min	-100 min	0 min	+30 min
Off-nest	VEH	2.2±1.2	4.4±2.8	5.2±1.8	2.2±1.4
	$CRF_{(6-33)}$	1.1±0.6	0.6±0.4	10.3±2.3	5.0±1.8
Locomotion	VEH	1.0±0.8	0.4±0.4	3.8±1.6	2.0±1.2
	$CRF_{(6-33)}$	1.0±0.7	0.4±0.4	6.0±1.6	2.4±0.8
Self-grooming	VEH	0.0±0.0	0.4±0.4	1.2±0.6	0.0±0.0
	$CRF_{(6-33)}$	0.1±0.1	0.0±0.0	3.6±1.1	1.3±0.5
Sleeping/ resting	VEH	1.2±1.2	3.6±2.9	0.2±0.2	0.2±0.2
	$CRF_{(6-33)}$	0.0±0.0	0.1±0.1	0.0±0.0	0.0±0.0

Curriculum vitae

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Education

Jun 2011 - date	PhD in Neuroscience in the group of Prof. Dr. Inga Neumann under supervision of PD Dr. Oliver Bosch, University of Regensburg
Oct 2010 - Apr 2011	Masterthesis: The role of corticotropin-releasing factor in maternal behavior and anxiety in lactating rats (Laboratory of Prof. Dr. Inga Neumann; final result: 1.1), University of Regensburg
Sep 2009 - May 2011	Elite Master's Programm "Experimental and Clinical Neuroscience" (final result: 1.6), University of Regensburg
Apr - Aug 2009	Bachelorthesis: The Oxytocin and Vasopressin system in the brain of pregnant and lactating rats: activation after confrontation with a female intruder (Laboratory of Prof. Dr. Inga Neumann; final result: 1.0), University of Regensburg
Oct 2006 - Aug 2009	B.Sc. Biology (final result: 2.3), University of Regensburg
Oct 2005 - Jul 2006	Teaching post for grammar school (French and Spanish), University of Regensburg
Jun 2005	High school diploma (Abitur) at St.- Gotthard-Gymnasium Niederalteich, final result: 2.1

Awards / Grants

Mar 2014	Travel Award to ECNP workshop on Neuropsychopharmacology, Nice, France
Nov 2013	DAAD Travel Grant to the 43th annual meeting of the Society for Neuroscience, San Diego, CA, USA

Publications

Klampfl SM, Brunton PJ, Bayerl DS, Bosch OJ 2014. Hypo-activation of CRF receptors, predominantly type 2, in the medial-posterior BNST is vital for adequate maternal behavior in lactating rats. J Neurosci 34(29):9665-76.

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