Unraveling Maternal Grief in Rats: From Neuroendocrine Dynamics to Neuroplasticity and Behavioral Adaptations

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Chapter 2: The brain oxytocin and corticotropin-releasing factor systems in grieving mothers: What we know and what we need to learn

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Luisa Demarchi:	first draft and revision of manuscript
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Chapter 3: Brief versus long maternal separation in lactating rats: Consequences on maternal behavior, emotionality, and brain oxytocin receptor binding

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Chapter 4: Neurobiological traces of grief: examining the impact of offspring loss after birth on rat mother's brain and behavior in the first week postpartum

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Chapter 5: Long-term consequences of child loss on the mother: Neurobiological insights from an animal model and therapeutic implications

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Alice Sanson:	experimental design, performance of experiments, revision of manuscript
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Oliver Bosch:	experimental design, performance of experiments, funding acquisition, revision of manuscript

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Abstract

The bond between a mother and her infant is widely recognized as one of the most powerful connections in mammals. However, the loss of a child can have devastating effects on parents, leading to immense distress and trauma. Unfortunately, our understanding of the impact of child loss on the maternal neurobiology remains limited. To address this knowledge gap, in my thesis I conducted three separate studies using different protocols of maternal separation in lactating Sprague-Dawley rats to investigate how offspring loss affects the mother's brain and behavior.

In the first study, rats were subjected to brief (BMS; 15 minutes) or long repeated maternal separation (LMS; 180 minutes) during the first postpartum week. LMS dams displayed higher levels of behavioral alterations, as shown by increased pup licking and grooming (LG), and decreased maternal motivation compared to BMS and non-separated control dams. Moreover, BMS mothers exhibited lower plasma CORT concentrations compared to control dams, and OXT-R binding in limbic brain regions was higher in LMS dams compared to BMS and control mothers.

The second study focused on the immediate effects of the permanent offspring loss over the first postpartum week; rat mothers experienced 1-, 3-, or 6- days of total offspring loss. Following 1 day of separation, the mother's neuronal activity increased in the limbic system resulting in a positive correlation between the prelimbic cortex and basolateral amygdala, while OXT-R binding was decreased in the central amygdala following up to 3 days of separation. While plasma CORT levels did not differ between groups either under basal conditions or following stressor exposure on any of the days, the mother's passive stress-coping was significantly increased after 6-days of offspring loss.

In the third study, I investigated the long-term impact of offspring loss on rat dams at molecular and behavioral levels compared to control lactating dams and Virgins. One day of motherhood experience followed by 19-days of offspring loss resulted in an increased OXT-R binding and a decreased dendritic spine density in limbic brain regions of separated mothers whereas the number of estrogen receptor α and calbindin cells were not altered. Moreover, separated dams' CORT plasma concentrations were back the level of Virgins. Importantly, the increased emotionality after long-term offspring loss as tested in the forced

swim test (FST) could be rescued by central blockade of corticotropin-releasing factor receptors, but not by oxytocin (OXT) infusion.

This thesis provides novel insight into the neurobiology of maternal grief and explore potential therapeutic interventions, which can offer better support for individuals going through the painful experience of grief.

-Chapter 1-

General introduction

1.1 Social bonds

Social relationships include the connections and interactions that individuals establish with others, constituting a fundamental aspect of the everyday life well-being not only for humans but also for other animal species, including rodents (Bosch and Young 2018; Pohl et al. 2019). Extensive research has suggested in fact that being in sociality with others could be linked to the feeling of being safe and protected from potential threats (Slavich 2020). Moreover, both the physical and mental health of individuals is also linked to the formation and maintenance of social relationships (Carter 1998; Insel and Young 2001). Social bonds acts as a positive buffer which offer many advantages, such as promoting reproductive success, mitigating stress and anxiety (Kikusui et al. 2006). Furthermore, a body of additional research underscores that experiencing intact and positive social bonds increase the overall longevity of the individuals (Pohl et al. 2019; Roach 2018; Shear and Shair 2005). For instance, studies have demonstrated a strong correlation between experiencing social relationships and reduced risk to develop cardiovascular and psychiatric diseases (Yang et al. 2016). Among the different social bonds, we can find for example parental bonds, social bonds between conspecifics, romantic bonds, and community social bonds (Feldman 2017). Notably, certain rodent species, like the prairie vole (*Microtus ochrogaster)* and California mice *(Peromyscus californicus),* exhibit exceptionally strong socially monogamous relationships, found in a minority of mammals (Carter and Getz 1993). Conversely, maternal care is widespread, with 95% of mammals engaging in this behavior (Lukas and Clutton-Brock 2013). I will describe in the next section the last-mentioned type of bond, the mother-infant bond.

1.1.1 **Maternal bond**

Among the different types of social bonds, the first and most persistent social connection is the bond between the mother and the infant (Bosch and Young 2018). The maternal bond, which is found in most of the mammalian species, is considered to be the evolutionary origin for the capacity to form social bonds later in life (Broad et al. 2006). Decades of research have investigated the repercussions of maternal care (MC) and attachment on the offspring development (Curley and Champagne 2016; Caldji et al. 1998). In both humans and rodents, mothers invest significant energy and effort to maintain the survival of the

offspring. In order to become maternal, the brain of the virgin female undergoes a multitude of neurobiological changes, which lastly allow the female to express maternal behavior (Pereira and Ferreira 2016). Among others, the OXT and corticotropin-releasing factor (CRF) systems are finely balanced to allow the healthy display of maternal behavior (Klampfl and Bosch 2019b). The peripartum period is a delicate period in which many different stimuli will increase and deepen the mother-infant bonding, for instance via breastfeeding, physical contact and caregiving (Rilling and Young 2014). In rats, dams express a strong maternal bond with the offspring, too. In fact, mother rats display a variety of maternal behaviors until the weaning, i.e., on lactation day 21 (LD 21). The quality of MC and the expression of maternal behavior is therefore crucial for the healthy development of the offspring and every kind of perturbation on the mother-infant dyad could have long-lasting effects on both the mother and the child (Bolukbas et al. 2020; Caldji et al. 1998; Numan and Young 2016; Rilling and Young 2014). While most of the past research focused on the investigation of the impact of early life stress on offspring development (for review see: (Nishi 2020)), further research is still needed to investigate the consequences of the mother-infant bond disruption from the mother's perspective.

1.1.2 **Maternal behavior in rats**

In order to explain why rat mothers might be a good model to study grief-related behaviors after offspring loss, I will first explain how the mother-infant bond is expressed by focusing on the mother. Rats exhibit a diverse range of maternal behaviors immediately following birth, all aiming towards enhancing the survival of their offspring and facilitating their journey to weaning, a process lasting until LD21 in rats (Cramer et al. 1990). These maternal behaviors can be classified into three main categories: maternal care (MC), maternal motivation, and maternal aggression. Each category is governed by distinct neural circuits and a complex network of neuropeptide signaling within the central nervous system (CNS) (Numan 2007). Notably, various cues from the pups, including olfactory, auditory, and tactile stimuli, serve as triggers for initiating and modulating these maternal behaviors (Kohl et al. 2018). Maternal Care (MC) encompasses behaviors such as licking and grooming (LG) of the pups, which are indicative of high-quality MC, and nursing. When the mother licks her pups, it serves not only to clean them but also to stimulate their urination and defecation. Nursing behavior can be further categorized into the blanket or

arched-back nursing position (ABN). ABN is considered the optimal form of nursing, characterized by an active posture in which the mother rat forms a kyphotic position over the pups, providing them with free access to the teats. Maternal Motivation is observed through pup retrieval behavior, which is particularly prominent during the first postpartum week (Numan 2007). Pup retrieval occurs when the mother locates her pups outside the nest and carries them back to the safety of the nest using her mouth. Pup retrieval plays a crucial role in maintaining the pups' body temperature and shielding them from potential threats (Numan 2007). Finally, maternal aggression towards intruders is another facet of maternal behavior essential for defending the offspring against potential threats, such as other conspecifics (Erskine et al. 1978). Throughout the peripartum period, the intensity of maternal aggression in rats undergoes significant changes, likely influenced by neuropeptide fluctuations (Caughey et al. 2011). It reaches its peak the day before parturition, diminishes immediately following parturition, peaks again during the early lactation phase around days 4 to 7, and eventually fades until weaning.

1.1.3 **Neural circuits underlying maternal behavior**

(This section has been adapted from my personal contribution to the book chapter: Sanson A; Demarchi L; Bosch OJ. 'Neuroendocrine basis of impaired mothering in rodents', submitted and accepted by Springer. 2023. To be published as part of the bookseries 'Masterclass in Neuroendocrinology').

Maternal behavior belongs to the big group of complex behaviors, involving multi-regions activity in the brain. Understanding the neural mechanism that underlies such behavior is one of primary goals in neuroscience. Complex social behaviors require the coordination of a wide variety of inputs coming from the context and from the internal state of the animal. As mentioned above, mothering requires the recognition of a variety of inputs, including the recognition and the interpretation of cues coming from the offspring, such as olfactory, auditive, tactile and visual cues. Each of these specific cues will determine a precise activation of a population of neurons in different brain regions, that will then consequently activate other neurons and regions allowing to express the specific maternal behavior as a final step. Previous research in the context of mothering behavior in rodents identified several key brain regions that promote specific aspects of maternal behavior. Most of these

studies employed mice and rat's animal models, due to their high suitably for the use of technologies of circuit manipulation and in-vivo monitoring. As a result of those studies, we have today access to a model of the brain circuits controlling maternal behavior (for a detailed overview of parental-related circuits, see (Dulac et al. 2014)). The developed model is far to be completed but it provides already a broad understanding of numerous anatomical and functional connectivity between brain regions required for adequate mothering (Fig. 1).

The first key regions that have been recognized to regulate maternal behavior are the medial preoptic area (MPOA) and the nearby bed nucleus of stria terminalis (BNST) thanks to conventional mapping studies (Numan and Numan 1997; Olazabal et al. 2013). More precisely, the MPOA has long been recognized as a central region for adequate mothering. It is in fact known that MPOA lesions can directly disrupt one of the most important maternal behaviors: the retrieval of pups (Lee et al. 2000). In addition to that, the MPOA express high levels of neuropeptide receptors which modulate mothering, and the direct stimulation of those receptors in the MPOA facilitates maternal behavior, too (Rosenblatt and Ceus 1998; Rosenblatt et al. 1998). Tracing techniques have been extremely useful to understand the input and output projections from the MPOA regions and now researchers can even better understand the role of this key region in controlling mothering (Simerly and Swanson 1986, 1988).

As previously mentioned, the odor is an important cue for the maternal drive. Indeed, the olfactory inputs can act on the MPOA, among other regions, to influence the maternal behavior. A possible route is that the accessory olfactory bulbs (AOB) that receives vomeronasal (VNO) input, projects directly to the medial amygdala (MeA), which in turn projects to the MPOA (Canteras et al. 1995). A second olfactory system, the main olfactory bulb (MOB), which receives input from the main olfactory epithelium (MOE), projects to the cortical amygdala. It is interesting also that both the olfactory systems (AOB and MOB) are directly linked to the MeA neurons (Pro-Sistiaga et al. 2007). MeA neurons are crucial then to integrate the olfactory information from both the circuits (Keshavarzi et al. 2015; Scalia and Winans 1975; Swanson and Petrovich 1998). This very complex circuit, which could converge in the MPOA, is able to give rise to the appropriate pup-directed maternal response.

Another important pup-cue that might influence maternal behavior is the tactile stimulation. The skin-to-skin contact between the mother and the pups was demonstrated to release OXT in pups (Kojima et al. 2012) and also to reduce the maternal anxiety (Lonstein 2005).

Among the tactile stimuli, suckling is one of the most common types of somatic sensory input experienced by lactating mammals. In particular, in lactating rats it was demonstrated that suckling activates a group of neurons in the posterior intralaminar complex of the thalamus (Cservenak et al. 2017), an area that has specific neurons projecting to the MPOA and BNST (Cservenak et al. 2017). Therefore, suckling stimuli could activate MPOA/BNST neurons via a neuronal pathway. Altogether, these findings sustain the idea that the MPOA acts as a crucial center that integrates different sensory stimuli coming from the pups to facilitate maternal responses.

Additionally, techniques such as pharmacological manipulation and surgery have been used to test the functionality of regions related to the MPOA in female rats. In particular, MPOA projections to the nucleus accumbens (NAc) or indirect NAc projections via the retrorubral field and/or ventral tegmental area (VTA) are hypothesized to mediate maternal response (Numan 2003). This motivational circuit is thought to be influenced by the paraventricular nucleus of the hypothalamus (PVN), the lateral habenula (LHb), and serotonergic inputs from the dorsal raphe nucleus (Insel and Harbaugh 1989; Corodimas et al. 1993).

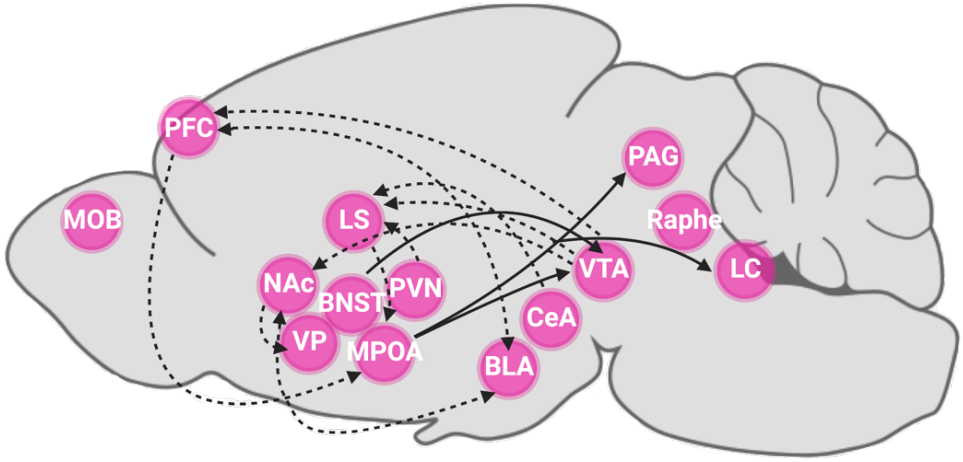


Fig. 1: Schematic representation of brain areas associated with parental care. Abbreviations of brain areas: BLA: basolateral amygdala, BNST: bed nucleus of stria terminalis, CeA: central amygdala, LC: locus coeruleus, LS: lateral septum, MOB: main olfactory bulb, MPOA: medial preoptic area, NAc: nucleus accumbens, PAG: periaqueductal gray, PFC: prefrontal cortex, PVN: paraventricular nucleus, Raphe: Raphe nucleus, VP: ventral pallidum, VTA: ventral tegmental area. Solid lines represent projections that are involved in the parental behavior, supported by direct evidence, and dotted lines denote known connections that exist between these areas and are potentially involved in the behavior. Adapted from: (Dulac et al. 2014).

1.2 Adaptations in motherhood

The transition to motherhood includes a series of transformations both at the psychological and physiological level. Those transformations for instance include changes in the hormonal and neuroendocrine systems (Bridges 2015) but significant changes have been also observed neuroanatomically (Pawluski et al. 2022). In particular, fundamental changes in the neuroendocrine OXT and stress response are necessary to prepare the virgin to became maternal and to ensure the mother to express the variety of maternal care and maternal behaviors towards the offspring (Bridges 2015). In the next sections I will introduce in general the OXT, the hypothalamic-pituitary-adrenal axis, the CRF systems and the neuroplastic changes occurring in mothers.

1.2.1 **The Oxytocin system**

The nonapeptide OXT is synthesized in magnocellular neurons located in the hypothalamic nuclei, specifically the paraventricular nucleus (PVN), accessory nucleus, and supraoptic nucleus (SON). These magnocellular neurons are the primary sites for OXT gene expression within the CNS.

Upon the occurrence of action potentials in these neurosecretory cells, OXT is released from their axon terminals situated in the neurohypophysis (also referred to as the posterior pituitary) (Poulain and Wakerley 1982). In the PVN, parvocellular neurons projects to various extrahypothalamic brain regions. Only a small fraction, approximately 0.2%, of OXT neurons possess axon collaterals connecting both the neurohypophysis and extrahypothalamic regions. OXT fibers and nerve endings have been observed in various brain areas of rats (Fig. 2) (Jurek and Neumann 2018).

In rats, OXT-R are notably abundant in numerous brain regions, encompassing cortical areas, the olfactory system, basal ganglia, the limbic system, thalamus, hypotalamus, brainstem, and the spinal cord. Notably, adult rats exhibit a heightened density of OXT-R in specific regions, such as the dorsal peduncular cortex, the anterior olfactory nucleus, the islands of Calleja, ventral pallidum cell groups, and various components of the limbic system, including the BNST, amygdala, ventral subiculum, and the ventromedial hypothalamus (VMH) (Tribollet et al. 1992).

Importantly, the regulation of OXT-R in the rat brain by gonadal steroids is a complex process. Estrogen exerts a modest influence on OXT synthesis in the brain but exerts a pronounced effect on the regulation of OXT-Rs. For instance, estrogen treatment enhances the affinity of OXT-R in specific brain areas, such as the MPOA, and increases both the density and area of OXT-R binding in the rat VMH (Coirini et al. 1991).

The OXT peripheral action includes stimulating myometrium contractions during labor and facilitating milk secretion during lactation. Importantly, OXT assumes a pivotal role in mediating both maternal care and maternal aggression in rodents (Bosch and Neumann 2012), with its levels increasing during the postpartum period in rat mothers (Landgraf et al. 1992). Notably, there is an elevation in the density of OXT-R around the time of parturition in brain regions well-established to be associated with maternal behavior, including the MPOA and the BNST (Bosch et al. 2010), moreover, the central blockade of OXT-R impedes the initiation of maternal care (van Leengoed et al. 1987).

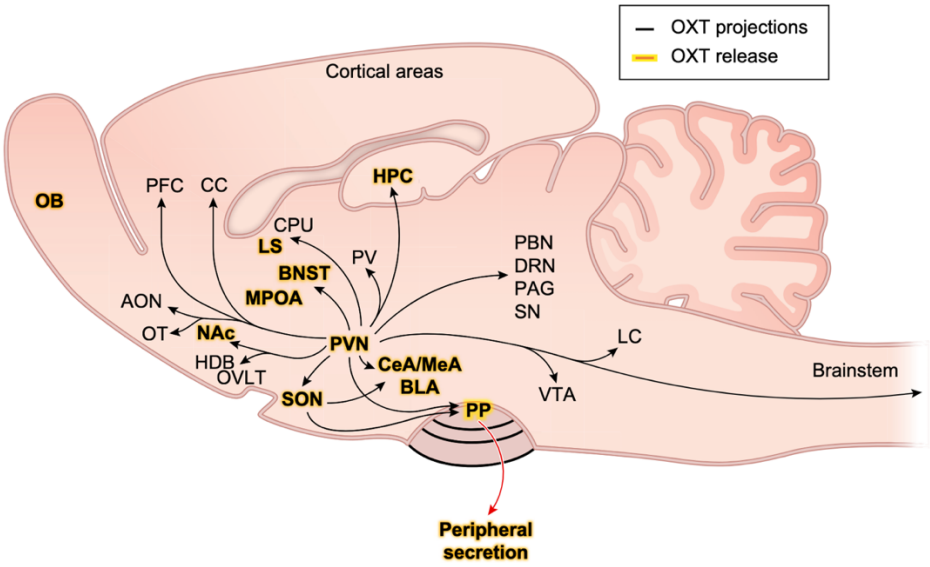


Fig. 2: Schematic illustration of the OXT system.

AON: anterior olfactory nucleus, OB: olfactory bulb, OT: olfactory tubercle, Nac: nucleus accumbens, OVLT: organum vasculosum laminae terminalis, SON: supraoptic nucleus, PVN: paraventricular nucleus of the hypothalamus, PP: posterior pituitary, PFC: prefrontal cortex, CC: cingulate cortex, MPOA: medial preoptic area, BNST: bed nucleus of the stria terminalis, LS: lateral septum, CPu: caudate putamen, PV: paraventricular nucleus of the thalamus, CeA: central amygdala, MeA: medial amygdala, BLA: basolateral amygdala, VTA: ventral tegmental area, LC: locus coeruleus, PBN: parabrachial nucleus, DRN: dorsal raphe nucleus, PAG: periaqueductal gray, SN: substantia nigra, HPC: hippocampus, HDB: nucleus of the horizontal limb of the diagonal band. From: (Jurek and Neumann 2018)

1.2.2 **The hypothalamic-pituitary-adrenal axis**

During motherhood, the physiological response to a stressor is impaired. Losing the offspring might represent a rather strong and even chronic stressor, which might further impact the grief-related outcomes in the mother. The stress response in both humans and rodents is driven by the hypothalamic-pituitary-adrenal (HPA) axis. This system plays a central role in the famous "fight or flight" response, finely tuning an individual's behavior when faced with a stressor (Sheng et al. 2020). The HPA axis consists of three key components: the hypothalamus, the pituitary gland, and the adrenal glands (refer to Fig. 3). At the onset of a stressor, a specific group of neurons releasing CRF located in the dorsomedial parvocellular division of the paraventricular nucleus (PVN) takes the lead (Antoni 1986). These neurons project to the median eminence, releasing CRF into the hypophysial portal plexus of veins. CRF then travels to the anterior pituitary gland, binding to receptors on corticotropes, which stimulates the release of adrenocorticotropic hormone (ACTH) (Aguilera 1994). ACTH, once synthesized, is packaged into vesicles and released into the systemic circulation via regulated exocytosis. In the zona fasciculata of the adrenal cortex, ACTH acts on melanocortin 2 receptors, increasing intracellular cAMP levels and cholesterol biosynthesis (Simpson and Waterman 1988). Cholesterol is a precursor for mineralocorticoids and glucocorticoids. Synthesized glucocorticoids (cortisol in humans, corticosterone (CORT) in rodents) are released into the bloodstream, traveling to different areas of the body and even back to the CNS. The final chemicals in the HPA axis are cortisol in humans and CORT in rats. The secretion of ACTH and glucocorticoids is precisely regulated through negative feedback mechanisms, i.e., when elevated levels of ACTH or cortisol/CORT are detected in the blood, the brain signals the hypothalamus to reduce CRF production.

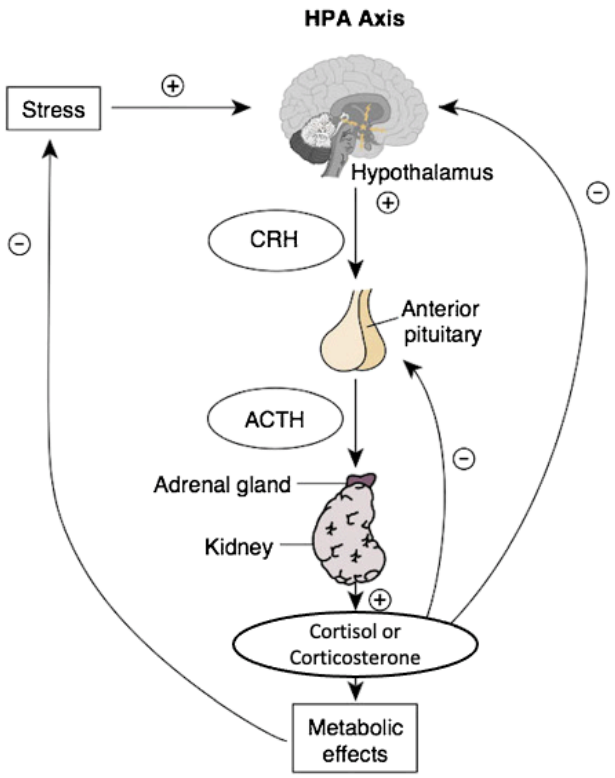


Fig. 3: Overview of the HPA axis. Adapted from: (Hoogendoorn et al. 2017).

1.2.3 **The CRF system: an overview**

The CRF system is a key component of the body's response to stress, but it is also involved in anxiety and depression. Furthermore, the CRF system plays a crucial role in regulating a variety of behaviors accompanying these conditions and has been extensively studied (Deussing and Chen 2018).

CRF is a 41-amino acid peptide that was discovered originally from ovine hypothalamus in 1981 (Vale et al. 1981). CRF has been demonstrated to be the primary regulator of the secretion of ACTH from the anterior pituitary gland, involved in the body's stress response (Vale et al. 1981). The CRF family includes also urocortin 1 (Ucn1) and Ucn2 (also known as stresscopin-related peptides), Ucn3 (stresscopin), and the CRF binding protein (CRF-BP), which can sequester the freely available CRF and Ucn1, preventing them from binding the receptor (Linton et al. 1990). The physiological effect of the CRF family of peptides are mediated through two receptors belonging to the class B of G-protein coupled receptors: CRF type 1 receptor (CRF-R1) and CRF type 2 receptor (CRF-R2) (Perrin and Vale 1999) (Fig. 4). CRF-R1 and CRF-R2 have differential binding affinities to each of the CRF family

members. CRF-R1 shows high affinity to CRF and Ucn1, but no appreciable binding affinity to Ucn2 and Ucn3. CRF-R2 primarily binds to Ucn1, Ucn2, and Ucn3 with greater affinity than to CRF (Fig. 4). The widespread anatomical distribution of CRF and Ucn1-2-3 correlates with the variety of functions associated with this peptide family. CRF is expressed both centrally and peripherally. Research has shown, for example, that the CRF system not only influences general stress and anxiety responses but also modulates social behaviors (Hostetler and Ryabinin 2013). Studies have investigated the relationship between the CRF system and social interaction, which is often used also as a measure of anxiety-like behavior (Lee et al. 2008; Dunn and File 1987). Importantly, maternal behaviors in rats and maternal stress can be modulated by the CRF system (Klampfl et al. 2018; Klampfl and Bosch 2019a).

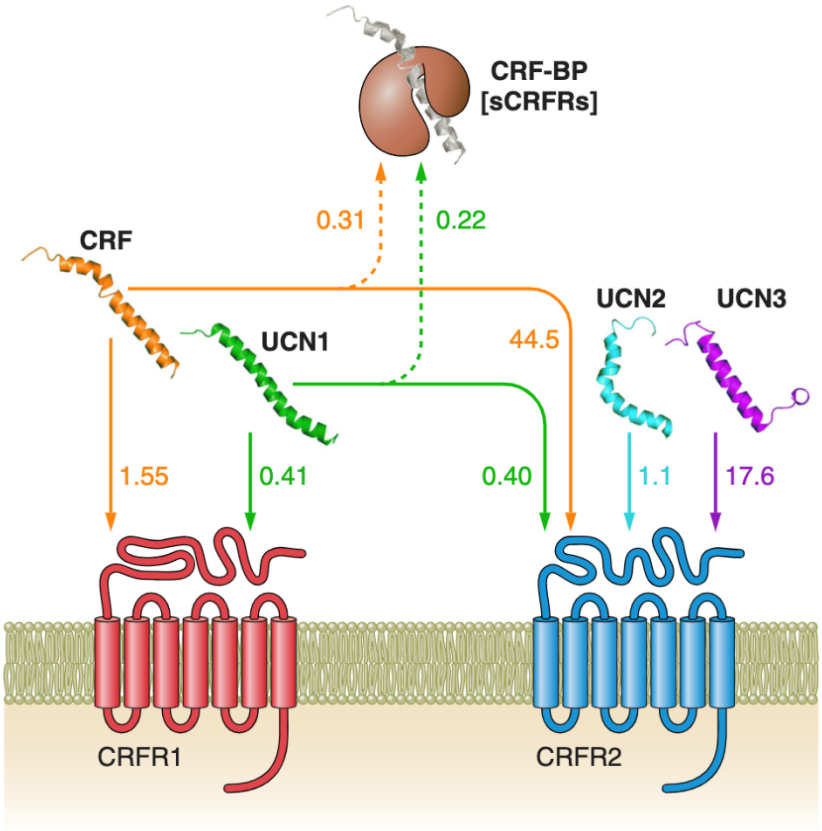


Fig. 4: Interactions of corticotropin-releasing factor (CRF)-related peptide ligands with their receptors and binding proteins. The neuropeptides CRF and Ucn1 (urocortin 1) can bind to CRF-R1 and CRF-R2. Ucn2 and Ucn3 are exclusive ligands of CRF-R2. CRF-binding protein (CRF-BP) can sequester CRF and Ucn1 and thereby controls their availability for receptor activation. From: (Deussing and Chen 2018).

1.2.4 **Neuroplasticity in motherhood**

The peripartum period is one of the most neuroplastic stages in females, together with the adolescence and the menopause (Leuner et al. 2010). In fact, dramatic hormonal fluctuations occur throughout pregnancy and postpartum, and the brain is exposed to rising levels of several hormones, especially steroid hormones (estradiol, progesterone, CORT) which levels increase during pregnancy and drastically drop after parturition (Fig. 5) (Duarte-Guterman et al. 2019). Steroid hormones can play crucial roles in neuroplasticity, influencing neurogenesis and synaptic plasticity (Been et al.2022).

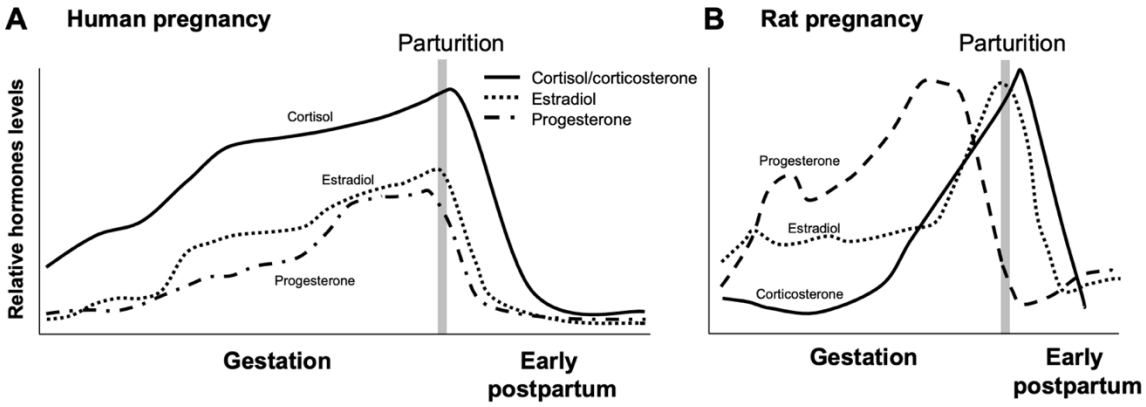


Fig. 5: Hormone profiles during human (A) and rat (B) pregnancy and early postpartum. Taken from (Duarte-Guterman et al. 2019).

Even though the neuroplastic changes have been extensively studied in rodent animal models (Pawluski et al. 2009a; Hillerer et al. 2014; Levy et al. 2011) only more recently, human research has started to investigate and understand the structural and functional neuroplasticity during the peripartum period (Servin-Barthet et al. 2023a). It is interesting that both human mothers and rodent animal models show parallelism. Interestingly, human mothers show a decreased amount of grey matter during pregnancy (Haim et al. 2017; Hoekzema et al. 2017; Pawluski et al. 2016) and postpartum (Martinez-Garcia et al. 2021; Zhang et al. 2019). In rats, similar findings have been found: a reduction of the whole brain size was seen in postpartum rat mothers compared to virgin (Hillerer et al. 2014). The reduced gray matter volume is not linked to behavioral deficits, but it could suggest that the maternal brain undergoes a specific tuning which could be beneficial for the female behavioral function (Pawluski et al. 2022). It is in fact interesting to note that a reduced

brain gray matter could be associated to a decreased neurogenesis and synaptic pruning, both important for healthy behavioral outcome (Lambert et al. 2019). Moreover, the affected brain areas overlap with the neural network regulating the 'theory of mind' (Hoekzema et al. 2017). The theory of mind is the ability to decode mental states in us and others, and it is crucial in maternal care (Schaafsma et al. 2015). In addition, in rat mothers, the MPOA and other brain areas such as the prefrontal cortex (PFC) and Hippocampus undergo neuronal morphological changes in the soma size, dendritic length, branch number and dendritic spine number during the peripartum period (Pawluski et al. 2022). In conclusion, several studies report neuroplastic changes, supporting the hypothesis that hormones may be directly or indirectly involved in those changes. However, further research is needed to better understand how hormonal levels could impact the mother's brain in both a healthy and unhealthy contest.

1.3 Animal models of grief

The arising neuroscience of the grief field predominantly relies on using rodent animal models such as the biparental and socially monogamous prairie voles *(Microtus ochrogaster)* and California mice *(Peromyscus californicus).* These animal models provide the unique opportunity to gain deeper knowledge about the grief-related biological mechanisms resulting from partner loss. Prairie voles, for example, form long-lasting pair-bonds, and previous research has in fact discovered important neuroendocrine changes and behavioral symptoms of grief when these bonded partners are separated (Bosch et al. 2009; Pohl et al. 2019; Sadino et al. 2023; Sun et al. 2014; Vitale et al. 2023). In addition to rodents, other examples of grieving animal models can be found among non-human primates, such as marmosets and titi monkeys (Bales et al. 2017; Miller et al. 2016), which have been studied regarding terms of social bond disruptions (Arias Del Razo et al. 2022; Norcross and Newman 1999). Moreover, there are less commonly studied animals that exhibit behaviors that resemble grief and mourning. For instance, elephants have been seen visiting the remains of dead family members (Pokharel et al. 2022), and non-human primates have been hypothesized to experience the awareness of death by showing specific behavior such as carrying, inspecting and retrieving the bodies of the dead conspecifics (for a detailed review see: Goncalves and Carvalho 2019). Lastly, rats demonstrate a strong mother-offspring bond, making them an interesting model to study consequences of

offspring loss on different levels. While much research has focused on offspring development, some studies importantly have shown that repeated separation from the offspring (Boccia et al. 2007; Maniam and Morris 2010) and even total offspring loss (Pawluski et al. 2009c; Rincon-Cortes and Grace 2021) may have long-lasting negative consequences on the mother's rat emotionality and stress-response. Together, those studies highlight that grief-like responses are expressed throughout the animal kingdom. In the subsequent sections, this exploration delves deeper into the maternal grief experience, shedding light on the neurobiological mechanisms involving the OXT and CRF systems that modulate grief-related symptoms. Understanding these mechanisms becomes imperative, particularly in the context of maternal grief, which remains understudied despite its significant impact on the well-being of individuals worldwide.

1.3.1 **Maternal grief**

When the maternal bond is disrupted, whether due to a temporary separation or the tragic loss of the child, it is not surprising that well-being of the caregiver(s) is profoundly affected. The global child mortality rate is estimated to be 4% (Roser 2013) but statistics on miscarriage rate are even higher, among women who are aware of their pregnancy, the miscarriage rate is 10 % – 20 % with 45 % of pregnancies ending in miscarriage in women over the age of 40 (Quenby et al. 2021).

Grief emerging from experiencing the loss of the child is a process that involves a range of emotional and physical responses and adaptations. In humans, the loss of a child due to accident, stillbirth or illness is considered one of the most traumatic events someone could experience in their life (Hobson; Charles 1998). This emotional trauma experienced by bereaved mothers highlights the considerable significance of the mother-child dyad. Unfortunately, there is a paucity of research investigating the neurobiological consequences of the maternal grief, even though this traumatic event is experienced by many individuals across the world (Lundorff et al. 2017).

When grief symptoms persist for more than 6 months, they can lead to prolonged grief disorder (PGD) (Lundorff et al. 2017). The PGD is characterized by severe and disabling grief reactions, including high emotional distress, depression, and anxiety, and it belongs to the DSM-5 and ICD-11. In fact, the loss of a child is considered the most vulnerable type of bereavement which is highly invaliding (Kreicbergs et al. 2004). The most common

therapies to treat grief are based on general anxiolytics and antidepressant drugs and psychotherapeutic approaches (Shear et al. 2016), but more recently clinical trials developed to also test a specific pharmacological treatment (Naltrexone, a drug to treat addiction) for PGD symptoms (Gang et al. 2021). Therefore, based on the still unavailable targeted therapy, it becomes imperative to deepen our knowledge of the neurobiological mechanisms of grief, especially through the study of animal models. The loss of offspring is a traumatic event that can severely impact the physiology and emotionality of the mother by altering the homeostasis of the stress system, the ability to cope with stressors as well as the central release of neuropeptides. Two neuropeptide systems, which are hypothesized to be dysregulated following the offspring loss, are the OXT and CRF systems. Both play a central role in the formation and maintenance of the maternal bond and have been found dysregulated in other rodent animal models of grief. To date, there are no studies in rats investigating the impact of permanent offspring loss in conjunction with OXT and CRF systems analysis in the mother's brain and no rat animal model is available for the investigation of the maternal grief. In the next section I will introduce the OXT and CRF potential role in modulating grief-related symptoms.

1.3.2 **The OXT and CRF systems in depression and grief**

The lifetime incidence of depression or anxiety-like disorders is about 20% of the population, with women being two times at higher risk than men (Kornstein et al. 2002). Depression is a complex psychiatric disorder that often coexists with grief and PGD. OXT plays a crucial role in the regulation of a variety of behaviors, acting as a neuromodulator in the brain. OXT has been implicated in various aspects of human social behavior, including trust, empathy, and the formation of social bonds (Kosfeld et al. 2005; Hurlemann and Scheele 2016). Moreover, research in humans has explored the potential therapeutic applications of this neuropeptide in the treatment of social impairments associated with psychiatric disorders, via intranasal administration (Kendrick et al. 2018). However, the methodologies for measuring OXT and the challenges associated with standardized measurements remain areas of active research (Tabak et al. 2023). The OXT system has been demonstrated to regulate the stress response (Bosch and Neumann 2012; Jurek and Neumann 2018), for example, chronic central OXT infusion has been shown to reduce the neuronal and neuroendocrine responses to stress in virgin females (Windle et al. 2004).

Importantly, alterations of the OXT signaling have also been associated with postpartum depression (Thul et al. 2020) and grief (Bui et al. 2019). Moreover, the stress system can modulate the OXT system as shown in an animal model of partner loss in the monogamous and biparental prairie vole (*Microtus ochrogaster*) (Bosch et al. 2016). With respect to mother-offspring bond disruption and the impact on the OXT system, for example, repeated short separation from pups from postnatal days 1–22 increases OXT-R expression in numerous brains regions involved in the response to stress and/or maternal behavior, including the hippocampus, CeA, PFC, mPOA, lateral septum, and NAcc shell compared to a control group (Stamatakis et al. 2015). Given the potential anxiolytic and antidepressant role of OXT, some research has investigated this further but, still, inconclusive research is found when OXT is tested as antidepressant drug (De Cagna et al. 2019; Slattery and Neumann 2010). Overall, the OXT plays a fundamental role in modulating the maternal bond and social bonds, making it a crucial neuropeptide to investigate in relation to maternal grief.

The past decades of human and rodent research have shown a clear link between the dysregulation of the CRF system and the pathophysiology of depression (for review see: Waters et al. 2015). For example, high levels of CRF in the cerebrospinal fluid have been found in depressed patients, suicide victims or grieving individuals (Arborelius et al. 1999). Also, specific brain regions expressing high levels of CRF mRNA have been found in postmortem tissues of depressed patients (Arborelius et al. 1999). Altogether, the human research literature highlights that increased central CRF system signaling is linked to depression and mood disorders (Binder and Nemeroff 2010). Rodent studies also show similar findings. For example, intracerebroventricular (icv) injection of CRF induces anxiety, anhedonia, decreased appetite, reduced libido, reduced slow wave sleep, and psychomotor alterations (Binder and Nemeroff 2010). Conversely, CRF-R1 knockout mice exhibit diminished stress responses to stressful stimuli (Contarino et al. 1999; Smith et al. 1998). Moreover, chronic subcutaneous administration of CORT in rats has been shown to induce depression-like changes (Johnson et al. 2006), as evidenced by does-dependent increased floating in the FST, indicative of depressive-like behavior (Slattery and Cryan 2012).

Considering the involvement of the CRF system in depression (Nemeroff 1988; Heuser et al. 1994), it has become a potential target for the development of antidepressant therapies. For example, studies have shown that CRF-R1 receptor antagonists, which block the binding of CRF to this receptor subtype, can improve the activity of tricyclic antidepressant

and selective serotonin reuptake inhibitor antidepressant drugs in animal models (Wrobel et al. 2017).

In conclusion, the CRF system is crucial in the modulation of psychiatric disorders and, therefore, is a potential candidate for the investigation of the neurobiology of (maternal) grief.

1.3.3 **Neuroplasticity and depression**

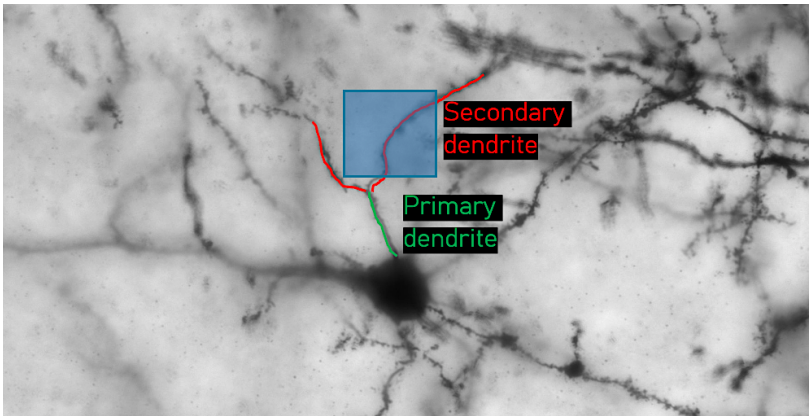
Neuroplasticity, which is also known as neural or brain plasticity, is a process which involves adaptive structural and functional changes to the brain. In this section, I will focus only on the first type of neuroplasticity: the structural plasticity. The structural plasticity is the brain's ability to change its anatomical properties, including changes in the number, location, and size of dendritic spines in response to different experiences (Lamprecht and LeDoux 2004). Dendritic spines are morphological specializations that protrude from the main shaft of neuronal dendrites. Most excitatory synapses in the adult brain of mammals occur on spines, so, dendritic spines represent the main postsynaptic compartment for excitatory input (Suratkal et al. 2021) (see Fig. 6). Normally, some brain regions are more plastic than others, for examples the brain regions involved in learning and memory are highly plastic due to their role in promoting and facilitating those functions. Importantly, it was recently shown that neuroplasticity is an ongoing process throughout life and it can occur also during adulthood (Chen et al. 2020b). Changes in neuroplasticity mechanisms can be associated also to the development of psychiatric disorders. For instance, depression is a complex mental health condition that, among other changes, involves alterations in neuroplasticity within specific regions of the brain (Radulescu et al. 2021).

While there is limited research specifically examining the relationship between grief and spine density, studies on depression provide valuable insights. Indeed, depressed individuals have been found to exhibit reductions in spine density, particularly in brain regions associated with mood regulation and emotion, belonging to the limbic system (Zhuang et al. 2019) and for a comprehensive review see (Tartt et al. 2022). These neuroplastic changes may contribute to the cognitive and emotional symptoms observed in depression, such as negative emotional rumination and impaired emotional regulation. Research from animal models demonstrate that depressive-like behaviors are accompanied by dendritic spines changes. For instance, animals exposed to chronic stress show spine

densities alterations which vary in different brain regions such as hippocampus, PFC and Amy (for a in depth review see: Qiao et al. 2016).

It is important to note that grief and depression are complex phenomena, and the relationship between spine density and these conditions is not fully understood. Thus, further research is needed to explore the specific effects of grief on spine density and how it relates to the emotional and cognitive aspects of the grieving process.

Fig. 6: Principal neuron dendritic arbors from Golgi-Cox staining. Image taken with Leica Thunder DM6 B, Camera Leica DFC9000 GT, 20X magnification.



1.4 The limbic system and emotion regulation

When referring to emotions, the modern neurobiological term defines them as a complex program of actions triggered by the presence of stimuli, external to the body or internal to the body, that activate certain neural systems. Emotions are commonly defined as a group of superior cerebral functions, which result from the different states of reward and punishment (Simic et al. 2021). For instance, rewarding situations reinforce certain reactions, which bring satisfaction and wellbeing. Normally, animals escape from and avoid punishment or harmful consequences (LeDoux 2000). As a result of extensive animal and human research, the best understood emotion is fear (LeDoux 2000). The past decades of research have highlighted the neural circuits associated with cortical and subcortical brain structures, which are responsible for the generation of emotions. Those regions are part of the limbic system (Fig. 7). Even though there is no universal agreement on the list

of brain regions that form the limbic system, it is currently accepted that the following areas are involved in the majority of the emotional processes: PFC, amygdala, anterior cingulate cortex (ACC), hippocampus, insula, septum, hypothalamus (Rajmohan and Mohandas 2007). I will briefly describe the three regions that were the main focus of this thesis: the PFC, the amygdala, and the hypotalamus.

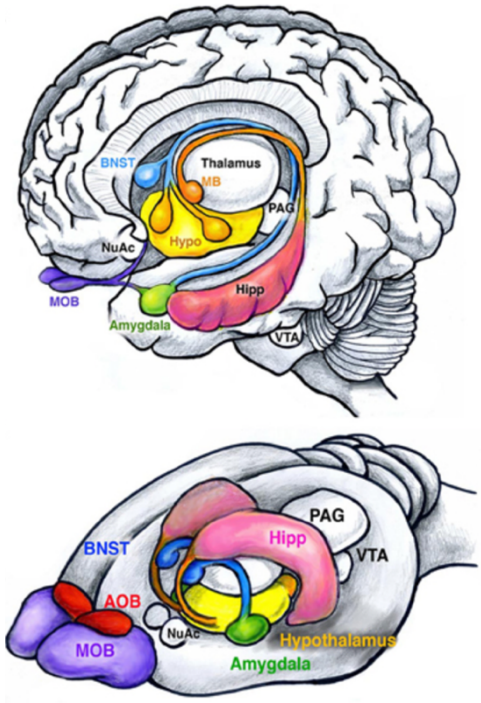


Fig. 7: Illustration of the human and rat limbic system. Abbreviations: BNST, bed nucleus of stria terminalis; Hipp, hippocampus; Hypo, hypothalamus; MB, mamillary bodies; PAG, periaqueductal gray; NuAc, nucleus accumbens; VTA, ventral tegmental area; MOB, main olfactory bulbs; AOB, accessory olfactory bulbs. From: (Sokolowski and Corbin 2012).

1.4.1 **Prefrontal cortex**

In the mammalian brain, the PFC is located in the frontal lobe of the cerebral cortex. The activity of the PFC is thought to be involved in the higher cognitive processes, such as the decision-making, emotion processing, working memory and executive functions, making the PFC the seat of the human personality (Miller et al. 2002; Sheng et al. 2020). The PFC is subdivided in the ventral, medial and lateral prefrontal cortex. In rodents, the prelimbic cortex (PL) is part of the medial prefrontal cortex (mPFC), adjacent to the infralimbic cortex (see Fig. 8), and is considered to be involved in various cognitive and emotional processes (Giustino and Maren 2015; Wellman and Moench 2019). Specifically, the PL can allow rodents to adapt their behavioral responses under changing experimental circumstances. The PL plays a crucial role also in behavior flexibility, in evaluating rewards

and punishments, and recently its role in parental behavior has also been shown (Sabihi et al. 2017; Alsina-Llanes and Olazabal 2020; Alsina-Llanes and Olazabal 2021). Furthermore, the PL is interconnected with other limbic structures, such as the amygdala and hippocampus. These connections allow for the integration of emotional and cognitive information, contributing to the regulation of emotional responses and memory processes (Coutureau and Killcross 2003). Research has shown that the PL is involved in stress-induced grooming behaviors in rats, suggesting its role in stress responses and coping mechanisms (Mu et al. 2020) and its role in depression (Hare and Duman 2020).

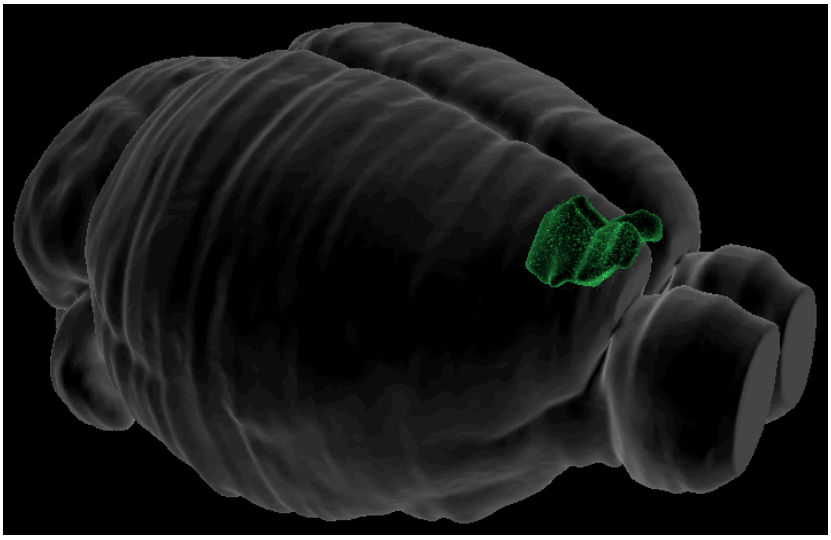


Fig. 8: Illustrative location of the right and left prelimbic cortex. (Adapted from: the blue brain atlas)

1.4.2 **Amygdala**

The amygdala was identified by Burdach in the 19th century (McDonald 2003). This region is an almond-shaped structure, found in the temporal lobe, at the anterior end of the hippocampus. The amygdala is structurally diverse and comprises different nuclei. The major nuclei groups are the basolateral nuclei, the central nuclei, and the medial nuclei (see Fig. 9) (McDonald 2003). The amygdala plays a crucial role in processing and regulating emotions, particularly fear and anxiety responses (Ressler 2010). The amygdala receives sensory information from various sensory systems and is involved in evaluating and assigning emotional significance to stimuli. It is interconnected with other limbic structures, such as the hippocampus, hypothalamus and PFC, allowing for the integration of emotional and cognitive information. In addition to its role in fear processing, the amygdala is involved in social behaviors and has been implicated in ultrasonic vocalizations, which are communication signals in rats (Chareyron et al. 2011). It plays

also a significant role in the formation and consolidation of fear memories and is crucial for fear conditioning and fear extinction processes (Davis et al. 1994). Also, the amygdala is important for the modulation of maternal behavior (Naeem et al. 2022). Each of the different nuclei and subregions the compose the amygdala contributes to specific aspects of emotional processing and behavioral responses. It receives inputs from sensory systems, such as the olfactory and vomeronasal systems, as well as inputs from cortical and thalamic regions (Janak and Tye 2015). Overall, the amygdala in rats is a central hub for emotional processing, fear conditioning, and social behaviors. It integrates sensory information, assigns emotional significance to stimuli, and plays a critical role in the regulation of emotional and behavioral responses.

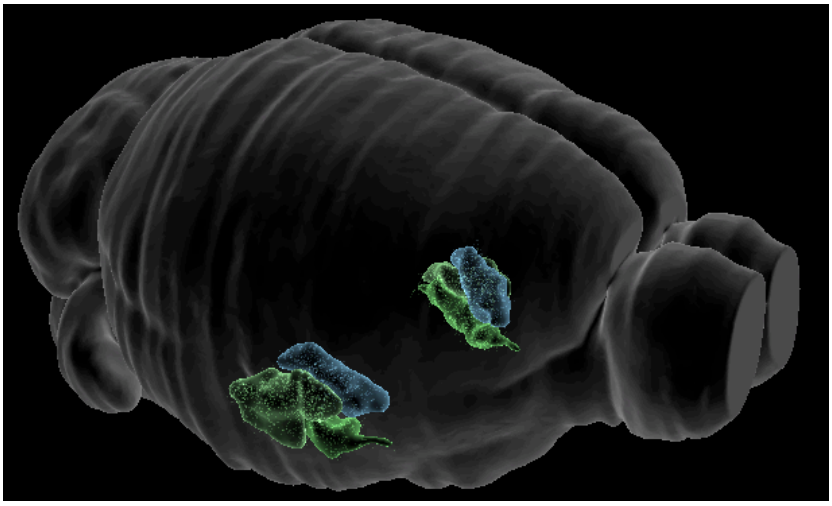


Fig. 9: Illustrative location of the right and left amygdala subnuclei. Central amygdala (blue) and basolateral amygdala (green). (Adapted from: the blue brain atlas)

1.4.3 **The ventromedial hypothalamus**

The VMH is a brain region located below the thalamus and within the hypothalamus, belonging to the limbic system (see Fig. 10). Among the different functions of the VMH, it is important to highlight its role in the modulation on feeding behavior and energy homeostasis (Abizaid et al. 2006). In addition, the VMH plays a crucial role in reproductive behaviors, especially in females (Flanagan-Cato 2011). Indeed, the VMH serves as a link between the CNS and the endocrine system, controlling the release of hormones that regulate these functions. Moreover, due to its anatomical connection with other limbic regions such as hippocampus and amygdala, the VMH can also participate in emotion

modulation and memory formation (Kunwar et al. 2015; Silva et al. 2016). The VMH has been shown to regulate the estrous cycle, due to its close connection with sexual hormones, resulting in being a key brain region in modulating reproductive behaviors (Inoue et al. 2019). Ovarian hormones, including estradiol, induce significant changes in the dendritic architecture of VMH neurons i.e., estrogen selectively induces dendritic spines within the dendritic arbor of VMH neurons, contributing to dendritic remodeling (Calizo and Flanagan-Cato 2000). In the VMH is a high expression of OXT-R, which suggest its role in social bonds (Bale et al. 2001). Recently, studies in rodents have found an important role of the VMH also in the regulation of alloparental care (Liu et al. 2019b) and lesions of the VMH results in altered maternal behaviors indicating the critical role of this brain region in coordinating the complex spectrum of maternal behaviors (Bridges et al. 1999; Mann and Babb 2004).

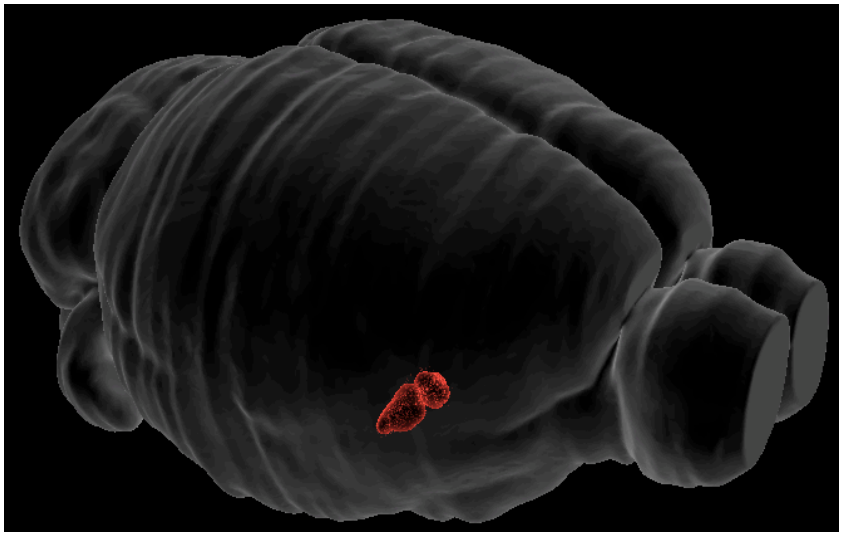


Fig. 10: Illustrative location of the right and left ventromedial hypothalamus. (Adapted from: the blue brain atlas)

1.5 Aim of the thesis

This thesis overall aim was to investigate the impact of breaking the maternal bond on the mother's behavior and emotionality, and to elucidate the underlying neurobiological mechanisms. The first aim, which I assessed in Chapter 2, was to collect the available rodent and human literature about the current knowledge of grief and neuropeptides, with the aim to identify the gaps in the neurobiology of grief.

The second aim was to investigate the effects of a short and long repeated separation of the offspring during the first post-partum week on the rat mother's brain and emotionality, which I conducted in Chapter 3.

The last aim was to develop an animal model of permanent removal of the offspring and identify possible brain alterations to find a potential neurobiological target for therapeutical interventions. Therefore, different periods of loss experience were assessed. A short-term offspring loss investigation was conducted in Chapter 4, while a long-term offspring loss investigation, including a potential therapeutical treatment for maternal grief, has been conducted in Chapter 5.

-Chapter 2-

The brain oxytocin and corticotropin-releasing factor systems in grieving mothers: What we know and what we need to learn

Authors' contribution:

Luisa Demarchi: first draft and revision of manuscript

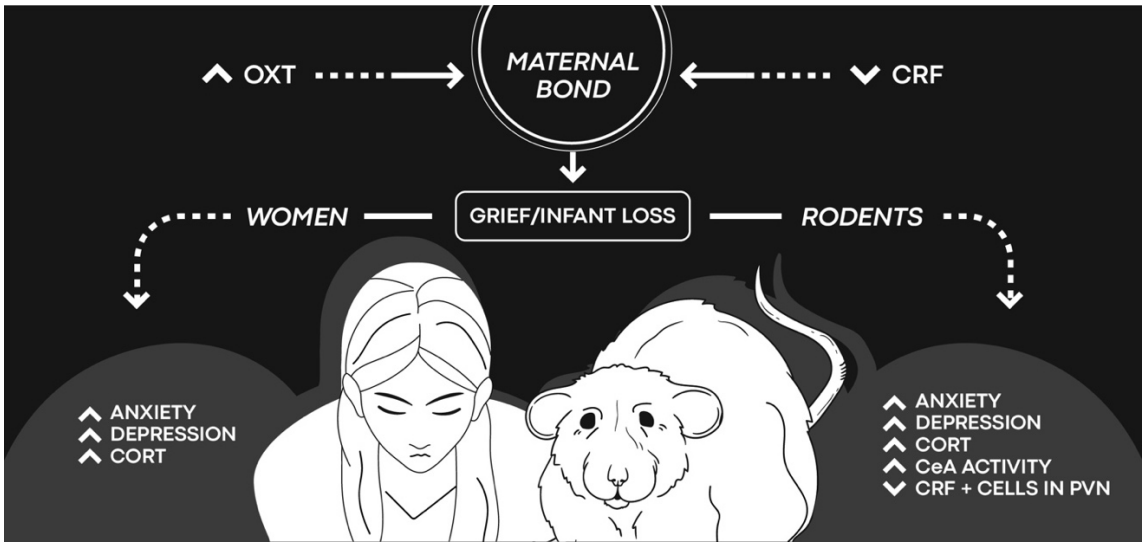
Jodi Pawluski: revision of manuscript

Oliver Bosch: revision of manuscript, funding acquisition

[Taken and partly adapted from: Demarchi L, Pawluski JL, Bosch OJ (2021) The brain oxytocin and corticotropin-releasing factor systems in grieving mothers: What we know and what we need to learn. Peptides 143:170593.]

2.1 Abstract

The bond between a mother and her child is the strongest bond in nature. Consequently, the loss of a child is one of the most stressful and traumatic life events that causes PGD in up to 94% of bereaved parents. While both parents are affected, mothers are of higher risk to develop mental health complications; yet, very little research has been done to understand the impact of the loss of a child, stillbirth and pregnancy loss on key neurobiological systems. The emotional impact of losing a child, e.g., PGD, is likely accompanied by dysregulations in neural systems important for mental health. Among those are the neuropeptides contributing to attachment and stress processing. In this review, I present evidence for the involvement of the brain OXT and CRF systems, which both play a role in maternal behavior and the stress response, in the neurobiology of grief in mothers from a behavioral and molecular point of view. I will draw conclusions from reviewing relevant animal and human studies. However, the paucity of research on the tragic end to an integral bond in a female's life calls for the need and responsibility to conduct further studies on mothers experiencing the loss of a child both in the clinic and in appropriate animal models.



Graphical abstract.

2.2 Introduction

Positive and intact social bonds are important in the social life of humans as well as of many other social species (Pohl et al. 2019); such positive social bonds ensure the individual's psychological and physiological well-being. Consequently, these can be dramatically impacted by the loss of a bond, i.e., due to separation or death. Among the emotional bonds found in nature, the maternal bond is arguably the most important and often the most long lasting in the course of our lives (Bosch and Neumann 2012; Bosch and Young 2018).The early mother-child bond not only shapes the future 'me', but has a great impact on the mother as she undergoes a number of peripartum adaptations in order to become and stay maternal (Barba-Muller et al. 2019). It is undisputed that a dramatic event in the peripartum period like the termination of the mother-child bond due to, e.g., pregnancy loss, stillbirth, crib death, illnesses or accidents, is considered an emotional trauma that devastates the psychophysical well-being of the mother and family (Burden et al. 2016; Daugirdaite et al. 2015; Kersting and Wagner 2012). The loss of a child, consequently, is a dramatic and stressful event that afflicts many families around the world. In fact, globally in 2019 it was estimated that for every 71 live births there was one stillbirth, which is defined as no sign of life at 28 weeks of pregnancy or later (UN and IGME 2020). In addition, the global child mortality rate is estimated to be 4% (Roser et al. 2013). Statistics on miscarriage are even higher, as this is the most common complication of early pregnancy (National Collaborating Centre for Women's and Children's Health., 2012). Among women who are aware of their pregnancy, the miscarriage rate is 10% to 20% (Wilkins 2012) with 45% of pregnancies ending in miscarriage in women over the age of 40 (Wilkins 2012). Therefore, with so many mothers experiencing the loss of a child there is an urgent need to understand the neurobiology of bereavement in motherhood. Conceptually, grief is an attachment reaction while bereavement is the period in which the person suffers with the loss (Zisook and Shear 2009). Psychological symptoms following the loss of a loved one can range from feeling insecure, agitated, aggressive, anxious and having difficulties accepting the loss to depressive states that can lead in 7% to 10% of the bereaved population to PGD (Prigerson et al. 2009; Shear 2012, 2015; Kersting et al. 2011) i.e., when the symptoms last more than 6 months. However, in bereaved mothers and fathers, up to 94% develop PGD (McCarthy et al. 2010; Huh et al. 2017). Moreover, PGD is frequently comorbid with depression, anxiety, and PTSD with up to 75% of individuals

with PGD developing symptoms of one or more of these disorders (He et al. 2014). Furthermore, recent research has shown that women who experience early pregnancy loss (loss before 12 weeks of gestation) have high levels of posttraumatic stress, anxiety and depression that remains clinically significant 9 months later (Farren et al. 2020). It is also not surprising that stillbirth is accompanied by high levels of anxiety and depression in the mother for up to 3 years (Cambpell-Jackson and Horsch 2014). The neurobiology of grief is an emerging research field that is constantly growing but unfortunately, scientific research has been influenced by a strong sex bias that has created a lack of knowledge of female physiology (Holdcroft 2007), thereby also leading to the little we know about grieving mothers' neurophysiology and emotionality. Hence, I aim to review the current literature on the neurobiological impact of losing a child on the mother with focus on neuropeptide systems known to facilitate (OXT) or to hinder (CRF) adequate mothering (Neumann 2008; Bosch and Neumann 2008; Klampfl and Bosch 2019a; Stolzenberg et al. 2019) (for a more general review of neuroendocrine mechanisms in grief and bereavement see: Hopf et al. 2020). Given the severe paucity of scientific research in this field, I decided to include examples of relevant studies on repeated forced mother-offspring separation. This repeated mother-offspring separation paradigm is not the same as permanent removal of the offspring but is a forced and often unpredictable separation that can provide knowledge of the neurophysiological systems at play when a mother-offspring bond is severed. The impact of repeated mother offspring separation on the mother spans from changes in behavior to molecular levels in experimental animal models and women and could further help to identify gaps and possible future research directions on neurobehavioral processes in grieving mothers.

The maternal bond

Among the central evolutionary force in life is the ability to develop specific and even long-lasting bonds (Bowlby 1982; Hofer 1994). In particular, the maternal bond is considered not only the basis to form social bonds, but also the strongest and most enduring bond in life for both participants in the mother-child dyad (Rilling and Young 2014; Numan and Young 2016). The experience of becoming a mother involves adaptations on hormonal, neuronal and, consequently, behavioral levels all of which ensure the survival of the offspring (Kinsley and Lambert 2008; Broad et al. 2006; Hillerer et al. 2012; Neumann 2003; Bosch and Neumann 2012; Klampfl and Bosch 2019a; Stolzenberg et al. 2019).

Maternal behavior is defined as the sum of all behaviors aimed at maintaining the offspring's well-being. After giving birth, the rodent mother is spontaneously attracted to her pups, which is triggered by numerous sensory stimuli (olfactory, auditory, visual, chemical), (Mennella and Moltz 1989; Peters and Kristal 1983; Stern 1985).

In rodents, the reproductive experience itself is capable of affecting the maternal brain on multiple levels, including emotionality, stress response and cognition (Macbeth and Luine 2010). Curiously, in women the experience of pregnancy causes neuroplastic changes, i.e., reducing gray matter volume in many brain areas associated with social cognition, that are predictive of greater maternal postnatal attachment (Hoekzema et al. 2017). In line with this, lactating rodents, with lower levels of neurogenesis in the hippocampus, show increased memory during the postpartum period and even after weaning (Gatewood et al. 2005; Kinsley et al. 1999; Love et al. 2005; Pawluski et al. 2006; Galea et al. 2008). This suggests a fine-tuning of neural systems with the transition to motherhood to ensure offspring survival. The relationship between the mother and the infant is directly linked to the neurobiological and behavioral development and well-being of both subjects (Pena et al. 2013; Nephew and Murgatroyd 2013). For example, a better quality of MC has long-term positive effects on pups, reducing anxiety and increasing sociability (Caldji et al. 1998; Rilling and Young 2014). Consequently, disrupting the bond, either repeatedly or permanently can have detrimental impact on the offspring, as has been shown over the past decades (for reviews see: Alves et al. 2020; Nishi 2020). For example, a reduced quality of MC has a negative effect on learning and memory and on the neuroendocrine response to stress in the adult offspring (Barha et al. 2007). The reduction in MC or repeated separation from the mother in the postnatal period causes severe long-lasting consequences, such as increased anxiety (Caldji et al. 2000), altered stress response (Ladd et al. 1996) and epigenetic changes in the CNS of the adult offspring (Holmes et al. 2005; Weaver et al. 2004). It is unfortunate, though, that only a few studies have characterized the infant-loss mediated impact on maternal neurobiology.

2.3 Role of the OXT and CRF systems in the healthy maternal brain

The aim of this review is to collect the information on the consequences of breaking the mother-child bond on the maternal brain with respect to the “pro-maternal” OXT and “anti-maternal” CRF neuropeptide systems (Klampfl and Bosch 2019a). As these neuropeptide

systems are also involved in the etiology of stress-related disorders in both rodents and humans, and that losing a child is a severely stressful experience that dramatically affects the maternal brain, these neuropeptide systems are promising candidates with respect to the underlying physiology of emotional and behavioral consequences in grieving mothers. Our first step here is to briefly summarize our knowledge of these neuropeptide systems in the maternal brain. See the following for more in-depth reviews on the brain OXT (Bosch and Neumann 2012; Jurek and Neumann 2018) and CRF systems (Deussing and Chen 2018; Klampfl and Bosch 2019a).

2.3.1 **The OXT system is activated in lactation and promotes maternal behavior**

OXT is a nonapeptide synthesized primarily in two hypothalamic nuclei, i.e., the PVN and the SON. The magnocellular OXTergic neurons project from these two nuclei to the neurohypophysis, where OXT is stored in the axon terminals and then secreted into the blood. The peripheral actions of OXT include the stimulation of the myometrium contractions during labor and secretion of milk during lactation (Jurek and Neumann 2018). Concurrently, OXT is also released in the CNS in regions such as the PVN, SON, septum, hippocampus and olfactory bulb (Kendrick et al. 1988; Landgraf et al. 1991; Moos et al. 1989; Moos et al. 1991; Neumann and Landgraf 1989; Neumann et al. 1993b). Central OXT acts as a modulatory factor of emotionality, reducing anxiety and the response to stress on one hand and favoring social and emotional bonds, and social behavior on the other hand (Leng et al. 2008; Neumann 2008; Donaldson and Young 2008; Lee et al. 2009; Jurek and Neumann 2018). In this context, OXT is involved in mediating both MC and maternal aggression as shown in rodents (Pedersen and Prange 1979; Pedersen et al. 1982; Champagne et al. 2001; Pedersen and Boccia 2003; Bosch et al. 2004; Bosch et al. 2005; for in-depth reviews see (Bosch and Neumann 2012; Bosch 2013; Jurek and Neumann 2018)). Therefore, it is not surprising that both central and peripheral OXT levels increase during the postpartum period as demonstrated in rat and sheep mothers (Landgraf et al. 1991; Landgraf et al. 1992; Keverne and Kendrick 1994; Leng et al. 2008). Additionally, OXT receptor (OXT-R) density increases around parturition in several brain areas known to be involved in maternal behavior, including the MPOA and BNST (Bosch et al. 2010; Meddle et al. 2010). In parturient rats, central administration of an OXT-R antagonist

blocks the onset of MC (van Leengoed et al. 1987). In lactating rats, icv infusion of OXT does not further increase MC (Fahrbach et al. 1985), while an OXT-R antagonist lowers the level of pup-directed behavior (Pedersen and Boccia 2003; Bosch and Neumann 2008). In support, mice with a knockout for the OXT-R show impaired MC (Takayanagi et al. 2005). In women, high levels of plasma OXT are associated with high levels of maternal attachment (Levine et al. 2007; Feldman et al. 2007). Conversely, low plasma OXT levels are thought to be a sign for an increased risk for postpartum depression (Moura et al. 2016; Stuebe et al. 2013; Skrundz et al. 2011). However, it is important to underline that the release of OXT into the peripheral blood stream can be independent of central release and, thus, does not necessarily reflect brain OXT levels (Neumann and Landgraf 2012). Recent genetic studies in humans reveal that the quality of mothering is influenced by single nucleotide polymorphisms in the genes for OXT (e.g., rs2740210; rs4813627; (Mileva-Seitz et al. 2013)) or OTXT-R (e.g., rs968389; (Mehta et al. 2016)).

2.3.2 **The CRF system activity is dampened in lactation and impairs maternal behavior**

The CRF system consists of four polypeptidergic ligands (CRF, Ucn 1-3), CRF binding protein (binds free CRF and Ucn 1 thereby reducing their availability for the receptors), and two receptors (CRF-R1 and CRF-R2). These members of the CRF system are expressed widely throughout the brain (Deussing and Chen 2018). One of the main roles of CRF is driving the stress response via the HPA axis. The response to stress is triggered in the parvocellular part of the PVN leading to CRF release into the portal blood. Here, it elicits the release of ACTH from the PP into the main blood stream. In the adrenal cortex, ACTH stimulates the release of cortisol (humans) or CORT (rats, mice), which in turn exerts a negative feedback on various levels including PVN, hippocampus, and pituitary gland. In the brain, increased CRF system activity is affecting numerous behavioral outcomes including impairment of maternal behavior. Therefore, it is not surprising that one of the peripartum adaptations is a dampening of the CRF system activity (for a comprehensive review see: Klampfl and Bosch 2019a).

The HPA axis activity is significantly reduced in lactating rats, mice and humans (Johnstone et al. 2000; Douglas et al. 2003; Schulte et al. 1990); the brain of lactating rats, in general, is less responsive to CRF compared to virgin females (da Costa et al. 1996; Da

Costa et al. 1997). This includes, e. g., decreased CRF mRNA in the CeA (Walker et al. 2001), and the parvocellular PVN (Johnstone et al. 2000; Walker et al. 2001; Klampfl et al. 2013; Lightman et al. 2001). Consequently, a centrally activated CRF system impairs maternal behavior in lactating mouse and rat mothers (Klampfl et al. 2013; Klampfl et al. 2014; Creutzberg et al. 2020), and can even induce pup-killing in ovariectomized female rats (Pedersen et al. 1991). In addition, the involvement of both CRF-R subtypes can differ. In the antero-dorsal BNST, e.g., CRF-R1 activation impairs maternal behavior to a greater extent compared to CRF-R2 activation (Klampfl et al. 2014), whereas opposite effects are observed in the medial-posterior BNST (Klampfl et al. 2016a; Klampfl and Bosch 2019b). Therefore, those and other studies (see review: Klampfl and Bosch 2019a) demonstrate that a downregulation of the CRF system peripartum is a prerequisite for mothers to exhibit adequate maternal behavior. In support, an upregulated CRF system is thought to be a risk factor for psychological disorders (Lebow et al. 2012; Schartner et al. 2017; Elharrar et al. 2013). Hence, any maladaptation of the CRF system in general - and in the peripartum period in particular - likely results in serious consequences like increased risk to develop emotional disorders, which affect at least 15% of mothers (Brummelte and Galea 2010b). Importantly, CRF is one of the factors known to contribute to such maladaptation (Magiakou et al. 1996; O'Keane et al. 2011).

2.4 Grief and bereavement of the offspring

Worldwide, approximately 2.6 million newborns die each year (Heazell et al. 2016). Infant loss is a tragic life-changing event for parents which often causes PGD, including symptoms of depression and anxiety (Li et al. 2002; Znoj and Keller 2002; Voss et al. 2020), e.g., the bereaved parents show higher levels of emotional distress and post-traumatic symptoms when compared to non-bereaved parents (Heazell et al. 2016; Gold et al. 2016). In fact, the loss of one's child is considered the most vulnerable type of bereavement (Singh and Raphael 1981; Johannesson et al. 2011; Kreicbergs et al. 2004). It can even cause physical pain, i.e. significantly increasing pain intensity, compared to the loss of a partner or parent (Wing et al. 2001). It is not surprising that the first six months following the death of a child are the most intense months of psychological stress for mothers (Wall-Wieler et al. 2018). The extent to which mothers suffer from the consequences of child loss can decline over time (Bonanno and Kaltman 2001; Shear 2012). A study reports that one

month after their child dies, 17% of parents (both the mother and father) suffer from post-traumatic stress disorder (PTSD), which decreases to 9% in the following 3-12 months (Jind et al. 2010). While both parents are affected by the loss of the child, mothers are of higher risk to develop mental health complications (Fernández-Alcantara and Zech 2017; Hunter et al. 2017; Lundorff et al. 2017) with the majority suffering from chronic depression (Singh and Raphael 1981). Surprisingly, the form of grief following the loss of a child is still underestimated and not well understood (Burden et al. 2016; Cacciatore and Bushfield 2007; Cacciatore et al. 2009). Studies on grieving mothers find no differences in the symptomatology across the various types of infant loss (e.g., prenatal versus postnatal) (Dyregrov 1990).

2.4.1 **The CRF system after offspring loss in rodents**

The HPA axis underlies peripartum adaptations; its activity is different compared to virgin female or male animals due to the role of components of the HPA axis in offspring care (Slattery and Neumann 2008; Almanza-Sepulveda et al. 2020). Under normal, non-stress conditions, lactating mothers show hypercortisolism, which is required to meet the constantly high energy demand in lactation (Stern et al. 1973; Walker et al. 1995; Windle et al. 1997c, Lightman et al. 2001) as well as form maternal memory (Almanza-Sepulveda et al. 2020). Under acute stress conditions, the HPA axis reactivity is altered to enable the mother to react adequately (Stern et al. 1973; Windle et al. 1997a; Neumann et al. 1998; Shanks et al. 1999; Lightman et al. 2001; Walker et al. 2001; Brunton and Russell 2003). In the case of breaking the maternal-offspring bond, it is likely that this acts as a chronic stressor, or abnormal event, thereby altering the function of the maternal HPA axis on many levels and for a longer period time (Brunton and Russell 2008; Brummelte and Galea 2010b; Dickens and Pawluski 2018). In support of this notion when looking at the studies that remove the litter completely from the mother shortly after birth, more analogous to the clinical situation of infant loss, it has been shown that these mother rats have increased depressive-like behavior, increased anxiety and an impairment in memory weeks later (Smith and Lonstein 2008; Pawluski et al. 2009c; Pawluski et al. 2006). These behavioral effects weeks after offspring removal occurred despite the fact that removal of the litter

shortly after birth resulted in serum levels of CORT returning to virgin levels 5 days later (Pawluski et al. 2009b).

Unfortunately, we lack detailed knowledge on the effects of infant or child loss on the maternal brain in rodent literature and how it may differ from both the maternal and virgin states. This is even though a large body of research has analyzed the consequences of mother-pup separation on neurobehavioral outcomes in offspring, perhaps most popularized with the research of Harry Harlow. Of the few papers that have investigated the effects of mother-offspring separation on the mother, most studies have adopted the maternal separation paradigm with slightly varying timelines and durations of separation.

Given that there are no studies of offspring loss that thoroughly investigate the neuroendocrine effects in the mother, I extend the discussion here to studies that investigated the OXT and CRF systems by using repeated mother-offspring separation paradigms. Such paradigms which consist of forced separation between mother and offspring are generally applied during the first postnatal days (PND), typically during the first 2-3 weeks after giving birth, either for a short (15-30 minutes per day) or a prolonged period (3-6 hours per day). The studies that adopt this paradigm demonstrate an impact of the separation on both behavioral and molecular levels in the mother (Table 1). For instance, MC is influenced by the duration of separation from the pups. Repeated short separation induces an increase in LG and ABN nursing (Orso et al. 2018), whereas repeated prolonged separation leads to reduced MC (Ivy et al. 2008; Boccia and Pedersen 2001). However, a recent study that applied daily 4 hour-separations from PND 2-20 reports increased licking and ABN nursing behaviors in rat dams (Bolukbas et al. 2020). In addition, the long-term separation can also impair the mothers' short-term memory (Aguggia et al. 2013). With respect to emotionality, repeated prolonged separation between 3 and 6 hours for 2-3 weeks postpartum results in increased depressive-like behavior (Boccia et al. 2007) and an anxious phenotype in the mothers (Eklund et al. 2009, Aguggia et al. 2013, Orso et al. 2018, Maniam and Morris 2010; but also see: Bolukbas et al. 2020). The latter is speculated to be due to an increased cellular activity of the CeA (Aguggia et al. 2013).

From a molecular point of view, the literature on neuroendocrine effects of mother-offspring separation on the maternal brain is limited and mostly focuses on glucocorticoids. Following repeated short separation, maternal plasma CORT levels increase (Eklund et al. 2009). Curiously, in another study, complete removal of the offspring from the postpartum

dams for 3 days decreased CORT to levels observed in virgin rats (Leuner et al. 2007). Repeated prolonged separation during the first week postpartum increases glucocorticoid receptor gene (Nr3C1) expression in the mothers' hippocampus (Orso et al. 2018; but also see: Bolukbas et al. 2020). Previous studies show that variations in the expression pattern of this gene in the hippocampus could induce a vulnerability to potential injuries following chronic stress (Conrad 2008). Furthermore, the expression of Morc1 mRNA, which is connected to chronic stress experience and depression (Nieratschker et al. 2014; Mundorf et al. 2018; Thomas et al. 2020), is increased in the mPFC of mothers after repeated prolonged pup-separation (Bolukbas et al. 2020). Repeated prolonged separation decreases the density of positive CRF cells in the PVN of the mother when compared to mothers exposed to repeated short separation (Baracz et al. 2020). These data demonstrate a strong impact of pup separation on the maternal stress axis and emotionality which may have life-long effects on the well-being of the mother. More concentrated research efforts are needed on the neurophysiology of grieving mothers to identify potential treatment targets to regulate the HPA system and ease the impact of the loss.

2.4.2 **The OXT system after offspring loss in rodents**

The OXT system plays a fundamental role in maternal behavior and at the same time in regulating the stress response (Bosch and Neumann 2012; Jurek and Neumann 2018). During pregnancy and lactation, OXT system activity is elevated (Bosch and Neumann 2012; Neumann and Slattery 2016); e.g., the expression of, and binding to, OXT-R as well as the number of OXT positive cells in hypothalamic regions is increased in non-stressed mothers (Insel 1990; Veenema et al. 2007). Chronic central OXT infusion attenuates the neuronal and neuroendocrine responses to stress in virgin female rats (Windle et al. 2004) further supporting the involvement of endogenous OXT in the attenuated stress responses during the peripartum period.

Importantly, the stress system is also capable of manipulating the OXT system as demonstrated in an animal model of partner loss in the monogamous, biparental prairie vole (*Microtus ochrogaster*) (Bosch et al. 2016), which have evolved as an important animal model to study social bonds (for detailed reviews on their social system and studies on loss see (Young et al. 2011; Young and Wang 2004; Sadino and Donaldson 2018; Bosch and

Young 2018). Briefly, losing the female partner, i.e. permanent separation, acts as a chronic stressor on the male (Bosch et al. 2009), thereby causing increased CRF-R2 activation, which suppresses OXT mRNA expression in the PVN and reduces local OXT signaling in the nucleus accumbens (NAcc) shell. The result is an adverse emotional state that shares common mechanisms with bereavement (Bosch and Young 2018; Pohl et al. 2019). Importantly, this state can be reversed by chronic administration of OXT or deactivation of CRF-R2 in the NAcc shell (Bosch et al. 2016). Interestingly, the OXT and CRF systems might interact directly via neuronal coexpression of OXT-R and CRF-R (Winter and Jurek 2019). For example, in the NAcc CRF positive parvocellular PVN neurons express OXT but not OXT-R, while magnocellular PVN neurons coexpress CRF, CRF-R2, OXT and OXT-R, allowing these positive CRF neurons to respond to OXT release and *vice versa* (Dabrowska et al. 2013; Winter and Jurek 2019). Similarly, separation from the male partner induces chronic stress in the lactating female prairie vole, increasing the anxious phenotype and passive stress-coping (Bosch et al. 2018). In socially separated female prairie voles, i.e. being isolated from a female sibling for 4 weeks, repeated subcutaneous administration of OXT buffered the separation-induced increase in depressive-like behavior and cardiac dysfunction (Grippo et al. 2009). However, the OXT systems' role in female prairie vole mothers following separation remains to be investigated.

With respect to mother-offspring bond disruption, most studies use the paradigm of repeated short or long maternal separation, as mentioned in section 3, to analyze potential dysregulations of the OXT system (Table 1). For example, repeated short separation from PND 1 to 22 increases OXT-R expression in numerous brains regions involved in the response to stress and/or maternal behavior, including the hippocampus, CeA, PFC, mPOA, lateral septum (LS), and NAcc shell compared to a control group (Stamatakis et al. 2015). Furthermore, repeated short separation from pups increases the number of OXT-immuno-reactive (ir+) cells in the PVN whereas repeated prolonged stress reduces it in the caudal PVN compared to non-lactating rats (Baracz et al. 2020). This is an interesting finding as it suggests that prolonged maternal separation stress is able to reverse the peripartum adaptation-associated increase in OXT-ir+ cells (Baracz et al. 2020). Different to those effects on the OXT system in the brain, plasma OXT levels are similar in rat mothers exposed to repeated short or prolonged separation from offspring (Eklund et al. 2009). Moreover, even total deprivation of pups for 20 hours over 4 consecutive days does not alter plasma OXT levels compared to control in rat mothers (Liu et al. 2019a). However, it is important to keep in mind that removal of pups from the mother also results in the

absence of the milk ejection reflex and, consequently, an absence of stimulation of the mammary glands, which can stop lactation. Therefore, it is not surprising that the same study reports a cessation of lactation in the long-term separated group (Liu et al. 2019a). The cause of this cessation of lactation is the abnormal electrical activity of OXT neurons, which do not respond adequately when not being stimulated regularly by suckling (Liu et al. 2019a). Another factor that needs to be considered when studying the maternal brain is the age of the pups or rather the progress of lactation. In the biparental, monogamous prairie voles, removal of the pups for 30 minutes strongly activates OXT neurons in the PVN on PND 2 and 9, whereas there is only minimal activity on PND 21 suggesting that the functional plasticity of the OXT system is associated with parental behavioral and the growth of the pups (Kelly et al. 2017).

2.4.3 **The CRF systems after infant loss in humans**

Studies in humans evaluating the endocrine effects following bereavement use blood cortisol levels as a primary indicator of stress or rather of chronic stress (Table 2). Cortisol is released from the adrenal gland, and activation of the HPA axis increases circulating cortisol levels. Moreover, high cortisol concentrations can cause a dysregulation of the HPA axis, a process that has been related to a state of chronic stress as seen, e.g., in major depressive disorders (MDD) (Deuschle et al. 1998). The first studies that have analyzed the relationship between psychological stress and adrenal gland activity date to more than 50 years ago. In 1964, Wolff and colleagues analyzed plasma levels of 17-hydroxycorticosteroid (17-OHCS) in parents of fatally ill children, affected by leukemia or other fatal childhood neoplasms. This study demonstrates that the more effectively a parent "fights" against the threat of loss, i.e. the more that control is exercised over feelings, the lower their chronic 17-OHCS excretion rate is (Wolff et al. 1964). A few years later, a study indicated that the 17-OHCS excretion levels during a pre-child loss period can be significantly different from the bereavement period and that the direction of the difference is an important characteristic of the individual subject (Hofer et al. 1972). An evaluation of pairs of parents who experienced sudden child death demonstrates no difference in cortisol levels compared with non-bereaved subjects, whereas the bereaved parents score higher on

the Beck Depression Inventory test and have increased helper T cells as an indicator of an immunological response (Spratt and Denney 1991).

Due to a lack of further data on grieving mothers, we also must consider studies on women suffering from the loss of close persons. In the literature, most studies have analyzed women and men in mourning as one group, without considering potential gender differences or even distinguishing the type of bereavement, i.e., whether a child, a partner, or a family member has deceased. In 2009, Buckley and colleagues analyzed the plasma cortisol level in non-bereaved versus bereaved men and women 2 weeks and 6 months after the loss of a loved one (a partner or a child). Comparing the two time points, the morning cortisol level increased in the bereaved compared to the non-bereaved participants, regardless of gender, suggesting an evident reaction of the HPA axis to the stress of loss. Furthermore, elevated depressive symptoms were found in the bereavement group at both time points (Buckley et al. 2009). Curiously, a study found that men show lower cortisol levels while women show higher levels at 6 months post bereavement (Richardson et al. 2015). In a small sample of widows, within the first 6 months of partner loss plasma cortisol levels were significantly higher compared to a sample of women who were still married (Irwin et al. 1988). In support of long-term effects, daytime cortisol levels were increased in men even many years after the loss of a loved one (Nicolson 2004). Furthermore, morning and daytime cortisol levels were higher in participants with PGD than in normal grieving or non-grieving participants, the latter two being no different from each other (Saavedra Perez et al. 2017). In a recent review, it emerged that the loss of a loved one can be associated with neuroendocrine alterations both in the early / acute stage and in the late / chronic stage of bereavement (Hopf et al. 2020). In general, people with PGD have flattened daytime cortisol curves and reduced morning cortisol levels when compared to people in normal bereavement. Furthermore, the closer the relationship to the deceased person was, the more the neuroendocrine system is dysregulated.

2.4.4 **The OXT systems after infant loss in humans**

Similar to rodents, the OXT system facilitates maternal behavior and the maternal bond in humans (Feldman et al. 2007). Research on the maternal bond identified a potential link between OXT and dopamine in the MeA, i.e., NAcc, amygdala, and PFC, assuming that

both neurotransmitters/modulators contribute to the attachment (Atzil et al. 2017). Recently, peripartum salivary OXT levels in mothers were found to be moderately associated with maternity blues and significantly correlated with postpartum fatigue (Shishido et al. 2019). However, only a few studies tried to investigate a potential link between the parental OXT system and separation from the children as a stressor (Table 2). In a study on the effects of intermittent separation, e.g. 15 minutes, and reunion with one's infant, near-infrared spectrometry demonstrated a positive correlation between parental activation of the PFC, parenting and OXT levels (Ito et al. 2017). This finding is in line with previous research demonstrating a connectivity to the medial PFC and orbitofrontal cortex from the limbic system, such as amygdala, NAcc, and hypo (Strathearn et al. 2009; Atzil et al. 2011), thereby suggesting that OXT activates the limbic system directly, and the PFC indirectly, via neural connections. In addition, a study by Rutherford and colleagues using event-related potentials (ERPs) showed that OXT administration can enhance the ERPs elicited by infant distress as compared to infant neutral faces in non-parent women giving more support to the OXT system's role in the modulation of attentional processing of infant stimuli (Rutherford et al. 2017).

With respect to grief, studies that have analyzed the association with OXT usually do not distinguish between men and women, nor the type of grief (see section 5). We do know that up to 94% of parents develop PGD after the death of a child (McCarthy et al. 2010, Huh et al. 2017), whereas mourning the death of a loved one causes PGD in 10% of individuals (Lundorff et al. 2017). Women are, in general, more vulnerable to developing PGD (Kersting et al. 2011). This gender difference is an important factor to consider when investigating OXT and PGD as OXT levels vary with gender (Bredewold and Veenema 2018). In individuals with PGD, plasma OXT is often measured due to its role as a 'social' neuropeptide and its role in social bonds. Although it is debatable whether plasma OXT reflects brain OXT levels, the direct correlation between peripheral and central OXT levels appears to be the prerogative of non-human animal studies, while in humans a direct correlation is apparent in high stress situations (Valstad et al. 2017). Indeed, the latter might be the case in PGD, and numerous studies have reported a link between plasma OXT and human behavior (for review see: Bartz et al. 2011). In a recent study, Bui and colleagues investigated plasma OXT levels in men and women with a primary PGD and they found that in participants with PGD plasma OXT levels were higher compared to participants with depression, but not different from participants with normal grief experiences. Secondary analyses revealed that the diagnosis of PGD is positively associated with plasma

OXT levels in men and women (Bui et al. 2019). Hence, it is speculated that the increased plasma OXT levels is reflecting the stress arising from the loss of a social bond, which is specific to PGD compared to depression (Shear et al. 2011). An additional study provides further support for a potential role of OXT signaling in the pathophysiology of PGD by reporting a gene x environment effect between a genetic variant of the OXT-R (single nucleotide polymorphism rs2254298) and greater PGD symptom severity (Schiele et al. 2018).

2.5 Summary and conclusions

This review proposes that the OXT and CRF systems are involved in the emotional impact of bereavement and grief in mothers. Rodent mothers that have experienced prolonged separation from their pups show an anxious and depressed phenotype accompanied by impaired short-term memory and reduced MC (Smith and Lonstein 2008; Pawluski et al. 2006; Pawluski et al. 2009c; Aguggia et al. 2013; Boccia and Pedersen 2001; Ivy et al. 2008). These phenotypic effects can be partly explained by the involvement of the OXT and CRF systems; alterations in the levels of CORT and mRNA of the NrC1 and Morc genes, respectively, in the hippocampus and in the PFC and a decrease in CRF positive cells in the PVN (Eklund et al. 2009; Leuner et al. 2007; Orso et al. 2018; Bolukbas et al. 2020; Baracz et al. 2020). Repeated short separation from pups induces an increase in OXT-R in numerous brain areas and an increase in OXT positive cells in the PVN while prolonged separation from pups decreases the density of OXT positive cells in the caudal PVN (Stamatakis et al. 2015; Baracz et al. 2020). There do not seem to be any alterations in plasma OXT following the separation of the pups while the activity of OXT positive neurons increase following the repeated short separation (Eklund et al. 2009; Liu et al. 2019a; Kelly et al. 2017).

Human studies also suggest the involvement of the two presented neuropeptide systems during grief and loss of a child. In particular, there is a direct correlation between 17-OHCS and cortisol levels with the severity of PGD symptoms (Wolff et al. 1964; Hofer et al. 1972; Nicolson 2004; Saavedra Perez et al. 2017). Bereaved parents have an increased susceptibility to develop depression (Spratt and Denney 1991; Buckley et al. 2009) and grieving women show higher cortisol levels than grieving men (Richardson et al. 2015).

Many studies support the role of OXT in increased emotionality and grief (Feldman et al. 2007; Atzil et al. 2017; Ito et al. 2017; Rutherford et al. 2017). Salivary OXT levels are related to maternity blues and postpartum fatigue (Shishido et al. 2019). At the molecular level, people with PGD have higher plasma OXT levels than depressed people, and the level of OXT is directly correlated with the severity of PGD symptoms; a severity that has also been shown to be genetically influenced by a single nucleotide polymorphism in the OXT-R gene (Bui et al. 2019; Schiele et al. 2018).

The review of the literature leads us to hypothesize that the OXT and CRF systems - and with the latter also glucocorticoids - are involved in bereavement and, more importantly, that further studies are desperately needed. For example, the lack of an adequate animal model for studying the emotional impact following the bereavement of a child is a limiting factor. Studies in such an animal model would open the door to a better understanding of the neurobiology of grief, helping to develop possible precision therapies for people suffering from PGD.

Human studies often do not differentiate between different types of bereavement when studying PGD patients and very few have investigated neurophysiological correlates of bereavement. Thus, future studies on humans should take these factors into account, e.g., conducting surveys among mothers who have lost their child and assessing cortisol and OXT levels in these women, thereby potentially distinguishing the types of loss, from pregnancy to after childbirth and the biomarkers they impact.

2.6 Outlook

Given the high number of child deaths, stillbirths and miscarriages every year and the resulting psychological impact especially in mothers, there is a need for better in-sight in the occurring dysregulations in the brain of grieving mothers. While psychotherapeutic interventions for PGD are already deployed (Shear et al. 2016; Smid et al. 2015) they are not specific to child loss. In addition, PGD-specific pharmacological treatments are not available. Thus, rodent research specific to offspring loss is needed to further understand the neurophysiological profiles mediating PGD symptoms such as anxiety and depression. I therefore suggest that future studies of infant loss and maternal grief use rodent models where complete offspring separation occurs. Furthermore, I believe that the use of rodent species such as rats and prairie voles are an excellent starting point for understanding the

neurophysiological mechanisms contributing to parental grief given that they are, respectively, the foundational animal models for the study of the neurobiology of maternal caregiving and bonding. In addition to this, and from what has emerged in this review, it is important that both the OXT and CRF systems are studied in these models, and clinical populations, as both systems are candidates in the emotional modulation of bereavement. Thus, treatments targeting the OXT and/or the CRF system could be promising for future research in the context of PGD and PGD after the loss of a child. Much more research is needed in clinical populations and in appropriate animal models to decipher the neuropeptide activities in relevant brain regions and determine how the CRF and OXT systems may be targeted for interventions to alleviate PGD when the mother-child bond is broken.

Table 1: Overview of studies related to the OXT and / or CRF systems in rodents after pup loss/separation (for abbreviations see Table 2).

MS DURATION	MS PERIOD	BEHAVIORAL TEST	BIOMARKERS	MAIN FINDINGS	STRAIN	REF
Permanent separation within 24 h after parturition	Dams tested 34 or 55 days after parturition	Radial arm maze		Litter removal induced memory impairment and decreased learning strategies	Sprague-Dawley rats	Pawluski et al., 2006
Permanent separation within 24 h after parturition	Dams tested 30 days after parturition	FST, OF, EPM		Litter removal induced depressive-like behavior without effect on anxiety	Sprague-Dawley rats	Pawluski et al., 2009c
Permanent separation within 24 h after parturition	Dams PND1, PND5, PND14, PND21, PND35		Cort, CBG	Litter removal reduced Cort levels and increased CBG levels by PD5	Sprague-Dawley rats	Pawluski et al., 2009b
Permanent separation of: 1) 60 min 2) 1 Day 3) 8 Days	Dams PND10-PND14 to PND 18-PND22		Cort	Litter removal reduced Cort levels as the pup separation duration increased	Sprague-Dawley rats	Kalyani et al., 2017
3 days of total separation	PND0 to PND3		Cell proliferation, Cort	Total litter removal reduced Cort levels compared to CTRL group	Sprague-Dawley rats	Leuner et al., 2007
20 h / day repeated separation	PND1 to PND4	Maternal care	ACTH, Cort, OXT	LMS induced depression and hypogalactia and reduced OXT activity. Intranasal OXT infusion rescued partially the impaired behavior	Sprague-Dawley rats	Liu et al., 2019a
180 min / day repeated separation	PND 2 to PND 7	Maternal behavior, OF, Light/Dark box, social interaction	Nr3c1 expression in Hip	LMS induced anxiety without effect on social behavior; LMS increased Nr3c1 mRNA in the Hip; LMS impaired maternal care	BALB/c mice	Orso et al., 2018
270 min / day repeated separation	PND 1 to PND 21	FST, maternal care, EPM, step down inhibitory avoidance task	C-Fos expression	LMS reduced maternal care, increased anxiety, no effect on depressive-like behavior and impaired memory LMS increased C-Fos+ cells in CeA	Wistar rats	Aguggia et al., 2013
240 min / day repeated separation	PND 2 to PND 20	Maternal behavior, EPM, marble burying	GABA and Glutamate in serum, Morc1 and Nr3c1 mRNA	LMS increased LG behavior and arched-back nursing; no effect on anxiety; LMS increased Morc1 mRNA in the Hip and increased the GABA serum levels	Sprague-Dawley rats	Bolukbas et al., 2020
240 min separation	PND 7 or 8	EPM	C-Fos expression	Litter removal increased anxiety and increased C-Fos+ cells in dorsal / ventral mPOA, LHb, SUM, VMH	Long- Evans rats	Smith et al., 2008
BMS (15 min/day)	PND1 to PND22	Maternal care, EPM	OXT+ cells, OXTR binding	BMS increased maternal care and reduced anxiety BMS increased OXTR+ cells in PFC, Hip, SL, mPOA, NAcc shell, CeA BMS increased OXTR binding in Hip, SL, mPOA, NAcc shell	Wistar rats	Stamatakis et al., 2015
BMS (15 min / day) LMS (360 min / day)	PND 1 to PND 21	Maternal care, FST	OXT and CRF IR cells in PVN	LMS increased maternal care compared to BMS LMS and BMS both increased anxiety in the dams BMS increased OXT-IR cells in PVN compared to LMS and CTRL groups	Long-Evans rats	Baracaz et al., 2013
BMS (15 min / day) LMS (240 min / day)	During 8 random days in the first 2 weeks postpartum	Spontaneous motor activity, defensive	Adrenal gland weight, ACTH, Cort, OXT	Only BMS induced anxiety Only BMS reduced adrenal glands weight Only BMS increased Cort levels	Wistar rats	Eklund et al., 2009
		withdrawal test		No changes in OXT between groups		
BMS (15 min / day) LMS (180 min / day)	PND 2 to PND 14	FST, sucrose preference test, EPM	Cort	LMS induced anxiety and depressive-like behavior LMS increased Cort levels	Sprague-Dawley rats	Maniam et al., 2010
BMS (15 min / day) LMS (180 min / day)	PND 3 to PND 14	FST, maternal care		LMS induced depressive-like behavior compared to BMS and CTRL groups	Wistar rats	Boccia et al., 2007

Table 2: Overview of studies related to the OXT and / or CRF systems in grieving humans.

GRIEF TYPE	PERIOD	SUBJECTS	METHODS	BIOMARKERS	MAIN FINDINGS	REF
Child loss	During 2 years after loss	N = 40 parents	Interviews, urine samples	17-OHCS	The adrenocortical excretion levels of an individual anticipating the death of their child is characteristic of that individual only in that situation and is not characteristic of them throughout their life or in another stressful situation	Hofer et al., 1972
Sudden death of the child	At 2, 4, 6 and 8 months after loss	N = 11 parents	Blood samples, Back Depression Inventory Questionnaire	Cortisol, T-suppressor cells	Depression in bereaved parents; decreased T-suppressor cells and increased T-helper cells	Spratt et al., 1991
Terminally ill children	During child illness	N = 31 parents	Interviews, urine samples	17-OHCS	17-OHCS excretion rates are related to the individual's response to threat	Wolff et al., 1964
Bereaved spouses or partners of the deceased and bereaved parents	Within 2 weeks and 6 months after loss	N = 62 bereaved individuals, N = 50 non-bereaved	Questionnaire	Cortisol, lipids	Acutely bereaved individuals had depression, anxiety and anger, as well as higher cortisol, reduced appetite, lower total cholesterol and lower sleep duration	Buckley et al., 2009
Partner, child, parent, sibling, friend	At 2 and 5 years after loss	N= 1922 person with no grief; N= 131	Saliva samples, interview, Dutch version of the Inventory of Complicated Grief	Cortisol	2 years after loss, the CG group had lower levels of morning cortisol and lower diurnal cortisol compared to the grief and no-grief groups	Saavedra Perez et al., 2017
Grandparent, parent, sibling, friend, relative, child	Average loss occurred 9.73 years back	N= 93 bereaved individuals	Questionnaire, Inventory of Complicated Grief, Hamilton Depression Rating Scale	OXTR rs2254298 variant	OXTR genotype is related to CG severity	Schiele et al., 2018
Bereaved spouses	At 6, 18 and 48 months after loss	N = 263 bereaved individuals; N = 293 non-bereaved	Interviews, blood and urine samples	Cortisol	Gender-specific effect: bereaved women had higher cortisol levels than bereaved men at 6 months post-bereavement	Richardson et al., 2015
Loss or separation during childhood	(1) Loss of husband < 6 months (2) husband terminally ill (3) healthy husband	N= 9 women (1); N= 11 women (2); N= 3 women (3)	Blood samples	Cortisol, natural killer cells	Bereaved women had increased cortisol levels and natural killer cell activity	Irwin et al., 1988
Parent, spouse	At least 6 months after loss	N= 47 adults with CG; N= 46 bereaved adults with MDD; N= 46 mentally healthy adults bereaved controls	Structured Clinical Interview, Inventory of Complicated Grief, blood samples	OXT plasma level	CG group had higher plasma OXT compared to MDD group	Bui et al., 2019
Parent loss	Loss or separation during childhood	N= 9 men experienced parent loss; N= 10 men experienced parent separation; N= 38 men control group	Saliva samples	Cortisol	Higher cortisol in men who experienced parental death during childhood	Nicolson et al., 2004

Abbreviations: BMS = brief maternal separation; CeA = central amygdala; CG = complicated grief ; Cort = corticosterone; CTRL = control; EPM = elevated plus maze; FST = forced swim test; Hip = hippocampus; IHC = immunohistochemistry; IR = immuno-reactive; LG = licking and grooming; LHb = lateral habenula; LMS = long maternal separation; MDD = major depressive disorder; mPOA = medial preoptic area; MS = maternal separation; NAcc = nucleus accumbens; OF = open field test; OXT = oxytocin; OXT-R = oxytocin receptor; PFC = prefrontal cortex; PND = postnatal day; SL= lateral septum; SUM = supramammilary nucleus; VMH = ventromedial hypothalamus; 17-OHCS= 17-hydroxycorticosteroid.

-Chapter 3-

Brief versus long maternal separation in lactating rats: Consequences on maternal behavior, emotionality, and brain oxytocin receptor binding

Authors' contribution:

Luisa Demarchi:	experimental design, performance of experiments, data analysis, first draft and revision of manuscript
Alice Sanson:	experimental design, performance of experiments, revision of manuscript
Oliver Bosch:	experimental design, performance of experiments, funding acquisition, revision of manuscript

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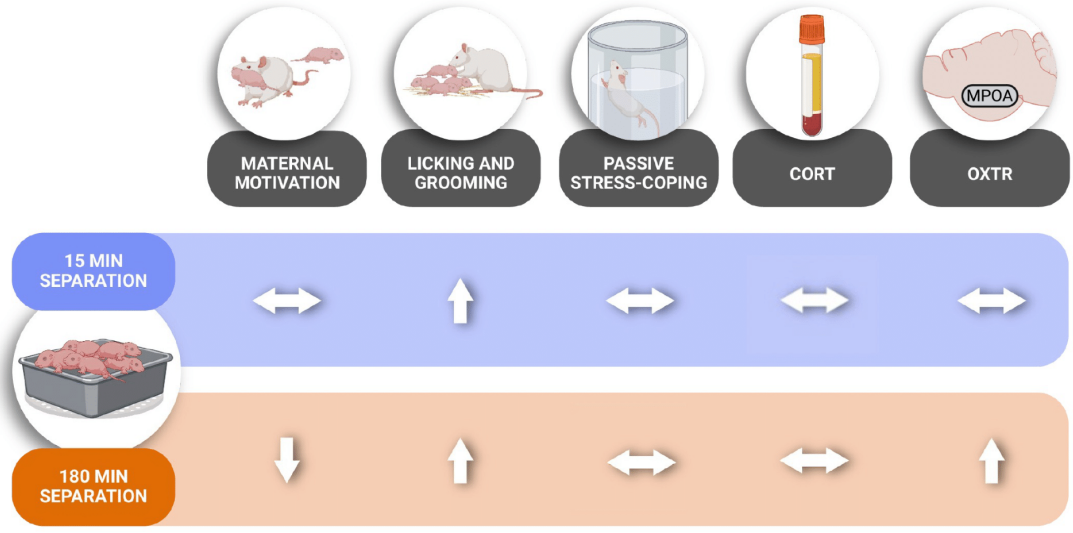
3.1 Abstract

Maternal separation is a widely used animal model to study early life adversity in offspring. However, only a few studies have focused on the impact of disrupting the maternal bond from the mother's perspective. Such studies reveal alterations in behavior, whereas the underlying neuroendocrine mechanisms remain largely unknown. In this work, I compared the consequences of daily brief maternal separation (BMS; 15-minutes) versus long maternal separation (LMS; 180-minutes) during the first week postpartum with respect to behavioral and neuroendocrine changes in lactating Sprague-Dawley dams. Mothers were tested for their MC before and after separation, maternal motivation to retrieve pups, as well as anxiety-related and stress-coping behaviors. In addition, I analyzed their basal plasma CORT levels and oxytocin receptor binding in selected brain regions of the limbic system and maternal network. LMS dams showed higher levels of behavioral alterations compared to BMS and non-maternally separated (NMS) dams, including increased LG of the pups and decreased maternal motivation. Anxiety-related behavior was not affected by either separation paradigm, whereas passive stress-coping behavior tended to increase in the LMS group. Plasma CORT concentrations were not different between groups. Oxytocin receptor binding was higher in the MPOA and tended to be higher in the PL of LMS dams, only. Our results demonstrate that especially daily prolonged maternal separation impacts on the mothers' behavior and OXT system, which suggests that enhanced oxytocin receptor binding could be a compensatory mechanism for potentially decreased central OXT release due to limited pup contact.



Graphical abstract.

3.2 Introduction

The maternal bond is the most important and long-lasting social bond in nature. The mother-child dyad is in fact interconnected, and it is critical to maintain a positive and intact bond for the mental and physical well-being of both subjects (Pohl et al. 2019). In recent decades, several studies have been conducted to investigate the consequences of a disturbed mother-offspring bond focusing on the development of the offspring; hence, the maternal separation paradigm became a well-known animal model for early life stress (Nishi 2020; Teissier et al. 2020; Veenema et al. 2007). Maternal separation is performed daily for short (15 - 30 minutes) or long periods of time (180 - 360 minutes) and is typically applied during the first PND after giving birth. In rodents, maternal separation acts as chronic stressor on the offspring (Ladd et al. 2000), impacting the offspring's behavior and brain (Aisa et al. 2007; Lehmann and Feldon 2000; Lippmann et al. 2007; Monroy et al. 2010). Indeed, it has been linked to increased emotionality (Tsotsokou et al. 2021; for review see: Wang et al. 2020), cognitive impairment (Aisa et al. 2007), altered stress responses (Abdelwahab et al. 2021; Bian et al. 2021), and epigenetic changes in the adult CNS (Holmes et al. 2005; Weaver 2007). Considering that the postpartum period is of potentially high-risk to develop psychiatric illnesses such as postpartum depression, it is surprising that only few studies investigated the effects of a disturbed mother-child bond from the mother's perspective (Brummelte and Galea 2010a; Brunton and Russell 2008; Jones et al. 2014). Those studies show that maternal separation affects the mothers' MC, and anxiety- and depressive-like behavior (Alves et al. 2020; Baracz et al. 2020; Bolukbas et al. 2020; Liu et al. 2019a; Orso et al. 2018; Smith et al. 2004). For example, daily 180-minute separation during the first two weeks postpartum increases passive stress-coping behavior in the forced swim test (FST) (Boccia et al. 2007) and anhedonia in the sucrose preference test (Maniam and Morris 2010). In other studies, the total removal of the offspring increases passive-stress coping behavior of the mother one month later (Pawluski et al. 2009c) and impairs cognition (Pawluski et al. 2006). However, many inconsistencies are still found when it comes to the phenotypic alterations following offspring separation, contributing to an unclear picture of separation-effects. Therefore, the reproduction of known data can help to confirm experimental setups and animal models used.

Even less studies have examined neuroendocrine alterations, mainly focusing on the OXT and the CRF systems demonstrating, e.g., that prolonged separation of 20 hours daily from

LD1 to LD4 decreases the OXT+ neurons activity in the SON of the mothers (Liu et al. 2019a). To our knowledge, no study has investigated the mechanisms of the OXT system to compensate for the decreased activity of OXT+ neurons following pup separation. Therefore, I aimed to reproduce some behavioral alterations and to add new insight into the neuroendocrine adaptations of the separated mothers, specifically over the first postpartum week, a time-window characterized by higher intensities and incidences of maternal responses (Bridges 2015).

The OXT system plays an important role in the onset and maintenance of maternal bonding and behavior (Ishak et al. 2011). OXT is primarily synthesized in and released from the hypothalamic PVN and SON. During the peripartum period, the activity of the OXT system is upregulated as it is critical for the expression of maternal behavior, maternal memory, and emotion modulation (Bosch and Neumann 2012; D'Cunha et al. 2011; Ferris et al. 2015; Lonstein et al. 2014; Pedersen and Boccia 2003; Sabihi et al. 2014b; Sanson and Bosch 2022). Furthermore, OXT-R binding is increased during the postpartum period (Insel 1990) and is involved in decreased anxiety-related behavior. Moreover, increased OXT system activity also dampens the stress response through modulation of the HPA axis (Neumann 2002; Slattery and Neumann 2008), which is triggered by the CRF system eliciting increased plasma ACTH and CORT levels. Increased brain CRF system activity has severe behavioral effects including impaired maternal behavior (Klampfl and Bosch 2019a). Therefore, it is not surprising that one of the peripartum adaptations is a marked decrease of the CRF system activity (Klampfl and Bosch 2019a). It is evident that a fine-tuning of both systems —increased OXT signaling in parallel to decreased HPA axis activity— is required for adequate maternal behavior.

To shed more light on a potential involvement of both systems on the phenotype of the separated mother (as recently reviewed in: Demarchi et al. 2021), I compared the most frequently used maternal separation paradigms, i.e., daily separation for 15 minutes (BMS) versus 180 minutes (LMS) in lactating Sprague-Dawley rat mothers. In addition, I included non-maternally separated (NMS) dams as a control group. I investigated whether brief versus long maternal separation during the first week postpartum differentially affects the mothers' behavior and physiology, i.e., MC, maternal motivation, anxiety-related behavior, passive stress-coping behavior, and OXT-R binding as well as basal plasma CORT levels and adrenal gland weight. This study sheds new light on potential compensatory mechanisms in the OXT system that are triggered by offspring separation.

3.3 Materials and methods

3.3.1 **Animals**

The experiments were conducted in female Sprague-Dawley rats (Charles River Laboratories, Sulzfeld, Germany), weighing 230-250 g at arrival. The rats were kept under standard laboratory conditions (change of bedding once per week, 12:12h light/dark cycle, lights on at 7 a.m., room temperature 22 ± 1°C, relative humidity 55 ± 5°C) with access to standard rat chow (ssniff-Spezialdiäten GmbH, Soest, Germany) and water *ad libitum*. After 7 days of habituation, females were mated with sexually experienced male Sprague-Dawley rats in standard laboratory cages (Eurostandard Type IV, 60 cm X 40 cm X 20 cm) for 10 days. From potential pregnancy day 18 onwards, pregnant females were single housed for undisturbed delivery either in standard laboratory cages (experiment A), or in observational cages (plexiglass, 38 cm x 22 cm x 35 cm; experiments B, C). On the day of delivery (LD0), litters were reduced to 8 pups. All experiments were performed in accordance with the European Union Directive (2010/63/EU) and were approved by the Government of Unterfranken, Bavaria, Germany. According to the 3-R principles, all efforts were made to minimize the number of rats and their suffering.

3.3.2 **Maternal separation procedure**

Dams were randomly assigned to one of the following three experimental groups: NMS (control group), BMS (15-minute separation), or LMS (180-minute separation). Separations occurred daily from 10.00 a.m. to 13.00 p.m. in LMS and from 12.45 p.m. to 13.00 p.m. in BMS mothers. During the separation, litters were kept in boxes containing their home cage bedding on a heating pad under constant temperature (32 °C). Mothers were left undisturbed in their home cage during the separation procedure. At the end of the separation protocol, pups were returned to their home cage and placed in the corner opposite of the mother.

3.3.3 **Experimental schedule**

For an overview of experiments A – C, see Fig. 11.

Experiment A: From LD1 to LD7, rat mothers underwent the maternal separation protocol except on LD3 when all three groups were tested in the pup retrieval test (PRT) in a novel arena. In addition, the PRT was performed in the home cage on LD1 and LD7 in the BMS and LMS groups after ending the separation. Number of rats were: NMS = 9; BMS = 9; LMS = 8.

Experiment B: From LD1 to LD6, a different cohort of rat mothers underwent the maternal separation protocol. On LD7, all three groups were tested in the light dark box (LDB) and on LD8 in the FST. Number of rats were: NMS = 8; BMS = 8; LMS = 7.

Experiment C: From LD1 to LD6, a different cohort of rat mothers underwent the maternal separation protocol. MC was monitored from LD1 to LD6 before and after the reunion with the pups. Body weight of mothers and of whole litters was taken from all groups at 5.00 p.m. from LD1 to LD6. On LD7, all rats were sacrificed, and blood, brains and adrenal glands were collected for further analysis. Number of rats were: n = 7 (in each group).

3.3.4 **Test for maternal motivation**

Maternal motivation to retrieve pups is an essential maternal behavior ensuring the survival of the offspring until their reproductive maturity (Numan and Woodside 2010). The motivation was tested in the PRT, either in the home cage or in a novel arena, and the number of pups retrieved every 10-sec noted.

Home cage: Maternal motivation to retrieve pups into the nest after placing them back into the home cage was recorded immediately after reunion following the separation paradigms (Baracz et al. 2020). Pups were placed in the corner opposite of the mother and the mother's behavior was videotaped for later analysis by an experimenter blind to the treatment. The latency to retrieve each of the 8 pups during the 10-minute test was analyzed.

Novel arena: The PRT was performed between 10.00 a.m. and 12.00 p.m. and lasted 15 minutes. One hour prior to testing, pups were removed from the mother into separate boxes containing bedding from their home cage under controlled temperature conditions (32 °C). For the PRT, pups were placed in the novel arena (54 cm x 34 cm x 60 cm) covered with

bedding from their home cage following a specific scheme of placement as described before (Bosch and Neumann 2008). Trials were video recorded for subsequent analysis by an experimenter blind to the treatment. The latency to retrieve each of the 8 pups during the 15-minute test was analyzed. Before each test, the arena was cleaned with tap water and dried thoroughly.

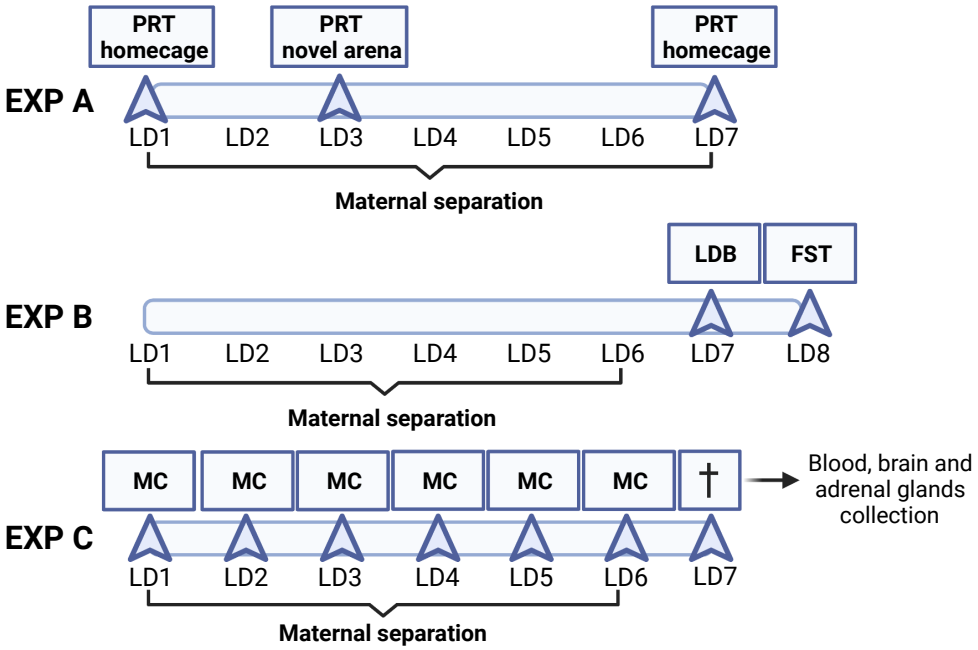


Figure 11. Experimental design and timeline. Experiment A was designed to study maternal motivation in two different settings. Experiment B aimed to reveal effects of maternal separation on anxiety-related and passive stress-coping behavior. Experiment C was designed to monitor maternal care before and after separation, and to collect basal samples from blood, brain, and adrenal glands. Experiment (EXP); Forced swim test (FST); lactation day (LD); light-dark box (LDB); maternal care (MC); pup retrieval test (PRT).

3.3.5 **Test for anxiety-related behavior**

Anxiety-related behavior was tested between 9.00 a.m. and 12.00 p.m. in the LDB (Crawley and Goodwin 1980). The arena is divided into a light (40 cm x 50 cm, 180 lux) and a dark compartment (40 cm x 30 cm, 0 lux) connected via an opening (7.5 cm x 7.5 cm). At the beginning of the test, dams were placed in the center of the light box and the behavior was recorded for 10 minutes for later analysis by an experimenter blind to the treatment with

EthoVision XT (Noldus, Wageningen, the Netherlands). Behaviors analyzed were: time spent in the light box, numbers of transitions from the light to the dark box and locomotor activity in the whole arena. Before each test, the arena was cleaned with tap water and dried thoroughly.

3.3.6 **Test for passive stress-coping**

Passive stress-coping behavior was tested in the FST (Ebner et al. 2005). Between 9.00 a.m. and 12.00 p.m., rat mothers were forced to swim for 10 minutes in a cylindrical tank (40 cm high, 18 cm diameter) filled with tap water (23 ± 1 °C) to a depth that rats could not touch the bottom with their hind paws or tail. Trials were recorded for later analysis using the software JWatcher (https://www.jwatcher.ucla.edu) by an experimenter blind to the treatment. The total time spent floating (passive stress-coping, indicative of depressive-like behavior (Slattery and Cryan 2012)) was analyzed.

3.3.7 **Observation of maternal care**

MC in the home cage was monitored and manually scored by an experimenter blind to the treatment according to an established protocol (Klampfl et al. 2016b; Klampfl et al. 2013; Bosch and Neumann 2008; Bayerl et al. 2014). Observation of MC was performed from 9.00 a.m. to 10.00 a.m. and from 13.00 p.m. to 14.00 p.m. every 2 minutes for 10 seconds prior the maternal separation (T1) and after reunion with the pups (T2) leading to a total count of 60 observation points per dam and per day. The scored behaviors to determine the quality of MC were LG of the pups and ABN (Bosch 2011).

3.3.8 **Blood collection and corticosterone ELISA**

Between 10.00 a.m. and 12.00 p.m., dams in their home cage were transported to a separate room, flash-anesthetized with isoflurane (in preparation for perfusion; see below), the thorax opened, and blood was collected immediately from the right atrium of the heart in EDTA-coated tubes (0.5M, pH 7.4; Sarstedt, Nümbrecht, Germany), which were maintained on ice until further processing. Blood samples were centrifugated for 10

minutes at 10,000 rpm, plasma was collected and processed with the ELISA kit for CORT analysis following the protocol of the manufacturer (Tecan IBL International GmbH, Hamburg, Germany).

3.3.9 **Brain sampling and OXT-R autoradiography**

After blood sampling (see above), dams underwent cardiac perfusion with ice-cold 1 x phosphate-buffered saline (PBS), decapitated, brains were flash-frozen in n-methylbutane, and stored at -20 °C until cutting into coronal sections of 16 µm using a cryostat (CM3050S Leica Microsystem GmbH, Nussloch, Germany). For each brain region of interest, i.e., agranular insular cortex (AIP), accessory olfactory bulbs (AOB), bed nucleus stria terminalis (BNST), central amygdala (CeA), lateral septum dorsal (dLS) and ventral (vLS), medial preoptic area (MPOA), nucleus accumbens shell (NAcc shell), prelimbic cortex (PL), ventral medial hypothalamus (VMH), six sections per rat were collected on SUPERFROST microscope slides and stored at -20 °C until further processing. The OXT-R autoradiography was performed following an established protocol (Oliveira et al. 2021; Bosch and Neumann 2008). Briefly, the ornithin vasotocin analogue [^{125}I]-OVTA [d(CH_2)$_5$[Tyr(Me)^2,Thr^4,Orn^8,[^{125}I]Tyr^9-NH_2]; Perkin Elmer, USA) was used as a tracer. First, the slides were thawed and allowed to dry thoroughly at room temperature. The tissue was shortly fixed via 0.1 % PFA, washed 2 x in Tris (50 mM, pH 7.4), covered with the tracer solution (50 mM tracer, 10 mM $MgCl_2$, 0.1 % BSA) for 60 minutes, washed 3 x in Tris / $MgCl_2$ buffer for 7 minutes, each, followed by 30-minute spinning in Tris / $MgCl_2$. Finally, slides were dipped into water and air dried before being exposed to Biomax MR films (Kodak, Cedex, France) for 15 days. The films were scanned using the EPSON Perfection V800 Scanner (Epson GmbH, Munich, Germany), and the optical density of each region of interest was analyzed using ImageJ (Schneider et al. 2012) by subtracting the background activity as previously described (Baskin and Stahl 1993). The analyses were performed simultaneously for 6 sections per rat and per region in the left hemisphere.

3.3.10 **Adrenal gland collection**

After brain removal, adrenal glands were collected and stored on ice in 1 x PBS. Adrenal glands were dissected from the surrounding fat and weighed to calculate the adrenal gland's relative weight (adrenal gland weight / body weight).

3.3.11 **Statistical analyses**

All statistical analyses were performed with GraphPad PRISM 9 (GraphPad Software, San Diego, USA). Normality and homoscedasticity were verified (Shapiro-Wilk or Kolmogorov–Smirnov test and Brown-Forsythe test, respectively) and analysis of outliers was run via the ROUT method. Maternal motivation, maternal behavior, and body weight were analyzed using a two-way RM ANOVA (factors: time x treatment) followed by Sidak post-hoc multiple comparisons if main effects were found. Latency to retrieve the first pup in the homecage was analyzed using the Mann-Whitney test. Latency to retrieve the first pup on LD3, behavioral parameters analyzed in the LDB and FST, OXT-Rs binding, and adrenal gland weight were analyzed using a one-way ANOVA. Plasma CORT concentration data did not meet the homoscedasticity and a Welch's ANOVA was run. Additional size effects between groups were calculated using the Cohen's *d* coefficient and eta squared h^2. Data are presented as mean ± SEM; $p < 0.05$ was considered significant and a trend was accepted up to $p = 0.08$.

3.4 Results

3.4.1 **Experiment A**

3.4.1.1 **LMS impaired maternal motivation in the home cage**

In BMS group, no differences were found in the latencies to retrieve the first pup between LD1 and LD7 (Mann-Whitney U = 28, n1 = n2 = 9, p = 0.296 two-tailed; Fig. 12a).
In BMS dams, the main effect of time on number of pups retrieved was significant (two-way RM ANOVA; [$F (59, 944) = 16.13$, $p<0.0001$, $h^2 = 0.16$]; Fig. 12b) but the main effect of LD1 and LD7 on the number of pups retrieved was not significant (two-way RM

ANOVA; [F (1, 16) = 0.7047, p = 0.414, h^2 = 0.03]; Fig. 12b). In BMS dams, there was not a significant interaction effect (two-way RM ANOVA; factors time x treatment [F (59,944) = 0.7729, p = 0.894, h^2 = 0.007]; Fig. 12b).

LMS dams displayed significantly lower latencies to retrieve the first pup on LD7 (Mann-Whitney U = 12, n1 = n2 = 8, p = 0.033 two-tailed; Fig. 12c). There was a significant main effect of time (two-way RM ANOVA; [F (1.97, 27.65) = 17.23, p<0.0001, h^2 = 0.12]; Fig. 12d) and of lactation day on the number of pups retrieved (two-way RM ANOVA; [F (1, 14) = 6.646, p = 0.022, h^2 = 0.25]; Fig. 12d). In LMS dams, an interaction effect was found when comparing LD1 and LD7 (two-way RM ANOVA; factors time x treatment [F (59,826) = 1.402, p = 0.028, h^2 = 0.009]; Fig. 12d).

3.4.1.2 **LMS impaired maternal motivation in a novel arena on LD3**

The treatment groups differed in the latency to retrieve the first pup (one-way ANOVA; [F (2,22) = 3.978, p = 0.034, h^2 = 0.27]; Fig. 13a). Post-hoc Sidak multiple comparisons test revealed that the time taken to retrieve the first pup was significantly greater in LMS dams than NMS dams (p = 0.029, 95% C.I. = [-538.9, -23.57]). However, neither NMS and BMS (p = 0.445) nor BMS and LMS (p = 0.374) differed from each other.

The main effect of 10-sec intervals of time on the number of retrieved pups was significant (two-way RM ANOVA; [F (2.90, 66.89) = 44.73, p = 0<0.0001, h^2 = 0.33]; Fig. 13b) but the main effect of treatment groups was not significant (two-way RM ANOVA; [F (2,23) = 2.155, p = 0.138, h^2 = 0.08]; Fig. 13b). There was no interaction effect comparing NMS, BMS and LMS dams (two-way RM ANOVA; [F (178, 2047) = 0.866, p = 0.891, h^2 = 0.01]; Fig. 13b).

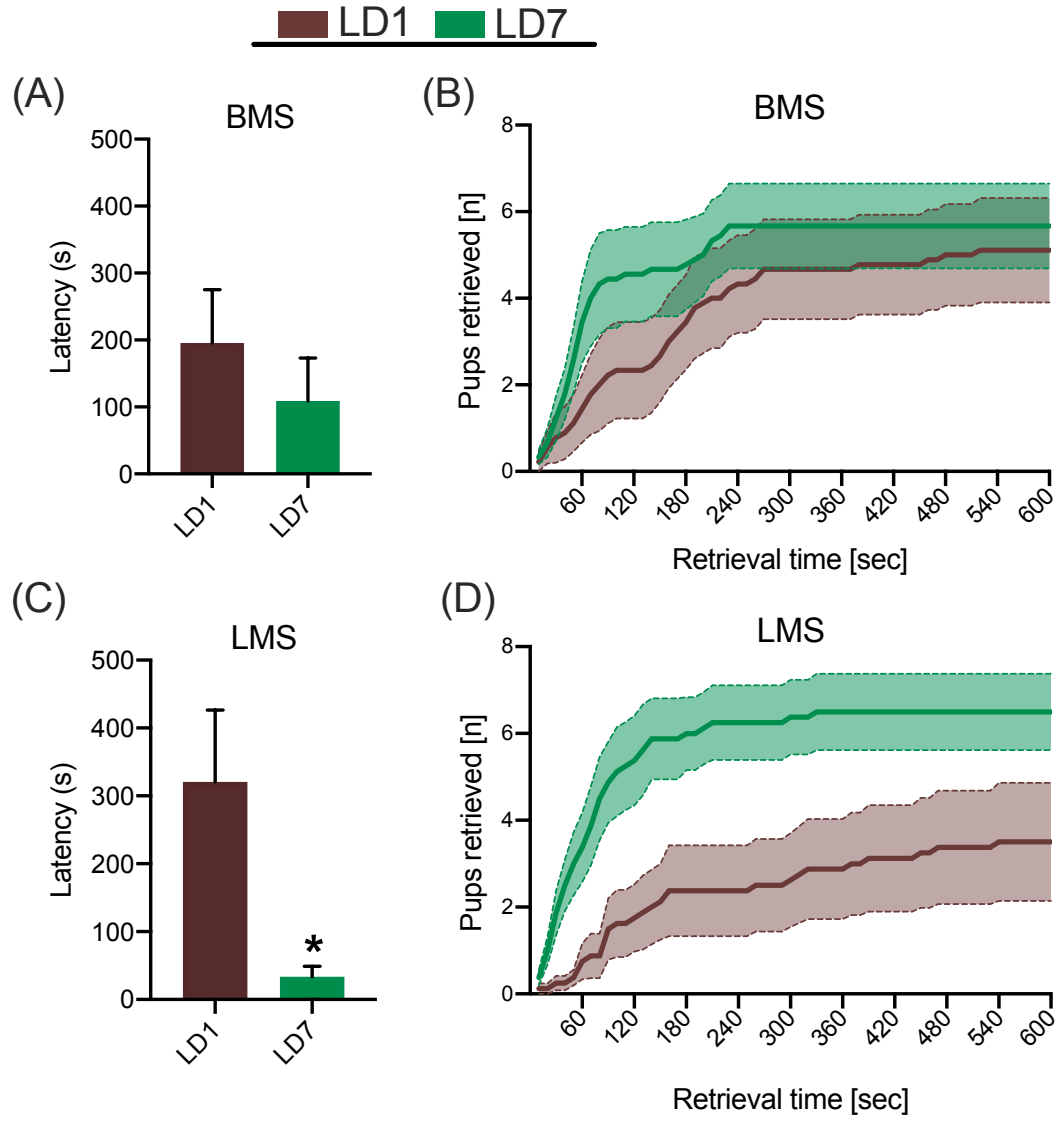


Fig. 12. Maternal motivation in the home cage on LD1 and LD7. (A) Time until retrieval of first pup in BMS (Mann-Whitney test). (B) Number of retrieved pups during the 600-sec test in BMS group on LD1 (brown line) versus LD7 (green line) (two-way RM ANOVA). (C) Time until retrieval of first pup in LMS (Mann-Whitney test). (D) Number of retrieved pups during the 600-sec test in LMS group on LD1 (brown line) versus LD7 (green line) (two-way RM ANOVA). Number of animals: BMS = 9, LMS = 8. Dashed lines represent SEM values. * $p<0.05$ LD1versus LD7

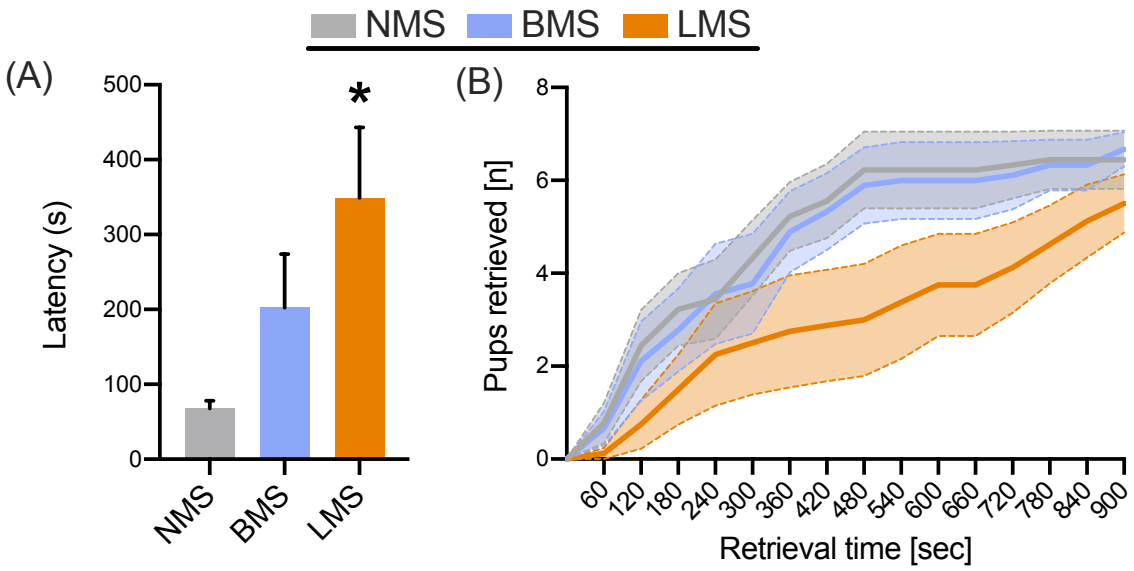


Fig. 13. Maternal motivation in a novel arena on LD3. (A) Time until retrieval of first pup (one-way ANOVA). (B) Number of retrieved pups during the 900-sec test (two-way RM ANOVA). Number of animals: NMS = 9, BMS = 9, LMS = 8. Dashed lines represent SEM values. * $p<0.05$ LMS versus NMS

3.4.2 **Experiment B**

3.4.2.1 **BMS and LMS had no effect on anxiety-related behavior**

On LD7, no significant differences between the groups were found in the time spent in the light box as a measure of anxiety-related behavior (one-way ANOVA; [$F(2,18) = 0.113$, $p = 0.895$, $h^2 = 0.01$]) (NMS: 42.6 ± 2.3 sec; BMS: 44.5 ± 4.6 sec; LMS: 41.9 ± 4.6 sec) nor in transitions between the compartments ([$F(2,18) = 1.169$, $p = 0.333$, $h^2 = 0.15$]) (NMS: 10.1 ± 1.3; BMS: 10.8 ± 3.5; LMS: 13.6 ± 2.2) or locomotion during the LDB test ([$F(2,18) = 0.94$, $p = 0.409$, $h^2 = 0.09$]) (NMS: 4436 ± 389 cm; BMS: 5188 ± 727 cm; LMS: 4163 ± 307 cm).

3.4.2.2 **LMS tended to increase passive stress-coping behavior**

On LD8, time spent floating during the FST over the first 5-minute test tended to differ between groups (one-way ANOVA; [$F(2,20) = 2.875$, $p = 0.079$, $h^2 = 0.22$]) (NMS: 20.93 ± 4.47 sec; BMS: 12.46 ± 2.87 sec; LMS: 28.78 ± 6.71 sec). Cohen's *d* coefficient revealed

the strongest effect size between LMS and BMS groups (d = 1.06) however, this did not reach statistical significance. Over the full 10-minute test, no significant differences were found between groups (one-way ANOVA; [F (2,20) = 2.083, p = 0.150, h^2 = 0.17]).

3.4.3 **Experiment C**

3.4.3.1 **BMS and LMS increased LG behavior**

The main effect of time (two-way RM ANOVA; [F (1.839, 18.3) = 27.64, p < 0.0001, h^2 = 0.36]; Fig. 14a) and of treatment (two-way RM ANOVA; [F (1, 10) = 25.69, p = 0.0005, h^2 = 0.01]; Fig. 14a) on the total mean LG frequency was significant. LG differed between the groups depending on time and treatment (two-way RM ANOVA; factors time x treatment: [F (2,20) = 12.69, p = 0.0003, h^2 = 0.17]; Fig. 14a). The post-hoc Sidak multiple comparisons revealed that from T1 (before separation) to T2 (after separation), BMS (p = 0.037) and LMS dams (p = 0.002) showed increased LG compared to NMS group.
ABN did not differ depending on time (two-way RM ANOVA; [F (2, 20) = 2.686, p = 0.093, h^2 = 0.15]; Fig. 14b) or treatment (two-way RM ANOVA; [F (1, 10) = 1.645, p = 0.228, h^2 = 0.04]; Fig. 14b). In addition, when calculating the delta LG frequencies between T2 (after separation) and T1 (before separation), there was a main time effect (two-way ANOVA: [F (5,90) = 15.97, p<0.0001, h^2 = 0.12]; Fig. 14c), a main treatment effect (two-way ANOVA: [F (2,90) = 212.3, p<0.0001, h^2 = 0.62]; Fig. 14c) and an interaction effect (two-way ANOVA, factors time x treatment: [F (10,90) = 9.553, p<0.0001, h^2 = 0.14]; Fig. 14c). Post-hoc Sidak multiple comparisons revealed increased LG in BMS vs NMS on LD1 (p = 0.006), LD2 (p = 0.0003), LD3 (p < 0.0001), LD5 (p < 0.0001), and LMS vs NMS from LD1 to LD6 (p < 0.0001, each; Fig. 14c), but no differences were observed when comparing BMS and LMS. No significant effect was found when calculating the delta ABN frequencies between T2 and T1 (Fig. 14d).

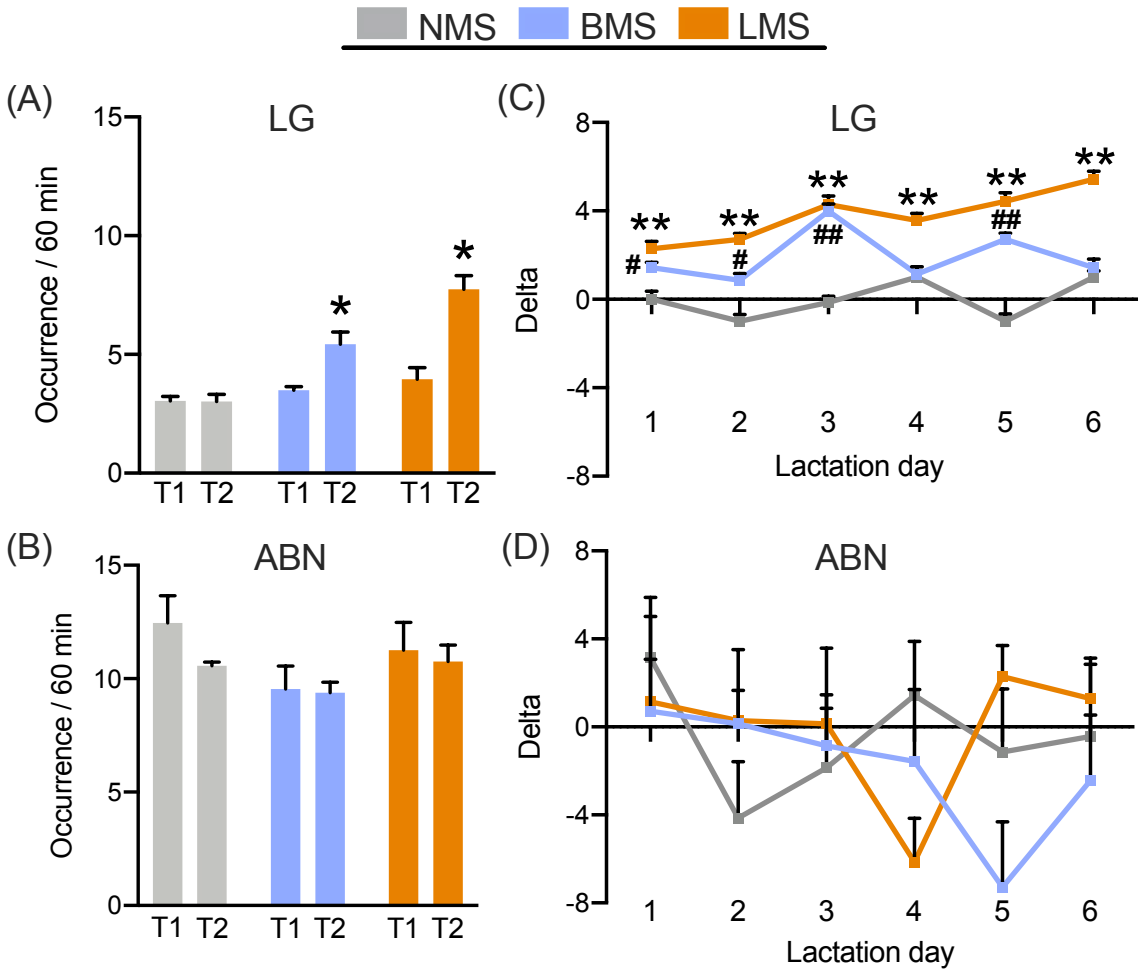


Fig. 14. Maternal care in the home cage before and after separation from LD1 to LD6. Mean overall frequency of (A) licking and grooming the pups (LG) and (B) arched back nursing (ABN) for 60 min at T1 (preceding the separation) and T2 (immediately after reunion with the pups). Delta scores (T2-T1) for (C) LG and (D) ABN from LD1 to LD6. (A – D) Two-way RM ANOVA, number of animals: n = 7 (each group). Results are expressed as mean + SEM. * $p<0.05$, ** $p<0.0001$ LMS versus NMS; T2 versus T1; # $p<0.05$, ## $p<0.0001$ BMS versus NMS

3.4.3.2 **BMS and LMS had no effect on basal plasma corticosterone concentrations**

The treatment groups did not differ in basal plasma CORT concentrations (Welch's ANOVA test; $p = 0.116$) (NMS: 419.3 ± 121.5 ng / ml; BMS: 150.1 ± 41.3 ng / ml; LMS: 720.3 ± 314.4 ng / ml).

3.4.3.3 **LMS increased OXT-R binding in the PL and MPOA**

In the PL region, OXT-R binding tended to differ between treatment groups (one-way ANOVA; [F (2,18) = 3.005, p = 0.075, h^2 = 0.25]; Fig. 15a-c). Cohen's *d* coefficient revealed the strongest effect size between NMS and LMS groups (*d* = 1.16). In the MPOA, OXT-R binding differed between treatment groups (one-way ANOVA; [F (2,17) = 3.726, p = 0.045, h^2 = 0.30]; Fig. 15a-c). Post-hoc Sidak multiple comparisons revealed a significant difference between the BMS and LMS groups (p = 0.043; Fig. 15a-c). No differences in OXT-R binding between the treatment groups were found in the other analyzed regions: CeA [F (2,18) = 0.346, p = 0.712], BNST [F (2,17) = 0.907, p = 0.422], AIP [F (2,18) = 1.836, p = 0.188], NAcc shell [F (2,17) = 0.647, p = 0.536], AOB [F (2,17) = 0.228, p = 0.798], LSd [F (2,17) = 0.004, p = 0.995], LSv [F (2,17) = 2.132, p = 0.149], VMH [F (2,18) = 0.952, p = 0.405].

3.4.3.4 **Adrenal glands and body weight**

Neither the relative adrenal gland weight on LD7 (one-way ANOVA; [F (2,18) = 0.1257, p = 0.883, h^2 = 0.01]; NMS: 0.247± 0.007 g; BMS: 0.257 ± 0.013 g; LMS: 0.253 ± 0.019 g) nor the change of body weight over the days in mothers (two-way RM ANOVA; factors time x treatment: [F (12,108) = 0.511, p = 0.904, h^2 = 0.06]; data not shown) or litters (two-way RM ANOVA; factors time x treatment: [F (10,90) = 0.286, p = 0.983, h^2 = 0.03]; data not shown) differed between the groups.

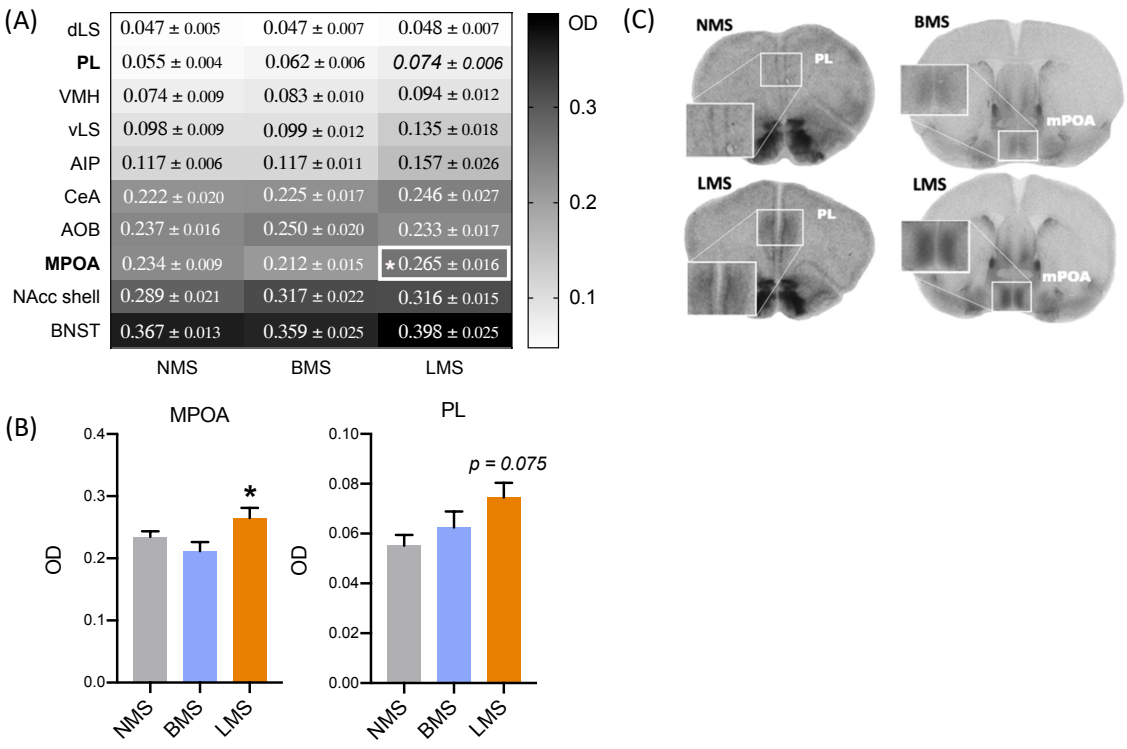


Fig. 15. Oxytocin receptor (OXT-R) binding in brain regions of the limbic system and maternal network on LD7. (A) Overview of gray densities (arbitrary units) for OXT-R binding in the analyzed brain areas. (B) Representative coronal brain sections of the prelimbic cortex (PL) and medial preoptic area (MPOA) demonstrating differences in OXT-R binding. (C) Optical gray densities (arbitrary units) for OXT-R binding in the MPOA and PL. Abbreviations: Agranular insular cortex (AIP), accessory olfactory bulbs (AOB), bed nucleus stria terminalis (BNST), central amygdala (CeA), lateral septum dorsal (dLS) and ventral (vLS), medial preoptic area (MPOA), nucleus accumbens shell (NAcc shell), prelimbic cortex (PL), ventral medial hypothalamus (VMH). (A, C) One-way ANOVA, number of animals: n = 7 (each group). Results are expressed as mean ± SEM. * $p<0.05$ LMS versus BMS

3.5 Discussion

The present study compared the consequences of daily brief versus long maternal separation in the first week postpartum on rat mothers' maternal, emotional, physiological, and neuroendocrine parameters (see Table 3 for an overview of the results). Overall, I was able to demonstrate that LMS had a stronger impact on various factors compared to BMS.

Group	Maternal care	PRT LD1 vs. LD7	PRT LD3	Passive stress-coping	Plasma cort	OXTR binding
NMS	=		=	=	=	=
BMS	↑	=	=	=	=	=
LMS	↑	↓	↓	=	=	↑ MPOA

Table 3: Summary of the behavioral and neuroendocrine results.

Abbr. Brief maternal separation (*BMS*), Corticosterone (CORT), lactation day (LD), long maternal separation (*LMS*), medial preoptic area (MPOA), oxytocin receptor (OXT-R), non-maternally separated (*NMS*), prelimbic cortex (PL), pup retrieval test (PRT). ↑ Increase, ↓ decrease, = no difference

LMS resulted in reduced maternal motivation both in the home cage on LD1 (Fig. 12c-d) and in a novel, and more challenging, arena (Fig. 13). Interestingly, maternal motivation was normalized after one week of separation thereby demonstrating that the mothers might have adapted to the daily separation distress (Joushi et al. 2021). On the contrary, BMS dams did not show alterations in maternal motivation (Fig. 12a-b and Fig. 13). Those findings are consistent with previous studies demonstrating a slower pup retrieval of dams that had been separated from their pups for 180-minutes or even 360-minutes versus 15-minutes (Maniam and Morris 2010; Baracz et al. 2020; Aguggia et al. 2013). Our results reveal that LMS reduced maternal motivation immediately, followed by an improvement over one week possibly due to coping strategies. As previously demonstrated by Stolzenberg and colleagues (Stolzenberg et al. 2012), the rescue of maternal motivation as a result of repeated distress experiences may even imply epigenetic alterations at the level of chromatin modifications, which in turn change the expression of genes that promote MC, particularly in the MPOA (Stolzenberg et al. 2012), one of the main brain regions where pup retrieval is processed (Numan and Stolzenberg 2009; Numan 2020).

With respect to MC, both LMS and BMS mothers showed more LG behavior compared to NMS dams. When analyzing the data day-by-day, it becomes clear that LMS dams tended to increase LG behavior over the course of the lactation days (Fig. 14c), peaking on LD6. These findings are supported by the literature's assertion that either a short or protracted maternal separation improves maternal behavior (Zimmerberg et al. 2003; Stamatakis et al. 2015; Pryce et al. 2001; Own and Patel 2013; Marmendal et al. 2004; Eklund et al. 2009; Bolukbas et al. 2020; Boccia et al. 2007). In fact, it has been suggested that separation stress serves as a catalyst for increased maternal behavior (Smotherman 1983; Liu et al. 1997). Thus, the enhanced pup-directed care following the reunion is likely an attempt to make up for the pups' lack of care during the separation (Baracz et al. 2020).

As maternal behavior is mediated by increased brain OXT signaling, among others (Stolzenberg DS 2019; Bridges 2015; Bosch and Neumann 2012), I speculated that maternal separation might interfere with the mothers' brain OXT system (Demarchi et al. 2021). In fact, alteration in OXT-R binding have been described in a different maternal separation model, i.e., 15-minutes per day lasting from LD1 to LD22 using Wistar rat mothers (Stamatakis et al. 2015). Therefore, I analyzed OXT-R binding in several limbic and maternal network regions following one week of separation. I found that LMS dams - in comparison to BMS and NMS mothers – tended to have higher OXT-R binding in the PL and significantly increased OXT-R binding in the MPOA, but not in any other region analyzed (CeA, BNST, AIP, NAcc shell, OB, LS, VMH; Fig. 15a-c). While the MPOA is commonly associated with pup retrieval behavior ((Jacobson et al. 1980; Bayerl et al. 2016); for reviews see (Numan and Stolzenberg 2009; Gammie 2005; Bosch 2011)), little is known about a potential role of the PL in maternal behavior. Recently, the PL has been shown to modulate the reward system through the OXT system (Everett et al. 2019). OXT neurons are highly activated during pup nursing and OXT is released not only into the periphery but also within the brain (Neumann et al. 1993b; Neumann et al. 1993a). Furthermore, nursing the pups is a reward for the mother that is even stronger than cocaine (Ferris et al. 2005) and pup retrieval requires the dopamine mesolimbic system activity (Numan and Stolzenberg 2009). Thus, I hypothesize that the mother is seeking to nurse the pups, which in turn could lead to their retrieval when outside the nest.

Interestingly, a study by Stamatakis et al. (Stamatakis et al. 2015) describes effects of maternal separation on OXT-R binding similar to ours, i.e., increased OXT-R binding in the prefrontal cortex and MPOA. In addition, the authors show higher OXT-R binding also

in the hippocampus, LS, and NAcc shell, which I did not find. This discrepancy could be explained by the extended maternal separation distress (22 days versus 7 days) as well as the different rat strain (Wistar versus Sprague-Dawley). Furthermore, different technical approaches had been used, i.e., analyses of sagittal brain sections (Stamatakis et al. 2015) while I used coronal sections resulting in a different volumetric area for each brain region. However, Stamatakis et al. (Stamatakis et al. 2015) also demonstrate that in separated rat dams increased OXT-R binding is paralleled by more LG of the pups, which is in line with studies on high LG-ABN mothers (Champagne et al. 2001) as well as with our present findings. Indeed, LMS dams exhibited the highest frequency of LG after one week of maternal separation (Fig. 14a, c). Therefore, I speculate that the increased OXT-R binding I found in LMS dams might be part of a compensatory strategy for potentially less brain OXT activity, consistent with other studies showing concomitant opposite central changes in OXT and OXT-R levels (Zanos et al. 2014; Rae et al. 2018). Indeed, the prolonged separation leads to less interactions with the pups, and consequently less milk ejection reflexes, which in turn might result in reduced brain OXT signaling. On the other hand, daily brief separation may not be sufficient to cause alterations at the level of the OXT system during the first postpartum week. Further research could test this hypothesis, especially as the current literature already suggests that the OXT system is modulated by maternal separation, e.g., reduced c-Fos expression in OXT+ neurons of the SON of prolonged separated mothers (Liu et al. 2019a) and less ir+ OXT neurons in the PVN of 180-minute separated mothers (Baracz et al. 2020).

While anxiety-related behavior is reduced in lactation (Neumann 2003; Lonstein 2007; Bosch 2011) neither BMS nor LMS had any effect compared with NMS. The literature is not consistent as some studies on prolonged maternal separation describe the same lack of effect on anxiety-related behavior (Pawluski et al. 2009c; Bolukbas et al. 2020), whereas other studies indicate higher anxiety in prolonged separated mothers (Maghami et al. 2018; Bousalham 2013; Aguggia et al. 2013). Differences in maternal separation procedures, rat strain, and anxiety-testing methods could explain these discrepancies. For example, prolonged separated Sprague-Dawley mothers did not show altered anxiety-related behavior in the EPM (Bolukbas et al. 2020), whereas separated Wistar rats had increased anxiety-related behavior (Maghami et al. 2018; Bousalham 2013; Aguggia et al. 2013). Therefore, more research with consistent paradigms is needed to better understand the impact of maternal separation on anxiety-related behavior in lactating mothers.

When tested for passive stress-coping in the FST, which is indicative of a depressive-like phenotype ((Slattery and Cryan 2012), but also see (von Mucke-Heim et al. 2022)), LMS but not BMS dams showed a tendency towards increased floating behavior in the first 5 min. Such increased floating behavior in LMS is in line with previous studies (Maniam and Morris 2010; Boccia et al. 2007). The lack of effect in BMS mothers further demonstrates that brief separation from the pups may not be sufficient to increase passive stress-coping, which might be explained by the fact that BMS could represent a safer and more natural early rearing condition than LMS (Eklund et al. 2009). Interestingly, passive stress coping in the FST is influenced by brain OXT signaling as shown, e.g., by intranasal and *icv* administration of OXT in male and female rats (Khodagholi et al. 2022; Ji et al. 2016). Hence, altered OXT signaling in separated mothers could be involved in altered passive stress-coping behavior as well. However, there is no direct evidence for such an effect of OXT in separated rat mothers, yet.

Basal plasma CORT levels did not differ between groups, which contrasts with a study where daily repeated brief separation in Sprague-Dawley dams (Maniam and Morris 2010) or the total removal of the offspring reduced basal CORT levels (Ulrich-Lai et al. 2006; Leuner et al. 2007; Kalyani et al. 2017). However, I have not tested the stress response of the HPA axis, which might give us a different picture regarding group differences. In a recent study, restraint stress induced higher CORT levels in virgin females and in one day separated dams, but not in control lactating dams (Kalyani et al. 2017). Future research could shed more light on the impact of offspring separation on the mother's basal as well as stress-induced HPA axis response. Since CORT is produced by and released from the adrenal glands, I also assessed their relative weight as an increase is thought to be an indicator for chronic stress (Ulrich-Lai et al. 2006). However, I could not detect any differences between groups, which is in line with our CORT result, but in contrast to previous studies that show increased adrenal gland weight in prolonged separated dams (Maniam and Morris 2010; Eklund et al. 2009). The longer maternal separation procedures (two weeks versus one week) may account for the discrepancies. In fact, one week of separation might not be a sufficient stimulus, as it was shown that chronic stress over two weeks induce adrenal gland hyperplasia and hypertropia (Ulrich-Lai et al. 2006).

Neither the body weight of the mothers nor the overall weight of their litters was different at any timepoint measured. This confirms previous studies demonstrating that the separation itself has no impact on the mothers' body weight (Maniam and Morris 2010; Eklund et al. 2009). In addition, a daily 3-h lack of milk ingestion did not affect the pup

weight, suggesting that the responsiveness of BMS and LMS dams to the pups was not altered, which is in line with our data of unaltered ABN nursing (Fig. 14 b-d). As with many studies, the design of the current study is subject to limitations. A small number of rats used for behavioral analysis as higher numbers might have revealed a slightly stronger behavioral outcome. Another limitation is the type of behavioral tests: additional anxiety- and depressive-like behavioral tests could have given deeper information about the phenotypes of the separated mothers. Finally, the duration of maternal separation treatment days could have limited of some molecular and /or behavioral outcomes.

In conclusion, our findings confirm previous studies demonstrating that one week of daily prolonged separation from the pups has a significant impact on the mother's maternal behavior. Our results suggest a potential compensatory mechanism of increased OXT-R expression in maternal brain regions due to the limited pup contact, underlining the complicated involvement of the OXT system when it comes to a break of the maternal bond.

-Chapter 4-

Neurobiological traces of grief: examining the impact of offspring loss after birth on rat mother's brain and behavior in the first week postpartum

Authors' contribution:

Luisa Demarchi:	experimental design, performance of experiments, data analysis, first draft and revision of manuscript
Alice Sanson:	performance of experiments, revision of manuscript
Oliver Bosch:	experimental design, performance of experiments, funding acquisition, revision of manuscript

4.1 Abstract

The bond between a mother and her infant is one of the strongest social bonds found in mammals. Consequently, the loss of an infant has immense psychological and physiological effects on the caregiver. Despite the significance of this bereavement, only a few studies have investigated the neurobiological impact of offspring loss in mothers. In an approach to fill this gap, I studied in lactating rat dams the effects of losing all pups the day after giving birth on the mothers' brain and behavior. Specifically, rat mothers experienced 1-, 3-, or 6-days of total offspring loss. I analyzed the neuronal activity and oxytocin receptor (OXT-R) binding in the brain limbic and maternal network regions, as well as the stress response and stress-coping strategies. Following 1 day of separation, the mothers' neuronal activity increased in the limbic system resulting in a positive correlation between the prelimbic cortex and basolateral amygdala, while OXT-R binding was decreased in the central amygdala following up to 3 days of separation. At all three timepoints, plasma corticosterone concentrations did not differ either under basal conditions or following stressor exposure. Interestingly, after 6 days of offspring loss the mothers' passive stress-coping behavior significantly increased. Our results provide novel insight into the short-term neurobiological traces of grief, emphasizing the significant impact of offspring loss on the mothers' neuronal activity and brain oxytocin system, and paves new avenues for future research in this field.

4.2 Introduction

The maternal bond is the crucial component of a wide array of social behaviors that is observed in numerous mammalian species. Indeed, the bond between a mother and her offspring is essential for the well-being of both subjects. Attachment behavior is mediated by a complex interplay of neurobiological factors, including the oxytocin (OXT) system (Bosch and Young 2018; Pohl et al. 2019). OXT plays a major role in the maternal bond, but also in the regulation of stress and anxiety (Neumann and Landgraf 2012). OXT acts as a pivotal regulator of emotional responses, dampening anxiety and mitigating the reactivity to stressors, and simultaneously, OXT promotes and facilitates social bonding, underlining its impact on both animal models and human emotions (Donaldson and Young 2008; Jurek and Neumann 2018; Leng et al. 2008; Neumann 2008). Beyond its involvement in the

maternal bond, OXT wields its influence over a broad spectrum of behaviors, including maternal care and aggression (Bosch and Neumann 2012; Rilling and Young 2014). In the postpartum period, increased OXT signaling, both centrally and peripherally, stands as a compelling phenomenon. Studies conducted on rodent mothers have consistently demonstrated such increased signaling, highlighting the pivotal role of OXT in the intricate interplay of emotions, social interactions, and maternal care (Baracz et al. 2020; Ng et al. 2023; Valtcheva et al. 2023; Yukinaga et al. 2022).

Previous research on rodents has shown that repeated maternal separation can cause chronic stress in the offspring, thereby affecting their behavior and brain functions (for a review see: Nishi 2020). Importantly, repeated maternal separation paradigms were found to also affect the mother's maternal care, anxiety, and depressive-like behavior (for a comprehensive review see: Alves et al. 2020). For example, studies in lactating rats report that repeated maternal separation induces passive stress-coping in the FST, as indicated by increased immobility (Boccia et al. 2007; Maniam and Morris 2010), which is reminiscent of depressive-like behavior (Slattery and Cryan 2012). Furthermore, other studies found a similar outcome after 3 weeks (Rincon-Cortes and Grace 2021) and 4 weeks (Pawluski et al. 2009c) of permanent separation from the offspring. With respect to mother-offspring bond disruption and OXT system, repeated short maternal separation increases the OXT-immunoreactive cells in the PVN while a repeated prolonged separation reduces their number (Baracz et al. 2020). Moreover, both repeated short and prolonged maternal separation increase OXT-R binding in brain regions associated with stress and maternal behavior and induces behavioral alterations in the rat mother (Demarchi et al. 2023; Stamatakis et al. 2015).

Rats represent an excellent animal model for investigating mother-infant bond perturbations from the mother's perspective. However, the repeated maternal separation paradigm is insufficient to investigate the effect of a total disruption of the mother-infant bond, i.e., losing the offspring. In humans, it is very well known that dissolution of the mother-infant relationship is, in fact, one of the most potent events for grief-like reactions (Averill 1968). The grieving process is complex and includes emotional, cognitive, and behavioral responses (Arizmendi and O'Connor 2015). Losing a child is one of the most traumatic events an individual can experience (Hobson 1998), and the neurobiological mechanisms behind the grieving process are still mostly unknown. Thus, a novel approach is necessary. Based on our previous hypothesis (Demarchi et al. 2021) that rats could serve as a potential novel animal model to study the neurobiology underlying grief, here I aimed

to assess, for the first time, the impact of experiencing the total loss of the offspring on the mother's brain and emotionality within the first postpartum week. It needs to be beard in mind that the physiological and psychological effects of offspring loss on rat mothers may be influenced by several factors, including the age of the offspring at the time of separation or the length of separation.

Key regions involved in stress responses and the regulation of emotions are the prelimbic cortex (PL) and the amygdala. The PL is a prefrontal region (PFC) that plays a major role in higher-order cognitive functions such as decision-making (Capuzzo and Floresco 2020; Jobson et al. 2021), while the amygdala is a subcortical region well known for its role in the processing and modulation of emotional responses, including fear, aggression, and social behavior (Grossman et al. 2022; LeDoux 2000). In recent years, there has been growing interest in the potential role of the amygdala in the grieving process. For example, a study using functional magnetic resonance imaging (fMRI) found that individuals who had recently experienced the death of a loved one show increased activity in the amygdala in response to emotional stimuli compared to healthy controls (Fernandez-Alcantara et al. 2020). Previous studies in rodents have also suggested that the connectivity between PL and amygdala may be involved in regulating stress responses, and that connectivity dysfunctions are associated with various mental health disorders, including depression and anxiety (Alexandra Kredlow et al. 2022). Therefore, I hypothesized that separation from the offspring affects the mothers on multiple levels. In the present study, I removed all pups the day after delivery and compared 1-day, 3-day, and 6-day experiences of total offspring loss in lactating female Sprague-Dawley rats. I examined the offspring loss-mediated impact on neuronal activation and OXT-R binding in brain regions involved in emotionality and maternal behavior, as well as plasma CORT concentration, and stress-coping strategies in the FST.

4.3 Materials and Methods

4.3.1 **Animals**

Sprague-Dawley rats were obtained from Charles River Laboratories (Sulzfeld, Germany) and housed under standard laboratory conditions (12:12h light/dark cycle, lights on at 7 a.m., room temperature 22 °C ± 1°C, relative humidity 55 % ± 5 %) with access to standard rat chow (ssniff-Spezialdiäten GmbH, Soest, Germany) and water *ad libitum*. After 7 days of habituation, females were mated with sexually experienced male Sprague-Dawley rats in standard laboratory cages (Eurostandard Type IV, 60 cm x 40 cm x 20 cm) for 10 days. From potential pregnancy day 18 on, pregnant females were single housed for undisturbed delivery in observational cages (plexiglass, 38 cm x 22 cm x 35 cm). On lactation day 0 (LD0; delivery day), each litter was adjusted to 8 pups. All experiments were performed in accordance with the European Union Directive (2010/63/EU) and were approved by the Government of Unterfranken, Bavaria, Germany. According to the 3-R principles, all efforts were made to minimize the number of rats and their suffering.

4.3.2 **Offspring loss procedure**

Lactating rats were randomly assigned to either one of the unseparated control groups, which were divided in C+1, C+3, C+6 or the separated groups, divided in S+1, S+3, S+6 (indicating the length of separation). Separation from the litter occurred on the day after delivery (LD1) for the S+1, S+3, S+6 groups at 10.00 a.m. Following the separation, mothers were left undisturbed in their home cage for the remaining days. Control dams were kept single-housed together with their litter without experiencing any separation.

4.3.3 **Experimental schedule**

For a graphical overview of the Experiments A-B, see Fig. 16.

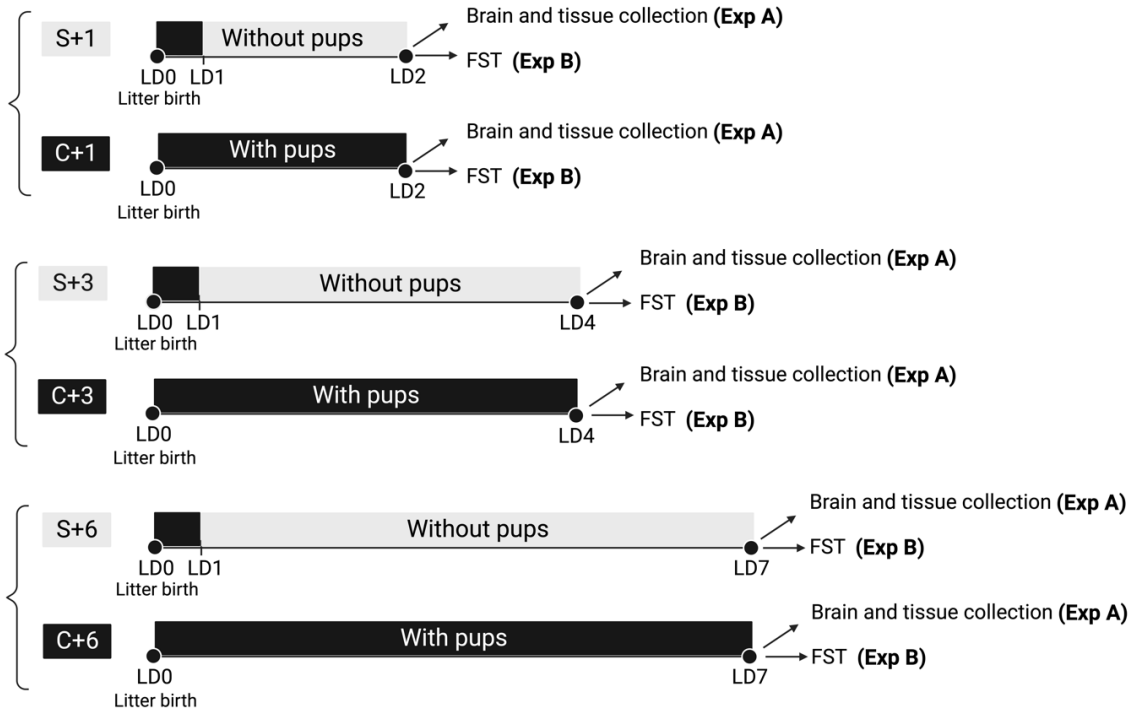


Fig. 16. Schematic timeline summarizing the experimental procedures.

4.3.4 **Experiment A**

Lactating rats of S+1, S+3, S+6 underwent the maternal separation protocol whereas the dams of the corresponding control groups C+1, C+3, C+6 remained with their pups in the home cage. Without further manipulations, blood and brains were collected at the time of sacrifice. Number or rats were: C+1 = 8; C+3 = 8; C+6 = 7; S+1 = 7; S+3 = 8; S+6 = 7.

4.3.5 **Experiment B**

A different cohort of rats was treated as described for Experiment A. Both control (C+1, C+3, C+6) and separated dams (S+1, S+3, S+6) were tested in the FST on the last day of separation, and directly afterwards trunk blood was collected for further analysis. Number or rats were: C+1 = 9; C+3 = 9; C+6 = 9; S+1 = 10; S+3 = 9; S+6 = 9.

4.3.6 **Immunofluorescence and image processing**

In experiment A, dams underwent cardiac perfusion with ice-cold 1x PBS. Brains were removed, flash-frozen in n-methylbutane, and stored at -20 °C until cutting into coronal sections of 16 µm using a cryostat (CM3050S Leica Microsystem GmbH, Nussloch, Germany). For each brain region of interest, i.e., PL, basolateral amygdala (BLA), CeA, medial amygdala (MeA), six coronal sections were collected on SUPERFROST microscope slides and stored at -20 °C until further processing. Slices were postfixed in 4% PFA for 30 min, washed in 1x PBS, and blocked with 5% normal goat serum (Vector Laboratories, Newark, USA) with 0.3% Triton X 100 to permeabilize the membrane. The slides were then incubated overnight at 4 °C with primary antibodies (Abcam, rabbit anti c-Fos 1:2,000; Merck, mouse anti-NeuN 1:1,000). The next day, slides were washed in 1x PBS and went through 1 h incubation with secondary antibodies (Alexa Fluor 488-conjugated anti-rabbit IgG and Alexa Fluor 594-conjugated anti-mouse IgG 1:800) at room temperature. The slides were finally mounted (ROTI®Mount FluorCare DAPI).
For image analysis, all stained images of the MeA, CeA, BLA, and PL were acquired using a Thunder Leica microscope (Leica Thunder DM6 B, Camera Leica DFC9000 GT). For the PL acquisition, superficial layers (II/III) and deep layers (V/VI) were separately captured. A total of 6 images per region and rat were taken at 20X magnification. Images were analyzed manually using ImageJ (Schneider et al. 2012) by an experimenter blind to the treatment conditions. The nuclei of the overall cells marked with DAPI, the nuclei of mature neurons marked with NeuN, and the cells marked with c-Fos were counted. The percentage of c-Fos co-labeled neurons were calculated for each region and rat.

4.3.7 **OXT-R autoradiography and image processing**

Brains sampled in Experiment A were also analyzed for OXT-R binding. For each brain region of interest, i.e., bed nucleus of the stria terminalis (BNST), nucleus accumbens shell (NAcc), medial preoptic area (MPOA), accessory olfactory nuclei (AOB), central amygdala (CeA), agranular insular cortex (AIP), ventral medial hypothalamus (VMH), lateral septum dorsal (dLS) and ventral (vLS), PL, six coronal sections of 16 µm per rat were collected on SUPERFROST microscope slides and stored at -20 °C until further processing. The OXT-R autoradiography was performed following an established protocol

(Bosch and Neumann 2008; Oliveira et al. 2021). Briefly, the ornithin-vasotocin analogue [^{125}I]-OVTA ([d(CH_2)$_5$[Tyr(Me)^2,Thr^4,Orn^8,[^{125}I]Tyr^9-NH_2]; Perkin Elmer, USA) was used as tracer. First, the slides were thawed and allowed to dry thoroughly at room temperature. The tissue was shortly fixed via 0.1 % PFA, washed 2 x in Tris (50 mM, pH 7.4), covered with the tracer solution (50 mM tracer, 10 mM $MgCl_2$, 0.1 % BSA) for 60 min, washed 3 x in Tris / $MgCl_2$ buffer for 7 min, each, followed by 30-min spinning in Tris / $MgCl_2$. Finally, slides were dipped in water and air dried before being exposed to Biomax MR films (Kodak, Cedex, France) for 15 days. The films were scanned using the EPSON Perfection V800 Scanner (Epson GmbH, Munich, Germany), and the optical density of each region of interest was analyzed by an experimenter blind to the experimental conditions using ImageJ (Schneider et al. 2012) by subtracting the background activity as previously described (Baskin and Stahl 1993). The analyses were performed simultaneously for 6 slices per rat and per region.

4.3.8 **Blood collection and plasma CORT measurement**

Experiment A: Between 10.00 a.m. and 12.00 p.m., dams were transported in their home cage to a separate room, flash-anesthetized with isoflurane (in preparation for perfusion; see above), the thorax opened, and blood was collected immediately from the right atrium of the heart in EDTA-coated tubes (0.5 M, pH 7.4; Sarstedt, Numbrecht, Germany) on ice.
Experiment B: After termination of the 10-min FST (see below), rats were rapidly sacrificed and trunk blood was collected in EDTA-coated tubes (0.5 M, pH 7.4; Sarstedt, Numbrecht, Germany) on ice.
Quantification of plasma CORT concentration was performed using enzyme-linked immunosorbent assay (ELISA). Blood samples were centrifuged at 4 °C (10,000 rpm, 10 min), aliquoted and stored at −20 °C until the assay was performed using a commercially available ELISA kit (Tecan IBL International GmbH, Hamburg, Germany) following the manufacturer's protocol.

4.3.9 **Forced swim test (FST)**

In experiment B, passive stress-coping behavior was tested in the FST (Ebner et al. 2005; Slattery and Cryan 2012). Between 9.00 a.m. and 12.00 p.m., rats were placed for 10 min in a cylindrical tank (50 cm high, 30 cm diameter) filled with tap water (23 ± 1 °C) to a depth that rats could not touch the bottom with their hind paws or tail. Trials were recorded for later analysis using JWatcher (https://www.jwatcher.ucla.edu) by an experimenter blind to the treatment. The total time spent on floating (passive stress-coping, indicative of depressive-like behavior (Slattery and Cryan 2012)) was analyzed.

4.3.10 **Statistical analysis**

All statistical analyses were performed with GraphPad PRISM 9 (GraphPad Software, San Diego, USA). Normality and homoscedasticity were verified (Shapiro-Wilk or Kolmogorov–Smirnov test and Brown-Forsythe test, respectively). Analysis of outliers was run via the ROUT method. Plasma CORT concentration, OXT-R binding, immunoreactive cells and passive stress-coping behavior were analyzed with a two-tailed unpaired Student's t test. Graphical data illustration was created to give a better overview between the different loss experiences, but the statistical analysis was performed within groups since the effect of different times of infant loss was not the scope of this study. The correlation of neuronal activation between different brain regions was additionally analyzed with Pearson' *r* correlation. Data are presented as mean + SEM; $p \leq .05$ was considered significant.

4.4 Results

4.4.1 Experiment A

4.4.1.1 Neuronal activity of PL superficial and deep layers was upregulated by 1-day offspring loss experience

To assess whether the offspring loss experience affects the basal neuronal activity in the superficial layers of the PL, I calculated the percentage of c-Fos co-labeling with NeuN cells (Fig. 17a). As shown in Fig.17b, S+1 dams revealed statistically significant higher % c-Fos / NeuN in the PL layers II/III (M = 4.903, SD = 2.346) than the control group C+1 [(M = 0.828, SD = 0.327), t (13) = 4.884, p = .0003]. Both S+3 (M = 4.646, SD = 1.334) and S+6 dams (M = 1.330, SD = 0.5760) did not differ in % c-Fos / NeuN from their respective controls, i.e., C+3 [(M = 2.279, SD = 1.490), t (10) = 1.829, p = .097] and C+6 [(M = 1.923, SD = 1.214), t (12) = 1.167, p = .266].

With respect to the basal neuronal activity in the deep layers of the PL (Fig. 18a), S+1 dams revealed statistically significant higher % c-Fos / NeuN in the PL layers V/VI (M = 5.833, SD = 2.042) than the control group C+1 [(M = 2.331, SD = 0.6116), t (13) = 4.641, p = .0005] (Fig. 18b). Both S+3 (M = 5.520, SD = 3.739) and S+6 dams (M = 1.831, SD = 0.7466) did not differ in % c-Fos / NeuN from their respective controls, i.e., C+3 [(M = 4.013, SD = 2.117), t (10) = 0.8945, p = .392] and C+6 [(M = 2.316, SD = 1.569), t (12) = 0.7375, p = .475)].

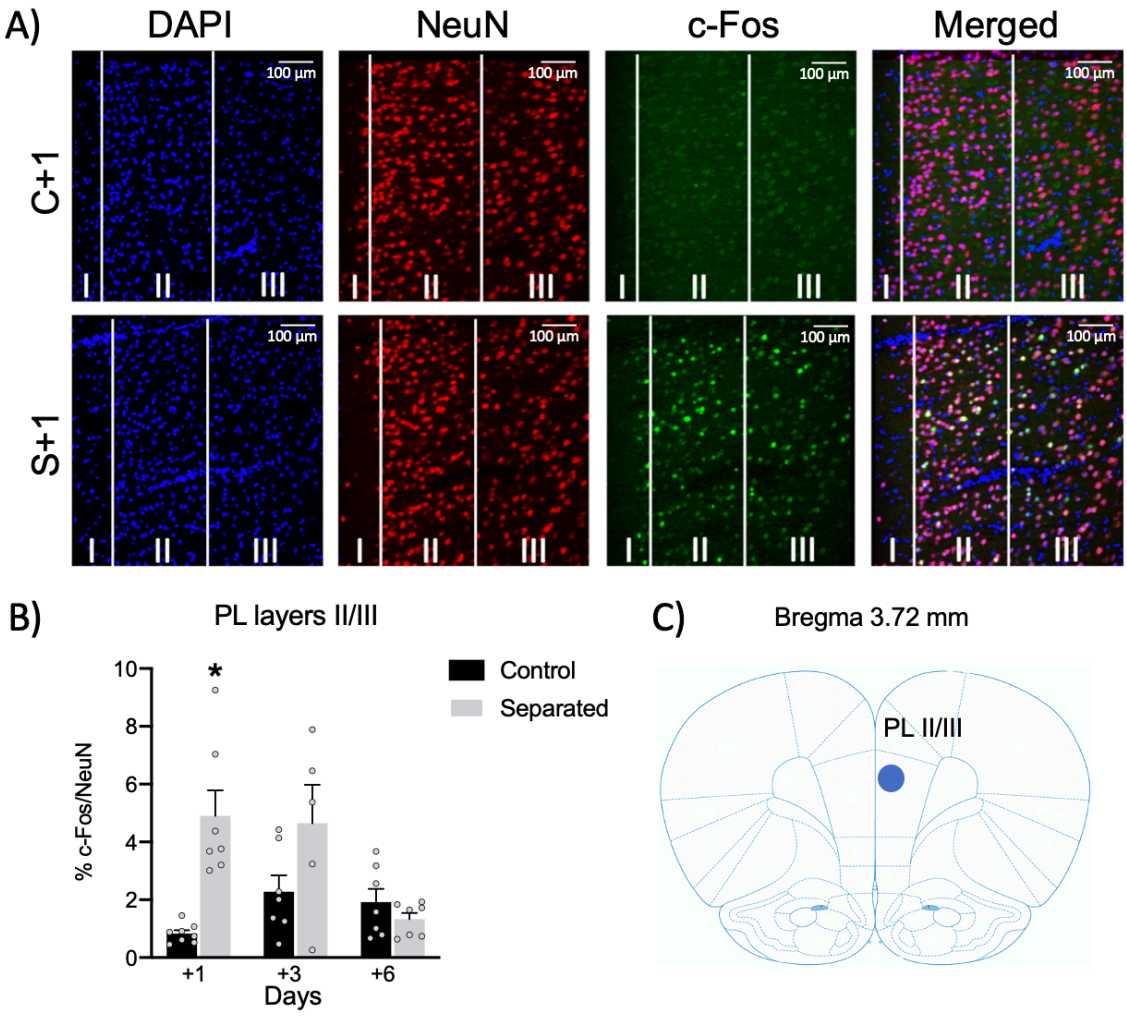


Fig. 17. Offspring loss activated neurons in the superficial layers of the prelimbic cortex (PL) in control and separated rat mothers. (A) Fluorescent images showing DAPI (blue), NeuN (red), and c-Fos immunopositive cells (green) in the I, II and III layers of the PL comparing control (C+1) and separated dams (S+1) (scale bar = 100 µm). (B) Percentage of neurons co-labeling with c-Fos following 1-, 3- or 6-days of separation from the offspring. (C) Coronal slice of rat brain showing the ROI analyzed (blue dot) for the superficial layers II/III in the PL (illustration adapted from (Paxinos 2007)). Two-tailed unpaired Student's t test. Results are expressed as mean + SEM. * $p \leq .05$ versus control at the same timepoint.

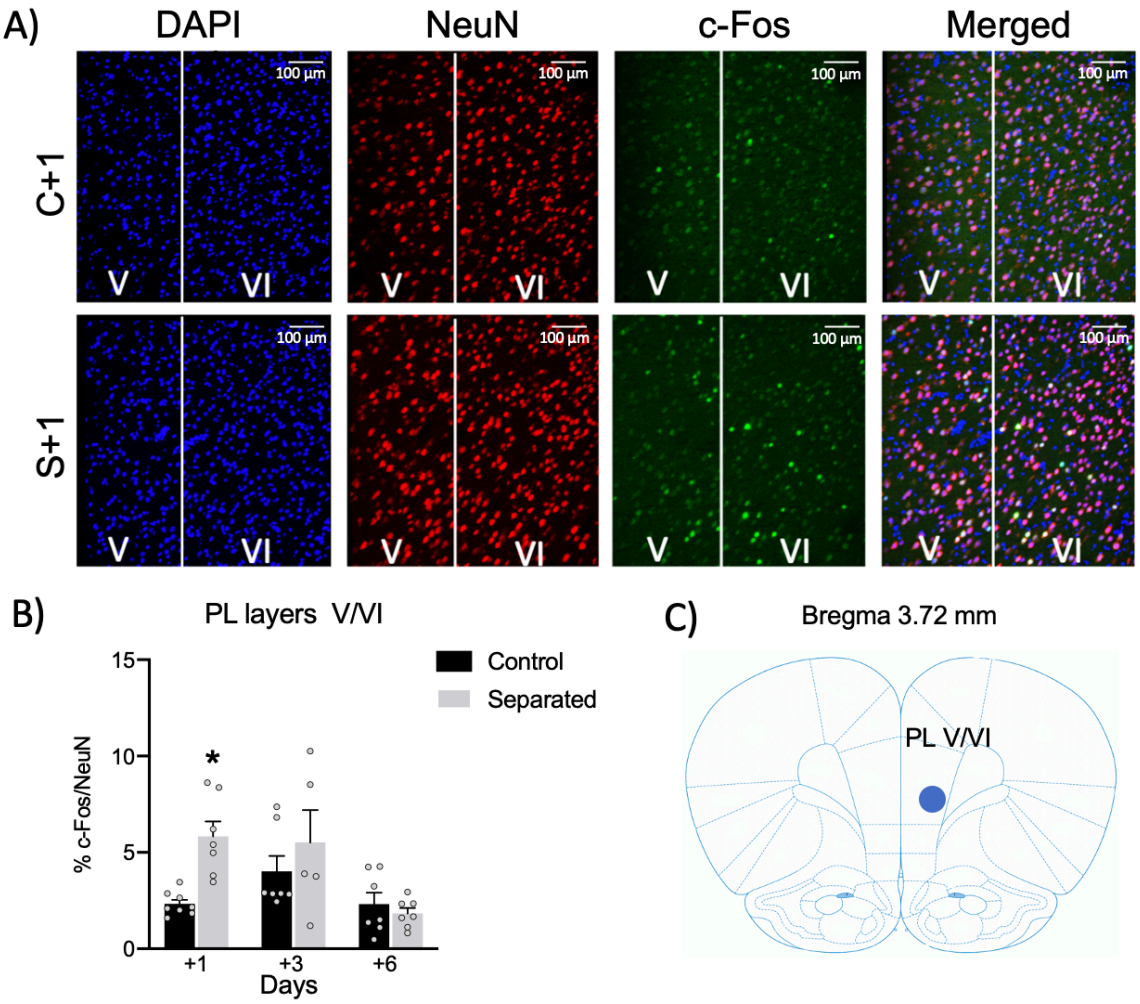


Fig. 18. Offspring loss activated neurons in the deep layers of the prelimbic cortex (PL) in control and separated rat mothers. (A) Fluorescent images showing DAPI (blue), NeuN (red), and c-Fos immunopositive cells (green) in the V and VI layers of the PL comparing control (C+1) and separated dams (S+1) (scale bar = 100 µm). (B) Percentage of neurons co-labeling with c-Fos following 1-, 3- or 6-days of separation from the offspring. (C) Coronal slice of rat brain showing the ROI analyzed (blue dot) for the deep layers V/VI in the PL (illustration adapted from (Paxinos 2007)). Two-tailed unpaired Student's t test. Results are expressed as mean + SEM. * $p \leq .05$ versus control at the same timepoint.

4.4.1.2 **Neuronal activity of BLA, but not of CeA or MeA, was upregulated by 1-day offspring loss experience**

I next evaluated the basal neuronal activity in the BLA, CeA and MeA (Fig. 19a). In the BLA, S+1 dams revealed statistically significant higher % c-Fos/ NeuN (M = 0.5867, SD = 0.1451) than the control group C+1 [(M = 0.1200, SD = 0.1095), t (9) = 5.904, p = .0002] (Fig. 19b). Both S+3 (M = 0.4833, SD = 0.4579) and S+6 dams (M = 0.1800, SD = 0.1304) did not differ in % c-Fos / NeuN from their respective controls, i.e., C+3 [(M = 0.3833, SD = 0.2994), t (10) = 0.4477, p = .664] and C+6 [(M = 0.3833, SD = 0.4262), t (9) = 1.020, p = .335] (Fig. 19b).

The % c-Fos / NeuN was not altered by any separation length in both the CeA and MeA (data not shown) [**CeA:** C+1 (M = 1.100, SD = 0.3742), S+1 (M = 1.183, SD = 0.8704), t (9) = 0.1980, p = .848; C+3 (M = 1.183, SD = 0.6145), S+3 (M = 0.6000, SD = 0.7071), t (10) = 1.525, p = .158; C+6 (M = 1.450, SD = 1.126), S+6 (M = 0.4000, SD = 0.4123), t (9) = 1.964, p = .081. **MeA:** C+1 (M = 1.010, SD = 0.4227), S+1 (M = 1.165, SD = 0.4295), t (9) = 0.6002, p = .563; C+3 (M = 1.617, SD = 1.363) S+3 (M = 1.437, SD = 1.171), t (10) = 0.2453, p = .811; C+6 (M = 0.6767, SD = 0.3543), S+6 (M = 0.8960, SD = 0.7049), t (9) = 0.6720, p = .519].

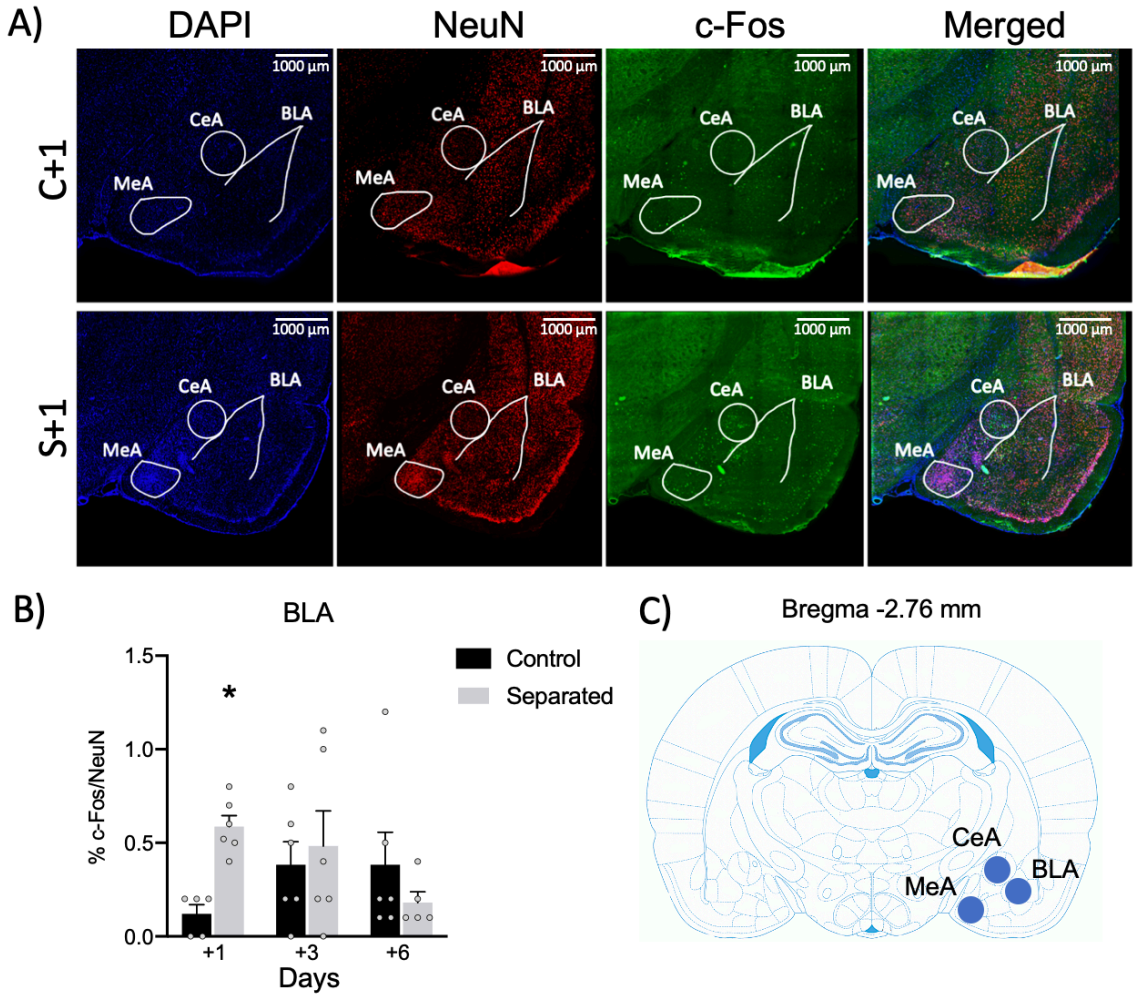


Fig. 19. Offspring loss increased neuronal activity in the basolateral (BLA), but not central (CeA) and medial (MeA), amygdala in control and separated rat mothers. (A) Fluorescent images showing DAPI (blue), NeuN (red), and c-Fos immunopositive cells (green) in the amygdala subnuclei (BLA; CeA; MeA) comparing control (C+1) and separated dams (S+1) (scale bar = 1,000 µm). (B) Percentage of neurons co-labeling with c-Fos in the BLA following 1-, 3- or 6-days of separation from the offspring. (C) Coronal slice of rat brain showing the ROIs analyzed (blue dots) in the BLA, CeA and MeA (illustration adapted from (Paxinos 2007)). Two-tailed unpaired Student's t test. Results are expressed as mean + SEM. * $p \leq .05$ versus control at the same timepoint.

4.4.1.3 **Offspring loss induced a positive correlation in the neuronal activity between PL and BLA**

To deepen our analysis, a Pearson correlation coefficient was computed to assess the linear relationship of the percentage of c-Fos co-labeling with NeuN between the PL and the BLA (Fig. 20). Indeed, the separated groups (S+1, S+3, S+6) showed a positive correlation between the two brain regions (r = 0.7783, p = .0006), whereas the control groups (C+1, C+3, C+6) had a negative correlation (r = -0.4834, p = .049) (Fig. 20b).

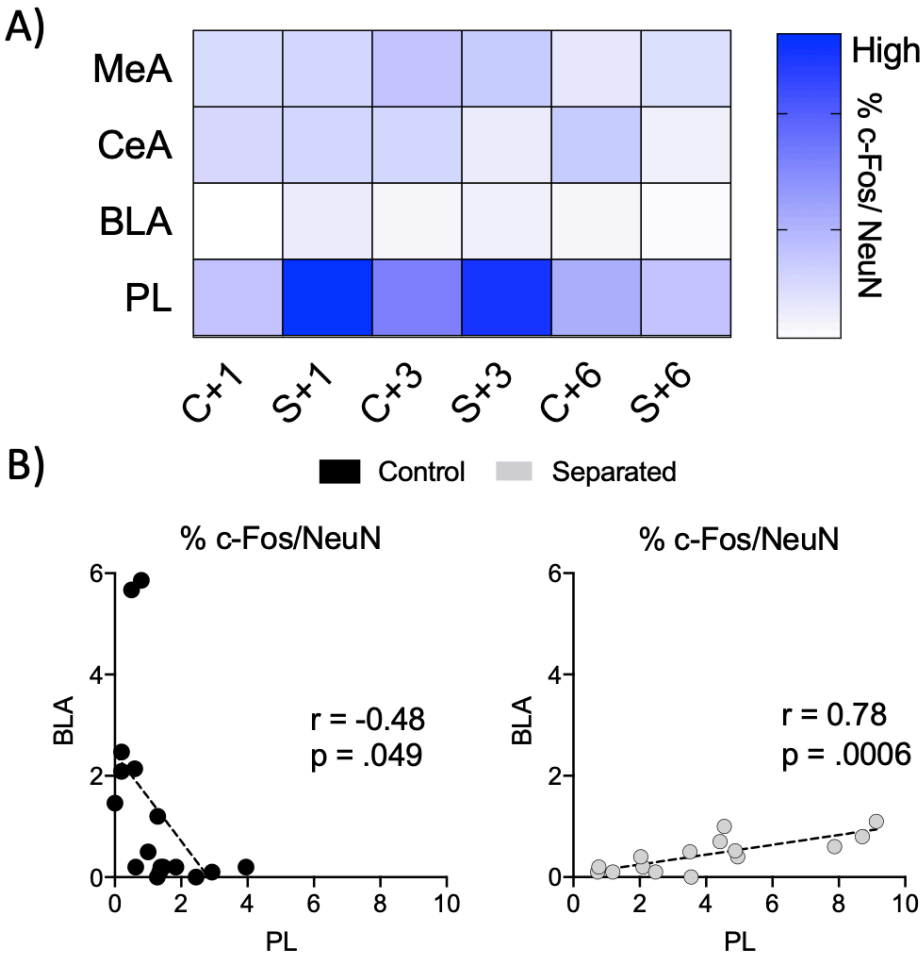


Fig. 20. Neuronal activation pattern in limbic regions of control and separated rat mothers. (A) Overview heatmap of percentage of activated neurons in the medial (MeA), central (CeA) and basolateral amygdala (BLA) as well as prelimbic cortex (PL) in control (C) and separated (S) rat mothers. (B) Correlation between the BLA and PL neuronal activity as shown in % c-Fos / NeuN in control (left graph) and separated groups (right graph). Pearson r linear correlation.

4.4.1.4 **Decreased OXT-R binding in the CeA, only, after 1- and 3-day offspring loss experience**

To evaluate if offspring loss experience could affect the OXT system, I analyzed the OXT-R binding in brain regions involved in emotionality and maternal behavior (Fig. 21; Tab.4). Both S+1 (M = 0.1616, SD = 0.0565) and S+3 dams (M = 0.1671, SD = 0.0406) revealed statistically significant lower OXT-R binding in the CeA compared to their corresponding controls, i.e., C+1 [(M = 0.2334, SD = 0.0569), t (10) = 2.163, p = .0558] and C+3 [(M = 0.2417, SD = 0.0605), t (11) = 2.645, p = .023] (Fig. 21a-b). However, OXT-R binding in the CeA of S+6 dams (M = 0.1354, SD = 0.0608) was not different from the control group C+6 [(M = 0.1987, SD = 0.0689), t (12) = 1.821, p = .094] (Fig. 21). No differences in OXT-R binding were found in the other regions analyzed between separated and control groups (see Tab. 4).

Table 4. Schematic table summarizing the OXT-R binding statistical p values across groups and regions as plotted in Figure 6a.

	BNST	**NAcc**	**MPOA**	**AOB**	**AIP**	**VMH**	**dLS**	**vLS**	**PL**
C+1 vs S+1	p = .46	p = .99	p = .28	p = .29	p = .54	p = .44	p = .08	p = .08	p = .99
C+3 vs S+3	p = .84	p = .95	p = .87	p = .08	p = .30	p = .79	p = .29	p = .72	p = .94
C+6 vs S+6	p = .47	p = .93	p = .77	p = .43	p = .11	p = .30	p = .53	p = .48	p = .74

Abbreviations: Agranular insular cortex (AIP), accessory olfactory nuclei (AOB), bed nucleus of the stria terminalis (BNST), central amygdala (CeA), lateral septum dorsal (dLS) and ventral (vLS), medial preoptic area (MPOA), nucleus accumbens shell (NAcc), prelimbic cortex (PL), ventral medial hypothalamus (VMH).

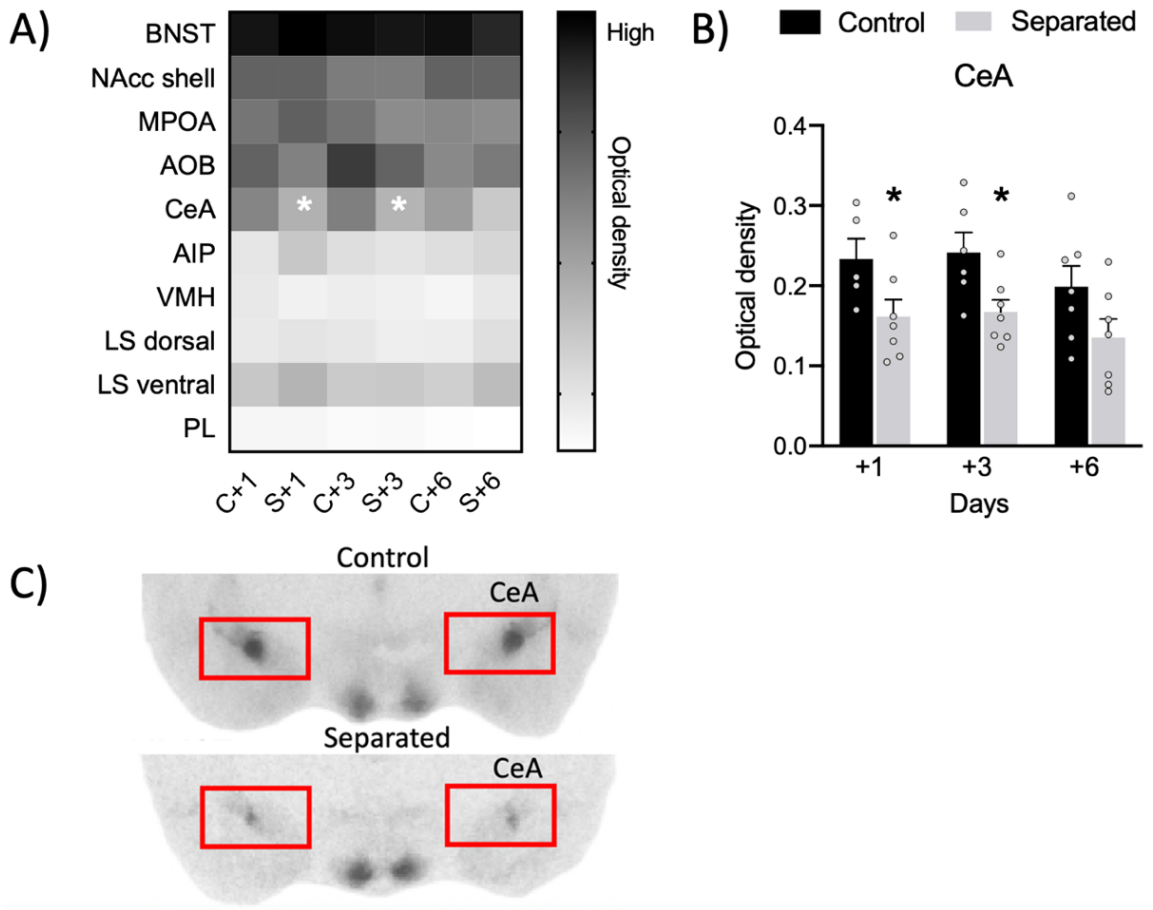


Fig. 21. Oxytocin receptor (OXT-R) binding in brain regions of the limbic system and maternal network in control and separated rat mothers. (A) Overview heatmap of gray density (arbitrary units) for OXT-R binding in the analyzed brain areas. (B) Optical density (arbitrary units) for OXT-R binding in the CeA following 1-, 3- or 6-days of separation from the offspring. (C) Representative coronal brain sections of the CeA demonstrating differences in OXT-R binding. Abbreviations: Agranular insular cortex (AIP), accessory olfactory nuclei (AOB), bed nucleus of the stria terminalis (BNST), central amygdala (CeA), lateral septum dorsal (dLS) and ventral (vLS), medial preoptic area (MPOA), nucleus accumbens shell (NAcc), prelimbic cortex (PL), ventral medial hypothalamus (VMH). Two-tailed unpaired Student's t test. Results are expressed as mean + SEM. * $p \leq$ .05 versus control at the same timepoint.

4.4.1.5 **Basal plasma CORT concentration was unaffected by offspring loss**

I next determined if offspring loss could affect basal CORT concentrations. In fact, none of the loss protocols affected basal plasma CORT concentrations (S+1: $M = 169.0$, $SD = 126.2$; C+1: $M = 392.2$, $SD = 316.8$, $t\ (13) = 1.741$, $p = .105$; S+3: $M = 282.3$, $SD = 152.3$; C+3: $M = 461$, $SD = 318.4$, $t\ (14) = 1.432$, $p = .174$; S+6: $M = 388.6$, $SD = 391$; C+6: $M = 419.3$, $SD = 321.6$, $t\ (12) = 0.1604$, $p = .875$) (Fig. 22a).

4.4.2 **Experiment B**

4.4.2.1 **Plasma CORT concentrations after acute stress were unaffected by offspring loss**

In addition to basal plasma CORT concentrations obtained in experiment A, I determined if offspring loss experience could affect stress-induced plasma CORT concentration (Fig. 22b). However, I did not find any significant differences between the groups [S+1: $M = 1017$, $SD = 453.2$; C+1: $M = 1041$, $SD = 346$, $t\ (16) = 0.1248$, $p = .902$; S+3: $M = 1775$, $SD = 510.9$; C+3: $M = 1408$, $SD = 402.8$, $t\ (16) = 1.692$, $p = .110$; S+6: $M = 1365$, $SD = 310.3$; C+6: $M = 1365$, $SD = 611.2$, $t\ (15) = 0.0005$, $p = .999$].

4.4.2.1 **Increased passive stress-coping following 6-days offspring loss experience**

To assess the impact of offspring loss on the dam's emotionality, I analyzed the percentage of time spent floating during the FST (Fig. 22c). In fact, with prolonged separation passive stress-coping increased, i.e., S+6 dams showed significantly more percentage of time floating ($M = 36.40$, $SD = 15.52$) compared to the control group C+6 [($M = 20.48$, $SD = 12.26$), $t\ (15) = 2.325$, $p = .035$]. However, neither S+1 ($M = 21.29$, $SD = 12.81$) nor S+3 ($M = 21.03$, $SD = 13.13$) differed from their corresponding controls C+1 [($M = 27.30$, $SD = 20.99$), $t\ (16) = 0.7338$, $p = .474$] and C+3 [($M = 22.28$, $SD = 12.67$), $t\ (16) = 0.2061$, $p = .839$].

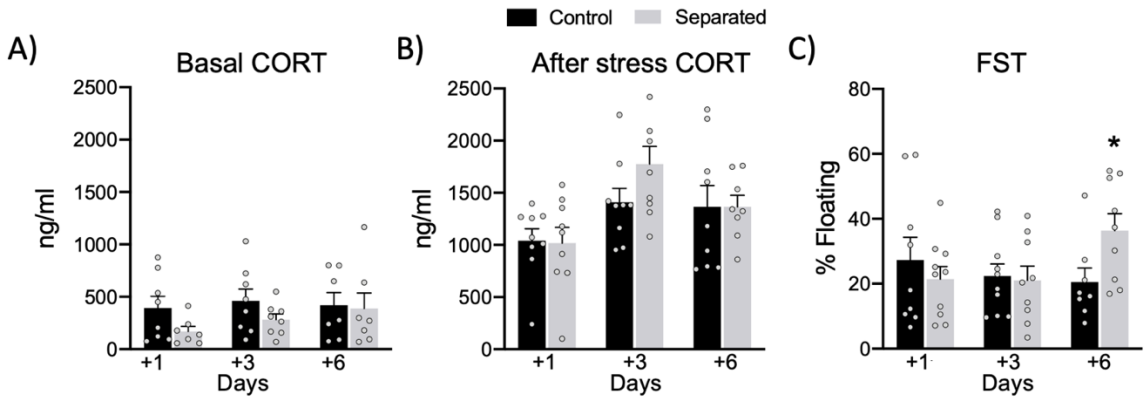


Fig. 22. Basal and stress-induced plasma corticosterone (CORT) concentration and passive stress-coping behavior in control and separated rat mothers. Plasma concentration of CORT under (A) basal conditions and (B) after acute exposure to the forced swim test (FST) following 1-, 3- or 6-days of separation from the offspring as well as (C) their percentage of floating during the 10-min FST. Two-tailed unpaired Student's t test. Results are expressed as mean + SEM. * $p \leq .05$ versus control at the same timepoint.

4.5 Discussion

The neurobiology of grief in general, and, more specifically, of losing the young have only been sparsely investigated to this date. This is rather surprising given the devastating and often life-long lasting negative impact on the orphaned parents. To shed more light on the underlying neurobiological mechanisms of offspring loss, I characterized the neuronal activity and OXT-R binding in the brains of recently orphaned rat dams, thereby providing insights into the potential neurobiological mechanisms through which infant loss influences both brain function and behavior. The findings indicate that experiencing offspring loss caused heightened basal neuronal activation after 1-day offspring loss specifically in the PL and BLA, which even correlated positively. Furthermore, OXT-R binding was decreased in the CeA, only, after 1- and 3-day offspring loss. Additionally, with growing duration of offspring loss, i.e., after 6-days the increase in passive stress-coping became inevitable in orphaned mothers.

Increased basal neuronal activity positively correlated between PL and BLA after 1-day offspring loss

Our study yielded significant impact of offspring loss on basal neuronal activity in specific brain regions. Already one day of offspring loss led to increased basal neuronal activity of both the superficial (Fig. 17) and deep layers (Fig. 18) of the PL. At the same time, the neuronal activity in the BLA was upregulated (Fig. 19). Notably, I observed a positive linear relationship between the neuronal activities of PL and BLA in the separated mothers, while a negative correlation was evident in the control mothers (Fig. 20). These findings suggest that the experience of offspring loss, ranging from one day up to six days, significantly modifies the basal neuronal activity of limbic regions in the mother's brain. Previous research has highlighted the involvement of the PL and BLA in modulating stress response and emotions in rodents (Barfield et al. 2020; Grossman et al. 2022; Lidhar et al. 2021; Sharp 2017). Interestingly, a recent study demonstrated that human mothers who experienced the loss of their child exhibited increased activity in the ACC (Kark et al. 2022), which is the analogous region to the PL in rodents (VanElzakker et al. 2014).

In support of a potential role of the PFC in offspring loss, this brain area is implicated in pup-directed maternal behavior. For example, inhibition of neurons in the medial PFC reduces maternal behavior in rats, particularly pup retrieval behavior (Febo et al. 2010). Moreover, increased neuronal activity can be observed in the infralimbic area of the medial PFC in maternal rats that developed a place preference for the pup-paired context (Mattson and Morrell 2005). Hence, those studies not only indicate the involvement of the medial PFC in the maternal brain but, furthermore, support our findings of a contribution to the effects of offspring loss on PL activity.

Strengthening our results, the amygdala exhibits reciprocal connections with the PFC, and studies in rats have explored their functional interactions in mediating reinforcement-based and emotional behaviors (Hintiryan et al. 2021; Saddoris et al. 2005; Schoenbaum et al. 2003). Furthermore, these structures mediate aspects of emotional processing, including emotional valence and intensity (Salzman and Fusi 2010). In a human study, researchers explored the neuronal mechanisms involved in grief regulation, focusing on PFC and amygdala brain activity (Chen et al. 2020a; Gundel et al. 2003). Their findings support a model suggesting that the amygdala-prefrontal connectivity reflects variations in how individuals cope with and regulate attention and sadness during intense grief (Freed et al. 2009). Another recent study on grieving participants highlights the role of amygdala-based

brain networks in explaining grief symptoms (Chen et al. 2020a). Considering the above-mentioned human studies, our data hold a potential translational value, proposing for the first time the use of rats as a suitable model to explore the rather immediate effects of infant loss on brain activity.

I must acknowledge that the use of c-Fos as an indirect measure of neuronal activity does not directly capture neuronal firing and can be influenced by other neuronal events, such as plasticity (Kovacs 2008; Minatohara et al. 2015). Furthermore, while c-Fos provides spatial information, its temporal resolution is relatively low, which can be significant in complex social situations that unfold over an extended period. Additionally, although I sampled c-Fos from four regions covering a substantial portion of the major limbic brain areas, it represents only a fraction of the entire limbic system. Therefore, our data provide a partial understanding of the neuronal response in rats, and these findings should be interpreted considering this limitation. Nonetheless, it is encouraging that the PL and BLA regions emerged as key areas in offspring-loss impact, which is in line with studies in humans.

Reduced OXT-R binding in the CeA after 1- and 3-day offspring loss

The brain OXT system plays a central role in social bonding in general (Blumenthal and Young 2023; Bosch and Young 2018) and after breaking a bond in particular (Bales and Rogers 2022; Pohl et al. 2019; Vitale and Smith 2022). Therefore, we speculated that offspring loss might interfere with OXT-R binding in the mothers' brain regions involved in emotionality and maternal behavior after offspring loss. We found OXT-R binding across all examined regions, including those that were previously studied in the context of an involvement of OXT in emotionality, i.e., BNST, PL and LS (Duque-Wilckens et al. 2018; He et al. 2019; Huang et al. 2020; Menon et al. 2018; Sabihi et al. 2014a). Furthermore, regarding an involvement of OXT in maternal bonding the NAcc shell, MPOA and AOB have been studied (D'Cunha et al. 2011; Klampfl et al. 2018; Pedersen et al. 1994; Yu et al. 1996), whereas other regions such as the AIP and VMH remain unexplored in the context of OXT and maternal bond (Valtcheva and Froemke 2019). For instance, the activity of OXT-R in the NAcc shell has been found essential to consolidate the maternal memory of postpartum lactating rats (D'Cunha et al. 2011); while OXT was found to promote maternal care both in the MPOA and AOB (D'Cunha et al. 2011; Klampfl et al. 2018; Pedersen et al. 1994; Yu et al. 1996).

Among the brain regions analyzed, only the CeA was significantly affected, with decreased OXT-R binding (Fig. 21) following 1- and 3-days of offspring loss. This suggests a potential role of the CeA in the context of offspring-loss, which was restored on the 6th day. Assuming that offspring loss is a stressful event for the mother, the amygdala is of certain interest; it plays a critical role in processing behavioral and neuroendocrine stress responses thereby involving the OXT system. Indeed, OXT-R and oxytocinergic fibers are abundant in the CeA, suggesting that locally released OXT may function as a potential modulator of the complex stress response (Bale et al. 2001; Knobloch et al. 2012; Neumann et al. 2000a; Sobota et al. 2015). Ebner and colleagues showed that an acute swim stressor leads to OXT release in the CeA and increased floating (Ebner et al. 2005) while Peters and colleagues showed that chronically released OXT results in less OXT-R binding in the CeA (Peters et al. 2014). Based on those findings, it is reasonable to suggest that offspring loss, as an emotionally stressful event, could trigger chronic OXT release in the CeA during the initial three days of the loss, leading to a compensatory decrease in OXT-R binding as seen after 6 days, paralleled by increased floating. In future studies, it would be important to also measure the local OXT release in the CeA.

Increased passive stress-coping behavior after 6-day offspring loss but no effect on plasma CORT concentrations

The findings revealed that 6-day offspring loss increased the mothers' passive stress-coping behavior in the FST, indicative of depressive-like behavior (Slattery and Cryan 2012) (Fig. 22c). This finding is in line with studies where rat mothers, which had pups removed within one day after birth, show increased floating in the FST even after 3 weeks (Rincon-Cortes and Grace 2021) and 4 weeks following the separation (Pawluski et al. 2009c). Moreover, studies using the repeated maternal separation protocol over the first two weeks postpartum describe increased passive stress-coping behavior in the FST (Boccia et al. 2007) and decreased sucrose preference (Maniam and Morris 2010). The altered emotionality might be triggered by dysfunctional OXT signaling. In fact, OXT signaling has been proven to influence passive stress-coping behaviors, as demonstrated in studies involving the administration of OXT agonists via intranasal or icv routes in both male and female rats (Ji et al. 2016; Khodagholi et al. 2022). Based on these results in addition to our findings of increased OXT-R binding in the CeA after offspring loss, I speculate that altered OXT signaling in separated dams is one of the factors contributing to their increased passive

stress-coping behavior. Moreover, the increased neuronal activity in PL and BLA after offspring loss, which even correlated positively, has been shown in various rodent studies to potentially trigger heightened passive stress-coping behavior (Becker et al. 2023; Grossman et al. 2022; Hare and Duman 2020). Therefore, it is plausible that the altered neuronal activity in these regions is directly involved in the increased emotionality of separated dams.

In human patients suffering from depression and grief symptoms, dysregulations of the HPA axis, in particular of increased basal cortisol concentrations, have been described (Mason and Duffy 2019; Roy et al. 1988). Similarly, repeated CORT injections in animal models are related to depressive-like behaviors (Johnson et al. 2006). Thus, I analyzed basal and post-stressor (FST) plasma CORT samples to understand if offspring loss could alter the mother's stress response. Surprisingly, there were no differences between the groups within the basal or post-stressor plasma CORT levels (Fig. 22a, b). This is in contrast to previous studies in Sprague-Dawley dams demonstrating that basal plasma CORT concentration is reduced following 1-day of offspring loss (Kalyani et al. 2017) and increases post-stress following 1-day and 8-day offspring loss (Pawluski et al. 2009b). The discrepancies between those studies and the present results may be due to different experimental protocols regarding the method and time of blood collection, and the timing of pup removal. For example, Pawluski et al. (Pawluski et al. 2009b) analyzed plasma CORT collected from tail nicks between 7.30 a.m. and 9.00 a.m. while I collected trunk blood between 9.00 a.m. and 12.00 p.m. However, Kalyani et al. (Kalyani et al. 2017) analyzed trunk blood samples between 7.30 a.m. and 9.30 a.m., but the pup removal happened on LD3, while in the present study the offspring were removed on LD1. In fact, natural diurnal variations occur in the CORT plasma level that might explain those discrepancies (McCarthy et al. 1960). Moreover, different technics of blood collection might induce higher stress which could affect physiological markers (Kim et al. 2018).

4.6 Conclusions and future directions

In conclusion, I provide insights into the rather short-term neuronal and behavioral consequences of total offspring loss in lactating rat mothers. I found that 1-day of offspring loss resulted in heightened - and positively correlating - basal neuronal activation in the PL and BLA. Furthermore, 1- and 3-days of offspring loss led to a reduction in OXT-R binding

in the CeA. After 6-days of offspring loss, passive stress-coping behavior was significantly increased. These findings suggest that the neuronal processes underlying maternal adaptation and stress-coping mechanisms are negatively influenced by the loss of offspring. The identified changes in neuronal activity and OXT-R binding highlight the involvement of specific brain regions, such as the PL, BLA, and CeA. These regions have been implicated in stress regulation, emotional processing, and parental care in previous studies, both in rodents and humans. The convergence of findings across species suggests a potential translational effect and reinforces the importance of these brain regions in maternal adaptation.

Future directions should consider investigating potential interventions targeting these neuronal processes, which could provide valuable targets for developing therapeutic strategies to, at least, alleviate the devastating and long-lasting impact of child loss in orphaned mothers.

-Chapter 5-

Long-term consequences of child loss on the mother: Neurobiological insights from an animal model and therapeutic implications

Authors' contribution:

Luisa Demarchi:	experimental design, performance of experiments, data analysis, first draft and revision of manuscript
Alice Sanson:	experimental design, performance of experiments, revision of manuscript
Anna-Lena Boos:	performance of experiments
Oliver Bosch:	experimental design, performance of experiments, funding acquisition, revision of manuscript

5.1 Abstract

The maternal bond is the strongest social bond in mammals, building the basis for future social bonds and warranting the wellbeing of the mother and her young. Hence, the sudden disruption of the mother-infant bond can have enduring consequences for both. When the bond is dissolved due to the loss of the infant, dramatic and life-changing consequences emerge in the grieving caregivers. However, the maternal grieving process remains poorly understood.

Here, I studied the long-term impact of offspring loss in Sprague-Dawley rat mothers on behavioral and molecular levels. Dams either cared for their pups for 20 days or underwent a 1-day motherhood experience followed by 19-days of offspring loss. Such treatment led to increased oxytocin receptor binding and decreased dendritic spine density in limbic brain regions, whereas estrogen receptor α and calbindin ir+ cells were unaltered compared to control dams. Furthermore, separated mothers showed increased passive stress-coping behavior in the forced swim test paralleled with higher plasma corticosterone concentrations. Importantly, the increased emotionality after offspring loss could be rescued by central blockade of corticotropin-releasing factor receptors, but not by oxytocin infusion.

The results suggest that rat dams represent a valuable animal model for studying the detrimental effects of offspring loss emphasizing the potential involvement of the corticotropin-releasing factor system in the grieving process. These findings offer potential directions for therapies and advance our knowledge of the complex neuroscience of maternal grief.

5.2 Introduction

The bond between a mother and her infant is widely recognized as one of the strongest social bonds in mammals (Rilling and Young 2014). In humans, the dissolution of the mother-infant bond triggers grief-like reactions in the bereaved mothers, encompassing a range of emotional, cognitive, and behavioral responses, including depression and altered stress response, often resulting in prolonged grief disorder (PGD) (O'Connor 2019; Alves et al. 2022; McCarthy et al. 2010). However, the impact of offspring loss on the mother's brain and behavior remains largely unknown.

Extensive research has focused on understanding the maternal bond in rats. Particularly, the negative effects of impaired maternal care on offspring development have been demonstrated as well as traumatic emotional reactions in the offspring (for review see: Nemeroff 2016). Importantly, significative behavioral consequences have also been found in the mothers experiencing repeated offspring separation (Demarchi et al. 2023; Bolukbas et al. 2020; Baracz et al. 2020).

While previous research mainly utilized paradigms of repeated maternal separation in rodents (for review see: Alves et al. 2020), these models do not fully replicate the experience of complete offspring loss, which could better mimic the human experience of maternal grief. So far, only few studies explored the impact of total offspring loss on rat mothers (Pawluski et al. 2009c; Rincon-Cortes and Grace 2021); for review see: Demarchi et al. 2021). As I hypothesized that the experience of offspring loss in rats could potentially trigger neurobiological changes paralleling aspects of grief in humans (Demarchi et al. 2021), I aimed to study the impact of long-term offspring loss on the maternal brain and behavior, and to elucidate the underlying neurobiological mechanisms focusing specifically on the corticotropin-releasing factor (CRF) and oxytocin (OXT) systems. Previous research on the neurobiology of social loss in rodents indicates that social bond disruptions lead to alterations in neurotransmitter systems, neuroplasticity, and behavior (Shirenova et al. 2023; Dimonte et al. 2023). For example, in the monogamous and biparental prairie vole (*Microtus ochrogaster*) partner-loss increases CRF signaling thereby leading to reduced OXT signaling in the nucleus accumbens (NAcc) as well as changes microglia activity and morphology in a brain region- and sex-specific manner (Bosch et al. 2009; Bosch et al. 2016; Pohl et al. 2021).

The CRF system has been extensively studied in the context of depression as well as the maternal bond in both humans and rodents (Klampfl et al. 2016a; Klampfl et al. 2018; Carpenter et al. 2004; Binder and Nemeroff 2010). The neuropeptidergic ligands CRF and Urocortin 1-3 as well as their receptors CRF-R1 and -R2 are expressed both peripherally and centrally, and they are involved in physiological, autonomic, and behavioral responses to stress (Binder and Nemeroff 2010; Deussing and Chen 2018). Depressed patients often have increased CRF levels in the cerebrospinal fluid and elevated CRF receptor binding in specific brain regions (Bissette et al. 2003; Pandey et al. 2019), suggesting an upregulated CRF system activity (Arborelius et al. 1999; Davis et al. 2018). In animal studies, the chronic overexpression of CRF in rats has been associated with various depressive symptoms, including dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis,

alterations in neurotransmitter systems, and changes in mood and behavior (Flandreau et al. 2012). Moreover, prolonged exposure to stress can result in the sensitization of the CRF system, which becomes hyperresponsive (Herman et al. 2016).

OXT is a neuropeptide that is crucially involved in social behavior, particularly in the formation of bonds like the maternal-infant bond (Marlin et al. 2015; Carcea et al. 2021; Sanson and Bosch 2022), among others (Menon and Neumann 2023). OXT-R are distributed throughout the brain, and OXT signaling facilitates social behavior in both humans and rodents (Jurek and Neumann 2018). Furthermore, OXT is thought to have antidepressant effects and to modulate the HPA axis (Arletti and Bertolini 1987; Windle et al. 1997b; Neumann et al. 2000c). Although the underlying mechanisms are not fully understood, the role of OXT in modulating social behavior and its potential to reduce stress reactivity have been proposed as possible contributors to its therapeutic effects in depression (Jurek and Neumann 2018; Menon and Neumann 2023) including postpartum depression (Rashidi et al. 2022).

Another factor that might be involved in depression and grief symptoms is brain neuroplasticity, i.e., the brain's ability to reorganize itself by forming new neural connections in response to environmental changes and experiences. Previous studies have indicated a potential link between depression and neuroplasticity, specifically regarding spine density, which refers to the number and distribution of dendritic spines on neurons that receive synaptic inputs (Ho and King 2021). While evidence suggests that changes in neuroplasticity and spine density may be involved in the pathogenesis of depression, the specific mechanisms underlying these relationships are still unclear (Price and Duman 2020).

Furthermore, estrogen receptor α (ESR1), a nuclear and membrane hormone receptor widely expressed in various tissues including the brain, has attracted significant attention due to its crucial role in regulating central physiological processes, such as dendritic spines, chromatin organization, learning and memory, and emotions (Sheppard et al. 2019; Jaric et al. 2019). Particularly, high density of ESR1 positive cells is found in calbindin ir+ cells of the ventromedial hypothalamus (VMH) (Mori et al. 2008) and calbindin ir+ cells are decreased in post-mortem depressed patients' occipital cortex (Maciag et al. 2010). Furthermore, ESR1 activation by estrogen is essential for the induction of OXT-R expression (Young et al. 1998).

Therefore, I hypothesized that early loss of offspring may have enduring effects on the mother's emotionality and brain neuroendocrine systems. To explore this hypothesis, I

studied lactating female Sprague-Dawley rats that experienced 1 day motherhood followed by the total loss of offspring for 19 days. I assessed the resulting impact on their emotionality and HPA axis reactivity as well as OXT-R binding, dendritic spine density, ESR1 and calbindin ir+ cells in various limbic and maternal network regions. Our study offers valuable insights into the potential use of lactating rats as an animal model for studying the complex phenomenon of grief and, furthermore, sheds light on the therapeutic approaches involving central CRF receptor manipulations for treating grief-related disorders.

5.3 Materials and methods

5.3.1 **Animals**

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

5.3.2 **Experimental groups**

Rats were randomly assigned to the following experimental groups: Virgin (control group), non-maternally separated for 20 days (NMS20; control group), mothers separated on lactation day 1 for 19 days (LD1+19). The period of 19 days was selected due to being just prior to the weaning time (LD21), the period until when most of the studies have demonstrated a strong mother-offspring bond (Numan and Young 2016). Each litter was

adjusted to 8 pups of mixed sexes on LD0 (delivery day). In the LD1+19 group, all pups were removed on LD1 at 10.00 a.m. Afterwards, LD1+19 mothers were left undisturbed in their home cage. For comparison of the single housing duration, virgin rats were kept single-housed for the same amount of time (Fig. 23).

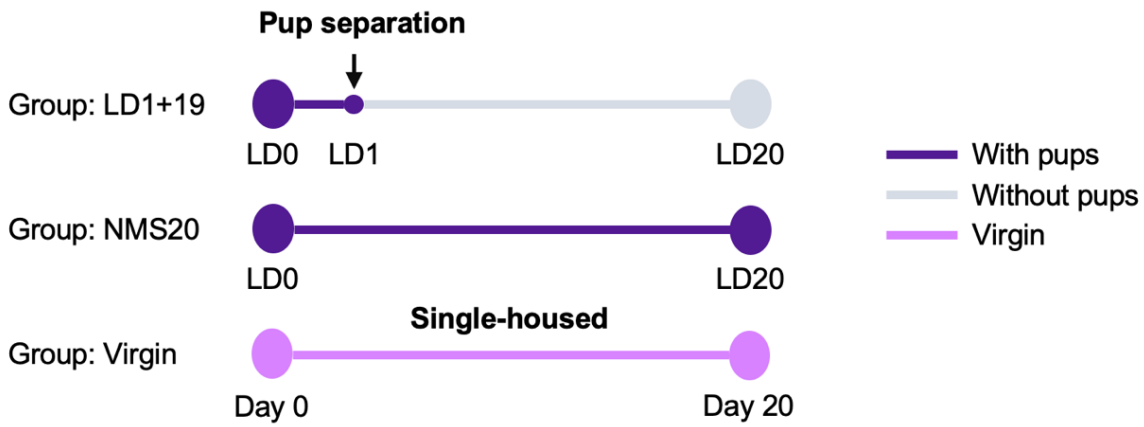


Fig. 23. Schematic timelines summarizing the experimental groups. Abbreviations: LD, lactation day; NMS, non-maternal separation.

5.3.3 **Experimental design**

Experiment 1: Rats were treated as described in 2.2 and sacrificed on LD20 without further manipulations (Fig. 23). Brains were taken for analysis of OXT-R binding (NMS20 = 6; LD1+19 = 6), for immunofluorescence analysis (NMS20 = 7; LD1+19 = 5), and for Golgi staining (Virgin = 5; NMS20 = 4; LD1+19 = 4).

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

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

Experiment 4 and 5: Rats were treated as described in 2.2. On LD15, they underwent stereotaxic implantation of an intracerebroventricular (icv) guide cannula for acute substance infusion. On LD19, rats were subjected to the pre-test of the modified FST

(mFST). On LD20, all rats were tested in the mFST after infusion of vehicle (VEH) or either the CRF-R1/2 antagonist D-Phe (Experiment 4: NMS20 VEH = 10; LD1+19 VEH = 8; LD1+19 D-Phe = 9) or synthetic OXT (Experiment 5: NMS20 VEH = 8; LD1+19 VEH = 8; LD1+19 OXT = 7). Afterwards, all rats were sacrificed.

5.3.4 **Brain analyses**

OXT-R autoradiography. Rats from NMS20 and LD1+19 were anesthetized and underwent cardiac perfusion with ice-cold 1x PBS, were decapitated, brains were flash-frozen in n-methylbutane, and stored at -20 °C until cutting into coronal sections of 16 µm using a cryostat (CM3050S, Leica Microsystem GmbH). For each brain region of interest, i.e., bed nucleus stria terminalis (BNST), nucleus accumbens shell (NAcc shell), medial preoptic area (MPOA), accessory olfactory nuclei (AOB), central amygdala (CeA), agranular insular cortex (AIP), ventral medial hypothalamus (VMH), lateral septum dorsal (LS), prelimbic cortex (PL), six slices per rat were collected on SUPERFROST microscope slides and stored at -20 °C until further processing. The OXT-R autoradiography was performed following an established protocol (Oliveira et al. 2021). Briefly, the ornithin-vasotocin analogue [^{125}I]-OVTA [d(CH_2)$_5$[Tyr(Me)^2,Thr^4,Orn^8,[^{125}I]Tyr^9-NH_2]; Perkin Elmer, USA) was used as a tracer. First, the slides were thawed and allowed to dry thoroughly at room temperature. The tissue was shortly fixed via 0.1 % PFA, washed 2 x in Tris (50 mM, pH 7.4), covered with the tracer solution (50 mM tracer, 10 mM $MgCl_2$, 0.1 % BSA) for 60 min, washed 3 x in Tris / $MgCl_2$ buffer for 7 min, each, followed by 30 min spinning in Tris / $MgCl_2$. Finally, slides were dipped into water and air dried before being exposed to Biomax MR films (Kodak, Cedex, France) for 15 days. The films were scanned using the EPSON Perfection V800 Scanner (Epson GmbH, Munich, Germany), and the optical density of each region of interest was analyzed using ImageJ (Schneider et al. 2012) by subtracting the background activity as previously described (Bosch and Neumann 2008; Oliveira et al. 2021). The analyses were performed simultaneously for 6 slices per rat and per region.

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

Golgi staining for spine density visualization. The spine density was assessed in Virgin, NMS20 and LD1+19 to investigate neuronal plasticity. Firstly, the Rapid GolgiStain™ Kit (FD NeuroTechnologies, Columbia, USA) was used and applied according to the manufacturer's protocol. Rats were sacrificed with CO_2 and brains were collected. Brains were cut into 100 µm slices using a cryostat (CM3050S, Leica Microsystem GmbH) at -28° and placed on gelatin-coated microscope slides (manufacturer). After complete drying, Golgi staining was performed, and sections were stored at room temperature in the dark before acquiring images using a brightfield microscope (Leica Thunder DM6 B, Camera Leica DFC9000 GT). Secondly, ImageJ (Schneider et al. 2012) was used to count the spines of secondary dendrites in the PL, AIP, BLA and VMH. Secondary dendrites from different neurons (between 4 and 12, depending on the brain region) were chosen from 10 X overview preview images followed by acquiring 40 X focus images for analysis. The ROI of each dendrite was selected, brightness and sharpening function were adjusted. Spines were counted on a 25 - 30 μm^2 section of the dendrite (each dendrite belonging to a different neuron) and the absolute spine density was calculated by using the following formula:
$(\mathrm{n} = \text{number of spines};\ \mu\mathrm{m} = \text{dendritic length})$: $\frac{n-1}{\mu m}$ (Gu et al. 2014).

5.3.5 **Behavioral experiments**

Light dark box (LDB). Rats were tested in the LDB to measure anxiety-like behavior (Crawley and Goodwin 1980). The apparatus consisted of a light (40 cm x 50 cm, 180 lux) and a dark compartment (40 cm x 30 cm, 0 lux) connected via an opening (7.5 cm x 7.5 cm). At the beginning of the test, rats were placed in the center of the light box, and the behavior was recorded for 10 min for later analysis by an experimenter blind to the treatment with EthoVision XT (Noldus, Wageningen, the Netherlands). The following behaviors were scored: (a) percentage of time in the light box; (b) latency to re-enter the light compartment; (c) locomotion. Before each test, the arena was cleaned with tap water and dried thoroughly.

Open-field (OF). Rats were tested in the OF to measure locomotor- and anxiety-like behavior (Gould 2009). The apparatus consisted of an empty rectangular arena (80 x 80 cm). Rats were placed in one corner of the apparatus and left to freely explore the arena for 10 min. The behavior was recorded for later analysis by an experimenter blind to the treatment with EthoVision XT (Noldus, Wageningen, the Netherlands). The following behaviors were scored: (a) locomotion; (b) velocity; (c) center entries. Before each test, the arena was cleaned with tap water and dried thoroughly.

Classic forced swim test (FST). Passive stress-coping behavior was tested in the classic FST (Slattery and Cryan 2014). Between 9.00 a.m. and 12.00 p.m., rats were placed for 10 min in a cylindrical tank (50 cm high, 30 cm diameter) filled with tap water (23 ± 1 °C) to a depth that rats could not touch the bottom with their hind paws or tail. Trials were recorded for later analysis using the software JWatcher (https://www.jwatcher.ucla.edu) by an experimenter blind to the treatment. The total time spent on floating, i.e., passive stress-coping behavior, indicative of depressive-like behavior (Slattery and Cryan 2014), was analyzed.

Modified forced swim test (mFST). The mFST was performed as it is best for testing antidepressant drugs (Slattery and Cryan 2014). On two consecutive days between 9.00 a.m. and 12.00 p.m., passive stress-coping behavior was tested in the mFST following two different central pharmacological manipulations, i.e., icv infusion of the CRF-R1/2

antagonist D-Phe or of synthetic OXT (for information on surgeries and treatment concentration, see below). On LD19, rats underwent a pre-test session for 15 min. On LD20, rats were tested in a 10 min FST following pharmacological manipulations. All other test conditions and behavioral analyses were as described above.

5.3.6 **Stereotaxic surgery**

On LD15, rats were implanted with a stainless steel guide cannula (21-G, length 12 mm), above the right ventricle (1.0 mm posterior, 1.6 mm lateral to bregma, 1.8 mm ventral) (Paxinos 1998; Klampfl et al. 2013) under isoflurane anesthesia as described before (Klampfl et al. 2013). The guide cannula was closed using a 25-G stainless steel stylet of the same length as the guide cannula. All rats received a subcutaneous injection of analgesics (0.05 mg/kg Buprenorphine, Bayer Vital GmbH, Leverkusen, Germany) pre-surgery to avoid post-surgical pain.

5.3.7 **Drug administration**

On LD20, rats received one acute icv infusion of either VEH (5 µL sterile Ringer's solution; pH adjusted to 7.4; Braun, Melsungen, Germany), the CRF-R1/2 antagonist D-Phe [(D-Phe^{12}, Nle$^{21-38}$, a-Me-Leu^{37})-CRF (12–41); human/rat; 10 µg / 5 µL]; Bachem, Bubendorf, Switzerland] or synthetic OXT (1 µg / 5 µL; Tocris, Nordenstadt, Germany), 10 min prior to the mFST on the second day of testing. Doses were chosen based on previous studies (Klampfl et al. 2013). For the acute icv infusion, a 25-G stainless steel infusion cannula (length 14 mm) was connected to a 10 µL micro syringe via PE-50 tubing (50 cm) and lowered into the guide cannula, where it was kept in place by a piece of silicon tubing during slow substance infusion for approximately 30s (Neumann et al. 2000c).

5.3.8 **Verification of cannula placement**

After the final behavioral test, rats were sacrificed with CO_2 and blue dye was injected via the infusion system into the guide cannula. Verification of the icv location of the infusion site was observed by dye spread throughout the ventricular system. Only animals with correct, histologically verified infusion sites were included in the statistical analyses.

5.3.9 **ELISA for plasma CORT**

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

5.3.10 **Statistical analysis**

All statistical analyses were performed with GraphPad PRISM 9 (GraphPad Software, San Diego, USA). Normality and homoscedasticity were verified (Shapiro-Wilk or Kolmogorov–Smirnov test and Brown-Forsythe test, respectively) and analysis of outliers was run via the ROUT method.
OXT-R binding, ESR1 and calbindin ir+ cells analyses were performed via a two-sample Student's t-test. Dendritic spine density, plasma CORT concentration, anxiety-like, locomotor and stress-coping behaviors were analyzed via one-way ANOVA followed by Sidak *post hoc* multiple comparisons. Data are presented as mean ± SEM; $p \leq .05$ was considered significant.

5.4 Results

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

To assess whether the offspring loss experience could affect OXT-R binding in limbic and maternal network regions, I performed a receptor autoradiography (Fig. 24a) comparing NMS20 and LD1+19. In detail, LD1+19 dams had significantly higher levels of OXT-R binding in the VMH (M = 0.257, SD = 0.083; Fig. 24b, c) and the PL (M = 0.070, SD = 0.005; Fig. 24b, d) than the NMS20 [VMH: M = 0.147, SD = 0.077, t (10) = 2.366, p < .039; PL: M = 0.066, SD = 0.002, t (10) = 2.203, p < .052]. No significant differences were found in any other region analyzed [MPOA (t (10) = 0.215, p < .834); BNST (t (10) = 0.828, p < .427); CeA (t (10) = 0.061, p < .952); OB (t (10) = 0.595, p < .565); NAcc shell (t (10) = 1.440, p < .180); AIP (t (10) = 1.212, p < .254); LS (t (10) = 0.613, p < .554)].

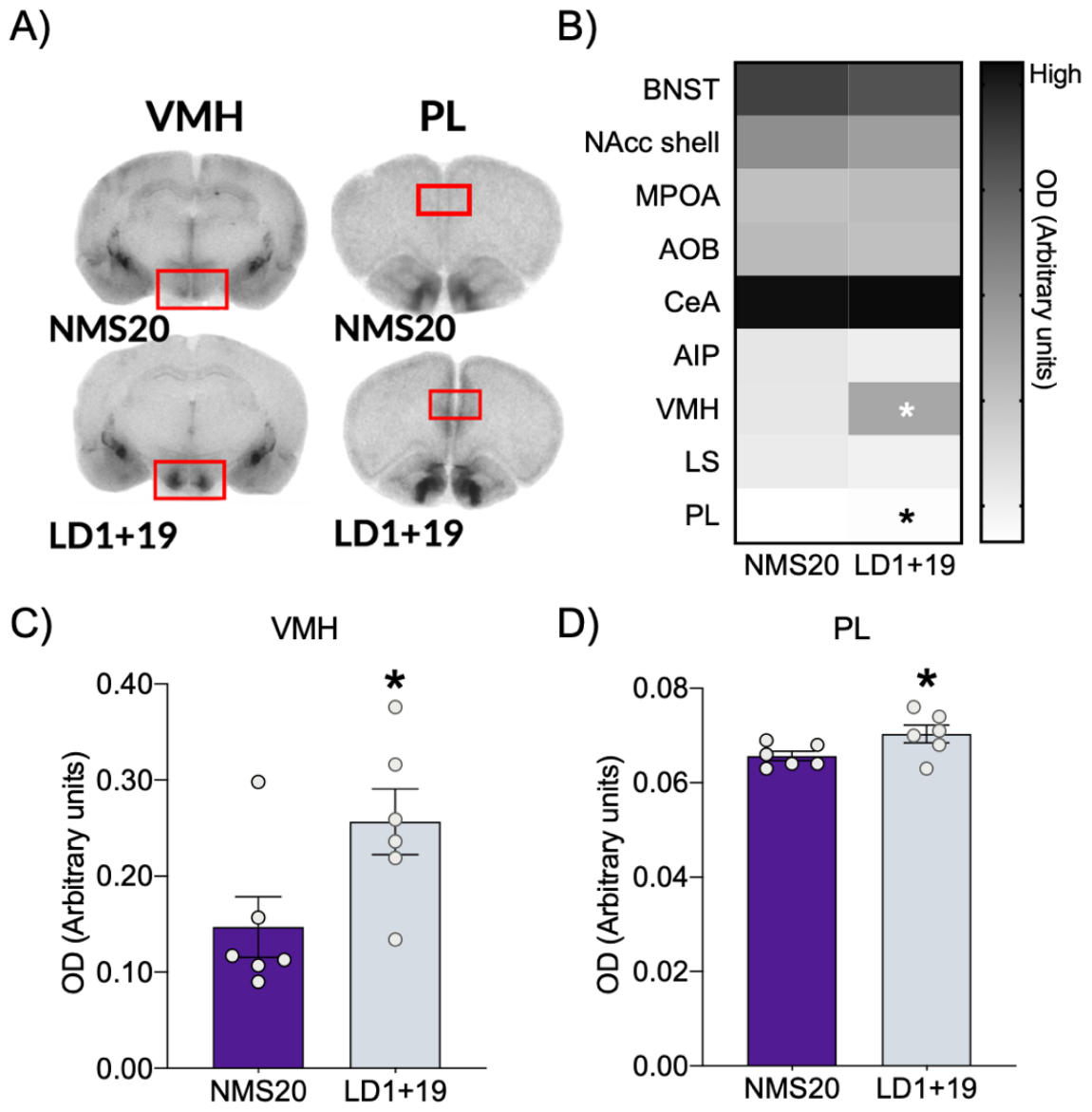


Fig. 24. **OXT-R binding in brain regions of the limbic system and maternal network.** (A) Representative coronal brain sections of the VMH and PL demonstrating differences in OXT-R binding. (B) Overview heatmap of gray density (arbitrary units) for OXT-R binding in the analyzed brain areas (* $p \leq .05$ versus NMS20). Optical density (arbitrary units) for OXT-R binding in the (C) VMH and (D) PL. Abbreviations: AIP, agranular insular cortex; AOB, accessory olfactory nuclei; BNST, bed nucleus of the stria terminalis; CeA, central amygdala; LS, lateral septum; MPOA, medial preoptic area; NAcc, nucleus accumbens; PL, prelimbic cortex; VMH, ventral medial hypothalamus. Student's t Test. Data are expressed as mean ± SEM. * $p \leq .05$ versus NMS20.

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

To determine if the differences in OXT-R binding were due to hormonal changes, I analyzed the expression of ESR1 and calbindin ir+ cells in the VMH of NMS20 and LD1+19 rats. There was no significant difference in the percentage of ESR1 immunoreactive cells (t (10) = 0.456, $p < .658$; Fig. 25a, b) or of calbindin ir+ cells (t (10) = 0.706, $p < .496$; Fig. 25a, c) between the groups.

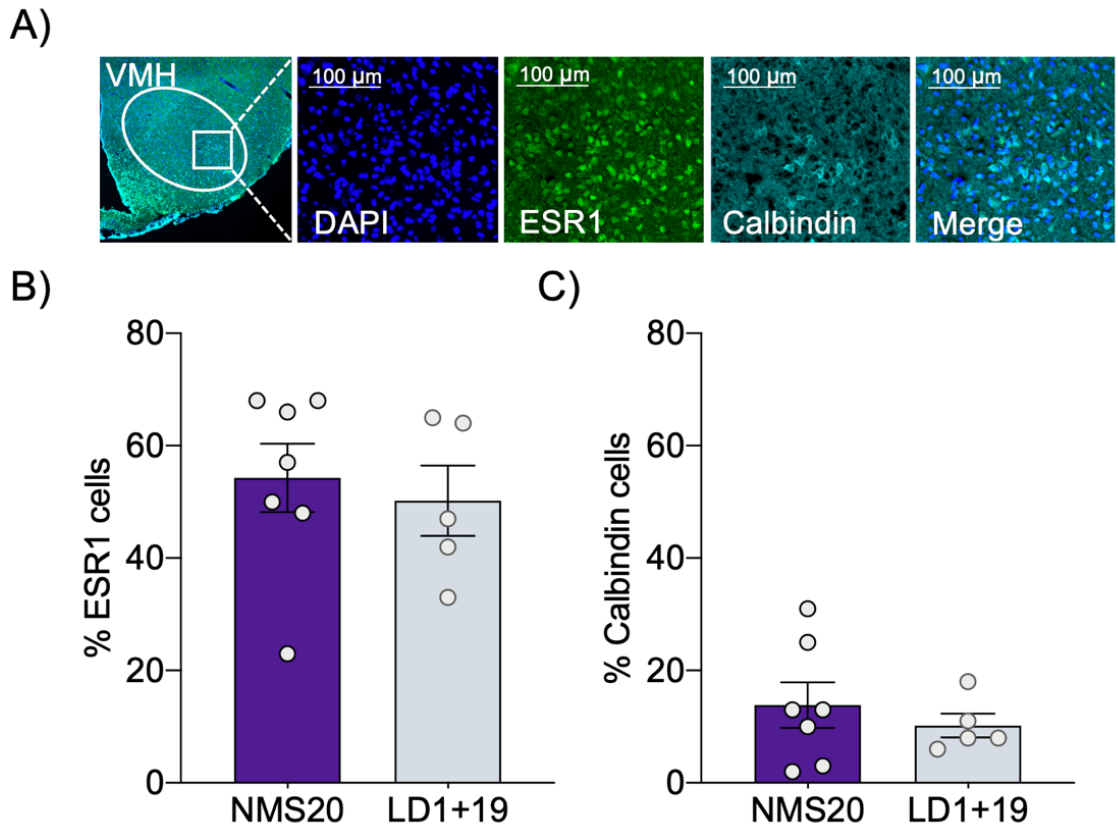


Fig. 25. **ESR1 and calbindin ir+ cells in the VMH.** (A) Representative fluorescent images showing DAPI (blue), ESR1 cells (green), calbindin (cyan) and merged channels in the VMH. Percentage of (B) ESR1 and (C) calbindin ir+ cells in the VMH. One-way ANOVA. Data are expressed as mean ± SEM.

5.4.3 **Reduced secondary dendritic spine density in the VMH after offspring loss**

To determine the effects of offspring loss on neuroplasticity, I analyzed the secondary dendritic spine density in the VMH, PL, BLA and AIP of NMS20 and LD1+19 and of an additional Virgin control group. The statistical analysis revealed a significant difference in secondary dendritic spine density between the groups in the VMH, only (one-way ANOVA: $F(2, 10) = 8.734$; $p < .006$; Fig. 26), and *post hoc* multiple comparisons showed significantly lower levels in LD1+19 dams compared to Virgin ($p < .024$) and NMS20 ($p < .009$). No significant differences were found in the PL [Virgin: 0.64 ± 0.07; NMS20: 0.53 ± 0.04; LD1+19: 0.62 ± 0.04; one-way ANOVA: $F(2,15) = 0.590$, $p < .356$], the BLA [Virgin: 0.61 ± 0.03; NMS20: 0.64 ± 0.009; LD1+19: 0.61 ± 0.02; one-way ANOVA: $F(2,16) = 0.158$, $p < .855$], and the AIP [Virgin: 0.62 ± 0.05; NMS20: 0.69 ± 0.04; LD1+19: 0.68 ± 0.04; one-way ANOVA: $F(2,15) = 0.599$, $p < .562$].

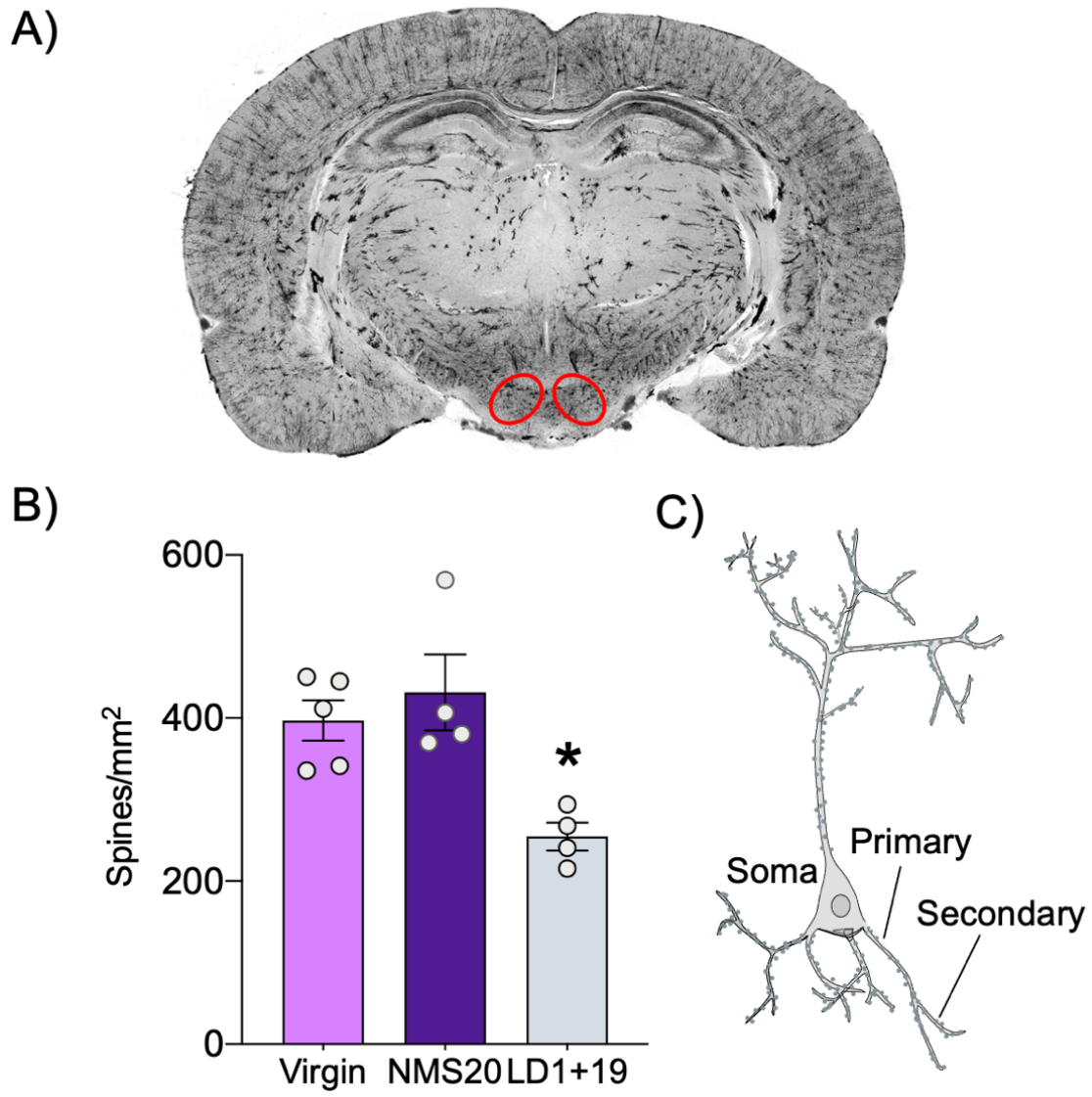


Fig. 26. **Secondary dendritic spine density in the VMH.** (A) Representative coronal whole brain section with Golgi staining. (B) Absolute spine density / mm^2 across experimental groups. (C) Schematic illustration of the neuronal dendritic arbor. One-way ANOVA. Data are expressed as mean ± SEM. * $p < .05$ versus all other groups.

5.4.4 **Anxiety-like behavior was not affected by offspring loss**

To evaluate if offspring loss could alter anxiety-like behavior, Virgin, NMS20 and LD1+19 rats were tested in the LDB on LD19. Groups differed in the latency to re-enter the light box as a measure of anxiety-like behavior [Virgin: 213.5 ± 77.5 sec; NMS20: 48.6 ± 15.9 sec; LD1+19: 121.9 ± 31.9 sec; Kruskal-Wallis test, $p < .045$] with the Virgin group tending to have a higher latency compared to NMS20 (Dunn's test, $p < .058$). No significant

differences were found in the time spent in the light box [Virgin: 34.0 ± 6.3 sec; NMS20: 36.6 ± 3.6 sec; LD1+19: 42.0 ± 5.1 sec; one-way ANOVA: F (2,36) = 0.676, $p < .515$] or in locomotion [Virgin: 3778 ± 326 cm; NMS20: 3733 ± 194 cm; LD1+19: 3851 ± 312 cm; one-way ANOVA: F (2,36) = 0.054, $p < .948$].

5.4.5 **Locomotor activity was not affected by offspring loss**

The same animals as in 5.4.5 were tested in the OF for locomotor activity on LD20. The groups significantly differed in the distance travelled (one-way ANOVA: F (2,36) = 3.773, $p < .032$; Fig. 27a) and the velocity (one-way ANOVA; [F (2,36) = 3.788], $p < .032$; Fig. 27b). In detail, Sidak *post hoc* test revealed increased locomotion ($p < .029$) and velocity ($p < .028$) in Virgin compared to NMS20. No significant differences were found in the number of entries in the center of the arena [Virgin: 21.4 ± 2.7; NMS20: 16.3 ± 1.5; LD1+19: 20.5 ± 2.0; one-way ANOVA: F (2,36) = 1.858, $p < .170$].

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

A different cohort of Virgin, NMS20 and LD1+19 rats were tested for passive stress-coping behavior in the classic FST on LD20. Groups significantly differed in the time spent floating during the 10-min test (one-way ANOVA: F (2,32) = 6.233, $p < .005$; Fig. 27c), and Sidak *post hoc* multiple comparisons revealed that LD1+19 spent more time floating compared to Virgin ($p < .019$) and NMS20 ($p < .014$).

The rats' plasma was collected for CORT measurement within 5 min following the FST. The statistical analysis showed that plasma CORT levels significantly differed between the groups (one-way ANOVA: F (2,28) = 13.50, $p < .0001$; Fig. 27d), and Sidak *post hoc* multiple comparisons revealed lower plasma CORT concentration in NMS20 compared to Virgin ($p < .002$) and LD1+19 ($p < .0001$). Interestingly, plasma CORT concentration in LD1+19 was not different from Virgin ($p > .05$ vs virgin group), suggesting that the separation experience re-instated the normal stress response.

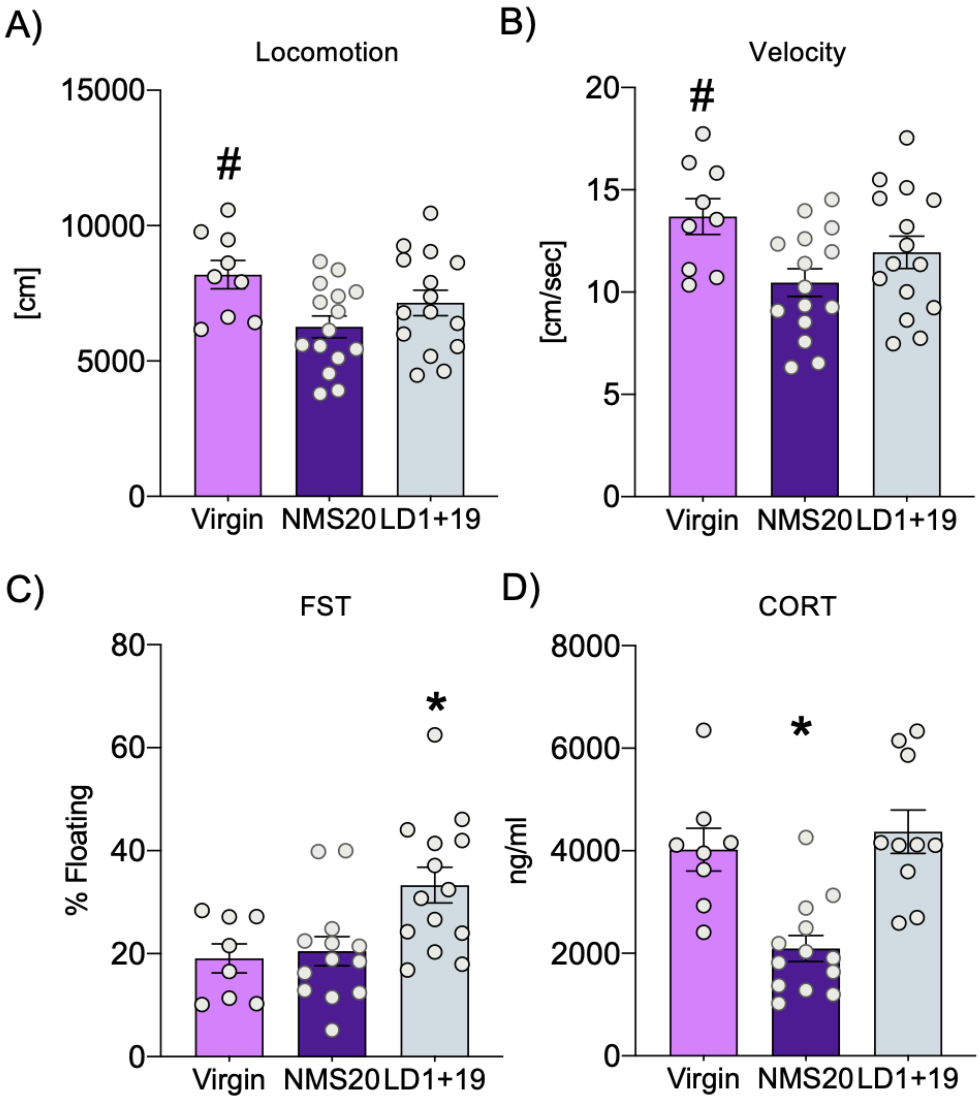


Fig. 27. Locomotor behavior in the OF, passive stress-coping behavior in the classic FST, and plasma CORT. (A) Total distance traveled in cm during and (B) mean velocity over the 10 min OF test. (C) Percentage of floating during the 10-min classic FST. (D) Plasma CORT concentration immediately after termination of the FST. One-way ANOVA. Data are expressed as mean ± SEM. # $p < .05$ versus NMS20; * $p < .05$ versus all other groups.

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

Following a pre-test on LD19, NMS20 and LD1+19 rats were tested in the mFST after central pharmacological manipulation. Dams received a single acute icv infusion of either VEH (NMS20, LD1+19) or D-Phe (LD1+19) 10 min prior to the mFST. The statistical analysis revealed a significant difference between the groups (one-way ANOVA: $F(2,23) = 5.828$, $p < .009$; Fig. 28a). In detail, LD1+19 VEH spent more time floating compared to NMS20 VEH (Sidak *post hoc* multiple comparisons, $p < 0.047$), thereby confirming our previous results in the classical FST. Most importantly, the percentage time spent on floating was significantly lower in LD1+19 D-Phe compared to LD1+19 VEH ($p < .014$), even reaching control levels ($p > .05$ vs NMS20 VEH).

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

A different cohort of rats were tested in the mFST (see 3.7) after receiving a single acute icv infusion of either VEH (NMS20, LD1+19) or OXT (LD1+19) 10 min prior to the test. The groups differed significantly in the percentage time spent on floating (one-way ANOVA: $F(2,20) = 5.273$, $p < .042$; Fig. 28b); I confirmed again our previous results demonstrating increased percentage time spent on floating in the LD1+19 VEH versus NMS20 VEH (Sidak *post hoc* multiple comparisons: $p < .046$), but LD1+19 OXT rats were no different from LD1+19 VEH ($p < .187$).

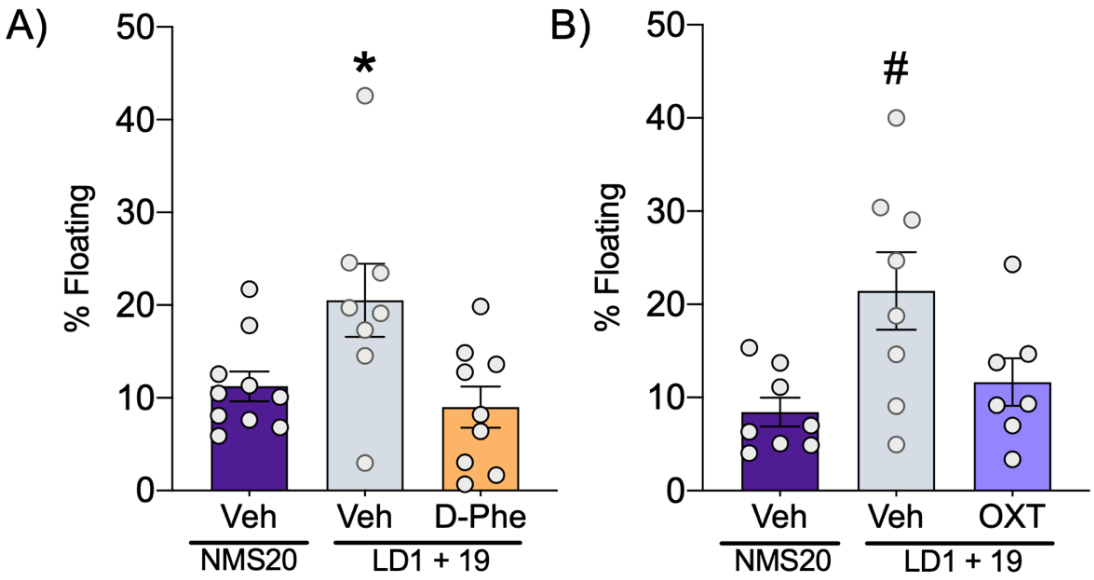


Fig. 28. Passive stress-coping behavior in the modified FST following central pharmacological manipulation of the brain CRF and OXT systems. Percentage of floating during the 10 min modified FST following icv infusion of (A) VEH or the CRF-R1/2 antagonist D-Phe, and (B) of VEH or synthetic OXT. One-way ANOVA. Data are expressed as mean ± SEM. * $p < .05$ versus all other groups; # $p < .05$ versus NMS20.

5.5 Discussion

Losing a child is one of the most devastating experiencing in life. When grieving over such loss, parents experience negative impact in their physical and mental well-being (Kersting and Wagner 2012; Burden et al. 2016) with a greater risk of developing an invaliding PGD (Huh et al. 2017; McCarthy et al. 2010). Adequate animal models are required to identify and comprehend the neurobiology of offspring loss to help develop effective treatments. In the present study, I used rat dams as a novel animal model with the potential to reconstitute the parallels to grieving mothers. Specifically, following 19-days of offspring loss I observed increased OXT-R binding and reduced dendritic spine density in the VMH, and increased OXT-R binding in the PL. Furthermore, our findings show that, compared to control mothers and virgins, the orphaned mothers' stress response and passive stress-coping behavior were increased. Importantly, the impaired phenotype was rescued by central CRF-R1/2 antagonism, but not by OXT agonism, providing significant insight for novel therapeutical approaches.

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

To prepare the female for the new challenges as a mother, dynamic changes occur peripartum with respect to the brain OXT system (Valtcheva et al. 2023; Ng et al. 2023). Maternal attachment is modulated by OXT-R distribution (Macbeth et al. 2010). Therefore, I examined whether the long-term offspring loss mirrored changes in OXT-R. Indeed, OXT-R binding was significantly higher in the VMH and PL in LD1+19 (Fig. 24), a finding I did not expect as I hypothesized that offspring loss would result in less OXT signaling.

The VMH is involved in regulating various social behaviors, including maternal behavior (Bridges et al. 1999) and female aggression (Hashikawa et al. 2017). Moreover, VMH neurons are essential in controlling defensive behaviors like maternal defense (Kunwar et al. 2015). Due to the high concentration of OXT-R positive cells in the VMH (Narita et al. 2016), especially in females (Mitre et al. 2017), it is plausible that OXT signaling in the VMH could modulate maternal behaviors (Mitre et al. 2017). However, an involvement of OXT within the VMH in maternal behavior has not been studied in detail, yet. The PL has been associated to cognitive and emotional functions (Marek et al. 2018; Capuzzo and Floresco 2020). Interestingly, OXT-R blockade in the PL in post-partum Sprague-Dawley rats impairs maternal behavior (Sabihi et al. 2014a). This highlights the role of the PL in the neural circuitry of OXT-mediated maternal bonding. Together, the data for PL and VMH suggests that the increased OXT-R binding of LD1+19 mothers reflect a compensatory mechanism triggered by forced maternal bond disruption.

Interestingly, I recently observed a trend towards increased OXT-R binding in the PL but a significant increase in the MPOA after one week of repeated 3-h maternal separation (Demarchi et al. 2023). In that line, other studies also found changes in OXT-R binding associated with altered maternal behaviors, further emphasizing the crucial role of OXT-R in regulating maternal behaviors (Champagne et al. 2001; Stamatakis et al. 2015). Prolonged separation from offspring results in reduced interaction with the pups and fewer occurrences of milk ejection reflexes (Li et al. 2020), potentially leading to dampened OXT signaling in the brain. Indeed, prior research has shown a decrease in c-Fos expression in OXT-positive (OXT+) neurons of the supraoptic nucleus and a decrease in the number of OXT immunoreactive (OXT ir+) neurons in the PVN of separated mothers (Liu et al. 2019a; Baracz et al. 2020). Therefore, I speculate that the sudden offspring loss may induce alterations in OXT signaling in the brain, and increasing OXT-R in certain brain areas might be a compensatory mechanism for the less available OXT. While no changes were detected in the other regions analyzed, I cannot exclude that relevant changes may occur in those

regions at the level of OXT release or mRNA expression, which is not captured by the receptor autoradiography. Future research could specifically analyze those parameters in certain brain areas to provide a better picture of their biodynamic changes.

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

The relationship between the observed increase of the OXT-R binding and the distress experienced from offspring loss is not yet fully understood, and it remains unclear whether these changes could be attributed to endocrine factors. Estrogen, a hormone primarily associated with female reproductive function, can trigger the expression and function of OXT-R via ESR1, potentially influencing social and reproductive behaviors (Young et al. 1998). Furthermore, estrogen concentrations are linked to calbindin (Wang et al. 2013), which is highly expressed in the VMH (Vishnyakova et al. 2021; Kalinowski et al. 2022). Therefore, I examined the percentage of ESR1 and calbindin ir+ cells in the VMH to see if these proteins underly the changes in local OXT-R binding after offspring loss (Fig. 25). As we did not observe any differences between the groups, the increase in OXT-R binding is unlikely to be caused by hormonal estrogen changes. However, at this point I cannot exclude that other neurohormonal systems, like the CRF system (as discussed below), might have contributed to the observed changes in OXT-R binding. Nevertheless, it is essential to acknowledge the potential presence of alterations in ESR1 or calbindin ir+ cells within the PL. Despite detecting increased OXT-R binding in this region, we did not conduct an analysis due to the absence of supporting evidence indicating a direct modulation of OXT-R binding by estrogen in that region.

Decreased spine density in the VMH after offspring loss

In rats, the maternal brain network undergoes neuronal morphological changes in the dendritic spine density during pregnancy and the postpartum period (Servin-Barthet et al. 2023b). Both human studies and rat animal models of postpartum depression demonstrate reduced dendritic spine density and neuronal changes in the hippocampus (Workman et al. 2013), prefrontal cortex (Leuner et al. 2014), amygdala and insular cortex (Wonch et al. 2016; Payne and Maguire 2019). Due to the substantial body of research indicating a connection between alterations in spine density and OXT-R (Becker et al. 2013; Pekarek et al. 2020), I analyzed secondary dendritic spine density, which revealed a notable decrease in the VMH, only (Fig. 26).

In response to chronic stress and social isolation, dendritic spine changes have been found in the BLA, PL and AIP (Sequeira and Gourley 2021; Radley et al. 2006; Mitra et al. 2005). More importantly, numerous studies have consistently highlighted the link between reduced spine density and depression (for review see: Qiao et al. 2016). Similarly, also in humans a low spine density correlates with depressive symptoms (Holmes et al. 2019). Therefore, I speculate that the VMH, renowned for its involvement in aggression, stress response, and pain attacks, in both humans and rats (Borszcz 2006; Wilent et al. 2010), likely plays a part in the adverse effects of offspring loss on maternal well-being. Further research is necessary to explore the functional implications of decreased spine density and its potential behavioral consequences.

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

While I confirmed decreased anxiety-like behavior in lactation (for review see: Bosch 2011; Bosch and Neumann 2012) when comparing NMS20 to Virgin, this postpartum adaptation was not apparent anymore in separated mothers (Fig. 27). In fact, the total absence of the nursing and caring experience in LD1+19 and, thus, the lack of high OXT brain levels might account for the return of their anxiety-like behavior to virgin levels as OXT is facilitating reduced anxiety levels in lactation (Neumann et al. 2000b). In a similar study, permanent removal of offspring for 4 weeks did not alter anxiety-like behavior in separated mothers compared to virgin and lactating dams (Pawluski et al. 2009c). This is different to repeated prolonged maternal separation, which can result in increased anxiety-like behavior (Orso et al. 2018; Aguggia et al. 2013; Smith and Lonstein 2008). It is worth noting that in the present study, the phenotype of the Virgin group could have been influenced by the experimental design. Indeed, Virgin rats underwent a period of social isolation to match this group with the pup-loss group, and previous research in male rats has linked such stress to increased anxiety and a hyperactive phenotype (Begni et al. 2020).

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

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

To examine the potential impact of the offspring loss experience on emotional behavior, I conducted the classic FST. Notably, the separated mothers displayed increased duration of floating compared to both the control Virgin group and the NMS20 lactating dams, which were not different from each other (Fig. 27c). Importantly, I was able to confirm the increased passive stress-coping after offspring loss twice in the following experiments where I aimed to manipulate the behavior with pharmacological interventions (see below). Our findings support and expand previous work demonstrating that mother-offspring separation leads to increased depressive-like behaviors, i.e., our results reconfirm two studies which demonstrate that 24-h of maternal experience followed by 3 weeks (Rincon-Cortes and Grace 2021) and 4 weeks (Pawluski et al. 2009c) of offspring loss increases floating behavior in the FST. Hence, offspring loss has a strong emotional impact on the mother leading to increased passive stress-coping behavior, which is reminiscent of depressive-like behavior (Slattery and Cryan 2014). Remarkably, I found no differences in locomotor activity between the control NMS20 mothers and LD1+19 mothers in the OF test, which endorses that any observed changes in the FST were likely due to emotional causes rather than altered body energy or locomotion impairments.

The analysis of plasma CORT concentrations following exposure to the classic FST (Fig. 27d) not only confirmed the dampened stress response in lactation when comparing Virgin with NMS20 but, more importantly, revealed that the CORT levels of LD1+19 mothers were back the level of Virgins. On one hand, this suggests that the offspring loss experience disrupts the dampened stress response in lactation (Douglas et al. 2003). It implies that separated mothers are more susceptible to an acute stressor (FST) compared to lactating dams. On the other hand, one cannot exclude that the stress physiology of the separated mothers came back to virgin levels due to the long separation from the offspring and, thus, the lack of mothering experience.

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

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

The activity of the brain CRF system is downregulated in lactation to enable the mother to adequately respond to a stressor (Klampfl et al. 2013; Klampfl et al. 2014). However, exposure to, e.g., severe chronic stress before / during parturition can lead to increased CRF system signaling with strong impact on the emotionality (Darnaudery et al. 2004; Zoubovsky et al. 2020). In fact, the CRF system is hypothesized to be a potential neuromodulator following the break of a bond as shown in, e.g., lactating prairie vole mothers following separation from the pair-bonded male partner (Bosch et al. 2018).

In the present study, central administration of the CRF-R1/2 antagonist D-Phe effectively reversed the offspring loss-associated high levels of passive stress-coping in the mFST (Fig. 28), which is recommended to assess anti-depressant drug effects (Slattery and Cryan 2014). Our finding suggests that permanent separation from the pups leads to increased activity of the brain CRF system. This is in line with previous studies demonstrating that acute central infusion of D-Phe rescues the increased emotionality after the loss of a social bond, e.g., in lactating (Bosch et al. 2018) and male prairie voles (Bosch et al. 2009) after losing the partner.

The brain CRF system can interact with the OXT system as has been shown in, e.g., lactating rats (Klampfl et al. 2018) and male prairie voles (Bosch et al. 2016). In the latter, when separated from their female partner, local OXT infusion into the NAcc shell rescues their passive phenotype (Bosch et al. 2016). This led us to hypothesized that the high levels of passive-stress coping behavior of separated mothers could implicate a lack of central OXT release due to the loss of the pups' stimuli. However, central OXT infusion did not alter the passive phenotype in separated mothers. The lack of an effect does not necessarily exclude the OXT system as a potential target. The central infusion, which acts in almost all brain regions at the same time, might mask positive effects happening in certain brain regions as seen, e.g., the PVN of lactating rats (Wang et al. 2018) or the NAcc shell in male prairie voles (Bosch et al. 2016) versus other brain regions.

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

5.6 Conclusions

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

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

-Chapter 6-

General discussion

6.1 Summary of results

The study of the behavioral and neurobiological consequences of loss in rat mothers as an animal model for bereaved human mothers is a rather new topic that has not received the necessary attention at this point. Therefore, I started to delve into the existing literature both human and animal studies alike, focusing on the correlation between loss, grief, and the link to the OXT and CRF systems (Chapter 2; Demarchi et al., 2021). The review revealed that clinical studies establish a connection between symptoms associated with grief, such as depression, anxiety, cognitive impairments, and social difficulties, and dysregulations in OXT signaling. Specifically, an elevation in plasma OXT and the presence of a genetic variant of the OXT-R (single nucleotide polymorphism rs2254298) are correlated with increased severity of PGD symptoms, as evidenced (Heinrichs et al. 2003; Bui et al. 2019; Schiele et al. 2018). Notably, the literature presents inconsistencies in the context of postpartum depression and OXT signaling, with frequent observations of reduced endogenous plasma OXT inversely related to postpartum depression, as highlighted in a systemic review by (Thul et al. 2020).

Simultaneously, investigations involving rodents experiencing partner loss or offspring separation have yielded comparable findings, indicating the potential involvement of the OXT system in modulating grief-like behaviors (Demarchi et al. 2021). For instance, brief repeated separations from postnatal days 1 to 22 lead to increased OXT-R binding in various brain regions associated with stress response and/or maternal behavior (Stamatakis et al. 2015). Additionally, repeated brief separation from offspring augments the number of OXT-immunoreactive cells in the PVN, while repeated prolonged stress diminishes it in the caudal PVN compared to non-lactating rats (Baracz et al. 2020). In contrast to these effects on the brain's OXT system, plasma OXT levels remain similar in rat mothers exposed to repeated short or prolonged separation from offspring (Eklund et al. 2009). Furthermore, even a total deprivation of pups for 20 hours over four consecutive days does not alter plasma OXT levels compared to the control group in rat mothers (Liu et al. 2019). Moreover, human research has also revealed a robust association between the CRF system and grief-related symptoms, especially a heightened CRF signaling and disturbances in the HPA axis following the traumatic loss of a loved one (Buckley et al. 2012; Hopf et al. 2020). Concurrently, in rodent models that focus on chronic stress and maternal separation, documented changes in CRF signaling within the brain have been observed (Perez-Tejada

et al. 2013; Planchez et al. 2019). Consequently, maternal plasma CORT levels increase following repeated short separation (Eklund et al. 2009).
In substance, Chapter 2 suggests that both the OXT and CRF systems may play pivotal roles in grief-related symptoms. Furthermore, it became apparent that there's currently no available rat animal model suitable for a more translational investigation of maternal grief.

Chapter 3 of my thesis set out to explore the effects of repeated maternal separations on the mother. I employed a standard protocol involving brief (15 minutes, BMS) or long (180 minutes, LMS) maternal repeated separation during the first postpartum week. The impact of this protocol was assessed in terms of maternal behavior and motivation, maternal stress-coping behaviors, plasma CORT levels, adrenal gland weight, and OXT-R binding. The results showed that both BMS and LMS significantly influenced maternal behavior, leading to increased licking and grooming. Mothers subjected to 180-min separation displayed reduced maternal motivation compared to control mothers and those who experienced brief separation. However, no significant effects were observed in the FST or adrenal gland weight and plasma CORT levels. Notably, LMS dams exhibited increased OXT-R binding in the MPOA and a trend towards significance of increased OXT-R in the PL. This study underscores how even temporary disruptions in the mother-offspring bond can impact a mother's behavior and the brain's OXT system.

Chapter 4 aimed to establish a more translational model of maternal grief. To do so, I investigated the effects of 1-day experience of motherhood followed by varying durations of permanent offspring removal on the maternal brain and behavior: 1-day, 3-days, and 6-days. In fact, 1-day of offspring loss induced increased neuronal activity in the PL and BLA of separated mothers, displaying a positive neuronal activity correlation between those regions, which contrasted with the negative correlation seen in control mothers. Additionally, 1- and 3-day offspring loss decreased OXT-R binding in the CeA. While no effects were noted in plasma CORT levels, an increased amount of time spent floating in the FST, indicative of passive stress-coping behavior, was observed in separated mothers after experiencing offspring loss for 6 days.

In Chapter 5, aligning with the previous findings from Chapter 4, I investigated the impact of an extended 19-day offspring loss experience on the maternal brain and behavior. This prolonged separation resulted in increased OXT-R binding, specifically in the PL and VMH

of orphaned mothers. Moreover, Golgi Cox staining revealed decreased spine density in the VMH of long-term separated dams. No significant differences were found between groups in the number of ESR1 cells or calbindin neurons in the VMH. Furthermore, no changes in locomotor or anxiety-like behaviors were detected when comparing long-term separated mothers and controls. Importantly, the 19-day offspring loss experience increased the percentage of floating in the FST. To further investigate potential interventions to rescue the passive-stress coping behavior, I conducted pharmacological experiments. The icv administration of a synthetic OXT failed to affect the impaired phenotype of separated dams in the FST. In a subsequent experiment, the administration of icv D-Phe, a CRF-R1/2 antagonist, successfully rescued the impaired phenotype.

Collectively, these studies offer a comprehensive exploration of the intricate relationships between maternal bond disruption and the underlying neurobiological mechanisms involving the OXT and CRF systems, neuroplasticity, and the resulting behavioral outcomes.

6.2 Towards the development of a rat animal model for maternal grief: a focus on behavior

In this section, I will discuss the findings of my thesis concerning the impact of offspring loss on maternal emotionality, reserving a detailed discussion of the other findings for subsequent sections.

In Chapter 2, I hypothesized the involvement of both the OXT and CRF systems in modulating grief-related symptoms. The review of existing literature, encompassing both human and rodent studies, revealed a significant gap in understanding the neurobiological mechanisms underlying maternal grief due to the absence of an appropriate rodent model (Demarchi et al. 2021). While other rodent species, such as the biparental and socially monogamous prairie voles (*Microtus ochrogaster*) and the California mice (*Peromyscus californicus),* have been repeatedly studied concerning the OXT and CRF systems in response to partner loss (Bosch et al. 2009; Valentino et al. 2021; De Jong et al. 2013), research on the specific neurobiology of offspring separation has been limited mostly to studies in mice and rats using a standard paradigm of repeated maternal separation. Furthermore, most of those studies do not examine neuroendocrine parameters.

Furthermore, it emerged that a scarcity of research was evident, and that a novel animal model of offspring loss was urgently required.

Initially, my focus in the opening study (Chapter 3), was to examine the consequences of a brief versus long maternal repeated separation on both the mother's brain OXT system, and her maternal and stress-coping behavior. Despite previous research (Boccia et al. 2007; Maniam and Morris 2010) indicating that long maternal repeated separation negatively affects maternal emotionality, I could only see a tendency towards increased floating behavior in the FST in the LMS group. Discrepancies in the duration of the paradigm (2 weeks ((Boccia et al. 2007; Maniam and Morris 2010) versus 1 week (Demarchi et al. 2023)) could explain why LMS dams only displayed a tendency and not a strong floating increase, suggesting that the paradigm duration could affect the mother's emotionality differently. The lack of effect in BMS mothers suggest that brief repeated separations from the pups may not be a sufficient stimulus to increase passive stress-coping behavior.

Given that the primary goal of my thesis was to determine whether the permanent loss of the offspring could adversely affect maternal emotionality, I designed a distinct protocol of loss, involving permanent offspring removal. In Chapter 4, the study aimed to investigate the effects of varying periods of permanent offspring loss during the first postpartum week. Each of the groups of separated mothers (S+1, S+3, S+6) were matched for the respective control mothers (C+1, C+3, C+6), which did not experience any separation. Lactation and nurturing behaviors play a crucial role in strengthening the maternal bond in rats. These behaviors not only ensure the physical well-being of the pups but also contribute to the emotional connection between the mother and her offspring (Rickenbacher et al. 2017). Moreover, maternal licking during lactation induces olfactory memory in both the mother and the pups, and this memory forms the basis of recognition and bonding between the mother and her offspring (Lee et al. 1999). The mother's odor becomes familiar and comforting to the pups, enhancing the bond with the mother (Lucion and Bortolini 2014). The activity of hormones like OXT in the mother is associated with maternal behavior, and lactation triggers hormonal changes that influence the mother's protective and caring instincts, strengthening the maternal bond (Rickenbacher et al. 2017). The separated groups of mothers were therefore experiencing 1-day motherhood following the partum, which allowed them to freely nurture the offspring and receiving the pup stimuli, which stimulate OXT release via the milk ejection reflex and induce a robust mother-offspring bond before being separated (Moos et al. 1989).

This study demonstrated that, depending on the duration of the loss experience, orphaned mothers exhibited specific brain (discussed in 6.3) and behavioral impairments. Notably, mothers experiencing 6-day offspring loss had increased floating time in the FST compared to the control group (C+6). No effects were observed when the period of offspring loss was shorter (1 or 3 days). These findings are in line with previous studies conducted in rat mothers, which had pups removed within 24-hours following partum, showing increased floating in the FST even after 3 weeks (Rincon-Cortes and Grace 2021) and 4 weeks following offspring separation (Pawluski et al. 2009b) but highlights that even shorter period of experiencing the loss are sufficient to impact on the mother's emotionality.

Additionally, I investigated the long-term effects of offspring loss on mothers and determined whether the loss of offspring could result in enduring emotional trauma. Therefore, in Chapter 5, I conducted a separate study aimed at exploring the neurobiology of maternal grief when mothers experienced 1-day of motherhood followed by a prolonged 19-day period of permanent offspring loss. Control groups of lactating dams and virgins were included for comparison. The selection of the 19-day offspring loss protocol was based on the hypothesis that mother rats maintain a strong maternal bond, expressing maternal care and behaviors, up to the weaning stage (LD21). The recognition of offspring by rat mothers typically lasts for a limited period, with the maternal bond being strongest during the early postpartum period. The weaning period, when the mother begins to discourage nursing and the pups start to eat solid food, marks a significant transition. During this time, maternal recognition may decrease further as the pups become more independent (Bridges 2015). Here, I successfully demonstrated that, even after experiencing a long-term 19-day period of offspring loss, separated mothers exhibited increased floating behavior compared to control groups being in line with previous studies (Rincon-Cortes and Grace 2021; Pawluski et al. 2009b; Boccia et al. 2007; Maniam and Morris 2010).

Taken together, all studies with different experimental designs (Chapter 3-5) validate the hypothesis (Chapter 2) that rats could be used as an animal model for investigating maternal grief. The various paradigms involving offspring separation and permanent loss underscore the pivotal role of timing in shaping maternal emotionality. Specifically, an extended and repeated period of separation (180 minutes) has been found to notably influence the emotional state of mother rats. Moreover, it has been observed that a complete absence of pup stimuli for a minimum of 6 days is necessary to instigate disruptions in the stress-coping mechanisms of orphaned mother rats. Significantly, these impairments in stress-

coping strategies endure for at least 19 days following the loss of their offspring. Collectively, these studies emphasize the postpartum period as a highly vulnerable phase for the development of emotional disturbances.

6.3 The OXT system and maternal grief

OXT, often known as the "love hormone" is recognized as a central player in the initiation and regulation of maternal behaviors and bonding (Servin-Barthet et al. 2023). It responds to social and reproductive cues, enabling maternal behaviors and nurturing the mother-infant relationship (Marlin et al. 2015; Valtcheva et al. 2023). OXT strengthens the attachment between rat mothers and their offspring. It encourages nurturing behaviors like licking, grooming, nursing, and retrieving pups, which are crucial for the well-being and survival of the offspring (Champagne et al. 2001).

OXT supports recognizing sensory cues, including the ultrasonic vocalizations emitted by the pups (Valtcheva et al. 2023). This communication helps the mother to comprehend her offspring's needs and to respond appropriately. Finally, OXT supports maternal rats in coping with the challenges of caregiving and reduces stress-related behaviors, creating a nurturing and supportive environment for the pups (Klampfl et al. 2018).

In my thesis, across Chapters 3 - 5, I explored the repercussions of distinct protocols of pup separation and permanent offspring loss on the maternal OXT system. In Chapter 3, I conducted a study involving three experimental groups: BMS (15 minutes), LMS (180 minutes), and NMS (control) dams. My aim was to investigate the effects of repeated brief versus long maternal separation on OXT-R binding in various brain regions linked to both the limbic system and the maternal network. The analyzed brain regions included the AIP, AOB, BNST, CeA, dLS, vLS, MPOA, NAcc shell, PL, and VMH. My results revealed a significant increase in OXT-R binding in the MPOA of LMS, but not BMS, dams, with a trend in the PL, compared to NMS control dams. This finding suggests that a 180-min repeated maternal separation experience during the first postpartum week is sufficient to induce alterations in the mother's OXT system, aligning with a study showing increased OXT-R binding in the PFC, MPOA, hipp, LS, NAc shell in rat mothers following 22 days of offspring repeated separation (Stamatakis et al. 2015).

Despite the absence of emotional alterations in LMS or BMS mothers compared to NMS controls, contrary to previous literature findings (Boccia et al. 2007), I observed reduced

maternal motivation in LMS dams. Furthermore, both BMS and LMS dams exhibited elevated levels of pup licking and grooming in comparison to NMS dams. This heightened licking behavior, coupled with an increase in OXT-R binding in the MPOA of LMS dams, suggests a potential compensatory mechanism. This mechanism may be triggered by the distress experienced during a prolonged 180-min offspring separation, causing the mothers to overcompensate for the lack of nurturing and maternal care after being reunited with the pups.

In Chapter 4, I delved into the impact of short-term (1-, 3- or 6-day) offspring loss experiences on the maternal OXT system. Like the approach in Chapter 3, I examined OXT-R binding in the same brain regions. Intriguingly, my findings highlighted a specific reduction in OXT-R binding in the CeA following 1-day and 3-day offspring loss experiences. This study indicates that even short-term pup deprivation, lasting just 1-day or 3-days, is sufficient to influence the brain OXT system. Notably, OXT and OXT-R within the CeA have been associated with the modulation of stress responses and passive-stress coping behaviors, supported by studies like (Ebner et al. 2005) which demonstrated increased OXT release in the CeA of stressed male rats and a study from (Peters et al. 2014), who showed that chronic administration of OXT in the CeA in mice led to a decrease in OXT-R binding. However, the increased OXT-R binding in the CeA following 1- and 3- days of offspring loss could suggest that permanent separation from the offspring acts as an acute stressor for the mother, leading to a potential increase of OXT release in the CeA and, therefore, to a decreased OXT-R binding.

The complex regulation of OXT-R expression and transcription deserves consideration (Jurek and Neumann 2018; Zingg and Laporte 2003). The observed reduction in OXT-R binding in the CeA in Chapter 4 could be attributed to several scenarios. First, altered OXT release following 1-day and 3-day offspring loss experiences might have contributed to decreased OXT-R binding in the CeA via compensatory mechanisms. Alternatively, 1-day and 3-day offspring loss might directly influence OXT-R binding, regardless of changes in OXT release. Future research should, thus, investigate whether the loss of pup stimuli increases OXT protein and mRNA levels in the CeA, specifically.

In Chapter 5, I aimed to explore the impact of long-term offspring loss experience, specifically 19-day offspring loss following 1-day of motherhood. By analyzing the same brain regions as in Chapters 3 and 4, I observed increased OXT-R binding in the PL and VMH of long-term separated mothers. Importantly, it became evident that different durations of offspring loss could modulate differently OXT-R binding in specific brain

regions. Specifically, 19-days of lack of pup stimuli (which encompasses the entire lactation period and the subsequent OXT release due to the milk-ejection reflex) could potentially lead to reduced OXT activity and release from the PVN and SON (Liu et al. 2019), specifically affecting the OXT-R binding of specific brain regions such as PL and VMH, integral components of the limbic system.

Both PL and VMH regions have established roles in modulating maternal behaviors (Fang et al. 2018; Febo 2012). Consequently, it is plausible that the observed increase in OXT-R binding implies a compensatory mechanism in response to the absence of pup stimuli. Further research should address and verify this by analyzing OXT concentrations in these regions and examining whether the activity of OXT + neurons in the PVN is affected by offspring loss, thus resulting in a reduced OXT release in the PL and VMH. In addition, analyses of OXT-R mRNA should be conducted to provide a better picture of the overall effects in all analyzed regions, which could not be visible via receptor autoradiography and cannot be excluded to be impacted by offspring loss.

Previous research has explored the antidepressant effects of OXT and its involvement in mood regulation and the modulation of the HPA axis, a central component of the stress response (Arletti and Bertolini 1987; Windle et al. 1997; Neumann et al. 2000). Although the precise mechanisms underlying OXT's antidepressant effects are not fully understood, its role in modulating social behavior and its potential to reduce stress reactivity have been proposed as possible contributors to its therapeutic effects in depression.

In Chapter 5, I sought to verify the potential antidepressant role of OXT in the 19-day separated mothers. Employing a modified FST, which is a better paradigm for assessing antidepressant drugs (Slattery and Cryan 2012), I administered icv OXT agonist to a group of separated mothers, comparing their responses to control vehicle mothers and separated vehicle mothers. While I successfully replicated in vehicle-treated mothers that separation increased floating behavior, the single-dose OXT administration was insufficient to rescue the impaired behavior. It's worth considering that future research should explore targeted OXT administration in specific brain regions, such as the PL and VMH where specific OXT-R alterations were observed. Additionally, the timing and dosage of OXT agonist administration should be considered. A multi-dose approach, for instance, may better mimic the OXT release induced by pup stimuli in the mother's brain.

In summary, my research, detailed in Chapters 3, 4, and 5, presents compelling evidence supporting OXT signaling impairments resulting from disruptions in maternal bonding. Different scenarios of offspring separation and permanent loss exert distinct effects on

OXT-R binding in specific brain regions. However, the absence of data on OXT levels or OXT-R mRNA expression in these regions constrains our interpretations. In Chapter 3, exposure to intermittent pup stimuli resulted in heightened OXT-R binding in the MPOA, associated with maternal care and licking behavior. Chapter 4 demonstrated that 1-day and 3-day permanent offspring loss reduced OXT-R binding in the CeA, potentially attributable to central OXT release triggered by acute stress induced by offspring loss. Chapter 5 unveiled that long-term offspring loss increased OXT-R binding in the VMH and PL, but a lack of extensive research on maternal grief limits our ability to draw definitive conclusions. This study pioneers the exploration of the impact of long-term offspring loss on the OXT system. Although literature on the VMH's role in maternal behavior is sparse, further investigations should delve into local OXT release and OXT-R mRNA levels in these regions to achieve a comprehensive understanding of OXT signaling in response to pup loss. The observed variations in OXT-R binding between short- and long-term experiences of offspring loss may mirror the distinctions between acute and chronic stress effects in the maternal brain.

6.4 The HPA axis and maternal grief

The activity of the HPA axis plays a pivotal role in the mother-offspring bond. For example, early-life stressors, such as maternal separation, significantly affect the HPA axis of offspring (Orso et al. 2020). However, maternal separation is also a stressor to the mother and can impact her HPA axis functioning and maternal behaviors, too ((Bolukbas et al. 2020; Baracz et al. 2020; Orso et al. 2018).

Dysregulation of the HPA axis has strong associations with the development of psychological disorders in rodents (Mello et al. 2003). The HPA axis comprises a complex network of glands and hormones, including the hypothalamus, pituitary gland, and adrenal glands. When rats face stressors like environmental challenges or social disruptions, the HPA axis gets activated. However, chronic or excessive HPA axis activation can have detrimental effects. In rodents, persistent stress can disrupt the HPA axis equilibrium, leading to elevated CORT levels and altered behavioral responses (Pitman et al. 1988). Such dysregulation is often associated with the development of psychological disorders, including anxiety- and depression-like behaviors as shown in both animal models and humans (Binder and Nemeroff 2010).

As summarized in Chapter 2, it is plausible that offspring loss could act as a stressor, subsequently influencing the activity of the CRF and other stress-related markers within the HPA axis (Demarchi et al. 2021). Consequently, one of the primary objectives of this thesis was to investigate how various protocols of maternal separation and loss might affect the maternal stress response.

In Chapter 3, the focus was to understand whether repeated brief (BMS) or long (LMS) maternal separation could impact the HPA axis. I assessed this by analyzing basal plasma CORT levels and the relative adrenal gland weights in mothers. Surprisingly, I did not observe any differences in basal plasma CORT levels between BMS, LMS, and control groups. This finding contrasts with a previous study in Sprague-Dawley dams where daily brief separations reduced basal CORT levels (Maniam and Morris 2010). However, it's essential to note that I solely collected basal samples and did not measure CORT levels after an acute stressor, which could provide a different perspective on the groups. When evaluating the relative weight of the adrenal gland, I found no significant differences between groups, which is in line with my CORT results, but in contrast to previous studies that show increased adrenal gland weight in prolonged separated mothers (Maniam and Morris 2010; Eklund et al. 2009). A longer maternal separation protocol (2 weeks versus 1 week) might account for the discrepancies.

In the second study, which is presented in Chapter 4, I explored the effects of 1-day, 3-day, or 6-day offspring loss experiences on the maternal HPA axis. I assessed plasma CORT levels under basal conditions and after exposure to the FST. Surprisingly, there were no differences between the groups within the basal or post-stressor plasma CORT levels. This is in contrast to previous studies in Sprague-Dawley dams demonstrating that basal plasma CORT concentration is reduced following 1-day of offspring loss (Kalyani et al. 2017) and increases post-stress following 1-day and 8-day offspring loss (Pawluski et al. 2009a). The discrepancies between those studies and the present results may be due to different experimental protocols regarding the method and time of blood collection, and the timing of pup removal. For example, Pawluski et al. (Pawluski et al. 2009a) analyzed plasma CORT collected from tail nicks between 7.30 a.m. and 9.00 a.m. while I collected trunk blood between 9.00 a.m. and 12.00 p.m. However, Kalyani et al. (Kalyani et al. 2017) analyzed trunk blood samples between 7.30 a.m. and 9.30 a.m. In fact, natural diurnal variations occur in the CORT plasma level that might explain those discrepancies (McCarthy et al. 1960). Moreover, different technics of blood collection might induce higher stress levels, which could affect physiological markers (Kim et al. 2018).

Furthermore, mothers experiencing 6-day loss showed increased passive-stress coping behaviors without alterations in CORT levels, implying that 6 days may be adequate to induce a depressive-like phenotype (Slattery and Cryan 2012) but insufficient to act as a chronic stressor impacting both basal and acute CORT release.

In the conclusive investigation (Chapter 5), I delved into the HPA axis response among mothers enduring prolonged offspring loss lasting 19 days. While these mothers did not manifest changes in anxiety-like behaviors, as evidenced by the LDB test, they did exhibit an increase in passive stress-coping behavior, spending more time in a floating state during the FST. Moreover, analyzing plasma CORT concentrations after exposure to the FST not only affirmed the attenuated stress response during lactation when comparing Virgin with NMS20, but crucially, it unveiled that the CORT levels of LD1+19 mothers had reverted to the levels observed in Virgin mothers. On one hand, this implies that the experience of offspring loss disrupts the mitigated stress response during lactation, as indicated by (Douglas et al. 2003), suggesting that mothers separated from their offspring are more vulnerable to an acute stressor like the FST compared to lactating dams. On the other hand, one cannot ignore the possibility that the stress physiology of separated mothers returned to virgin levels due to the prolonged separation from their offspring and, consequently, the absence of mothering experience.

Moreover, I tested the hypothesis that the altered HPA axis response and emotionality was due to the increased central CRF signaling. Importantly, the pharmacological intervention targeting the CRF system by administering a synthetic CRF-R1/2 general antagonist (D-Phe) via icv infusion in separated mothers effectively rescued the impaired behavior. These findings align with previous research in our lab, demonstrating the reversal of increased passive-stress coping observed in abandoned prairie vole mothers following partner loss (Bosch et al. 2018) and reduced anxiety in rat dams (Klampfl et al. 2013) with D-Phe icv infusion.

Specifically, Chapter 3-5 show that (i) BMS and LMS mothers did not display altered HPA axis responses, (ii) up to 6- day of permanent offspring loss did not alter HPA axis responses of the mothers, (iii) 19-day of permanent offspring loss disrupted the typically dampened stress response observed during lactation, and (iv) central blockade of CRF-R1/2 rescued the impaired phenotype in the FST.

Overall, the HPA axis response was found robustly impaired following a long-term permanent offspring loss, only. The altered passive stress-coping behavior was found strictly associated with a heightened central CRF signaling. This is the first study showing

a direct link in rescuing the impaired emotionality via CRF-R1/2 central blockage in orphaned mothers, providing further insight into novel directions for pharmacological therapies to attenuate grief-related symptoms.

6.5 The neuroplasticity and maternal grief

The maternal brain network in rats undergoes remarkable neuroanatomical transformations, primarily characterized by alterations in dendritic spine numbers, across pregnancy and the postpartum period (Servin-Barthet et al. 2023). This phenomenon is particularly pronounced in regions such as the PFC, Hipp, MPOA, and NAcc, where increased dendritic spine density has been documented during the postpartum phase in comparison to nulliparous females (Kinsley et al. 2006; Hillerer et al. 2018; Shams et al. 2012; Haim et al. 2014).

In Chapter 5, I explored the enduring impact of offspring loss on maternal brain neuroplasticity. My primary focus was to investigate structural brain plasticity by evaluating dendritic spine density. Previous research has established a robust association between depression and a reduced number of dendritic spines, as evidenced in post-mortem human brain tissues and animal models (Duman and Duman 2015). Therefore, it was crucial to concentrate on specific brain regions interconnected with the limbic system, leading me to focus on the PL, BLA, VMH, and AIP.

Examining dendritic spines in lactating rats introduced complexity into the study's objective due to documented structural brain changes during the peripartum period when compared to virgin females (Servin-Barthet et al. 2023). Consequently, I introduced a cohort of virgin females as an additional control group. Intriguingly, at LD20 (the time of sacrifice for all groups of dams), no disparities were detected among control lactating dams, Virgin group, or separated mothers in the BLA, AIP, or PL regions. The significant distinction emerged in the VMH, only, where the group of separated mothers (LD1+19) exhibited decreased spine density compared to both the control lactating dams and virgins. In the face of chronic stress and social isolation, alterations in dendritic spine morphology have been identified in the BLA, PL, and AIP (Sequeira and Gourley 2021; Radley et al. 2006; Mitra et al. 2005). Significantly, an extensive body of research highlights a consistent association between reduced spine density and depression, as evidenced by studies such as Qiao and colleagues (Qiao et al. 2016). Human studies further affirm this link, establishing

a connection between low spine density and depressive symptoms (Holmes et al. 2019). Considering the well-established role of the VMH in various physiological processes such as aggression, stress response, and pain attacks in both humans and rats (Borszcz 2006; Wilent et al. 2010), I propose that the VMH likely contributes to the detrimental effects of offspring loss on maternal well-being. It's noteworthy that the VMH stands out as one of the few anatomically preserved brain structures across mammalian species. Its involvement in the regulation of diverse aspects of energy homeostasis, as demonstrated by (Hirschberg et al. 2020), coupled with its intricate interplay with sexual hormones, including progesterone and estrogen (Correa et al. 2015), underscores its significance in understanding the complexities of the mother's well-being. Estrogen, in particular, play a crucial role in modulating the dendritic structure of neurons in the VMH. Studies have demonstrated that ovarian hormones, including estradiol, induce significant changes in the dendritic architecture of VMH neurons, i.e., estrogen selectively induces dendritic spines within the dendritic arbor of VMH neurons, contributing to dendritic remodeling (Calizo and Flanagan-Cato 2000). Therefore, in the VMH, my investigation extended to scrutinize the expression of cells positive for the ESR1 receptor and calbindin. Remarkably, no significant differences were observed among the groups, leading to the conclusion that the alterations in spine density are likely unrelated to variations in the number of these ir+ cells. I hypothesize that factors beyond hormonal changes, possibly including increased OXT-R binding, may contribute to the observed reduction in spine density. In fact, prior research has already unveiled associations between OXT and spine density in the same region (Ferri and Flanagan-Cato 2012).

6.6 The limbic system and maternal grief

The limbic system is a complex network of brain structures that plays a crucial role in the regulation of emotions and emotional responses in rats (Mu et al. 2020). Emotion dysregulation in rats refers to the impaired ability to modulate and manage emotional responses effectively. The core objective of my thesis was to unravel the neurobiological traces of grief. To accomplish this, I chose to investigate specific brain regions known for their involvement in emotional processes.

In the first study (Chapter 3), I subjected mothers to an extended 180-minute repeated separation protocol (LMS). This manipulation resulted in a noteworthy increase in OXT-R

binding in the MPOA and a trend towards increased binding in the PL among separated mothers. Both the MPOA and PL have been involved in the modulation of maternal behaviors and anxiety-like behaviors ((Zhang et al. 2021); (Stern et al. 2010); (Sabihi et al. 2014)). In the subsequent investigation (Chapter 4), I extended my analysis to mothers experiencing various durations of offspring loss. I conducted an analysis of basal neuronal activity in the PL and amygdala, encompassing the BLA, CeA, and MeA. Already one day of offspring loss led to increased basal neuronal activity of both the superficial and deep layers of the PL. At the same time, the neuronal activity in the BLA was upregulated. Notably, a positive linear relationship between the neuronal activities of PL and BLA was observed in the separated mothers, while a negative correlation was evident in the control mothers. These findings suggest that the experience of offspring loss, ranging from up to six days, significantly modifies the basal neuronal activity of limbic regions in the mother's brain. Previous research has highlighted the involvement of the PL and BLA in modulating stress response and emotions in rodents (Barfield et al. 2020; Grossman et al. 2022; Lidhar et al. 2021; Sharp 2017). Interestingly, a recent study demonstrated that human mothers who experienced the loss of their child exhibited increased activity in the anterior cingulate cortex (Kark et al. 2022b), which is the analogous region to the PL in rodents (VanElzakker et al. 2014). PL and BLA are reciprocally connected, and, together, participate in the regulation of depression. As discussed above, in the third study (Chapter 5), I conducted an analysis of OXT-R binding in limbic regions of mothers experiencing long-term offspring loss. The investigation unveiled increased OXT-R binding in the PL and VMH in the orphaned mothers. Notably, among LD1+19 mothers, a reduction in secondary dendritic spine density was observed specifically in the VMH. This discovery aligns with prior research highlighting the significant role of OXT in modulating dendritic spines in the VMH, as demonstrated by Ferri and colleagues (Ferri and Flanagan-Cato 2012).

These findings provide valuable insights into how offspring loss affects the mother's limbic system, with particular emphasis on the PL, CeA, BLA, and VMH regions. Notably, some of these observations parallels with human studies employing fMRI. For example, grieving mothers, when exposed to images of their deceased children during fMRI scans, exhibited heightened neuronal activity in the amygdala and the anterior cingulate cortex (Kark et al. 2022a; Fernandez-Alcantara et al. 2020).

6.7 Translational aspects

In the studies presented in this thesis, several findings hold significant translational implications for humans. Notably, alterations in the OXT system have captured primary interest. In humans, alterations in maternal attachment, such as postpartum blues or grief, are often associated with fluctuations in plasma OXT levels (Skrundz et al. 2011; Chen et al. 2022), yet data regarding OXT-R binding in post-mortem human tissues remain limited. Moreover, a recent study showed for the first time an elevated plasma OXT level specifically in PGD patients (Bui et al. 2019). While psychotherapeutic interventions for PGD are available (Shear et al. 2016) no pharmacotherapy is currently available for PGD, and the OXT system may be a potentially promising target or future research. In Chapter 3, LMS dams showed increased OXT-R binding in the MPOA and PL, together with increased licking behavior. Moreover, orphaned mothers exhibited reduced OXT-R binding in the CeA after short-term offspring loss (1-day and 3-day) and increased binding in the VMH and PL following long-term loss (19-days). These results suggest a nuanced connection between OXT system dysregulations and the presence or absence of pup stimuli, with distinct limbic regions showing varying responses over time. Together, this thesis provides insights on the possible involvement of altered OXT signaling and maternal mood disorders in response to mother-offspring bond disruption in rats.

Secondly, dysregulations leading to postpartum mood disorders have often been attributed to hyperactivity of the HPA axis (Slattery and Neumann 2008; Brummelte and Galea 2010). In Chapter 5, long-term separated mothers exhibit altered stress responses, evidenced by elevated plasma CORT levels following an acute stressor (FST). This finding led to the hypothesis of an increased CRF central signaling. Notably, the pharmacological blockade of CRF-R1/2 ameliorated impaired passive-stress behavior, clearly demonstrating the hyperactivity of the CRF central system in orphaned dams. This finding holds clinical relevance, as numerous studies have linked grief to increased cortisol levels (Mason and Duffy 2019; Hopf et al. 2020; O'Connor et al. 2012). For instance, Roy and colleagues (Roy et al. 1988) provided the first evidence of PGD affecting cortisol levels, in fact, participants with PGD showed significantly higher basal plasma cortisol levels and significantly smaller ACTH responses to CRF then controls. Consequently, the CRF-R general antagonist examined in Chapter 5 holds promise for mitigating the negative emotional symptoms associated with offspring loss, offering a potential treatment avenue for individuals coping

with grief. Several clinical studies are in fact already exploring different drugs that selectively block CRF-R (O'Brien et al. 2001; Ding et al. 2021).

6.8 Conclusions and perspective

In summary, this thesis has delved into the intricate connection between maternal bond disruption and maternal emotionality. Chapter 2 laid the groundwork by proposing the involvement of the OXT and CRF systems in shaping grief-related symptoms, revealing a critical gap in the existing research on the neurobiology of maternal grief in rodent models. Chapter 3, while not replicating previous findings, prompted a shift toward a more refined experimental approach involving permanent offspring removal. This strategic pivot proved crucial in Chapter 4, where the examination of varying periods of offspring loss unveiled a nuanced understanding—the duration of loss emerged as a key determinant in maternal emotional impairment. The exploration continued in Chapter 5, where an extended 19-day period of permanent offspring loss revealed the enduring effects of maternal grief. The observation that mothers displayed heightened floating behavior even after this prolonged separation underscores the persistence of maternal distress long after the initial loss in mother rats, replicating previous studies (Rincon-Cortes and Grace 2021; Pawluski et al. 2009b).

These findings underscore the necessity of considering the duration of offspring loss when assessing its impact on maternal emotionality and stress responses in rat dams. Future research should expand on this by delving into specific brain regions and circuitries implicated in distress and emotion regulation. This includes a deeper understanding of the PL-BLA circuitry, exploring subpopulations of neurons and neurochemicals involved. Additionally, an in-depth analysis of the VMH, a region significantly affected by loss, and its role in modulating passive stress-coping behavior is warranted. Lastly, a focused examination of neuropeptide release within specific brain regions could offer a more comprehensive picture of the biodynamic changes beyond OXT-R binding and increased CRF signaling.

The implications of this research extend to the broader context of maternal experiences, shedding light on the profound emotional journey that mothers undergo when faced with the heart-wrenching reality of offspring loss. In a wider scope, the insights gained from studying the complex emotional experiences of mothers may contribute to a better

understanding of human maternal grief, potentially informing therapeutic approaches and interventions.

As this thesis concludes, the hope is that the findings presented here will inspire further research, fostering a deeper understanding of the emotional lives of mothers — both within the animal kingdom and among humans. Through compassionate consideration and continued investigation, we may uncover new avenues for support and intervention, ultimately contributing to the well-being of mothers facing the challenges of maternal grief.

Abbreviations

ABN	Arched-back nursing
ACTH	Adrenocorticotropic hormone
AIP	Agranular insular cortex
AOB	Accessory olfactory bulbs
AON	Anterior olfactory nucleus
BLA	Basolateral amygdala
BMS	Brief maternal separation
BNST	Bed nucleus of the stria terminalis
CeA	Central amygdala
ACC	Anterior cingulate cortex
CNS	Central nervous system
CORT	Corticosterone
CPu	Caudate putamen
CRF	Corticotropin-releasing factor
CRF-R	Corticotropin-releasing factor receptor
DRN	Dorsal raphe nucleus
ELISA	Enzyme-linked immunosorbent assay
EPM	Elevated plus maze
ERPs	Event-related potentials
ESR1	Estrogen receptor α
fMRI	Functional magnetic resonance imaging
FST	Forced swim test
HDB	Nucleus of the horizontal limb of the diagonal band
HPA	Hypothalamic-pituitary-adrenal axis
Icv	Intracerebroventricular
IHC	Immunohistochemistry
Ir+	Immuno-reactive
LC	Locus Coeruleus
LD	Lactation Day
LDB	Light dark box test

LG	Licking and grooming
LHb	Lateral habenula
LMS	Long maternal separation
LS	Lateral septum
MC	Maternal care
MDD	Major depressive disorder
MeA	Medial amygdala
MOB	Main olfactory bulb
MOE	Main olfactory epithelium
mPFC	Medial prefrontal cortex
MPOA	Medial preoptic area
NAcc	Nucleus accumbens
NMS	Non-maternally separated
OF	Open field
OT	Olfactory tubercle
OVLT	Organum vasculosum laminae terminalis
OXT	Oxytocin
OXT-R	Oxytocin receptor
PAG	Periaqueductal gray
PBN	Parabrachial nucleus
PBS	Phosphate-buffered saline
PFC	Prefrontal cortex
PGD	Prolonged grief disorder
PL	Prelimbic cortex
PND	Postnatal day
PP	Posterior pituitary gland
PRT	Pup retrieval test
PTSD	Post-traumatic stress disorder
PV	Paraventricular nucleus of the thalamus
PVN	Paraventricular nucleus of the hypothalamus
SN	Substantia nigra
SON	Supraoptic nucleus
SUM	Supramammilary nucleus

Ucn	Urocortin
VMH	Ventromedial hypothalamus
VNO	Vomeronasal nucleus
VP	Ventral Pallidum
VTA	Ventral tegmental area

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Curriculum Vitae

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Education

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2017-2020 — M.Sc. in Biology, University of Turin, Turin, Italy
Thesis: *Extracellular components in neuroplasticity and behavior.* Supervised by Prof. Carola Eva and Dr. Ilaria Bertocchi

2013-2017 — B.Sc. in Biology, University of Turin, Turin, Italy
Thesis: *Quantification of BRAFV600E mutation in melanoma.* Supervised by Prof. Tiziana Venesio.

2008-2013 — High school diploma, Liceo Scientifico G. Peano, Cuneo, Italy

Awards/Grants

International conference travel grant 2023 British Society of Neuroendocrinology
Parental brain 2022 Young Investigator Award, sponsored by the Journal of Neuroendocrinology
Winner of the Wiki Science Competition Italy 2019 for the best microscopy image
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Conferences

2023 Neurobiology of grief international network (NOGIN) workshop (talk)
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2022 Parental Brain 7th International meeting of neuroscience (talk)
2022 Neurobiology of grief international network (NOGIN) virtual conference
2022 The 3rd Munich Winter Conference on Stress (poster)
2021 Neurobiology of grief international network (NOGIN) virtual conference
2020 FENS virtual conference

Publications

Demarchi L, Sanson A, Bosch OJ (2023) Neurobiological traces of grief: examining the impact of offspring loss after birth on rat mothers' brain and behavior in the first week postpartum. In preparation, submission in December 2023

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Demarchi L, Sanson A, Bosch OJ (2023) Brief versus long maternal separation in lactating rats: Consequences on maternal behavior, emotionality, and brain oxytocin receptor binding. J Neuroendocrinol e13252.

Sanson A, **Demarchi L**, Bosch OJ (2023) Neuroendocrinology of Behavior and Emotions. Book chapter. Submitted and accepted by Springer. 2023. To be published as part of the bookseries 'Masterclass in Neuroendocrinology'.

Delgado-García JM, Cambiaghi M, Bertocchi I, González A, Sánchez Ruiz A, Carretero-Guillén A, Kortabarria G, Torres Durán N, Leal-Campanario R, Dogbevia GK, **Demarchi L**, Larkum ME, Sprengel R, Perea G, Gruart A, Hasan MT (2023) Astrocyte NMDA receptors differentially modulates negative and positive reinforcement in learning and memory processes. Submitted

Demarchi L, Pawluski JL, Bosch OJ (2021) The brain oxytocin and corticotropin-releasing factor systems in grieving mothers: What we know and what we need to learn. Peptides 143:170593.

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