The brain vasopressin system mediates maternal behaviour in lactating rats - impact of V1b receptors in hypothalamic and limbic brain regions

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“It doesn’t matter how beautiful your theory is, it doesn’t matter how smart you are.
If it doesn’t agree with experiment, it’s wrong.”

– Richard Feynman, physicist, Nobel laureate (1918–1988)

Declaration of included Manuscripts

Chapter 2: Central V1b receptor antagonism in lactating rats impairs maternal care but not maternal aggression.

Authors' contribution:

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

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Chapter 3: Maternal behaviour in two breeding lines for extremes in anxiety – past and present observations in HAB/LAB dams with special focus on V1b receptors

Authors' contribution:

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Chapter 4: Antagonism of V1b receptors promotes maternal motivation to retrieve pups in the MPOA and impairs pup-directed behaviour during maternal defence in the mpBNST of lactating rats.

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Chapter 5: Vasopressin V1a, but not V1b, receptors within the PVN of lactating rats mediate maternal care and anxiety-related behaviour

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Chapter 6: More than reproduction - central blockade of GnRH and kisspeptin decrease maternal behaviour in lactating rats

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Abstract

Adequate maternal behaviour offers the best chance for the offspring to survive to maturity. This pro-social behaviour is known to be regulated by the nonapeptide arginine-vasopressin (AVP) amongst other peptidergic and non-peptidergic systems in rodents as well as in humans. Most research regarding the AVP system in maternal behaviour has focussed on the peptide itself and its V1a receptor (V1aR), and revealed a pivotal involvement in the regulation of different aspects of maternal behaviour. In addition, the involvement of AVP and its V1aR was investigated in the animal model of rats selected for extremes in anxiety-related behaviour. Highly anxious dams (HAB) have a single nucleotide polymorphism in the AVP promoter-region, resulting in increased expression and release of AVP. Regarding the phenotype, HAB dams show a hyper-protective mothering style in contrast to dams selected for low anxiety-related behaviour (LAB), which only provide a low amount of maternal behaviour. Since the maternal phenotype of HAB and LAB dams can be partly reversed by blockade or infusion of V1aR or synthetic AVP, respectively, these rats provide a good model to observe the behavioural consequences after pharmacological manipulation of the AVP system on maternal behaviour.

The influence of V1b receptors (V1bR) on mediating maternal behaviour has only been investigated in a single study in V1bR knockout mice to date. This study showed that V1bR knockout dams show decreased maternal aggression compared to wildtype dams.

Therefore, I aimed to investigate in more detail the role of central V1bR on the maternal repertoire *via* acute central application of V1bR agonist and antagonist during lactation in non-selected rats (Chapter 2) as well as in the animal model of rats selected for extremes in anxiety-related behaviour (Chapter 3). Although the V1bR is widely distributed within the brain, I aimed to investigate the involvement of the receptor subtype in brain-regions known to be involved in maternal behaviour, including the medial preoptic area (MPOA), the bed nucleus of the stria terminalis (BNST) and the paraventricular nucleus (PVN) *via* acute local infusion of V1bR antagonist. In addition, I determined whether endogenous V1bR protein and/or mRNA expression differ in lactation within these brain regions (Chapter 4 -5).

Further, in a pilot study co-expression of AVP and gonadotropin-releasing hormone (GnRH) immune-reactivity in the organum vasculosum of the lamina terminalis (OVLT) in lactating rats was observed. Since mice with a 30 % decrease of GnRH neurons show impaired pup retrieval, and the co-localization suggests an interaction with the AVP system known to be

involved in maternal behaviour, I was interested in the influence of the GnRH and the interconnected kisspeptin system in the regulation of maternal behaviour in rodents (Chapter 6).

To investigate my aims I used a variety of cellular, molecular and behavioural approaches like quantitative real-time PCR, western blotting, central or local acute pharmacological manipulation of V1bR, GnRH receptors or kisspeptin receptors followed by observations of maternal care, maternal motivation to retrieve pups, maternal aggression and anxiety-related behaviour.

In this thesis I showed that central blockade of V1bR in non-selected rats impaired maternal care but did not affect maternal motivation, maternal aggression or anxiety-related behaviour. V1bR manipulation in HAB and LAB dams had no effect on any maternal behaviour. When investigating the role of V1bR in specific brain regions, I could show that blockade of V1bR within the MPOA increased maternal motivation whereas within the BNST maternal aggression, more precisely pup-directed behaviour, was decreased. Additionally, V1bR blockade in both brain regions decreased nursing, but only under stress conditions. Interestingly, in the PVN only V1aR, but not V1bR blockade decreased nursing under non-stress conditions in addition to maternal anxiety.

Thus, both V1R subtypes seem to be involved in the regulation of maternal behaviour in a brain region-dependent and context-specific manner, thereby acting exclusively, as complement or as counterpart of the other receptor subtype. Interestingly, this was not associated with alterations in mRNA or protein expression in any brain-region, suggesting that either transport of the receptor-subtype to the membrane, signal transduction or agonist sensitivity may be altered in lactation, which remains to be investigated.

Regarding the involvement of GnRH and kisspeptin in the regulation of maternal behaviour, blocking GnRH decreased maternal aggression, whereas kisspeptin receptor (GPR54) blockade resulted in impaired maternal care, in a dose-dependent manner. These results provide evidence, that the GnRH and kisspeptin system are not only involved in reproduction, but also in various aspects of maternal behaviour. Further investigation of their role, especially in maternal brain circuits, is essentially required.

In conclusion, the presence of V1bR in brain regions involved in maternal behaviour are mandatory in lactating rodents to show adequate maternal behaviour, especially in stressful situations. Further, maternal behaviour is regulated by a variety of hormones acting centrally

within the brain like oxytocin and AVP, whereby hormones like GnRH and kisspeptin, thought to be mainly responsible in reproduction also influence maternal behaviour.

Taken all these results into account I could provide further knowledge of the impact of central hormonal systems on the complexity of regulatory mechanism in maternal behaviour.

Zusammenfassung

Adäquates mütterliches Verhalten ermöglicht den Nachkommen die bestmögliche Chance bis zum Erwachsenenalter zu überleben. Dieses prosoziale Verhalten wird sowohl im Nager als auch im Menschen, unter anderem durch das Nonapeptid Arginin-Vasopressin (AVP), reguliert.

Im Zusammenhang mit mütterlichem Verhalten lag der Forschungsschwerpunkt bezüglich des AVP-Systems bis jetzt auf dem Peptid selbst, sowie seinem im Gehirn exprimierten V1a Rezeptor (V1aR). Beide sind ein essentieller Bestandteil in der Regulierung von verschiedenen mütterlichen Verhaltensweisen. Des Weiteren wurde der Einfluss von AVP und seinem V1aR im Tiermodell für Ratten, die auf die Extreme im Angstverhalten selektiert sind, untersucht. Rattenmütter mit einem hohen Maß an Angstverhalten (HAB) haben einen Einzelnukleotid-Polymorphismus (*single nucleotide polymorphism*, engl.) in der AVP Promoter-Region, der zu einer erhöhten AVP Expression und Ausschüttung führt. Bezüglich des Phänotypen, kennzeichnen sich HAB-Mütter durch ein Übermaß an Fürsorge, im Gegensatz zu Rattenmüttern die auf niedriges Angstverhalten selektiert sind (LAB). Diese zeigen nur ein geringes Maß an mütterlichen Verhaltensweisen. Da der mütterliche Phänotyp der HAB und LAB Ratten durch eine Blockade von V1aR bzw. eine Injektion von synthetischem AVP verändert werden kann, eignet sich dieses Tiermodell besonders gut, um die Auswirkungen pharmakologischer Manipulationen des AVP Systems auf mütterliches Verhalten zu beobachten.

Der Einfluss von V1b Rezeptoren (V1bR) auf mütterliches Verhalten wurde bis jetzt nur in einer einzigen Studie untersucht. Diese zeigt, dass V1bR Knockout-Mäuse im Vergleich zu Wildtyp-Mäusen eine verringerte mütterliche Aggression zeigen.

Aufgrund dieser unzureichenden Studienlage war es ein Ziel meiner Arbeit, den Einfluss der zentralen V1bR auf die unterschiedlichen mütterlichen Verhaltensweisen während der Laktation in nicht-selektierten Ratten (Kapitel 2), sowie im Tiermodell für Ratten, die auf die Extreme im Angstverhalten selektiert sind (Kapitel 3), mit Hilfe akuter zentraler Verabreichung eines V1bR Agonisten sowie Antagonisten genauer zu untersuchen. Obwohl der V1bR weitflächig im Gehirn exprimiert wird, war es ein weiteres Ziel meiner Arbeit den Einfluss des Rezeptors auf mütterliches Verhalten, mit Hilfe von akuten lokalen Injektionen eines V1bR Antagonisten in das mediale präoptische Areal (MPOA), in den *bed nucleus* der *Stria terminalis* (BNST) und in den paraventrikulären Nukleus (PVN), zu untersuchen. Diese

Gehirnregionen sind nachweislich an der Regulation von mütterlichem Verhalten beteiligt. Des Weiteren, habe ich erforscht, ob sich die Menge an endogenem V1bR Protein und/oder die mRNA Expression in diesen Gehirnregionen während der Laktation unterscheiden (Kapitel 4 und 5).

Auf Basis einer Pilotstudie wurde außerdem im *organum vasculosum* der *lamina terminalis* (OVLT) laktierender Ratten eine Koexpression von AVP und des Gonadotropin-releasing Hormons (GnRH) beobachtet. Da Mäuse mit 30 %iger Reduzierung von GnRH Neuronen ein vermindertes Einsammeln ihres Nachwuchses zeigen, und die Kolokalisation einen Zusammenhang mit dem AVP System, das nachweislich mütterliches Verhalten beeinflusst, vermuten lässt, war ich ausserdem am Einfluss des GnRH- und des eng verbundenen Kisspeptin-Systems auf mütterliches Verhalten im Nager interessiert (Kapitel 6).

Zur Untersuchung meiner Ziele habe ich eine Reihe an zellulären, molekularen sowie verhaltensbasierten Methoden angewendet, wie z.B. *quantitative real-time PCR*, Western Blot, zentrale oder lokale akute pharmakologische Manipulation von V1bR, GnRH- oder Kisspeptin-Rezeptoren, gefolgt von Verhaltensbeobachtungen (mütterliche Fürsorge, mütterliche Motivation zum Einsammeln der Jungen, mütterliche Aggression und Angstverhalten).

In meiner Arbeit konnte ich zeigen, dass die zentrale Blockade von V1bR in nicht-selektierten Ratten zu einer verringerten mütterlichen Fürsorge führt, ohne dabei die mütterliche Motivation, mütterliche Aggression oder das Angstverhalten zu beeinflussen. Die Manipulation von V1bR in laktierenden HAB und LAB Ratten hatte keine Auswirkungen auf etwaige mütterliche Verhaltensweisen. Eine gehirnregionspezifische Blockade von V1bR in der MPOA führte zu einer gesteigerten mütterlichen Motivation zum Einsammeln der Jungen, während die Blockade im BNST die mütterliche Aggression, genaugenommen die Aufmerksamkeit, die dem Nachwuchs während des Aggressionstests entgegengebracht wurde, verringert war. Zusätzlich führte die V1bR Blockade in beiden Gehirnregionen zu einem verminderten Säugeverhalten, jedoch ausschließlich unter Stressbedingungen. Interessanterweise veranlasste im PVN nur die Blockade von V1aR, jedoch nicht von V1bR, ein niedrigeres Säugeverhalten unter stressfreien Bedingungen, sowie ein geringeres Angstverhalten.

Daraus lässt sich folgern, dass beide V1R-Subtypen in einer gehirnregions- sowie kontextspezifischen Art und Weise an der Regulation von mütterlichem Verhalten beteiligt

sind. Das resultierende Verhalten erfolgt daher aus der Wirkungsweise eines einzelnen Rezeptorsubtypen, dem Zusammen- oder aber auch dem Gegenspiel beider Rezeptorsubtypen. Allerdings standen die Verhaltensänderungen nicht im Zusammenhang mit der mRNA- oder Protein-Expression. Dies lässt eine Anpassung im Transport des Rezeptors zur Zellmembran, in der Signalweiterleitung oder in der Sensibilität des Agonisten vermuten, was untersucht werden muss.

Hinsichtlich des Einflusses von GnRH und Kisspeptin auf mütterliches Verhalten führte die Blockade von GnRH zu verminderter mütterlicher Aggression, während die Blockade von Kisspeptin Rezeptoren (GPR54) dosisabhängig zu einer verringerten mütterlichen Fürsorge führte. Diese Ergebnisse zeigen deutlich, dass sowohl das GnRH- als auch das Kisspeptin-System nicht nur in der Fortpflanzung, sondern auch in verschiedenen Aspekten von mütterlichem Verhalten eine Rolle spielen. Die weiterführende Erforschung beider Systeme, insbesondere im Zusammenhang mit mütterlichen Verhaltensweisen, ist von großem Interesse.

Es ergibt sich die Schlussfolgerung, dass die V1bR in Gehirnregionen, die mütterliches Verhalten modulieren, zwingend notwendig sind, um angemessenes mütterliches Verhalten vor allem unter Stressbedingungen zu garantieren. Des Weiteren wird mütterliches Verhalten von zahlreichen Hormonen im Gehirn, wie Oxytocin und AVP, reguliert, wobei aber auch Hormone wie GnRH und Kisspeptin, die bis jetzt hauptsächlich dem Fortpflanzungsverhalten zugesprochen wurden, mütterliches Verhalten beeinflussen.

Somit konnte ich mit meiner Arbeit das Wissen über den Einfluss zentraler Hormonsysteme auf die Regulation komplexer Mechanismen, die mütterliches Verhalten regulieren, erweitern.

Chapter 1

General Introduction

One of the most important objectives in the life cycle of humans and other mammalian species is reproduction and the successful rearing of the young. The latter is promoted by parental behaviour, which was defined by Numan and Insel as “any behaviour of a member of a species towards a reproductively immature conspecific that increases the probability that the recipient will survive to maturity” (for review see Numan and Insel, 2003). About 90 % of species show uniparental care, with the mother taking care for the offspring which is due to the fact that in mammals only the females provide milk. This is essential for the young as they would die without the nutrition. In preparation for motherhood the female undergoes numerous physiological, cellular and molecular adaptations to be prepared for the upcoming birth, lactation and defence of the young against potential threats. These changes occur as a direct consequence of both hormonal and non-hormonal alterations that occur throughout the peripartum period. All the more remarkable as in most mammalian species females are not maternal *per se* I want to give insight in these changes that lead to maternal behaviour in more detail. Due to the fact that these mechanisms are most studied in laboratory rodents, the following descriptions refer to rats unless otherwise stated.

1.1 Maternal Behaviour

1.1.2 Non-hormonal and hormonal basis of maternal behaviour in rats

Nulliparous rodent females naturally avoid pups (Fleming and Rosenblatt, 1974; Rosenblatt, 1967) or even show infanticide (Mennella and Moltz, 1989). Interestingly, these virgins show less infanticide after a distinct co-habituation period with the actual mother of the test pups; after a co-habituation period of 5 to 10 consecutive days 55 % of nulliparous female exhibit pup killing, whereas after 20 days of co-habituation only 9 % show infanticide (Mennella and Moltz, 1989). Also the co-habituation of nulliparous rats with foster pups over a period of 4 to 7 days, leads to tolerance of the pups, which is followed by spontaneous maternal interest, expressed as increased licking of the pups, before retrieving them to a nest and

crouching over them (Fleming and Rosenblatt, 1974; Rosenblatt, 1967; Stern, 1983). This process is known as “sensitization” and occurring as a response to pup stimuli.

In contrast to virgins, primiparous female rats show spontaneous maternal behaviour starting around 3.5 hours before parturition by licking (foster) pups, retrieving them to a nest site and crouching over them to provide milk (Mayer and Rosenblatt, 1984). Such preterm maternal interest in pregnant rats was linked to the hormonal changes, concomitant to non-hormonal adaptations, occurring during the peripartum period and inducing maternal behaviour.

The steroid hormones oestradiol and progesterone - together with lactogenic hormones like prolactin (PRL) and placental lactogens (see below) - are the major factors implicated in the regulation of maternal behaviour in rats (Bridges et al., 1996; Grattan et al., 2001; Southard and Talamantes, 1991). Thereby, oestradiol levels are low during the first trimester, before they start rising on pregnancy day (PD) 10 and peak at a level, 3- to 4-fold higher than at the start of pregnancy, from day 15 until the day of parturition in rats (see Figure 1). In contrast, progesterone is already high during the first two weeks of pregnancy and declines with a robust drop (to 1/6 of magnitude) during the last days of gestation. Finally, a reversal of the oestradiol-to-progesterone ratio occurs leading from long progesterone dominance to a short oestradiol dominance (for reviews see Bridges, 2014; Numan and Insel, 2003).

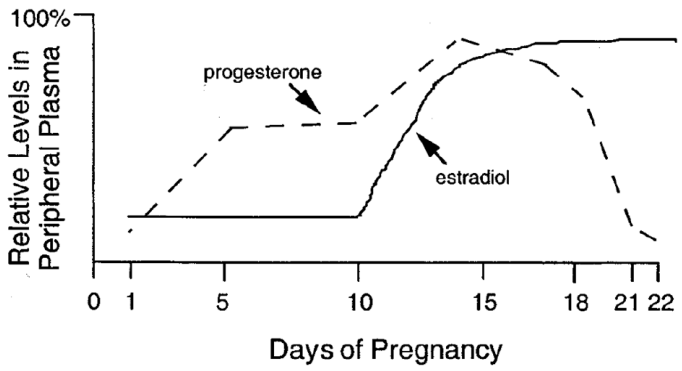

Figure 1 Relative levels of oestradiol and progesterone in blood plasma throughout pregnancy in rats.
The percentage of the maximum value detected during pregnancy is shown.
Adapted from Numan, 2003.

Lactogenic hormones are secreted throughout pregnancy and are either derived from the anterior pituitary (PRL) or from the placenta (placental lactogens e.g. placental lactogen I and II) (Grattan, 2001). PRL is released in two daily surges within the first half of pregnancy and on the last day of pregnancy, whereas during the second half of pregnancy plasma levels of placental lactogens increase (Cohick et al., 1996; Southard and Talamantes, 1991). The

dominance of oestradiol, together with the drop of progesterone and increase of PRL at the end of pregnancy are important hormonal adaptations which prime the neural circuits within the medial preoptic area (MPOA) and the bed nucleus of the stria terminalis (BNST) among others. These brain regions are also termed “maternal circuits” and facilitate the appropriate onset of maternal behaviour (Bridges and Ronsheim, 1990; Bridges et al., 1997, 1996).

Besides PRL, the neuropeptides oxytocin (OXT) and arginine-vasopressin (AVP) are released within this maternal circuits around parturition, facilitating the adequate onset of maternal behaviour (Numan et al., 1977; Pedersen and Prange, 1979; Pedersen et al., 1994, 1982). The OXT system also adapts during pregnancy and lactation in order to decrease stress responsiveness of the HPA axis (Slattery and Neumann, 2008), i.e. with elevated OXT release within the hypothalamus in response to a stressor (Bosch et al., 2004). In addition, changes in sizing and branching patterns of dendritic trees within the supraoptic (SON) and paraventricular nuclei (PVN), alterations of electrophysiological properties and changes in the synthetic and secretory activity of oxitocinergic neurons take place (for review see Neumann, 2003). All these adaptations provide a dynamic OXT system, which is highly responsive to stimuli in the context of reproduction, i.e. labour and suckling of the pups, but attenuated in response to pharmacological, emotional or physical stressors (for reviews see Neumann, 2003; Slattery and Neumann, 2008).

Similar adaptations during the peripartum period occur within the AVP system, described in more detail below (Chapter 1.8). Before going into detail with the AVP system, I will introduce the distinct maternal parameters, which can be observed in lactating laboratory rodents, and will especially refer to brain regions, which are involved in the modulation of maternal behaviour.

1.1.3 Maternal care and “brain circuitry”

Rodent mothers show a variety of distinct behaviours towards their pups to provide them with milk, warmth and protection against potential threats (for review see Bosch and Neumann, 2012). This care provided by the mother, i.e. maternal care, is one of the most important pro-social behaviours displayed by mammalian mothers and helps to raise the litter to maturity (for review see Bosch, 2011). Therefore, nest-building is an active,

voluntary aspect of maternal behaviour initiated by the dam and mediated *via* oestradiol, PRL, OXT and dopamine within the MPOA in rodents (for review see Numan and Insel, 2003). By licking and grooming their pups, rat mothers enable them to urinate and defecate. Further, it positively influences the social and emotional development of the offspring (Caldji et al., 1998; Champagne, 2008). This behaviour occurs very frequently during the first weeks of life, declining with the growth of the pups and their move to independence (for review see Numan and Insel, 2003). Another important parameter is nursing, which can be subdivided into various distinct nursing postures. The dams show blanket posture to keep the pups warm and safe while lying in a flat position above them. Furthermore, dams will also lay on their side or back during nursing, especially when the pups grow older. Sometimes dams also hover over their litter by resting on their hind limbs while being engaged in licking the pups or mouthing them. The most intense nursing position is termed arched back nursing (ABN; Figure 2), the only active nursing posture with the dam crouching over the pups in a quiescent kyphosis (Stern and Johnson, 1990). Depending on the angle of her back, one can distinguish between low crouch with a flat or slightly arched back, or high crouch with an intensely arched back and splayed legs (Stern, 1996), thereby ensuring the best possibility for all young (12 on average in Wistar rats (Rosen et al., 1987)) to have access to the 12 nipples (for review see Numan and Insel, 2003).

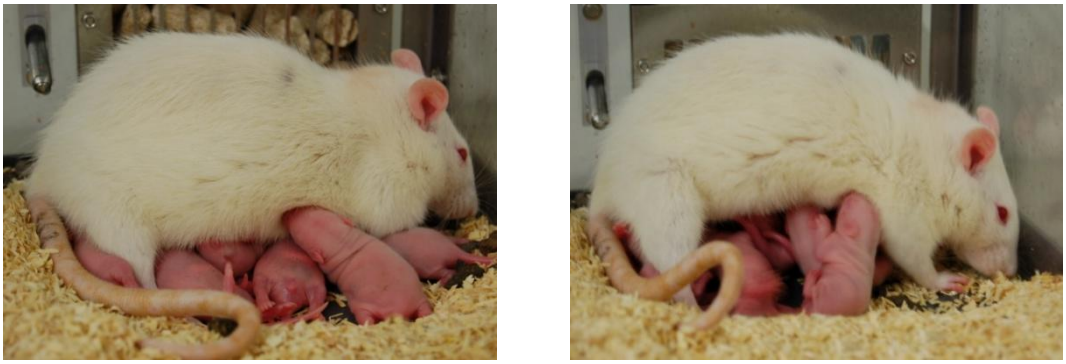

Figure 2 Lactating rat engaged in arched back nursing (ABN).

Dam showing ABN in either low crouch (left) or high crouch (right) position during quiescent kyphosis.

Kyphosis can be seen as a reflex, which is induced by ventral stimulation caused by the nuzzling, rooting pups searching for the nipples (for review see Numan and Insel, 2003). Maintenance of this behaviour, including its relatively long upright crouch durations, depends of the suckling stimulus of the pups, which itself is not essential for the initiation of kyphosis (Stern and Johnson, 1990; Stern, 1991).

The tactile stimulus from the pups provides positive afferent input to the MPOA/BNST region, which facilitates maternal behaviour. Neurons within the dorsolateral MPOA and the ventral BNST (vBNST) form a functional system, called “maternal super-region” (for reviews see Bosch and Neumann, 2012; Numan and Insel, 2003), and are important for the regulation of maternal behaviour in rats (Numan and Numan, 1996). These neurons express oestrogen receptors, which bind oestradiol and lactogenic hormones, thereby facilitating the onset of maternal behaviour in rats (see above)(Bakowska and Morrell, 1997; Numan and Insel, 2003; Pfaff and Keiner, 1973). Further, the maternal super-region receives input from the medial amygdala, which is the relay site of olfactory information from the pups, and thus leading to a fear/avoidance response of pups in virgins or an attraction/approach circuit in dams (for reviews see Numan and Insel, 2003; Numan and Woodside, 2010). Another brain region projecting to the “maternal super-region” is the PVN (Ingram and Moos, 1992). The PVN is known to be important for the onset, but not the maintenance of established maternal care (Consiglio, 1996; Insel and Harbaugh, 1989; Numan and Corodimas, 1985). In addition, it is the main source of oxitocinergic, but also vasopressinergic projections to other brain regions (de Vries and Buijs, 1983). As both the OXT and the AVP system are increased in the peripartum period (see Chapter 1.8), they implicate a role in the regulation of maternal behaviour (Bosch and Neumann, 2008; Bosch et al., 2010; Neumann et al., 1993, 2000; for review see Bosch and Neumann, 2012).

1.1.4 Pup retrieval and “brain circuitry”

During the period when pups depend on their mother, dams show retrieval behaviour if a pup is lying outside the nest. This describes mouthing the pups and carrying them back to the nest if they got scattered or if they are transported to a new nesting site. Pup retrieval is thought to reflect the dam’s motivation to retrieve the offspring to a single nesting-site and is described as a voluntary, proactive and goal-directed appetitive response of the mother (for review see Numan and Insel, 2003). As motivation is referred to an internal process that regulates changes in responsiveness to a constant stimulus, pup cues evoke this response in mothers. A study focussing on the brain regions involved in mediating maternal motivation found that lesions of the MPOA disrupts pup retrieval (Kalinichev et al., 2000). Further, the processing of information occurs via dopaminergic neurons projecting from the

MPOA/vBNST to the nucleus accumbens (NAc) (Numan and Numan, 1996) both directly and indirectly via synapses in the ventral tegmental area (for review see Numan and Insel, 2003). More specifically, lesion of the dorsolateral connections of the MPOA/vBNST by knife-cuts extinguishes pup retrieval (Numan et al., 1990). These findings provide insight in brain regions important in maternal motivation to retrieve pups, which are regulated via the neuropeptides OXT and AVP within the MPOA (Bosch and Neumann, 2008; Bosch et al., 2010; Meddle et al., 2007)(see below for more details).

1.1.5 Maternal aggression and "brain circuitry"

Another adaptation occurring in the postpartum female is increased maternal aggression. This behaviour is not directed towards the pups (Peters et al., 1991), but against any potential threat, which could harm the offspring, like conspecifics.

Maternal aggression can be distinguished from other forms of aggression, like predator aggression, territorial aggression, intermale aggression, defensive aggression or competitive aggression as the trigger to attack the opponent is a different one (Blanchard et al., 2003; Moyer, 1968). This can also be seen by the fact that lactating females show higher aggression towards a male intruder than non-lactating females (Erskine et al., 1980, 1978a).

Maternal aggression first occurs at the end of pregnancy, vanishes at parturition and peaks during the first week of lactation before declining afterwards (Caughey et al., 2011). During this time the dam shows a variety of offensive and defensive aggressive behaviours when confronted with an opponent (Bosch et al., 2005; Erskine et al., 1978a; for review see Lonstein and Gammie, 2002). In the laboratory, this can be tested in the maternal defence test (Neumann et al., 2001). The lactating mother, as resident, is confronted with an unfamiliar virgin female or male intruder, ideally about 10 % smaller than the resident (to act as an stressor but to prohibit a dominance over the resident; for review see Bosch, 2013). This encounter leads to a defensive reaction of the intruder followed by aggressive behaviours of the residents (Blanchard et al., 2001; Haney et al., 1989). Aggressive behaviours shown by the resident include offensive behaviours like attacks, which are normally directed towards the neck or the back of an intruder, sometimes paired with bites. Further, the dam displays threat behaviours, e.g. pushing the intruder from the side termed "lateral threat", or standing with all four paws on top of the intruder, termed "keep down"

(Figure 3). Also boxing the intruder in an “offensive upright” position and “aggressive grooming” of the intruder are considered threat behaviours. Species-specific cues lead to termination of aggression, i.e. defensive behaviour in rats. Thus, as soon as the intruder shows submissive behaviour, the residents’ attacks and threats decrease/stop.

Figure 3 Lactating dam engaged in threat behaviours during the maternal defence test.
Resident dam showing lateral threat (left), keep down (middle) and offensive upright (right) against a virgin female intruder (marked with black stripes).

A variety of extrinsic and intrinsic factors contribute to the occurrence of maternal aggression (for review see Bosch, 2013). The main extrinsic factor is the offspring; the presence of the pups is essential for the initiation of maternal aggression (Gandelman and Simon, 1980). Short-term removal (4 to 5 hours) of the young decreases, but does not suppress, maternal aggression, whereas 24 hours or more do so (Erskine et al., 1978a; Ferreira and Hansen, 1986; for review see Lonstein and Gammie, 2002). Importantly, it is not the suckling stimulus *per se*, but the ventral somato-sensory stimulation (Stern and Kolunie, 1993) and olfactory cues (Ferreira and Hansen, 1986; for review see Lonstein and Gammie, 2002) from the pups, which are important for the onset and the maintenance of maternal aggression.

Another main extrinsic factor is the intruder, which acts as a potential threat to the offspring and, therefore, is a critical threat which evokes maternal aggression in lactating rats to protect the offspring from infanticide (for review see Bosch, 2013). Depending on the age and size of the intruders, the mothers attack smaller intruders up to 80 %, whereas larger males receive only 30 % attacks (Erskine et al., 1978b; Flannelly and Flannelly, 1985). Also the sex of the intruder plays a role, with females being attacked more often than males (Haney et al., 1989); independent of their reproductive status (Neumann et al., 2001).

In addition to these factors, intrinsic factors also influence aggressive behaviour in lactating mothers. On the last days before parturition, aggression already increases, which depends on rising oestrogen, but not dropping progesterone, levels (Mayer and Rosenblatt, 1987; for review see Lonstein and Gammie, 2002). Adaptations in neuropeptidergic AVP (see below Chapter 1.8) and OXT systems during the peripartum period play a crucial role in the modulation of maternal aggression. In response to the maternal defence test, OXT release is increased within the PVN, central amygdala (CeA), BNST and lateral septum (LS), whereas increased neuronal activity and OXT receptor binding is found within the BNST and LS compared to non-aggressive controls immediately after the end of the test, lasting up to 45 min (Caughey et al., 2011; Gammie and Nelson, 2001; for review see Bosch, 2013). Noteworthy, adaptations in the neuropeptide systems, i.e. increased OXT release in the LS in response to the maternal defence test strongly depends on the rat line used with Sprague Dawleys, but not Wistar, dams showing the effect (Bosch et al., 2004; for review see Bosch, 2013).

1.1.6 Maternal anxiety and "brain circuitry"

The decreased anxiety in postpartum females is important to allow the dam to protect the offspring at the nest site (for review see Numan and Insel, 2003). A reduction in anxiety occurs directly after parturition and continues until mid-lactation, while being more anxious in late pregnancy, compared to virgin rats (Neumann et al., 2000; for review see Lonstein, 2007). In order to achieve this hypoanxious state the dam must be able to assess the stressful stimulus or scenario and adequately cope with it (for review see Neumann et al., 2010). Similar to maternal aggression, it is pup contact and not the suckling stimulus *per se* that facilitates this adaptation (Lonstein, 2005). Thus, dams separated 4 hours or more from their pups show no reduction in anxiety (Lonstein, 2005). Further, changes in the PRL, OXT and AVP systems are involved in the regulation of anxiety during lactation. For example, increased levels of central PRL (Torner et al., 2002), as well as of central OXT (Neumann et al., 2000; for review see Bosch, 2011), act anxiolytically, whereas central AVP exerts an anxiogenic action (Bosch and Neumann, 2008) during the postpartum period.

Regarding the fact that dams are less anxious but highly aggressive during the first week postpartum, the increased expression of maternal aggression and protection of the offspring

may require a concomitant reduction in anxiety when facing the intruder (Hansen et al., 1985; Lonstein, 2005). The brain regions and circuitries involved in the regulation of anxiety in lactating rats are still not completely understood but studies suggest an involvement of the periaqueductal gray (Lonstein et al., 1998) as well as the extended amygdala (Davis and Shi, 1999). Indeed, OXT receptor (Figueira et al., 2008) and γ-amino butyric acid (GABA)a receptor (Miller et al., 2010) antagonism in the ventrocaudal PAG increases anxiety-related behaviour in lactating rats. But also pharmacological blockade of V1aR within the BNST tended to decrease anxiety on the elevated plus maze (EPM) in lactating dams, suggesting an involvement of this part of the extended amygdala in the regulation of anxiety-related behaviour in rat dams (Bosch et al., 2010). Further, the corticotropin-releasing factor (CRF) system is known to be involved in the regulation of anxiety in the peripartum period, with central CRF receptor blockade eliciting an anxiolytic effect (Klampfl et al., 2013). In more detail, antagonism of the CRF receptors within the medial-posterior part of the BNST (mpBNST) leads to anxiolysis (Klampfl et al., 2014). Also the noradrenergic system is involved in the regulation of anxiety by releasing noradrenalin into the BNST in response to aversive stimuli. In lactating female rats this response is blunted by disinhibiting GABAergic output cells as response to pup contact, resulting in attenuated anxiety (Smith et al., 2013; for review see Lonstein, 2007).

Although other neurotransmitter systems are implicated in the regulation of maternal anxiety and the other maternal parameters, the main interest in my thesis is the impact of the AVP system on maternal behaviour; therefore, I will focus on AVP in the remainder.

1.2 The brain AVP system

In the last decades AVP has not only been linked to the regulation of the hypothalamo-pituitary-adrenal (HPA) axis and anxiety, but also to the facilitation of a wide range of social behaviours including pair bonding, parental care and aggression in rodents.

1.2.1 AVP

AVP was firstly isolated and synthesized in 1953, together with OXT (for review see Ottenhausen et al., 2015). Both are nonapeptides forming a ring structure, linked by a disulphide bridge connecting two cysteines. AVP differs only in two amino acids at position 3 and 8 from OXT; AVP contains phenylalanine instead of isoleucine in the ring and arginine instead of leucine in the side branch (Figure 4).

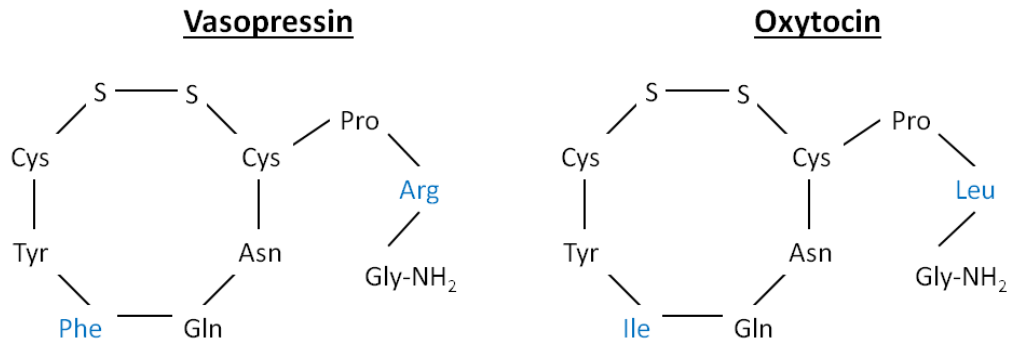

Figure 4 Molecular structure of the nonapeptides vasopressin and oxytocin

The peptides only differ in two out of nine amino acids (highlighted in blue) formed to ring structure by a disulphide bridge (S-S).

Arg = arginine, Asn = asparagine, Cys = cysteine, Gln = glutamine, Gly = glycine, Ile = isoleucine, Leu = leucine, NH_2 = amidogen, Phe = phenylalanine, Pro = proline, Tyr = tyrosine.

Both hormones are products of several enzymatic cleavage steps. In case of AVP, the primary protein precursor preprovasopressin is cleaved into a signal peptide and provasopressin. After translation and several modification steps, provasopressin is further cleaved into the active nonapeptide itself, neurophysin II protein and the copeptin glycopeptide (Gainer, 1983; Russell et al., 1980; Sachs et al., 1969). Neurophysin II ensures the proper targeting, packing and storage of AVP before release into the bloodstream (Ginsburg and Ireland, 1966). The exact function of the copeptin remains unknown although

it is a stable biomarker for pathologies linked to AVP like stress, diabetes, heart failure and myocardial infarction in human diagnostics (Bolignano et al., 2014; Pozsonyi et al., 2015; Reinstadler et al., 2015; Urwyler et al., 2015; Wannamethee et al., 2015; for review see Yilman et al., 2015).

The cleavage process takes place within the Golgi complex of neurone cell bodies in the hypothalamic SON and PVN, where neurosecretory granules containing the prohormone are formed. The cleavage products pass down nerve axons to be stored in the posterior pituitary, where they are released from nerve terminals after stimulation into the blood (Brownstein et al., 1980) but also centrally from cell bodies and dendrites of magnocellular neurons of the SON (Ludwig, 1998). In addition, AVP is also synthesized centrally within the suprachiasmatic nucleus (SCN), the olfactory bulb and the LS, among others. In the brain, AVP mediates a wide range of behaviours linked to social behaviours (see Chapter 1.7), emotionality, learning and memory but also circadian rhythms (Laycock et al., 2010).

Further, its importance in HPA axis regulation in response to stress is generally accepted (Aguilera et al., 2008). Thereby, AVP exerts its effect on all three different levels of the HPA axis, the hypothalamus, the pituitary as well as the adrenals (for review see Zelena et al., 2015). Whereas former studies in AVP-deficient Brattleboro rats supported the theory of AVP being the predominant regulator of the HPA axis response during chronic stress (Aguilera et al., 2008, 1994), recent studies also hint to a role of AVP after acute stressor exposure (Makara et al., 2012; for review see Zelena et al., 2015). On the level of the hypothalamus an increased AVP mRNA concentration was observed within the PVN during chronic stress (Aguilera et al., 1994). In addition, AVP was found to compensate for CRF effects in HPA axis regulation (for review see Scott and Dinan, 2002). Interestingly, the response of the HPA axis to stressors is attenuated in human, but also in rodent, mothers towards the end of pregnancy and in lactation (Heinrichs et al., 2001; Neumann et al., 2000, 1998a). Instead, these females show a basal hyper-corticism (Brunton and Russell, 2003; Neumann et al., 1998a), enhanced sensitivity of the pituitary to AVP (Toufexis et al., 1999) and a down-regulation in the brain CRF system (Klampfl et al., 2013; Neumann et al., 1998b; Walker et al., 2001) among other factors involved in the change in stress sensitivity (Slattery and Neumann, 2008).

Besides central and neurohypophysial functions, AVP is synthesized in the periphery, i.e. within the sympathetic ganglia, the adrenals, ovaries and testis among others (Laycock et al.,

2010). Indeed AVP was originally known as antidiuretic hormone, leading to increased blood pressure and water permeability in renal nephrons. In addition, AVP was found to play a role in thermoregulation (Richmond, 2003) and in cardiac function (Wasilewski et al., 2015).

1.2.2 AVP receptors

AVP mediates its action *via* three receptors, the V1a, V1b (formerly V3) and the V2 subtypes. Whereas the V1 receptor subtypes are mostly found both centrally and peripherally, the V2 receptor is distributed exclusively in the periphery; mainly within the kidney. There, V2 receptors in the collecting ducts and the loop of Henle are responsible for free water reabsorption (for reviews see Laycock and Hanoune, 1998; Verbalis, 2009). The main focus in this thesis is on the V1 receptors, which I want to further describe in the following.
V1a receptors (V1aR) are mainly distributed in the central nervous system, where they have been identified within the hippocampus, BNST and PVN among others (Figure 5).
V1b receptors (V1bR) are mainly found within the adenohypophysis on corticotroph cells, where they mediate adrenocorticotropic hormone (ACTH) secretion, especially under chronic stress conditions (Volpi et al., 2004a, 2004b). Within the brain the receptor-subtype is distributed throughout brain regions like the hippocampus, thalamus, MPOA and amygdala (Hernando et al., 2001; Lolait et al., 1995) among others (Figure 5).

Both V1 receptors are G-protein coupled receptors (GPCRs) of the 1b family. Thus, they have a similar basic structure, which consists of seven transmembrane helices, connected by three intracellular and three extracellular loops, with two cysteine residues forming a disulfide bridge (for review see Bockaert and Pin, 1999).
The primary signalling pathway linked to the receptors has been well-characterised: Gq/G11 for both V1 receptors (Laycock et al., 2010; Thibonnier et al., 1997; Wange et al., 1991).
In detail, the Gαq protein activates phospholipase C, which results in the hydrolysis of phosphatidylinsositol-4,5-bisphosphate into two 2nd messengers (Kirk et al., 1981; Michell et al., 1979); namely inositol triphosphate (IP3) and diacylglycerol (Laycock et al., 2010). IP3 can diffuse into the cytosol and bind to calcium channels in the plasma membrane of the endoplasmatic reticulum, followed by the release of calcium from its intracellular storage or *via* calcium dependent channels (Dayanithi et al., 2000; Levy et al., 1990). In return, in the presence of calcium, diacylglycerol can activate protein kinase C which than phosphorylates

further protein substrates. Additionally, the released calcium can bind to calmodulin, which activates calcium/calmodulin-dependent kinases (Laycock et al., 2010). These kinases have the ability to phosphorylate cyclic adenosine monophosphate response element binding protein (CREB), which stimulates the expression of genes by recruiting a coactivator, thus resulting in new protein synthesis.

Regarding the V1bR, the signalling pathway is similar to that of V1aR. In addition to coupling to a Gq/G11 protein, V1bR have also been found to recruit Gs and Gi proteins, but only in transfected cells, rendering the physiological importance of such mechanisms unclear (Birnbaumer, 2000; Laycock et al., 2010; Thibonnier et al., 2001).

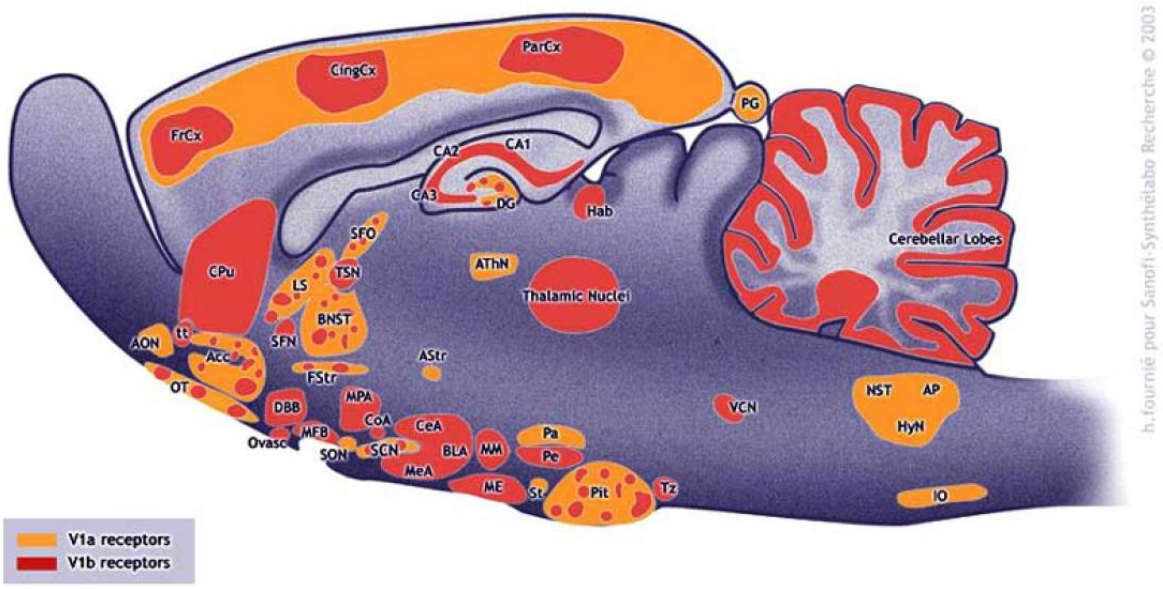

Figure 5 Distribution of V1 receptors in the rat brain

Adapted from (Griebel et al., 2005)

Acc = nucleus accumbens, AON = anterior olfactory nucleus, AP = area postrema, Astr = amygdalostriatal area, AthN = anteroventral thalamic nucleus, BlA = basolateral amygdala, BNST = bed nucleus of the stria terminalis, CA1 = hippocampal field CA1, CA2 = hippocampal field CA2, CA3 = hippocampal field CA3, CeA = central amygdala, CingCx = cingulate cortex, CoA = cortical amygdaloid nucleus, Cpu = caudate/putamen, DBB = diagonal band of Broca, DG = dentate Gyrus, FrCx = frontal cortex, FStr = fundus striati, Hab = medial habenula, HyN = hypoglossal nucleus, IO = inferior olive, LS = lateral septum, ME = median eminence, MeA = median amygdala, MFB = medial forebrain bundle, MM = medial mammillary nucleus, MPA = medial preoptic area, NST = nucleus of the solitary tract, OT = olfactory tubercle, Ovasc = organum vasculosum laminae terminalis, Pa = paraventricular hypothalamic nucleus, ParCx = parietal cortex, Pe = periventricular hypothalamic nucleus, PG = pineal gland, Pit = pituitary gland, SCN = suprachiasmatic nucleus, SFN = septofimbrial nucleus, SFO = subfornical organ, SON = supraoptic nucleus, St = stigmoid nucleus, TSN = triangular septal nucleus, tt = taenia tecta, Tz = trapezoid body, VCN = ventral cochlear nucleus

Under physiological conditions, V1 receptors are thought to be expressed in a steady state within the membrane. Thus, the combination of receptor synthesis, proteolytic degradation, internalisation and recycling back to the membrane occurs until binding of the hormone or an analogue (Bowen-Pidgeon et al., 2001; Fishman et al., 1985; Koshimizu et al., 2012; Laycock et al., 2010). This cycle was indeed shown to be true for V1aR, being primarily located in the plasma membrane (Innamorati et al., 2001). A very recent study on the V1bR showed an adapted mechanism for the receptor-subtype in cell culture (Kashiwazaki et al., 2015); a high number of V1bR within the cytoplasm, as well as an agonist-dependent mechanism of V1bR internalization was found. Thus, the binding affinity, receptor expression and internalization of V1bR is different to V1aR, which seems to be necessary during stress conditions to deal with the augmented AVP release (Kashiwazaki et al., 2015).

1.2.3 The central AVP system in social behaviours

AVP and its V1 receptors have been implicated in various social behaviours (Bosch and Neumann, 2008; Insel and Young, 2001; Landgraf et al., 1995). In male prairie voles, AVP is responsible for the pair bond formation; inhibition of AVP actions by blocking V1aR within the ventral pallidum of male prairie voles can lead to a suppression of pair bond formation (Barrett et al., 2013; Pitkow et al., 2001; Winslow et al., 1993; for review see Young and Wang, 2004). In addition, a recent study showed a link between epigenetic regulation of the V1aR gene in the retrosplenial cortex of prairie voles (which is linked to spatial memory) in relation to sexual fidelity trade-offs, i.e. if males stay closer to home and the partner, being more faithful (high V1aR expression) or if they enter neighboring territories to mate with additional females (low V1aR expression)(Okhovat et al., 2015).

The role of AVP in social recognition was not only proved in prairie voles, but also in mice and rats (for review see Bielsky and Young, 2004). Antagonism of the V1aR in rats leads to social recognition deficits, and further studies evaluated primarily the septum to be the brain region mediating this effect (Engelmann and Landgraf, 1994; Engelmann et al., 1994; Everts and Koolhaas, 1997). V1aR downregulation *via* antisense oligodeoxynucleotides in the mediolateral septum of male rats leads to the inability to discriminate between a known and a novel conspecific (Landgraf et al., 1995). Further, the facilitatory role of AVP in intermale aggression is widely studied (Ferris, 2005; for review see Neumann et al., 2010). Orally

administered V1aR (Ferris et al., 2006) or V1bR antagonist (Blanchard et al., 2005) decreased aggression in a resident-intruder test in male Syrian hamsters. A study by Wersinger et al. in V1bR knockout mice found, that these mice show reduced attack behaviour without deficits in predatory or defensive aggression (Wersinger et al., 2007). Thus, both V1aR and V1bR seem to be important for the coupling of social context to the display of the appropriate aggressive behaviour.

AVP is also found to mediate parental behaviour in voles, rats, and mice (Bosch and Neumann, 2010, 2008; Bosch et al., 2010; Kessler et al., 2011; Wang et al., 1994). Due to the main focus on maternal behaviour in my thesis I want to introduce this pro-social behaviour in context with AVP in the following.

1.2.4 The central AVP system in maternal and anxiety-related behaviour

The AVP system in rats is activated around parturition when AVP mRNA expression (Landgraf et al., 1991; Walker et al., 2001) and AVP-ir (Caldwell et al., 1987) are increased. After birth, AVP is important for the onset (Pedersen et al., 1994, 1982) as well as the maintenance of maternal care in rodents (Bosch and Neumann, 2008; Bosch et al., 2010; Kessler et al., 2011). Further, increased AVP release during mother-pup interactions within the MPOA and BNST is observed in lactation (Bosch et al., 2010).

Besides AVP itself, the brain V1aR adapt during the peripartum period. This takes the form of increased V1aR binding in brain areas important for the expression of maternal care in lactating rats compared to virgins (Bosch and Neumann, 2008; Bosch et al., 2010). In addition, activation or up-regulation of the V1aR facilitates maternal care, especially in brain regions like the MPOA and the BNST. In contrast, blockade or down-regulation of the V1aR *via* an receptor subtype-specific antagonist or antisense oligodeoxynucleotides decreases maternal care (Bosch and Neumann, 2008). Besides maternal care, V1aR are also involved in maternal motivation as down-regulation of V1aR within the MPOA decreases pup retrieval (Bosch and Neumann, 2008). Further, activation of V1aR results in increased maternal aggression in rat dams (Bosch and Neumann, 2010; Bosch et al., 2010; for review see Bosch and Neumann, 2012). The other way around, maternal aggression also causes increased V1aR-binding in the MeA in mid-lactation compared to parturition, but decreased V1aR-binding in the PVN or CeA in early or late lactation compared to parturition, respevtively

(Caughey et al., 2011). The AVP system is also implicated in the regulation of maternal anxiety; infusion of synthetic AVP was shown to be anxiogenic whereas blockade of V1aR act anxiolytic on the EPM (Bosch and Neumann, 2008).

As indicated above, the V1bR has only scarcely been investigated in relation with maternal behaviour to date. A single study showed decreased attack behaviour in V1bR knockout mice when confronted with an unfamiliar male intruder (Wersinger et al., 2007).

1.3 Animal model for extremes in anxiety-related behaviour

Given the detrimental effects of postpartum mood disorders on both mothers and the offspring, investigating the underlying pathophysiology in animal models appears a beneficial approach.

In 1993, the breeding of two rat lines for extremes in anxiety-related behaviour was started out of unselected Wistar rats (Liebsch et al., 1998). The most and least anxious rats, validated on the EPM at the age of 10 weeks, are re-bred within the same phenotype for following generations (avoiding sibling pairing). After several generations, rats showing a stable high anxiety-related behaviour (HAB) or low anxiety-related behaviour (LAB) phenotype on the EPM have established. In contrast to non-selected for anxiety-related behaviour (NAB) rats, which spent around 25-35 % on the open arms of the EPM, HABs spent less than 10 %, LABs more than 40 % on the open arms of the EPM, independent of sex (Neumann et al., 2011). The highly anxious phenotype of HAB males (Murgatroyd et al., 2004) as well as females (Bosch and Neumann, 2008; Neumann et al., 2005) was found to rely on a single nucleotide polymorphism in the AVP promoter region (Murgatroyd et al., 2004; for review see Landgraf et al., 2007), leading to increased AVP synthesis and release within the PVN (Keck et al., 2002; Wigger et al., 2004). This leads to increased AVP mRNA expression within the PVN (Bosch and Neumann, 2008; Bosch et al., 2006; Keck et al., 2002; Wigger et al., 2004), the BNST and the medial amygdala (Bosch and Neumann, 2010) compared to NAB and LAB rats. In addition, female HAB rats show increased CRF mRNA within the PVN compared to LAB rats (Bosch et al., 2006), which might also contribute to the hyper-anxious phenotype.

The HAB and LAB phenotype has been stable over years (Beiderbeck et al., 2012; Bosch and Neumann, 2008; Landgraf and Wigger, 2002; Liebsch et al., 1998) and also between several laboratories (Salomé et al., 2002) thereby providing an ideal animal model for the investigation of the neurochemical basis of behaviour (Neumann et al., 2011).

However, HAB rats do not only differ in their anxiety phenotype, but also in their stress-coping strategy and depressive-like behaviour. In detail, HAB rats are characterized by a hyper-responsive HPA axis activity (Landgraf et al., 1999) and a more passive stress-coping strategy (Keck et al., 2003; Neumann et al., 2005), which is indicative of higher depressive-like behaviour (Landgraf and Wigger, 2002). Thus, HABs show more floating in the forced swim test, increased risk assessment, and less exploration in a novel environment compared

to LAB rats (Bosch et al., 2006; Liebsch et al., 1998; Neumann et al., 2005; Ohl et al., 2001; Slattery and Neumann, 2010).

Regarding social behaviours, the two breeding lines differ in their aggressive behaviour and also in maternal behaviour. In virgin females, HABs have been found to be more aggressive in the female intruder test compared to LABs or NABs (de Jong et al., 2014). In lactating females, HAB dams are more aggressive in the maternal defence test (Neumann et al., 2001) compared to LABs (Bosch et al., 2005; for review see Bosch, 2013). In contrast to HAB and LAB males, only HAB dams show higher aggression compared to NABs (and also to LABs), as seen by decreased attack latency, an increased number of attacks and threat behaviour (for review see Bosch, 2011). Importantly, HAB and LAB dams have not been described to attack vulnerable body parts of the intruder as this was seen in males (Beiderbeck et al., 2012). Interestingly, a correlation between the intensity of maternal behaviour and the dam's innate anxiety was found in the HAB/LAB breeding lines (for review see Bosch, 2011).

HAB dams are highly maternal compared to LAB dams (Bosch and Neumann, 2008; Bosch et al., 2010, 2006, 2005; Neumann et al., 2005); the high innate anxiety of HAB dams leads to a more protective mothering style, i.e. leaving the nest less often and showing more ABN as well as total nursing (Bosch and Neumann, 2008; Bosch et al., 2006; Neumann et al., 2005). Further, HAB dams retrieve their pups faster to the nest and also retrieve more pups in a novel environment (for review see Bosch, 2011), even under challenging conditions, for instance pups are located behind a metal curtain or the dams need to cross an unpleasant floor surface (Neumann et al., 2005).

With the different anxiety and maternal phenotypes relying on the adaptation in the AVP system, studies in lactating HAB/LAB females help to understand the involvement of the neuropeptide AVP in the regulation of maternal behaviour.

1.4 The gonadotropin-releasing hormone (GnRH) and kisspeptin system in maternal behaviour

In addition to OXT, PRL and AVP mediating maternal behaviour, also other neuropeptide systems like the GnRH system influence reproductive behaviour, e.g. the onset of puberty and mating behaviour (Yoon et al., 2005). The main function of GnRH is to facilitate the action of the hypothalamo-pituitary-gonadal (HPG) axis as it mediates sexual and reproductive behaviour by releasing steroids into the blood stream (Ellis, 2013). In detail, GnRH is synthesized from neurons in the hypothalamus and released at the median eminence into the hypophyseal portal system. At the pituitary level, GnRH facilitates the release of luteinising hormone (LH) and follicle stimulating hormone (FSH), which stimulate the testes or ovaries for steroid production, respectively.

GnRH is released in pulses before binding to its receptor, GnRH receptor type I (shortly referred to as GnRH receptor), which is present in both humans and rats (Millar, 2005). Interestingly, GnRH neurons have been found in many of the brain areas described above in connection to maternal behaviour including the MPOA, the amygdala, and the arcuate nucleus (ARC; Bosch and Neumann, 2010; Bosch et al., 2010; Ehret et al., 1993; Moss and Foreman, 1976; Numan and Insel, 2003; Pi and Grattan, 1999; Yoon et al., 2005). However, there is only one study to date describing a role of GnRH in maternal behaviour. In detail, transgenic mice with a 30 % reduction in GnRH neurons have severe impairments not only in reproduction and parturition (Tsai et al., 2005), but also fail to retrieve and nurse their pups appropriately (Brooks et al., 2012).

A pilot study performed in transgenic rats expressing an AVP-enhanced green fluorescent protein (eGFP) fusion gene (Ueta et al., 2005) showed increased AVP immunoreactivity (ir) within the organum vasculosum of the lamina terminalis (OVLT) of lactating dams compared to virgins (Y. Ueta, V. Grinevich, O. Bosch, unpublished observation). Interestingly, when staining the same region for GnRH expression, and creating an overlay, a co-expression of AVP and GnRH seems to exist (Figure 6), suggesting that both systems may interact or oppose each others effects.

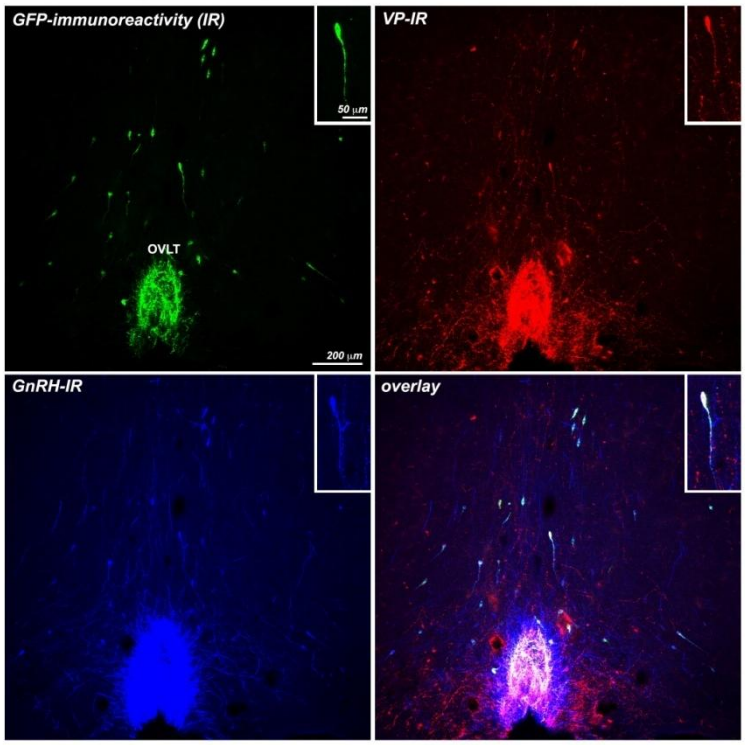

Figure 6 Expression of AVP and GnRH-ir in the organum vasculosum of the lamina terminalis (OVLT) of a lactating AVP-eGFP reporter rat.
Single stainings for eGFP signal (upper left), AVP (VP; upper right) and GnRH (lower left) in the OVLT of a lactating rat. The overlay (lower right) shows a co-expression of AVP- and GnRH-positive cells.

Furthermore, in non-transgenic ovariectomized, hormonally primed rats priming induced an increased AVP-ir within the OVLT compared to control rats (Figure 7). These preliminary results further strengthen the implication of GnRH in mediating maternal behaviour.

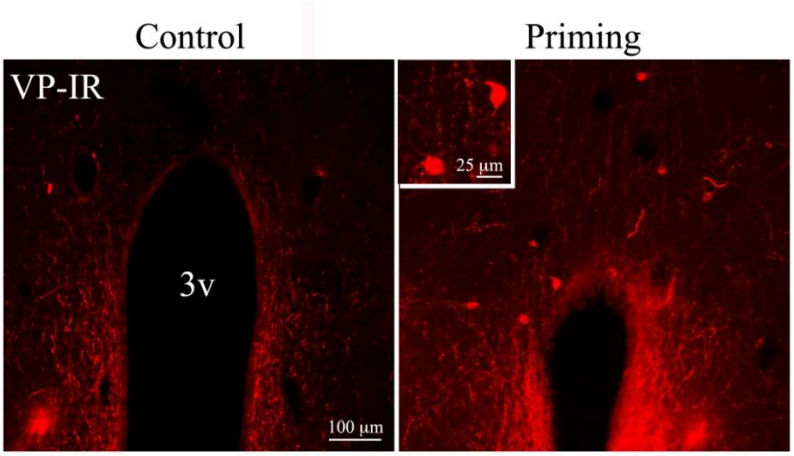

Figure 7 AVP immunoreactivity within the organum vasculosum of the lamina terminalis of primed female rats
Ovariectomized, hormone-treated virgin rats show increased AVP-immunoreactivity (ir) compared to control rats.
3v: third ventricle, VP-IR: AVP-ir

In addition to the overlap with the AVP system, GnRH interacts with the kisspeptin system. Kisspeptin has been shown to stimulate GnRH neurons to release the neuropeptide into the plasma (Guerriero et al., 2012; Han et al., 2005; Liu et al., 2008). Kisspeptin neurons are also innervated by GnRH neurons, thus being a potential target for a role in maternal behaviour

(Han et al., 2005; Kalló et al., 2013; Liu et al., 2008). Thus, co-expression of GnRH and AVP neurons within the OVLT of lactating rats may also be triggered by the kisspeptin system.

Interestingly, kisspeptin was not investigated in the context of maternal behaviour so far. It is known to mainly influence reproduction and especially integrates signals about the metabolic status to the reproductive axis (for review see d'Anglemont de Tassigny and Colledge, 2010).

Taken together, GnRH and kisspeptin represent strong candidates to promote maternal behaviour besides the neuropeptides OXT and AVP.

1.5 Aim of the thesis

Given the outlined knowledge on peripartum adaptations occurring in preparation for birth and lactation, the critical involvement of the different components of the AVP system in maternal behaviour in rats is obvious. Although AVP and its V1aR are highly studied, the contribution of V1bR is only poorly investigated so far. In addition, the GnRH and kisspeptin system seems to be potentially linked to play a role in maternal behaviour, but is hardly investigated in this context at all.

Therefore, the aim of my thesis was:

1. To outline the involvement of brain V1bR on different aspects of maternal behaviour in lactating Wistar rats.
2. To investigate the distinct role of V1bR in the maternal super-region, i.e. the MPOA and the BNST in the regulation of maternal behaviour.
3. To assess the effects of both, V1aR and V1bR antagonism in the PVN on maternal behaviour.
4. To determine a potential role of the GnRH as well as the kisspeptin system in maternal behaviour of lactating Wistar rats.
5. To study the involvement of V1bR in maternal behaviour of lactating HAB and LAB dams.

Chapter 2

Central V1b Receptor Antagonism in Lactating Rats: Impairment of Maternal Care But Not of Maternal Aggression

Authors' contribution:

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

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2.1 Abstract

Maternal behaviour in rodents is mediated by the central OXT and AVP systems, amongst others. The role of AVP, acting via the V1aR, on maternal care and maternal aggression has recently been described. However, a potential involvement of the V1bR in maternal behaviour has only been demonstrated in knockout mice.

The present study aimed to examine the effects of central pharmacological manipulation of the V1bR on maternal behaviour in lactating Wistar rats. On PD 18, female rats were implanted with a guide cannula targeting the lateral ventricle. After parturition, dams received an acute central infusion of a specific V1bR agonist (d[Leu4,Lys8]VP) or V1bR antagonist (SSR149415) once daily, followed by observations of maternal care [lactation day (LD) 1], maternal motivation in the pup retrieval test (PRT; LD 2), anxiety-related behaviour on the EPM (LD 3) and maternal aggression in the maternal defence test followed by maternal care monitoring (LD 4).

Our data demonstrate that, under non-stress conditions, the V1bR antagonist decreased the occurrence of both nursing and mother–pup interaction, whereas the V1bR agonist did not affect either parameter. Under stress conditions (i.e. after the maternal defence test), mother–pup interaction was decreased by infusion of the V1bR antagonist. During the maternal defence test, neither treatment affected aggressive or non-aggressive behaviour. Finally, neither treatment altered maternal motivation or anxiety.

In conclusion, central V1bR antagonism modulates aspects of maternal care but not of maternal aggression or maternal motivation in lactating rats. These findings further extend our knowledge on the AVP system as a vital mediator of maternal behaviour.

2.2 Introduction

Maternal behaviour is crucial for the well-being and development of the offspring, as well as for protection against potential threats (e.g. infanticide by conspecifics) (for reviews see Bosch and Neumann, 2012; Numan and Insel, 2003). In addition to the neuropeptide OXT, the closely-related AVP has also been shown to mediate maternal care and maternal aggression in lactating rats (for review see Bosch and Neumann, 2012). The majority of these effects have been linked to activation of the V1aR (for reviews see Bosch and Neumann, 2012; Bosch, 2013) but not of the V1bR.

Around parturition, the brain AVP system in rats becomes activated, as indicated by increased AVP mRNA expression (Landgraf et al., 1991; Walker et al., 2001) and AVP-ir (Caldwell et al., 1987). During mother–pup interactions, the release of AVP is increased within the MPOA and the BNST (Bosch et al., 2010), which are brain areas that are importantly involved in mediating maternal behaviour (Bosch et al., 2010; for reviews see Bosch and Neumann, 2012; Bosch, 2011; Numan and Insel, 2003). AVP facilitates the onset (Pedersen et al., 1994, 1982), as well as the maintenance, of maternal care in both rats (Bosch and Neumann, 2008; Bosch et al., 2010) and mice (Kessler et al., 2011). Furthermore, V1aR binding is increased in brain areas important for the expression of maternal care in rat dams compared to virgins (Bosch and Neumann, 2008; Bosch et al., 2010). Moreover, V1aR activation promotes maternal aggression in lactating rats (Bosch and Neumann, 2010; Bosch et al., 2010; for review see Bosch and Neumann, 2012). However, whether the V1bR plays a role in maternal care and/or maternal aggression has not been studied so far.

The V1bR was initially found in the adenohypophysis on the surface of corticotroph cells, which mediate ACTH secretion (Koshimizu et al., 2012). Here, the main function of the V1bR is regulation of hypothalamic-pituitary-adrenal axis activity at the pituitary level, under both resting and stress conditions (Tanoue et al., 2004). In the brain, the V1bR is expressed in various regions of the hypothalamus and limbic system (Laycock et al., 2010). Some of these regions have been implicated in the regulation of maternal care and maternal aggression, such as the BNST and the MPOA, as described above (Caughey et al., 2011; for review see Bosch, 2013). Interestingly, maternal aggression is reduced in lactating V1bR knockout mice (Wersinger et al., 2007), which indicates a potential involvement of the V1bR in mediating maternal behaviour. However, compensatory mechanisms may underlie the observed phenotype, such as OXT acting on the V1bR (Koshimizu et al., 2012), meaning that a

pharmacological approach is necessary to determine the role of the V1bR in maternal behaviour. In addition to the BNST and MPOA, the V1bR is also found in the septum and the amygdala (Hernando et al., 2001), which play an active role not only in maternal behaviour, but also in anxiety-related behaviour. Indeed, the V1bR antagonist SSR149415 increases the time mice spend on the open arms of the EPM, which is indicative of an anxiolytic effect (Griebel et al., 2002).

In the present study, we aimed to characterise the role of the central V1bR in maternal and anxiety-related behaviour in lactating rats *via* bidirectional pharmacological manipulation. One day after parturition (i.e. LD 1), dams received once daily acute intracerebroventricular (icv) infusions of vehicle (VEH), a specific V1bR agonist (d[Leu4, Lys8]VP), or a specific V1bR antagonist (SSR149415) for four consecutive days. Maternal care was monitored under non-stress conditions (LD 1), as well as after an acute psychosocial stressor, namely the maternal defence test (LD 4). Moreover, maternal motivation in the PRT (LD 2), maternal aggression during the maternal defence test (LD 4) and anxiety-related behaviour on the EPM (LD 3) were assessed.

2.3 Material and methods

2.3.1 Animals

All rats were maintained under a 12 : 12 h light/dark cycle (lights on 07.00 h) at 22 ± 1 °C and 55 ± 5% relative humidity, with free access to water and standard rat chow.
Virgin female Wistar rats (12–14 weeks, 220–250 g; Charles River, Sulzfeld, Germany) were mated with sexually experienced stud Wistar males and pregnancy was confirmed the next day by the presence of sperm in vaginal smears [assigned as PD 1]. Rats were housed in groups of three to four until PD 18, when they underwent surgery. Subsequently, they were single-housed in plexiglass observation cages (38 x 22 x 35 cm^3) to ensure undisturbed parturition. Prior to (PD 16–17) and after (PD 19–21) surgery, rats were handled twice daily to familiarise them with the infusion procedure and to minimise stress-related behavioural reactions (Neumann et al., 1998a). All of the rats gave birth on PD 22 or 23. On the day of parturition, offspring were culled to eight pups of mixed sexes. At the same time, half of the bedding was replaced with new bedding to avoid any disturbance of the mother during the experiment. Virgin female Wistar rats (10 weeks, 180–220 g; Charles River) at random stages of their oestrous cycle were used as intruders in the maternal defence test. They were kept group-housed in a separate room until the behavioural testing to avoid olfactory recognition. All experiments were conducted in accordance with the European Communities Council Directive (86/609/EEC) and were approved by the local government of the Oberpfalz, Bavaria.

2.3.2 Implantation of an icv guide cannula

On PD 18, female rats were implanted with a stainless steel guide cannula (21 G, length 12 mm), 2 mm above the right lateral ventricle (1.0 mm posterior, 1.6 mm lateral to bregma, 2.0 mm ventral) (Paxinos and Watson, 1998) under semi-sterile conditions. Briefly, rats were anaesthetised with the inhalation narcotic isoflurane (Baxter Germany GmbH, Unterschleißheim, Germany) and placed on a thermo pad (32 °C) to minimise core body temperature loss. The guide cannula was fixed with dental cement (Kallocryl, Speiko, Münster, Germany) to two stainless steel screws, which were inserted into the skull. The cannula was closed with a stainless steel dummy cannula of the same length as the guide

cannula. At the end of the surgery, rats received 0.12 ml of the antibiotic Enrofloxacin (Baytril 2.5%; Bayer Vital GmbH, Leverkusen, Germany) subcutaneously to prevent inflammation.

2.3.3 Infusion of receptor-specific V1bR agonist or V1bR antagonist

Starting on LD 1, dams received daily one acute infusion of VEH (5 µl; Ringer's solution + 5% dimethyl sulphoxide), the receptor-specific V1bR agonist d[Leu4,Lys8]VP (50 ng/5 µl) (Pena et al., 2007a) or the receptor-specific V1bR antagonist SSR149415 (500 ng/5 µl) (Serradeil-Le Gal et al., 2002) 10 min prior to behavioural testing. The dose of the V1bR antagonist was based on previous studies (Salomé et al., 2006; Stemmelin et al., 2005), whereas the dose of the V1bR agonist matched one-tenth of the V1bR antagonist. The treatments were administered over 20 s *via* a 10-µl Hamilton syringe connected to an injection system consisting of an infusion cannula (25 G, length 14 mm, i.e. 2 mm exceeding the guide cannula) on a polyethylene tube 20 cm in length.

2.3.4 Experimental schedule

The experimental schedule is presented in Figure 8.

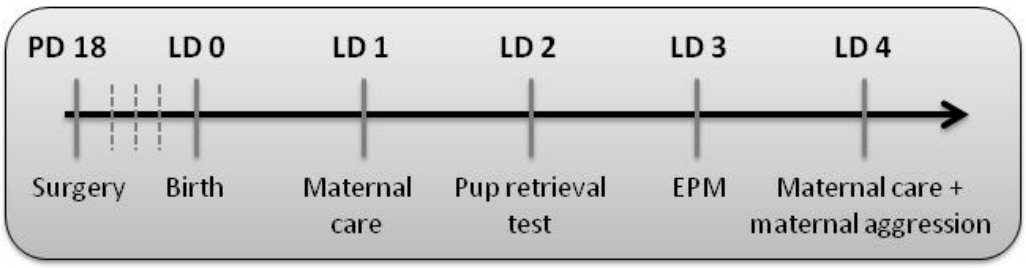

Figure 8 Experimental schedule.

PD, pregnancy day; LD, lactation day; EPM, elevated plus maze. Dotted lines represent PD19 until PD21.

On LD 1, maternal care was observed in the home cage under 'non-stress' conditions before and immediately after treatment infusion at 10.00 h for 1 h, as well as in the afternoon between 15.00 h and 16.00 h. On LD 4, maternal care was monitored for 1 h in the morning before moving the rats to the maternal defence test room, as well as 1 h immediately after termination of the maternal defence test, which served as an acute psychosocial stressor for

the lactating mother (‘stress’ conditions) (Neumann et al., 2001). To distinguish LD 4, on which the stress of the maternal defence test was included, from LD 1, we use the term ‘non-stress’ conditions for LD 1, although we are aware of the minimal amount of stress associated with the infusion procedure. Additionally, dams were tested for maternal motivation in the PRT on LD 2, for anxiety-related behaviour on the EPM on LD 3 and for maternal aggression in the maternal defence test on LD 4 (for details, see below). Importantly, the dams were moved to separate rooms and given 1 h for habituation before treatment infusion and testing. All tests were performed in the light cycle between 09.00 h and 12.00 h.

2.3.5 Behavioural tests

2.3.5.1 Maternal care observation in the home cage

Maternal care was observed in the home cage according to an established protocol (Bosch and Neumann, 2008; Klampfl et al., 2013). In detail, the behaviour of the mother was monitored every 2 min for approximately 10 s at 30-min intervals. The behaviours scored were nursing including ABN as the only active nursing posture of the mother (Stern and Johnson, 1990) and blanket posture (i.e. lying passively on top of the pups) (for review see Bosch, 2011), as well as mother–pup interaction, which includes all nursing postures, licking/grooming the pups and carrying the pups.

2.3.5.2 Maternal motivation in the PRT

In the PRT, dams were tested for their motivation to retrieve their own eight pups in a novel environment (van Leengoed et al., 1987) according to an established protocol (Bosch and Neumann, 2008; Neumann et al., 2005). Naturally, the motivation to retrieve the pups during this early phase of lactation is very high (for review see Bosch, 2011). One hour prior to testing, pups were separated from their mothers at the time when dams were transferred to the experimental room. During the separation period, pups were kept on a thermo pad (32 °C) in a different room than the dams. Subsequently, pups were brought to the experimental room and distributed in a white plastic box (54 x 34 x 32 cm^3) prepared with a handful of bedding from the home cage. Ten minutes after infusion of the treatment, the

dam was placed in the middle of the box and the time of retrieval and number of retrieved pups was scored during the 15-min test period. After termination of the test, the dam was placed back into her home cage together with her pups.

2.3.5.3 Maternal aggression in the maternal defence test

In the maternal defence test, the dams (residents) received their respective treatment infusion 10 min prior to introducing a virgin female rat (intruder) into their home cage in the presence of the pups (Bosch et al., 2005; Neumann et al., 2001; for review see Bosch, 2013). During the 10-min testing period, the behaviour was video-taped for subsequent analysis by an experienced observer blind to the treatment. The behaviours scored were attacks and threat behaviours, which consist of offensive upright (the dam stands in an upright position in front of the intruder), keep down (the mother keeping the intruder down with her front paws), lateral threat (the mother engages to push the intruder aside by approaching laterally with her whole body) and aggressive grooming (for review see Bosch, 2013). Further, non-aggressive behaviours were scored, including sniffing the intruder, exploration of the cage, maternal care, eating/drinking and self-grooming.

One resident did not show any aggressive behaviour and was excluded from the statistical analysis. All other residents showed attacks and keep down behaviour, whereas two residents from the VEH group did not show any lateral threat behaviour.

After termination of the test, the virgin intruders were removed from the residents' home cage. Intruders were used only once per day, as well as only twice during the experiment, with at least 1 day in between for recovery.

2.3.5.4 Anxiety-related behaviour on the EPM

Anxiety-related behaviour was tested on the EPM as described previously (Bosch and Neumann, 2008; Liebsch et al., 1998; Pellow et al., 1985). Briefly, the plus-shaped maze consisted of two open arms (50 x 10 cm^2; 90 lux) and two closed arms (50 x 10 cm^2; height of walls: 30 cm; 10 lux) connected by a neutral zone (10 x 10 cm^2; 65 lux) and was elevated 80 cm over the floor. This test is based on the rats' aversion of open areas combined with their exploratory drive, which results in an approach–avoidance conflict behaviour (Handley and McBlane, 1993; Pellow et al., 1985). Ten minutes after infusion of the treatment, the dams

were placed in the neutral zone facing a closed arm and were allowed to freely explore the maze for 5 min. Behaviours were scored live by an observer who was blind to treatment *via* a camera located above the EPM. The percentage of time spent on the open arms (time on the open arms versus all arms) and the percentage of entries into the open arms (entries into the open arms versus all arms) were counted as measures of anxiety-related behaviour, whereas the number of closed arm entries accounted for locomotion (Neumann et al., 2000).

2.3.6 Verification of guide cannula placement

At the end of the experiments, rats were sacrificed with CO2 and infused with 5 µl of ink with an infusion cannula via the implanted guide cannula. Brains were removed and cut with a razor blade at the implantation site of the cannula. If the ventricles were coloured blue, the cannula placement was considered as correct.

2.3.7 Statistical analysis

Statistical tests were performed using SPSS, version 20 (IBM, Ehningen, Germany). Only rats with correctly implanted cannula were included in the statistical analysis.
Maternal care and maternal motivation were analysed using a two-way ANOVA for repeated measures (factors: treatment x time). Maternal aggression, number of retrieved pups and anxiety-related behaviour were analysed using a one-way ANOVA (factor: treatment). The ANOVAs were followed by Sidak post-hoc correction if main effects were found.
Data are presented as the mean + SEM. $p < 0.05$ was considered statistically significant.

2.4 Results

2.4.1 Maternal care was reduced by V1bR antagonism under non-stress conditions

On LD 1 under non-stress conditions, ABN significantly differed over time (two-way ANOVA for repeated measures, factor time: $F_{2,36} = 4.786$; $p = 0.014$; Figure 9A) but not depending on treatment, nor was there a treatment x time interaction. The post-hoc test revealed that V1bR antagonist-treated mothers showed increased ABN at t +60 min compared to t +30 min ($p = 0.028$).

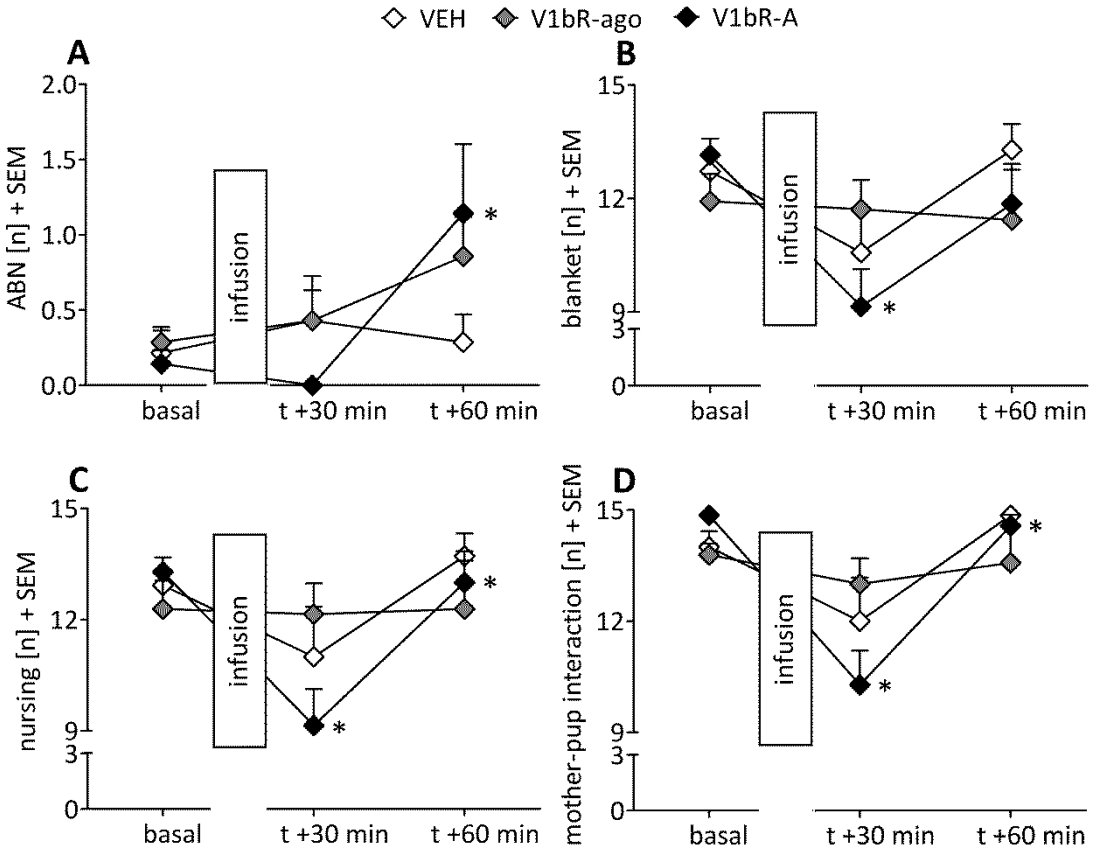

Figure 9 Effects of V1b receptor (V1bR) manipulation on maternal care under non-stress conditions.

The scored behaviours were arched back nursing (ABN; A), blanket nursing posture (blanket; B), as well as the sum of all nursing postures (nursing; C) and of all mother–pup interactions (D). The behaviours were observed before (basal) and for 60 min (t +30 min and t +60 min) after icv infusion of vehicle (VEH) (5 µl; Ringer's solution + 5% dimethyl sulphoxide), V1bR agonist d[Leu4,Lys8]VP (V1bR-ago; 50 ng/5 µl) or V1bR antagonist SSR149415 (V1bR-A; 500 ng/5 µl).

Data are presented as the mean + SEM (n = 7 per group). *$p < 0.05$ versus previous sample.

Blanket nursing differed over time (two-way ANOVA for repeated measures, factor time: $F_{2,36}$ = 4.786; p = 0.014; Figure 9B) but was not affected by treatment, nor did we find a treatment x time interaction. The post-hoc test revealed a decrease at t +30 min compared to pre-injection (p = 0.008) in the V1bR antagonist-treated dams only.

Nursing behaviour significantly differed over time (two-way ANOVA for repeated measures; factor time: $F_{2,36}$ = 5.558; p = 0.008; Figure 9C) but was not affected by treatment, nor was there a treatment x time interaction. At t +30 min, the post-hoc test showed decreased nursing in the V1bR antagonist-treated dams compared to pre-injection (p = 0.008) and increased nursing at t +60 min compared to t +30 min (p = 0.05). The infusion did not influence nursing in the other groups.

Mother–pup interaction significantly differed over time depending on treatment (two-way ANOVA for repeated measures; factor treatment x time: $F_{4,36}$ = 2.651; p = 0.049; Figure 9D). In detail, the post-hoc test showed that, after injection of the V1bR antagonist, mother–pup interaction was significantly lower at t +30 min compared to pre-injection (p = 0.001). At t +60 min, it increased compared to t +30 min (p = 0.006) and therefore was no longer different from basal values. The infusion did not change mother–pup interaction in the other groups.

We did not detect changes in any other pup-directed behaviour, and maternal care observation in the afternoon did not reveal any differences (data not shown). Furthermore, V1bR agonist injection did not alter any behaviour.

2.4.2 Maternal care was altered by V1bR antagonism and agonism under stress conditions

On LD 4 under stress conditions, the combination of treatment with the maternal defence test revealed a significant effect on ABN over time (two-way ANOVA for repeated measures, factor time: $F_{2,36}$ = 13.242; p < 0.001; Figure 10A) but no effect of treatment. Statistical analysis did not show a treatment x time interaction. The treatment/maternal defence test combination did not affect the occurrence of ABN directly after infusion at t +30 min but was increased at t +60 min in the VEH- and the V1bR antagonist-treated mothers compared to the previous time-point.

However, blanket nursing significantly changed over time (two-way ANOVA for repeated measures, factor time: $F_{2,36} = 23.342$; $p < 0.001$; Figure 10B) but not depending on treatment. Statistical analysis did not show a treatment x time interaction. The post-hoc test revealed that after treatment/maternal defence test blanket nursing is reduced at t +30 min in V1bR antagonist- ($p = 0.037$) and V1bR agonist- ($p = 0.015$) but not in the VEH-treated mothers. The amount of blanket nursing increased in both V1bR-manipulated groups at t +60 min compared to the previous time-point (V1bR antagonist: $p = 0.009$, V1bR agonist: $p = 0.004$).

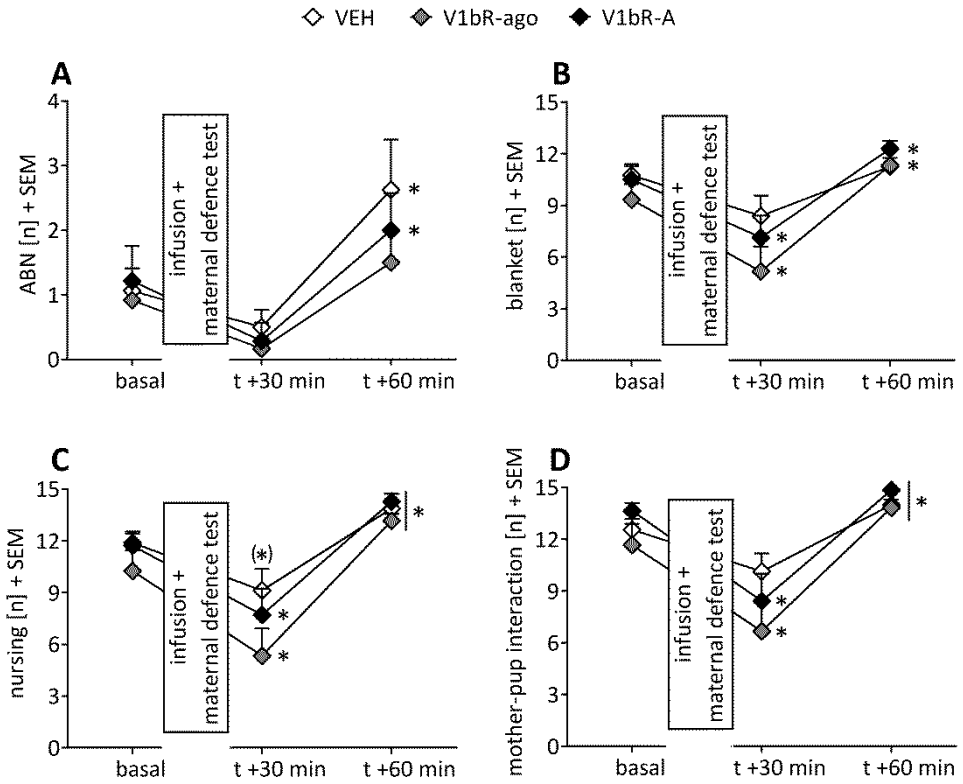

Figure 10 Effects of V1b receptor (V1bR) manipulation on maternal care under stress conditions.

The scored behaviours were arched back nursing (ABN; A), blanket nursing posture (blanket; B), as well as the sum of all nursing postures (nursing; C) and of all mother–pup interactions (D). The behaviours were observed before (basal) and for 60 min (t +30 min and t +60 min) immediately after the combined icv infusion and maternal defence test (for details, see Section 2.3.5.3). Dams received a 5 µl icv infusion of vehicle (VEH), V1bR agonist (V1bR-ago) or V1bR antagonist (V1bR-A) (for details, see Figure 9) 10 min prior to the maternal defence test.

Data are presented as the mean + SEM (n = 7 per group). *$p < 0.05$; (*)$p = 0.06$ versus previous sample.

Nursing significantly changed over time (two-way ANOVA for repeated measures, factor time: $F_{2,38} = 36.722$; $p < 0.001$; Figure 10C) but not depending on treatment. There was no significant treatment x time interaction. The post-hoc test revealed that after treatment/ maternal defence test at t +30 min nursing decreased (V1bR antagonist: p = 0.01; V1bR agonist: p = 0.003) or tended to decrease (VEH: p = 0.066) compared to basal values. At t +60 min, nursing increased again compared to t +30 min in all treatment groups (VEH: p = 0.016; V1bR antagonist: p = 0.002; V1bR agonist: p = 0.001).

The occurrence of a mother–pup interaction significantly differed between the groups over time (two-way ANOVA for repeated measures, factor time: $F_{2,36} = 36.035$; $p < 0.001$; Figure 10D) but not depending on treatment. Statistical analysis did not show a treatment x time interaction. The post-hoc test revealed that mother–pup interaction was significantly reduced after treatment/maternal defence test at t +30 min in V1bR antagonist- (p = 0.001) and V1bR agonist- (p = 0.004) but not in the VEH-treated dams. The occurrence of a mother–pup interaction returned to pre-stress values at t +60 min (VEH: p = 0.036; V1bR antagonist: p = 0.001; V1bR agonist: p = 0.001) compared to t +30 min.

We did not detect any other changes in pup-directed behaviour after treatment/maternal defence test (data not shown).

2.4.3 Maternal motivation was not affected by V1bR manipulation

Maternal motivation as measured by number of retrieved pups in the PRT on LD 2 increased over time (two-way ANOVA for repeated measures, factor time: $F_{14,266} = 15.34$; $p < 0.001$; Figure 11) but was not altered by any treatment. Statistical analysis did not show a treatment x time interaction.

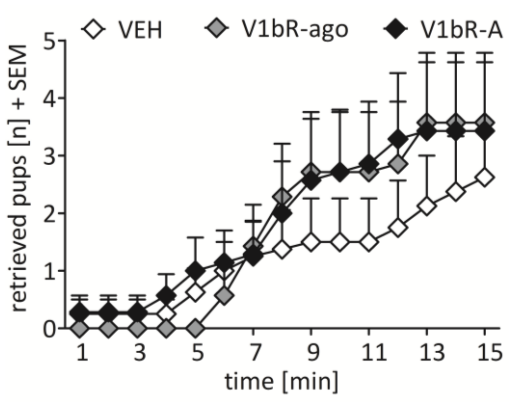

Figure 11 Effects of V1b receptor (V1bR) manipulation on maternal motivation in the pup retrieval test.

Following a 60-min separation, pups were distributed in a new cage and the retrieval of pups was recorded (for details, see Section 2.3.5.2). The dams received a 5 µl icv infusion of vehicle (VEH), V1bR agonist (V1bR-ago) or V1bR antagonist (V1bR-A) (for details, see Figure 9) 10 min prior to the test.

Data are presented as the mean + SEM (n = 7–8 per group).

There was no difference in the latency to initiate pup retrieval (VEH: 590 ± 120 s, V1bR antagonist: 450 ± 128 s, V1bR agonist: 577 ± 98 s), nor in the total number of retrieved pups (VEH: 2.6 ± 1.0, V1bR antagonist: 3.4 ± 1.2, V1bR agonist: 3.6 ± 1.2).

2.4.4 Maternal aggression was not altered by V1bR manipulation

During the maternal defence test on LD 4, the number of attacks, as well as the occurrence of threat behaviours (keep down, lateral threat; Figure 12), did not differ between the groups.

Furthermore, no effects of treatment were seen in the occurrence of offensive upright posture or aggressive grooming (data not shown). Other aggressive, as well as non-aggressive, behaviours (exploration, sniffing, self-grooming, maternal care, eating/drinking) did not differ between the groups (data not shown).

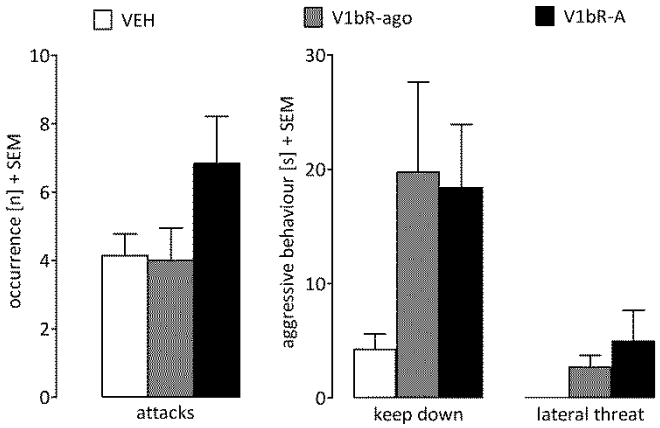

Figure 12 Effects of V1b receptor (V1bR) manipulation on maternal aggression in the maternal defence test.
Lactating mothers (residents) were exposed to virgin female rats (intruders) in the presence of their pups for 10 min. The number of attacks (left) and the time spent on the threat behaviours keep down (middle) and lateral threats (right) are shown. The dams received a 5 µl icv infusion of vehicle (VEH), V1bR agonist (V1bR-ago) or V1bR antagonist (V1bR-A) (for details, see Figure 9) 10 min prior to the test.
Data are presented as mean + SEM (n = 5–7 per group).

2.4.5 Anxiety-related behaviour was not altered by V1bR manipulation

None of the treatments altered anxiety-related behaviour on LD 3 as measured as percentage of time spent on the open arms (VEH: 47.1 ± 6.6; V1bR antagonist: 59.3 ± 6.3;

V1bR agonist: 46.4 ± 9.9) or percentage of entries into the open arms of the EPM (VEH: 50.7 ± 4.4; V1bR antagonist: 50.2 ± 4.4; V1bR agonist: 47.4 ± 4.6). Furthermore, the number of closed arm entries as a measure of locomotion was not affected by any treatment (VEH: 9.5 ± 1.4; V1bR antagonist: 11.0 ± 1.4; V1bR agonist: 10.4 ± 1.3).

2.5 Discussion

In the present study, we demonstrate that central V1bR play a critical role in the mediation of maternal care but probably not maternal aggression. Under both non-stress and stress conditions, central blockade of the V1bR reduced the occurrence of nursing and mother–pup interactions. By contrast, neither manipulation altered maternal motivation, maternal aggression or anxiety-related behaviour.

In the peripartum period, AVP system activity is up-regulated in the maternal brain. In lactating rats, AVP mRNA expression and/or AVP-ir in the PVN (Bosch et al., 2007; Caldwell et al., 1987; Walker et al., 2001) and AVP release within distinct brain regions are increased (Bosch and Neumann, 2012; Landgraf et al., 1991). These adaptations are necessary to provide adequate maternal behaviour (Caldwell et al., 1987; Landgraf et al., 1991; Walker et al., 2001). An involvement of AVP in the regulation of maternal-like behaviour was first suggested by Pedersen et al. (Pedersen et al., 1982), who demonstrated that an acute icv AVP infusion in ovariectomized, steroid-primed virgin female rats is capable of inducing pup retrieval and promotes maternal care, as indicated by crouching over the pups as well as licking/grooming. They extended their finding to V1aR in the MPOA, where local infusion of a specific V1aR antagonist in parturient rats delays the onset of maternal care (Pedersen et al., 1994). Furthermore, our group demonstrated a peptide-specific involvement of the brain AVP system both centrally and locally within the MPOA in maternal care (Bosch and Neumann, 2008). This result confirmed that such effects are mediated directly via the V1aR, rather than an off-target effect via the OXT receptor (Pedersen et al., 1994). For example, maternal care is impaired after local down-regulation of V1aR expression *via* antisense oligodeoxynucleotide infusion, and improved after local V1aR up-regulation via an adeno-associated viral vector within the MPOA (Bosch and Neumann, 2008). Regarding maternal aggression, central (for review see Bosch and Neumann, 2012) or local manipulations in the CeA (Bosch and Neumann, 2010) or BNST (Bosch et al., 2010) could demonstrate that such pup-defence behaviour is impaired after blocking V1aR. By contrast, V1aR blockade during late lactation in multiparous Sprague–Dawley rats revealed an increased attack duration (Nephew et al., 2010) suggesting that parity may be important for the role of the V1aR in such behaviours. However, there are almost no data available on a potential role of the V1bR in the regulation of maternal care and maternal aggression.

V1bR are widely distributed in the brain, and have been identified in the hypothalamus and numerous limbic areas in male rats (Hernando et al., 2001) where V1bR are assumed to mediate social behaviour (Lolait et al., 1995; Wersinger et al., 2002; for review see Stevenson and Caldwell, 2012). Importantly, some of these brain regions form part of the maternal brain circuitry, such as the PVN, BNST and MPOA (for reviews see Bosch and Neumann, 2012; Numan and Insel, 2003). To date, only one study has examined the role of central V1bR in maternal behaviour (Wersinger et al., 2007). Wersinger et al. demonstrated that female V1bR knockout mice are less maternally aggressive than wild-type mice (Wersinger et al., 2007). However, compensatory mechanisms involving, for example, the OXT system (Koshimizu et al., 2012) are likely to determine the phenotype of the V1bR knockout mice. Therefore, and as a result of the importance of the AVP system for the behavioural expression of maternal behaviour, we aimed to investigate the role of central V1bR in maternal care and maternal aggression of lactating Wistar rats using a pharmacological approach.

In the present study, we could show that maternal care was reduced by central blockade of V1bR under non-stress conditions. More specifically, blanket nursing, total nursing and mother–pup interactions were significantly reduced after acute V1bR blockade.

These findings are similar to the observation that central V1aR antagonism impairs ABN (Bosch and Neumann, 2008). These studies suggest a crucial but subtly different role for both V1R subtypes in the regulation of maternal care. Additionally, we investigated the effects of a psychosocial stressor, the maternal defence test (Neumann et al., 2001), in combination with central V1bR manipulation on maternal care. Exposure to the maternal defence test is known to reduce maternal care immediately after termination of the test (Klampfl et al., 2013). Indeed, when comparing the pre-stressor exposure with the 30 min immediately after the combined treatment/maternal defence test, we found that central administration of the V1bR antagonist reduced the amount of blanket nursing, total nursing and mother–pup interaction. A similar trend was also observed in the VEH group. Therefore, inhibition of V1bR under stress conditions may prohibit adequate stress coping, which leads to decreased mother–pup interaction. Moreover, these dams returned to pre-stress levels of nursing and interaction with their pups 60 min after termination of the maternal defence test. This could be explained by the short half-life of the V1bR antagonist SSR149415, which is active for at least 30 min when administered i.v. in rats (Oost et al., 2011). Although we

used a different route of administration and central half-life might not be identical with peripheral half-life, the duration of central actions (at least on maternal care) might be restricted to a similar time frame.

In addition to its vital effects on maternal care, the brain AVP system is also involved in the regulation of maternal aggression (for review see Bosch, 2013). AVP release within the CeA is increased during the maternal defence test in dams showing high levels of maternal aggression (Bosch and Neumann, 2010). In confirmation, blockade of V1aR within the CeA reduces the aggression level in these dams, whereas V1aR activation increases such behaviour in low aggressive dams (Bosch and Neumann, 2010) (additional details are provided elsewhere (for review see Bosch, 2013, 2011)). Furthermore, similar effects were found within the BNST, where V1aR blockade decreases maternal aggression in lactating rats compared to VEH-treated mothers (Bosch et al., 2010). By contrast, we did not find significant changes in aggressive behaviour via central V1bR activation or blockade, suggesting no (or at most a minor) role of V1bR in mediating pup defence. Interestingly, there is evidence for the involvement of V1bR in aggressive behaviour from studies in lactating V1bR knockout mice; such dams show less attacks, as well as a longer attack latency, towards a male conspecific compared to wild-type mice (Wersinger et al., 2007). In male Syrian hamsters, orally administered V1bR antagonist reduces aggression in a resident–intruder paradigm (Blanchard et al., 2005). However, these studies reflect aggression either in knockout mice or in males, which cannot be compared to maternal aggression in rats because the genetic background and the reason for attacking the opponent are different (Blanchard et al., 2003; for reviews see Bosch, 2013; Neumann et al., 2010).

Similar to maternal aggression, we did not find any effect of treatment on maternal motivation in the PRT. However, this does not exclude a role for V1bR in maternal motivation. For example, central blocking of V1aR does not change pup retrieval, whereas local reduction of MPOA V1aR expression *via* antisense reduces pup retrieval (Bosch and Neumann, 2008). Hence, the lack of behavioural effects not only on maternal motivation, but also on maternal aggression might be a result of the icv approach; the V1bR antagonist and V1bR agonist might not have reached brain regions involved in the tested maternal behaviours in sufficient amounts, highlighting the need to perform studies with various doses tested for their behavioural relevance. Furthermore, treatments infused locally act in a highly restricted area, whereas icv infused drugs diffuse almost throughout the entire

brain, thereby acting on various brain regions, which might influence the behaviour in opposing directions. Consequently, the behavioural outcome reflects the integration of all the effects in those brain regions, which might mimic a lack of behavioural changes (for review see Bosch, 2013, 2011).

In the present study, no differences were found in anxiety-related behaviour on the EPM. However, systemic administration of the V1bR antagonist, SSR149415, has been described to result in an anxiolytic phenotype (Griebel et al., 2005). This discrepancy appears to depend on the animal model, route of administration, experimental design and also on the targeted brain area because previous studies were conducted in males and via local or oral administration (Griebel et al., 2002; Litvin et al., 2011; Salomé et al., 2006; Serradeil-Le Gal et al., 2002; Stemmelin et al., 2005). For example, in male Sprague–Dawley rats, SSR149415 acts anxiolytically when applied orally (Griebel et al., 2002; Serradeil-Le Gal et al., 2002) or infused into the basolateral amygdala (Salomé et al., 2006) but not into the LS (Stemmelin et al., 2005). These findings suggest that SSR149415 needs to be applied peripherally to act on the pituitary V1bR, thereby affecting the stress axis and acting anxiolytically (Shimazaki et al., 2006), or locally into a specific brain region (Salomé et al., 2006).

Because this is one of the first behavioural studies using both the V1bR antagonist SSR149415 (Serradeil-Le Gal et al., 2002) and V1bR agonist d[Leu4, Lys8]VP (Pena et al., 2007a), it is important to note that both treatments are highly specific for the V1bR in rats (Griebel et al., 2002; Pena et al., 2007a, 2007b; Serradeil-Le Gal et al., 2002) (see also a study on human cell culture (Griffante et al., 2005)). This is of particular interest because blocking or activating the V1bR might influence other neuropeptide systems, which may underlie the observed outcomes. For example, if V1bR are insufficiently expressed or knocked out, thereby resembling receptor inhibition, OXT receptors can partly take over the stress-induced ACTH release (Nakamura et al., 2008). Furthermore, studies in V1bR knockout mice suggest that OXT can act on V1bR (Koshimizu et al., 2012). Because OXT is a major regulator of maternal behaviour (for review see Bosch and Neumann, 2012), these studies show a partial incorporation of other receptors with AVP, which could also be the case in the present study. This strongly suggests that central V1bR manipulation might not be sufficiently specific to assess the role of this receptor in all aspects of maternal behaviour. Therefore, the role of this system needs further investigation with respect to local manipulations of V1bR in lactating rats.

In conclusion, this is the first study to demonstrate a role for V1bR in lactating rats with respect to maternal care, further emphasizing the importance of the brain AVP system in the regulation of maternal behaviour. V1bR antagonism reduced various parameters of maternal care but not of maternal aggression, suggesting an exclusive role of V1bR in direct mother–pup interaction. Interestingly, the behavioural effects after V1bR manipulation were not as prominent as they were after V1aR activation/inhibition (Bosch and Neumann, 2008). Furthermore, studies investigating local manipulations of the V1bR in specific brain areas crucially involved in mediating maternal behaviour are warranted.

Chapter 3

Maternal behaviour in two breeding lines for extremes in anxiety – past and present observations in HAB/LAB dams with special focus on V1b receptors

Doris Bayerl: experimental design, performance of experiments, data analysis

Stefanie Klampfl: performance of experiments

Oliver Bosch: experimental design, performance of experiments

3.1 Abstract

The animal model of extremes in trait anxiety is commonly used to investigate neurobiological mechanisms of anxiety disorders and depression not only in males, but also in females. In lactating HAB and LAB rats studies provide evidence for increased maternal behaviour in hyper-anxious HAB compared to hypo-anxious LAB dams, which is connected to the elevated AVP expression and release in HAB dams. Blockade of the V1aR within HAB dams lead to an impairment of maternal behaviour. To investigate the involvement of V1bR in both breeding lines, we infused lactating HAB and LAB dams acutely icv with specific V1bR agonist and antagonist and observed subsequent maternal behaviour. Overall, no effect of treatment was detected in any group on any maternal behaviour. Maternal care, pup retrieval and maternal anxiety were higher in HAB dams compared to LAB dams, as described before. Surprisingly, maternal aggression was not higher in HAB dams, but equal in both breeding lines, with elevated threat behaviour in LAB dams.

Follow-up studies investigating maternal behaviour in non-manipulated HAB and LAB dams of subsequent generations show a shift in maternal aggression, with LAB dams becoming hyper-aggressive, even showing forms of abnormal aggression. Maternal care, pup retrieval and maternal anxiety were not changed. Potential changes in AVP and V1aR remain to be investigated.

In summary, V1bR seem not to be involved in the regulation of maternal behaviour in rats bred for extremes in trait anxiety; although site specific manipulations should be performed to confirm this. In addition, the maternal aggression phenotype of LAB dams changed in subsequent breedings over the last 3 years, now exceeding the aggression level of HAB dams.

3.2 Introduction

The successful rearing of offspring strongly depends on the mothers' amount of care giving, i.e. nursing and protection of her young. Especially in mothers suffering of postpartum depression or anxiety disorders, insufficient attention and a loss of interest towards the offspring is commonly observed. Among 10 – 22 % of mothers experience postpartum depression whereas postpartum anxiety disorders have an incidence of 5 – 12 %. These forms of postpartum mood disorders are hard to distinguish as they have most symptoms in common. Postpartum anxiety disorder is mainly diagnosed by a hyper-protective mothering-style, also called "helicopter-parenting" (for review see Hillerer et al., 2014).

This over-protective mothering style is characteristic in the HAB rat breeding line. The counterpart, LAB dams provide only low levels of maternal behaviour. These different phenotypes are referred to differences in the AVP system. The highly anxious phenotype of the HAB rats is based on an a single nucleotide polymorphism (SNP) in the AVP promoter region (Murgatroyd et al., 2004; for review see Landgraf et al., 2007), leading to increased AVP synthesis and release within the PVN (Keck et al., 2002; Wigger et al., 2004). This results in increased AVP mRNA expression within the PVN (Bosch and Neumann, 2008; Bosch et al., 2006; Keck et al., 2002; Wigger et al., 2004), the BNST and the medial amygdala (Bosch and Neumann, 2010) of male and female HAB rats compared to NAB and LAB rats. Thus, both breeding lines serve as animal models to investigate the neuronal basis of dysregulation in neuropeptides and the resulting maternal behaviour.

Regarding maternal behaviour, the hyper-anxious HAB rats differ in almost all aspects from hypo-anxious LAB dams (Bosch and Neumann, 2008; Bosch, 2011; Bosch et al., 2010, 2006, 2005; Neumann et al., 2005). Despite their hyper-anxiety, HAB dams, show higher amount of maternal care, which is reflected by increased ABN and total nursing compared to LAB dams (Bosch and Neumann, 2008; Bosch et al., 2006; Neumann et al., 2005). In the conventional PRT (see 3.2.5.2), HAB dams retrieve their scattered pups faster and also generally retrieve more pups compared to LAB dams (Neumann et al., 2005; for review see Bosch, 2011). Due to the hyper-protective mothering style of HAB dams, an aggressive phenotype in the maternal defence test is described by a decreased attack latency, an increased number of attacks and more threat behaviour compared to LAB dams (Bosch et al., 2005; for review see Bosch, 2013, 2011). Noteworthy, LAB dams were even shown to be less aggressive than NAB dams, similar to what was seen in maternal care (for review see Bosch and Neumann, 2012;

Bosch, 2011). This is in contrast to males, where both breeding lines are more aggressive than NAB males, LAB males even showing “abnormal” forms of aggression, e.g. attacking vulnerable body parts of the intruder (Beiderbeck et al., 2012).

Taking all these differences of HAB/LAB dams together, a correlation between the intensity of maternal behaviour and the dam’s innate anxiety was found in each breeding line (for review see Bosch, 2011).

Maternal behaviour is known to be mediated, at least in part, via AVP and its two distinct brain receptors, the V1aR and the V1bR (Bayerl et al., 2016, 2014; for review see Bosch and Neumann, 2012, 2008). Due to the increased AVP system in HAB rats, targeting the V1R in lactating dams is of special interest. In earlier studies an involvement of the V1aR was already demonstrated, as central infusion of a V1aR antagonist decreases the high levels of maternal care in HAB dams. In addition, central administration of synthetic AVP in less maternal LAB dams increases ABN (Bosch and Neumann, 2008). The level of maternal aggression was found to be correlated with AVP release within the amygdala in HAB, but not in LAB dams, i.e. AVP release increased during the maternal defence test in HAB dams only (Bosch and Neumann, 2010). However, local administration of a V1aR antagonist within the CeA decreases maternal aggression in HAB dams, whereas local synthetic AVP in LAB dams increases their aggressive behaviour towards an virgin female intruder (Bosch and Neumann, 2010).

So far, an involvement of V1bR in the differences of maternal behaviour in HAB and LAB dams was not investigated. Due to our results obtained from icv manipulated NAB dams (Chapter 2), an involvement of V1bR in the modulation of maternal behaviour was proved. Therefore, our aim was to determine the effects of central V1bR manipulation on maternal behaviour in lactating HAB/LAB dams, predicting effects on maternal care, especially by using V1bR antagonist in HAB dams and V1bR agonist in LAB dams. As we found similar aggression levels within the VEH-treated HAB and LAB dams, we analyzed data of maternal defence tests in non-manipulated dams of subsequent generations to observe the potential changes in maternal behaviour, with special focus on maternal aggression, over the past three years.

3.3 Material & Methods

3.3.1 Animals

Virgin female HAB and LAB rats of our own breeding (12–14 weeks, 220–250 g; University of Regensburg, Regensburg, Germany) were mated with sexually experienced NAB stud Wistar males and pregnancy was confirmed the next day by the presence of sperm in vaginal smears (assigned as PD 1). Rats were housed in groups of three to four until PD 18, when rats underwent surgery. On PD 18, rats were single-housed in plexiglass observation cages (38 x 22 x 35 cm^3) to ensure undisturbed parturition. All of the rats gave birth on PD 22 or 23. On the day of parturition, offspring were culled to eight pups of mixed sexes. At the same time, half of the bedding was replaced with new bedding to avoid any disturbance of the mother during the experiment.

Virgin female Wistar rats (10 weeks, 180–220 g; Charles River) at random stages of their oestrous cycle were used as intruders in the maternal defence test. They were kept group-housed in a separate room until the behavioural testing to avoid olfactory recognition.

All rats were maintained under a 12:12 h light/dark cycle (lights on 07.00 h) at 22 ± 1 °C and 55 ± 5% relative humidity, with free access to water and standard rat chow

All experiments were conducted in accordance with the European Communities Council Directive (86/609/EEC) and were approved by the local government of the Oberpfalz, Bavaria.

3.3.2 Implantation of an icv guide cannula

On PD 18, female rats were implanted with a stainless steel guide cannula (21 G, length 12 mm), 2 mm above the right lateral ventricle (1.0 mm posterior, 1.6 mm lateral to bregma, 2.0 mm ventral) (Paxinos and Watson, 1998) under semi-sterile conditions under isoflurane (Baxter Germany GmbH, Unterschleißheim, Germany) inhalation anaesthesia as described before (Bayerl et al., 2014; Klampfl et al., 2013)(also see 2.3.2). To prevent inflammation, rats received 0.12 ml of the antibiotic Enrofloxacin (Baytril 2.5%; Bayer Vital GmbH, Leverkusen, Germany) subcutaneously at the end of surgery.

3.3.3 Infusion of receptor-specific V1bR agonist or V1bR antagonist

Prior to (PD 16–17) and after (PD 19–21) surgery, rats were handled twice daily to familiarise them with the infusion procedure (Neumann et al., 1998a) Starting on LD 1, dams received one acute infusion of VEH (5 µl; Ringer's solution + 5% dimethyl sulphoxide), the receptor-specific V1bR agonist d[Leu4,Lys8]VP (50 ng/5 µl) (Pena et al., 2007a) or the receptor-specific V1bR antagonist SSR149415 (500 ng/5 µl) (Serradeil-Le Gal et al., 2002) on each experimental day 10 min prior to behavioural testing (Bayerl et al., 2014). The treatments were administered over 20 s *via* a 10-µl Hamilton syringe connected to an injection system consisting of an infusion cannula (25 G, length 14 mm, i.e. 2 mm exceeding the guide cannula) on a polyethylene tube 20 cm in length.

3.3.4 Experimental schedule

The experimental schedule was equivalent to testing V1bR agonist and antagonist in non-selected Wistar rats (see Chapter 2). Briefly, on LD 1, maternal care was observed in the home cage under 'non-stress' conditions before and immediately after treatment infusion. On LD 4, maternal care was monitored for 1 h in the morning before moving the rats to the maternal defence test room, as well as 1 h immediately after termination of the maternal defence test, which served as an acute psychosocial stressor for the lactating mother ('stress' conditions) (Neumann et al., 2001). To distinguish LD 4, on which the stress of the maternal defence test was included, from LD 1, we use the term 'non-stress' conditions for LD 1, although we are aware of the minimal amount of stress associated with the infusion procedure. Additionally, on LD 2 the dam's maternal motivation was tested in the PRT. Anxiety-related behaviour on the EPM was assessed on LD 3 and maternal aggression in the maternal defence test was measured on LD 4. All tests were performed in the light cycle between 09.00 h and 12.00 h.

3.3.5 Behavioural tests

3.3.5.1 Maternal care observation in the home cage

Maternal care was observed in the home cage according to an established protocol (Bayerl et al., 2014; Bosch and Neumann, 2008). In detail, the behaviour of the mother was

monitored every 2 min for approximately 10 s at 30-min intervals. The behaviours scored were nursing including ABN (high crouch posture + low crouch posture) as the only active nursing posture of the mother (Stern and Johnson, 1990) and blanket posture (i.e. lying passively on top of the pups) (for review see Bosch, 2011), as well as mother–pup interaction, which includes all nursing postures, licking/grooming the pups and carrying the pups.

3.3.5.2 Maternal motivation in the PRT

In the modified PRT (also see 4.3.5.2 and (Bayerl et al., 2016)), dams were tested for their motivation to retrieve their own eight pups in a novel environment (van Leengoed et al., 1987) according to an established protocol (Bosch and Neumann, 2008; Neumann et al., 2005). Naturally, the motivation to retrieve the pups during this early phase of lactation is very high (for review see Bosch, 2011).

In the afternoon of LD 2, dams were provided in their homecage with a potential nesting site, i.e. a red Perspex plastic house (13 x 17 x 11 cm^3, opening 6 x 8.5 cm^2; PLEXIGLAS® GS Rot 3C33, ThyssenKrupp Plastics GmbH, Regensburg, Germany; transparency: 13 %), which they were allowed to explore and to familiarize with for 150 min. On LD 3, pups were separated from their mothers one hour prior to testing, at the time when the red house was re-introduced and dams were transferred to the experimental room. During the separation period, pups were kept on a thermo pad (32 °C) in a different room than the dams. Subsequently, pups were brought to the experimental room and distributed in a black plastic box (54 x 34 x 60 cm^3) prepared with a handful of bedding from the home cage. Ten minutes after infusion of the treatment, the house and subsequently the dam was placed in the box (t0) and the time of retrieval and number of retrieved pups was scored during the 15-min test period. After termination of the test, the dam was placed back into her home cage together with her pups.

3.3.5.3 Maternal aggression in the maternal defence test

The dams were transported to the test room 60 min before testing. The dams (residents) received their respective treatment infusion 10 min prior to introducing a virgin female rat (intruder) into their home cage in the presence of the pups (Bosch et al., 2005; Neumann et

al., 2001; for review see Bosch, 2013). Intruders were used only once per day as well as only twice during the experiment with at least one day in between for recovery. During the 10-min testing period, aggressive behaviours, i.e. attacks and threat behaviours, which consist of offensive upright (the dam stands in an upright position in front of the intruder), keep down (the mother keeping the intruder down with her front paws), lateral threat (the mother engages to push the intruder aside by approaching laterally with her whole body) and aggressive grooming (for review see Bosch, 2013), were scored. Further, non-aggressive behaviours, including sniffing the intruder, exploration of the cage, maternal care, eating/drinking and self-grooming were assessed. After termination of the test, the virgin intruders were removed from the residents' home cage.

Due to the results of the maternal defence test (see below, Chapter 3.4.1.5.), we further controlled for maternal behaviour in HAB and LAB dams of subsequent breedings. These dams were observed under non-manipulated conditions, i.e. rats did not undergo surgery but were tested under the same experimental conditions as described above.

3.3.5.4 Anxiety-related behaviour on the EPM

Anxiety-related behaviour was tested on the EPM (Liebsch et al., 1998; Pellow et al., 1985) as described before (Bayerl et al., 2014; Bosch et al., 2010; Neumann et al., 2000). Briefly, the dams were transported to the separate EPM room 60 min prior to the test to allow habituation. The substances were infused 10 min before starting the test, when the dams were placed in the neutral zone of the EPM facing a closed arm and were allowed to freely explore the maze for 5 min. The percentage of time spent on the open arms (time on the open arms versus all arms) was taken as indicator for anxiety-related behaviour whereas the number of closed arm entries reflected locomotion (Neumann et al., 2000). Behaviours were scored live by an observer who was blind to treatment via a camera located above the EPM.

3.3.6 Verification of guide cannula placement

At the end of the experiments, rats were sacrificed with CO_2 and those being implanted with icv guide cannula were infused with 5 µl of ink. Brains were removed and cut with a razor blade at the implantation site of the cannula. If the ventricles were coloured blue, the

cannula placement was considered correctly, and only those rats were included in statistical analysis.

3.3.7 Statistical analysis

Statistical tests were performed using SPSS, version 20 (IBM, Ehningen, Germany).

For manipulated rats, maternal care and maternal motivation were analysed using a three-way ANOVA for repeated measures (factors: breeding line x treatment x time). In cases where we a priori predicted specific outcomes, planned comparisons of specific contrasts within a breeding line were also performed (two-way ANOVA for repeated measures; factors: time x treatment).

Maternal aggression and anxiety-related behaviour were analysed using a two-way ANOVA (factors: breeding line x treatment).

For non-manipulated rats, maternal motivation was analysed using a two-way ANOVA for repeated measures (factors: breeding line x time). Maternal care, maternal aggression and anxiety-related behaviour were analysed using independent t-tests. If data were not distributed normally (Kolmogorov-Smirnov Test), a Mann-Whitney-U test was performed.

The ANOVAs were followed by Sidak post-hoc correction if main effects were found. Data are presented as the mean + SEM. $p < 0.05$ was considered statistically significant.

3.4 Results

3.4.1 V1bR manipulation in lactating HAB/LAB dams of generation #47

3.4.1.1 Effects of V1bR agonism and antagonism on maternal care under non-stress conditions

ABN differed significantly between breeding lines (three-way ANOVA for repeated measures; $F_{1,32}$ = 42.15, p < 0.001; Figure 13A), but not depending on treatment or on time; no interactions were found. Planned comparisons, analyzing potential treatment effects in each breeding line separately revealed a time effect in LABs only ($F_{4,72}$ = 2.72, p = 0.036); no treatment effect or time x treatment interaction was found.

Splitting ABN in its two crouching behaviours, high crouch differed between breeding lines ($F_{1,32}$ = 17.50, p < 0.001; Figure 13B), but not depending on treatment or on time; no interactions were found. Planned comparisons, analyzing potential treatment effects in each breeding line separately did not reveal any further differences.

Low crouch also differed depending on breeding line ($F_{1,32}$ = 42.54, p < 0.001) and over time ($F_{4,128}$ = 3.06, p = 0.019), but not depending on treatment; no interactions were found (not shown). Planned comparisons, analyzing potential treatment effects in each breeding line separately revealed a time effect in LABs only ($F_{4,72}$ = 4.27, p = 0.004); no treatment effect or time x treatment interaction was found.

Blanket differed over time ($F_{4,128}$ = 4.83, p = 0.001; Figure 13C), but not depending on breeding line or on treatment; no interactions were found. Planned comparisons, analyzing potential treatment effects in each breeding line separately revealed a time effect in LABs only ($F_{4,72}$ = 4.60, p = 0.002); no treatment effect or time x treatment interaction was found.

Nursing differed significantly depending on breeding line ($F_{1,32}$ = 7.07, p = 0.012; Figure 13D) and over time ($F_{4,128}$ = 6.62, p < 0.001), but not depending on treatment; no interactions were found. Planned comparisons, analyzing potential treatment effects in each breeding line separately revealed a time effect in LABs only ($F_{4,72}$ = 5.94, p < 0.001); no treatment effect or time x treatment interaction was found.

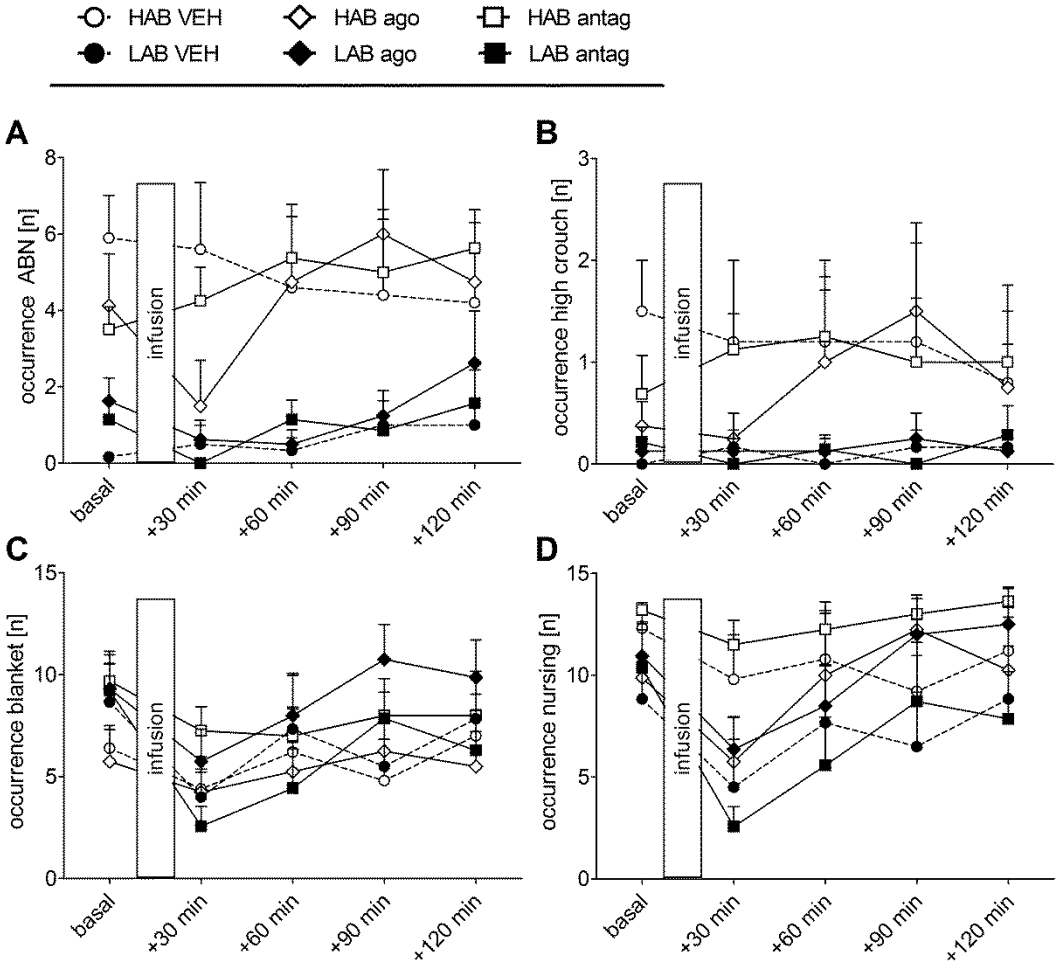

Figure 13 Effects of V1b receptor (V1bR) manipulation in HAB and LAB dams on maternal care under non-stress conditions.

The behaviours shown are arched back nursing (ABN; A), high crouch (B), blanket nursing posture (blanket; C), as well as the sum of all nursing postures (nursing; D). The behaviours were observed before (basal) and for 120 min in 30 min intervals after icv infusion of vehicle (VEH) (5 µl; Ringer's solution + 5% dimethyl sulphoxide), V1bR agonist d[Leu4,Lys8]VP (V1bR-ago; 50 ng/5 µl) or V1bR antagonist SSR149415 (V1bR-A; 500 ng/5 µl).

Data are presented as the mean + SEM (n = 4 - 8).

3.4.1.2 Effects of V1bR agonism and antagonism on maternal care under stress conditions

ABN differed significantly under stress conditions between breeding lines (three-way ANOVA for repeated measures; $F_{1,22}$ = 9.98, p = 0.005; Figure 14A) and over time ($F_{2,44}$ = 6.04, p = 0.005), but not depending on treatment; no interactions were found. Planned comparisons, analyzing potential treatment effects in each breeding line separately revealed a time effect

by trend in HABs only ($F_{2,14}$ = 3.67, p = 0.052); no treatment effect or time x treatment interaction was found.

Splitting ABN in its two crouching behaviours, high crouch differed between breeding lines ($F_{1,22}$ = 7.35, p = 0.013; Figure 14B) and over time ($F_{2,44}$ = 4.06, p = 0.024), but not depending on treatment; no interactions were found. Planned comparisons, analyzing potential treatment effects in each breeding line separately did not reveal any further differences

Low crouch also differed between breeding lines ($F_{1,22}$ = 9.14, p = 0.006) and over time ($F_{2,44}$ = 8.49, p = 0.001), but not depending on treatment; no interactions were found. Planned comparisons, analyzing potential treatment effects in each breeding line separately revealed a time effect in HABs only ($F_{2,14}$ = 4.35, p = 0.034); no treatment effect or time x treatment interaction was found (not shown).

Blanket differed between breeding lines ($F_{1,22}$ = 4.96, p = 0.037; Figure 14C) and over time ($F_{2,44}$ = 17.65, p < 0.001), but not depending on treatment; no interactions were found. Planned comparisons, analyzing potential treatment effects in each breeding line separately revealed a time effect in both breeding lines (HAB: $F_{2,14}$ = 7.66, p = 0.006; LAB: $F_{2,30}$ = 12.11, p < 0.001); no treatment effect or time x treatment interaction was found in any breeding line.

Nursing differed significantly between breeding lines ($F_{1,22}$ = 12.19, p = 0.002; Figure 14D) and over time ($F_{2,44}$ = 19.82, p < 0.001), but not depending on treatment; no interactions were found. Planned comparisons, analyzing potential treatment effects in each breeding line separately revealed a time effect in both breeding lines (HAB: $F_{2,14}$ = 15.12, p < 0.001; LAB: $F_{2,30}$ = 9.51, p = 0.001); no treatment effect or time x treatment interaction was found in any breeding line.

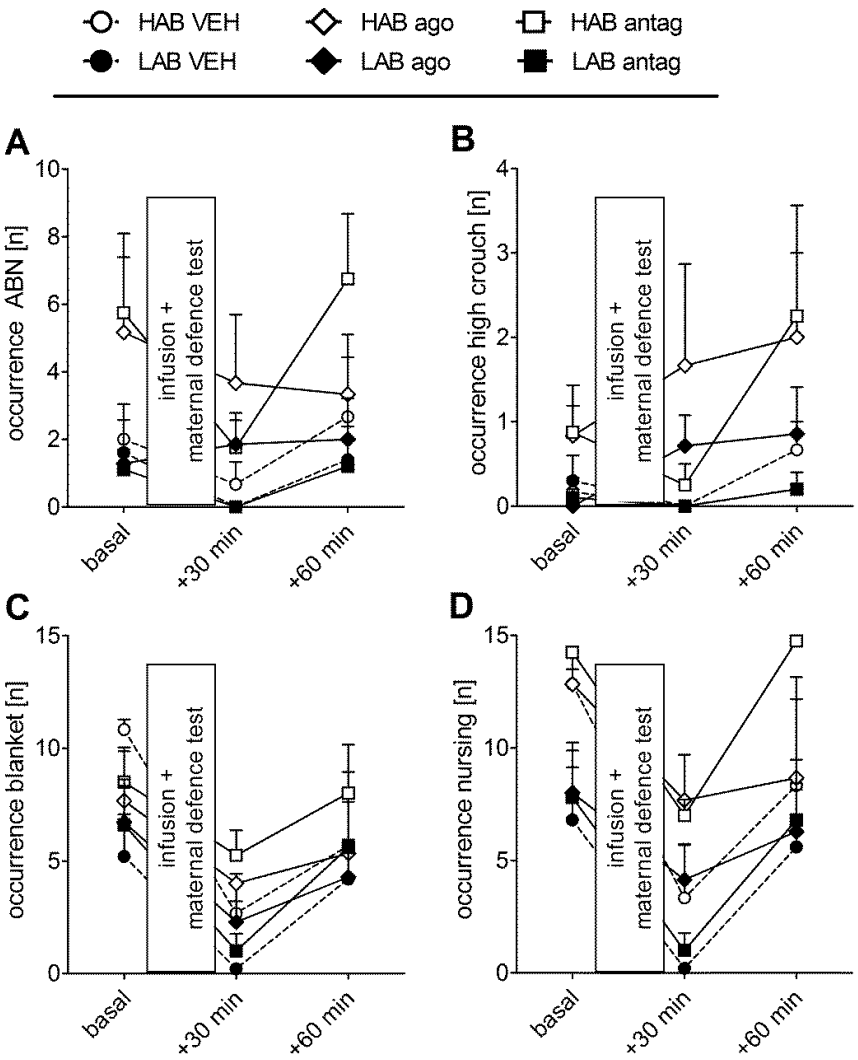

Figure 14 Effects of V1b receptor (V1bR) manipulation in HAB and LAB dams on maternal care under stress conditions.

The behaviours shown are arched back nursing (ABN; A), high crouch (B), blanket nursing posture (blanket; C), as well as the sum of all nursing postures (nursing; D). The behaviours were observed before (basal) and for 60 min (t +30 min and t +60 min) immediately after the combined icv infusion and maternal defence test (for details, see Section 3.3.5.3). Dams received a 5 µl icv infusion of vehicle (VEH), V1bR agonist (V1bR-ago) or V1bR antagonist (V1bR-A) (for details, see Figure 13) 10 min prior to the maternal defence test.

Data are presented as the mean + SEM (n = 3 - 7).

3.4.1.3 Effects of V1bR agonism and antagonism on maternal motivation

Maternal motivation measured in the modified PRT on LD 2 differed over time (three-way ANOVA for repeated measures; $F_{15,435} = 102.29$, $p < 0.001$), but not depending on breeding line or treatment; a time x breeding line interaction was found ($F_{15,435} = 7.26$, $p < 0.001$). No further interactions were analyzed (not shown).

Focussing on the VEH groups only (due to a missing treatment effect), retrieval behaviour differed over time (two-way ANOVA for repeated measures; $F_{15,135} = 51.49$, $p < 0.001$; Figure 15), but not depending on the breeding line; a significant time x breeding line interaction was found ($F_{15,135} = 6.27$, $p < 0.001$). In detail, HAB dams retrieved significantly more pups from t10 onwards, whereas LAB dams already retrieved more pups from t7 onwards, compared to t0.

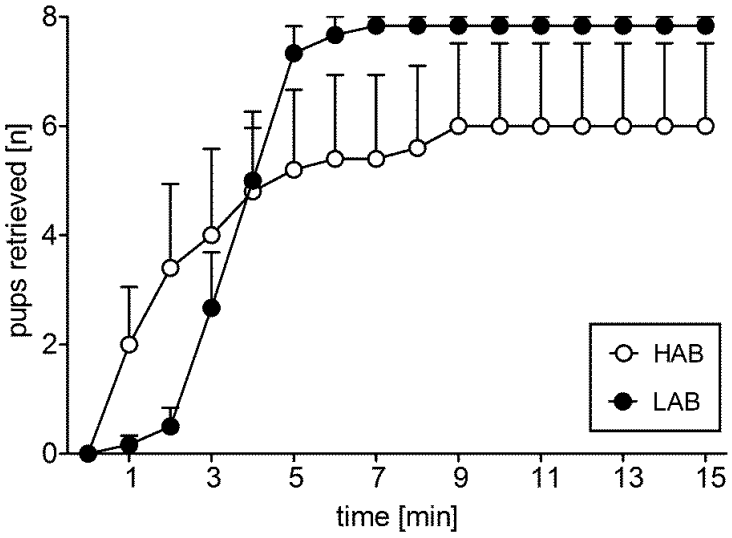

Figure 15 Maternal motivation to retrieve pups of HAB and LAB dams (VEH groups) in the pup retrieval test.
Following a 60-min separation, pups were distributed in the testing arena and the retrieval of pups was recorded (for details, see Section 3.3.5.2).
Data are presented as the mean + SEM (n = 4 - 8).

3.4.1.4 Effects of V1bR agonism and antagonism on maternal aggression

Aggressive and non-aggressive behaviours were not influenced by any treatment during the maternal defence test in any breeding line.

Depending on breeding line, differences were found in the latency for the first attack (two-way ANOVA; $F_{1,26} = 21.21$, $p < 0.001$), keep down ($F_{1,26} = 4.48$, $p = 0.046$), lateral threat ($F_{1,26} = 14.72$, $p = 0.001$), aggressive grooming ($F_{1,26} = 9.24$, $p = 0.006$) and sniffing ($F_{1,26} = 11.18$, $p = 0.003$) independent of treatment; no time x treatment interaction was found.

Focussing on the VEH groups only (due to a missing treatment effect), separate statistics showed an increase in number of attacks by trend in LAB dams compared to HAB dams ($t_6 = -$

2.48, p = 0.067; Figure 16A). Further, significantly increased lateral threat behaviour was found in LAB dams compared to HAB dams (independent t-test; t_6 = -2.46, p = 0.049; Figure 16B).

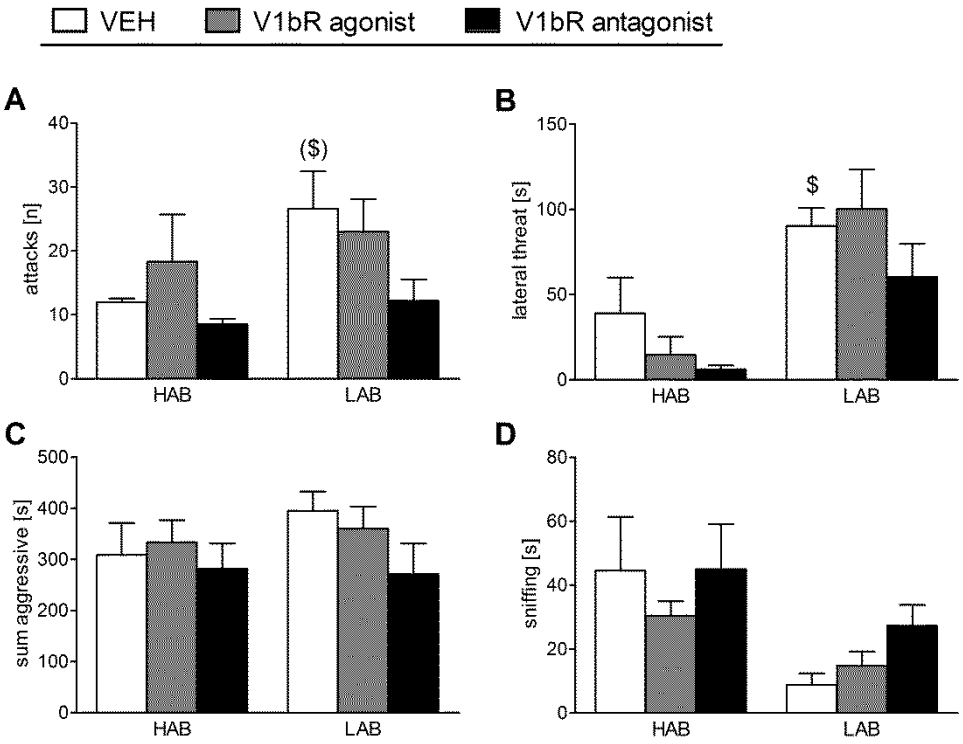

Figure 16 Effects of V1b receptor (V1bR) manipulation in HAB and LAB dams on maternal aggression in the maternal defence test.

Lactating mothers (residents) were exposed to virgin female rats (intruders) in the presence of their pups for 10 min. The number of attacks (A), the time spent on showing lateral threats (B) as well as the time spent on attacks plus threat behaviour as reflected in the sum of all aggressive behaviours (C) is shown. Additionally, sniffing the intruder was measured (D). The dams received a 5 µl icv infusion of vehicle (VEH), V1bR agonist (V1bR-ago) or V1bR antagonist (V1bR-A) (for details, see Figure 13) 10 min prior to the test.

Data are presented as mean + SEM (n = 3 - 7). $ p ≤ 0.05, ($) = 0.067 vs. HAB VEH in separate statistics.

3.4.1.5 Effects of V1bR agonism and antagonism on maternal anxiety

Anxiety-related behaviour on the EPM differed depending on breeding line (three-way ANOVA; $F_{1,31}$ = 24.94, p < 0.001; see Table 1), but was not affected by treatment; no breeding line x treatment interaction was found.

Locomotion did not differ between the breeding lines or depending on treatment; no breeding line x treatment interaction was found.

Table 1 Effect of central V1bR manipulation in HAB and LAB dams on maternal anxiety

	treatment	dams [n]	% time on open arm	closed arm entries [n]
HAB	VEH	5	11.6 ± 1.9	7.0 ± 1.8
	agonist	4	7.9 ± 1.5	6.8 ± 1.2
	antagonist	5	18.9 ± 5.8	8.0 ± 1.3
LAB	VEH	5	48.9 ± 7.4	7.2 ± 0.7
	agonist	8	36.9 ± 7.4	6.1 ± 0.9
	antagonist	5	40.1 ± 9.5	6.6 ± 0.8

Data are presented as mean ± SEM.

3.4.2 Maternal behaviour in non-manipulated HAB/LAB dams of generation #50

Maternal care was observed one hour in the morning on LD 1. We found that HAB and LAB dams differed significantly in their nursing behaviour. HAB dams showed more ABN compared to LAB dams ($t_{39} = 4.14$, $p < 0.001$; Figure 17A). Dividing ABN in high crouch and low crouch, both nursing positions are increased in HAB compared to LAB dams (high crouch: $U = 67.5$, $W = 320.5$, $Z = -3.85$, $p < 0.001$; Figure 17B; low crouch: $t_{39} = 2.61$, $p = 0.013$; Figure 17C). Blanket nursing did not differ between the breeding lines (Figure 17D). Total nursing was higher in HAB compared to LAB dams ($t_{39} = 3.05$, $p = 0.004$; Figure 17E).

Maternal motivation in the conventional PRT was higher in HAB dams compared to LAB dams. When testing the dams in the modified PRT, LAB dams improved their retrieval behaviour, what makes them indistinguishable from HAB dams (see Chapter 4).

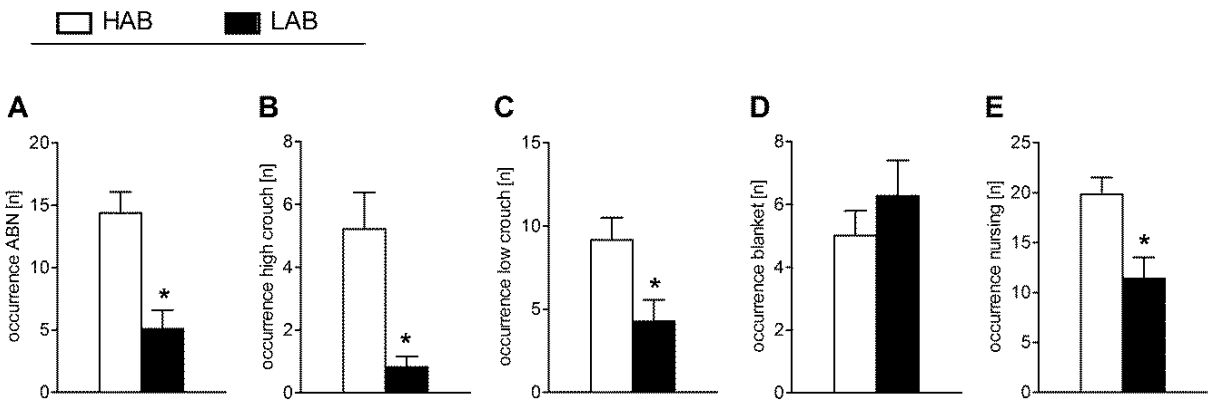

Figure 17 Maternal care under non-stress conditions in non-manipulated HAB and LAB dams of generation #50.

The behaviours shown are arched back nursing (ABN; A), high crouch (B), low crouch (C), blanket nursing posture (blanket; D), as well as the sum of all nursing postures (nursing; E). The behaviours were observed for 60 min in the morning (for details see Section 3.3.5.1).

Data are presented as the mean + SEM (n = 19 – 22 per group). * $p \leq 0.05$ versus HAB.

Due to the fact that rats were also involved in another subsequent experiment which is not part of this thesis, only a smaller cohort of HAB and LAB dams underwent maternal aggression testing. Therefore the rat numbers differ in the maternal defence test substantially from those above.

Maternal aggression was higher in LAB dams compared to HAB dams (sum aggressive: $t_{13} = 2.80$, $p = 0.015$; Figure 18).

LAB dams showed more attacks ($t_{13} = -2.35$, $p = 0.063$), duration of attack behaviour ($t_{13} = -2.73$, $p = 0.041$: Figure 18A) and threat behaviours like keep down ($t_{13} = -3.34$, $p = 0.021$; Figure 18B), lateral threat ($t_{13} = -2.61$, $p = 0.022$; Figure 18C) and offensive upright ($t_{13} = -2.75$, $p = 0.039$; Figure 18D) compared to HAB dams. Further, time sniffing the intruder was decreased in LABs compared to HABs ($t_{13} = 4.18$, $p = 0.001$). No further differences in any other aggressive or non-aggressive behaviour were found between breeding lines.

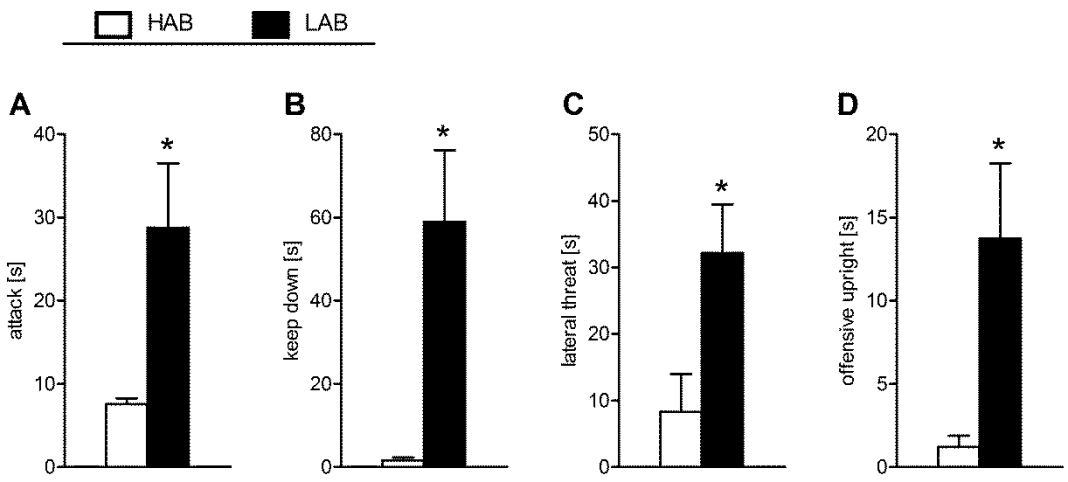

Figure 18 Maternal aggression in non-manipulated HAB and LAB dams of generation #50 in the maternal defence test.

Lactating mothers (residents) were exposed to virgin female rats (intruders) in the presence of their pups for 10 min. The number of attacks (A) as well as the time spent with the threat behaviours keep down (B), lateral threat (C) and offensive upright (D) are shown.

Data are presented as mean + SEM (n = 6 – 9 per group). * $p \leq 0.05$ versus HAB

Maternal anxiety was higher in HAB dams compared to LAB dams by spending less time on the open arm ($t_{39} = -8.71$, $p < 0.001$; Figure 19 left) and showing less locomotion ($t_{39} = -3.08$, $p = 0.004$; Figure 19 right).

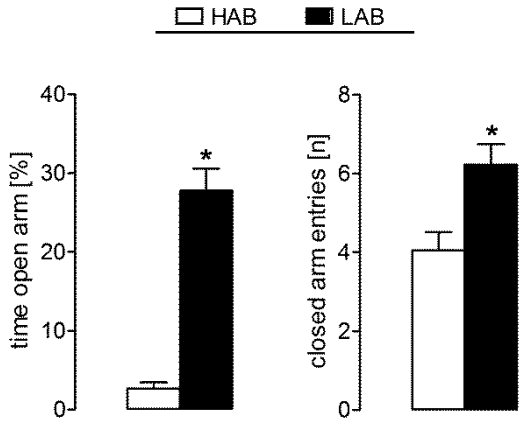

Figure 19 Anxiety-related behaviour in HAB and LAB dams of generation #50 on the EPM

Time spent on the open arms (left) and locomotion reflected by closed arm entries (right) of HAB and LAB dams.

Data are presented as the mean + SEM (n = 19 – 22 per group). * $p \leq 0.05$ versus HAB.

3.4.3 Maternal behaviour in non-manipulated HAB/LAB dams of generation #51

Maternal care was observed one hour in the morning on LD 1. We found that HAB and LAB dams differed significantly in their nursing behaviour. Although ABN did not differ between the breeding lines (Figure 20A), HAB dams showed more high crouch compared to LAB dams (t_{12} = 3.15, p = 0.008; Figure 20B). Low crouch did not differ between HAB and LAB dams (Figure 20C). Blanket nursing was higher in LAB compared to HAB dams (t_{12} = -2.70, p = 0.023; Figure 20D). Total nursing did not differ between the breeding lines (Figure 20E).

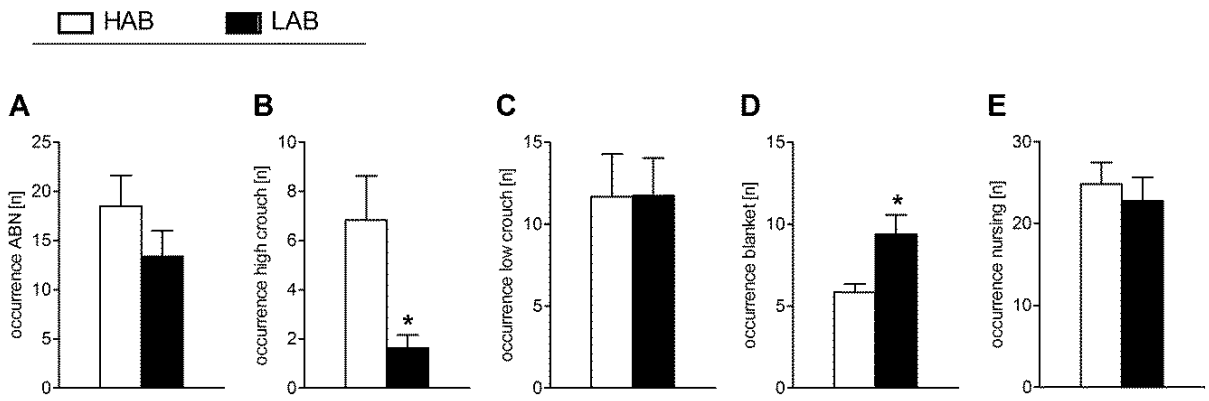

Figure 20 Maternal care under non-stress conditions in non-manipulated HAB and LAB dams of generation #51.

The behaviours shown are arched back nursing (ABN; A), high crouch (B), low crouch (C), blanket nursing posture (blanket; D), as well as the sum of all nursing postures (nursing; E). The behaviours were observed for 60 min in the morning (for details see Section 3.3.5.1).

Data are presented as the mean + SEM (n = 6 – 8 per group). * p ≤ 0.05 versus HAB.

Maternal aggression was not analyzed in the dams of this breeding as maternal defence tests in LABs have to be interrupted after a maximum of three minutes due to abnormal high aggression of the dams towards the intruders resulting in serious injuries of the intruders (attacks of vulnerable body parts).

Maternal anxiety was higher in HAB dams compared to LAB dams by spending more time on the open arm (t_{12} = -5.11, p < 0.001; Figure 21 left). Locomotion did not differ between breeding lines (see Figure 21 right).

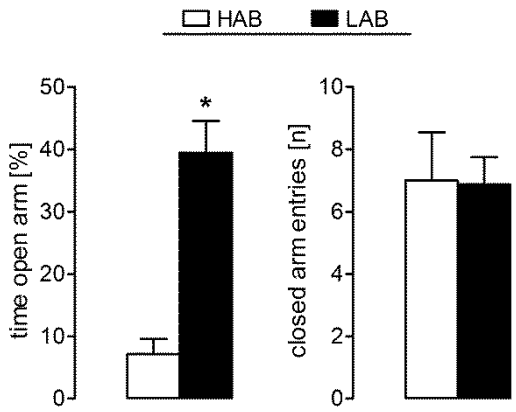

Figure 21 Anxiety-related behaviour in HAB and LAB dams of breeding #51 on the EPM

Time spent on the open arms (left) and locomotion reflected by closed arm entries (right) of HAB and LAB dams.

Data are presented as the mean + SEM (n = 6 - 8).

Analysis of breeding EPM data compared to experimental EPM data showed significant differences between HAB and LAB rats under different reproductive stages (Supplementary Figure S1). Thus LAB rats showed significantly decreased % time on the open arm in the experimental EPM compared to the breeding EPM (independent t-test; $t_{14} = 3.12$, $p = 0.013$), whereas no differences between the two tests were found in HAB rats. Comparing HAB and LAB rats in each test separately, the two breeding lines differ in the breeding EPM ($t_{12} = -24.96$, $p < 0.001$) as well as in the experimental EPM ($t_{12} = -5.11$, $p < 0.001$), with HAB rats being more anxious compared to LAB rats.

3.5 Discussion

Central manipulation of V1bR in lactating HAB and LAB rats did not show any effect on maternal behaviour, unlike to lactating NAB rats (Chapter 2). In line with previous studies, LAB dams improved their retrieval behaviour up to levels of HAB dams in the modified PRT as shown before (Chapter 4). Surprisingly, the aggressive phenotype of the dams was not as expected in this experiment. LAB dams had equivalent levels of aggressive behaviours compared to HAB dams, threat behaviour of LAB dams even exceeded the amount of HAB dam's threats. Nevertheless, we found the typical HAB phenotype described by elevated maternal care and maternal anxiety. With subsequent breeding, LAB dams showed an abnormal aggressive phenotype, leading to serious injuries of the intruders. Therefore, HAB/LAB rats do not provide a good tool for investigations of postpartum adaptations and dysregulations any longer.

Various studied have shown the involvement of AVP and its V1 receptors in the regulation of maternal behaviour in rats and mice (Chapter 2, 4 and 5)(Bayerl et al., 2014; Bosch and Neumann, 2010, 2008; Wersinger et al., 2007). The differences in the HAB/LAB phenotype have been proven to rely, at least in part, on increased levels of AVP in HAB rats. The hyper-maternal phenotype of HAB dams has been shown to be stable over years, with only mild fluctuations in the last generation (#51). In more detail, high crouch was still shown in a higher frequency in HAB dams, whereas low crouch levels appeared to be equal. This difference may depend on environmental conditions. High crouch position reflects a highly sensitive maternal behaviour as barely perceptible changes (at least for humans) may not disrupt, but decrease the quality of ABN, by performing low instead of high crouch (personal observations SMK, OJB, DSB). Further, noise disturbance during the behavioural observations, which may not be audible for researchers, may interfere with high crouch, as rats have better, and wider frequency range of hearing than humans (Lauer et al., 2009; Milligan et al., 1993).

In contrast to a stable maternal phenotype of HAB dams, regarding increased ABN, pup retrieval and maternal anxiety (Bosch and Neumann, 2010, 2008; Kruszynski et al., 1980) compared to LAB dams, we found changes in maternal aggression in our study.

Maternal aggression not only serves to protect the offspring of potential threats but also comes along with high energy costs and health risks (for reviews see Bosch and Neumann, 2012; Numan and Insel, 2003). Therefore, defensive behaviour can be seen by the

subordinate, which helps to reduce injuries or killing (Blanchard et al., 2003). Further, defensive behaviours of the intruder lead to a limitation of attacks by the resident, supported by vibrissae-contact between the opponents (Blanchard and Blanchard, 1977). With subsequent breeding, LAB dams seem to ignore defensive behaviours of the intruders, resulting in serious attacks and bites on vulnerable body parts like the nose, anogenital region, and belly. These observations on changed frequency and type of aggression are similar to what was seen in LAB males already several years ago (Beiderbeck et al., 2012). Normally, residents attack the back or flanks of an intruder rat, according to species-specific rules (Blanchard et al., 2003). Aggression that exceeds a distinct level or species-specific pattern is called escalated, pathological or abnormal aggression (Takahashi and Miczek, 2014). This abnormal aggression differs quantitatively (decreased attack latency, higher number and duration of attacks and threat behaviour) as well as qualitatively (attacks of high intensity towards vulnerable body parts, like head and throat, of the intruder) from normal aggression levels (for review see Haller and Kruk, 2006).

Most likely, the reason of this abnormal aggression is due to selective breeding over years. Selective breeding is described to lead to a stabilisation of traits (for review see Neumann et al., 2010). However, aggression in females, especially maternal aggression, was not assessed in all HAB/LAB generations (in contrast to anxiety as phenotype readout parameter), therefore, it may have increased over the last generations. This shift to heightened aggression was also seen in HAB and LAB males, with LAB males showing abnormal aggression (for review see Neumann et al., 2010).

In addition, beside the SNP for AVP, further neuropeptide systems involved in the regulation of aggression, i.e. the serotonin system (De Almeida et al., 2005; Ferris, 1996), may have adapted over years with subsequent breeding. To investigate possible adaptations a genetic screening of the AVP plus other neurotransmitter systems would provide the next step.

Another reason for abnormal aggressive behaviour may be the loss or impairment of receiving social cues and behavioural control. According to this hypothesis, LAB males show a decreased neuronal activation (c-fos) of the LS (Beiderbeck et al., 2007; Veenema et al., 2007), which is associated with increased aggression as seen in mice (for review see Haller and Kruk, 2006). Further, the LS is known to regulate anxiety and social recognition also in females (Engelmann et al., 1996; Sheehan et al., 2004). This might explain the higher amount of aggression that LAB dams display due to impaired recognition of social cues. Studies

investigating the neuronal activation in lactating HAB and LAB dams during maternal aggression, may shed light on a possible involvement of brain regions important for behavioural control, like the LS, the prefrontal cortex or the amygdala (for review see Haller and Kruk, 2006).

In addition, we observed decreased sniffing in LAB dams of generation #50 compared to HAB dams. This is in line with observations in male LABs, which also show less social investigation of conspecifics (Ohl et al., 2001; for review see Lukas and Neumann, 2013) but increased aggression also in situations without a potential threat, i.e. towards a female or anaesthetized rat (Beiderbeck et al., 2012; de Boer et al., 2003). However, data on social investigation in virgin females during the FIT does not reflect a difference between HAB and LAB rats (de Jong et al., 2014). Both strains show decreased sniffing compared to NAB females in adolescence as well as in adulthood. However, social investigation includes not only sniffing, but also approach behaviour. Data on social investigation and/or sniffing in non-manipulated lactating females during the maternal defence test are incomplete/ scarcely reported over generations. Therefore, future assessment of social investigation with special focus on sniffing in following generation of HAB and LAB rats can provide further insight in the ability of the breeding lines in receiving social cues properly. A lack in this ability could explain the abnormal levels of aggression in LAB dams towards an intruder in the maternal defence test.

Regarding the changes in maternal aggression, one also has to take seasonal fluctuations into account. Seasonal variations even under constant laboratory conditions in (inter-male) aggression have been observed before (de Boer et al., 2003). In our laboratory we also observe decreased (male) aggression during winter, compared to autumn and summer (for review see Neumann et al., 2010). These observations are in line with human studies, showing the lowest level of aggression during winter compared to other seasons (Miczek et al., 2002). Testing of generation #47 and #50 took place in spring, whereas experiments with generation #51 were performed in autumn. The increased aggression levels of generation #51 are in contrast to iterature; therefore our results cannot be referred to annual fluctuations, as a steadily increasing aggression was observed instead. Thus, changes due to other factors are more likely.

Interestingly, when comparing data of the EPM testing of rats used as virgins (breeding EPM) with the data of the dams (experimental EPM) of breeding #51, LAB dams show higher

anxiety in the latter, whereas anxiety in HAB rats is unchanged. This stands in contrast to anxiolytic effects, which are described in general in lactation (for reviews see Neumann et al., 2010; Numan and Insel, 2003). Due to the fact that LAB rats already show very little anxiety, a further drop might not be possible. But interestingly, also in HAB females the anxiolytic effect of lactation is missing, which could not be due to a floor effect. It might be that changes in maternal aggression are concomitant to these changes in maternal anxiety or vice versa, as a correlation of both behaviours have been shown in HAB and LAB rats before (for review see Bosch, 2011).

Interestingly, V1bR antagonism was ineffective in both breeding lines. This stands in contrast to the findings in NAB dams, where V1bR blockade decreased maternal care under non-stress as well as under stress conditions (Chapter 2). The lack of an effect in HAB/LAB dams might be due to changes in the AVP system and the resulting phenotype. Low levels of maternal care in LAB rats might hinder further decrease of nursing and mother-pup interaction after V1bR blockade. Chronic administration of the V1bR agonist/antagonist and/or local manipulation, i.e. within the PVN might help to rule out potential effects of the V1R subtype.

The change in aggression was rather suprising, as other maternal parameters were not affected. Due to observed changes in maternal aggression focusing especially on the abnormal aggressive behaviour in LAB dams, we decided to halt the use of HAB/LAB dams as animal model for investigating maternal behaviour. Studies investigating the influence of V1bR in maternal behaviour were conducted in Wistar rats instead (see Chapter 4 & 5).

Nevertheless, female HAB/LAB rats show a stable phenotype in anxiety- and depressive like behaviour, providing a good animal model for further investigations of neurobiological maladaptations in mood disorders, leading to anxiety or depression.

Chapter 4

Antagonism of V1b receptors promotes maternal motivation to retrieve pups in the MPOA and impairs pup-directed behaviour during maternal defence in the mpBNST of lactating rats.

Authors' contribution:

Doris Bayerl:	experimental design, performance of experiments, data analysis, first draft of manuscript
Veronika Kaczmarek:	performance of experiments
Benjamin Jurek:	performance of experiments, revision of manuscript
Erwin van den Burg:	primer-design for qPCR
Inga Neumann:	revision of manuscript
Barbara Gaßner:	performance of experiments
Stefanie Klampfl:	performance of experiments, revision of manuscript
Oliver Bosch:	experimental design, performance of experiments, revision of manuscript

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4.1 Abstract

Recent studies using V1bR knockout mice or central pharmacological manipulations in lactating rats highlighted the influence of this receptor for maternal behavior. However, its role in specific brain sites known to be important for maternal behavior has not been investigated to date. In the present study, we reveal that V1bR mRNA (quantitative real-time PCR; qPCR) and protein levels (Western Blot) within either the MPOA or the mpBNST did not differ between virgin and lactating rats. Furthermore, we characterized the effects of V1bR blockade *via* bilateral injections of the receptor subtype-specific antagonist SSR149415 within the MPOA or the mpBNST on maternal behavior (maternal care under non-stress and stress conditions, maternal motivation to retrieve pups in a novel environment, maternal aggression) and anxiety-related behavior in lactating rats. Blocking V1bR within the MPOA increased pup retrieval, whereas within the mpBNST it decreased pup-directed behavior, specifically licking / grooming the pups, during the maternal defense test. In addition, immediately after termination of the maternal defense test, V1bR antagonism in both brain regions reduced nursing, particularly ABN. Anxiety-related behavior was not affected by V1bR antagonism in either brain region. In conclusion our data indicate that V1bR antagonism significantly modulates different aspects of maternal behavior in a brain region-dependent manner.

4.2 Introduction

Maternal behavior serves to increase the probability that the offspring survive to maturity (for review see Numan and Insel, 2003). This behavior is comprised of different activities directed towards the young like nursing, licking / grooming, retrieving the pups into the nest but also defending them from any potential threat. Numerous studies have shown the importance of AVP and its V1aR in the regulation of maternal behavior of rats (Bosch and Neumann, 2008; Nephew and Bridges, 2008; for review see Bosch and Neumann, 2012). Shortly before parturition, the activity/expression of AVP and V1aR is up-regulated (Bosch et al., 2007; Caldwell et al., 1987; Caughey et al., 2011; Landgraf et al., 1991) in preparation for the forthcoming challenge of motherhood. This up-regulation continues into lactation (Bosch and Neumann, 2008; Van Tol et al., 1988; Walker et al., 2001), where the AVP system facilitates maternal behavior as demonstrated by central (Pedersen and Prange, 1979; Pedersen et al., 1982) and local manipulations within the MPOA (Bosch and Neumann, 2008; Pedersen et al., 1994) and the BNST (Bosch et al., 2010). Both brain regions are important regulators of maternal behavior and form the so-called maternal “super-region” (for review see Numan and Insel, 2003). Specifically, the MPOA plays a distinct role in maternal motivation as measured by pup retrieval behavior (Kalinichev et al., 2000; Neumann et al., 2005; Numan, 1990; Pedersen et al., 1994) whereas the BNST is involved in maternal aggression (Bosch et al., 2010; Consiglio et al., 2005; Klampfl et al., 2014). Accordingly, in the MPOA and BNST endogenous AVP release increases during mother-pup interaction (Bosch et al., 2010). Inhibition of the V1aR in the MPOA of the lactating mother *via* local infusion of a V1aR antagonist or receptor down-regulation by antisense oligodeoxynucleotides decreases pup retrieval (Bosch and Neumann, 2008; Pedersen et al., 1994). Blocking V1aR within the BNST decreases aggression against a virgin female intruder rat during the maternal defense test without affecting maternal care (Bosch et al., 2010).

To date, most studies investigating the function of the AVP system in maternal behavior have focused on the V1aR. Considering the expression of the second brain V1 receptor, the V1bR, in the maternal super-region (Hernando et al., 2001), it is very likely that the effects of AVP on maternal behavior are brought about by the two different AVP receptor subtypes. However, studies investigating the role of V1bR have been hindered due to a lack of specific pharmacological tools. An initial study in V1bR knockout mice demonstrated reduced maternal aggression in knockout compared to wild-type mothers (Wersinger et al., 2007).

Recently, using the receptor subtype-specific V1bR antagonist SSR149415 (Serradeil-Le Gal et al., 2002), we revealed that in Wistar rats central blockade of the receptor reduced nursing and mother-pup interaction, whereas it did not affect pup retrieval or maternal aggression (Bayerl et al., 2014).

In the present study, we investigated the role of V1bR in various aspects of maternal behavior in more detail by focusing on the two maternal brain regions MPOA and BNST. Firstly, we assessed whether lactation is associated with altered V1bR mRNA (experiment 1) and/or protein level (experiment 2) by comparing lactating and virgin rats. Secondly, we studied the behavioral effects of acute local infusion of the V1bR antagonist SSR149415 within the MPOA (experiment 3) or mpBNST (experiment 4) on maternal care under basal condition (non-stress) and following exposure to the maternal defense test (stress condition), as well as on maternal motivation to retrieve pups, maternal aggression and anxiety-related behavior.

4.3 Material and Methods

4.3.1 Animals

Female Wistar rats (12 - 14 weeks, 220 - 250 g, Charles River Laboratories, Sulzfeld, Germany) were kept under standard laboratory conditions (12 h / 12 h light-dark cycle, with lights on at 07:00 h; 22 ± 1 °C; 55 ± 5 % relative humidity; free access to water and standard rat chow).

In experiments 1 and 2, half of the rats were kept as virgins, whereas the other half was mated with sexually experienced stud Wistar males. Pregnancy was confirmed the next day by the presence of sperm in vaginal smears (assigned as PD 1). Rats were kept in mixed groups (pregnant/ virgin) of 3 to 4 rats before they were single-housed on PD 18 (or equivalent in virgins) to ensure undisturbed parturition.

For experiments 3 and 4 virgin female Wistar rats were mated as described above. Pregnant rats were group-housed up to four rats per cage until surgery on PD 18. After surgery, pregnant rats were single-housed in plexiglass observation cages (38 x 22 x 35 cm^3) to ensure undisturbed parturition. On the day of birth, offspring were culled to eight pups of mixed sexes and half of the bedding was replaced by new bedding. Dams were randomly assigned to one of the treatment groups on LD 1 and received the same treatment throughout the experiment.

For the maternal defense test, naïve virgin female Wistar rats (10 weeks, 180 - 220 g; Charles River) at random stages of their estrous cycle were used as intruders. They were kept group-housed in a separate room until behavioral testing to avoid olfactory recognition by the lactating mothers (for review see Bosch, 2013).

All experiments were performed in accordance with the European Communities Council Directive (86/609/EEC) and were approved by the local government of the Oberpfalz, Bavaria, Germany.

4.3.2 Experiment 1: qPCR for V1bR mRNA in the MPOA and BNST

Non-manipulated, undisturbed rats were sacrificed on LD 4 (or equivalent in virgins at random stages of the estrous cycle; n = 3 – 5 per group). Brains were removed, snap-frozen in n-methylbutane on dry ice and stored at -80 °C until further processing. Next, brains were

cut into 2x 200 µm slices at the regions of interest, which were identified with the aid of a rat brain atlas (Paxinos and Watson, 2007). The MPOA and BNST were dissected with a puncher (inner diameter: 1 mm) and side-pooled; thus, a total of 4 punches of each brain region were taken per rat. Total RNA was extracted using RNeasy Micro Kit (Qiagen, Hilden, Germany) in combination with QIAshredder columns (Qiagen) and RNase-Free DNase Set (Qiagen) according to the manufacturer's instructions. RNA content was determined with the aid of a NanoDrop photospectrometer. Next, 100 ng of RNA were reverse transcribed into cDNA by use of SuperScript III according to manufacturer's instructions (Invitrogen, Darmstadt, Germany). SYBR Green-based (Qiagen) qPCR for V1bR (forward primer: 5'-CAT ACC TCC ATC CAC CTT CC-3'; reverse primer: 5'-TCT TCA TCC CTA CCT AGC CA-3'; Metabion, Planegg/ Steinkirchen, Germany) relative to ribosomal protein L13A (Rpl13A; NM_173340; forward primer: 5'-ACA AGA AAA AGC GGA TGG TG-3'; reverse primer: 5'-TTC CGG TAA TGG ATC TTT GC-3'; Metabion) and Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, zeta (Ywhaz; NM_013011; forward primer: 5'-TTG AGC AGA AGA CGG AAG GT-3' ; reverse primer: 5'-GAA GCA TTG GGG ATC AAG AA-3'; Metabion) as reference genes (Bonefeld et al., 2008) was performed on the 7500 Fast Real Time PCR Systems v2.0.6 (Applied Biosystems, Darmstadt, Germany) and mRNA levels were quantified with the comparative C_T method ($\Delta\Delta C_T$). Specificity of the primers was assured by omitting reverse transcription and by using ddH_2O as template. The PCR protocol consisted of an initial denaturation step of 5 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 10 s, and annealing/extension at 60 °C for 45 s. At the end of the protocol a melting curve was generated and PCR products were analyzed by agarose gel electrophoresis to confirm the specificity of the primers. All samples were run in duplicate.

4.3.3 Experiment 2: Western Blot analysis for V1bR protein in the MPOA and BNST

Rats were treated and brain punches were taken as described for experiment 1 (n = 3 – 5 per group). Proteins were extracted from punches in 50 µl lysis buffer (containing 0.5 mM of 0.02 % EDTA (Sigma Life Science, Steinheim, Germany), 250 mM of NaCl (powder; VWR Chemicals, Leuven, Belgium), 50 mM of 1 M HEPES (Sigma Life Science), 0.5 % of Igepal (Sigma-Aldrich, Steinheim, Germany), 10 % of Halt-Protease & Phosphatase Inhibitor 10x Cocktail (Thermo Scientific, Rockford, USA)) in which the probes were homogenized with a

sterile handheld pestle. After gentle rotation (1 h, 4 °C) and centrifugation (15 min, 13.000 rpm, 4 °C) the supernatant containing the protein-fraction was collected on ice. The protein concentrations were determined using the BCA Protein Assay Kit (Pierce/Thermo Scientific) and the Optima plate reader (BMG Labtech GmbH, Ortenberg, Germany). Fifteen µg of each protein sample were electrophoretically separated on a 12 % SDS-gel (Criterion TGX Stain Free Precast Gel, Bio-Rad, Munich, Germany) and subsequently transferred onto a 0.2 µm nitrocellulose membrane (Bio-Rad) by means of the Turbo Trans Blot unit (Bio-Rad). Equal loading of protein samples was verified using the stain free technology provided by Bio-Rad, which quantifies the amount of total protein loaded in one lane by staining tryptophans, instead of using a single band of one reference protein. This technique was preferred over traditional methods of loading control, since pregnancy and lactation are known to induce substantial changes in protein levels in the brain, which might bias relative gene expression (Gilda and Gomes, 2013; Rivero-Gutiérrez et al., 2014). Non-specific binding sites on the nitrocellulose membranes were blocked with 5 % milk-powder (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) in TBST for 1 h at room temperature and incubated with a specific antibody against the rat V1bR (1:5000, Alpha Diagnostic, San Antonio, USA; for verification of specificity see Supplementary Information and Figure S2) in TBS-T overnight at 4 °C. Bands were visualized by a secondary HRP-conjugated anti-rabbit IgG antibody (1:2000 in 5 % milk-powder, 1h at room temperature; Cell Signaling Technology) and Clarity Western ECL Substrate (Bio-Rad). Images were taken with the ChemiDocTM XRS+ system (Bio-Rad). Optical density of the protein bands was measured and related to total protein binding with Image Lab 5.1 according to the manufacturer's protocol (Bio-Rad).

4.3.4 Experiments 3 & 4: Local blockade of V1bR within the MPOA (experiment 3) and mpBNST (experiment 4)

4.3.4.1 Implantation of local guide cannulae.

On PD 18, rats were implanted bilaterally with two stainless steel guide cannulae (23 G, 12 mm length) 2 mm above the MPOA (0.4 mm posterior, ±0.8 mm lateral to bregma, 6.8 mm ventral) or the mpBNST (0.5 mm posterior, -3.0 mm and +3.1 mm lateral to bregma, 4.9 mm ventral, angle 12.5°) (Paxinos and Watson, 2007) under semi-sterile conditions. Briefly, rats were anesthetized with the inhalation-narcotic isoflurane (Baxter Germany GmbH,

Unterschleißheim, Germany) and placed on a thermo pad (32 °C) to minimize core body temperature loss throughout anesthesia. Two stainless steel screws were inserted into the skull for better adhesion of the cannulae; the first cannula was fixed with light-hardening dental glue (Heraeus Kulzer GmbH, Hanau, Germany) to avoid an unnecessary elongation of anesthesia and for better precision while the second cannula was fixed (Kallocryl, Speiko, Münster, Germany). The wound was closed with dental cement (Kallocryl). The cannulae were closed with stainless steel dummy cannulae of the same length as the guide cannulae. At the end of the surgery, rats received 0.12 ml of the antibiotic Enrofloxacin (Baytril 2.5 %, Bayer Vital GmbH, Leverkusen, Germany) subcutaneously to prevent inflammation. After surgery, rats were placed individually in an observation cage to guarantee undisturbed parturition.

4.3.4.2 Infusion of receptor subtype-specific V1bR antagonist.

Rats were handled twice daily on PD 15 - 17 and 19 - 21 to familiarize them with the local infusion procedure and to minimize non-specific stress responses during the experiments (Bayerl et al., 2014; Klampfl et al., 2014). Starting on LD 1, lactating dams received on each experimental day acute bilateral infusions of VEH (0.5 µl per side; sterile Ringer's solution + 5 % dimethyl sulphoxide; pH 7.4; B. Braun Melsungen, Melsungen, Germany) or the receptor subtype-specific V1bR antagonist SSR149415 (100 ng / 0.5 µl VEH per side (Serradeil-Le Gal et al., 2002)) 10 min prior to the respective test. The concentration was chosen based on previous studies (Bayerl et al., 2014; Salomé et al., 2006; Stemmelin et al., 2005); binding of the V1bR antagonist to the structurally similar OXT and V1aR is rather unlikely as SSR149415 binds with high specificity to the rat V1bR (Griebel et al., 2002; Griffante et al., 2005; Serradeil-Le Gal et al., 2002). The treatments were administered over 20 s *via* a 10 µl Hamilton syringe connected to an injection system consisting of an infusion cannula (30 G, length 14 mm, i.e. 2 mm longer than the guide cannula) on a polyethylene tube of 20 cm length.

4.3.5 Behavioural tests

The behavioral tests from experiments 3 and 4 were conducted every other day based on an established protocol (Klampfl et al., 2014). Briefly, on LD 1, maternal care was observed in the home cage under 'non-stress' conditions. We are aware that the infusion *per se* creates a minimal amount of stress, though the term 'non-stress' is used here to distinguish maternal care without previous exposure to the maternal defense test from maternal care following the maternal defense test, which is a strong psychosocial stressor ('stress' condition, LD 7, see below; Neumann et al., 2001). On LD 3, maternal motivation to retrieve pups in a novel environment was assessed in the PRT. On LD 5, anxiety-related behavior was assessed on the EPM. For the PRT and EPM test dams were transported to separate rooms 60 min prior to the tests to allow for habituation. On LD 7, maternal care was monitored in the home cage before and after the maternal defense test, which was also used to measure maternal aggression (Bosch et al., 2005; Neumann et al., 2001; for review see Bosch, 2013).

4.3.5.1 Maternal care observation.

The mother's behavior was monitored every second minute for approximately 10 s in 30 min intervals, leading to a maximum of 15 counts per interval. On LD 1, dams were observed 60 min before (9:00 – 10:00 a.m.), and two hours immediately after (10:00 a.m. – 12:00 p.m.) infusion of V1bR antagonist or VEH (MPOA: n = 11 – 15 per group, mpBNST: n= 18 – 19 per group). On LD 7, dams were observed 60 min in the morning (9:00 – 10:00 a.m.) and subsequently brought to the maternal defense test room. After infusion and the maternal defense test, dams were observed for another 60 min to assess possible behavioral effects of stressor exposure. Behaviors were scored according to an established protocol (Bayerl et al., 2014; Klampfl et al., 2014) and included nursing, which is comprised of ABN as the only active nursing posture of the mother, blanket posture and lying on side or back. All nursing postures together with licking / grooming of the pups and carrying the pups were taken as mother-pup interactions. Furthermore, we assessed non-maternal behaviors like eating / drinking, self-grooming, resting / sleeping away from the offspring, digging and locomotion, which were summed up as "off pups" behavior.

4.3.5.2 Maternal motivation in the PRT.

The motivation of the dams to retrieve their pups is very high during the early phase of lactation (for review see Numan and Woodside, 2010) and was measured in the PRT. The pups were separated from the mothers 60 min prior to the test and kept on a thermo pad (32 °C) to maintain body temperature, while at the same time dams were transported to the experimental room. Ten minutes before starting the test, dams were infused with either V1bR antagonist or VEH; subsequently, pups were brought to the experimental room and distributed in the retrieval box (see Figure 22), which was additionally covered with a handful of home cage bedding.

In order to characterize pup retrieval under different challenging conditions, we tested the dams in one of two different experimental setups of the PRT (for details see below and Figure 22). Half of the dams were tested in the conventional setup of our laboratory ('no house' condition; MPOA: n = 6 – 7 per group, mpBNST: n = 6 per group; Figure 22A; Bayerl et al., 2014; Bosch and Neumann, 2008; Bosch et al., 2010; Klampfl et al., 2014; Neumann et al., 2005), the other half in the modified setup ('house' condition; MPOA: n = 6 – 8 per group, mpBNST: n = 13 – 15 per group; Figure 22B; also see Supplemental Information).

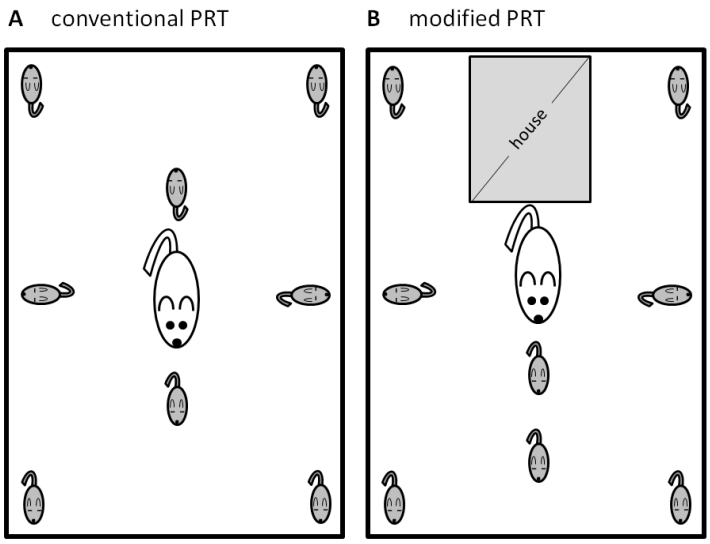

Figure 22 Conventional (A) and modified (B) pup retrieval test (PRT) setup.

Schematic representation of the conventional ('no house' condition; A) or the modified ('house' condition; B) PRT and distribution of the pups (grey). The dam (white) is placed in the middle of the box, facing away from the entrance of the house (gray rectangle) within the modified PRT setup.

The conventional PRT was performed according to an established protocol using a retrieval box made of white plastic (54 x 34 x 32 cm^3; Bosch and Neumann, 2008; Neumann et al., 2005). The dams were placed in the middle of the box (t0) and retrieval behavior (time and number of retrieved pups as well as latency to retrieve the first pup) was scored for a

maximum of 15 min (t15). After termination of the test, dams were put back into their home cages together with their pups. The modified setup consists of a black plastic box (54 x 34 x 60 cm^3), made out of the same material as the home cage floor and an additional red Perspex plastic house (13 x 17 x 11 cm^3, opening 6 x 8.5 cm^2; PLEXIGLAS® GS Rot 3C33, ThyssenKrupp Plastics GmbH, Regensburg, Germany; transparency: 13 %); based on our preliminary study in lactating HAB and LAB rats. While in HAB dams maternal motivation to retrieve pups is high, it is significantly lower in LAB dams when tested in the conventional PRT (Figure S3; (Neumann et al., 2005); interestingly, this is also the case in HAB and LAB mice (Kessler et al., 2011; for review see Bosch, 2011). Furthermore, especially LAB dams often retrieve their pups not to a single place in the conventional retrieval box but into two or even more corners (unpublished observation DSB, SMK, BMG, OJB). Therefore, we have chosen to provide the mothers with a red house (see above) as potential nesting site, as an enclosed dim area is known to be preferred to a light arena, similar to the conditions in the light-dark box test (Crawley and Goodwin, 1980). The result was that non-manipulated LAB dams retrieved their pups significantly faster as well as more pups during the 15-min test (HAB/LAB dams: n = 9 – 10 per group; Figure S3), elevating the LAB dams motivation to levels of HAB dams. Therefore, the modified test enables us to increase pup retrieval and, conversely, to detect potential impairing effects of any manipulation of the lactating mothers on maternal motivation to retrieve pups, which increases the significance of the test. For the modified PRT, dams were allowed to explore the house in their home cage for 150 min to familiarize them with the new object in the afternoon of LD 2. On LD 3, the house was re-introduced to the dam's home cage during the 60 min separation from the pups as well as in the PRT arena during the test (Figure 22B). Maternal motivation to retrieve pups was measured as described above.

4.3.5.3 Anxiety-related behaviour on the EPM.

Dams were tested for their anxiety-related behavior on the EPM (Liebsch et al., 1998; Pellow et al., 1985) as described before (Bayerl et al., 2014; Bosch et al., 2010; Neumann et al., 2000). Briefly, the dams were transported to the separate EPM room 60 min prior to the test to allow habituation. The substances were infused 10 min before starting the test (MPOA: n = 14 – 16 per group, BNST: n = 17 – 19 per group), when the dams were placed in the neutral

zone of the EPM (65 lux) facing a closed arm (10 lux) and were allowed to freely explore the maze for 5 min. The percentage of time spent on the open arms (90 lux; time on the open arms versus all arms) was taken as indicator for anxiety-related behavior whereas the number of closed arm entries reflected locomotion (Neumann et al., 2000).

4.3.5.4 Maternal aggression in the maternal defence test.

The dams were transported to the test room 60 min before testing. The dams (residents) received their infusion (MPOA: n = 4 – 7 per group, BNST: n = 18 per group) 10 min prior to introducing a virgin female rat (intruder) into their home cage in the presence of the pups (Bosch et al., 2005; Neumann et al., 2001; for review see Bosch, 2013). Intruders were used only once per day as well as only twice during the experiment with at least one day in between for recovery. The behavior during the 10-min testing period was video-taped for later analysis by an experienced observer blind to the treatment. The aggressive behaviors scored were: number of attacks, latency until first attack, offensive upright (the dam stands in an upright position in front of the intruder), keep down (the dam keeps the intruder down with her front paws), lateral threat (the dam engages to push the intruder aside by approaching laterally with her whole body), and aggressive grooming (for review see Bosch, 2013). Further, we distinguished between the following non-aggressive behaviors: pup-directed behavior (direct pup contact including nursing, licking / grooming, carrying), sniffing the intruder, exploration of the cage, self-grooming, resting offside the pups as well as eating / drinking. After the 10-min test period, the intruders were removed from the resident's home cages.

4.3.6 Verification of cannulae placement

At the end of the behavioral experiments, rats were sacrificed with CO_2 and infused with 0.5 µl ink with an infusion cannula *via* the implanted guide cannulae. Brains were taken out and snap-frozen in n-methylbutane on dry ice. Brains were cut in 40 µm cryosections, slide mounted, and a quick Nissl staining was performed. Correct placement was verified using the rat brain atlas (Paxinos and Watson, 2007), and only rats with both cannulae placed correctly in the target brain region were included in the statistical analysis (see Figure 23).

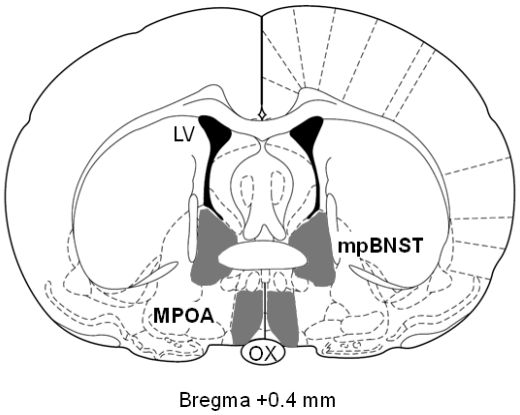

Figure 23 Verification of cannulae placement sites in the MPOA and mpBNST

Areas of the MPOA and mpBNST (grey shaded areas) in which the placement of the guide cannulae were counted as successful implantation (modified from Paxinos and Watson, 2007); the number indicates the distance in millimetres posterior to Bregma.

Abbreviations: LV, lateral ventricle; ox, optic chiasm.

4.3.7 Statistical analysis

Differences of mRNA and protein levels were statistically evaluated with an independent t-test for each brain region. Maternal care and maternal motivation to retrieve pups were analyzed using two-way ANOVA for repeated measures (factors: time x treatment). The ANOVAs were followed by Sidak *post-hoc* correction if main effects were found. If only a time effect was found, additional separate one-way ANOVA for repeated measures (factor: time) with subsequent Sidak *post-hoc* correction was performed for each treatment group. Maternal aggression, number of retrieved pups, latency to retrieve the first pup and anxiety-related behavior was analyzed using independent t-tests. If data were not distributed normally (Kolmogorov-Smirnov Test), a Mann-Whitney-U test was performed. A statistical significant difference was considered at $p \leq 0.05$ and effect size estimations were indicated by *eta squared* values for ANOVAs or *Cohens d* for independent t-tests. Data are presented as mean ± SEM. Statistical analysis was performed using the software SPSS, Version 20 (IBM, Ehningen, Germany).

4.4 Results

4.4.1 Experiments 1 & 2: Expression of V1bR in the MPOA and BNST of virgin versus lactating rats

The expression of V1bR as measured at the mRNA and protein level within the MPOA (independent t-test; mRNA: $t_7 = 0.31$, $p = 0.76$, $d = 0.21$; protein: $t_7 = -1.49$, $p = 0.18$, $d = 1.06$) or the BNST (mRNA: $t_5 = 0.75$, $p = 0.49$, $d = 0.57$; protein: $t_7 = -0.53$, $p = 0.61$, $d = 0.37$) did not differ between virgin and lactating rats (Table 2).

Table 2 Expression of V1bR mRNA and protein within the MPOA and the BNST

		MPOA	BNST
mRNA (relative to virgin)	Virgin	1.00 ± 0.13	1.00 ± 0.56
	lactating	0.87 ± 0.34	0.58 ± 0.25
protein (relative to total protein)	Virgin	1.00 ± 0.11	1.00 ± 0.20
	lactating	1.24 ± 0.04	1.17 ± 0.17

Abbreviations: BNST, bed nucleus of the stria terminalis; MPOA, medial preoptic area; V1bR, V1b receptor.
Data are presented as mean ± SEM (n = 3-5 per group).

4.4.2 Experiment 3: Local blockade of V1bR within the MPOA

4.4.2.1 Maternal care under non-stress conditions.

ABN was not affected by the treatment at any time point (Figure 24A).

Nursing tended to differ depending on time (two-way ANOVA for repeated measures; $F_{4,112} = 2.42$, $p = 0.053$, $\eta^2 = 0.05$; Figure 24B) but not on treatment; no time x treatment interaction was found. Separate one-way ANOVA for repeated measures did not reveal any further differences between time-points.

Mother-pup interaction differed significantly depending on time ($F_{4,112} = 4.45$, $p = 0.002$, $\eta^2 = 0.09$; Figure 24C) but not on treatment; no time x treatment interaction was found. Separate one-way ANOVA for repeated measures did not reveal any further differences between time-points.

4.4.2.2 Maternal care under stress conditions.

ABN differed significantly depending on time (two-way ANOVA for repeated measures; $F_{2,46}$ = 13.08, $p < 0.001$, $\eta^2 = 0.24$; Figure 24D) but not on treatment; no time x treatment interaction was found. Separate one-way ANOVA for repeated measures revealed a significant effect of time (VEH: $F_{2,20} = 4.28$, $p = 0.028$, $\eta^2 = 0.30$; V1bR antagonist: $F_{2,26} = 9.81$, $p = 0.001$, $\eta^2 = 0.43$); ABN tended to be decreased at t+30 compared to basal in the V1bR antagonist-treated dams only ($p = 0.059$), whereas at t+60, ABN significantly increased compared to t+30 in both groups (VEH: $p = 0.015$, V1bR antagonist: $p < 0.001$).

Nursing differed significantly depending on time ($F_{2,46} = 12.01$, $p < 0.001$, $\eta^2 = 0.16$; Figure 24E), but not on treatment; no time x treatment interaction was found. Separate one-way ANOVA for repeated measures revealed a significant effect of time for V1bR antagonist-treated dams ($F_{2,26} = 11.18$, $p < 0.001$, $\eta^2 = 0.46$) and an effect by trend for VEH dams ($F_{2,20}$ = 3.43, $p = 0.052$, $\eta^2 = 0.26$). In detail, nursing in the V1bR antagonist-treated dams was decreased at t+30 compared to basal ($p = 0.003$) and increased at t+60 compared to t+30 (p = 0.004).

Mother-pup interaction differed significantly depending on time ($F_{2,46} = 7.17$, $p = 0.002$, η^2 = 0.10; Figure 24F), but not on treatment; no time x treatment interaction was found. Separate one-way ANOVA for repeated measures revealed a significant effect of time for V1bR antagonist-treated ($F_{2,26} = 7.25$, $p = 0.003$, $\eta^2 = 0.36$), but not for VEH-treated, dams; mother-pup interaction dropped at t+30 compared to basal ($p = 0.002$) and increased at t+60 compared to t+30 ($p = 0.037$) in the V1bR antagonist-treated dams.

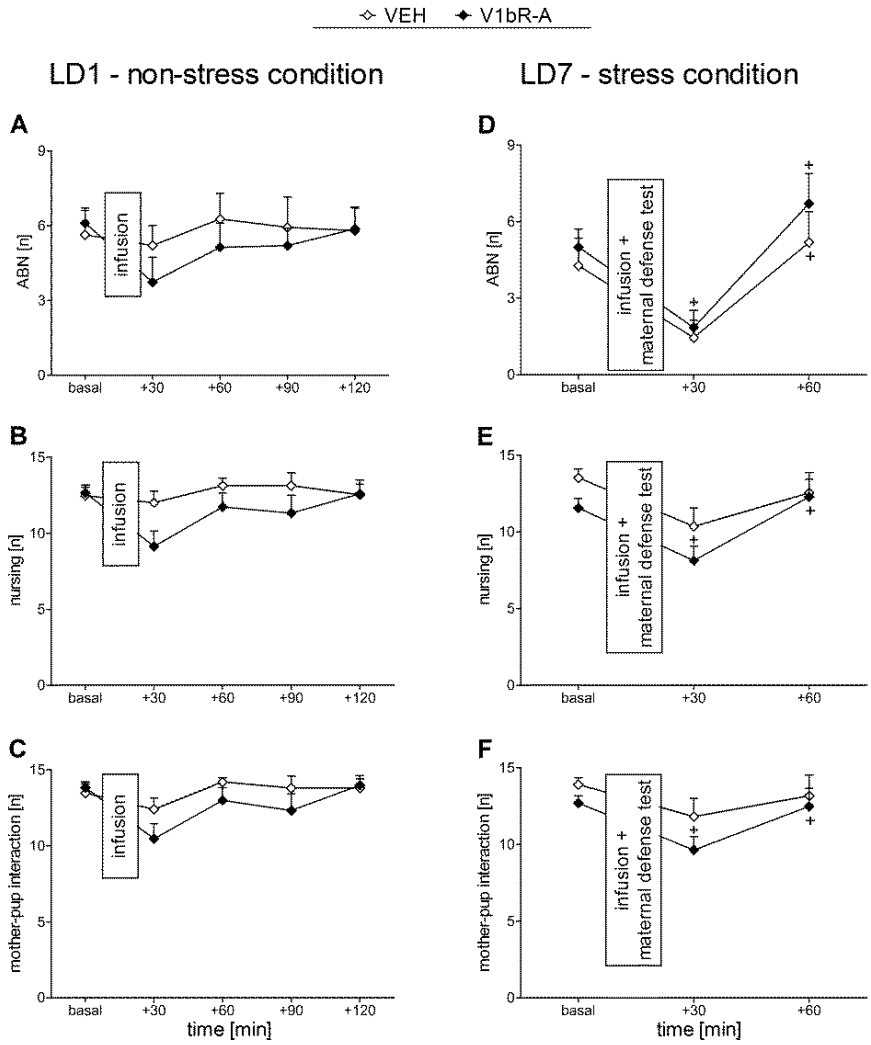

Figure 24 Effect of V1bR antagonism within the MPOA on maternal care under non-stress and stress conditions.

Maternal care was monitored before (basal) and after treatment infusion (non-stress condition; A-C) on lactation day (LD) 1 or after combined treatment infusion / exposure to the maternal defence test (stress condition; D-F) on LD 7. The occurrence of arched back nursing (ABN; A, D), sum of all nursing postures (nursing; B, E) and mother-pup interaction (C, F) is presented. Dams were acutely bilaterally infused with vehicle (VEH; 0.5 µl Ringer's solution containing 5 % dimethyl sulphoxide per side) or the V1bR antagonist SSR149415 (V1bR-A; 100 ng / 0.5 µl Ringer per side). Data are presented as means + SEM (n = 11 - 15 per group). + $p \leq 0.05$ versus previous sample of the same group.

4.4.2.3 Maternal motivation in the PRT.

In the conventional PRT ('no house' condition), the number of retrieved pups increased over the 15-min test (two-way ANOVA for repeated measures; $F_{15,165} = 14.86$, $p < 0.001$, $\eta^2 = 0.18$;

Figure 25A), was affected by treatment ($F_{1,11}$ = 8.19, p = 0.015, η^2 = 0.27), and showed a time x treatment interaction ($F_{15,165}$ = 4.39, p < 0.001, η^2 = 0.05); V1bR antagonist-treated dams retrieved more pups compared to VEH starting at 4 min (t4: p = 0.05, t5: p = 0.048, t6: p = 0.023, t7: p = 0.022, t8: p = 0.022, t9: p = 0.013, t10: p = 0.008, t11: p = 0.007, t12: p = 0.002, t13: p = 0.01, t14: p = 0.02, t15: p = 0.02). Consequently, the total number of retrieved pups was higher in V1bR antagonist-treated dams compared to VEH (independent t-test; t_{11} = -2.72, p = 0.02, d = 1.51). Further, the latency to retrieve the first pup was decreased by trend in V1bR antagonist-treated dams (t_{11} = 2.22, p = 0.072, d = 1.33; VEH: 490.0 ± 171.5 s, V1bR antagonist: 100.0 ± 37.0 s).

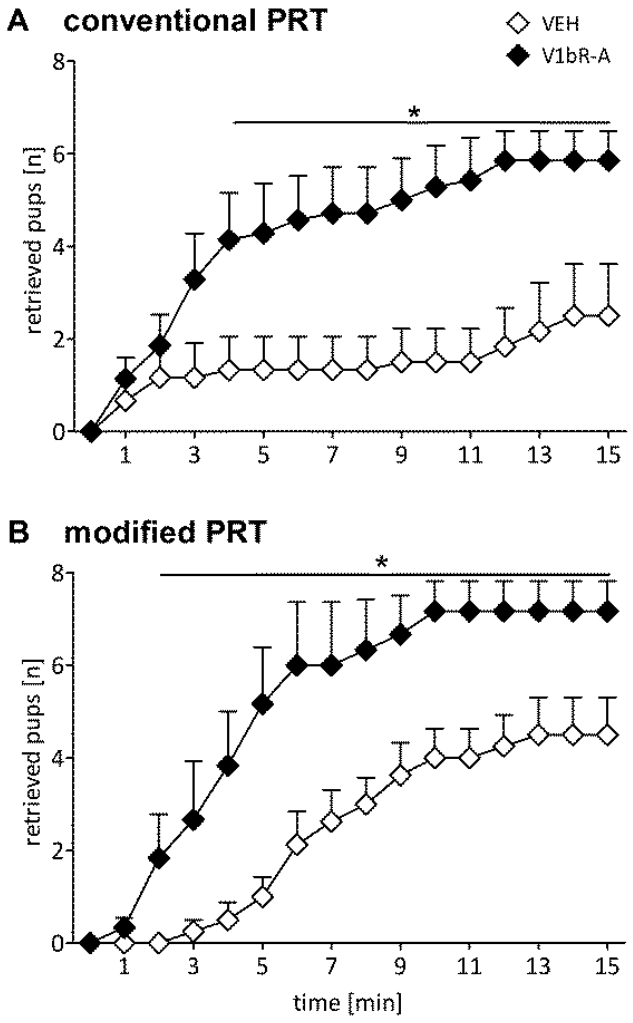

Figure 25 Effect of V1bR antagonism within the MPOA on maternal motivation.

Maternal motivation was measured either in the conventional ('no house' condition; A) or the modified ('house' condition; B) pup retrieval test (for details, see Section 4.3.5.2). The time until retrieval of each pup was monitored for 15 min following acute bilateral infusion of vehicle (VEH) or the V1bR antagonist SSR149415 (V1bR-A; for details see Figure 24).

Data are presented as means + SEM (n = 6 - 8 per group). * p ≤ 0.05 versus VEH.

In the modified PRT ('house' condition), the number of retrieved pups increased over the 15-min test (two-way ANOVA for repeated measures; $F_{15,180}$ = 30.78, p < 0.001, η^2 = 0.45; Figure 25B), was affected by treatment ($F_{1,12}$ = 14.26, p = 0.003, η^2 = 0.19) , and showed a time x

treatment interaction ($F_{15,180}$ = 2.10, p = 0.012, η^2 = 0.03); V1bR antagonist-treated dams retrieved more pups compared to VEH starting at 2 min (t2: p = 0.042, t3: p = 0.05, t4: p = 0.01, t5: p = 0.004, t6: p = 0.019, t7: p = 0.034, t8: p = 0.013, t9: p = 0.017, t10: p = 0.005, t11: p = 0.005, t12: p = 0.011, t13: p = 0.031, t14: p = 0.031, t15: p = 0.031). Consequently, the total number of retrieved pups was higher in V1bR antagonist-treated dams compared to VEH (independent t-test; t_{12} = -2.45, p = 0.031, d = 1.33). Further, the latency to retrieve the first pup was decreased in V1bR antagonist-treated dams (t_{12} = 2.30, p = 0.041, d = 1.24; VEH: 310.0 ± 35.5 s, V1bR antagonist: 153.3 ± 63.6 s).

Comparing pup retrieval of the VEH groups of both PRT setups confirmed our finding in LAB dams (Figure S3); in the modified PRT, mothers retrieved their pups faster compared to the conventional PRT (two-way ANOVA for repeated measures; $F_{15,180}$ = 14.33, p < 0.001, η^2 = 0.28); further a time x condition interaction was present ($F_{15,180}$ = 4.98, p < 0.001, η^2 = 0.10).

4.4.2.4 Maternal aggression in the maternal defence test.

The treatment groups did not differ in any aggressive or non-aggressive behaviors in the maternal defense test (data not shown).

4.4.2.5 Anxiety-related behaviour on the EPM.

The treatment groups did not differ in any parameter on the EPM (data not shown).

4.4.3 Experiment 4: Local blockade of V1bR within the mpBNST

4.4.3.1 Maternal care under non-stress conditions.

ABN differed significantly depending on time (two-way ANOVA for repeated measures; $F_{4,136}$ = 8.50, p < 0.001, η^2 = 0.13; Figure 26A) but not on treatment; no time x treatment interaction was found. Separate one-way ANOVA for repeated measures revealed a significant effect of time (VEH: $F_{4,68}$ = 3.22, p = 0.018, η^2 = 0.16; V1bR antagonist: $F_{4,68}$ = 6.19, p < 0.001, η^2 = 0.27); ABN increased at t+60 compared to t+30 in both treatment groups (VEH: p = 0.003, V1bR antagonist: p = 0.015).

Nursing ($F_{4,136}$ = 5.12, p = 0.001, η^2 = 0.09; Figure 26B) and mother-pup interaction ($F_{4,136}$ = 4.81, p = 0.001, η^2 = 0.08; Figure 26C) differed significantly depending on time, but not on treatment; no time x treatment interactions were found. Separate one-way ANOVA for repeated measures did not reveal any further differences between time-points.

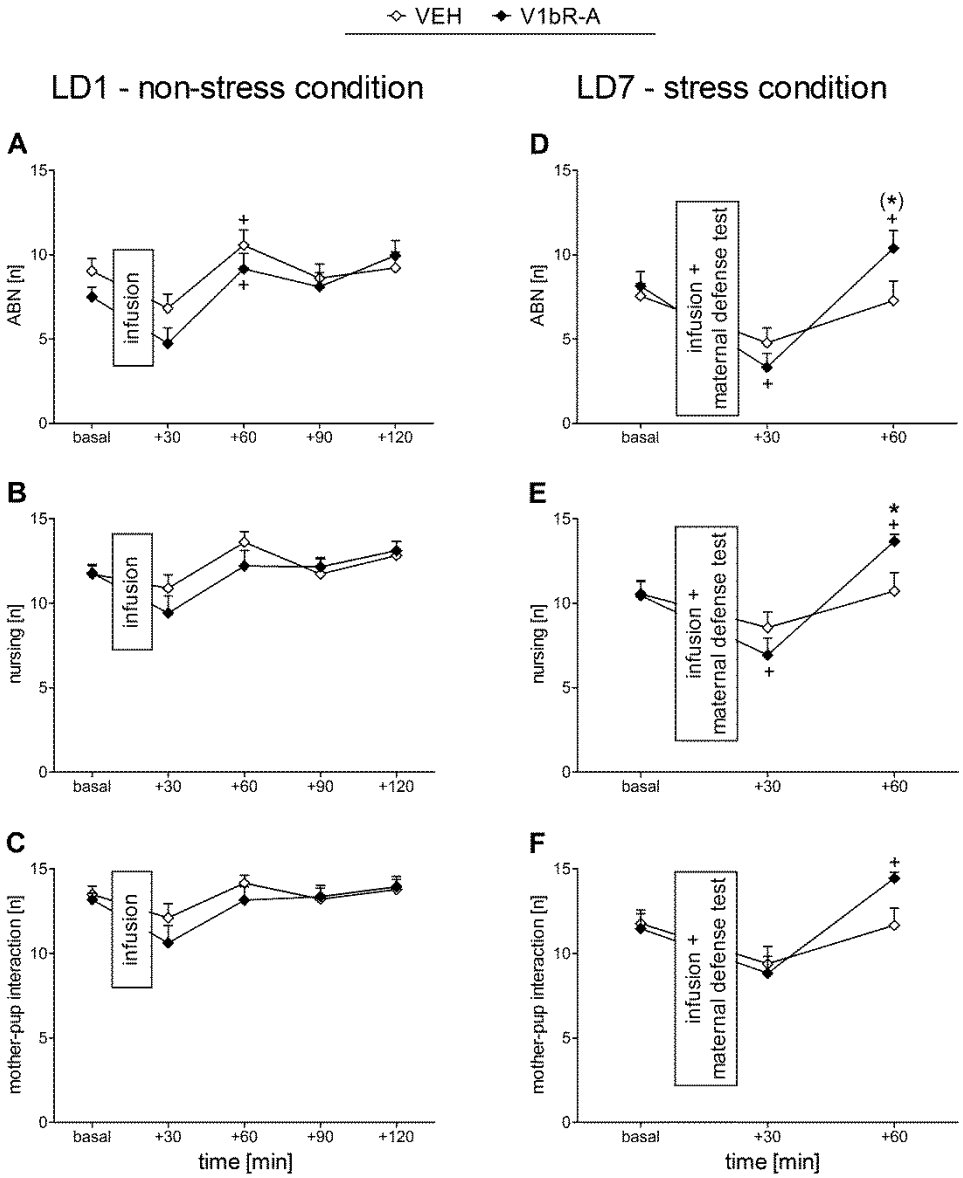

Figure 26 Effect of V1bR antagonism within the mpBNST on maternal care under non-stress and stress conditions.

Maternal care was monitored before (basal) and after treatment-infusion (non-stress condition; A-C) on LD 1 or after combined treatment-infusion / exposure to the maternal defence test (stress condition; D-F) on LD 7. The occurrence of arched back nursing (ABN; A, D), sum of all nursing postures (nursing; B, E) and mother-pup interaction (C, F) is presented. Dams were acutely bilaterally infused with vehicle (VEH) or the V1bR antagonist SSR149415 (V1bR-A; for details see Figure 24). Data are presented as means + SEM (n = 18 - 19 per group). + p ≤ 0.05 versus previous sample of the same group; * p ≤ 0.05, (*) p = 0.054 versus VEH.

4.4.3.2 Maternal care under stress conditions.

ABN differed significantly depending on time (two-way ANOVA for repeated measures; factor time: $F_{2,68} = 16.18$, $p < 0.001$, $\eta^2 = 0.21$; Figure 26D) but not on treatment; a significant time x treatment interaction ($F_{2,68} = 3.31$, $p = 0.042$, $\eta^2 = 0.04$) was found. The *post-hoc* test revealed that in the V1bR antagonist group ABN dropped at t+30 compared to basal ($p = 0.002$) and increased at t+60 compared to t+30 ($p < 0.001$). Furthermore, at t+60 V1bR antagonist-treated dams showed by trend more ABN compared to VEH dams ($p = 0.054$).
Nursing differed significantly depending on time ($F_{2,68} = 14.30$, $p < 0.001$, $\eta^2 = 0.19$; Figure 26E) but not on treatment; a significant time x treatment interaction ($F_{2,68} = 3.83$, $p = 0.027$, $\eta^2 = 0.05$) was found. The *post-hoc* test revealed a drop in nursing at t+30 compared to basal ($p = 0.03$) and an increase at t +60 compared to t+30 ($p < 0.001$) in the V1bR antagonist-treated group, resulting in significantly higher nursing compared to basal ($p = 0.025$). Furthermore, at t+60 V1bR antagonist-treated dams showed significantly more nursing compared to VEH dams ($p = 0.018$).
Mother-pup interaction differed significantly depending on time ($F_{2,68} = 12.20$, $p < 0.001$, $\eta^2 = 0.16$; Figure 26F) but not on treatment; no time x treatment interaction was found. Separate one-way ANOVA for repeated measures revealed a significant effect of time within the antagonist-treated dams only ($F_{2,34} = 11.80$, $p < 0.001$, $\eta^2 = 0.41$); mother-pup interaction increased at t+60 compared to t+30 ($p < 0.001$), resulting in significantly higher mother-pup interaction compared to basal ($p = 0.016$).

4.4.3.3 Maternal motivation in the PRT.

The treatment groups did not differ in any parameter, neither in the conventional nor the modified PRT (data not shown).

4.4.3.4 Maternal aggression in the maternal defence test.

During the maternal defense test, aggressive behavior towards the female virgin intruder was not different between the groups. Pup-directed behavior was decreased in the V1bR antagonist-treated group compared to VEH (independent t-test; $t_{34} = 2.15$, $p = 0.045$, $d = 0.72$; Table 3). In detail, licking / grooming of the pups ($t_{34} = 2.34$, $p = 0.031$, $d = 0.78$), but no

other maternal parameter, was lower in the V1bR antagonist-treated dams compared to VEH. No other non-aggressive behavior was affected by any treatment.

4.4.3.5 Anxiety-related behaviour on the EPM.

The treatment groups did not differ in any parameter on the EPM (data not shown).

Table 3 Effect of V1bR blockade within the mpBNST on maternal aggression on LD 7

	VEH	V1bR antagonist
aggressive behaviour		
number of attacks	5.2 ± 1.0	5.9 ± 1.0
attack duration	3.5 ± 0.7 s	3.6 ± 0.6 s
attack latency	181.0 ± 41.0 s	156.8 ± 38.0 s
keep down	13.0 ± 4.7 s	24.6 ± 6.9 s
lateral threat	7.2 ± 3.2 s	7.7 ± 2.1 s
offensive upright	2.3 ± 0.8 s	3.9 ± 1.6 s
aggressive grooming	24.3 ± 4.6 s	27.6 ± 5.2 s
sum aggressive	228.0 ± 31.0 s	279.5 ± 22.3 s
non-aggressive behaviour		
pup-directed behaviour	58.8 ± 18.8 s	17.4 ± 4.4 s *
direct contact	25.2 ± 12.3 s	8.0 ± 3.4 s
licking / grooming	33.2 ± 10.7 s	7.7 ± 2.1 s *
carrying	0.4 ± 0.2 s	1.7 ± 1.4 s
Sniffing	64.8 ± 7.7 s	58.0 ± 4.4 s
Exploration	136.2 ± 18.6 s	96.7 ± 14.3 s
self-grooming	75.8 ± 7.7 s	81.3 ± 12.9 s
Resting	14.5 ± 4.1 s	30.9 ± 8.7 s
Other	17.8 ± 5.0 s	23.1 ± 8.3 s
sum non-aggressive	367.9 ± 30.8 s	307.4 ± 23.7 s

Abbreviations: mpBNST, medial-posterior part of the bed nucleus of the stria terminalis; V1bR, V1b receptor; VEH, vehicle.

Data are presented as mean ± SEM (n = 18 per group). * $p \leq 0.05$ versus VEH.

4.5 Discussion

In the present study we show that, although neither V1bR mRNA or protein levels in the MPOA or the BNST of lactating rats undergo peripartum-associated changes, V1bR play a role in mediating various aspects of maternal behavior in a brain region-specific manner. Within the MPOA, V1bR blockade increased pup retrieval, whereas in the mpBNST the same treatment reduced pup-directed behavior during the maternal defense test.

V1bR are distributed across numerous areas of the rat brain as demonstrated in male rodents (Hernando et al., 2001), where they are implicated in mediating social behaviors (Caldwell et al., 2008) like social recognition and memory, social motivation and inter-male aggression (Pagani et al., 2015; Wersinger et al., 2008, 2004, 2002; for review see Stevenson and Caldwell, 2012). Importantly, V1bR are also expressed in brain areas known to be involved in the regulation of maternal behavior, i.e. the MPOA and BNST (for reviews see Bosch and Neumann, 2012; Numan and Insel, 2003). Here, V1aR binding is up-regulated during lactation as part of peripartum adaptations (Bosch and Neumann, 2008; Bosch et al., 2010; for review see Bosch and Neumann, 2012). Our current study reveals that neither V1bR mRNA nor protein levels differed within the MPOA or the BNST between lactating and virgin rats. Here, the use of virgins at random estrous stages is a limitation of the study, since the estrous stage can affect gene expression (Nakamura et al., 2010). Hence, a potential variability in virgins' V1bR expression may mask differences between virgin and lactating rats. However, the current data suggest that any involvement of V1bR in maternal behavior is not regulated at the gene expression or protein synthesis level, but rather relies on a preexisting pool of V1bR or changes in receptor binding as described for V1aR (Bosch and Neumann, 2008; Bosch et al., 2010).

Investigations of the impact of V1bR on maternal behavior started a few years ago and are still sparse to date. Wersinger et al. (2007) were the first to demonstrate that the lack of V1bR lowers maternal aggression in lactating V1bR knockout mice compared to wildtype mice. Recently, we showed that acute central infusion of the V1bR antagonist SSR149415 impairs mother-pup interaction in lactating Wistar rats (Bayerl et al., 2014). Due to this central approach, reflecting a global rather than a site-specific manipulation, we now aimed to investigate the behavioral role of V1bR within the MPOA and mpBNST.

The importance of the MPOA in maternal behavior has been shown in various studies starting in the 1950s; Fisher described that intracranial chemical stimulation of the MPOA

induces maternal behavior (Fisher, 1956). Later, Numan and colleagues demonstrated that knife cuts of the lateral projections of the MPOA disrupt maternal behavior (Numan and Callahan, 1980; Numan, 1990). With respect to the AVP system in the MPOA, V1aR have been shown to facilitate the onset (Pedersen et al., 1994) as well as the maintenance of established maternal care in lactating rats (Bosch and Neumann, 2008). Furthermore, depending on the interaction with the offspring, AVP is locally released within the MPOA (Bosch et al., 2010) and, thus, binding to both the V1aR and the V1bR. In the present study, local infusion of the V1bR antagonist into the MPOA decreased maternal care under stress, but not under non-stress conditions. This is partly in line with our results on central V1bR antagonism and stressor exposure where especially nursing and mother pup-interaction were reduced (Bayerl et al., 2014). Hence, our study suggests that V1bR activation within the MPOA seems to be beneficial in order to reinstate adequate maternal care after exposure to an acute stressor.

In addition to maternal care, the MPOA is highly important for mediating maternal motivation to retrieve pups (for reviews see Numan and Insel, 2003; Olazábal et al., 2013). For example, excitotoxic or electrical lesions of the MPOA, as well as knife cuts that sever the dorsolateral connections of the MPOA impair pup retrieval (Franz et al., 1986; Kalinichev et al., 2000; Lee et al., 2000; Numan and Corodimas, 1985; for review see Numan, 1988). This maternal behavior has been attributed to the activity of the AVP system within the MPOA; preventing V1aR activation significantly reduces pup retrieval in lactating rats (Bosch and Neumann, 2008; Pedersen et al., 1994). In contrast, blocking V1bR facilitated pup retrieval independent of the PRT setup (Figure 25). Thus, it is feasible that the behavioral outcome of parallel activation of V1aR and V1bR within the MPOA reflects the integration of facilitating and impairing maternal motivation to retrieve pups, respectively. Notably, as non-manipulated lactating rats show high levels of motivation to retrieve pups, it can be assumed that V1aR is the predominant receptor subtype in this brain region. In this context it would be interesting to determine the ratio of V1aR / V1bR expression in the MPOA of lactating rats. Based on the fact that within the MPOA V1aR binding is increased in lactation (Bosch and Neumann, 2008) whereas V1bR expression is unchanged compared to virgin rats (this study), we speculate that in lactating rats activation of V1aR overrule V1bR, thereby facilitating pup retrieval. This might also be the cause for higher pup retrieval in lactating HAB dams (for review see Bosch, 2011); the higher AVP synthesis and release due to a single

nucleotide polymorphism in the promotor region of the gene might result in excessive activation of V1aR over V1bR. Conversely, in virgin rats V1aR binding is lower than in lactation, which would increase the power of V1bR activation thereby potentially causing avoidance to retrieve pups in virgin rats (Rosenblatt, 1975). Future studies investigating V1bR binding could serve to clarify this hypothesis.

In addition to the MPOA, the BNST is also involved in the regulation of maternal behavior (for reviews see Bosch and Neumann, 2012; Bosch, 2011; Numan and Insel, 2003). For example, bilateral knife-cuts disrupting the dorsolateral connections of the MPOA/BNST region (Numan and Callahan, 1980) as well as excitotoxic lesions of the BNST (Numan and Numan, 1996) impair aspects of maternal care in lactating rats. Regarding the AVP system in the BNST, V1aR do not mediate maternal care or pup retrieval as demonstrated by local V1aR blockade (Bosch et al., 2010). In the present study, we extend these findings by showing that V1bR in the mpBNST are not involved in those behaviors either, at least under non-stress conditions (Figure 3). However, under stress condition, a preceding intra-mpBNST infusion of the V1bR antagonist, but not VEH, resulted in decreased ABN and nursing during the 30 min immediately after termination of the maternal defense test. This is in line with our previous study, thereby strengthening our hypothesis that V1bR might be involved in adequate stress coping (Bayerl et al., 2014).

The BNST is also involved in maternal aggression (Bosch et al., 2010; Consiglio et al., 2005; Klampfl et al., 2014; for review see Bosch, 2011). For example, injections of OXT in the anterior part of the BNST (Consiglio et al., 2005) as well as V1aR antagonism in the posterior BNST significantly decreases maternal aggression towards the female virgin intruder rat (Bosch et al., 2010). Interestingly, as OXT receptor and V1aR binding in the BNST are unchanged in response to the maternal defense test (Caughey et al., 2011) it is suggested that local neuropeptide release rather than changes on the receptor level modify the extent of maternal aggression, as described for the CeA (Bosch et al., 2005). However, since local infusion of the V1bR antagonist in the mpBNST did not alter maternal aggressive behavior (Table 3 top) we assume that only V1aR in this region are involved in mediating maternal aggression itself. Interestingly, during the maternal defense test dams treated with the V1bR antagonist displayed less pup-directed behavior (Table 3 bottom), which is not apparent in V1aR antagonist-treated dams (Bosch et al., 2010). These findings suggest a V1 receptor subtype-specific role, at least during the maternal defense test; AVP released within the

mpBNST facilitates aggressive behavior towards the intruder via V1aR, whereas activation of V1bR during maternal aggression induces pup-directed behavior to protect the offspring from threats by the intruder and, thus, to provide maternal care even under stressful situations.

Treatment with the V1bR antagonist in the MPOA or the mpBNST did not alter anxiety-related behavior. While the MPOA has not been related to anxiety, a role of the BNST has been described in various studies in virgin (female and male; Pezuk et al., 2008; Sahuque et al., 2006; Walker et al., 2003) and lactating rats (Klampfl et al., 2014; for review see Lonstein, 2007) independent of the AVP system. However, V1 receptors in the mpBNST are seemingly not involved in anxiety-related behavior given local inhibition of V1bR (this study) or V1aR (Bosch et al., 2010) did not affect this behavior.

In conclusion, our data demonstrate that preventing AVP to bind to V1bR in the MPOA promotes maternal motivation to retrieve pups, suggesting an opposing role compared to V1aR (Bosch and Neumann, 2008). Furthermore, within the mpBNST both V1aR (Bosch et al., 2010) and V1bR (this study) complement each other, as receptor blockade impairs defense and protection of the offspring, respectively. Hence, we provide strong evidence for a brain-site specific involvement of V1bR in the MPOA and mpBNST for various aspects of maternal behavior in lactating rats. These peripartum functions seem to be essential for the proper display of maternal behavior, thereby further advancing our knowledge of the involvement of the AVP system in maternal brain and consequently on potential adaptations during the postpartum period.

Chapter 5

Vasopressin V1a, but not V1b, receptors within the PVN of lactating rats mediate maternal care and anxiety-related behaviour

Authors' contribution:

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

[in preparation to be submitted to *Behavioural Brain Research* as Short Communication]

5.1 Abstract

The brain neuropeptide AVP mediates a wide range of social behaviours via its V1a (V1aR) but also its V1bR.With respect to maternal behaviour, V1bR are still less investigated, whereas V1aR have been shown repeatedly to trigger maternal behaviour, depending on the brain region. Here, we aimed to study the role of both V1aR and V1bR within the PVN, a major source of AVP, in maternal care (LD 1), maternal motivation in the PRT (LD 3) and anxiety-related behaviour on the EPM (LD 5) by acute local infusion of receptor subtype-specific antagonists for V1aR ($d(CH_2)_5Tyr(Me)^2AVP$) or V1bR (SSR149415). Furthermore, we compared V1bR expression in the PVN of virgin versus lactating rats (LD 4).

Our results demonstrate that intra-PVN V1bR mRNA (qPCR) or protein (Western Blot) content did not differ between virgin and lactating rats. Furthermore, antagonism of V1aR, but not of V1bR, decreased nursing and anxiety-related behaviour. Maternal motivation was not affected by any treatment.

In summary, we demonstrate subtype-specific involvement of V1 receptors within the PVN in various parameters of maternal behaviour and anxiety. The lack of effects after V1bR blockade demonstrates that AVP acts mainly via V1aR in the PVN, at least in lactating rats, to mediate maternal care and anxiety.

5.2 Introduction

The brain neuropeptides AVP and OXT are synthesized and released within the PVN (for review see Landgraf and Neumann, 2004; Veenema and Neumann, 2008), and facilitate maternal behaviour in lactating rats (for reviews see Bosch and Neumann, 2012, 2008). Central AVP acts on maternal care, maternal motivation and maternal aggression via two receptor subtypes in the brain, namely V1aR and V1bR (Bayerl et al., 2014; Bosch and Neumann, 2008; Nephew and Bridges, 2008a). Furthermore, the AVP system is upregulated around parturition and during lactation (Caldwell et al., 1987), as indicated by higher V1aR binding and increased AVP release during mother-pup interaction in the MPOA and the BNST (for review see Bosch and Neumann, 2012) as well as increased AVP mRNA expression within the PVN (Bosch et al., 2007; Walker et al., 2001). Interestingly, the PVN is also involved in the expression of maternal behaviour as shown by lesion studies (Insel and Harbaugh, 1989; Numan and Corodimas, 1985). Furthermore, pup-presentation increases c-fos expression only within the anterior magnocellular part of the PVN (amPVN) but not the parvocellular subdivision of the PVN (pPVN) in maternal rats (for review see Numan and Insel, 2003). We now aimed to study the impact of the AVP system in the PVN on maternal and anxiety-related behaviour by receptor subtype-specific antagonism of V1aR and V1bR in lactating rats.

5.3 Material and Methods

5.3.1 Animals

In all experiments, female Wistar rats (12 - 14 weeks, 220 - 250 g, Charles River, Sulzfeld, Germany) were kept under standard laboratory conditions (12 h / 12 h light-dark cycle, with lights on at 07:00 h; 22 ± 1 °C; 55 ± 5 % relative humidity; free access to water and standard rat chow). For the lactating groups, virgin females were mated with stud males and pregnancy was confirmed the next day by the presence of sperm in vaginal smears (assigned as PD 1). Rats were kept in groups of 3 to 4 rats before they were single-housed on PD 18 (or equivalent in virgins) to ensure undisturbed parturition. All experiments were performed in accordance with the European Communities Council Directive (86/609/EEC) and were approved by the local government of the Oberpfalz, Bavaria, Germany.

5.3.2 V1bR mRNA and protein in the PVN

To determine V1bR mRNA and protein content within the PVN, lactating rats were sacrificed on LD 4 (or equivalent in virgins at random stages of the oestrous cycle), brains were removed, snap-frozen in n-methylbutane on dry ice and stored at -80 °C until punches from the PVN were taken to measure mRNA *via* qPCR and protein content *via* Western Blot analysis as described previously (Bayerl et al., 2016).

5.3.3 Local blockade of V1aR and V1bR within the PVN

5.3.3.1 Implantation of local guide cannulae.

For behavioural analysis, on PD 18 females were implanted bilaterally with stainless steel guide cannulae (23 G, 12 mm length) 2 mm above the PVN (1.4 mm posterior, 1.8 / 2.1 mm lateral to bregma, 6.0 mm ventral, angle 10°; (Paxinos and Watson, 2007)) under inhalation anaesthesia and semi-sterile conditions as described before (Bayerl et al., 2014; Bosch and Neumann, 2008). At the end of the surgery, rats received 0.12 ml of the antibiotic Enrofloxacin (Baytril 2.5 %, Bayer Vital GmbH, Leverkusen, Germany) subcutaneously to prevent inflammation. Afterwards, pregnant rats were single housed and treated as described previously (Bayerl et al., 2016, 2014).

5.3.3.2 Infusion of receptor subtype-specific V1aR or V1bR antagonist.

On experimental days, dams received acute bilateral infusion of V1aR antagonist $d(CH_2)_5Tyr(Me)^2AVP$ (100 ng / 0.5 µl VEH / side (Kruszynski et al., 1980)), V1bR antagonist SS149415 (100 ng / 0.5 µl VEH / side (Serradeil-Le Gal et al., 2002)) or respective VEH (0.5 µl / side; V1aR: sterile Ringer's solution; V1bR: sterile Ringer's solution + 5 % dimethyl sulphoxide; pH 7.4; B. Braun Melsungen, Melsungen, Germany) 10 min prior to the test *via* an infusion cannula (27 G, length 14 mm, i.e. 2 mm longer than the guide cannula) as described previously (Bayerl et al., 2016). All behavioural experiments were conducted between 9:00 A.M. and 1:00 P.M.

5.3.4 Behavioural tests

5.3.4.1 Maternal care observation.

On LD 1, maternal care was observed 60 min before treatment infusion at 10:00 A.M. and two hours immediately afterwards for approximately 10 s every second minute in 60 min intervals according to an established protocol (Bayerl et al., 2014; Bosch and Neumann, 2008). The quality of nursing was reflected by the amount of ABN, the only active nursing posture in which the dam is engaged in a quiescent kyphosis (for review see Bosch and Neumann, 2012). Other parameters scored were nursing, in which all nursing positions (ABN, blanket posture and lying on side or back) are summed up. On LD 2, maternal care was observed one additional hour in the morning from 9:00 – 10:00 A.M. to control for potential long-term effects of the antagonists.

5.3.4.2 Maternal motivation to retrieve pups in the PRT.

On LD3, the motivation of the dams to retrieve their pups after a 60-min separation period was measured in the conventional PRT as described previously (Bayerl et al., 2014). Ten minutes before the test, dams were infused with their respective treatment, pups were brought to the experimental room and distributed in a white plastic box (54 x 34 x 32 cm^3) covered with home-cage bedding. Subsequently, the dams were placed in the middle of the box and the number of retrieved pups was scored for a maximum of 15 min.

5.3.4.3 Anxiety-related behaviour on the EPM.

On LD 5 dams were tested for their anxiety-related behaviour on the EPM as described previously (Bosch and Neumann, 2008; Pellow et al., 1985). The EPM consists of two open arms (50 x 10 cm²; 90 lux) and two closed arms (50 x 10 cm²; height of walls: 30 cm; 10 lux) connected by a neutral zone (10 x 10 cm²; 65 lux) and is elevated 80 cm over the floor. Dams were infused with their respective treatment 10 minutes before testing, placed subsequently in the neutral zone facing a closed arm and allowed to freely explore the maze for 5 min. The percentage of time spent on the open arms (versus all arms) and the percentage of open arm entries (versus all arm entries) is indicative for anxiety-related behaviour; the number of closed arm entries is a measure for locomotion (Bosch and Neumann, 2008).

5.3.5 Verification of cannulae placement

At the end of the behavioural experiments, rats were sacrificed and blue ink was infused *via* the implanted cannulae. Brains were removed, snap-frozen in n-methylbutane on dry ice, cut into 40 µm coronal sections, slide mounted, and stained *via* quick Nissl staining. Correct placement was considered using the rat brain atlas (Paxinos and Watson, 2007); only rats with both cannulae placed correctly were included in the statistical analysis (see Figure 27).

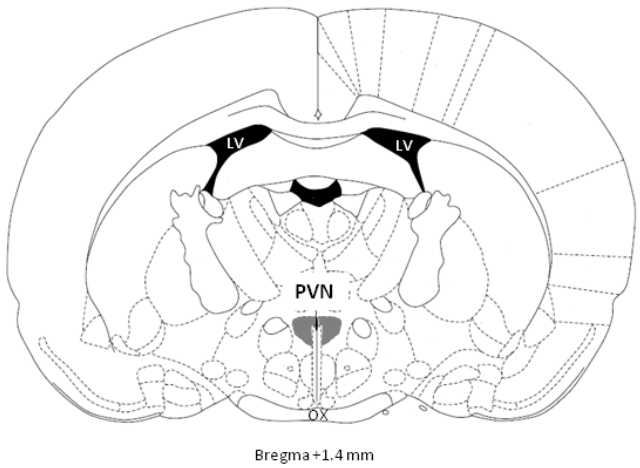

Figure 27 Verification of cannulae placement sites in the PVN

Areas of the PVN (grey shaded areas) in which the placement of the guide cannulae were counted as successful implantation (modified from Paxinos and Watson, 2007); the number indicates the distance in millimetres posterior to Bregma.

Abbreviations: LV, lateral ventricle; ox, optic chiasm.

5.3.6 Statistical analysis

Maternal care and maternal motivation was analyzed using two-way ANOVA for repeated measures (factors: time x treatment). The ANOVAs were followed by Sidak *post-hoc* correction if main effects were found. When only a time effect was found, an additional separate one-way ANOVA for repeated measures (factor: time) with subsequent Sidak *post-hoc* correction was performed for each treatment group. Anxiety-related behaviour as well as V1bR mRNA and protein content were analyzed using independent t-test, after testing for normal distribution (Kolmogorov-Smirnov test). Data are presented as mean + SEM, and significance was considered at $p \leq 0.05$. For all analysis the software package SPSS, Version 20 (IBM, Ehningen, Germany) was used.

5.4 Results

5.4.1 Expression of V1bR in the PVN of virgin versus lactating rats

V1bR mRNA as well as protein expression were similar between the groups (independent t-test; mRNA: t7 = 2.048, p = 0.08, protein: t7 = -1.399, p = 0.204; Table 4).

Table 4 Expression of V1b receptor mRNA and protein within the PVN

		expression values
mRNA	virgin	1.00 ± 0.38
(relative to virgin)	lactating	0.28 ± 0.10
protein	virgin	1.00 ± 0.14
(relative to total protein)	lactating	1.34 ± 0.20

Data are presented as mean ± SEM (n = 3-6).

5.4.2 Local blockade of V1aR or V1bR within the PVN

5.4.2.1 Maternal care under non-stress conditions

Nursing was altered depending on time (V1aR antagonist: $F_{2,18}$ = 4.313, p = 0.029; V1bR antagonist: $F_{2,36}$ = 4.554, p = 0.017; Figure 28), but not on treatment; no time x treatment interaction was detected. Separate one-way ANOVA showed a significant time effect for both antagonist-treated groups (V1aR antagonist: $F_{2,10}$ = 9.18, p = 0.005; V1bR antagonist: $F_{2,16}$ = 3.962, p = 0.04), but not for VEH-treated dams. In detail, the *post hoc* test revealed for V1aR antagonist-treated dams a decrease in the 60 min after infusion compared to basal (p = 0.043). No further differences in V1bR antagonist-treated dams were found.
Maternal care was not affected by treatment on LD 2.

5.4.2.2 Maternal motivation in the PRT

Maternal motivation was not affected by antagonism of any V1 receptor (not shown).

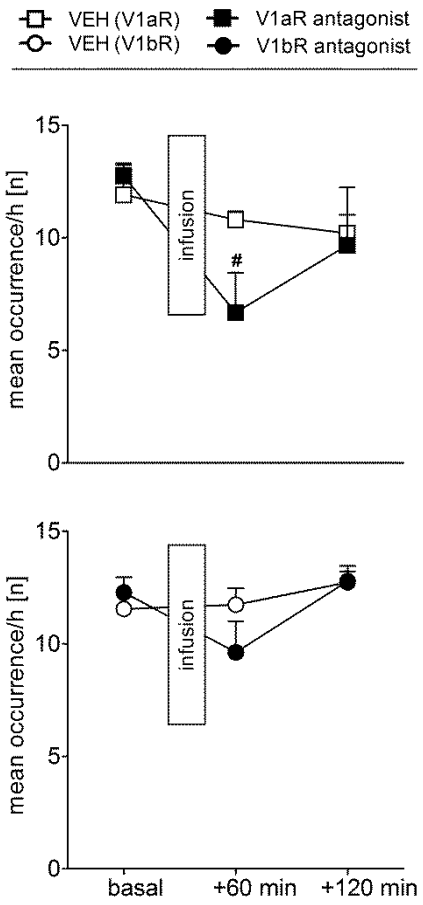

Figure 28 Effect of V1aR or V1bR antagonism within the PVN on nursing

The mean occurrence of nursing before (basal) and in the two hours after treatment infusion (+60min and +120 min) is presented. Dams were bilaterally infused with the V1aR antagonist $d(CH_2)_5Tyr(Me)^2AVP$ (100 ng/0.5 µl vehicle per side), the V1bR antagonist SSR149415 (100 ng/0.5 µl Ringer per side) or the respective vehicle (VEH; V1aR: 0.5 µl Ringer's solution per side; V1bR: 0.5 µl Ringer's solution containing 5 % dimethyl sulphoxide per side).

Data are presented as means + SEM (V1aR antagonist = 5 – 6; V1bR antagonist = 9 - 11).

$p \leq 0.05$ versus previous sample of the same group.

5.4.2.3 Anxiety-related behaviour on the EPM.

Anxiety-related behaviour on the EPM was decreased after blocking V1aR in the PVN (% time on the open arms: $t_9 = -2.681$, $p = 0.025$; % open arm entries: $t_9 = -2.399$, $p= 0.04$; Figure 29), whereas locomotion was unchanged (data not shown). Blockade of V1bR did not affect anxiety-related behaviour (not shown).

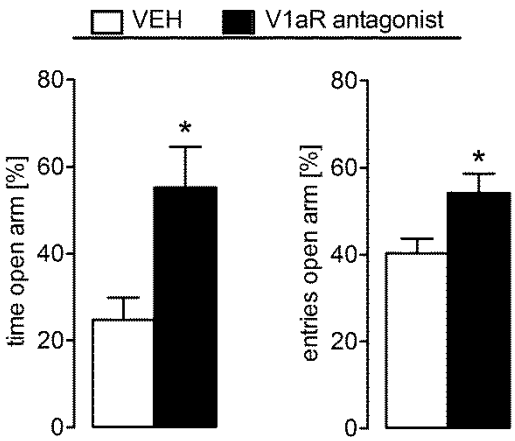

Figure 29 Effect of V1aR antagonism within the PVN on anxiety-related behaviour on the EPM

The percentage of time spent in (left) and percentage of entries into (right) the open arms of the EPM after V1aR antagonist infusion (for details see Figure 28) as assessed 10 min after treatment infusion on LD 5 are presented.

Data are presented as means + SEM (n = 5 - 6).

* $p \leq 0.05$ versus VEH.

5.5 Discussion

In the present study we demonstrate that intra-PVN V1aR modulate maternal care and anxiety-related behaviour whereas V1bR were not found to modulate any maternal behaviour. In line, V1bR mRNA and protein expression within the PVN do not adapt due to lactation.

Similar to unchanged AVP mRNA expression and V1aR binding within the PVN of lactating versus virgin rats (Bosch and Neumann, 2010), V1bR mRNA as well as protein expression were similar between virgin and lactating rats. Thus, both receptors are seemingly not adapted during the peripartum period within the PVN as seen, for example, in the MPOA or BNST (Bosch and Neumann, 2008; Bosch et al., 2010). These findings indicate that a putative role of intra-PVN V1 receptors in maternal behaviour is probably based on changes in their activation *via* local AVP release or in the receptor signalling cascades, which should be addressed in future studies.

In the behavioural assessment of V1 subtype-specific manipulation within the PVN, we found that nursing on LD 1 was decreased only after infusion of the V1aR antagonist. This might indicate a specific role of local V1aR activation for the adequate expression of maternal care, e.g. after experiencing a mild stressor such as the infusion procedure (Klampfl et al., 2013). However, exposure to a stronger stressors like the maternal defence test (Neumann et al., 2001) could further highlight the involvement of the V1 receptor subtypes in maternal behaviour, as intra-PVN AVP release is elevated during the maternal defence test in lactating females (Bosch, O.J. & Meddle, S.L., unpublished data). Since none of the behaviours differed on LD 2 in any group, a long-term effect of either V1 antagonist can be excluded, which is in accordance with previous studies (Bayerl et al., 2016, 2014; Bosch and Neumann, 2008).

None of the antagonists affected maternal motivation to retrieve pups in the PRT. These results were somewhat predicted since lesion of the PVN before - but not after - delivery impair pup retrieval (Insel and Harbaugh, 1989) and in the present study the V1 receptors were antagonised postpartum immediately before performing the test. Furthermore, pup retrieval is mainly mediated via the MPOA (for review see Numan and Insel, 2003), where they, indeed, depend upon the simultaneous local activation and deactivation of V1aR (Bosch and Neumann, 2008) and V1bR (Bayerl et al., 2016), respectively.

Anxiety-related behaviour on the EPM was decreased after blocking V1aR in the PVN. This is in line with an earlier study on HAB males, where intra-PVN V1aR antagonist tended to act anxiolytic (Wigger et al., 2004). In contrast, blockade of V1bR did not affect anxiety-related behaviour, similar to central (Bayerl et al., 2014) as well as local infusion of the V1bR antagonist into the BNST and the MPOA of lactating rats (Bayerl et al., 2016). The current findings are in contrast to studies in male rats where orally administered V1bR antagonist acts anxiolytic (Serradeil-Le Gal et al., 2002). However, both the administration route and the gender differences might underlie the differences in the findings. Hence, in lactating females the anxiolytic effect of AVP is rather mediated via V1aR than V1bR.

Interestingly, lactating mothers of the HAB breeding line are characterized by an increased synthesis of AVP, which is positively correlated to the high levels of maternal care (for review see Bosch, 2011). Central blockade of V1aR within HAB dams decreased ABN and anxiety-related behaviour, whereas synthetic AVP in LAB dams increased these behaviours. In our Wistar rats, we found decreased nursing and anxiety-related behaviour after V1aR antagonism. The different outcomes of V1aR blockade demonstrate the brain region specific involvement in the regulation of distinct aspects of maternal behaviour. In addition, breeding line differences might be responsible for opposing outcomes.

Interestingly, the subregions of the PVN are activated differently, depending on the stimulus. While the amPVN is activated by pup presentation, the pPVN is linked to activation of CRF and AVP neurons resulting of HPA axis activation (for review see Numan and Insel, 2003). As we cannot distinguish between the subdivisions with our intra-PVN infusions, the different results might be obtained by activation/blockade of both PVN subdivisions in parallel.

Taken together, our data provide further insight in the complex, brain region-specific regulation of different aspects of maternal behaviour via V1 receptor subtypes. As uncovered in this study, only one of the two V1 receptors might play a role for a certain behaviour within a specific brain area, whereas in other regions both V1aR and V1bR may act in a similar manner or even counteract each other. Our data advances the understanding of postpartum adaptations and, therefore, of possible maladaptations with severe consequences for the offspring.

Chapter 6

More than reproduction - central blockade of GnRH and kisspeptin decrease maternal behaviour in lactating rats

Authors' contribution:

Doris Bayerl:	experimental design, performance of behavioural experiments, data analysis, first draft of manuscript
Stefanie Klampfl:	performance of behavioural experiments
Yoichi Ueta:	experimental design, performance of experiments with eGFP rats/brains
Valery Grinevich:	experimental design, performance of IHC staining
Ferdinand Althammer:	performance of IHC staining, data analysis of IHC staining
Oliver Bosch:	experimental design, performance of behavioural experiments, revision of manuscript

[in preparation to be submitted to the *Journal of Neuroendocrinology*]

6.1 Abstract

GnRH is a major regulator and activator of the HPG axis. Many studies proofed the importance of GnRH in reproduction and sexual behaviour. However, to date only one study shows an involvement of GnRH in maternal behaviour; 30% reduction of GnRH neurons abolishes the mother's motivation to retrieve pups. On this basis, we aimed to investigate the effects of central GnRH receptor blockade on maternal care under non-stress and stress conditions, maternal motivation in the PRT and maternal aggression in the maternal defence test in lactating rats. We found reduced maternal aggression in dams infused with the GnRH antagonist (luteneizing hormone-releasing hormone antagonist); no other behaviour was affected. Due to the fact that GnRH also binds to the kisspeptin receptor GPR54, and behavioural effects may be mediated via kisspeptin, a second set of lactating rats were centrally infused with three doses of the GPR54 antagonist kisspeptin-234 (0.002 µg/ µl; 0.02 µg/ µl; 0.2 µg/ µl) followed by observation of maternal care under non-stress and stress conditions, as well as maternal aggression. While maternal care under non-stress conditions was decreased by the low dose of GPR54 antagonist, maternal care under stress conditions was decreased by the medium dose. Maternal aggression was not affected by any dose.

Taken together we extend the knowledge on GnRH modulating maternal behaviour; GnRH receptor activation is necessary for protection of the offspring. Further, we demonstrate for the first time that maternal care is lowered by antagonism of GPR54. These findings shed new light on two hormones of the reproductive axis, extending their functions to the maternal behaviour.

6.2 Introduction

Throughout all mammalian species, the hormones of the HPG-axis play a distinct role in the endocrine control of reproduction. The HPG-axis is triggered by GnRH, which is synthesized from neurons in the hypothalamus and released at the median eminence into the hypophyseal portal system. At the pituitary level, GnRH facilitates the release of LH and FSH (for review see Ellis, 2013). Thus, GnRH in the brain acts as a neurohormone, important for the release of steroids into the bloodstream, which in turn facilitate sexual behaviour. GnRH neurons are also found in brain areas like the amygdala, the MPOA and ARC (Moss and Foreman, 1976; Yoon et al., 2005), where GnRH is thought to process information about olfactory cues and circadian rhythm (Wen et al., 2011), to facilitate puberty and mating behaviour (Yoon et al., 2005). The GnRH system consists of the hormone itself, from which more than 20 isoforms are known to date (Millar, 2005), and two GPCR subtypes: GnRH-I and GnRH-II, with only GnRH-I being active in rats and humans (Millar, 2005). GnRH is released in pulses (Millar, 2005) and follows a similar circadian rhythm as AVP (Funabashi et al., 2000). Further, *in vivo* studies showed that the release of AVP into the hypothalamo-neurohypophysial system of rats can be stimulated by icv infusion of a GnRH agonist (Boczek-Leszczyk et al., 2010). Interestingly, both GnRH and AVP can modulate the expression of maternal behaviour; transgenic lactating mice with systemically 30% less GnRH neuron show decreased pup retrieval (Brooks et al., 2012), and AVP and its V1 receptors directly impact on the various components of maternal behaviour (Bayerl et al., 2016, 2014; Bosch and Neumann, 2008; Nephew and Bridges, 2008a; Pedersen et al., 1994, 1982; for review see Bosch, 2013, 2011; Bosch and Neumann, 2012).

GnRH neurons also innervate kisspeptin neurons (Kalló et al., 2013) as well as kisspeptin can activate GnRH neurons (Han et al., 2005; Liu et al., 2008), thereby triggering the release of GnRH (Guerriero et al., 2012; for review see d'Anglemont de Tassigny and Colledge, 2010). Kisspeptin is secreted from neurons of the anteroventral periventricular nucleus (AVPV) and the ARC (Clarkson and Herbison, 2006; Han et al., 2005) and mediates its action via binding to GPR54 (also known as KISS1 receptor or AXOR12; Lee et al., 1999), a member of the Gq11 family of GPCRs (Kotani et al., 2001; Muir et al., 2001). In most studies the kisspeptin system is linked to sexual and reproductive behaviour (de Roux et al., 2003; Funes et al., 2003; Seminara et al., 2003), in which the neuropeptides AVP and OXT are also involved (for review see Bosch and Neumann, 2012). Interestingly, both neuropeptides not only modulate

maternal behaviour (for review see Bosch and Neumann, 2012) but, moreover, can be stimulated by kisspeptin (Rao et al., 2011; Scott and Brown, 2013).

In confirmation, a pilot study showed co-expression of AVP and GnRH after immuno-fluorescent staining of slices containing the OVLT of lactating eGFP rats (unpublished data, Ueta Y., Grinevich V.; also see Section 1.4). The OVLT is part of the anterioventral third ventricle (AV3V) region, which is known to be responsible for a balanced AVP secretion (McKinley et al., 2004). Furthermore, it is interconnected with the median preoptic nucleus, which is located near the MPOA, known to be involved in maternal behaviour (for review see Numan and Insel, 2003).

Due to those complex interactions as described above as well as the evidence that the GnRH system affects pup retrieval we aimed to further investigate the role of kisspeptin and GnRH in maternal behaviour. In two independent experiments, early lactating rats were acutely infused icv with either a GnRH receptor antagonist (Experiment 1) or a GPR54 antagonist (Experiment 2), followed by obseravtions of maternal care, maternal motivation in the PRT and maternal aggression.

6.3 Material & Methods

6.3.1 Animals

Rats were kept under standard laboratory conditions (12 h / 12 h light-dark cycle, with lights on at 07:00 h; 22 - 25 °C; 55 ± 5 % relative humidity; free access to water and standard rat chow). Virgin female Wistar rats (12 - 14 weeks, 220 - 250 g, Charles River, Sulzfeld, Germany) were mated with sexually experienced stud Wistar males. Pregnancy was confirmed the next day by the presence of sperm in vaginal smears (assigned as PD 1). Rats were kept in groups of 3 to 4 rats until PD 18, after which rats were single housed in plexiglass observation cages (38 x 22 x 35 cm^3) to ensure undisturbed parturition. The following days until PD 21, rats were handled twice daily to avoid unspecific stress responses during the experiments. On the day of birth offspring were culled to eight pups of mixed sexes and half of the bedding was replaced by new one.

Naive virgin female Wistar rats (10 weeks, 180 - 220 g; Charles River) at random stages of their oestrous cycle were used as intruders in the maternal defence test. They were kept group-housed up to 4 rats in a separate room until the behavioural testing to avoid olfactory recognition by the lactating mothers (for review see Bosch, 2013).

All experiments were performed in accordance with the European Communities Council Directive (86/609/EEC) and were approved by the local government of the Oberpfalz, Bavaria, Germany.

6.3.2 Experimental schedule

On PD 18, females were implanted with a stainless steel guide cannula (21 G, 12 mm length) 2 mm above the right lateral ventricle (1.0 mm posterior, 1.6 mm lateral to bregma, 2.0 mm ventral; Paxinos and Watson, 2007) under inhalation anaesthesia (Isoflurane; Baxter Germany GmbH, Unterschleißheim, Germany) and semi-sterile conditions as described before (Bayerl et al., 2014; Bosch et al., 2010). At the end of the surgery, rats received 0.12 ml of the antibiotic Enrofloxacin (Baytril 2.5 %, Bayer Vital GmbH, Leverkusen, Germany) subcutaneously to prevent inflammation.

6.3.2.1 Experiment 1: Icv infusion of GnRH antagonist

Starting on LD 1, lactating dams received daily an acute infusion of 5 µl VEH (sterile Ringer's solution; pH 7.4; B. Braun Melsungen AG, Melsungen, Germany) or GnRH antagonist (10^{-6} M; Luteneizing Hormone-Releasing Hormone Antagonist, Trifluoroacetate, Bachem, Bubendorf, Switzerland). The dose was chosen based on previous studies (Csabafi et al., 2013) and infused with an infusion cannula (25 G, length 14 mm, i.e. 2 mm longer than the guide cannula) as described previously (Neumann et al., 2000). Maternal care of lactating dams was observed on LD 1 under non-stress conditions, followed by assessment of maternal motivation in the PRT on LD 2. On LD 4, maternal care was monitored under stress conditions, i.e. directly after the maternal defence test, which itself was used to assess maternal aggression (see below). The dams received the same respective treatment throughout the experiment.

6.3.2.2 Experiment 2: Icv infusion of different doses of GPR54 antagonist

Starting on LD1, dams received a single infusion of either 5 µl VEH (Aqua ad iniectabilia, B. Braun Melsungen AG, Melsungen, Germany) or GPR54 antagonist (Kisspeptin 234; Tocris bioscience, Bristol, UK) on testing days. Due to the lack of behavioural data on the substance, a dose-response study with three different doses was performed (0.002 µg/ µl (low dose), 0.02 µg/ µl (middle dose), 0.2 µg/ µl (high dose)).
On LD1 we observed maternal care under non-stress conditions, followed by maternal care observations under stress conditions, i.e. the maternal defence test on LD 3, which was also used to assess maternal aggression. The dams received the same respective treatment on both testing days.

6.3.3 Behavioural tests

6.3.3.1 Maternal care observation.

Maternal care was observed in the home cage of the dams before and after treatment infusion under "non-stress" conditions. Additionally, we observed maternal care before and after treatment infusion followed by the maternal defence test (stress conditions; Bosch et al., 2005; Neumann et al., 2001; for review see Bosch, 2013). We use the term "non-stress"

as we want to distinguish between observations after infusion alone on LD 1 (non-stress condition) or infusions followed by the maternal defence test, which is a strong psychosocial stressor (Neumann et al., 2001), on LD 4 (stress condition). However, we are aware that the infusion procedure *per se* creates a limited amount of stress (Bayerl et al., 2014; Klampfl et al., 2014).

Under non-stress conditions, dams were observed 60 min before treatment infusion (9:00 – 10:00 A.M.) and two hours immediately after treatment infusion. Observations under stress conditions took place 60 min under basal conditions in the morning (9:00 – 10:00 A.M.), before dams were subsequently brought to the maternal defence test room. After the test for maternal aggression another 60 min observation period was assessed to rule out possible behavioural effects of stressor exposure.

The behaviour of the mother was monitored by an experienced observer blind to the treatment for approximately 10 s every second minute in 30 min intervals according to an established protocol (Bayerl et al., 2014; Bosch and Neumann, 2008; Klampfl et al., 2013). ABN was used as a measure for the quality of nursing, as it reflects the only active nursing posture in which the dam is engaged in a quiescent kyphosis (Stern and Johnson, 1990). Other parameters counted were “total nursing”, in which the different nursing postures ABN, blanket posture and lying on side or back are summed up. All nursing postures including licking / grooming the pups and carrying the pups is reflected by the occurrence of mother-pup interaction.

6.3.3.2 Maternal motivation in the PRT

The modified PRT was performed as described earlier (Bayerl et al., 2016). Briefly, dams were separated from their pups 60 min prior to testing. At the same time, a red plastic house (13 x 17 x 11 cm^3, entrance 6 x 8.5 cm^2), providing a potential nesting site, was introduced, which the dams were allowed to explore for 150 min the day before. Ten minutes before the test, dams were infused with their respective treatment. The pups were brought to the experimental room and distributed in a black plastic box (54 x 34 x 60 cm^3) covered with bedding. After placing the red house on one side of the box, the dams were put in the middle of the box (t0) and retrieval behaviour (time and number of retrieved pups) was scored for a maximum of 15 min (t15) by an experienced observer blind to the treatment.

6.3.3.3 Maternal aggression in the maternal defence test.

Lactating mothers (residents) received their respective treatment infusion 10 min prior to introducing a smaller virgin female rat (intruder) into their home cage in the presence of the pups (Bosch et al., 2005; Neumann et al., 2001; for review see Bosch, 2013). The behaviour during the 10-min testing period was video-taped for later analysis by an experienced observer blind to the treatment. The following behaviours were scored: number of attacks, latency until first attack, offensive upright (the dam stands in an upright position in front of the intruder), keep down (the mother keeping the intruder down with her front paws), lateral threat (the mother engages to push the intruder aside by approaching laterally with her whole body), aggressive grooming (for review see Bosch, 2013) and non-aggressive behaviour. At the end of the test the intruder was removed from the residents' home cage and maternal care was observed for another hour as described above. Intruders were used only once per day as well as only twice during the experiment with at least one day in-between for recovery.

6.3.4 Verification of guide cannula placement

At the end of experiments, rats were euthanized *via* CO_2 asphyxiation and infused with 5 µl ink with an infusion cannula *via* the implanted guide cannula. Brains were removed and cut with a razor blade at the implantation site of the cannula. Correct placement was considered if ventricles were coloured blue. Only rats with cannula placed correctly were included in the statistical analysis.

6.3.5 Statistical analysis

Maternal care and pup retrieval were analyzed using two-way ANOVA for repeated measures (factors: time x treatment). The ANOVAs were followed by Sidak *post-hoc* correction if main effects were found. If only a time effect was found, separate one-way ANOVA for repeated measures was performed for detailed description of the time differences within the treatment groups.

Maternal aggression, number of retrieved pups and house entrance latency was analyzed using independent t-test after testing for normal distribution (Kolmogorov-Smirnov Test). If data were not distributed normally, Mann-Whitney-U test was performed. A statistically significant difference was considered at $p \leq 0.05$. Data are presented as means + SEM. Statistical analysis was performed using the software SPSS, Version 20 (IBM, Ehningen, Germany).

6.4 Results

6.4.1 Experiment 1: Icv infusion of GnRH antagonist

6.4.1.1 Maternal care under non-stress conditions

ABN, blanket and total nursing were not affected by the treatment at any time point (Supplementary Table S4). Mother-pup interaction differed significantly depending on time ($F_{4,48}$ = 3.02, p = 0.027), but not on treatment; no time x treatment interaction was found. Separate one-way ANOVA for repeated measures revealed a significant effect of time in the VEH-treated dams only ($F_{4,28}$ = 4.19, p = 0.009). The post-hoc test did not give any further differences.

6.4.1.2 Maternal care under stress conditions

None of the observed behaviours differed at any time point (not shown).

6.4.1.3 Maternal motivation in the PRT.

Pup retrieval increased over time ($F_{15,180}$ = 25.90, p < 0.001), but not depending on treatment; no time x treatment interaction was found. Number of retrieved pups and house entrance latency was not affected by any treatment (not shown).

6.4.1.4 Maternal aggression in the maternal defence test.

During the maternal defence test, GnRH antagonist-treated dams were less aggressive than VEH-treated dams (Figure 30). In detail, the number (independent t-test; t_{12} = 2.54, p = 0.026; Figure 30A) and duration (t_{12} = 2.44, p = 0.031; Figure 30B) of attacks as well as offensive upright (t_{12} = 2.85, p = 0.023; 30C) were reduced in the GnRH antagonist-treated dams; although the sum of all aggressive behaviours did not differ between treatment groups (Figure 30D). None of the other aggressive or none-aggressive behaviours were altered (Supplementary Table S5).

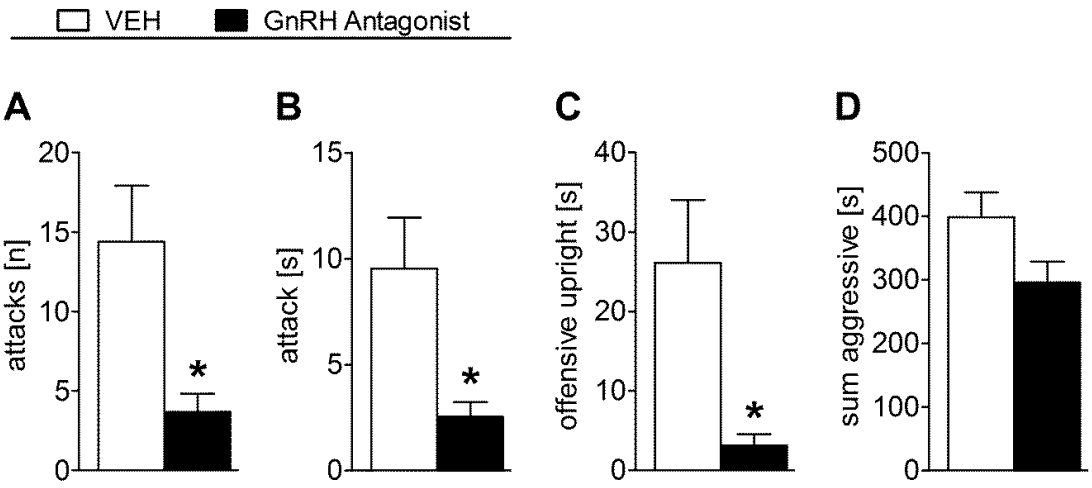

Figure 30 Effect of central GnRH antagonism on maternal aggression

The number (A) and duration of attacks (B), offensive upright (C) and the sum of aggressive behaviour (D) were measured on lactation day (LD) 4 in the maternal defence test 10 min after infusion of 5 µl vehicle (VEH; sterile Ringer's solution; pH 7.4) or GnRH antagonist (10^{-6} M; Luteneizing hormone-releasing hormone antagonist, Trifluoroacetate). Data are presented as mean + SEM, n = 6 - 8 per group. * $p \leq 0.05$ versus VEH.

6.4.2 Experiment 2: Icv infusion of different doses of GPR54 antagonist

6.4.2.1 Maternal care under non-stress conditions

ABN differed significantly depending on time ($F_{4,72} = 7.20$, $p < 0.001$; Figure 31A), but not on treatment; no time x treatment interaction was found. Separate one-way ANOVA for repeated measures revealed a significant effect of time for the low ($F_{4,20} = 6.07$, $p = 0.002$) and medium dose ($F_{4,16} = 3.31$, $p = 0.037$), but not for the high dose. The *post-hoc* test did not show any further difference.

Nursing differed significantly depending on time ($F_{4,72} = 5.24$, $p = 0.001$; Figure 31B), but not on treatment; no time x treatment interaction was found. Separate one-way ANOVA for repeated measures revealed a significant effect of time for the low dose only ($F_{4,20} = 4.84$, $p = 0.007$). *Post-hoc* test did not show any further difference.

Mother-pup interaction differed significantly depending on time ($F_{4,72} = 10.15$, $p = 0.005$), but not on treatment; no time x treatment interaction was found. Separate one-way ANOVA for repeated measures revealed a significant effect of time for the low dose only ($F_{4,20} = 4.62$, $p = 0.008$). *Post-hoc* test did not show any further difference.

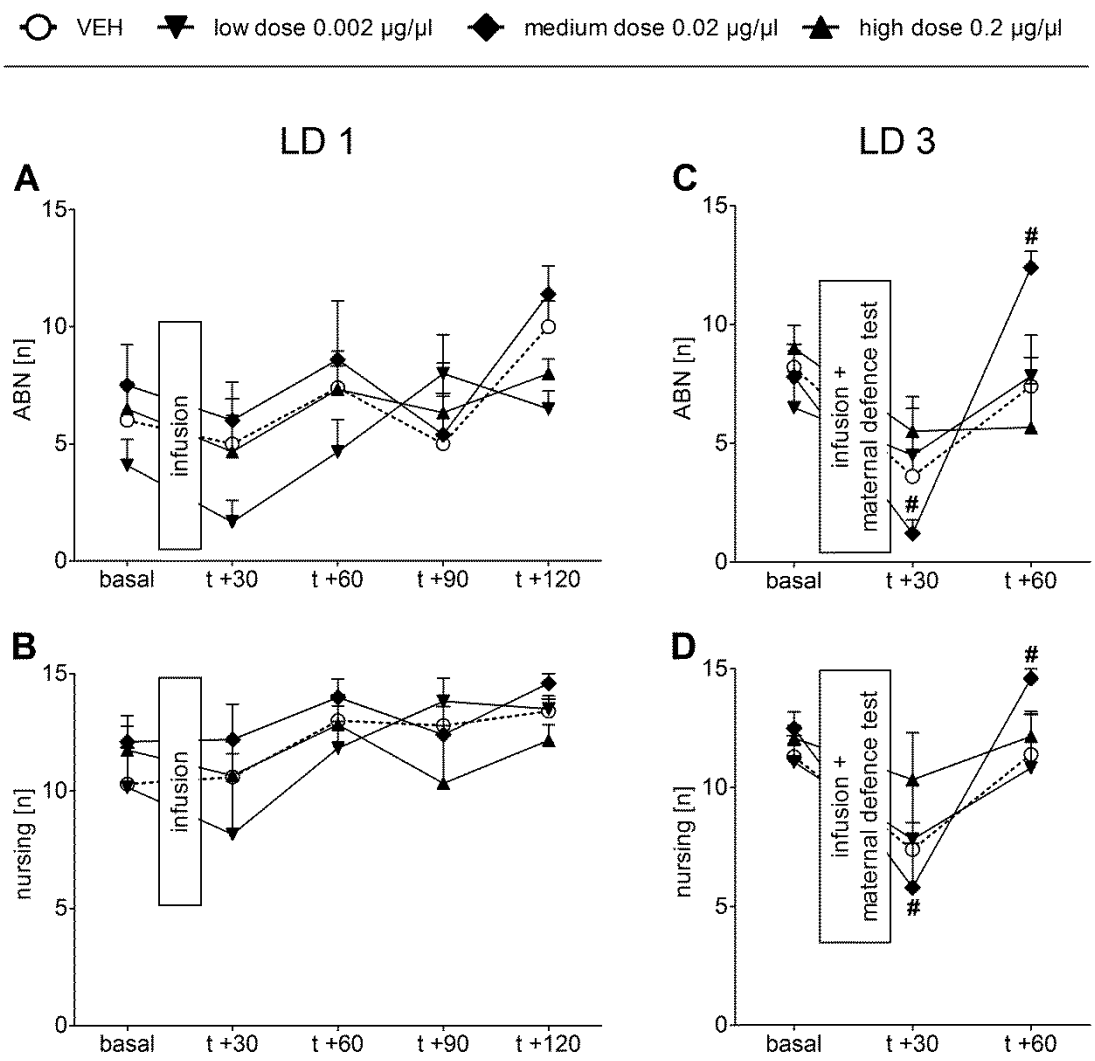

Figure 31 Effect of different doses of central kisspeptin antagonist on maternal care

Maternal care was monitored 60 min before (basal) and 120 min after treatment infusion on lactation day (LD) 1 (A, B) as well as 60 min before (basal) and 60 min immediately after treatment infusion in combination with the maternal defence test (C, D) on LD 3. The mean occurrence of arched back nursing (ABN; A, C) and all nursing postures (B, D) is presented. Dams were infused with 5 µl vehicle (VEH; Aqua ad iniectabilia) or 0.002 µg/ µl (low dose), 0.02 µg/ µl (medium dose) or 0.2 µg/ µl (high dose) of a kisspeptin antagonist (Kisspeptin 234). Data are presented as mean + SEM, n = 5 – 6 per group. # $p \leq 0.05$ versus previous sample of the same group (separate statistics).

6.4.2.2 Maternal care under stress condition

ABN differed significantly depending on time ($F_{2,36} = 13.29$, $p < 0.001$; Figure 31C), but not on treatment; a significant time x treatment interaction was found ($F_{6,36} = 3.08$, $p = 0.015$). When treated with the medium dose, ABN dropped in the first 30 min after the maternal

defence test (p = 0.019) and increased at t +60 (p < 0.001) compared to the previous time point, respectively. No effects for any other dose were found.

Nursing differed significantly depending on time ($F_{2,36}$ = 12.47, p < 0.001; Figure 31D), but not on treatment; no time x treatment interaction was found. Separate one-way ANOVA for repeated measures revealed a significant effect of time for the medium dose ($F_{2,8}$ = 22.27, p = 0.001). Nursing decreased at t +30 compared to basal (p = 0.023), whereas it increased at t +60 compared to the previous time point (p = 0.024). No effects for any other dose were found.

Mother-pup interaction differed significantly depending on time ($F_{2,36}$ = 13.659, p < 0.001), but not on treatment; no time x treatment interaction was found. Separate one-way ANOVA for repeated measures revealed a significant effect of time for the medium dose ($F_{2,8}$ = 12.01, p = 0.004). Mother-pup interaction tended to increase at t +60 compared to the previous time point (p = 0.052). No effects for any other dose were found.

6.4.2.3 Maternal aggression in the maternal defence test

None of the observed behaviours differed at any time point (not shown).

6.5 Discussion

This study is the first approach to assess maternal behaviour in the context of the GnRH and kisspeptin system using receptor antagonists in lactating rats. We demonstrate that central GnRH receptor antagonism decreases attacks and one aspect of threat behaviour, i.e. offensive upright, during maternal aggression testing, whereas central GPR54 antagonist affects maternal care under non-stress and stress conditions in a dose-dependent manner. Thus, the GnRH and kisspeptin system seem to be play different roles in virgin versus lactating rats, mediating a bunch of reproductive functions especially in virgin rats but also influencing different aspects of maternal behaviour in dams.

So far, there is only one study providing evidence of the involvement of GnRH in maternal behaviour. This initial study used transgenic mice, which had systemically 30% less GnRH neurons (Brooks et al., 2012). Although these mice did not have a total depletion of GnRH neurons, maternal behaviour is significantly impaired, i.e. dams leave their pups scattered in the cage and need longer to retrieve them into the nest compared with non-transgenic control mothers (Brooks et al., 2012). The reduction of GnRH neurons could result in decreased binding of GnRH receptors, but also in decreased GnRH release, both potentially having negative effects on maternal motivation to retrieve pups.

In accordance, mice dams with a $G_{q/11}$ fore-brain knockout failed to retrieve their pups and had no pups that survived to weaning age (Wettschureck et al., 2004). As the GnRH-I receptor subtype present in rats, but also in humans, is a GPCR mediating its action through $G_{q/11}$ pathway (Millar, 2005), the appropriate functioning of the receptor seems to be necessary for adequate maternal behaviour, i.e. protection of the offspring.

In our study, central GnRH antagonism resulted in reduced aggression. Although we do not see changes in maternal motivation to retrieve the pups, both, pup retrieval and aggression, serve to protect the pups from threat, i.e. hypothermia because of laying outside the nest or infanticide/injuries by a conspecific during the maternal defence paradigm. One has also to take in account that our central approach may overcome brain region specific effects, as i.e. the MPOA is importantly involved in pup retrieval (Kalinichev et al., 2000; Numan, 1990) and is further known to express GnRH neurons (Caligioni et al., 2007).

Interestingly, GnRH neurons within the MPOA have been shown to co-express OXT receptors (Caligioni et al., 2007). As both, the MPOA itself as well as the neurotransmitter are known to be involved in the regulation of maternal behaviour (for reviews see Bosch and Neumann,

2012; Numan and Insel, 2003), a disruption of proper signalling within this area could lead to disrupted maternal behaviour. Future studies directly targeting the MPOA may help to specify the role of GnRH in the regulation of the different maternal behaviour aspects.

As maternal aggression was affected in our study, an additional involvement of the BNST is likely. The BNST was linked to maternal aggression in various studies before (Bosch et al., 2010; Caughey et al., 2011; Consiglio et al., 2005) and is known to be innervated by GnRH neurons (Boehm et al., 2005). Further studies are necessary, i.e. by targeting the BNST directly with the GnRH antagonist, to rule out the effects on maternal aggression more specifically.

In the past years, the GnRH system was also found to be closely linked to the kisspeptin system. Studies have shown that neurons of one system innervate neurons of the other system and vice versa (Han et al., 2005; Kalló et al., 2013; Liu et al., 2008). In addition, GPR54 receptors were found on GnRH neurons (for review see Roseweir and Millar, 2013). Thus we hypothesized a negative influence of a GPR54 antagonist on maternal behaviour in lactating rats. To date, most studies on the kisspeptin system were investigated in the context of puberty, reproduction and metabolism (Han et al., 2005; Pineda et al., 2010; Stengel et al., 2011; Tolson et al., 2014; for review see Reynolds et al., 2009). In our initial study, we found a decrease in ABN under non-stress conditions directly after infusion of the low GPR54 antagonist dose. Earlier studies have shown that intravenous administration of a kisspeptin agonist increases OXT plasma levels in female rats (Kotani et al., 2001). Further, an icv infusion of kisspeptin increased the firing rate of OXT neurons in the SON in late pregnant and early lactating rats (Scott and Brown, 2013). As OXT is known to mediate maternal care (for review see Bosch and Neumann, 2012), a blockade of kisspeptin receptors which may negatively influence the firing of OXT neurons could lead to the observed decrease in ABN. Further studies have shown, that the activity of kisspeptin neurons within the MPOA of mice is mediated by AVP (Piet et al., 2015). In general, icv administered kisspeptin agonist increases AVP plasma concentration (Ten et al., 2010). As not only OXT, but also AVP is known to mediate maternal behaviour (for review see Bosch and Neumann, 2012), further studies unravelling a connection between kisspeptin, OXT and AVP, maybe also in relation to the GnRH system, are necessary.

Given the fact that under non-stress conditions the low dose, whereas under stress-conditions the medium dose shows effects, a direct proportional effect of the dose to the

type of stressor seems likely. Future studies unravelling an intermittent dose of our low and medium values could be useful in further investigating the role of the kisspeptin system in maternal behaviour of lactating rats. The lack of effect with the high dose might be due to compensatory mechanisms occurring in lactating rats. During lactation, females normally do not show an estrous cycle in order to protect themselves of another pregnancy, since this might endanger the female itself but also the offspring (Ohkura et al., 2009). The absence of the estrous cycle during lactation is considered to be due to inhibited pulse mode of GnRH and LH secretion. In detail, the LH surge in lactating females is hypothesized to be suppressed due to neural inputs associated with the suckling stimulus which inhibit the activity of kisspeptin neurons in the ARC and in turn may control pulsatile GnRH and LH release (Ohkura et al., 2009; Tsukamura et al., 1988). In addition to physiological suppression of kisspeptin release, maybe also decreased receptor sensitivity may occur in lactation, which has not been investigated so far.

Another fact one must consider is the complexity of adaptations occurring in lactating rodents (Neumann, 2001; Neumann et al., 1998a, 1998b; for review see Russell et al., 2001) and also kisspeptin and GnRH seem to adapt with the reproductive status. A study conducted in rhesus monkeys showed a switch from an ovarian steroid-independent mechanism in prepubertal to an ovarian steroid-dependent mechanism in pubertal individuals in the response of GnRH to kisspeptin (Guerriero et al., 2012). So far, nothing is known about this mechanism in lactation, implicating the necessity of further studies.

In summary, we provide evidence that a GnRH receptor antagonist impairs the protection of the offspring by reducing attacks and threat behaviour of the mother towards an intruder, whereas a GPR54 antagonist impairs ABN under non-stress and stress conditions in a dose-dependent manner.

In conclusion, blockade of GnRH as well as of kisspeptin receptors impairs maternal behaviour, thereby indicating a complex role in aspects of reproduction and maternal behaviour.

Chapter 7

General Discussion

7.1 Summary of results

The overall aim of my thesis was to provide further insight in the involvement of the AVP system, especially of V1bR, in hypothalamic and limbic brain regions in the different aspects of maternal behaviour in lactating rats.

In an initial approach I manipulated Wistar dams centrally with a specific V1bR agonist or antagonist to investigate the impact of the AVP receptor subtype in different aspects of maternal behaviour (Chapter 2). I could show that blocking V1bR centrally in the brain impaired maternal care directly after infusion under non-stress as well as under stress conditions. There were no effects of V1bR manipulation found in maternal motivation to retrieve pups, maternal aggression or maternal anxiety. Taken together, these results indicate a role of central V1bR in the modulation of certain aspects of maternal behaviour. However, local approaches are requested to reveal the specific location of the effects (see below).

In order to determine the role of V1bR manipulation in an animal model for extremes in anxiety and nursing behaviour, I repeated the experiment in HAB and LAB dams (Chapter 3). Due to the increased AVP expression and release in HAB dams, I hypothesised an influence of central V1bR antagonism especially in HAB dams, and the other way round, of central V1bR agonism in LAB dams on maternal care. Surprisingly, neither treatment had any effect. Instead, both breeding lines showed a stable phenotype regarding maternal care, maternal motivation and maternal anxiety as described before. Analysis of the maternal defence test showed for the first time a shift of increased maternal aggression from HAB dams to LAB dams. Non-manipulated LAB dams showed increased aggression compared to HAB dams in the maternal defence test, which was confirmed in subsequent generations. This change might have occurred due to breeding over many years, although sibling pairing was strictly avoided. An analysis of changes in neuropeptide and hormonal systems could contribute to

clarify the shift of a single parameter of maternal behaviour without changes in other aspects of the phenotype.

Due to the fact that opposing mechanisms mediated *via* single brain regions might be overruled by the central manipulation of V1bR, I elucidated the involvement of the V1 receptor subtypes specifically within distinct brain regions, i.e. the MPOA, the BNST (Chapter 4) and the PVN (Chapter 5), all known to be involved in the regulation of maternal behaviour. First, I investigated potential lactation-induced changes in V1bR mRNA and protein expression; I did not find any difference within any brain region. However, as central manipulation of V1bR indicated a behavioural relevance of the receptor subtype in maternal behaviour I continued with acute local infusion of V1bR antagonist. My findings are summarized in Figure 32 and Figure 33.

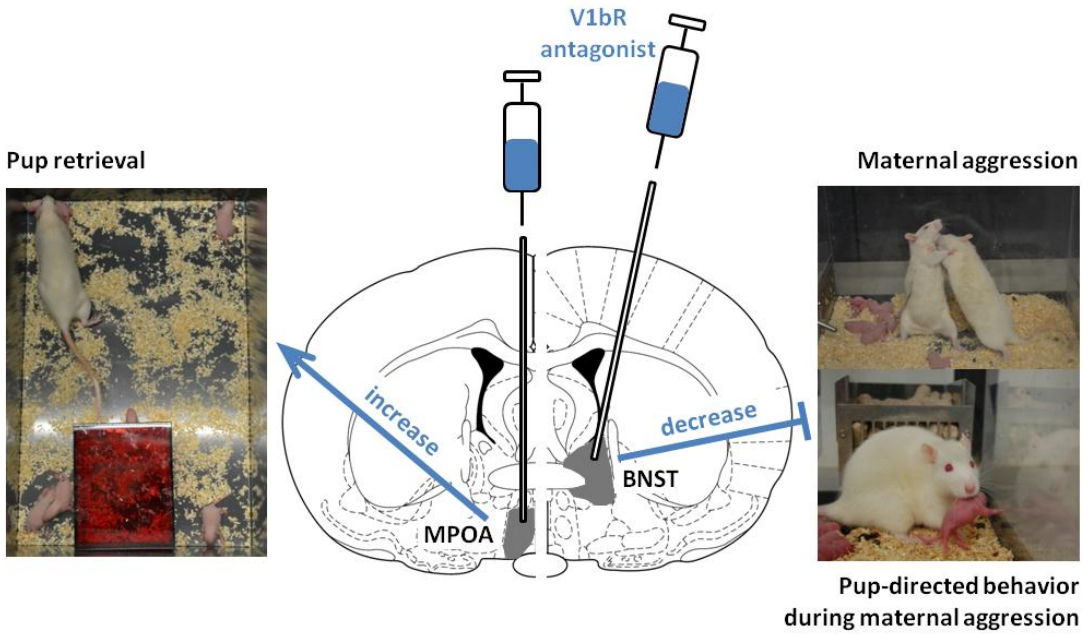

Figure 32 Graphical summary of local V1bR antagonism within the MPOA and the BNST

Local infusion of V1bR antagonist within the MPOA increased the motivation of the dams to retrieve their pups in a novel environment, whereas within the BNST decreased pup-directed behaviour during the maternal defence test was found.

In detail, I found increased maternal motivation to retrieve pups in a novel environment after V1bR antagonism within the MPOA. Within the mpBNST, V1bR blockade impaired pup-directed behaviour during the maternal defence test, with dams showing less licking and grooming of the pups in the presence of the intruder. Furthermore, V1bR antagonism

decreased maternal care under stress conditions in both brain regions. In the PVN, V1bR blockade had no effect on any maternal parameter, implicating its minor role in maternal behaviour within this brain region. In contrast, V1aR blockade decreased nursing under non-stress conditions and anxiety-related behaviour on the EPM, without affecting pup retrieval. In summary, V1R mediate different aspects of maternal behaviour in a brain region dependent manner.

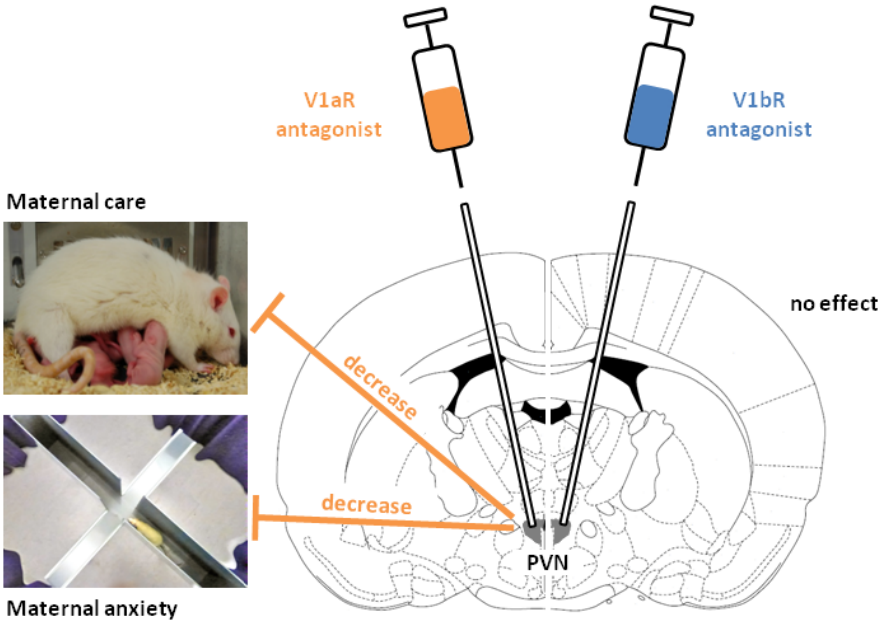

Figure 33 Graphical summary of local V1aR or V1bR antagonism within the PVN

Local infusion of V1aR antagonist (orange) within the PVN decreased maternal care and anxiety-related behaviour on the EPM, whereas local V1bR antagonist infusion (blue) had no effect on maternal behaviour.

During research on the source of AVP within the "maternal super-region" I could detect, in collaboration with Dr. Ueta (Kitakyushu, Japan) and Dr. Grinevich (Heidelberg, Germany), co-expression of AVP-GnRH in the OVLT of lactating, but not virgin, AVP eGFP rats. On the basis of this pilot study, I investigated the involvement of GnRH in the regulation of maternal behaviour in lactating rats (Chapter 6). Central antagonism of GnRH decreased aggression in the maternal defence test but did not affect any other maternal parameter. As GnRH neurons are closely linked to the kisspeptin system, I further blocked central GPR54 in another cohort of lactating dams (Chapter 6). As this is the first study investigating kisspeptin in the context of maternal behaviour, I performed a dose-response study with a GPR54 antagonist. Indeed, the blockade with the low dose of 0.002 µg/µl decreased maternal care under non-stress conditions, whereas the medium dose of 0.02 µg/µl kisspeptin antagonist

decreased maternal care under stress conditions. No further changes were detected on any other maternal parameter. These observations provide evidence of two further neuropeptide systems involved in the regulation of maternal behaviour.

7.2 Involvement of the brain AVP system, especially of V1bR, in maternal care

In most mammals, mothers take care of the offspring and provide them with the essential nutrition which guarantees their survival and growth. Various neurotransmitter systems are described to promote nursing behaviour, with OXT and AVP being among the most studied (for review see Bosch and Neumann, 2012).

An involvement of AVP was already suggested in the 1980s by Pedersen et al., who found that central AVP administration leads to an promotion of crouching over and licking/grooming of pups in ovariectomized, hormone-primed virgin female rats (Pedersen et al., 1982). In line with this finding, repeated central infusion of AVP in lactating rats increased ABN, whereas central V1aR blockade decreased ABN and mother-pup interaction (Bosch and Neumann, 2008). Also central V1bR antagonism decreased mother-pup interaction besides nursing (Chapter 2). These observations suggest a crucial role for both V1R subtypes in maternal care under non-stress condition. In addition, V1bR antagonism under stress conditions leads to similar findings of impaired nursing and mother-pup interaction (Chapter 2). This brings up the hypothesis that V1bR are necessary to provide adequate maternal care after disruption of the homeostasis by the infusion procedure *per se* or in combination with the strong stressor of the maternal defence test.

The effects can be clearly linked to V1bR, as the antagonist used is highly selective for the rat V1bR (Griebel et al., 2002; Serradeil-Le Gal et al., 2002). Thus, parallel inactivation of V1aR or OXT receptors is unlikely. Although, a compensatory effect of the OXT system might contribute to the behavioural outcome; studies in V1bR knockout mice have shown that OXT can act on V1bR (Koshimizu et al., 2012), thereby promoting maternal care. In confirmation, OXT receptors have been found to partly take over the stress-induced ACTH release in these mice (Nakamura et al., 2008). Nevertheless the antagonist provides a highly selective pharmacological tool to investigate the effects of V1bR in the different aspects of maternal behaviour.

Due to the fact that central manipulation of V1R might be too unspecific for distinct effects of brain regions known to be involved in maternal care, or to reflect the integration of various effects, region-specific antagonism was used to further elucidate potential effects of V1R subtypes.

Here, the MPOA is one of the brain regions of major interest, known to facilitate maternal care after chemical stimulation (Fisher, 1956). Lesion studies of the dorsolateral connections of the MPOA which disrupt maternal care in lactating rats underline the importance of the brain region (Numan and Callahan, 1980; Numan, 1990). Focussing on the AVP receptor subtypes, V1aR antagonism or down-regulation *via* antisense oligodeoxynucleotides in this brain region has an impairing effect on maternal care by delaying the onset in parturient rats (Pedersen et al., 1994) and decreased ABN as well as mother-pup interaction in lactating rats (Bosch and Neumann, 2008). In contrast, intra-MPOA up-regulation of V1aR *via* an adeno-associated viral vector improved maternal care (Bosch and Neumann, 2008). In addition, AVP is released in response to mother-pup interaction (Bosch et al., 2010) and thus, it binds to both AVP receptor subtypes. Under stress conditions, V1bR antagonism within the MPOA decreased ABN, total nursing and mother-pup interaction (Chapter 4), similar to central blockade. This underlines the suggested role of V1bR to reinstate adequate maternal care after exposure to a stressor, i.e. after disruption of the homeostasis in order to provide sufficient care for the offspring.

The mpBNST, which is inter-connected with the MPOA, is also involved in the regulation of maternal care, as initially shown by lesion studies (Numan and Numan, 1996). In contrast to the MPOA, intra-mpBNST V1aR blockade does not disrupt maternal care under non-stress conditions (Bosch et al., 2010), suggesting the involvement of another AVP receptor or neuropeptide system in the regulation of this behaviour. Indeed, antagonism of V1bR within the mpBNST only decreased ABN and total nursing after the maternal defence test, but not under non-stress conditions (Chapter 4). This confirms the importance of V1bR in maternal brain regions ensuring the ability of adequate stress coping in dams. Further it implies a region-specific involvement based on experimental conditions, i.e. the role of the mpBNST during stress. This was also shown to be the case regarding another neuropeptide system, i.e. CRF (Klampfl et al., 2015, 2014). Blockade of both CRF receptors (CRF-R), CRF-R1 and CRF-R2, improves nursing, specifically ABN, under stress conditions in the mpBNST. In contrast, only CRF-R1 blockade tended to increase ABN in the adBNST. Future studies

elucidating the role of V1bR within the adBNST could provide further insight in the specific involvement of the AVP system in the regulation of maternal behaviour aspects in the subnuclei of the BNST.

Besides the maternal super-region, also the PVN is implicated in mediating maternal care. In addition to lesion studies showing the involvement of the PVN in maternal care (Insel and Harbaugh, 1989; Numan and Corodimas, 1985), the presence of the pups was found to increase *c-fos* expression in the amPVN of dams (for review see Numan and Insel, 2003). After antagonism of V1aR within the PVN we found a decrease in maternal care under non-stress conditions, whereas V1bR antagonism had no effect. This might indicate that V1aR within the PVN are important in the adequate expression of maternal care under basal conditions.

Thereby, a brain-region dependent but also behaviour-specific and context-dependent mechanism seems to regulate central V1R: they either modulate maternal behaviour alone (V1aR, but not V1bR, blockade within the PVN impairs nursing under non-stress conditions), as counterparts (V1aR blockade within the MPOA impairs/ V1bR blockade improves pup retrieval within the MPOA) or in complement (blockade of both receptor subtypes within the mpBNST impairs pup defence/protection).

One also must not forget the potential influence of the closely related OXT in all these brain regions. Besides AVP, OXT is known to positively correlate with maternal behaviour (for review see Bosch and Neumann, 2012). Thereby, the dorso-medial BNST projects to the PVN and the SON, by targeting regions with a high density of oxytocinergic and vasopressinergic cell bodies, respectively (Dong and Swanson, 2006). Thus, manipulation of receptors within the mpBNST could also affect the projections to the PVN and mediate the neuropeptide signalling in this target region.

These distinct roles of the V1aR and V1bR on maternal behaviour in different brain regions might also be linked to the distribution of the receptor subtypes (see Figure 5). Whereas the MPOA is dominated by V1bR, the PVN mostly expresses V1aR. Within the BNST both receptor subtypes can be found (Griebel et al., 2005). Thus, the lack of behavioural effect after V1bR blockade within the PVN might be due to the very low amount of receptors in this brain region. In contrast, manipulation of both V1aR and V1bR in the mpBNST resulted in alterations of maternal behaviour. Within the MPOA V1bR are expressed in a higher amount than V1aR, suggesting additional mechanisms leading to the behavioural outcome. As the

distribution of receptors (see Figure 5) was investigated in male rats (Griebel et al., 2005; Hernando et al., 2001; Orcel et al., 2002), gender or lactation specific adaptations may occur, which has to be determined more specifically in future studies. Interestingly, in a first cellular approach, I did not find alterations of V1bR mRNA or protein expression within the MPOA, BNST and PVN in lactating rats compared to virgins. This suggests adaptations in the signal transduction or sensitivity to the agonist in lactation. Since V1bR are internalized after agonist binding in a higher amount than V1aR, also this internalization process might be adapted in lactation and/or under stress conditions (Kashiwazaki et al., 2015). In addition, an increased sensitivity of V1bR in lactation might be responsible. It is known that high levels of AVP binding to V1bR stimulate not only the IP3 pathway (Gq/G11), but also cyclic adenosine monophosphate (Gs) signalling (Orcel et al., 2009). Studies in cell culture have described, that a drug increasing the sensitivity of the V1bR, decreases the amount of AVP which is necessary to stimulate both signalling cascades (Orcel et al., 2009). Since the release of AVP is known to be increased in lactation (for review see Bosch and Neumann, 2012), additional mechanisms increasing the sensitivity of V1bR might lead to simultaneous activation of the Gq11 and Gs, i.e. IP3 and cyclic adenosine monophosphate pathway. Future studies are necessary to confirm this suggestion and the resulting behavioural consequences.

However, changes in maternal behaviour after V1bR manipulation occur without adaptation on mRNA or protein expression, a distinct role of V1bR in lactation can be suggested. This is supported by the fact, that virgin rats are not maternal. Future studies investigating if V1bR manipulation accelerates pup sensitisation in virgins could further help to understand the role of the AVP system in maternal behaviour.

Taken together, brain region specific blockade of V1R shows a pivotal role of V1aR within the PVN under non-stress conditions, and V1bR within the MPOA and the mpBNST under stress-conditions. Therefore, both V1R subtypes seem to be important for homeostasis, adequate stress-coping and thus adequate maternal care.

Regarding V1bR manipulation within HAB and LAB dams no differences due to treatment could be found (Chapter 3). In line with previous studies, HAB dams were highly maternal compared to less maternal LAB dams. Interestingly, among generation #51, a shift regarding the different nursing positions was observed. So far, HAB dams are described to be more engaged in ABN, i.e. showing more high and low crouch, as well as higher amount of nursing in general compared to LAB dams, with blanket position being equally provided in both

breeding lines. HAB dams of generation #51 still show increased high crouch than LAB dams, but low crouch position was equally shown in both breeding lines. Consequently, this diminishes the difference of ABN, which is also about the same value in HAB and LAB dams of this generation. In contrast, LAB dams show more blanket position compared to HAB dams, also adjusting the values of total nursing to the same level in both breeding lines. Taken together, LAB dams of generation #51 seem to show increased low crouch and blanket nursing compared to LAB dams of foregoing generations. Acoustic disturbances in the observation rooms (Lauer et al., 2009; Milligan et al., 1993), which might have impaired low crouch position in HAB dams thereby mimicking an increase in low crouch of LAB dams are unlikely, as low crouch and blanket levels in LAB dams seem to have increased between generation #50 and #51. As several other factors, like smell irritation (due to several unknown students) could be the reason for this change in maternal care, observations in dams of new pairings are necessary. Additionally, as maternal care is highly variable, V1bR manipulations should be repeated, to rule out receptor-subtype specific effects on this aspect of maternal behaviour.

7.3 Involvement of the brain AVP system, especially of V1bR, in maternal motivation

Maternal motivation reflects “the investment of time and resources to seek and maintain contact with the young” (Olazábal et al., 2013). This behaviour is measured in the laboratory in the PRT, where it reflects the dams’ motivation to retrieve her pups in a novel environment (Neumann et al., 2005; van Leengoed et al., 1987). In this test the dams’ drive to protect their pups by retrieving them to one place, which is in most cases the nest, has to overcome the fear of the unknown arena (for reviews see Numan and Insel, 2003; Olazábal et al., 2013). Maternal motivation was found to be mediated by the MPOA in rats, with lesions of the dorsolateral connections disrupting the behaviour (Franz et al., 1986; Kalinichev et al., 2000; Numan and Corodimas, 1985). Further, during pup retrieval the release of AVP within the MPOA is increased (Bosch et al., 2010). Thus, central manipulation of V1bR might be insufficient or lead to a minor effect, which behavioural outcome is not visible.

This is supported by our local blockade of V1bR restricted to the MPOA, which led to increased pup retrieval. In contrast, pup retrieval is decreased by blocking V1aR within the same region (Bosch and Neumann, 2008; Pedersen et al., 1994), proposing an opposing role of both V1R subtypes within the MPOA on maternal motivation. Further, V1aR activation, but simultaneous decreased V1bR signalling within the MPOA seems to be important for the adequate behaviour. Due to unchanged V1bR mRNA and protein expression (Chapter 4) but increased V1aR signalling (Bosch and Neumann, 2008) a dominance of V1aR within the MPOA can be suggested. Focussing on the AVP system, this V1aR dominance provides the basis of proper maternal motivation to retrieve pups in lactating rats. An increased activation of V1aR might also be the reason for increased pup retrieval in HAB dams compared to LAB dams. Due to their SNP in the AVP promoter region, elevated levels of AVP can bind to V1aR and results in the high motivational state of HAB dams. However, as the neuropeptide can also bind to the V1bR, the adaptations of the receptor subtypes in lactation and their involvement in the regulation of maternal motivation needs to be reconfirmed, e.g. by manipulation of V1bR within the MPOA.

However, pup retrieval behaviour of LAB dams could be improved by providing them a familiar nest site during the PRT (Chapter 3 and 4). LAB dams retrieve their pups very poorly in the conventional PRT (Neumann et al., 2005). Although retrieval behaviour is known to be very high in early lactation (for review see Bosch, 2011), the reason for the low number of pups retrieved by LAB dams might be due to a lack in social investigation. This hypothesis is supported by findings in the modified hole board test although performed in males; LAB rats prefer to halt social contact in order to investigate a novel environment, whereas HAB rats prefer to stay in contact with cage mates (Ohl et al., 2001). Importantly this study was performed in males and needs to be repeated in females to verify the hypothesis of a lack in social abilities. Indeed, first indices of differences in sociability between HAB and LAB dams were observed in the conventional PRT. HAB rats retrieved their pups quickly to a single nest and stayed in close contact to the young independent of the novel environment. In contrast, LAB dams retrieved their pups only poorly and spent more time sniffing and rearing in the box. Although their motivation should be high during this early phase of lactation (for review see Bosch, 2011), they preferentially investigate the novel environment instead of keeping contact to their offspring and protecting them of potential threats (reflected by decreased pup retrieval compared to HABs). Personal observations (DSB, SMK, OJB) show that LAB

dams even try to escape from the box (not as a flight reflex rather than being interested in the surrounding), which is in accordance to the observation of the modified hole board test in males (Ohl et al., 2001). Thus, LAB dams seem to be in a conflict: on the one hand they want to leave the pups in order to investigate the environment, which can be referred to their lack of sociality. On the other hand, they want to stay with their pups and retrieve them to a single nesting site, which is due to their maternal status. With the modified PRT, which consists of a box with higher walls, escape got impossible. In addition, the house provides a safe place for the pups and the dam, thus shifting the "interest", especially from LAB dams, to pup retrieval. Since pup retrieval is already very high in HAB dams, further improvement is hardly possible. Nevertheless, comparing the retrieval behaviour of VEH dams in the conventional PRT to the modified PRT (Chapter 4) an improvement in the number of pups retrieved can be observed, confirming the improvement not only of LAB, but also of NAB dams. Since these NAB dams were manipulated, confirmation of the PRT setup in non-manipulated lactating Wistar rats is warranted.

Blockade of V1bR within either the BNST (Chapter 4) or the PVN (Chapter 5) did not affect maternal motivation, similar to previous studies showing no effect on maternal motivation after V1aR blockade within the BSNT (Bosch et al., 2010). Although both brain regions have been shown to be involved in the regulation of maternal behaviour, pup retrieval seems to rely mainly on the MPOA. Further, the time-point of manipulation plays a role; lesions of the PVN before giving birth, but not postpartum, are known to disrupt maternal care and pup retrieval (Insel and Harbaugh, 1989). Thus, our infusions start in lactation, the results are in line with the previous studies. Future studies blocking V1R within the PVN at different time-points may strengthen the role of the AVP system in accordance to previous findings.

7.4 Involvement of the brain AVP system, especially of V1bR, in maternal aggression

Maternal aggression is an essential behaviour to protect the offspring from any potential threat (for review see Bosch, 2013). Under laboratory conditions, the threat is mimicked by an unfamiliar intruder, in my case a virgin female, about 10 % smaller than the resident. The opponents are not equal in size/body weight, as I want to stress the mother and evoke maternal aggression, but prohibit pup killing by the intruder. Nevertheless, the dams

respond with a repertoire of attack and threat behaviours, which can be manipulated by substance infusion.

Central agonism and antagonism of V1bR did not affect aggressive behaviour of the NAB dams (Chapter 2) nor in HAB and LAB dams (Chapter 3) during the maternal defence test. Brain region specific blockade of V1bR elucidated a V1R-subtype specific implication in maternal aggression within the mpBNST. While V1aR blockade reduced attacks and threat behaviour towards the intruder (Bosch et al., 2010), V1bR blockade reduced pup directed behaviour during the aggressive encounter, especially licking and grooming (Chapter 4). Studies on both the AVP (Bosch et al., 2010) and the CRF-system (Klampfl et al., 2014) show a specific implication of the mpBNST in maternal aggression. Interestingly, and in contrast to the other studies, V1bR blockade led to changes in non-aggressive behaviour, i.e. pup-directed behaviour. This could hint to the specific role of V1bR by mediating the caretaking of the offspring during stressful situations. Thus, in order to get the required balance between attacking the intruder and taking care of the offspring, both V1R subtypes need to be activated concomitantly. Only if both receptors are activated under stressful conditions, adequate defence and protection of the young is possible.

In contrast, a misbalance of adequate attacking of the intruder and protection of the offspring could lead to excessive forms of aggression, which was found with subsequent breeding in LAB dams (Chapter 3). Surprisingly, the LAB dams, which have low levels of anxiety and display lower levels of maternal care, reflected by decreased ABN and aggression during the maternal defence test (Bosch and Neumann, 2008; Bosch et al., 2010, 2006, 2005; Neumann et al., 2005; for review see Bosch, 2011), appeared to turn into abnormally aggressive dams (Chapter 3). During the maternal defence test they attacked the head, nose and belly of the intruders and injured the defensively behaving counterparts seriously, similar to observations in LAB males (Beiderbeck et al., 2012; Neumann et al., 2010). This behaviour already fulfils two out of three criteria for abnormal aggression (for review see Haller and Kruk, 2006): (1) disregarding species-specific rules by attacking vulnerable body parts and (2) insensitivity towards social signals of the opponent, by ignoring the submissive behaviour of the intruder and continuing attacking. The last criteria, (3) mismatch between provocation and response, is hard to distinguish in maternal defence test. Any other rat, although slightly smaller than the dam, is a potential risk for the offspring, thus, a dam aggressively defending her offspring is quite normal. In case of

maternal aggression, the other two criteria seem to be more important than the third one, as maternal aggression is triggered by different cues than other types of aggressive encounters like intermale or territory aggression (Wersinger et al., 2007). In addition, aggression was negatively correlated with AVP release within the LS in males (Everts et al., 1997). The AVP system, in detail V1aR, within the LS is also linked to social memory in male rats (Everts and Koolhaas, 1997). Infusion of a V1aR antagonist impairs social memory, which is not confirmed in lactating rats so far. If so, decreased AVP within the LS of LAB dams and consequently less binding of V1R, leading to decreased sociability might be an explanation for elevated aggression within these dams. Thus, measurement of AVP release within the LS during the maternal defence test might rule out differences in HAB/LAB dams, which micht be linked to inter-LS AVP levels. Importantly, this might not be true in lactating females, as maternal aggression has different triggers compared to intermale aggression (Wersinger et al., 2007). Another important factor seems the decreased sniffing in LAB dams compared to HAB dams of generation #50 during the maternal defence test. As anogenital sniffing is part of the social investigation pattern in rats, the decreased sniffing hints to an impairment of social abilities in LAB rats, as described before (Ohl et al., 2001). Nevertheless, the reason of the changing aggressive phenotype in LAB rats still remains unknown. Studies in male V1bR knockout mice found decreased social memory in these mice, compared to wildtype (Wersinger et al., 2002). Interestingly, a lack of social memory was also recognized in pregnant female V1bR knockout mice; these mice failed to terminate their pregnancy when they or cohabituated with an unfamiliar novel male (Bruce effect) (Wersinger et al., 2008; for review see Stevenson and Caldwell, 2012). In male rats, antagonism of V1aR impairs maternal memory, indicating the importance of V1aR in social behaviour and the proper receiving of pup cues (Nephew and Bridges, 2008b). Therefore, the lack of social abilities observed in LAB rats might also rely on changes in the AVP system, more specifically, in the V1R. Besides the AVP system, also dopamine, serotonin, and glucocorticoids have been found to be involved in the regulation of high levels of aggression (Ferris, 2005; Miczek et al., 2002; Neumann et al., 2010; for reviews see Haller and Kruk, 2006; Haller, 2013). Studies in LAB males have confirmed the role of dopamine and the reward system in the regulation of aggression (Couppis and Kennedy, 2008; Ferrari et al., 2003); the high levels of aggression in LAB males could be decreased by local infusion of a dopamine D2 receptor antagonist within the NAc (Beiderbeck et al., 2012). The NAc has been closely related to the dopaminergic

reward system, which is known to be hyper-active not only in rats, but also in psychopathic patients (Buckholtz et al., 2010). Thus, high levels of aggression could have a rewarding effect in LAB dams, which needs further investigation. Besides the dopamine system also the serotonin system has been shown to be linked to aggression. Thereby, systemic administration of serotonin or a specific serotonin receptor agonist (either for 5-HT1A or 5-HT1B) reduce maternal aggression in an unspecific manner (since also general activity can be reduced) (Olivier et al., 1995; for review see Lonstein and Gammie, 2002). Due to the fact that breeding over years might have changed the AVP system further, but also several petidergic and non-peptidergic systems in LAB rats, a genetic screening may outline these adaptations. Understanding the potential changes in these systems could help to understand the neurobiological mechanisms behind abnormal levels of aggression in lactating LAB dams.

7.5 Involvement of the brain AVP system, especially of V1bR, in maternal anxiety

In order to provide adequate maternal care for the young anxiety-related behaviour in lactating rats has to be reduced (Hansen et al., 1985; Lightman and Young, 1989; Neumann et al., 1998a; Windle et al., 1997). This adaptation occurs due to facilitation of the PRL, OXT and CRF-system during lactation (Klampfl et al., 2015, 2014; Neumann et al., 2000; Torner et al., 2001). Further, AVP is implicated in the regulation of anxiety related behaviour, by exhibiting an anxiogenic effect in male and virgin female rats (Landgraf et al., 1995; Wigger et al., 2004; for review see Ring, 2005) as well as in mice (Bunck et al., 2009). Also in lactating rats central chronic synthetic AVP infusion increased whereas repeated central V1aR antagonist infusion decreased anxiety-related behaviour on the EPM (Bosch and Neumann, 2008). This is similar to findings in male V1aR knockout mice, which show decreased anxiety on the EPM, in the light-dark box and the open field (Bielsky et al., 2004). In contrast, male V1bR knockout mice do not differ in anxiety related behaviour from wild-type controls (Wersinger et al., 2002). However, no data on anxiety-related behaviour of female virgin and lactating V1bR knockout mice are available to date, which remains to be investigated. In more natural animal models, intraperitoneally or orally administered V1bR antagonist was shown to act anxiolytic on the EPM, the open field (Amikishieva et al., 2011; Griebel et al., 2002) but also in a social defeat paradigm (Litvin et al., 2011) in male rodents.

Central blockade of V1bR in lactating rats did not affect anxiety-related behaviour (Chapter 2), suggesting that differences in administration route but also gender can influence the behavioural outcome. Further, a brain-region specific effect is likely, which may be masked by the central approach. Lonstein (for review see Lonstein, 2007) suggested an involvement of the BNST in the regulation of postpartum anxiety. In detail, pup contact may decrease noradrenaline release in the ventral BNST (vBNST) when dams experience an anxiogenic situation. Consequently, GABAergic projections from the vBNST to brain regions like the PVN, amygdala and the ventrocaudal PAG are decreased. Potentially excitatory projections from the PVN and the amygdala to the ventrocaudal PAG are also inhibited. The release of OXT and PRL within and from the PVN may also be induced by vBNST disinhibition to exert anxiolytic properties in the lactating dam.

This hypothesis is strengthened by the results of intra-BNST blockade of V1aR, which tends to decrease anxiety-related behaviour in lactating rats (Bosch et al., 2010). In contrast, no changes were observed after blocking V1bR within the mpBNST or within the MPOA. In case of the latter, this is not surprising, as the MPOA has not been referred to mediate anxiety in rats (Bosch et al., 2010). However, the role of the BNST, more specific the subnuclei of the BNST, in the modulation of maternal anxiety is not fully understood yet; the obtained effects are thought to be mediated by activation of inhibitory projections from the BNST to subnuclei of the amygdala (Walker et al., 2003), leading to suppressed anxiety in lactation (for review see Lonstein, 2007). Therefore, investigation of V1bR blockade within the subnuclei of the amygdala may help to understand the involvement of the receptor subtype in the regulation of anxiety-related behaviour in lactating dams.

Local V1aR, but not V1bR, blockade within the PVN of Wistar rats decreased anxiety-related behaviour on the EPM. This is in line with studies in hyper-anxious HAB males and, more importantly, dams; the increased anxiety due to elevated AVP expression and release within the PVN can be reversed by repeated central infusion of a specific V1aR antagonist (Bosch and Neumann, 2008; Wigger et al., 2004). Thus, although the V1R within the BNST seem not to be main regulators of maternal anxiety, projections from the BNST to the PVN might be involved in the regulation of anxiety of V1aR within the PVN.

In addition, due to diffusion of the antagonist, all subdivisions of the PVN might have been targeted. As the pPVN and the amPVN are known to be activated by different neuropeptide systems, i.e. the AVP and CRF system, respectively (for review see Numan and Insel, 2003),

their concomitant activation might have decreased maternal anxiety. Indeed there has been evidence, that V1bR and CRF-R1, both GPCRs, can build heterodimers (Milligan et al., 2006). These heterodimers can show synergistic action, e.g. on catecholamine release of bovine chromaffin cells or trigger ACTH release from corticotroph cells in the pituitary (Murat et al., 2012). As CRF may also potentiate the AVP-induced signalling pathway by increased IP3 production, the behavioural effects may reflect down-stream adaptations of V1bR/CRF-R1 heterodimers. In addition, CRF neurons within the pPVN and amPVN are known to express V1bR (Dabrowska et al., 2013). Thus, they could respond to local AVP release *via* activation of V1bR. As a simultaneous activation of AVP and CRF neurons within the PVN in lactation my blunt behavioural outcomes, future studies targeting the V1bR and the CRF system, e.g. by targeting CRF-R1, in parallel may shed further insight in the complex network of maternal circuits.

Intriguingly, an initial study investigated the involvement of the V1bR in context of antidepressant treatment on anxiolytic actions of the drug. In detail, V1bR knockout mice chronically infused intraperitoneally with a selective serotonine-noradrenaline-reuptake inhibitor spent more time on the open arm of the EPM compared to V1bR knockout mice infused with VEH (Ishizuka et al., 2010). Notably, these studies have been conducted in males. Nevertheless, anxiety-related behaviour seems to be regulated by a variety of synergistic neuropeptid systems. Since a decrease in anxiety-related behaviour on the EPM is only visible in V1bR knockout-, but not wildtype mice, treated with a SNRI, blockade of V1bR alone, seems not efficient enough to create a visible behavioural effect. In addition, as non-peptidergic and peptidergic systems are adapted in lactation, a potential effect of V1bR blockade might be overruled by effects of other systems, e.g. decreased noradrenergic input from the PVN during early lactation (for reviews see Lonstein, 2007; Slattery and Neumann, 2008).

Thus, future studies investigating the role of V1bR more specifically in anxiety-related but also in depressive-like behaviour in lactating females could further help to clarify the subsequent roles of the V1R subtypes. Taken together, I provide evidence that the regulation of anxiety-related behaviour on the level of the hypothalamus, exactly within the PVN, in lactating rats seems to be mediated rather by V1aR than V1bR. Importantly, synergistic effects of the AVP system with other neuropeptide systems are likely and can affect the behavioural outcome.

7.6 Correlation of the AVP, GnRH and kisspeptin system in the view of maternal behaviour

On the basis of the co-expression of AVP and GnRH neurons within the OVLT of lactating rats, I further investigated the role of GnRH in maternal behaviour. Due to an initial study in mice with a 30% reduction of GnRH neurons, leading to impaired maternal behaviour (Brooks et al., 2012), I assessed the GnRH system in a more natural animal model. Indeed, antagonism of GnRH decreased maternal aggression in the maternal defence test. In contrast to the study by Brooks et al., I did not find changes in maternal care or maternal motivation to retrieve pups. As the study was conducted in genetically modified mice whereas I used Wistar rats, the differences of the target organisms may explain the different outcomes. However, blockade of GnRH receptors leads to decreased activity of GnRH neurons as GnRH binding is prohibited. This is similar to a decrease in GnRH neurons, which leads to a decrease in GnRH release and following decreased receptor stimulation (Brooks et al., 2012). Thus, the different effects of decreased GnRH signalling on maternal behaviour might also be due to the central/systemic approaches, reflecting a downstream effect of stopping the activity of the GnRH neurons on projection sites. In addition, the behavioural outcome could also hint to a local effect, e.g. of the MPOA; the MPOA is known to be critically involved in maternal behaviour and represents the predominant area of GnRH neuron expression (Brooks et al., 2012; for review see Numan and Insel, 2003). Future studies investigating the role of GnRH within the MPOA might provide further insight in the complex regulation of different aspects of maternal behaviour. In addition, GnRH neurons also have been found to express dopamine, serotonin and OXT receptors (Li and Pelletier, 1995; for review see Numan and Stolzenberg, 2009), which are known to mediate maternal behaviour. This leads to the suggestion, that GnRH within the MPOA acts as a neuromodulator on maternal circuits (Brooks et al., 2012).

Regarding the comprised amount of GnRH neurons in the study of Brooks et al. (Brooks et al., 2012), another reason for the behavioural outcome could be the decreased stimulation of GPR54, known to be co-expressed on GnRH neurons (Irwig et al., 2004). This in turn would lead to altered activation of kisspeptin neurons (Han et al., 2005; Liu et al., 2008). So far, the involvement of the kisspeptin system in maternal behaviour was not investigated. In my initial approach I found an influence of kisspeptin receptor blockade on maternal care. In detail, central kisspeptin receptor antagonism decreased ABN and nursing under non-stress

and stress conditions. Due to unchanged maternal care after GnRH blockade (see above), the involvement of other neuropeptide systems facilitating maternal care concomitant with kisspeptin is likely. Indeed, centrally administered kisspeptin or kisspeptin agonist has been shown to trigger the firing of OXT neurons within the SON of lactating rats (Scott and Brown, 2013) and to increase AVP plasma concentrations (Ten et al., 2010), respectively. As both neuropeptides are critically involved in the regulation of maternal care (for review see Bosch and Neumann, 2012), a cross-link between the kisspeptin and OXT or/and AVP system is hypothesised. Future studies are necessary to address the relationship of kisspeptin with these systems and their influence in maternal behaviour. Importantly, the obtained effects after kisspeptin receptor antagonism seem to be dose-dependent; whereas the low dose was effective under non-stress conditions (referring to the mild stress of infusion), the medium dose showed effects under stress-conditions (referring to the strong psychosocial stressor of maternal defence testing). Consequently, kisspeptin receptor antagonist exerts its effect in a dose-dependent way in response to the stress type in the experiments. It has been shown before that different doses of a specific drug affect different behavioural parameters, e.g. grooming and crouching in male prairie voles appears dependent on different intra-LS AVP doses (Wang et al., 1994).

Taken together I could provide important insights into the involvement of the GnRH and kisspeptin system in the regulation of maternal behaviour. Future studies are warranted to investigate in brain regions involved in the regulation of different maternal aspects by both neuropeptide systems.

7.7 Conclusions, translational aspects and outlook

In my thesis I could reveal for the first time a role of the V1bR in the regulation of maternal behaviour in lactating Wistar rats. V1bR mediate specific maternal aspects depending on the brain-region. Thereby, the V1bR subtype exerts its action alone, in a concomitant or an opponent manner to V1aR in the modulation of maternal behaviour, also depending on the brain region.

Whereas V1bR have a minor/unknown role within the PVN, they interfere with appetitive maternal behaviour within the MPOA. Due to the fact that the MPOA is a rely station of the behavioural responses to pup stimuli and exerts a variety of efferent projections to other brain regions, the behavioural outcome may include downstream effects as well. Within the mpBNST, V1bR modulate pup-directed behaviour during stressful conditions, i.e. the maternal defence test. This indicates a role of V1bR within the mpBNST in modulating social interactions. With the pups being the social counterpart for interaction, the activation of intra-mpBNST V1bR seem to be important to provide adequate maternal care also in stressful situations. Within the mpBNST, V1bR seem to complement V1aR, as blockade of each receptor subtype impairs defence and protection of the offspring. Regarding the PVN, V1aR seem to dominate over V1bR. Since the AVP system is also known to be implicated in maternal aggression within the PVN, future studies investigating the effects of V1R antagonism after maternal defence testing are warranted. Results seem promising, as studies in males prove evidence of a stress induced increase of AVP release within the PVN in response to social defeat (Wotjak et al., 1996).

Another aspect which is worth to be investigated in future studies is the effect of chronic V1bR manipulation in maternal behaviour. Studies in virgin male and female V1bR knockout mice as well as AVP-deficient Brattleboro rats showed a stressor dependent involvement of the V1bR in normal ACTH and CORT responses (Lolait et al., 2007; Zelena et al., 2004). These findings suggest a different involvement of V1bR in behavioural outcomes dependent on acute or chronic manipulation of the receptor.

Importantly, all these behavioural adaptations within the distinct brain regions (MPOA, BNST and PVN) occur although mRNA and protein expression of V1bR are not altered in lactation compared to virgins, proposing alterations in receptor density or binding as well as in signalling pathways. Additionally, lactation induced changes in the recycling of V1bR from

the cytoplasm back to the membrane are likely. To test this hypothesis, highly specific antibodies are needed, to guarantee reliable results. Future studies approaching the binding or the recycling pathway of V1bR within lactating rats could further elucidate its role in the different aspects of maternal behaviour. In contrast, V1aR binding seems to adapt in lactation, showing increased levels compared to virgins (Bosch and Neumann, 2008; Bosch et al., 2010). This leads to the conclusion that both V1R subtypes are adapted in lactation to contribute to the modulation of maternal behaviour in lactation, but on the basis of different regulatory mechanisms. Future studies on adaptations of receptor density in the cytoplasm, receptor recycling or signalling transduction may outline potential adaptions, especially of V1bR.

Due to the expression pattern of V1bR throughout the brain, other brain regions implicated in distinct maternal aspects like the CeA, the PAG and the LS could further clarify the role of V1bR within the mediation of maternal behaviour. During maternal aggression increased AVP release in CeA was measured in HAB, but not in LAB, dams (Bosch and Neumann, 2010). In this line, V1aR antagonism reduces aggression in resident HAB dams, whereas synthetic AVP increases the attack and threat behaviour in low aggressive LAB dams (Bosch and Neumann, 2010; for review see Bosch, 2013). According to this knowledge of the involvement of AVP and its V1aR in the animal model of extremes in anxiety, further investigations of the V1bR in NAB rats would shed light on the link between receptor subtypes and the behavioural outcome.

A further brain region expressing V1bR is the SCN, which is known to be responsible for circadian rhythm of diverse hormonal fluctuation, like AVP, melatonin, and growth hormone (for reviews see Challet, 2015; Kalsbeek et al., 2010; Kim et al., 2015; Moore, 2013), as well as of motivated behaviours like maternal behaviour, eating, and exercise (Antle and Silver, 2015; for review see Johnston, 2014). Further, AVP neurons from the SCN project to the MPOA but also to the BNST and PVN (Kalsbeek and Buijs, 2002), known to mediate maternal care. Interestingly, also maternal care shows a rhythmic pattern over 24 hours (Antle and Silver, 2015), which might be due to different release pattern of AVP within the SCN and projections to the maternal brain regions. In order to approach this hypothesis activation and inactivation of the SCN with subsequent observation of maternal care could help to understand this correlation.

So far, these findings help to increase our understanding of the complex changes and systems that are involved in the maintenance and control of appropriate maternal care. Initial studies in humans showed a link between a polymorphism in the V1aR gene, specifically on the RS3 allel, and decreased maternal structuring and supportive behaviour during a free-play session with their children (Avinun et al., 2012). In more detail, mothers with two copies of the RS3 "risk" allel were less sensitive in perceiving and responding to the infants' cues promptly and appropriatly than mothers with one or zero copies of the allele (Bisceglia et al., 2012). Thus, genetic identification could identify mothers which are carrier of a "risk" allele. Depending on the individual personality and the severity of insensitivity of the mother towards her child, child abuse or maternal neglect might be prevented in time. Future studies on other potential alleles, maybe on the V1bR gene, may help to understand genetic modifications which lead to pathological phenotypes in maternal behaviour.

In addition to the AVP system, also the GnRH as well as the kisspeptin systems are involved in the modulation of maternal behaviour in the postpartum period. Both systems have mainly been attributed to reproduction, except one single study; genetically modified mice with a partial reduction in GnRH neurons have been found to show impairments in pup retrieval. Thus, I could provide the first insight in two further hormonal systems implicated in the regulation of maternal behaviour of lactating Wistar rats.

Due to the fact that the co-expression of GnRH and AVP-ir was found within the OVLT, further investigation of this specific brain region in the modulation of maternal behaviour by administration of selective GnRH or/and AVP (receptor) antagonists could be a future step.

In humans, GnRH is already used to treat reproductive diseases, e.g. hypogonadotropic hypogonadism (Maggi et al., 2015). Similar to the closely linked kisspeptin system, the GnRH system has not been related to maternal behaviour in humans to date. Nevertheless, studies on plasma kisspeptin in pregnant women provide a link between kisspeptin mRNA expression and labour. Kisspeptin mRNA is decreased in women with in term vaginal delivery compared to women receiving a cesarean section. In addition, the expression is dramatically increased when delivery occurs preterm (Torricelli et al., 2008). Furthermore, studies during pregnancy indicated a link between low kisspeptin-levels and preeclampsia, pregestational insulin-dependent diabetes mellitus (type 1) and gestational diabetes (Cetković et al., 2012). In addition decreased levels of kisspeptin indicate placental dysfunction, often followed by adverse perinatal outcome. Future studies investigating plasma kisspeptin levels during

mother-child interaction could help to understand the wide range of physiological actions of the kisspeptin system. Nevertheless, these studies in pregnant women and my studies in lactating rats show evidence for the importance of the GnRH- and kisspeptin system not only in reproduction and pregnancy, but also in lactation. Therefore both systems may provide potential targets in treatment of reproductive, as well as maternal behaviour deficits.

Thus, in my thesis I showed a crucial involvement of V1bR in the regulation of maternal behaviour. Thereby, V1R subtypes act in a brain region dependent manner, including hypothalamic and limbic areas, on distinct aspects of the maternal repertoire. This provides evidence for a central effect of V1bR in the complex regulation of maternal behaviour.
In addition, I showed a GnRH-AVP co-expression within the OVLT of lactating rats and an influence of the GnRH- and kisspeptin system on the regulation of maternal aggression and maternal care, respectively. This suggests an interaction between the neuropeptide systems, which has to be confirmed in future studies.

Abbreviations

ABN	arched back nursing
ACTH	adrenocorticotropic hormone
amPVN	anterior magnocellular part of the paraventricular nucleus of the hypothalamus
ANOVA	analysis of variance
ARC	arcuate nucleus
AVP	arginine-vasopressin
BNST	bed nucleus of the stria terminalis
CeA	central amygdala
CREB	cyclic adenosine monophosphate response element binding protein
CRF	corticotropin-releasing factor
eGFP	enhanced green fluorescent protein
EPM	elevated plus-maze
FSH	follicle stimulating hormone
GABA	γ-amino butyric acid
GnRH	gonadotropin-releasing hormone
GPCR	G-protein coupled receptor
GPR54	kisspeptin receptor
HAB	high anxiety-related behaviour
HPA axis	hypothalamo-pituitary-adrenal axis
HPG axis	hypothalamo-pituitary-gonadal axis
icv	intracerebroventricular
IP3	inositol triphosphate
ir	immunoreactivity
LAB	low anxiety-related behaviour
LD	lactation day
LH	luteineizing hormone
LS	lateral septum
mpBNST	medial-posterior bed nucleus of the stria terminalis
MPOA	medial preoptic area
mRNA	messenger ribonucleic acid

NAB	non-selected for anxiety-related behaviour
NAc	nucleus accumbens
OVLT	organum vasculosum of the lamina terminalis
OXT	oxytocin
PD	pregnancy day
pPVN	parvocellular part of the paraventricular nucleus
PRL	prolactin
PRT	pup retrieval test
PVN	paraventricular nucleus of the hypothalamus
qPCR	quantitative real-time polymerase chain reaction
SCN	suprachiasmatic nucleus
SON	supraoptic nucleus
V1aR	V1a receptor
V1bR	V1b receptor
vBNST	ventral bed nucleus of the stria terminalis
VEH	Vehicle

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Supplementary

Chapter 3

Comparison of breeding EPM to experimental EPM of HAB and LAB rats of generation #51

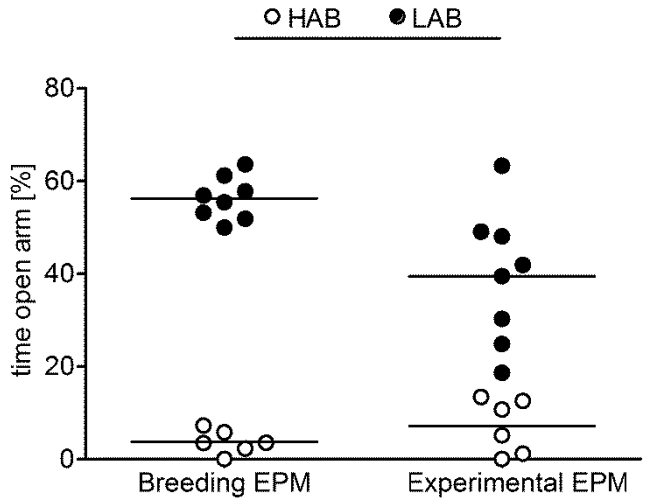

Figure S1 Anxiety-related behaviour of HAB and LAB rats of generation #51 as virgins on the breeding EPM and as lactating dams on the experimental EPM

Time spent on the open arms of HAB and LAB dams under two different reproductive conditions.

Chapter 4

Supplementary Information: Verification of V1bR antibody specificity in Western Blot analysis

Material & Methods

To verify the specificity of the antibody against the rat V1bR (1:5000, Alpha Diagnostic, San Antonio, USA), we tested maternal (cortex) and embryonic (day (d)12 and d16) brain tissue for V1bR protein expression. AVP receptors are not expressed in neonates before embryonic d16 (Tribollet et al., 1991); hence, we predicted a protein band at 55 KDa in both maternal brain tissue and embryonic d16, but not in d12, tissue.

At day 12 or 16 of pregnancy, dams were deeply anesthesized, decapitated, and the embryos were removed. Tissue was taken from the head of the embryos and proteins were isolated according to the protocol for adult brain regions (for details see main manuscript). Western Blot analysis was performed as described in the main manuscript. Loading was controlled by staining for ß-tubulin (1:1000).

Results

Our results show a clear negative result for d12 fetal tissue but a positive V1bR protein band in d16 embryonic and maternal tissue (Figure S2) thereby proofing the specificity of the V1bR antibody.

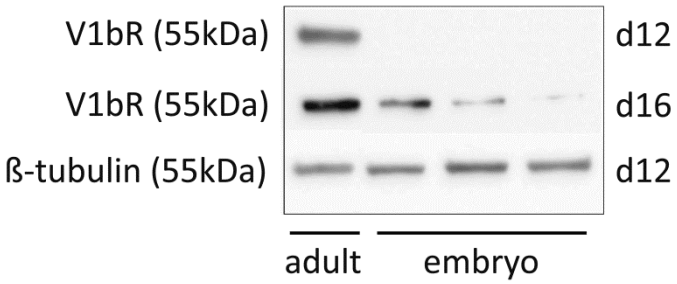

Figure S2 V1bR antibody specificity in protein analysis

Representative Western Blot for V1bR protein of adult (maternal brain cortex, lactation day 4; column 1) and embryonic (days d12 and d16; columns 2 - 4) brain tissue to verify the specificity of the antibody for rat V1bR. ß-tubulin from adult and embryonic (d12) tissue was used as loading control.

Supplementary Information: Verification of a modified setup in the PRT

Material & Methods

Animals. Female HAB and LAB rats (12 - 14 weeks, 220 - 250 g) were used (Landgraf and Wigger, 2002; Liebsch et al., 1998; Mathys et al., 2004; Neumann et al., 2005a; Neumann et al., 2005b; for review see Bosch, 2011). Rats were mated with sexually experienced stud Wistar males and pregnancy was confirmed the next day by the presence of sperm in vaginal smears. Pregnant females were kept in groups of 3 to 4 rats before they were single-housed in plexiglass observation cages (38 x 22 x 35 cm^3) on PD 18 to ensure undisturbed parturition. On the day of birth, offspring were culled to eight pups of mixed sexes and half of the bedding was replaced by new bedding.

Rats were kept under standard laboratory conditions (12 h / 12 h light-dark cycle, with lights on at 07:00 h; 22 ± 1 °C; 55 ± 5 % relative humidity; free access to water and standard rat chow).

All experiments were performed in accordance with the European Communities Council Directive (86/609/EEC) and were approved by the local government of the Oberpfalz, Bavaria, Germany.

Maternal motivation in the PRT. On LD 3, HAB and LAB dams were tested on their motivation to retrieve their pups in a novel environment. Lactating dams were moved to the test room 60 min prior to the test, pups were removed from the home cage and kept on a thermo pad (32 °C) in another room to avoid auditory recognition. To characterize retrieval behavior under different challenging conditions, two different PRT setups were used (see Figure 21, Chapter 4). Females of both breeding lines were tested either in the conventional setup of our laboratory (‘no house’ condition; Figure 21A; Bayerl et al., 2014; Bosch and Neumann, 2008; Bosch et al., 2010; Neumann et al., 2005a), or in the modified setup (‘house’ condition; Figure 21B).

➔ For details on the PRT setups please see Material and Methods of Chapter 4.

Statistical analysis. Maternal motivation was analyzed using three-way ANOVA for repeated measures (factors: time x condition x breeding line). The number of retrieved pups as well as the latency to retrieve the first pup was analyzed using two-way ANOVA (factors: condition x breeding line). The ANOVA was followed by Sidak *post-hoc* correction if main effects were

found. A statistical significant difference was considered at $p \leq 0.05$ and effect size estimations were indicated by eta squared values. Data are presented as mean + SEM. Statistical analysis was performed using the software SPSS, Version 20 (IBM, Ehningen, Germany).

Results

Pup retrieval in HAB and LAB dams significantly differed over the 15-min test (three-way ANOVA for repeated measures; $F_{15,510} = 124.50$, $p < 0.001$, $\eta^2 = 0.32$; Figure S3), between the breeding lines ($F_{1,34} = 31.55$, $p < 0.001$, $\eta^2 = 0.16$) as well as between the PRT setups ($F_{1,34} = 24.60$, $p < 0.001$, $\eta^2 = 0.13$). A significant time x breeding line x setup interaction was found ($F_{15,510} = 3.93$, $p < 0.001$, $\eta^2 = 0.10$). The *post-hoc* test revealed that in the conventional PRT HAB dams retrieved their pups faster than LAB dams from t1 on ($p < 0.001$ in each case). In the modified PRT, HAB and LAB dams only differed in the first 2 min (t1: $p = 0.006$, t2: $p = 0.009$). Moreover, when comparing only the LAB groups, it becomes evident that LAB dams retrieved their pups faster in the modified compared to the conventional PRT from t3 on ($p \leq 0.001$ in each case).

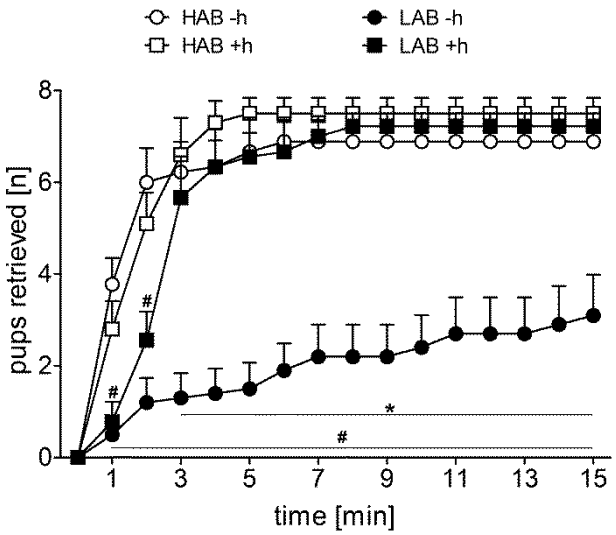

Figure S3 Maternal motivation of lactating rats bred for high (HAB) and low (LAB) anxiety-related behaviour in two different pup retrieval test (PRT) setups.

Maternal motivation was tested in HAB and LAB dams either in the conventional ('no house' condition; -h) or the modified ('house' condition; +h) PRT. The time until retrieval of each pup was monitored for 15 min. Data are presented as means + SEM (n = 9 - 10 per group). * $p \leq 0.05$ versus same breeding line with house; # $p \leq 0.05$ versus equivalent in HAB group.

Consequently, the total number of retrieved pups at t15 differed significantly between HAB and LAB (two-way ANOVA; $F_{1,37} = 13.34$, $p = 0.001$, $\eta^2 = 0.18$), depending on the PRT setup ($F_{1,37} = 18.07$, $p < 0.001$, $\eta^2 = 0.24$); additionally a breeding line x setup interaction was found ($F_{1,37} = 9.94$, $p = 0.003$, $\eta^2 = 0.13$). The *post-hoc* test revealed that LAB dams retrieved the least number of pups in the conventional PRT compared to all other groups ($p < 0.001$ in each case) and, importantly, LAB dams in the modified PRT did not differ from HAB dams in any PRT setup making these LABs indistinguishable from HABs.

Further, the latency to retrieve the first pup differed significantly between HAB and LAB mothers (two-way ANOVA; $F_{1,37} = 9.89$, $p = 0.003$, $\eta^2 = 0.19$), depending by trend on the PRT setup ($F_{1,37} = 3.92$, $p = 0.056$, $\eta^2 = 0.07$); additionally a breeding line x setup interaction was found ($F_{1,37} = 5.59$, $p = 0.024$, $\eta^2 = 0.11$). The *post-hoc* test revealed that in LAB dams the latency to retrieve the first pup is higher in the conventional PRT compared to the modified PRT ($p = 0.004$) and that in the conventional PRT LAB dams show a higher latency to retrieve their first pup compared to HAB dams ($p < 0.001$).

➔ For discussion of the results please see Discussion Chapter 4.

Chapter 6

Table S4 Maternal care under non-stress conditions before and after central GnRH antagonist infusion on LD 1

		Occurrence [n]				
Behaviour	Treatment	basal	t +30	t +60	t +90	t +120
ABN	VEH	3.4 ± 0.9	3.4 ± 1.1	3.6 ± 0.8	2.0 ± 1.0	2.8 ± 0.6
	GnRH ant	4.0 ± 1.1	3.7 ± 1.8	3.2 ± 1.0	4.3 ± 1.3	2.2 ± 0.9
Blanket	VEH	9.9 ± 0.9	8.0 ± 1.3	10.0 ± 0.8	12.1 ± 1.2	11.9 ± 0.7
	GnRH ant	8.2 ± 0.7	9.0 ± 1.9	10.7 ± 0.9	9.0 ± 1.3	10.3 ± 0.9
Nursing	VEH	13.3 ± 0.5	11.4 ± 1.1	13.8 ± 0.5	14.1 ± 0.4	14.6 ± 0.2
	GnRH ant	12.2 ± 0.7	12.7 ± 0.8	13.8 ± 0.7	13.3 ± 1.1	12.5 ± 1.4
Mother-pup	VEH	14.3 ± 0.6	12.0 ± 1.1	14.6 ± 0.2	14.5 ± 0.3	15.0 ±0.0
interaction	GnRH ant	13.8 ± 0.6	13.5 ± 0.8	15.0 ± 0.0	14.5 ± 0.3	13.5 ± 1.1
Off pups	VEH	0.8 ± 0.6	3.0 ± 1.1	0.4 ± 0.2	0.5 ± 0.3	0.0 ± 0.0
	GnRH ant	1.2 ± 0.6	1.5 ± 0.8	0.0 ± 0.0	0.5 ± 0.3	1.5 ± 1.1

Abbreviations: VEH, vehicle; ant, antagonist; ABN, arched back nursing.

Data are presented as mean ± SEM (n = 6 - 8 per group).

Table S5 Aggressive and non-aggressive behaviours during the maternal defence test after central GnRH antagonist infusion on LD 4

	VEH	GnRH antagonist
aggressive behaviour		
number of attacks	14.4 ± 3.5	3.7 ± 1.1 *****
attack duration	9.5 ± 2.4 s	2.5 ± 0.7 s *****
attack latency	76.3 ± 21.8 s	132.3 ± 46.7 s
keep down	35.6 ± 16.3 s	16.2 ± 9.0 s
lateral threat	39.5 ± 13.5 s	20.4 ± 11.0 s
offensive upright	26.1 ± 7.9 s	3.2 ± 1.4 s *****
aggressive grooming	19.4 ± 2.9 s	21.9 ± 9.7 s
other aggressive	268.4 ± 29.1 s	231.6 ± 24.8 s
sum aggressive	398.6 ± 39.1 s	295.8 ± 32.7 s
non-aggressive behaviour		
pup-directed behaviour	32.1 ± 22.6 s	39.8 ± 23.2 s
sniffing	46.1 ± 5.8 s	70.5 ± 16.1 s
exploration	72.0 ± 10.2 s	79.6 ± 17.1 s
self-grooming	36.0 ± 11.0 s	99.8 ± 27.9 s
resting	5.9 ± 3.0 s	11.6 ± 3.6 s
other	8.0 ± 6.9 s	2.0 ± 2.0 s
sum non-aggressive	200.2 ± 38.7 s	303.2 ± 32.6 s

Abbreviations: VEH, vehicle.

Data are presented as mean ± SEM (n = 6 - 8 per group). * $p \leq 0.05$ versus VEH.

Curriculum vitae

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Publications

Bayerl DS, Kaczmarek V, Jurek B, van den Burg EH, Neumann ID, Gaßner BM, Klampfl SM, Bosch OJ (2016) Antagonism of V1b receptors promotes maternal motivation in the MPOA and impairs pup-directed behaviour during maternal defence in the mpBNST of lactating rats. Hormones & Behavior 79:18-27. doi:10.1016/j.yhbeh.2015.12.003

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