Underlying neurobiological mechanisms of high and abnormal aggression in male rats: link to trait anxiety

DISSERTATION

ZUR ERLANGUNG DES DOKTORGRADES
DER NATURWISSENSCHAFTEN (DR. RER. NAT.)
DER NATURWISSENSCHAFTLICHEN FAKULTÄT III
BIOLOGIE UND VORKLINISCHE MEDIZIN
DER UNIVERSITÄT REGENSBURG

vorgelegt von

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Februar 2009

Promotionsgesuch eingereicht am: 09.02.2009

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Dissertation

durchgeführt am Institut für Zoologie der Universität Regensburg

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1. Introduction

The expression of emotions like aggression and anxiety is important for social communication between individuals. Intraspecific signals transmitting the emotional state of an individual are very prominent in humans and animals living in herds, cohorts or any kind of social community.

Intermale aggression is necessary for acquisition and maintenance of nutrition, territory or mating partners. Both the initiator and the recipient of aggressive signals have to obey species-specific rules to guarantee that the communication is effective. Mistakes in the communication involving aggression between conspecifics can lead to serious injuries or even death of one or more individuals. Aggression includes many different behaviours ranging from subtle threatening to severe attacks. Threats start with signals that do not need direct social contact, such as intimidating the opponent by appearing as large as possible. Male rats therefore usually show piloerection as a first sign of aggressive behaviour. Afterwards, they switch over to threats with direct body contact followed by lateral threat, which represents a very active form of threat. During this phase of aggression, the opponent has the possibility to escape or to show submissive behaviour to terminate aggression or, alternatively, attacks will follow the threats.

Exaggerated aggression disregarding species-specific rules is rarely seen in animal communities. In humans, abnormal forms of aggression can occur as symptom of psychological diseases, and every year, numerous people worldwide die because of an aggressive assault and many more become hurt physically or psychologically. This causes immense costs and is a great burden for the human society.

There are several types of drugs – most of them originally not developed for treatment of excessive aggression – to reduce aggression, but the efficacy is still low. Moreover, these drugs can elicit diverse side-effects with general sedation being the most prominent one. Finally, some drugs, like for example benzodiazepines, are even addictive. Thus, a better

understanding of the neurobiological mechanisms underlying aggression, and especially its abnormal forms, is essential for the development of novel drugs with better efficacy, less side-effects and a lower probability to be addictive. Therefore, appropriate animal models need to be established in order to study the neuronal mechanisms of normal and abnormal forms of aggression.

In the present thesis, high and abnormal aggressive behaviours have been investigated in great detail in rats (*Rattus norvegicus*), in particular in a rat model for low and high trait anxiety, and its adequacy as an animal model for aggression has been tested. First, an overview of the current state of research on aggression and the underlying neurobiological mechanisms is given. Furthermore, the two rat lines selectively bred for low or high anxiety-related behaviour are introduced and their behavioural and neurobiological characteristics are described.

2. Aggression

2.1 Aggression in humans

Every year, more than 700,000 people worldwide die because of assault (Bartolomeos *et al.*, 2007). Additionally, many more become victims of aggressive behaviour and get physically or psychologically injured. Besides the suffering of the affected persons and their families, a large financial burden for the society emerges. Forms of excessive or violent aggression can occur as a symptom of different types of psychopathologies, such as antisocial personality disorder (Woodworth & Porter, 2002), depression (Fava, 1998), Alzheimer's disease (Paveza *et al.*, 1992), schizophrenia (Eronen *et al.*, 1998), and post traumatic stress disorder (Beckham & Moore, 2000). Excessive aggression can also be caused by brain lesions, like for example of frontal or temporal cortical areas (Hawkins & Trobst, 2000).

Generally, there are two types of excessive and violent aggression in humans. The so-called hypo-arousal-driven type of aggression is accompanied by low affective reactions, low autonomic responses, low skin conductance and low cortisol levels (Virkkunen, 1985; Raine, 1996; Brennan *et al.*, 1997; Dolan *et al.*, 2001; Haller & Kruk, 2006). Hypo-arousal-driven aggression is often associated with instrumental aggression and seen, for example, in conduct and antisocial personality disorders. In contrast, the hyper-arousal-driven aggression is characterized by high affective reactions, high autonomic and high cortisol responses (Mazur, 1994; Cohen *et al.*, 1996). Hyper-arousal-driven aggression leads to uncontrollable outbursts and is rather associated with mood and intermittent explosive disorders (Haller & Kruk, 2006).

Drugs acting on major neurotransmitter systems in the brain, such as the dopamine, the γ-aminobutyric acid (GABA) or the serotonin system are used for the pharmacotherapy of violent persons. However, they have diverse side-effects, among them sedation, headaches, motor coordination impairment, sleep disturbance, cardiac arrhythmia, blood pressure changes and impotence - and are only effective when aggression is linked to specific psychological diseases (Brady *et al.*, 1998; Rodriguez-Arias *et al.*, 1998; Netter, 2001; Swann, 2003). For instance, benzodiazepines, a class of drugs primarily acting on the GABAergic system, reduce aggression (and lead to sedation) in some patients, but may induce aggression in others (DiMascio, 1973; Jonas *et al.*, 1992; Bond *et al.*, 1995; Cherek & Lane, 2001; de Almeida *et al.*, 2005). Moreover, chronic treatment with benzodiazepines is not advisable due to their addictive potential. Taken together, there is a need to improve pharmacotherapy for patients suffering from aggressive disorders.

2.2 Aggression in animals

Aggression belongs to the natural ethogram of most mammals. It is principally an adaptive behaviour and important for the survival and reproductive success of the individual. One has

to distinguish between different types of aggression, including offensive, defensive, maternal and predatory aggression.

Offensive aggression is displayed in fights for acquisition or maintenance of nutrition, territory or mating partners. In general, offensive aggression underlies strict species-specific rules resulting in a lower number of animals killed during offensive fighting. Threat behaviour is an important and distinct part of aggression and allows the inferior animal to submit or to escape even without the need for an attack by the superior conspecific. Thus, social hierarchies are established in order to reduce fighting within a group of socially living animals. Certainly, some fights will still occur to maintain or upgrade the position within the hierarchy, but the total number of fights is reduced to an essential minimum.

In rats, attacks displayed in the course of offensive aggression will mainly be directed towards less vulnerable body parts of the opponent that are covered with muscles and a thick layer of skin in contrast to defensive attacks that are directed towards vulnerable body parts like head, throat or belly (Blanchard & Blanchard, 1977; Blanchard *et al.*, 2003). Display of attacks towards a vulnerable target region of the opponent in a non life-threatening situation is considered to be abnormal (Haller *et al.*, 2001).

Defensive aggression is displayed in a life-threatening situation, for example when an animal has to defend itself against a predator or a conspecific (Blanchard & Blanchard, 1981). Defensive attack targets in rats are the head or other vulnerable body parts of the attacking animal with the intention to injure the opponent (Blanchard & Blanchard, 1977; Blanchard *et al.*, 2003). Defensive aggression seems to be related to an acute fear response, and attacks towards vulnerable body parts of the opponent are the last chance for self-defence of the individual. Therefore, these kinds of attacks are rather unpredictable.

To study the regulation of aggression, and especially its abnormal forms, appropriate animal models are needed. These animal models may include the selection for high levels of aggression. For example, male house mice (*Mus musculus domesticus*) caught from the wild

have been selectively bred for short (SAL) or long (LAL) attack latency towards an unfamiliar male intruder mouse (van Oortmerssen & Bakker, 1981). The high aggression in SAL mice is part of their 'proactive' stress coping strategy, which is further reflected by more active behaviours in, for example, the forced swim test and the shock-probe burying test. In contrast, the low level of aggression seen in LAL mice is part of their 'reactive' stress coping strategy further involving higher levels of immobility and freezing behaviours (For review see: Koolhaas *et al.*, 1999). SAL mice not only show high levels of aggression, but also abnormal forms of aggressive behaviour, as demonstrated by a high percentage (> 25 %) of attacks towards vulnerable body parts of a male intruder mouse (Haller & Kruk, 2006), the display of aggression towards a male opponent in a neutral environment or even in the home cage of the opponent, and attacks towards female mice (Sluyter *et al.*, 2002). Thus, SAL mice are proposed to be an animal model for antisocial behaviours (Sluyter *et al.*, 2003). Other mouse lines selected for high- and low-aggressive behaviour are the Turku aggressive and Turku non-aggressive mice (Sandnabba, 1996) as well as the North Carolina 900 and North Carolina 100 mice (Gariepy *et al.*, 1996).

In addition to mice, rats have also been bred for differences in aggression, but in this case towards humans rather than conspecifics. The Novosibirsk rats selected for high or low aggressiveness towards humans (Naumenko *et al.*, 1989; Plyusnina & Oskina, 1997) likely represent a form of high and low defensive aggression. Additionally, an outbred laboratory strain of wild-type rats (*Rattus norvegicus*) consists of individuals showing a wide range of aggression from almost no aggression to a very high level of aggression (de Boer *et al.*, 2003), but these rats have not been selectively bred for differences in aggressive behaviour.

Other animal models of aggression use either frustration (deprivation of an expected reward), instigation (pre-exposure to a possible opponent) or alcohol to induce heightened aggressive behaviour in male rats (Miczek *et al.*, 2002; de Almeida *et al.*, 2005). Finally, rats with a glucocorticoid-deficiency, induced by adrenalectomy combined with an implanted pellet

releasing low levels of corticosterone, show an enhanced level of attacks directed to vulnerable body parts (Haller *et al.*, 2004). Acute treatment with corticosterone suppresses this rise in aberrant attacks (Haller *et al.*, 2001). Taken together, there are several rodent models of aggression, but a rat model providing high and/or abnormal aggression based on the genetic background is missing so far.

2.3 Brain regions involved in aggression

In rodents, olfactory information from the olfactory bulbs directly reaches the medial amygdala. After processing, the medial amygdala transmits the information to the lateral septum, the bed nucleus of the stria terminalis (BNST) and the anterior hypothalamic area. These brain regions project to the periaqueductal grey, which is one of the major brain regions that may initiate species-specific aggressive behaviour (Fig. 1) (Nelson & Trainor, 2007).

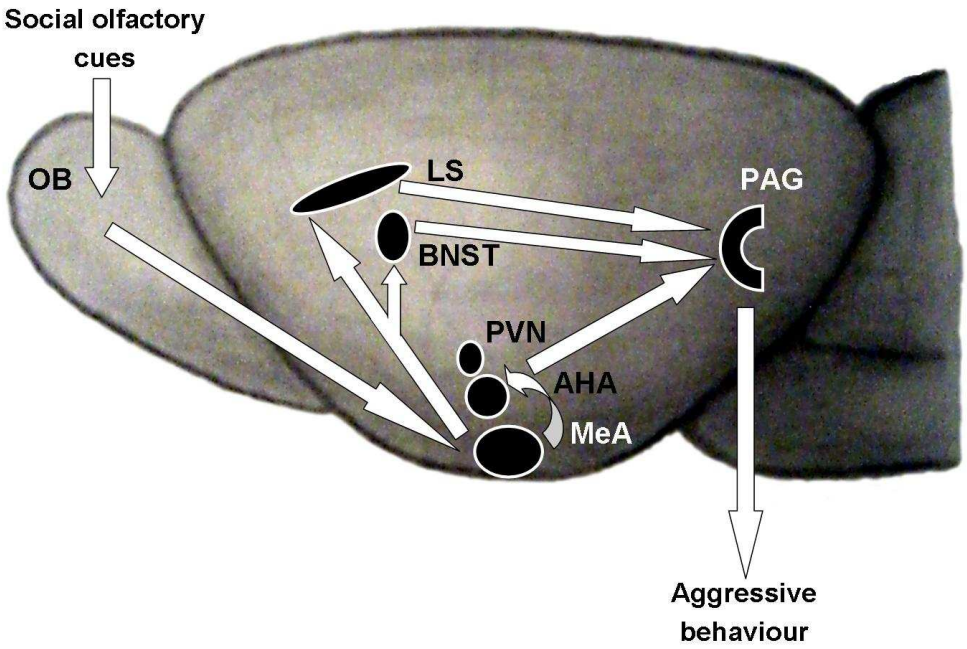

Fig. 1: Neuroanatomical circuits of aggression in rodents. Social cues reach the medial amygdala (MeA) via the olfactory bulb (OB). The medial amygdala projects to the anterior hypothalamic area (AHA), the lateral septum (LS) and the bed nucleus of the stria terminalis (BNST). These brain areas project to the periaqueductal grey (PAG) which than can promote aggressive behaviour. A variety of factors, such as fear or stress, could influence these pathways by acting on the AHA via the paraventricular nucleus (PVN). Adapted from Nelson & Trainor (2007).

The two approaches that most frequently have been used to study the involvement of distinct brain regions in the regulation of aggression are: (i) lesioning the respective brain region, and (ii) investigating neuronal activation in response to the display of aggressive behaviour. Lesions of the lateral septum, BNST, anterior hypothalamus or medial amygdala result in a strong reduction of intermale aggression (Kruk, 1991), whereas lesioning the orbitofrontal cortex increases aggression in male rats (de Bruin *et al.*, 1983). Neuronal activation, quantified by the expression of the immediate-early gene *c-fos*, in response to the display of aggression, is found in the lateral septum, BNST, anterior hypothalamus and medial amygdala in hamsters (Kollack-Walker & Newman, 1995; Delville *et al.*, 2000). Experiments in rats showed aggression-induced neuronal activation of the cortex, septum, BNST, hypothalamus, amygdala, dorsal premammillary nucleus and locus coeruleus, and in particular of the hypothalamic attack area, the medial amygdala and the periaqueductal grey (Halasz *et al.*, 2002; Veening *et al.*, 2005). Interestingly, rats showing abnormal aggression due to glucocorticoid-deficiency demonstrate normal activation of aggression-related brain regions, but show additional activation of two brain regions involved in stress coping, namely the paraventricular nucleus of the hypothalamus (PVN) and the central amygdala (Halasz *et al.*, 2002). This implicates that differences in neuronal activation in the aggression-related regions investigated do not lead to the display of glucocorticoid-deficiency-induced abnormal aggression, but changes within the neuronal systems regulating stress coping and/or fear responses might be involved. Another study showed neuronal activation of the prefrontal cortex in response to an aggressive encounter, but not in response to a psychosocial encounter without direct contact of the rats. In detail, both the infralimbic and the medial orbital cortices were activated in response to aggression (Halasz *et al.*, 2006). Furthermore, in SAL mice selected for high-aggressive behaviour, a strong neuronal activation is found in response to an agonistic encounter in the central amygdala and the lateral and ventrolateral periaqueductal grey (Haller *et al.*, 2006).

The above mentioned studies suggest an involvement of the lateral septum, BNST, anterior hypothalamus, periaqueductal grey, PVN, central and medial amygdala, and prefrontal cortex in the regulation of aggressive behaviour with the PVN and central amygdala being involved especially in abnormal forms of aggression.

2.4 Hormones, neuropeptides and neurotransmitters involved in aggression

An important step towards understanding the neurobiological regulation of aggression is to identify the hormones, neurotransmitters and neuromodulators like, for example, neuropeptides that are involved. Research over the last decades has implicated many transmitters in the regulation of aggressive behaviour, including dopamine, GABA, testosterone, arginine vasopressin (AVP) and serotonin. The latter three will be briefly discussed below.

2.4.1 Testosterone

Testosterone, the principal male sex hormone, has been implicated in the regulation of aggression long ago in both animals and humans (Dijkstra *et al.*, 1992; Banks & Dabbs, 1996; Lucion *et al.*, 1996; Wingfield *et al.*, 2001). Nevertheless, the relationship between testosterone and aggression is much more complex than just a general positive correlation (Archer, 2006). Testosterone is a steroid hormone and derives from cholesterol. In mammals, testosterone is mainly synthesized and secreted in the testes, which are controlled by the hypothalamus via the hypothalamic-pituitary-gonadal axis. After secretion, testosterone can exert its effects on different parts of the body by either acting on the androgen receptor or it can be converted to oestradiol and thereafter activate oestrogen receptors. Besides its effect on spermatogenesis, testosterone amongst others also has anabolic effects on muscles and can directly influence neurotransmitter systems in the brain and behaviour. There are several facts pointing towards a direct positive relationship between the amount of testosterone and

aggression in male mammals. Like in many mammals, testosterone levels increase at puberty in rats accompanied by an increase in aggression (Koolhaas *et al.*, 1980). Moreover, males are generally more aggressive and have a much higher testosterone level than females (Hyde, 1984; Knight *et al.*, 1996; Giammanco *et al.*, 2005). Finally, castration reduces aggressive behaviour, an effect which is reversible by testosterone administration (Beeman, 1947; Barfield *et al.*, 1972; Luttge, 1972; Barr *et al.*, 1976). However, at least in some animals (especially birds), aggressive behaviour in the non-breeding season is testosterone-independent and persists after castration (Logan & Carlin, 1991; Wingfield, 1994b; a). In male rats, castration induces a decrease in aggression accompanied by a decrease in the androgen-dependent extrahypothalamic AVP immunoreactivity (De Vries *et al.*, 1992; de Vries & Miller, 1998). Following castration, the number of AVP messenger ribonucleic acid (mRNA) containing cells in the BNST and of AVP fibres projecting to the lateral septum is reduced by about 90 %. Treatment of castrated rats with testosterone reverses these changes (de Vries et al., 1986; Miller et al., 1989), and administration of AVP into the lateral septum or medial amygdala facilitates aggressive behaviour in castrated rats (Koolhaas *et al.*, 1990; Koolhaas *et al.*, 1991). These results implicate that testosterone, at least partly, influences intermale aggression via changes in the extrahypothalamic AVP system.

2.4.2 Arginine vasopressin

AVP is a nonapeptide mainly synthesized in the PVN and in the supraoptic nucleus. After axonal transport to the pituitary, AVP is released into the blood. Its main peripheral effects are vasoconstriction via V1 receptors and water retention via V2 receptors. Among the three types of AVP receptors, namely V1a, V1b and V2 receptors, the V1a receptor is the most prominent one in the central nervous system (Jard, 1983; van Leeuwen *et al.*, 1987; Barberis & Tribollet, 1996). AVP synthesized in the BNST, medial amygdala or suprachiasmatic nucleus can also be locally released in response to certain stimuli in different brain regions,

where it exerts effects on learning and memory, thermoregulation, cardiovascular and circadian functions (de Wied *et al.*, 1993; Engelmann *et al.*, 1996; de Vries & Miller, 1998). Additionally, both clinical and preclinical studies support the view that AVP is an important regulator of aggressive behaviour (Koolhaas *et al.*, 1990; Ferris, 1992; Albers & Bamshad, 1998; Coccaro *et al.*, 1998; Ferris, 2005). In humans, the AVP concentration in the cerebrospinal fluid correlates positively with the level of aggression in personality-disordered subjects (Coccaro *et al.*, 1998). Furthermore, AVP has been shown to influence social communication in men. AVP administered intranasally decreases the perception of friendly faces and increases the perception of threat and anger when neutral faces are presented (Thompson *et al.*, 2004; Thompson *et al.*, 2006). In rodents, AVP is known to be released within distinct brain regions acting as neurotransmitter or neuromodulator (Landgraf & Neumann, 2004). Particularly the AVP pathway originating in the BNST and in the medial amygdala and projecting to the lateral septum (Fig. 2) (De Vries *et al.*, 1992; de Vries & Miller, 1998) has been shown to influence aggressive behaviour.

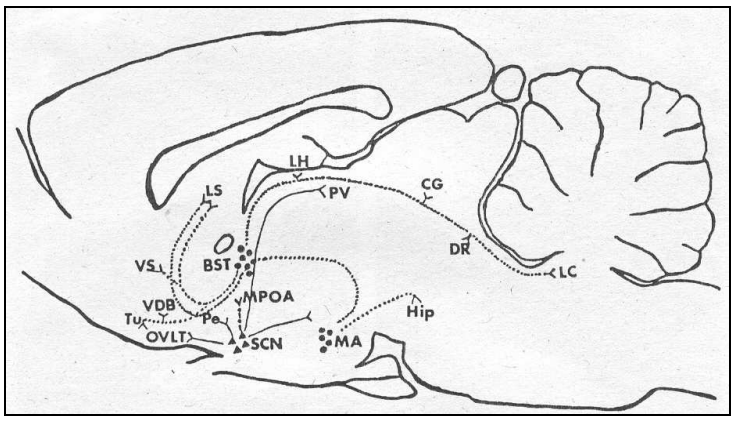

Fig. 2: AVP system in the rat brain (De Vries *et al.*, 1992): The main sites of origin of central AVP are cell bodies (indicated by dots and triangles) in the bed nucleus of the stria terminalis (BST), medial amygdala (MA) and suprachiasmatic nucleus (SCN); AVP projections are found to the midbrain central grey (CG), dorsal raphe nucleus (DR), ventral hippocampus (Hip), locus coeruleus (LC), lateral habenular nucleus (LH), lateral septum (LS), medial preoptic/anterior hypothalamic area (MPOA), organum vasculosum lamina terminalis (OVLT), periventricular nucleus (Pe), dorsomedial nucleus of the thalamus (PV), olfactory tubercle (Tu), perimeter of the diagonal band of Broca (VDB) and ventral septum (VS)

These AVP fibres are sensitive to gonadal hormones (de Vries *et al.*, 1984; De Vries *et al.*, 1985) and more dense in male compared with female rats (De Vries *et al.*, 1981). Injections of AVP into the ventrolateral hypothalamus, anterior hypothalamus, BNST or lateral septum promote aggressive behaviour in hamsters (*Mesocricetus auratus*) (Irvin *et al.*, 1990; Delville *et al.*, 1996a; Ferris *et al.*, 1997). In rats, injection of AVP into the medial amygdala or lateral septum of castrated males leads to an increase in aggression to a level seen in intact males (Koolhaas *et al.*, 1990; Koolhaas *et al.*, 1991). However, the results concerning the involvement of AVP particularly in the septal area are contradictory. More AVP and more AVP receptors were found in the lateral septum of the more aggressive male California mice (*Peromyscus californicus*) compared with the less aggressive White-footed mice (*Peromyscus leucopus*) (Bester-Meredith *et al.*, 1999). In contrast, there are less AVP projections from the BNST to the lateral septum in aggressive SAL mice compared with low-aggressive LAL mice (Compaan *et al.*, 1993). Similar results have been found in aggressive versus low-aggressive wild-type rats (Everts *et al.*, 1997). However, direct measurement of AVP locally released in the lateral septum or other regions of the AVP circuit during the display of aggressive behaviour has not been performed.

2.4.3 Serotonin

Serotonin (5-hydroxytryptamine) is a monoamine neurotransmitter that is synthesized in the central nervous system of mammals from the amino acid L-tryptophan mainly in the dorsal raphe nuclei, which send projections to several brain regions, including prefrontal cortex, hippocampus and nucleus accumbens (Ferrari et al., 2005). Serotonin can influence several behaviours, such as food intake, sensory processing, motor activity and cognition, by acting on one of at least 14 serotonin receptors described until now (Olivier & van Oorschot, 2005). Jørgensen et al. (2003) showed that the serotonin system influences local AVP release in the hypothalamus. Furthermore, earlier studies suggested a direct relationship between serotonin

and AVP in the regulation of intermale aggression in hamsters (Ferris, 1996; Ferris et al., 1997). In general, serotonin is thought to reduce aggression and there are many studies contributing to this serotonin-deficiency theory of aggression by showing that elevated levels of serotonin decrease aggression in different species (Miczek *et al.*, 2002; de Almeida *et al.*, 2005), including humans (Linnoila & Virkkunen, 1992; Coccaro *et al.*, 1994; Kavoussi *et al.*, 1997). For example, lower levels of the serotonin metabolite 5-hydroxyindoleacetic acid have been found in patients with a higher score for their life history of aggressive behaviour (Brown *et al.*, 1979) or with antisocial personality disorder (Linnoila *et al.*, 1983). Preclinical research showed that pharmacological activation of serotonin 1_A or 1_B receptors leads to a reduction in aggressive behaviour (Olivier *et al.*, 1995; Miczek *et al.*, 1998; de Boer *et al.*, 1999; Fish *et al.*, 1999; for review see: Miczek *et al.*, 2002). Recent studies that found a positive correlation between serotonin and the display of aggression challenged the serotonin-deficiency theory (Olivier, 2004; de Boer & Koolhaas, 2005). The current view is that abnormal aggression is associated with a chronic low level of serotonin (Miczek *et al.*, 2002), whereas normal and adaptive forms of aggression are accompanied by an activation of the serotonin system (van der Vegt *et al.*, 2003a; van der Vegt *et al.*, 2003b; Summers *et al.*, 2005a).

2.5 Aggression and the hypothalamic-pituitary-adrenal axis

The hypothalamic-pituitary-adrenal (HPA) axis (Fig. 3) is one of the main systems activated during the stress response to cope with stressors. Perception of a stressful situation causes an activation of parvocellular neurons of the PVN which produce corticotropin releasing hormone (CRH) and AVP (Tramu *et al.*, 1983; Whitnall *et al.*, 1987). After axonal transport, both neuropeptides are stored at the presynapse and released into the hypophyseal portal blood circulation at the median eminence in response to a stressor.

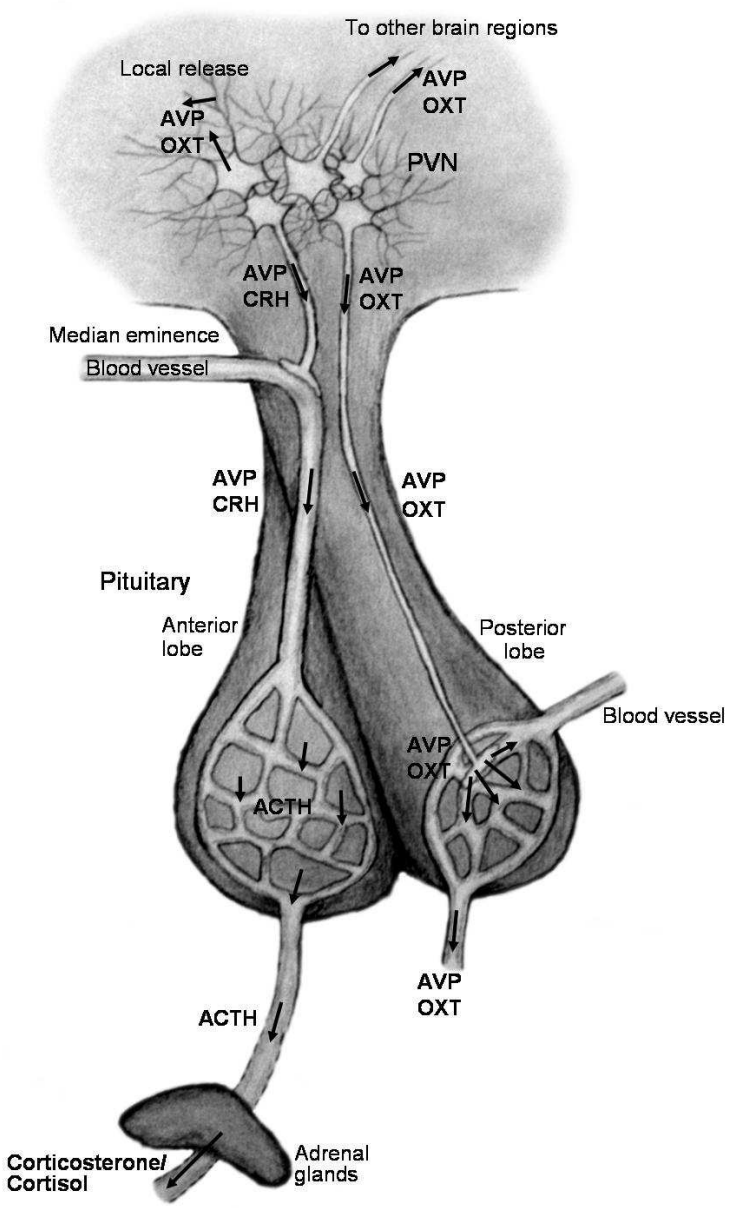

Fig. 3: Schematic drawing of the hypothalamic-pituitary-adrenal (HPA) axis and the arginine vasopressin (AVP)/oxytocin (OXT) system originating from the hypothalamus. Neurons in the hypothalamus (especially in the paraventricular Nucleus [PVN]) synthesize diverse neurotransmitters, amongst them AVP, corticotropin releasing hormone (CRH) and OXT. CRH is released in response to a stressor into the blood at the median eminence. When CRH reaches the corticotropic cells in the anterior lobe of the pituitary, adrenocorticotropic hormone (ACTH) is released into the blood. ACTH stimulates the adrenals to produce and release glucocorticoids (corticosterone in rats, cortisol in humans). AVP released at the median eminence can amplify the effect of CRH on ACTH release in the anterior lobe of the pituitary. When transported to and released at the posterior lobe of the pituitary, AVP and OXT get into the peripheral bloodstream and can thereby elicit peripheral effects. AVP and OXT can also be transported to and released in other brain regions or released locally within the PVN.

The hypophyseal portal circulation directly links the median eminence with the anterior lobe of the pituitary, where CRH and AVP stimulate the production and secretion of adrenocorticotropic hormone (ACTH). Although CRH is thought to be the main stimulator of ACTH release, AVP can amplify this effect of CRH (Gillies *et al.*, 1982; Rivier & Vale, 1983). ACTH stimulates the release of glucocorticoids (corticosterone in rats, cortisol in humans) from the adrenal cortex into the blood. Due to their lipophilic properties, glucocorticoids can cross the blood-brain barrier and also pass cell membranes to reach their intracellular receptors, which exert their effects directly as transcription factors. In the periphery, glucocorticoids are important for providing energy by enhanced catabolism which mobilises lipid and glucose reserves of the body. Additionally, glucocorticoids influence the immune system and cardiovascular responses, and they exert a negative feedback on the pituitary and the hypothalamus to reduce the release of CRH and ACTH. Furthermore, glucocorticoids are implicated in the circadian rhythm.

Besides these versatile effects, HPA axis parameters have also been linked with aggression. An accompanying symptom of several stress-related diseases is excessive aggression (Plotsky *et al.*, 1998; Mello *et al.*, 2003; de Kloet *et al.*, 2005), and preclinical studies confirmed HPA axis activity as an important regulator of aggressive behaviour (Haller et al., 1998; Haller et al., 2001; Summers et al., 2005b). Circulating glucocorticoids can exert opposite effects on aggression, depending on the time point. Chronic low levels are rather thought to reduce aggressive behaviour, whereas glucocorticoids rather promote the acute display of aggression (Summers & Winberg, 2006). Accordingly, acute treatment of rodents with glucocorticoids stimulates aggressive behaviour (Hayden-Hixson & Ferris, 1991; Kruk *et al.*, 2004). Paradoxically, chronic low as well as high levels of glucocorticoids can be associated with excessive aggression (for review see: Haller *et al.*, 2005a). Thus, the role of glucocorticoids in aggression seems to be complex and is far from being clear. Furthermore, ACTH as well as CRH and its receptors have been implicated in the regulation of aggression (Elkabir *et al.*,

1990; Ebner *et al.*, 2005; Gammie & Stevenson, 2006). Generally, the circadian onset of the active period in mammals is accompanied by the highest HPA axis activity which is linked to an increase of aggressive behaviour in rats (Haller *et al.*, 2000a; Haller *et al.*, 2000b).

2.6 Normal versus abnormal forms of aggression

Although aggressive behaviour can be adaptive and is essential for individual and reproductive success, uncontrolled and abnormal forms of aggression are not. Maladaptive exaggerated aggression can be found in humans as well as in animals. Haller & Kruk (2006) proposed three characteristics for discriminating abnormal from adaptive forms of aggression in rodents: (i) Mismatch between provocation and response, (ii) disregarding species-specific rules (e.g. attacking females, attacking vulnerable body parts), (iii) insensitivity towards the social signals of the opponent (e.g. ignoring submissiveness by continuing attacking). Similar behaviours can be discriminated in humans.

Preclinical research on abnormal aggression has focused on the role of glucocorticoids, serotonin, GABA and dopamine (for reviews see: de Almeida *et al.*, 2005; Haller *et al.*, 2005a). For example, high as well as low levels of glucocorticoids have been associated with abnormal forms of aggression. Hyper-arousal by frustration or instigation leads to exaggerated aggressive behaviour during the resident-intruder (RI) test in rodents (de Almeida & Miczek, 2002; Miczek *et al.*, 2002) and acute glucocorticoid treatments increase aggressive behaviour in different rodent species (Hayden-Hixson & Ferris, 1991; Brain & Haug, 1992; Haller *et al.*, 1997; Mikics *et al.*, 2004). As display of aggression increases glucocorticoid levels and glucocorticoids facilitate aggressive behaviour (Kruk *et al.*, 2004), a self-stimulating feedback-loop is likely. On the other hand, also glucocorticoid-deficiency can be associated with abnormal aggressive behaviour. In mice, selective breeding for short or long attack latencies resulted in a reduced stress response in the SAL line (Veenema *et al.*, 2003a; Veenema *et al.*, 2003b). Similar results were found in birds selected for an active

coping strategy, where heightened aggression correlated with lower glucocorticoid responses (Carere *et al.*, 2003). Furthermore, chronic low levels of glucocorticoids lead to abnormal aggression reflected by mismatched attack targeting, diminished autonomic arousal and social deficits in rats (Haller *et al.*, 2004).

There are studies suggesting a close interaction of the HPA axis with the serotonin system and, generally, serotonin has been implicated in the regulation of abnormal aggression (Olivier & van Oorschot, 2005). Furthermore, serotonin seems to exert an effect on glucocorticoid-deficiency-induced abnormal aggression (Haller *et al.*, 2005b). Clinical studies confirmed the link between serotonin and aggression, as low levels of the serotonin metabolite 5-hydroxyindoleacetic acid have been found in the cerebrospinal fluid of patients showing elevated levels of aggression and violent behaviour (Berman *et al.*, 1997).

2.7 Genetic and environmental factors influencing aggression

Environmental factors include early life stress, such as child abuse, neglect or parental loss, which lead to impulsive aggression, violence and antisocial personality symptoms in adulthood (Widom, 1989; Dodge *et al.*, 1990; Loeber & Stouthamer-Loeber, 1998; Barnow, 2001; Barnow & Freyberger, 2003; Barnow *et al.*, 2004). Preclinical research confirmed long-lasting changes in aggression as well as of anxiety and stress response in rodents exposed to early life stress (Plotsky & Meaney, 1993; Ladd *et al.*, 1996; Wigger & Neumann, 1999; Kalinichev *et al.*, 2002; Romeo *et al.*, 2003; Veenema *et al.*, 2006). Interactions between genes and environment play an important role in the regulation of aggression and violence (Seroczynski *et al.*, 1999; Moffitt, 2005). For example, there is a great variability in the length of tandem repeats in the regulatory region of the gene for monoamine oxidase A (MAOA), an enzyme that catalyses the oxidative deamination of serotonin, noradrenaline and dopamine. Abused children with a low MAOA activity show increased antisocial behaviour and violence compared to abused children with higher MAOA activity (Caspi et al., 2002). These results

suggest that a certain genetic predisposition can determine the effects of negative environmental factors. The influence of genetic factors on aggressive behaviour is in a first approach often studied by using knockout mice, which are lacking a specific gene product due to a spontaneous or induced mutation of their genome. Reduced aggression has been found in male knockout mice lacking the long form of the dopamine D2 receptor (Vukhac et al., 2001), the α-isoform of the oestrogen receptor (Ogawa *et al.*, 1997; Scordalakes & Rissman, 2003), the AVP V1b receptor (Wersinger et al., 2002) or a functional dopamine β-hydroxylase (Marino et al., 2005). In contrast, elevated aggressive behaviour is seen in mice lacking serotonin 1_B receptors (Saudou et al., 1994), dopamine transporter (Rodriguiz et al., 2004), nitric oxide synthase (Nelson et al., 1995) or MAOA (Cases et al., 1995). Interestingly, several males of a Dutch family, who are lacking MAOA due to a point mutation that caused a stop codon in the *maoa* gene, show impulsive aggression (Brunner et al., 1993). Thus, knockout studies may indicate the importance of several gene products for the regulation of aggressive behaviour. However, knockout mice are lacking the respective molecule not only when they are adult, but also during development. Therefore, behavioural abnormalities in adulthood could be due to changes that occurred during development. To avoid this possibility, approaches that acutely and specifically block or activate a neurotransmitter system in adult animals, such as local cerebral injection of agonists or antagonists, respectively, can be used for further research.

2.8 Aggression and anxiety

Aggression is a complex social and emotional behaviour and is closely linked to and influenced by other emotional behaviours, in particular anxiety. Anxiety is important for the survival of the individual, to escape predators or other dangerous situations. Although adequate levels of anxiety are beneficial for an individual, exaggerated and uncontrolled forms of anxiety are maladaptive. The lifetime prevalence for anxiety disorders, like panic

disorder or general anxiety disorder, is about 17 % (Somers et al., 2006). In wildlife animals, individuals with too low or too high levels of anxiety are in danger of either starving because they are not able to find enough food in the presence of predators or of getting killed by a predator because of taking too much risk. In the laboratory, animals selectively bred for high and low anxiety can be used to study the neuronal mechanisms regulating anxiety and its influence on the regulation of aggression.

Interestingly, several neurotransmitters that are implicated in the regulation of aggression also play a role in the regulation of anxiety, like for instance AVP.

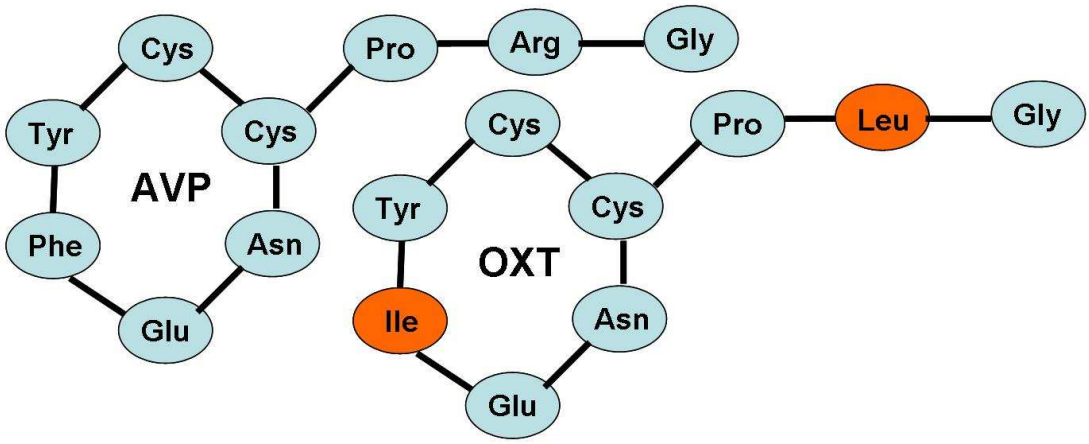

Fig. 4: Molecular structure of the two closely related nonapeptides arginine vasopressin (AVP) and oxytocin (OXT) consisting of amino acids: arginine (Arg), asparagine (Asn), cysteine (Cys), glutamic acid (Glu), glycine (Gly), isoleucine (Ile), leucine (Leu), phenylalanine (Phe), proline (Pro), tyrosine (Tyr). Only two out of nine amino acids are different between AVP and OXT.

The structurally related nonapeptides AVP and oxytocin (Fig. 4) have been shown to regulate anxiety-related behaviour in an opposite manner. When applied centrally, AVP exerts anxiogenic actions in rats (Williams *et al.*, 1983; de Wied *et al.*, 1993; Landgraf *et al.*, 1995a) and mice (Bielsky *et al.*, 2005), whereas oxytocin has anxiolytic properties (McCarthy *et al.*, 1996; Windle *et al.*, 1997; Neumann *et al.*, 2000; Bale *et al.*, 2001; Ring *et al.*, 2006). So far, there are only a few rather conflicting studies on the influence of oxytocin on intermale aggression (DeVries *et al.*, 1997; Winslow *et al.*, 2000; Ferris, 2005).

Another important regulator of both aggression and anxiety is the main inhibitory neurotransmitter GABA. Many drugs used for the treatment of anxiety (like diazepam) act via $GABA_A$ receptors (Whiting, 2006). $GABA_A$ receptors are chloride ion channels and opening of these channels results in hyperpolarisation and, thus, inactivation of the cell. Benzodiazepines have strong anxiolytic, muscle-relaxant and anticonvulsant effects. One of their side-effects is general sedation and chronic use of these drugs may lead to addiction (Bateson, 2002).

So far, research on the link between aggression and anxiety resulted in ambivalent findings. For example, Turku aggressive mice show less anxiety-related behaviour than their non-aggressive counterparts as measured in several anxiety tests like the elevated plus-maze (EPM), light-dark box and staircase test (Nyberg *et al.*, 2003). In contrast, there is no correlation between the level of aggression and the level of anxiety in wild-type rats as tested on the EPM (de Boer *et al.*, 2003). Likewise, the high-aggressive SAL mice and the low-aggressive LAL mice do not differ in their level of anxiety-related behaviour measured on the EPM (Veenema *et al.*, 2003a) or in the light-dark box (Hogg *et al.*, 2000). These results argue against a strict co-selection of aggression and anxiety, and both low anxiety as well as high anxiety may result in a more frequent and faster attack behaviour, the latter for example to defend oneself in a life-threatening situation. Additionally, it is not known, if anxiety tested on the EPM correlates with social anxiety during an aggressive encounter.

3. Wistar rats selected for low and high anxiety-related behaviour as animal model

3.1 History

Starting in 1993, commercially available Wistar rats (*Rattus norvegicus*) (Charles River, Sulzfeld, Germany) were bred for low (LAB) or high (HAB) anxiety-related behaviour

measured on the EPM in the animal facilities of the Max Planck Institute of Psychiatry in Munich, Germany (Liebsch *et al.*, 1998b). One year later, Wistar rats selectively bred for high or low behavioural performance in an active avoidance task at the University of Leipzig, Germany, were crossbred into the LAB and HAB lines, respectively, in order to improve the fitness and to intensify anxiety-related behaviour in HAB rats, in particular the freezing behaviour that indicates anxiety (Hess *et al.*, 1992). In 2003, LAB and HAB rats were transferred to the laboratories of Professor Inga D. Neumann at the University of Regensburg, Germany, where breeding has been continued. Throughout the years, the LAB and HAB rat lines were treated identical in terms of housing, animal care, mating and behavioural testing (Landgraf & Wigger, 2002; 2003).

3.2 Behavioural differences (Tab. 1)

3.2.1 Anxiety-related behaviour

Basically, one has to distinguish between trait and state anxiety. Trait anxiety is personality-based (long-term), whereas state anxiety is an acute, fear-induced type of anxiety. Anxiety-related behaviour measured on the EPM was taken as the selection criterion for LAB and HAB rats which have been selected for low and high levels of trait anxiety. The EPM is a nonconditioned test based on creating a conflict between the rat's exploratory drive and its innate fear of open spaces (Pellow *et al.*, 1985). An anxious animal will rarely enter the open arms of the maze, whereas less anxious animals spend more time on the open arms. Therefore, the percentage of time spent on the open arms has been used as the main selection parameter for LAB and HAB breeding. For example, in 2002, the average time spent on the open arms of the maze was above 50 % in LAB and less than 5 % in HAB rats (Landgraf & Wigger, 2002). The robust differences in anxiety-related behaviours between LAB and HAB rats have been confirmed in other established tests. For instance, in the open field and the modified hole board test, anxious animals avoid the centre zone of the testing arena and prefer

to stay in the outer zone. Accordingly, the ratio of time spent as well as the distance travelled in the centre zone compared to the outer zone is higher in LAB than in HAB rats in both tests (Liebsch et al., 1998b; Ohl et al., 2001). Furthermore, in the black-white box, LAB rats enter the white compartment more often and spend more time in the white compartment compared with HAB rats, which is also indicating a lower level of anxiety in LAB rats (Henniger *et al.*, 2000). It has to be mentioned that HAB rats show a reduced locomotor activity in some of the tests for anxiety. Therefore, it is possible that the difference in anxiety between the two breeding lines is based on a difference in locomotor activity. Otherwise, the reduced locomotor activity could be caused by the high level of anxiety of the HAB rats (Rodgers *et al.*, 1997; Escorihuela *et al.*, 1999). The latter hypothesis is supported by several findings. First, there is no line difference in locomotor activity in single-housed LAB and HAB rats tested in their home cage under undisturbed conditions (Liebsch *et al.*, 1998a). Second, HAB pups show more ultrasonic vocalization when separated from their mother compared with LAB pups (Wigger *et al.*, 2001). Ultrasonic vocalisation is an indicator of anxiety independent of locomotion (Tornatzky & Miczek, 1994). Third, the locomotor activity does not correlate with the level of anxiety in LAB, HAB and non-selected Wistar (NAB) rats when tested in the modified hole board (Ohl *et al.*, 2001). Finally, a study by Salome *et al.* (2002) also showed by principal component analysis that the difference between LAB and HAB rats is rather based on anxiety than on locomotion. Testing of LAB and HAB rats in different laboratories confirmed that the behavioural profiles of these breeding lines are robust and consistent (Salome *et al.*, 2002).

3.2.2 Stress coping

Besides their opposing inborn level of anxiety, LAB and HAB rats show clear differences in stress coping strategies. For example, LAB rats show a reduced level of immobility in the forced swim test (Liebsch et al., 1998b) as well as reduced risk assessment accompanied by

more exploratory behaviour in the modified hole board and open field test (Ohl et al., 2001). In general, LAB rats display a rather active stress coping style, whereas HAB rats are characterized by a passive, depression-like stress coping behaviour. Therefore, HAB rats have been established as animal model for anxiety- and depression-related disorders (for review see: Landgraf & Wigger, 2002). Links between emotionality and stress coping were also found in other animal models (Overstreet *et al.*, 1992; Steimer *et al.*, 1997). However, they are often based on non-social stressors like physical exercise or novel environment, whereas social, emotional stressors are rarely used. A study by Frank et al. (2006) showed that there is also a line difference in stress coping behaviour in the context of social stressors. When exposed to an aggressive resident rat as intruder in the social defeat paradigm, LAB rats spend less time immobile and more time rearing and self grooming compared with HAB rats.

3.2.3 Social behaviour

Social behaviour consists amongst others of social investigation, social interaction as well as defensive and aggressive behaviours. In the social interaction test, LAB rats spend more time in active social interaction accompanied by a higher number of line crossings compared with HAB rats (Henniger *et al.*, 2000). These findings suggest also a more active stress coping strategy in the context of social stressors in LAB rats. In contrast, male HAB rats show a tendency towards a higher amount of passive social interaction compared with LAB rats (Henniger *et al.*, 2000). Furthermore, in the modified hole board, HAB rats spend more time in social contact to their cage mates than LAB and NAB rats, indicating that social isolation in a novel test situation is more stressful for HAB rats than for LAB and NAB rats (Ohl *et al.*, 2001). Moreover, group-housed male LAB rats tend to show more aggressive interactions between the cage mates than male HAB rats (Henniger *et al.*, 2000). The latter finding is a first indication for differences in aggression between the lines. Studies in females showed that lactating LAB and HAB dams differ in maternal aggression which occurs when the pups are

protected against an intruder rat. The higher level of maternal aggression in HAB dams compared with LAB dams is thought to be part of an elevated level of maternal behaviour displayed by HAB dams (Bosch et al., 2005; Neumann et al., 2005a).

Tab. 1: Behavioural differences between rats selectively bred for high (HAB) or low (LAB) anxiety-related behaviour. > higher in HAB rats, < higher in LAB rats, ~ no difference between HAB and LAB rats; BWB = Black-white box, EPM = Elevated plus-maze, FS = Forced swim test, HC = Home cage, mHB = Modified hole board, OF = Open field; PND = Postnatal day; SD = Social defeat; SI = Social interaction test

Anxiety	HAB vs. LAB	Reference(s)
% Time open arms (EPM)	<	Liebsch *et al.* 1998b, Landgraf & Wigger 2002, Salome *et al.* 2002
Time central zone (OF)	<	Liebsch *et al.* 1998b
Time central zone (mHB)	<	Ohl *et al.* 2001
Time spent in white box (BWB)	<	Henniger *et al.* 2000
Basal locomotion (single-housed, HC)	~	Liebsch *et al.* 1998a
Ultrasound isolation calls (PND 11)	>	Wigger *et al.* 2001
Stress coping	**HAB vs. LAB**	**Reference**
Immobility (FS)	>	Liebsch *et al.* 1998b
Struggling (FS)	<	Liebsch *et al.* 1998b
Risk assessment (mHB, EPM)	>	Ohl *et al.* 2001
Immobility (SD)	>	Frank *et al.* 2006
Rearing/Grooming (SD)	<	Frank *et al.* 2006
Social behaviour	**HAB vs. LAB**	**Reference(s)**
Active social interaction (SI)	<	Henniger *et al.* 2000
Passive social interaction (SI)	>	Henniger *et al.* 2000
Time contact to cage mates (mHB)	>	Ohl *et al.* 2001
Aggressive interactions with cage mates (home cage, undisturbed)	<	Henniger *et al.* 2000
Maternal aggression	>	Bosch *et al.* 2005, Neumann *et al.* 2005a
Maternal care	>	Neumann *et al.* 2005a

3.3 Neuroendocrine and neurobiological differences (Tab. 2)

3.3.1 Arginine vasopressin system

Besides behavioural differences, LAB and HAB rats also differ in neuroendocrine and neurobiological parameters. Male HAB rats show a higher level of AVP mRNA expression in the PVN under basal conditions as well as in response to open arm exposure (Wigger et al.,

2004). Additionally, AVP release in the PVN is higher in HAB rats compared with LAB rats in response to ten minutes of forced swimming (Wigger *et al.*, 2004). Interestingly, in the same brain region, neither mRNA expression nor release of the structurally and functionally related neuropeptide oxytocin differed between LAB and HAB rats (Wigger *et al.*, 2004). In several brain regions investigated, including amygdala, BNST, septum and PVN, no line differences in AVP V1a receptor binding between HAB and LAB rats were found (Wigger *et al.*, 2004). It has been shown that the difference in the AVP system is based on a single nucleotide polymorphism in HAB rats. This single nucleotide polymorphism [A(-1276)G], which is located in the *cis*-regulatory element of the AVP gene, leads to a change of one base pair in the desoxyribonucleic acid. Due to this change, the binding of the transcriptional repressor CArG binding factor A is reduced. This results in an increased transcription of the respective gene. Therefore, the single nucleotide polymorphism [A(-1276)G] leads to a region-specific overexpression of AVP *in vitro* (in the PVN) and *in vivo* (Murgatroyd *et al.*, 2004). However, it remains unclear, what mechanisms are involved to inhibit overexpression of AVP in related brain regions like the supraoptic nucleus.

3.3.2 Hypothalamic-pituitary-adrenal axis

LAB and HAB rats were found to show robust differences in HPA axis responsiveness. HAB rats have a higher HPA axis response to acute mild, non-social stressors, such as exposure to a novel environment, indicated by elevated ACTH as well as corticosterone secretion compared with LAB rats (Landgraf *et al.*, 1999; Landgraf & Wigger, 2002). In the dexamethasone/CRH test, HAB rats show a reduced dexamethasone-induced suppression of ACTH release compared with LAB rats (Keck *et al.*, 2002) indicating an over-reactive HPA axis. Prior intravenous administration of an AVP receptor antagonist normalizes the pathological outcome of the dexamethasone/CRH test in HAB rats confirming the influence of the endogenous AVP in the over-reactive HPA axis in response to non-social stressors in these

rats (Keck *et al.*, 2002). In contrast, confrontation with a social stressor, such as a dominant conspecific, results in a higher corticosterone response in LAB than in HAB rats (Frank *et al.*, 2006).

Tab. 2: Neuroendocrine/neurobiological differences between rats selectively bred for high (HAB) or low (LAB) anxiety-related behaviour. > higher in HAB rats, < higher in LAB rats, ~ no difference between HAB and LAB rats; ACTH = Adrenocorticotropic hormone, Amg = Amygdala, AVP = Arginine vasopressin, BNST = Bed nucleus of the stria terminalis, DEX = Dexamethasone, Hippo = Hippocampus, HPA axis = Hypothalamic-pituitary-adrenal axis, LS = Lateral septum, mRNA = Messenger ribonucleic acid, N-stressor = Non-social stressor, PVN = Paraventricular nucleus of the hypothalamus, S-stressor = Social stressor

AVP/Oxytocin	HAB vs. LAB	Reference
AVP V1a receptor binding (in several brain regions, incl. BNST, septum, Amg and PVN)	~	Wigger *et al.* 2004
AVP mRNA PVN (basal/N-stressor)	>	Wigger *et al.* 2004
AVP release PVN (basal/N-stressor)	>	Wigger *et al.* 2004
Oxytocin mRNA PVN (basal/N-stressor)	=	Wigger *et al.* 2004
Oxytocin release PVN (basal/N-stressor)	=	Wigger *et al.* 2004
HPA axis	**HAB vs. LAB**	**Reference(s)**
ACTH/Corticosterone response to N-stressor	>	Landgraf *et al.* 1999, Landgraf & Wigger 2002
Corticosterone response to S-stressor	<	Frank *et al.* 2006
DEX-induced suppression of ACTH release	<	Keck *et al.* 2002
Serotonin system	**HAB vs. LAB**	**Reference**
Release Hippo in response to N-stressor	<	Keck *et al.* 2005
mRNA Hippo basal	<	Keck *et al.* 2005
Serotonin transporter binding sites Hippo	>	Keck *et al.* 2005
Release LS/Amg in response to N-stressor	<	Salome *et al.* 2006
Release PVN basal	=	Umriukhin et al. 2002
Release PVN in response to N-stressor	>	Umriukhin et al. 2002

3.3.3 Serotonin system

There were also differences in the serotonin system found between LAB and HAB rats. In response to a stressor, only in LAB rats there was an increase in serotonin release within the hippocampus, whereas basal hippocampal release did not differ between the two breeding lines (Keck *et al.*, 2005). Chronic treatment with paroxetine, a serotonin reuptake inhibitor, results in a rise in stress-induced serotonin within the hippocampus in HAB rats (Keck *et al.*,

2005). Furthermore, the serotonin 1_A receptor mRNA level is higher and the amount of serotonin transporter binding sites is lower in the hippocampus of LAB rats than in HAB rats (Keck *et al.*, 2005). Exposition to an acute stressor leads to a higher level of serotonin release in the lateral septum and the amygdala (Salome *et al.*, 2006), but a lower increase in serotonin release in the PVN (Umriukhin *et al.*, 2002) in LAB rats compared with HAB rats. In conclusion, in most brain regions, serotonin neurotransmission seems to be higher in LAB compared with HAB rats.

4. Aim of the present thesis

Exaggerated aggression is a burden for the society and for the individuals involved. However, the neurobiological mechanisms underlying excessive and abnormal forms of aggressive behaviour are largely unknown, but suitable animal models are relatively scarce. Therefore, the aim of the present study is to establish LAB and HAB rats as an animal model to reveal the neurobiological systems relevant for the regulation of aggression.

In chapter 2, the behavioural profile and the time course of anxiety-related and aggressive behaviours of LAB and HAB rats and NAB rats collected over the last six years are presented. Furthermore, seasonal effects on anxiety-related and aggressive behaviours were studied in these rat lines. Additionally, the occurrence of abnormal forms of aggression, including attacks towards vulnerable body parts as well as aggression towards a female or a narcotised rat, was investigated. Finally, the role of the serotonin system in the regulation of normal and abnormal forms of aggression in LAB rats was investigated by administration of the serotonin 1_A autoreceptor agonist S-15535.

In chapter 3, it was investigated whether there is a link between the innate level of anxiety and HPA axis responsiveness as well as neuronal activation in response to the display of

aggression. Therefore, blood sampling during the RI test and subsequent staining for c-Fos protein has been performed.

In chapter 4, I investigated the role of the AVP system in the regulation of intermale aggression in LAB and HAB rats by using intracerebral microdialysis to assess the *in vivo* AVP release in the lateral septum during the display of aggressive behaviour. Furthermore, retrodialysis was used to investigate the effects of pharmacological manipulation of the AVP system in the septum on aggression and anxiety.

In chapter 5, the region-specific release patterns of AVP in the septum and the BNST were studied during the display of aggression in non- selected male Wistar rats. Furthermore, the causal link between AVP release and aggressive behaviour was studied by local pharmacological manipulation of the AVP system.

Chapter 2

Paradox of anxiety and aggression: both low and high trait anxiety are linked with high and abnormal forms of intermale aggression

[adapted from: Beiderbeck DI, Neumann ID, Veenema AH; Paradox of anxiety and aggression: both low and high trait anxiety are linked with high and abnormal forms of intermale aggression; In preparation]

Abstract

Excessive aggression and violence are a major problem in human society. A better understanding of the mechanisms underlying high and abnormal aggression is essential for novel therapy, treatment and prevention strategies. Here, we demonstrate that selective breeding of rats for extremes in anxiety-related behaviour resulted in two behavioural phenotypes with high and abnormal forms of intermale aggression. Data collected over the last six years reveal a stable and robust line difference in anxiety. Moreover, rats bred for low anxiety-related behaviour consistently show high levels of aggression and low levels of social investigation in the RI test compared with non-selected rats, whereas rats bred for high anxiety also show a relatively high, but rather intermediate level of aggression. Accordingly, a significant U-shaped correlation between anxiety and intermale aggression was found. In addition to their elevated aggressiveness, both LAB and HAB rats display abnormal forms of aggression, i.e. they attack vulnerable body parts of the intruder. Moreover, LAB residents had a shorter attack latency and showed a higher number of attacks towards a non-oestrus female and towards a narcotised male compared with NAB and HAB residents. Treatment of LAB rats with the preferential somatodendritic serotonin 1_A receptor agonist S-15535 [4 mg/kg subcutaneously (s.c.)] significantly reduced the number of attacks towards a conscious or narcotised male intruder. In conclusion, extremes in trait anxiety are linked to high and abnormal aggression, with highest levels observed in LAB rats, making them an interesting model to investigate neurobiological mechanisms underlying excessive aggression.

Introduction

Aggressive behaviour is an important factor for the survival of an individual to obtain nutritional resources, territory and mating partners. However, energetic and health costs of aggression are high. Therefore, aggressive behaviour underlies strict species-specific rules in

order to minimize injuries and killing. In human society, excessive aggression is a major health, social and financial problem. More than 700,000 people worldwide die each year because of an aggressive assault, and an additional number of people become victims of physical or psychological injury (Bartolomeos *et al.*, 2007). Exaggerated, violent forms of aggressive behaviour can occur as a symptom of several diseases like personality disorders, schizophrenia or depressive illnesses (Eronen *et al.*, 1998; Haller & Kruk, 2006), and can be classified as either hyper- or hypo-arousal driven. Hyper-arousal-driven aggression is associated with high affective reactions, and with high autonomic and cortisol responses, whereas people who display hypo-arousal-driven aggression show low affective reactions, low autonomic responses, low cortisol levels, and low skin conductance (Haller & Kruk, 2006).

We have recently reported a negative correlation between trait anxiety and intermale aggression (Veenema et al., 2007b). Rats selectively bred for low anxiety-related behaviour are more aggressive than rats bred for high anxiety-related behaviour and NAB rats during the RI test (Beiderbeck et al., 2007; Veenema et al., 2007b). The pronounced aggressive behaviour of LAB rats is accompanied by an elevated level of neuronal activity within the PVN and an elevated ACTH response to social stimuli such as the RI test (Veenema et al., 2007b). This suggests an association between an elevated level of aggression and high social stress responsiveness in LAB rats, and makes them an interesting animal model for studying the regulation of high or abnormal aggression.

In rats, abnormal forms of aggression include, for example, aberrant attack targeting. During an encounter between a resident and an intruder male rat, the resident will normally direct its attacks towards the back and the flanks of the intruder (Blanchard *et al.*, 2003). Attacks towards more vulnerable body parts like the head, throat or belly of the intruder indicate abnormal aggression, as species-specific rules are ignored (Haller & Kruk, 2006). Other

forms of abnormal aggressive behaviours include attacks towards females or unconscious males (de Boer *et al.*, 2003; Sluyter *et al.*, 2003; Natarajan *et al.*, 2008) as they are no threat to the resident's territory.

Diverse neurotransmitters and neuromodulators have been shown to be involved in the regulation of aggressive behaviour (for review see: Nelson & Trainor, 2007) including serotonin (Olivier & van Oorschot, 2005). For example, patients with high levels of aggression and impulsive violence are characterized by a low level of the serotonin metabolite 5-hydroxyindoleacetic acid in the cerebrospinal fluid (Berman *et al.*, 1997). Reduced aggressive behaviour after treatment with serotonin receptor agonists suggests a negative correlation between serotonin and aggression (Olivier *et al.*, 1995; Millan *et al.*, 1997; de Boer *et al.*, 1999; 2000). In contrast, more recent studies found a positive correlation between serotonin and the display of aggressive behaviour (Olivier, 2004; de Boer & Koolhaas, 2005). It is therefore hypothesized that abnormal forms of aggression can be characterized by a chronically low level of serotonin activity (Miczek *et al.*, 2002), whereas normal expression of aggression is accompanied by an acute increase in serotonin activity (van der Vegt *et al.*, 2003a; van der Vegt *et al.*, 2003b; Summers *et al.*, 2005a).

In the present study, we investigated whether the difference in anxiety-related behaviour between LAB and HAB rats has been stable over the last six years, and can be consistently linked to the respective aggression phenotype in comparison with Wistar rats that have not been selected for anxiety. It was further analysed, whether the high level of intermale aggression of LAB and HAB rats is accompanied by forms of abnormal aggression, including attacks directed towards vulnerable body parts of a male intruder, or towards a non-oestrus female or a narcotised male. Finally, we determined the effect of the preferential

somatodendritic serotonin 1_A autoreceptor agonist S-15535 on aggressive behaviour in LAB rats.

Materials and Methods

Animals and breeding procedure

Experiments were carried out on male LAB and HAB rats selectively bred for low or high anxiety-related behaviour on the EPM since 1993 (Liebsch *et al.*, 1998b; Landgraf & Wigger, 2002) and on male NAB rats (Charles River, Sulzfeld, Germany). LAB and HAB rats have been bred in the animal facilities of the University of Regensburg, Germany, since 2003. Rats were constantly kept under controlled laboratory conditions (12:12 h light/dark cycle with lights on at 6:00 a.m., 21 ± 1°C, 60 % ± 5 % humidity, standard rat chow and water *ad libitum*) and were housed in groups of 3-5 of same sex and line in standard rat cages (56 × 36 × 20 cm). For the breeding, one male was mated with 2-3 females of the same breeding line for a period of one week. Mating of siblings was avoided. During the last week of gestation, females were single-housed in standard rat cages. Pups were weaned at the age of three weeks and kept in groups of 4-6 of same sex and line in standard rat cages. At the age of nine weeks, all LAB and HAB rats were tested on the EPM to verify their inborn level of anxiety and to select rats for further breeding. The selection criteria for the breeding are based on the percentage of time spent on the open arms of the EPM, which is set at more than 30 % for LAB rats and less than 10 % for HAB rats, as well as the number of entries into closed arms as indication of locomotor activity. As a clear line-dependent divergence of locomotor activity has been found over the years with high activity in LAB and low activity in HAB rats, we try to select LAB rats with relatively low (below 6 entries) and HAB rats with rather high (above 2 entries) activity. After primary behavioural testing, rats were housed in groups of 3-5 of same sex and line in standard rat cages until the start of the experiments. The experiments were approved by the Committee on Animal Health and Care of the Government of the

Oberpfalz and are in accordance with the *Guide for the Care and Use of Laboratory Animals* by the National Institute of Health.

Elevated plus-maze

The EPM is based on creating a conflict between the rat's exploratory drive and its innate fear of open spaces (Pellow *et al.*, 1985). The apparatus consisted of a plus-shaped platform elevated 80 cm above the floor, with two opposing open (50 × 10 cm; 100 lux) and two opposing closed (50 × 10 × 40 cm; 20 lux) arms. A raised edge (0.5 cm) on the open arms provided extra grip for the rats. Rats were placed individually in the centre square facing a closed arm and were allowed to explore the maze for five minutes. The following parameters were recorded by means of a video/computer system (Plus-maze version 2.0; Ernst Fricke): entries into closed and open arms, percentage of entries into open arms, time spent in closed and on open arms, percentage of time spent on open arms, latency to enter an open arm. Here, statistical analysis is only presented for the percentage of time spent on the open arms.

Resident-intruder test

Aggressive behaviour has been quantified during the RI test (Koolhaas et al., 1980; Veenema et al., 2007b). Adult LAB, HAB and NAB male rats (16-22 weeks of age) were housed in an observational cage (40 × 24 × 35 cm) together with a female Wistar rat (Charles River, Sulzfeld, Germany) for ten days to stimulate territorial behaviour (Flannelly & Lore, 1977). At the same time, the 12:12 h light/dark cycle was switched to lights off at 13:00 p.m. Bedding was not changed during the last three days prior to the RI test. The female cage mate was removed 30 minutes before an unfamiliar, lighter (-10 %) male Wistar rat (housed under the same light conditions in another room) was placed into the resident's home cage for ten minutes. The behaviour of the rats was videotaped, and the following parameters were scored by an experienced observer blind to breeding line and treatment according to Veenema *et al.*

(2007b): Aggressive behaviour (sum of attack, lateral threat, offensive upright, keep down, threat, aggressive grooming), social investigation (investigating opponent, anogenital sniffing), exploration, self grooming, defensive behaviour, and immobility. Behaviour was scored in real-time using pre-set keys on a PC (Eventlog; Version 1.0, 1986, R. Hendersen). All behaviours were calculated as percentage of time. Additionally, the attack latency time and the number of attacks were measured.

Experiment 1: Anxiety and aggression in LAB and HAB rats: Time-course, seasonal effects and correlation

Anxiety-related behaviour

We assessed the percentage of LAB and HAB male rats that met the selection criteria on the EPM between 2003 and 2008. We then randomly included 10 % of all tested LAB and HAB males (resulting in a total number of 98 LAB and 88 HAB rats) to generate a time course for anxiety-related behaviour over the last six years and to assess possible seasonal effects.

Aggressive behaviour

Data concerning all male LAB (n = 102), HAB (n = 73) and NAB (n = 176) rats which were tested in the RI test between 2004 and 2008 were summarised to obtain an aggression profile over time (years) and over season. Moreover, a detailed behavioural analysis during the RI test was performed. To determine the distribution of aggression within each line, rats were divided according to their level of aggression displayed during the RI test in either low (< 15 % of time occupied with aggressive behaviour during the 10-minute RI test), medium (between 15 and 55 % of time), or high (> 55 % of time) aggressive groups.

Correlation between anxiety and aggression

To investigate a correlation between anxiety and aggression, LAB (n = 30), HAB (n = 30) and NAB (n = 30) rats were tested for anxiety (EPM; 9 weeks of age) and for aggression (RI test; 16 weeks of age).

Experiment 2: Abnormal aggression in LAB, HAB and NAB rats

Abnormal aggression towards a male intruder

Aggressive behaviour during the RI test (n = 10 in each LAB, HAB and NAB males) was analysed on video-tape at low speed and the percentage of attacks towards the following attack targets were calculated: back, flank, as well as the vulnerable targets, i.e. head, throat and belly (Haller *et al.*, 2001).

Aggression towards a non-oestrus female intruder rat

To test whether LAB, HAB or NAB rats show aggressive behaviour towards a non-oestrus female rat, which normally does not elicit aggressive or sexual behaviour in male rats (Blanchard *et al.*, 1984), male LAB (n = 10), HAB (n = 11) and NAB (n = 10) rats were allowed to habituate to an observational cage (40 × 24 × 35 cm) for 30 minutes before a non-oestrus female rat was introduced into the cage for ten minutes. The attack latency and the number of attacks towards the female intruder were measured. Vaginal smears were taken one hour before the RI test to verify the non-oestrus state of the females.

Aggression towards a narcotised male intruder rat

To test for aggressive behaviour of LAB (n = 11), HAB (n = 12) and NAB (n = 8) residents towards a narcotised male intruder rat, the male intruder (10 % less body weight than resident) was narcotised with pentobarbital [55 mg/kg, intraperitoneally (i.p.)] 30 minutes before being placed into the resident's home cage for ten minutes. The behaviour was

videotaped, and the attack latency, the number of attacks, and the percentage of attacks directed towards the head (compared to total attacks) were measured.

Experiment 3: Effects of S-15535 on aggressive behaviours in male LAB rats

To determine the role of serotonin 1_A autoreceptors in the modulation of high and abnormal forms of aggression in LAB rats, rats received either vehicle (distilled water; 1 ml/kg s.c.) or the highly selective serotonin 1_A receptor agonist S-15535 (4 mg/kg s.c.; kindly provided by Dr. S. F. de Boer) 30 minutes prior to the RI test. S-15535 (4-(benzodioxan-5-yl)1-(indan-2-yl)piperazine) exerts low intrinsic activity and acts in vivo as an agonist at serotonin 1_A autoreceptors and as a competitive antagonist at postsynaptic serotonin 1_A receptors (Millan *et al.*, 1993; Millan *et al.*, 1994). To minimize unspecific stress responses due to the injection procedure, rats were handled several times prior to the experiment. Thirty minutes after vehicle- or S15535-treatment, male LAB rats were exposed to a male intruder in their home cage for ten minutes. Three days later, the rats received the same treatment, but were exposed to a narcotised male intruder for ten minutes.

Statistics

Statistical analyses were performed using the software package SPSS (version 13). Behavioural data of experiment 1 and 2 were analysed using one way analysis of variance (ANOVA) (factor line) or two way ANOVA (factor line × factor season; factor line × factor year). ANOVA was followed by Bonferroni *post hoc* test, when appropriate. A linear and nonlinear (quadratic) regression analysis was carried out to calculate the correlation coefficient that best fitted the correlation between anxiety (percentage of time spent on open arms) and aggression (percentage of time) using simple regression analysis. Behavioural data of experiment 3 were analysed using the Student's *t*-test. Statistical significance was set at $p < 0.05$. Data are presented as means + standard error of the mean (SEM).

Results

Experiment 1: Anxiety and aggression in LAB and HAB rats: Time-course, seasonal effects and correlation

Anxiety-related behaviour

Between 2003 and 2008, 980 LAB and 880 HAB males were tested on the EPM for anxiety-related behaviour at the age of nine weeks. On average, 74 % of the LAB and 84 % of the HAB males met the selection criteria defined above (percentage of time spent on the open arms: LAB > 30 %; HAB < 10 %). Moreover, random selection of 10 % of all LAB and HAB males tested in this period demonstrates a robust and stable line difference in the level of inborn anxiety, i.e. in the percentage of time spent on the open arms (factor line: $F_{(1,174)} = 436$; $p < 0.001$; Fig. 5A). There was no change in the level of anxiety over the years in LAB or HAB rats (factor year: $F_{(5,174)} = 1.71$; $p = 0.135$). In LAB rats, a seasonal effect for anxiety-related behaviour was found (factor season: $F_{(3,173)} = 5.15$; $p < 0.01$), with a further reduced anxiety in summer as revealed by a higher percentage of time on the open arms in summer compared with the other seasons ($p < 0.01$; Fig. 6A).

Aggressive behaviour

Data collected over the past five years between 2004 and 2008 demonstrate a consistent line difference in total aggressive behaviour (factor line: $F_{(2,339)} = 33.0$; $p < 0.001$), with LAB rats showing the highest level of aggression compared with NAB rats ($p < 0.05$ for each year; Fig. 5B). HAB rats showed an intermediate level of aggression with significantly less aggressive behaviour than LAB rats in 2004 ($p < 0.01$) and more aggressive behaviour than NAB rats in 2005/2006 and 2007 ($p < 0.001$; Fig. 5B).

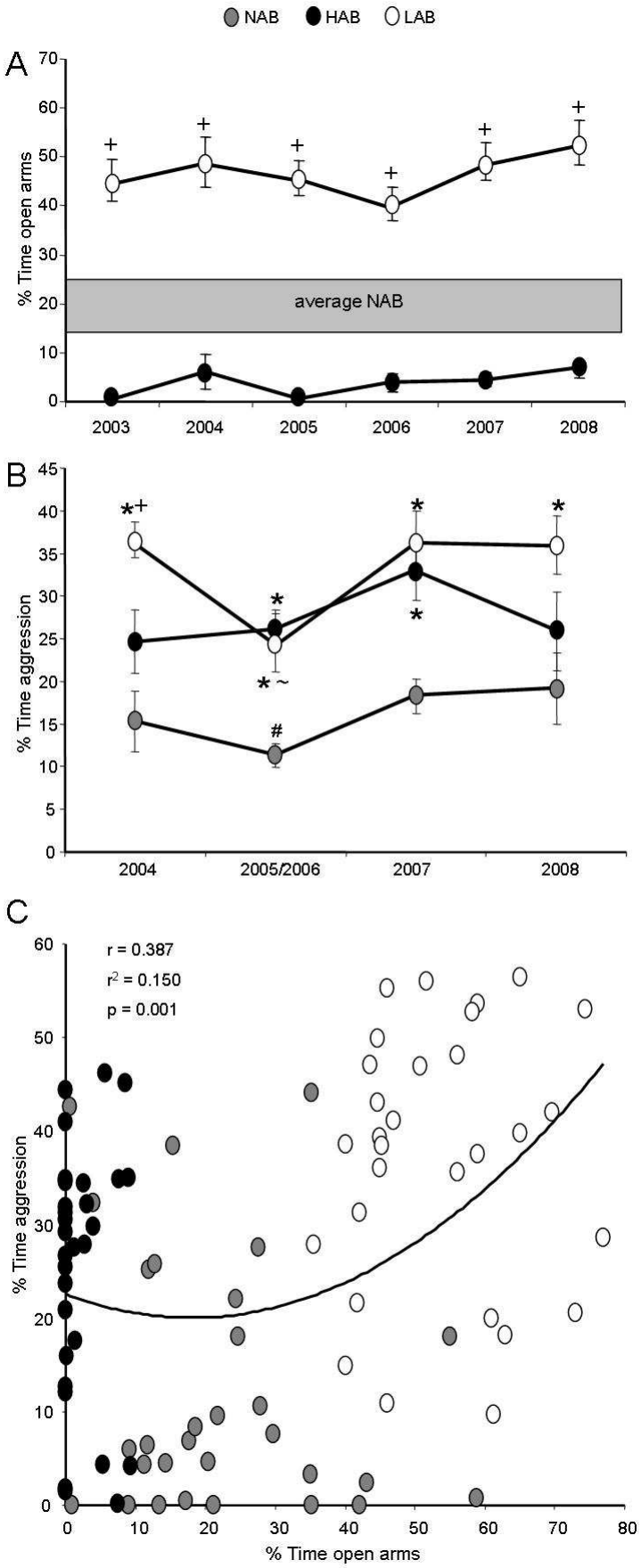

Fig. 5: (A) Time course of anxiety-related behaviour (percentage of time spent on the open arms) measured on the EPM in 9-week old male LAB and HAB rats; **(B)** Time course of aggressive behaviour in 16- to 22-week old male LAB, HAB and NAB rats. * $p < 0.05$ vs. NAB, + $p < 0.05$ vs. HAB, ~ $p < 0.05$ vs. 2004/2007, # $p < 0.05$ vs. 2007. Data are presented as means ± SEM; **(C)** U-shaped correlation of anxiety (percentage of time spent on the open arms of the EPM) with aggression (percentage of time of aggressive behaviour during the RI test) in male LAB (n = 30), HAB (n = 30) and NAB (n = 30) rats. $r = 0.387$, $r^2 = 0.150$, $p < 0.005$

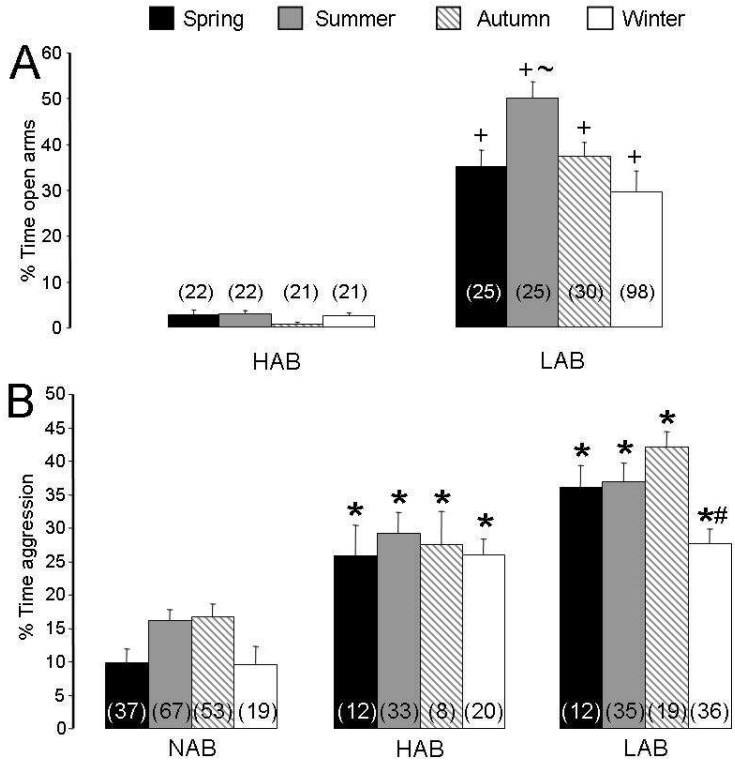

Fig. 6: Seasonal effects on **(A)** Anxiety-related behaviour (percentage of time on the open arms) measured on the EPM in 9-week-old male LAB and HAB rats, and **(B)** Aggressive behaviour of 16- to 22-week-old male NAB, HAB and LAB rats. * $p < 0.05$ vs. NAB, + $p < 0.05$ vs. HAB, ~ $p < 0.05$ vs. spring/autumn/winter, # $p < 0.05$ vs. summer/autumn. Numbers in parentheses indicate group size. Data are presented as means + SEM.

Behavioural profile during the RI test

Collected data from the last five years demonstrate a significant line difference in total aggressive behaviour ($F_{(2,348)} = 66.6$; $p < 0.001$) with LAB rats showing the highest level of aggression compared with NAB ($p < 0.001$) and HAB ($p < 0.01$) rats, and higher aggression of HAB compared with NAB rats ($p < 0.001$; Fig. 7A). Among the elements of aggressive behaviour, line-dependent differences were found in the display of lateral threat ($F_{(2,348)} = 76.7$; $p < 0.001$; LAB > NAB), offensive upright ($F_{(2,348)} = 15.9$; $p < 0.001$; LAB > NAB/HAB) and threat ($F_{(2,348)} = 25.0$; $p < 0.001$; LAB/HAB > NAB) (data not shown). Calculations of elements of aggressive behaviour as percentage of total time spent with aggressive behaviour revealed more lateral threat in LAB and HAB (almost 50 % of the time) versus NAB rats (< 20 % of the time; $F_{(2,323)} = 76.7$; $p < 0.001$; Bonferroni: $p < 0.001$; Fig. 7B). Finally, LAB rats showed less non-aggressive social investigation ($F_{(2,348)} = 77.4$; $p < 0.001$) than NAB ($p < 0.001$) and HAB ($p < 0.001$) rats (Fig. 7A).

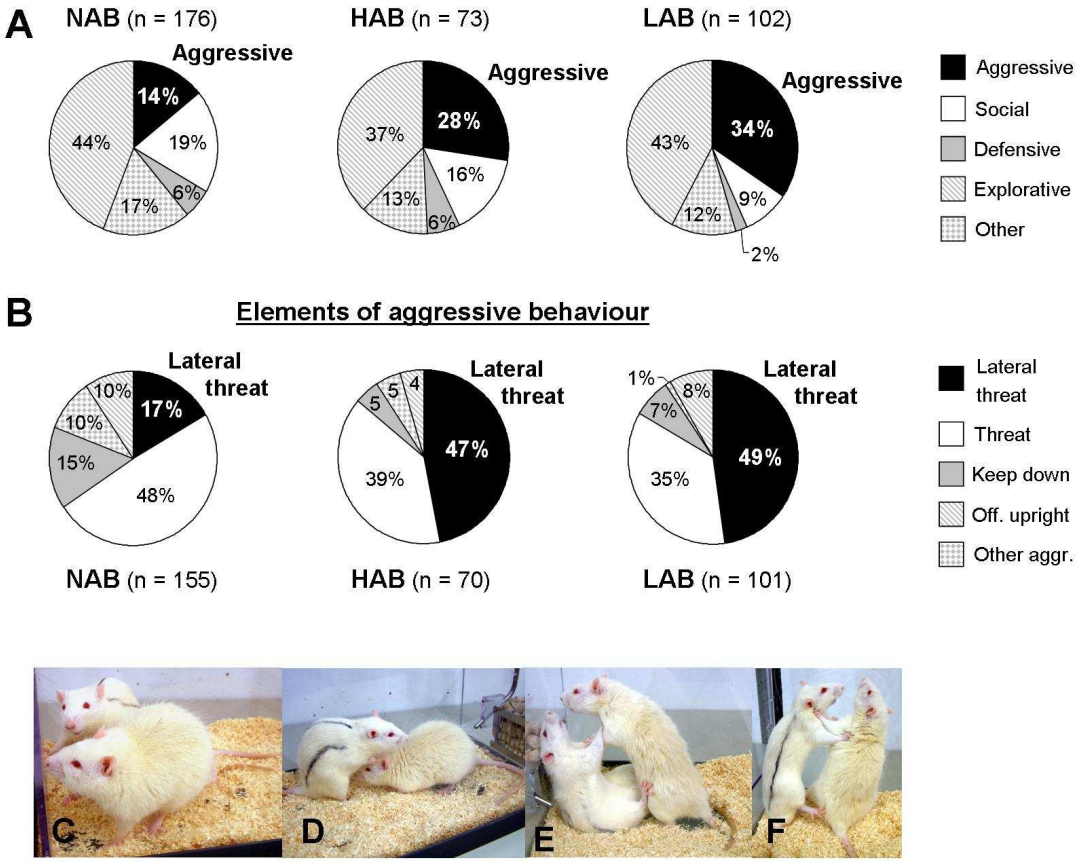

Fig. 7: Behavioural profile of male NAB, HAB, and LAB residents during the RI test performed at the age of 16-22 weeks. **(A)** Aggressive behaviour, social investigation, defensive, explorative, and other behaviours are presented as percentage of time. **(B)** Elements of aggressive behaviour are calculated as percentage of total aggressive behaviour. Data are presented as means. **(C)** Lateral threat, **(D)** Threat, **(E)** Keep down, and **(F)** Offensive upright displayed by a male resident rat towards a male intruder rat (marked with black stripes) during the RI test

An effect of time (factor year: $F_{(3,339)} = 5.00$; $p < 0.01$) was found for aggressive behaviour in LAB and NAB rats. LAB rats showed a lower level of aggression in 2005/2006 compared with 2004 and 2007 ($p < 0.05$), and NAB rats had a higher level of aggression in 2007 compared with 2005/2006 ($p < 0.05$; Fig. 5B).

An overall effect of season on aggressive behaviour (factor season: $F_{(3,339)} = 3.90$; $p < 0.01$) was reflected by a lower level of aggressive behaviour in LAB rats during winter compared

with autumn ($p < 0.01$) and summer ($p < 0.05$; Fig. 6B). The seasonal effect on aggression in winter may explain the lower level of aggression in LAB rats in 2005/2006 (Fig. 5B) as LAB rats were only tested in winter during these two years. Neither NAB nor HAB rats showed significant changes in aggressive behaviour across the four seasons.

Correlation between anxiety and aggression

A non-linear quadratic regression analysis ($r = 0.387$, $r^2 = 0.150$; ANOVA: $p = 0.001$) yielded a higher correlation coefficient than a linear regression analysis ($r = 0.292$, $r^2 = 0.085$; ANOVA: $p = 0.005$). Thus, a rather U-shaped correlation characterizes the relationship between anxiety (as measured on the EPM) and intermale aggression (as measured during the RI test) in LAB, HAB and NAB rats (Fig. 5C).

Distribution of LAB, HAB and NAB rats in low-, medium- and high- aggressive groups

Rats were classified according to their level of total aggressive behaviour displayed during the RI test in either low- (< 15 %), medium- (between 15 and 55 %) or high-aggressive (> 55 %) groups. Almost 80 % of the LAB and HAB rats are categorized as medium-aggressive (Fig. 8), and almost 10 % of the LAB rats, but none of the NAB or HAB rats, belonged to the most aggressive group. In contrast, more than 50 % of NAB rats were categorized as low-aggressive (Fig. 8).

Experiment 2: Abnormal aggression in male LAB, HAB and NAB rats

Abnormal aggression towards a male intruder

Line differences were observed for attack latency ($F_{(2,27)} = 9.10$; $p < 0.005$; Fig. 9A), the percentage of attacks ($F_{(2,19)} = 7.22$; $p < 0.01$; Fig. 9B) and the number of attacks ($F_{(2,27)} = 3.91$; $p < 0.05$; Data not shown) towards vulnerable body parts of the intruder. In detail, LAB residents showed a shorter attack latency ($p < 0.005$; Fig. 9A), a higher percentage of attacks

($p < 0.01$; Fig. 9B), and a higher number of attacks ($p < 0.05$; data not shown), compared with NAB rats. Interestingly, a higher percentage of attacks directed towards vulnerable body parts was also found in HAB compared with NAB rats ($p < 0.05$; Fig. 9B).

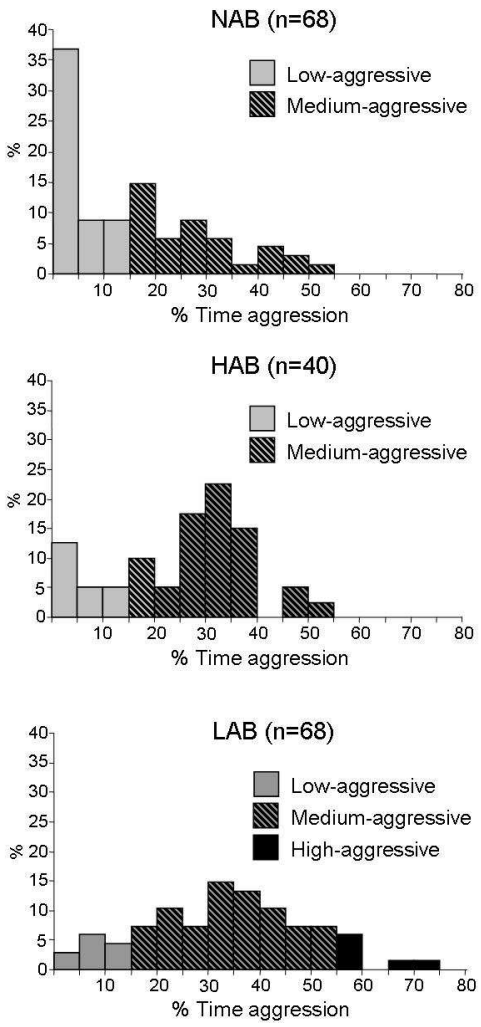

Fig. 8: Distribution of male NAB, HAB, and LAB residents according to their aggression level (% time aggressive behaviour towards a male intruder) displayed during the RI test.

Aggression towards a non-oestrus female intruder

A line difference was found for the latency to attack a female intruder ($F_{(2,28)} = 8.61$; $p < 0.005$), with LAB residents attacking faster than NAB ($p < 0.01$) and HAB ($p < 0.005$) residents (Fig. 9A). Moreover, differences in the number of attacks ($F_{(2,28)} = 6.55$; $p < 0.01$) were found, with more attacks displayed by LAB compared with NAB ($p < 0.05$) and HAB ($p < 0.01$) rats (Fig. 9B).

Aggression towards a narcotised male intruder

Line differences were found for attack latency ($F_{(2,28)}$ = 7.04; $p < 0.005$) and for the percentage of attacks towards the head of the intruder ($F_{(2,28)}$ = 6.41; $p < 0.01$). In detail, LAB rats had a shorter attack latency than NAB rats ($p < 0.005$; Fig. 9A) and showed a higher percentage of attacks directed towards the head of the intruder compared with NAB ($p < 0.01$) and HAB ($p < 0.05$) rats (Fig. 9B).

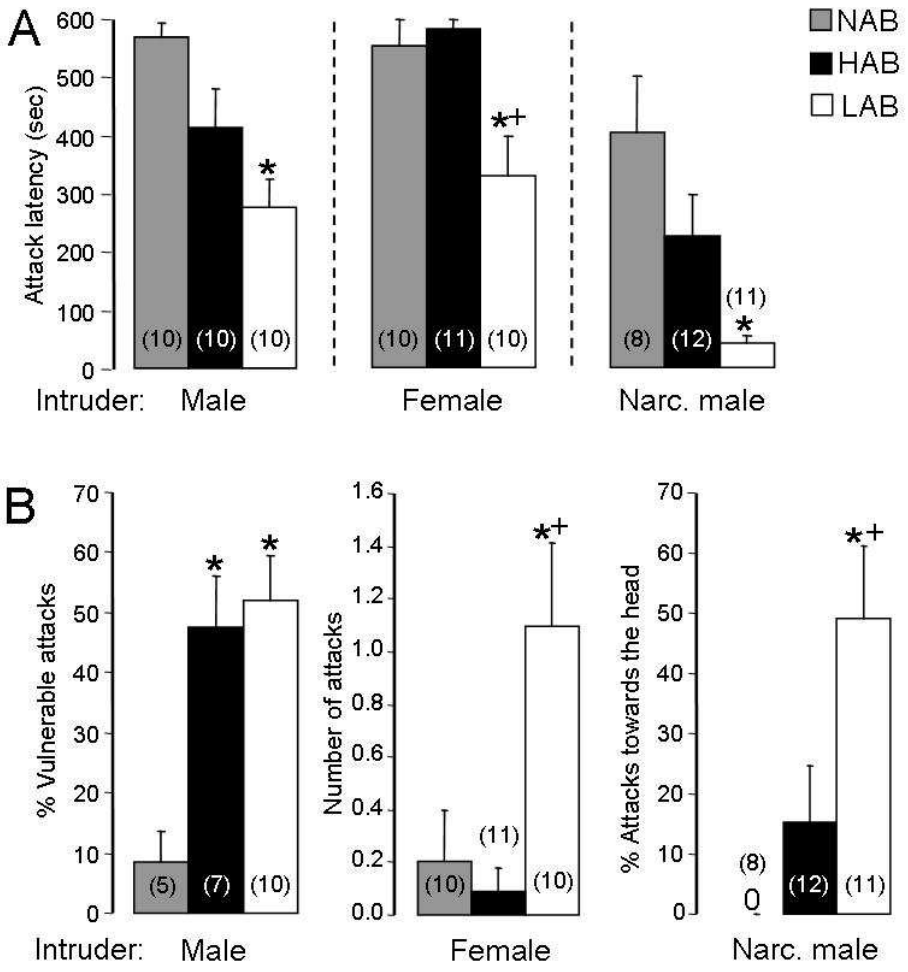

Fig. 9: Abnormal aggression in NAB, HAB and LAB rats. **(A)** Attack latency towards a vulnerable body part of a male intruder, towards a non-oestrus female intruder, and towards a narcotised male intruder. **(B)** Percentage of attacks directed at vulnerable body parts of the male intruder, number of attacks towards a non-oestrus female intruder, and percentage of attacks towards the head of a narcotised male intruder. * $p < 0.05$ vs. NAB, + $p < 0.05$ vs. HAB. Numbers in parentheses indicate group size. Data are presented as means + SEM.

Experiment 3: Effects of S-15535 on aggressive behaviours in male LAB rats

Treatment of LAB residents with the selective serotonin 1_A autoreceptor agonist S-15535 thirty minutes before the RI test decreased the number of attacks towards a male intruder ($p < 0.01$; Fig. 10A) as well as the level of lateral threat ($p < 0.01$) and offensive upright ($p < 0.05$; data not shown) compared with vehicle-treated LAB rats. The total level of aggressive behaviour was not altered. Moreover, when exposed to a narcotised male intruder, three days later, LAB rats treated with S-15535 showed a reduction in the number of attacks compared with vehicle-treated LAB rats ($p < 0.01$; Fig. 10B) without affecting the total level of aggressive behaviour.

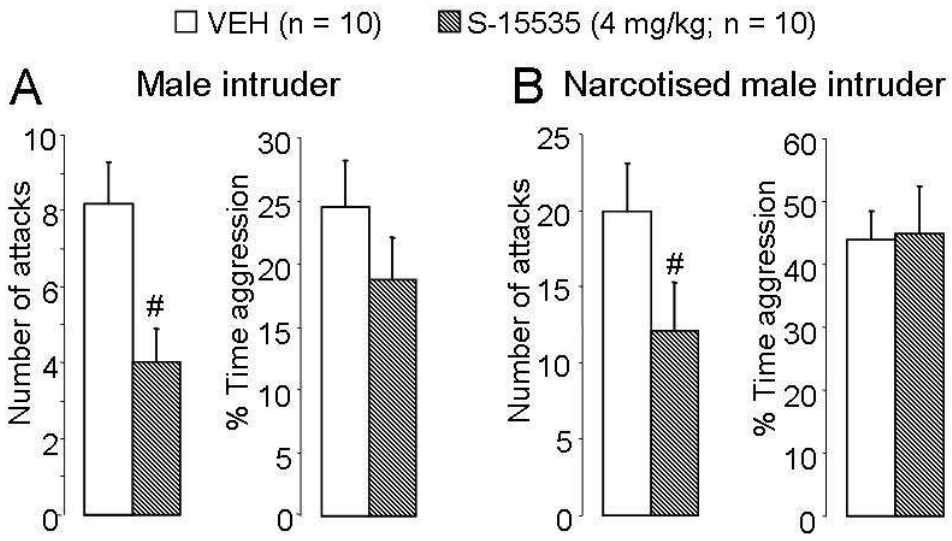

Fig. 10: Behavioural consequences of an acute injection with the serotonin 1_A autoreceptor, S-15535 (4 mg/kg, s.c.), in male LAB rats; controls received vehicle (distilled water; VEH). **(A)** Number of attacks and percentage time of aggressive behaviour towards a male intruder. **(B)** Number of attacks and percentage time of aggressive behaviour towards a narcotised male intruder. # $p < 0.05$ vs. vehicle. Data are presented as means + SEM.

Discussion

Data collected over the last six years (i.e. between 2003 and 2008) show that - as a result of the selective breeding process - the difference in the level of innate anxiety-related behaviour

between LAB and HAB rats is robust and stable. We could further demonstrate that low trait anxiety of LAB rats is linked to high intermale aggression when compared with NAB rats, whereas high trait anxiety of HAB rats is linked to a rather intermediate level of aggression. This resulted in a U-shaped correlation between anxiety and intermale aggression. Further, both LAB and HAB male rats showed signs of abnormal aggression, as reflected by a high percentage of attacks aimed at vulnerable targets of a male intruder. LAB rats displayed also a shorter attack latency towards a non-oestrous female and a narcotised male intruder. We further demonstrated the involvement of the brain serotonin system in the high and abnormal aggression of LAB rats.

Originating from outbred Wistar rats, LAB and HAB rats have been selectively bred for low and high anxiety-related behaviour, respectively, on the EPM since 1993 (Liebsch *et al.*, 1998b; Landgraf & Wigger, 2002; Keck *et al.*, 2003), but it remained largely unknown, how trait anxiety and aggression are associated with each other. The difference in trait anxiety between LAB and HAB rats is accompanied by differences in several stress coping behaviours, including risk assessment in a novel environment and immobility behaviour during the forced swim test (Liebsch *et al.*, 1998a; Henniger *et al.*, 2000; Ohl *et al.*, 2001; Neumann *et al.*, 2005a; Bosch *et al.*, 2006). Moreover, LAB and HAB rats differ in several aspects of social behaviours, including submissive/defensive behaviour during the social defeat test (Frank et al., 2006), and aggressive behaviour either during the RI test as demonstrated in the present study or during the maternal defence test performed in lactating dams (Bosch et al., 2005). In general, male HAB rats prefer a passive stress coping style and have been validated as a model for anxiety- and depression-related behaviours (Landgraf & Wigger, 2002), whereas male LAB rats have a more active stress coping style and have been proposed as a model to study aggression-related behaviours (Veenema & Neumann, 2007). Since 2003, when LAB and HAB rats were transferred to and bred at the University of Regensburg, Germany, their respective level of innate anxiety has remained robust and highly

consistent as demonstrated in the present and earlier studies (Bosch *et al.*, 2005; Neumann *et al.*, 2005a; Neumann *et al.*, 2005b; Bosch *et al.*, 2006; Beiderbeck *et al.*, 2007; Veenema *et al.*, 2007b; Bosch & Neumann, 2008). Moreover, data collected over the same time period demonstrate that LAB males have a consistently higher level of intermale aggression than NAB males, whereas HAB males are intermediate (Beiderbeck et al., 2007; Veenema et al., 2007b).

The observation that not only LAB, but also HAB rats, show relatively high levels of intermale aggression compared with NAB rats indicates that selective breeding for both low and high trait anxiety may result in a shift towards higher aggressiveness. This is further supported by the U-shaped correlation that characterizes the relationship between anxiety and intermale aggression. The negative linear correlation described before between inborn anxiety and intermale aggression was largely due to the individual variance in anxiety and aggression levels within the LAB line (Veenema et al., 2007b). Importantly, in our previous study, LAB rats also showed the highest level of aggression, while HAB rats had intermediate aggression levels, with NAB rats showing the lowest level of aggression (Veenema et al., 2007b), thus, confirming the present findings.

The paradoxical finding that both a low as well as a high level of anxiety correlates with high levels of intermale aggression may explain, in part, the inconsistent findings in the literature regarding the relationship between anxiety and aggression in rodents. For example, some reported a negative correlation between intermale aggression and anxiety (Nyberg et al., 2003), whereas others found that a high level of intermale aggression is associated with a high level of anxiety in mice (Ferrari et al., 1998; Veenema et al., 2006; Veenema et al., 2007a). Our present study includes data of the behavioural extremes in anxiety as well as of non-selected rats, which resulted in a sufficient individual variation in anxiety and in aggression enabling the demonstration that low as well as high trait anxiety can underlie high aggressiveness.

High aggressiveness in humans and in rodents has been associated with opposing states of arousal. For example, patients with mood disorders show a hyper-arousal-driven aggression, which is associated with high autonomic and HPA axis responses (Mazur, 1994; Cohen *et al.*, 1996). High aggressiveness associated with a state of hyper-arousal has also been found in different animal models (Hayden-Hixson & Ferris, 1991; de Almeida & Miczek, 2002; Kruk *et al.*, 2004; Mikics *et al.*, 2004). In contrast, a state of hypo-arousal has been associated with heightened aggression in animal models (Halasz et al., 2002; Haller et al., 2006) and is also seen in patients with personality disorders (Virkkunen, 1985; Raine, 1996; Brennan *et al.*, 1997; Dolan *et al.*, 2001; Haller & Kruk, 2006). Could the elevated level of aggression in both LAB and HAB rats be explained by such opposing physiological responses? HPA axis and autonomic responses of LAB and HAB rats do indeed differ, depending on a social versus a non-social context. For example, compared with HAB rats, male LAB rats show a lower HPA axis response when exposed to non-social stressors, such as a novel environment (Landgraf *et al.*, 1999; Salome *et al.*, 2004; Neumann *et al.*, 2005b), but a higher HPA axis response when exposed to a social stressor such as the RI test either as intruder (Frank *et al.*, 2006) or as resident (Veenema et al., 2007b). Thus, the context of the stressor (i.e. social versus non-social) may determine the extent of the stress response, and is dependent on the genetically-determined level of emotionality (Veenema & Neumann, 2007). The finding that aggression in LAB rats is associated with high physiological and neuronal responses, whereas aggression in HAB rats is associated with a rather moderate response, may shed some light onto different mechanisms underlying a high level of aggression. Therefore, LAB and HAB rats represent a unique animal model for studying distinct neurobiological principles regulating high aggressiveness.

During a fight between two adult males, only a minority of attacks is aimed at vulnerable body parts, such as head, throat, and belly (Blanchard *et al.*, 2003), which is a trait that has probably evolved and established in order to protect individuals of a given species against

dangerous forms of competitiveness (Haller & Kruk, 2006). Accordingly, NAB rats displayed less than 10 % of the attacks towards vulnerable body parts of the male intruder. In contrast, LAB and HAB rats showed a significantly higher percentage of attacks directed at vulnerable targets (51.8 % and 47.5 %, respectively). This abnormal attack targeting suggests alterations in threat perception, an impairment in the processing of social cues and/or a lack of behavioural control (Haller & Kruk, 2006). Accordingly, we hypothesize changes within the prefrontal cortex and septum, as well as in autonomic and/or HPA systems. A lack of prefrontal cortex and lateral septal activation has been associated with high and violent forms of aggression indicating a lack of behavioural control both in humans and rodents (Raine *et al.*, 2000; Blair, 2004; Haller *et al.*, 2006; Caramaschi *et al.*, 2008; Centenaro *et al.*, 2008). In confirmation, LAB rats have a lower neuronal activation and a reduced AVP release within the lateral septum and a higher plasma ACTH level in response to the RI test than HAB rats (Beiderbeck et al., 2007; Veenema et al., 2007b). However, in a previous study, a higher neuronal activation in the prefrontal cortex has been found in LAB compared with HAB rats in response to open arm exposure and social defeat (Kalisch et al., 2004). Neuronal activation patterns could differ if the experimental rat is the resident in the RI test, but so far, data are lacking.

Interestingly, LAB rats also showed a shorter attack latency and a higher number of attacks than NAB and HAB rats when confronted with a non-oestrus female intruder. Aggressive behaviour towards a non-oestrous female is rare, likely maladaptive and may indicate social communication deficits (Sluyter *et al.*, 2003; Nyberg *et al.*, 2004). Moreover, as another indication of their abnormal aggression, LAB rats attacked a narcotised male intruder more rapidly, and more attacks were directed against its vulnerable body parts. During agonistic fights, attacks are normally limited by submissive and defensive behaviours of the opponent, and by vibrissae-contact between attacker and defender (Blanchard *et al.*, 1977). The lack of appropriate agonistic reactions of the narcotised animal may have promoted high and

abnormal aggression in LAB males. Nevertheless, even in highly aggressive mice, attacks towards a narcotised intruder were observed in only one out of three mice (Natarajan *et al.*, 2008). Taken this into account, LAB rats clearly disregard species-specific rules by attacking females and narcotised males and are an interesting model to study abnormal aggressive behaviour.

In LAB rats, an unexpected seasonal effect on anxiety was found, resulting in even lower levels of anxiety in summer. There are several studies in humans suggesting seasonal effects on anxiety in mental disorders (Marriott *et al.*, 1994; de Graaf *et al.*, 2005; Ohtani *et al.*, 2006), but to our knowledge there are no comparative rodent studies so far. Moreover, LAB rats show a lower level of aggression in winter. Seasonal variations in RI aggression, even within laboratory settings with constant diurnal lighting and temperature conditions, have been found before (Miczek *et al.*, 2002; de Boer *et al.*, 2003). Interestingly, also in humans there is an annual rhythm of aggression with lower levels in winter (Miczek *et al.*, 1992; de Boer *et al.*, 2003). Future clinical and preclinical research should take into account possible seasonal effects on anxiety and aggression.

Despite other candidates, the serotonin system remains the primary molecular determinant of aggression identified so far (Michael & Zumpe, 1983; Maes *et al.*, 1995; Tiihonen *et al.*, 1997; Tellez *et al.*, 2006). Pharmacological activation of serotonin 1_A autoreceptors by S-15535 potently suppressed the display of aggressive behaviour in male rats (Miczek *et al.*, 2002; Olivier, 2004; de Boer & Koolhaas, 2005; Popova, 2006). This suggests that the reduction in aggression is predominantly based on reduction of serotonin neurotransmission during intermale aggression. Administration of S-15535 to the high-aggressive LAB males decreased attacks towards a male or narcotised male intruder. However, the overall level of aggressive behaviour was not altered, which suggests that pharmacological reduction in serotonin neurotransmission by activation of serotonin 1_A autoreceptors of LAB males selectively reduces the attack component in models of both normal (male intruder) as well as

abnormal (narcotised intruder) aggression. However, more detailed pharmacological studies are essential to elucidate the neurobiological mechanisms underlying high and abnormal levels of intermale aggression in more depth.

In conclusion, bi-directional selection based on anxiety-related behaviour of Wistar rats resulted in a shift towards higher intermale aggression compared with NAB rats. Furthermore, LAB rats showed the highest levels of aggression and most robust signs of abnormal aggressive behaviour, which could partly be reduced by pharmacological inactivation of the brain serotonin system. Both LAB and HAB rats are attractive animal models in understanding different neurobiological mechanisms underlying elevated aggressive behaviour.

Chapter 3

Low inborn anxiety correlates with high intermale aggression: link to ACTH response and neuronal activation of the hypothalamic paraventricular nucleus

[adapted from: Veenema AH, Torner L, Blume A, Beiderbeck DI, Neumann ID; 2007; Hormones and Behavior 51: 11-19]

Abstract

Aggression constitutes a central problem in several psychopathologies, including anxiety and depression disorders and antisocial behaviours. In particular, the activity of the HPA axis has been associated with aggression-related disorders. The present study assessed whether genetically-determined levels of anxiety-related behaviour influence the level of intermale aggression and whether this is associated with differences in neuroendocrine responsiveness and neuronal activation in the brain. Adult male Wistar rats bred for high or low anxiety-related behaviour were used, as well as non-selected rats with an intermediate anxiety level. LAB residents displayed more aggressive behaviour than HAB and NAB residents during the RI test. Moreover, an inverse correlation was found between the level of anxiety and the level of aggression. The plasma ACTH response to RI test exposure was significantly higher in LAB than in HAB and NAB rats, indicating that a higher level of aggression was linked to an elevated hormonal stress response. Furthermore, LAB residents showed more neuronal activation in the parvocellular part of the PVN than HAB residents one hour after the RI test. In addition, a tendency towards a higher number of c-Fos-positive cells in LAB compared with HAB rats was observed in the medial amygdala, hypothalamic attack area and central amygdala, areas relevant for the regulation of aggression. These data demonstrate that low trait anxiety is correlated with high intermale aggression. Furthermore, the increased neuronal activation of the PVN along with the higher ACTH responsiveness might underlie the display of high aggression.

Introduction

Aggressive behaviour has been associated with numerous neurological and psychiatric conditions, including anxiety and depression disorders (Apter *et al.*, 1990; van Praag, 1998; Fehon *et al.*, 2001). In preclinical research, aggressive behaviour has been predominantly

studied by utilizing the natural drive of animals to defend their territory (Blanchard *et al.*, 2003). Furthermore, to mimic abnormal forms of male aggression, and to reveal the underlying brain mechanisms, animal models, such as glucocorticoid hypofunction (Haller *et al.*, 2001; Haller *et al.*, 2004), olfactory bulbectomy (Leonard & Tuite, 1981; Mucignat-Caretta *et al.*, 2004), alcohol administration (Miczek et al., 1997), social instigation and frustrative non-reward (de Almeida et al., 2005), have been developed. Additionally, the etiology of psychiatric disorders, and the appearance of behavioural abnormalities, are influenced by the genetic background (Caspi *et al.*, 2002; Caspi *et al.*, 2003). Therefore, selective breeding of animals for extremes in behaviour constitutes a powerful research tool to analyse genetic and neurobiological correlates of specific psychopathologies (Steimer *et al.*, 1997; Landgraf & Wigger, 2002; Sluyter *et al.*, 2003; Veenema *et al.*, 2004; Overstreet *et al.*, 2005).

In our laboratories, Wistar rats have been selectively bred for high or low anxiety-related behaviour (Liebsch *et al.*, 1998a; Landgraf & Wigger, 2002; Neumann *et al.*, 2005a). HAB and LAB rats also differ in other parameters of stress coping strategies in addition to their difference in anxiety. HAB rats show a more passive strategy, as indicated by, e.g., increased freezing during social defeat, more floating in the forced swim test, increased risk assessment and decreased exploration in the modified hole board and open field (Liebsch *et al.*, 1998b; Ohl *et al.*, 2001; Bosch *et al.*, 2005; Frank *et al.*, 2006). These behavioural characteristics of HAB rats are genetically determined (Murgatroyd et al., 2004), and represent some symptoms prevalent in human anxiety and depression disorders (Keck et al., 2003). In contrast to HAB rats, LAB rats are less anxious and show a more active coping style when exposed to non-social and social stimuli, also compared with Wistar rats not selected for anxiety-related behaviour (Liebsch *et al.*, 1998b; Ohl *et al.*, 2001; Bosch *et al.*, 2005). Moreover, LAB rats show a lower reactivity of the HPA axis to non-social stressors (Landgraf *et al.*, 1999; Salome *et al.*, 2004) and a lower level of social interaction compared with HAB rats (Henniger *et al.*,

2000; Ohl *et al.*, 2001). In humans, low resting salivary cortisol levels have been associated with antisocial behaviour, conduct problems and persistent aggressive behaviour (McBurnett & Lahey, 1994; McBurnett *et al.*, 2000; Shoal *et al.*, 2003). Cortisol reactivity was also found to relate to antisocial and aggressive behaviours. Some studies reported an attenuated (Moss *et al.*, 1995; van Goozen *et al.*, 1998), while others reported an elevated (Susman *et al.*, 1997; McBurnett *et al.*, 2005; van Bokhoven *et al.*, 2005a) cortisol reaction to experimental stressors. Information about HPA axis responses during the display of aggression in animal models is very limited. Therefore, the LAB rats are an interesting candidate for studying intermale aggression and their underlying neuroendocrine mechanisms.

In the present study HAB, LAB and NAB rats were used in order (i) to investigate the influence of genetic variation in anxiety-related behaviour on the display of intermale aggression, (ii) to reveal whether differences in aggressive behaviour are accompanied by differences in neuroendocrine responsiveness (by measuring plasma ACTH, corticosterone, and testosterone concentrations), and (iii) to study whether different levels of aggressive behaviour are accompanied by a different neuronal activation within brain regions implicated in aggressive behaviour.

Materials and Methods

Animals

Experiments were carried out with male rats (400-450 g body weight) selectively bred for either high or low anxiety-related behaviour on the EPM over the last decade (Landgraf & Wigger, 2002). In addition, male NAB rats (400-450 g body weight, Charles River, Sulzfeld, Germany) were included to confirm selective breeding for both high and low anxiety-related behaviour. The rats were kept under standard laboratory conditions (12:12 light/dark cycle with lights on at 6:00 a.m., 22°C, 60 % humidity, and food and water *ad libitum*). All rats

were housed in groups of 3-5 unless mentioned otherwise. All experimental procedures were approved by the local government of Bavaria, Germany.

Anxiety-related behaviour

At the age of ten weeks, HAB (n = 9), LAB (n = 7), and NAB (n = 7) males were tested on the EPM in order to assess their level of anxiety. The EPM test is based on creating a conflict between the rat's exploratory drive and its innate fear of open and exposed areas. This test has been validated for the detection of emotional responses to anxiogenic and anxiolytic substances (Pellow et al., 1985). The EPM consists of a plus-shaped platform elevated 70 cm above the floor, with two closed (50 × 10 × 40 cm) and two open arms (50 × 10 cm). During the 5-minute test, the following parameters of anxiety-related behaviour were scored using a video/computer system: the percentage of open arm entries [open arm entries/(open + closed entries) x 100 %; an entry is defined as both fore-paws of the rat being on the arm], the percentage of time spent on the open arms [time on open arms/(time on open arms + time on closed arms) x 100 %], the number of full open arm entries (which is defined as the entire body of the rat being on the open arm). Moreover, the number of closed arms was recorded as parameter of locomotor activity.

Intermale aggression

Two weeks after EPM testing, each HAB, LAB and NAB male was housed with a female Wistar rat (Charles River, Sulzfeld, Germany) for 18 days in an observational cage (40 × 24 × 36 cm) to increase territorial behaviour. Male rats were tested twice for aggression, at day 18 (one day prior jugular vein catheterisation) and day 24 (five days after surgery). Aggressive behaviour was tested between 10:00 a.m. and noon using the RI test. The female rats were removed 30 minutes prior the first RI test and were not returned. At the time of testing, a smaller (40-60 g lighter) unfamiliar male Wistar rat (intruder, Charles River, Sulzfeld,

Germany) was introduced into the home cage of the male HAB, LAB or NAB resident for a period of ten minutes. The RI tests were videotaped for subsequent behavioural scoring (Eventlog, version 1.0, October 1986, R. Hedersen) by an experienced researcher blind to the treatment condition. The following parameters related to male aggression were measured: attack latency time, number of attacks, duration of lateral threat, clinch, offensive upright, and keep down. The duration of the latter four behavioural parameters was also summarised as the total duration of aggressive behaviour. The durations of social behaviour (investigating opponent, anogenital sniffing, mount), exploration, self grooming, and immobility were also quantified, see Koolhaas et al. (1980) for a detailed description of agonistic behaviour.

Surgery and blood sampling

One day after the first RI test, the resident HAB, LAB and NAB males were implanted with a chronic jugular vein catheter under isoflurane anesthesia under sterile conditions (Neumann et al., 1998). Briefly, the jugular vein was exposed and a silicone tubing catheter (4 cm; Dow Corning, USA) connected to a PE-50 polyethylene tube was inserted approximately 3 cm into the vessel until the tip reached the right atrium; the catheter was then exteriorised dorsally in the cervical region and filled with sterile saline (0.9 %) containing gentamicin (30,000 i.u./ml; Centravet, Germany). Following surgery, the males were handled carefully each day to familiarize them with the blood sampling procedure and to reduce non-specific stress responses during the experimental procedures. Five days after surgery, ACTH, corticosterone and testosterone responses to the RI test were measured. At 8:00 a.m., the jugular vein catheter was attached to an extension tube connected to a 1-ml plastic syringe filled with sterile heparinized 0.9 % saline (30 IU/ml, Heparin-Natrium, Ratiopharm, Ulm, Germany). The rats were then left undisturbed for the next two hours. Blood samples of 0.3 ml, substituted immediately by sterile 0.9 % saline, were taken under basal conditions, and 15 and

60 minutes after the onset of the 10-minute RI test. The catheter was disconnected from the extension tube and closed before the males were exposed to the RI test and reattached thereafter.

Hormone assays

All blood samples were collected in chilled ethylenediaminetetraacetic-acid-coated tubes (Sarstedt, Nümbrecht, Germany) containing 10 µl aprotinin (Trasylol, Bayer AG, Leverkusen, Germany) and centrifuged at 2600×g for five minutes at 4°C. Plasma aliquots (70 µl for ACTH, 10 µl for corticosterone, 50 µl for testosterone) were stored at –20°C until assayed. Plasma ACTH, corticosterone and testosterone were determined using commercially available radioimmunoassays (ICN Biomedicals, Inc., Costa Mesa, CA). Detection limits for ACTH, corticosterone and testosterone were 4 pg/ml, 10 ng/ml and 0.6 ng/ml, respectively.

c-Fos immunocytochemistry

c-Fos expression is a widely used marker of neuronal activity (Sagar et al., 1988). To investigate whether line differences in aggression are accompanied by differences in neuronal activation of selected brain regions, another set of male HAB (n = 5) and LAB (n = 6) rats was housed in observational cages as described above (NAB resident males were not included, as most NAB males did not display aggressive behaviour). At day 18, males were exposed to the 10-minute RI test and one hour later, they were deeply anaesthetised with pentobarbital (0.1 ml/100 g, i.p.) and perfused intracardially with 150 ml ice-cold phosphate-buffered saline, followed by 300 ml 4 % paraformaldehyde in ice-cold phosphate buffer (pH 7.4). Brains were removed, post-fixed overnight in 4 % paraformaldehyde and immersed in 30 % sucrose for cryoprotection for three days. Immunocytochemistry was performed on coronal, cryostat-cut, free floating slices (50 µm). Incubation with anti-c-Fos rabbit antiserum

(Oncogene; Cambridge, MA, USA) was followed by enhancement of the signal by binding of a secondary antibody and detection using the conventional avidin-biotin complex peroxidase reaction with diaminobenzidine as chromogen (vectastain ABC kit and DAB chromogen purchased from Alexis; Gruenberg, Germany). The dilution of the anti-c-Fos antiserum was 1:10.000. For quantification of c-Fos-positive cells, the number of stained neurons of the brain regions listed below was counted by two investigators in a blinded manner for each group on at least two sections from each rat brain. Sections were taken from the same level of each brain region and the specific brain nuclei using the rat brain atlas of Paxinos and Watson (1996). The following areas were analysed, as c-Fos induction was found in these areas after RI test exposure (Halasz et al., 2002) or after stressor exposure (Salome *et al.*, 2004; Frank *et al.*, 2006): the cingulate cortex, BNST, medial preoptic area, paraventricular thalamic nucleus, nucleus of the lateral habenula, supraoptic nucleus, anterior hypothalamus, parvo- and magnocellular parts of the PVN, arcuate nucleus, hypothalamic attack area, medial amygdala, central amygdala.

Statistics

All statistical analyses were performed using the software package SPSS (version 12). Behaviour and c-Fos expression were analysed using one way ANOVA. Plasma ACTH, corticosterone and testosterone concentrations were analysed using ANOVA for repeated measures (factor time × factor line). ANOVA was followed by Bonferroni *post-hoc* test when appropriate. Correlation analysis of anxiety (percentage of time spent on open arms) with aggression (percentage of time) was carried out using simple regression analysis. Data are presented as means ± SEM. Statistical significance was set at $p < 0.05$.

Results

Anxiety-related behaviour

The difference in innate anxiety between male HAB, LAB and NAB rats was confirmed on the EPM (percentage of open arm entries: $F_{(2,20)} = 22.71$, $p < 0.001$; percentage of time spent on the open arms: $F_{(2,20)} = 68.34$, $p < 0.001$; number of full open arm entries: $F_{(2,20)} = 13.83$, $p < 0.001$). Open arm entries (two fore-paws on the arm) and full open arm entries (entire body of the rat on the arm) correlated significantly ($r = 0.431$, $p < 0.05$, simple regression analysis). HAB males were more anxious than LAB and NAB males as reflected by a lower percentage of open arm entries ($p < 0.001$; Fig. 11) and lower percentage of time on open arms ($p < 0.001$ vs. LAB, $p < 0.05$ vs. NAB; Fig. 11). Bidirectional breeding was confirmed as NAB males were more anxious than LAB males (time on the open arms, $p < 0.001$; full entries onto the open arms, $p < 0.005$; Fig. 11). Locomotor activity, as measured by the entries onto the closed arms ($F_{(2,20)} = 12.19$, $p < 0.001$), was significantly lower in HAB than in LAB ($p < 0.01$) and NAB ($p < 0.001$) males (Fig. 11). Thus, HAB and LAB males represented the extremes in anxiety-related behaviour, whereas NAB males displayed an intermediate level of anxiety-related behaviour.

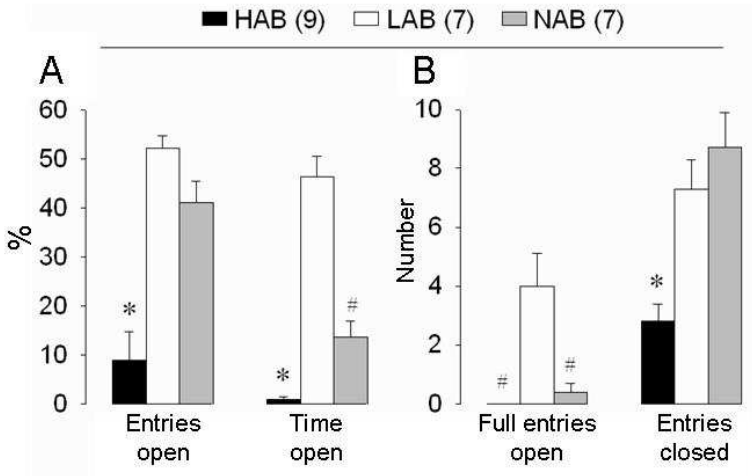

Fig. 11: Anxiety-related behaviour measured on the EPM in HAB, LAB and NAB males. **(A)** Percentage of open arm entries (both fore-paws on the arm) and percentage of time spent on the open arms. **(B)** Number of full open arm entries (entire body of the rat being on the open arm) and entries into the closed arms (indicates locomotor activity). * $p < 0.01$ versus LAB and NAB, # $p < 0.05$ versus LAB, ANOVA.

Intermale aggression

First RI test

HAB, LAB and NAB males differed significantly in aggression and attack latency (see Tab. 3 for statistical details). In detail, LAB males showed significantly more aggressive behaviour than HAB as well as NAB males, and had a shorter attack latency than NAB males (Tab. 3). Among the elements of aggressive behaviour, LAB males showed significantly more lateral threat and offensive upright posture than HAB and NAB males (Tab. 3). Furthermore, LAB males showed significantly less non-aggressive social behaviour than HAB males and less exploration than NAB males (Tab. 3).

Tab. 3: Behavioural parameters of HAB, LAB and NAB rats during the first 10-minute RI test. Aggressive behaviour, social behaviour and exploration were calculated as percentage of time.

	HAB	LAB	NAB	$F_{(2,20)}$ / p
Aggressive behaviour				
Total	4.2 ± 1.6	11.5 ± 3.0*	0.7 ± 0.5	6.91 / 0.005
Lateral threat	2.1 ± 0.8	8.9 ± 2.8*	0.1 ± 0.1	6.99 / 0.005
Offensive upright	0.1 ± 0.1	0.9 ± 0.2*	0.1 ± 0.1	9.77 / 0.001
Keep down	1.9 ± 1.0	1.7 ± 0.8	0.5 ± 0.5	0.74 / 0.492
Clinch	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	1.97 / 0.165
Attack latency (sec)	359 ± 80	277 ± 78$^{\#}$	598 ± 1	5.25 / 0.015
Number of attacks	2.3 ± 1.1	2.0 ± 0.5	0.1 ± 0.1	2.15 / 0.143
Social behaviour	34.6 ± 4.2	18.1 ± 2.1§	24.0 ± 0.9	6.25 / 0.008
Exploration	49.7 ± 3.5	46.4 ± 3.6$^{\#}$	61.3 ± 4.0	3.95 / 0.036

Data are presented as means ± SEM. F: ANOVA for line effects, * $p < 0.05$ versus HAB and NAB, § $p < 0.01$ versus HAB, $^{\#}$ $p < 0.05$ versus NAB, Bonferroni *post hoc* test.

Second RI test

Five days after surgery, HAB, LAB and NAB males underwent a second RI test, which recapitulated the results of the first RI test, in which LAB males showed a higher level of aggression than HAB and NAB males (aggressive behaviour: $F_{(2,20)} = 20.36$, $p < 0.001$; attack latency: $F_{(2,20)} = 13.06$, $p < 0.001$; number of attacks: $F_{(2,20)} = 6.62$, $p < 0.01$).

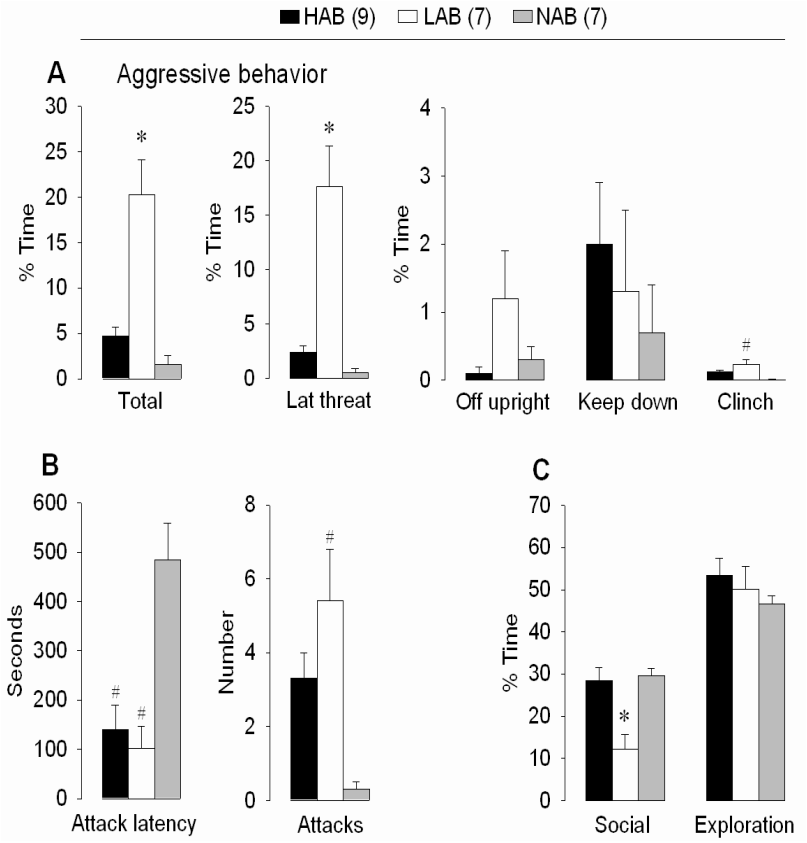

Fig. 12: Behavioural parameters of HAB, LAB and NAB males during the second RI test. **(A)** The duration of aggressive behaviour, **(B)** The attack latency time and the number of attacks, and **(C)** The duration of social behaviour and exploration. * $p < 0.005$ versus HAB and NAB, # $p < 0.01$ versus NAB, ANOVA followed by Bonferroni *post hoc* test.

In detail, LAB males showed more aggressive behaviour than both HAB and NAB males ($p < 0.001$; Fig. 12), displayed more attacks than NAB males ($p < 0.005$; Fig. 12), and LAB as well as HAB males had shorter attack latencies than NAB males ($p < 0.005$; Fig. 12). Among the elements of aggressive behaviour, differences were found for lateral threat ($F_{(2,20)} = 21.19$, $p < 0.001$) and clinch ($F_{(2,20)} = 6.14$, $p < 0.01$). LAB males showed more lateral threat than HAB and NAB males ($p < 0.001$; Fig. 12) and more clinch than NAB males ($p < 0.01$; Fig.

12). Furthermore, a difference was found for non-aggressive social behaviour ($F_{(2,20)} = 10.22$, $p < 0.005$), with the LAB males showing less non-aggressive social behaviour than HAB and NAB males ($p < 0.005$; Fig. 12).

Correlation of anxiety with aggression

A significant correlation was found between the percentage of time spent on the open arms and the percentage of time of aggressive behaviour during the second RI test in HAB, LAB and NAB rats ($r = 0.76$, $r^2 = 0.562$, $p < 0.001$). Here, a low level of anxiety correlated with a high level of aggression (Fig. 13).

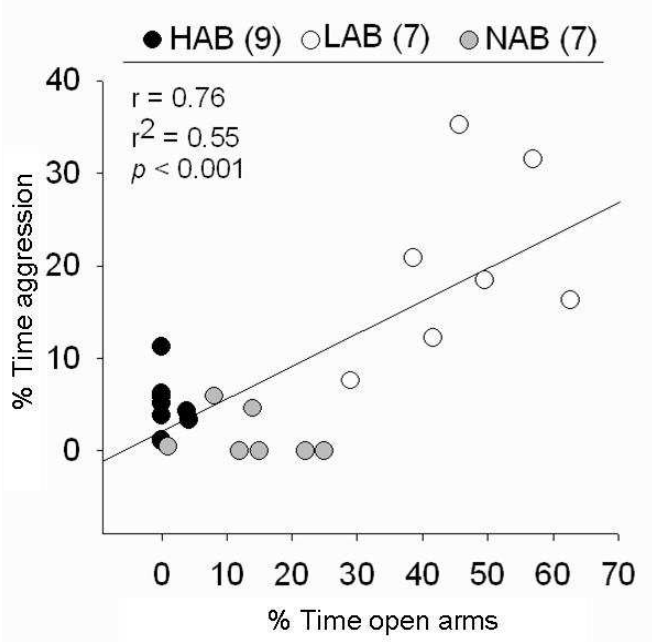

Fig. 13: Correlation of anxiety (percentage of time spent on the open arms) with aggression (percentage of time of aggressive behaviour during the second RI test) in HAB, LAB and NAB males.

Plasma hormone responses

ACTH

Line differences were found for plasma ACTH responsiveness to the second RI test (time × line: $F_{(4,40)} = 6.75$, $p < 0.001$). No difference was found for basal ACTH concentrations between HAB, LAB and NAB males. However, 15 minutes after the onset of the 10-minute

RI test, a rise in plasma ACTH concentrations was found in HAB ($p < 0.005$; Fig. 14) and LAB ($p < 0.001$; Fig. 14) males, but not in NAB males. Furthermore, the plasma ACTH response was significantly higher in LAB compared with HAB males ($p < 0.05$; Fig. 14).

Corticosterone

Significant time ($F_{(2,40)} = 59.94$, $p < 0.001$) and line ($F_{(2,20)} = 3.74$, $p < 0.05$) effects were found for plasma corticosterone concentrations. Basal plasma corticosterone concentrations were higher in LAB than in HAB males ($p < 0.005$; Fig. 14). A rise in plasma corticosterone was found in response to the second RI test in HAB, LAB and NAB males ($p < 0.001$; Fig. 14), with no significant differences in corticosterone responses between the lines.

Testosterone

Plasma testosterone concentrations were significantly altered in response to the second RI test (factor time: $F_{(1,20)} = 6.93$, $p < 0.05$). No significant differences were found for basal plasma testosterone concentrations between the three lines. However, a rise in plasma testosterone was found in response to the RI test in LAB ($p < 0.05$; Fig. 14) and NAB (tendency: $p = 0.054$; Fig. 14) males but was absent in HAB males.

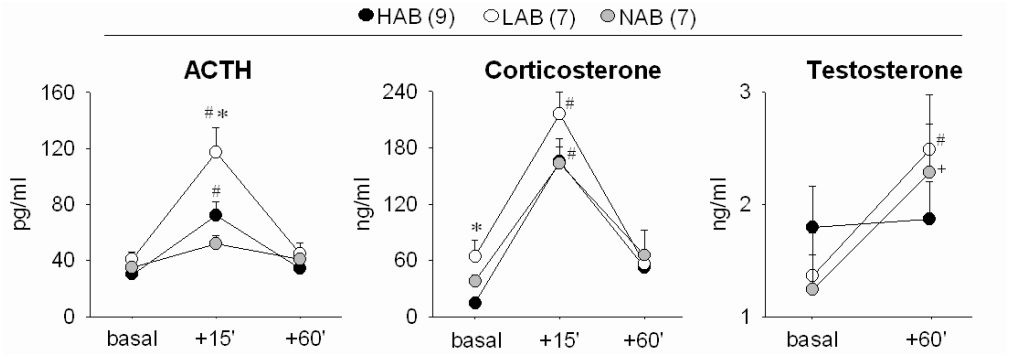

Fig. 14: Plasma ACTH, corticosterone, and testosterone concentrations in HAB, LAB and NAB males under baseline conditions and 15 (except testosterone) and 60 minutes after the onset of the second 10-minute RI test. * $p < 0.05$ versus HAB males, $^{\#}$ $p < 0.05$ and $^{+}$ $p = 0.054$ versus respective baseline, ANOVA for repeated measures followed by Bonferroni *post hoc* test.

c-Fos immunocytochemistry

Exposure of a separate set of HAB and LAB males to a single 10-minute RI test induced c-Fos activation in several brain regions relevant for the regulation of stress responses and the control of aggression, such as the PVN, the central amygdala, the BNST, the anterior hypothalamus, the hypothalamic attack area and the medial amygdala. LAB males had a significantly higher number of c-Fos-positive cells in the parvocellular part of the PVN ($p < 0.05$; Tab. 4) and a tendency towards a higher number of c-Fos-positive neurons in the central amygdala ($p = 0.080$; Tab. 4), the medial amygdala ($p = 0.067$; Tab. 4), and the hypothalamic attack area ($p = 0.067$; Tab. 4) compared with HAB males. Brains of an additional control group of HAB and LAB males housed under identical conditions but not exposed to the RI test, contained negligible c-Fos-positive cells in all of the brain regions studied (data not shown).

Tab. 4: Number of c-Fos-positive cells in selected brain regions of HAB (n = 5) and LAB (n = 6) resident male rats one hour after the onset of a 10-minute RI test.

	CC	BNST	MPO	PThN	LatHab	SON	AH
HAB	13.1 ± 4.1	18.6 ± 3.9	11.8 ± 4.3	17.9 ± 4.5	18.3 ± 3.2	12.3 ± 6.8	16.0 ± 4.5
LAB	8.6 ± 1.9	10.7 ± 4.2	13.6 ± 3.2	12.3 ± 1.6	16.6 ± 2.8	17.6 ± 3.1	14.4 ± 5.3

	pPVN	mPVN	ArcN	HAA	MeA	CeA
HAB	8.3 ± 4.7	14.2 ± 9.0	13.3 ± 4.9	21.0 ± 3.8	22.3 ± 5.1	3.7 ± 3.7
LAB	31.7 ± 5.0*	7.9 ± 2.7	15.3 ± 2.8	39.6 ± 8.7	43.2 ± 9.2	17.9 ± 6.6

Data are presented as means ± SEM. AH = anterior hypothalamus, ArcN = arcuate nucleus, BNST = bed nucleus of the stria terminalis, CC = cingulate cortex, CeA = central amygdala, HAA = hypothalamic attack area, LatHab = nucleus of the lateral habenula, MeA = medial amygdala, MPO = medial preoptic area, mPVN = magnocellular part of the hypothalamic paraventricular nucleus, pPVN = parvocellular part of the hypothalamic paraventricular nucleus, PThN = paraventricular thalamic nucleus, SON = supraoptic nucleus. * $p < 0.05$ versus HAB, ANOVA.

Discussion

The present study demonstrates that intermale aggression is dependent on the genetically-determined level of anxiety-related behaviour. More specifically, rats selectively bred for low levels of anxiety display a higher level of intermale aggression, accompanied by an elevated plasma ACTH response and a more pronounced neuronal activation of the parvocellular part of the PVN compared with high-anxious rats.

Anxiety and aggression

On the EPM, HAB and LAB males present the extremes in anxiety-related behaviour, while NAB males show a more intermediate level of anxiety-related behaviour, confirming previous findings (Ohl et al., 2001). Although the HAB rats showed less closed arm entries, which reflects reduced locomotor activity, HAB rats did not show less activity in the RI test (present study) nor did they differ from LAB rats in home cage locomotion (Liebsch et al., 1998a).

It has been shown that the innate level of anxiety in rats determines various forms of social behaviour, including social interaction and the search for social contact with cage mates (Henniger *et al.*, 2000; Ohl *et al.*, 2001). Here, we extend these findings by showing an inverse correlation between the inborn level of anxiety-related behaviour and adult intermale aggression during the defense of their territory. These findings are supported by previous studies showing that anxiety and aggression in rodents are inversely related and may be influenced by the same genes (Svare & Leshner, 1973; Fujita *et al.*, 1994; Nyberg *et al.*, 2003). In contrast, a positive relationship (Guillot & Chapouthier, 1996; Ferrari *et al.*, 1998) or a lack of relationship (Berton et al., 1997) between anxiety and aggression has been reported in inbred mouse or rat strains. Thus, in order to reveal a correlation between complex emotional states, such as anxiety and aggression, the choice of the animal model seems to be important. For example, in the above mentioned study of Berton et al. (1997), inbred rat

strains showed only a minor variation in anxiety levels as tested on the EPM (percentage of time spent on open arms varied between 0 and 7 %). Indeed, the range of individual behavioural variability was found to be significantly reduced in domesticated or inbred strains of rodents (de Boer *et al.*, 2003). Additionally, the individual variation in measures of aggression was found to be smaller in NAB compared with LAB rats. Thus, to demonstrate behavioural correlations, the use of animals with extremes in their behavioural performance, such as the outbred HAB and LAB rats, may be of advantage.

The level of male aggression may be related to the way individuals respond to environmental challenges in general (Benus *et al.*, 1991; Koolhaas *et al.*, 1999; de Boer *et al.*, 2003). For example, mice genetically selected for a short attack latency, display an active coping style, while long attack latency mice show a more passive strategy (Sluyter *et al.*, 1996; Veenema *et al.*, 2003a; Veenema *et al.*, 2003b; Veenema *et al.*, 2005a). HAB and LAB rats are also characterized by differences in their passive and active coping styles, respectively, as indicated by their behavioural responses in the modified hole board, the forced swim test and during social defeat (Liebsch *et al.*, 1998b; Ohl *et al.*, 2001; Bosch *et al.*, 2005; Frank *et al.*, 2006). Thus, the higher level of intermale aggression in LAB rats is likely to be part of their active behavioural strategy.

Our study further confirmed the finding that LAB rats generally show less non-aggressive social behaviour, as they make less non-aggressive social contacts with the male intruder than HAB males. In contrast, it has been shown that HAB males spend more time in proximity to their cage mates in a novel environment indicating that individuals with a high level of trait anxiety require more social contact or social support, particularly under stressful circumstances (Ohl et al., 2001). Another indication of the abnormal social behaviour of LAB males is the finding that they seem to persist in their attack behaviour when the intruder adopted a submissive posture. Interestingly, others reported that some high-aggressive rats and mice developed a more violent form of aggression, including decreased social

investigation (de Boer *et al.*, 2003) and attacking females (Sluyter et al., 2003). High violence and social conduct problems are core symptoms in human antisocial personality disorder (Kaylor, 1999; Hill, 2003). Further research is required to fully understand, whether the high level of aggression and low level of social investigation seen in LAB males reflects abnormal forms of social behaviour.

It needs to be mentioned that, in contrast to HAB and LAB males, lactating HAB dams displayed more aggression toward a virgin female intruder than LAB dams (Bosch et al., 2005). This higher level of maternal aggression in HAB dams is in line with their higher level of maternal behaviour (Neumann et al., 2005a). These opposing findings suggest that intermale and maternal aggression represent two distinct varieties of aggressive behaviour. Intermale aggression is thought to be purely offensive, serving to establish and hold territory and/or social rank, where the ultimate goal is to gain exclusive mating rights (Brain, 1981). In contrast, maternal aggression serves to protect the offspring from potentially dangerous conspecifics, and may consist of more defensive patterns of attack (Parmigiani et al., 1988).

Aggression and hormonal responses

In the high-aggressive LAB residents higher basal plasma corticosterone concentrations and higher plasma ACTH responses to RI test exposure were found compared with the low-aggressive HAB residents. In support, in clinical studies, elevated glucocorticoid levels/responses were found in individuals with high aggression levels (Gerra *et al.*, 1997; McBurnett *et al.*, 2005; van Bokhoven *et al.*, 2005a). Furthermore, an acute activation of the HPA axis was found to promote aggressive behaviour in rodents (Haller *et al.*, 2000b; Kruk *et al.*, 2004; Mikics *et al.*, 2004). In addition, ACTH was found to stimulate aggressive behaviour, likely via an extra-adrenal pathway (Brain & Evans, 1977; Clarke & File, 1983). Thus, the higher basal corticosterone levels and the elevated ACTH responsiveness of LAB males during the RI test might be related to their predisposition to display a higher level of

aggression. There are also preclinical (Haller *et al.*, 2001; Haller *et al.*, 2004) and clinical (McBurnett & Lahey, 1994; McBurnett *et al.*, 2000; Shoal *et al.*, 2003) studies reporting an inverse relationship between pituitary-adrenocortical (re)activity and aggressive behaviour. However, in these studies, high levels and abnormal forms of aggression were associated with a chronic glucocorticoid deficiency.

Interestingly, an elevated ACTH response in LAB compared with HAB males has recently been described when they were exposed as intruders in a social defeat paradigm (Frank et al., 2006). LAB intruders not only showed more offensive behaviour, even as intruders, but they also showed reduced signs of emotional distress (less freezing and ultrasound vocalizations) compared with HAB intruders (Frank et al., 2006). Thus, in a social conflict situation like the RI test, LAB males, either being the resident or the intruder, are generally more offensive, which is associated with a hyperresponse of the HPA axis. In contrast, when exposed to a non-social stressor (e.g. novel environment) LAB rats responded with an attenuated rise in plasma ACTH compared with HAB rats (Landgraf *et al.*, 1999; Salome *et al.*, 2004; Neumann *et al.*, 2005b), which reflects a generally lower stressor susceptibility accompanying low trait anxiety.

In both human (Banks & Dabbs, 1996) and animal (Dijkstra *et al.*, 1992; Lucion *et al.*, 1996; Wingfield *et al.*, 2001) studies, male aggression has been associated with plasma levels of testosterone; although a simple causal relationship seems to be rather unlikely (Archer, 2006). In the present study, basal plasma testosterone did not differ between HAB, LAB and NAB residents. LAB and NAB, but not HAB, males showed an increase in plasma testosterone in response to the RI test, which could have affected neuronal networks regulating aggression (Delville *et al.*, 1996b). However, as this rise was seen in both highly aggressive LAB males and low to non-aggressive NAB males, it is less likely to be directly related to the display of aggression.

Neuronal activation and aggression

Exposure of HAB and LAB residents to an unknown male intruder induced c-Fos expression in several brain regions, including the PVN, the central amygdala, the BNST, the anterior hypothalamus, the medial amygdala, and the hypothalamic attack area. These regions are particularly relevant for the regulation of stress and fear (Davis & Shi, 1999; Walker *et al.*, 2003; Herman *et al.*, 2005), and/or territorial aggression (Vochteloo & Koolhaas, 1987; Siegel *et al.*, 1999; Delville *et al.*, 2000; Halasz *et al.*, 2002). Importantly, LAB males showed a significantly higher neuronal activation in the parvocellular part of the PVN, and a tendency towards a higher neuronal activation in the central amygdala. Both the PVN and the central amygdala are strongly implicated in anxiety and fear mechanisms (Davis *et al.*, 1994; Van de Kar & Blair, 1999; Herman *et al.*, 2002), and c-Fos induction was found in both regions in response to anxiety- or fear-evoking stimuli (Duncan *et al.*, 1996; Campeau *et al.*, 1997). In addition, increased c-Fos expression in the PVN and central amygdala was found to be associated with abnormal forms of aggressiveness displayed by adrenalectomized male rats (Halasz et al., 2002). These rats showed attacks toward the head and the belly, which is an inappropriate response during territorial aggression (Blanchard et al., 2003). However, in our study, none of the male rats (HAB, LAB nor NAB) showed such an attack pattern. Therefore, the higher c-Fos expression in the PVN of LAB males is likely to reflect their elevated ACTH response. Indeed, neuronal activation in the PVN has repeatedly been linked to HPA axis activation (Figueiredo *et al.*, 2003; Salome *et al.*, 2004; Windle *et al.*, 2004). Considering the more pronounced neuronal activation in the PVN and HPA axis reactivity, our and previous (Frank *et al.*, 2006) data suggest that LAB males perceive social stimuli as more stressful, while HAB males perceive non-social stimuli as more stressful (Landgraf *et al.*, 1999; Salome *et al.*, 2004). LAB males also tended to show higher c-Fos expression in the medial amygdala and the hypothalamic attack area than HAB males, which might be associated with their higher level of aggression.

In conclusion, the present study demonstrates that innate anxiety is inversely related to the level of intermale aggression. The high level of aggression, as displayed by LAB males, was accompanied by high plasma ACTH responsiveness and increased neuronal activation in the PVN. Future studies need to reveal whether this pattern of activation underlies the display of enhanced aggression.

Chapter 4

Differences in intermale aggression are accompanied by opposite vasopressin release patterns within the septum in rats bred for low and high anxiety

[adapted from: Beiderbeck DI, Neumann ID, Veenema AH; 2007; European Journal of Neuroscience **26**: 3597-3605]

Abstract

Several studies suggest a role for AVP, particularly in the lateral septum, in the regulation of intermale aggression. We used intracerebral microdialysis to monitor the local *in vivo* AVP release within the mediolateral septum of adult male Wistar rats bred for low or high anxiety-related behaviour during exposure to the RI test. LAB residents showed a significantly higher level of aggression than HAB residents as reflected by more time spent with lateral threat, offensive upright and total aggressive behaviour as well as by more attacks and a shorter attack latency. Septal AVP release was significantly decreased in high-aggressive LAB males, while septal AVP release tended to increase in HAB males during RI test exposure. Moreover, LAB residents showed a reduced neuronal activation of the lateral septum, as indicated by fewer c-Fos positive cells, one hour after the RI test. Pharmacological manipulation of the septal AVP system by local application of either synthetic AVP to LAB residents or the selective AVP V1a receptor antagonist (V1a-A) $d(CH_2)_5$Tyr(Me)AVP to HAB residents did not change the level of aggression. However, application of AVP into the septum enhanced anxiety-related behaviour on the EPM in LAB males, while local administration of the V1a-A reduced social investigation in HAB males during the RI test. In conclusion, although AVP release patterns within the septum are dependent on the level of aggression, locally released AVP does not seem to be directly involved in the regulation of aggression, but rather modulates non-aggressive social and anxiety-related behaviours.

Introduction

Clinical and preclinical studies suggest an important role for AVP in the regulation of aggressive behaviour (Koolhaas *et al.*, 1990; Ferris, 1992; Albers & Bamshad, 1998; Coccaro *et al.*, 1998; Ferris, 2005). In humans, a positive correlation was found between the AVP concentration in the cerebrospinal fluid and aggression in personality-disordered subjects

(Coccaro *et al.*, 1998). Recently, AVP has been implicated in social communication in men, as intranasal AVP administration decreased perception of friendly faces and increased perception of anger and threat to neutral human facial expressions (Thompson *et al.*, 2004; Thompson *et al.*, 2006). In rodent studies, there are contradictory reports on the involvement of AVP in the regulation of aggression particularly in the septal area. Infusion of AVP into the lateral septum facilitates offensive aggression in castrated male rats (Koolhaas *et al.*, 1991), and increases flank marking, an aggressive display, in golden hamsters (Ferris & Delville, 1994). Furthermore, more aggressive California male mice have higher AVP-immunoreactive staining in the BNST and more AVP V1a receptors in the lateral septum than less aggressive White-footed mice (Bester-Meredith *et al.*, 1999). In contrast, high-aggressive wild-type rats have lower levels of AVP and a lower AVP fibre density in the lateral septum than low-aggressive wild-type rats (Everts *et al.*, 1997). Likewise, high-aggressive wild house mice have fewer AVP-immunoreactive cells in the BNST and a lower AVP-immunoreactive staining in the lateral septum than low-aggressive wild house mice (Compaan *et al.*, 1993). Additionally, prairie voles show a mating-induced reduction in the density of AVP-immunoreactive fibres in the lateral septum accompanied by the emergence of aggressive behaviour towards intruders (Bamshad *et al.*, 1994; Insel *et al.*, 1995). Thus, data so far are inconclusive about the precise role of the septal AVP system in intermale aggression.

Wistar rats bred for low or high anxiety-related behaviour on the EPM (Liebsch *et al.*, 1998b) differ in various aspects of behavioural stress coping (Landgraf & Wigger, 2002; Veenema & Neumann, 2007). Differences in hypothalamic AVP system activity, due to a single nucleotide polymorphism in the promoter region of the AVP gene identified in HAB rats (Murgatroyd *et al.*, 2004), are likely to underlie, at least partially, the behavioural phenotypes of LAB and HAB rats. Thus, HAB rats have a higher AVP mRNA expression and release within the PVN compared with LAB rats (Keck *et al.*, 2003; Wigger *et al.*, 2004). Recently, we demonstrated that LAB male residents are more aggressive than HAB male residents

during the RI test (Veenema et al., 2007b). Therefore, these rats provide a unique model to study the involvement of brain AVP in the regulation of aggression. In the present study, we used intracerebral microdialysis to locally monitor the *in vivo* release of AVP within the mediolateral septum of LAB and HAB males exposed to the RI test. Moreover, we aimed to reveal the causal involvement of septal AVP in the regulation of aggression by local application of synthetic AVP or a selective V1a-A.

Materials and Methods

Animals

Experiments were carried out with male Wistar rats (350 – 450 g) selectively bred for either low or high anxiety-related behaviour on the EPM (Liebsch *et al.*, 1998b; Landgraf & Wigger, 2002). The LAB and HAB rats used in the present study were bred in the animal facilities of the University of Regensburg, Germany. Rats were housed in groups of 4-6 under standard laboratory conditions (12:12 h light/dark cycle with lights on at 6:00 a.m., 21 ± 1°C, 60 % humidity, standard rat chow and water *ad libitum*) unless mentioned otherwise. The experiments were approved by the Committee on Animal Health and Care of the Government of Bavaria and are in accordance with the *Guide for the Care and Use of Laboratory Animals* by the National Institute of Health.

Resident-intruder test

Adult LAB and HAB male rats were each housed in an observational cage (40 × 24 × 35 cm) together with a female Wistar rat (Charles River, Sulzfeld, Germany) for ten days to stimulate territorial behaviour (Flannelly & Lore, 1977). At the same time, the 12:12 h light/dark cycle was switched to lights off at 13:00 p.m. in order to perform the RI test in the early dark phase, which is the most active phase in rats. As our previous experiments on aggressive behaviour

in male LAB and HAB rats were performed in the early light phase (Veenema et al., 2007b), we first confirmed the line differences in aggression during the RI test in the early dark phase in LAB (n = 8) and HAB (n = 8) resident rats. The female cage mate was removed 30 minutes before the RI test and was returned afterwards. For measuring aggressive behaviour during ongoing microdialysis, the female cage mate was removed just prior surgery, i.e. two days before the RI test. The RI test consists of placing an unfamiliar, lighter (10 %) male Wistar rat (Charles River, Sulzfeld, Germany) in the resident's home cage for ten minutes. The behaviour of the LAB and HAB residents was videotaped and the following behaviours were scored by an experienced observer blind to breeding line and treatment: Aggressive behaviour (attack, lateral threat, offensive upright, keep down, threat, aggressive grooming), social investigation (investigating opponent, anogenital sniffing), exploration, self grooming, defensive behaviour, and immobility (Koolhaas et al., 1980; Veenema et al., 2007b). Behaviour was scored in real-time using pre-set keys on a PC (Eventlog; Version 1.0, 1986, R. Hendersen). All behaviours were calculated as percentage of time. Additionally, the attack latency time and the number of attacks were measured.

Elevated plus-maze

In order to quantify the effects of pharmacological manipulation of the septal AVP system on anxiety-related behaviour, HAB and LAB resident rats were tested on the EPM. This test is based on the natural conflict of the rat between its exploratory drive and its innate fear of elevated, open and novel spaces (Pellow *et al.*, 1985). The EPM consisted of two opposing open (50 × 10 cm; 100 lux) and two opposing closed (50 × 10 × 40 cm; 20 lux) arms, which are connected by a common central area (10 × 10 cm). A raised edge (0.5 cm) on the open arms provided additional grip for the rats. The apparatus was made of dark grey plastics and was elevated to a height of 80 cm above the floor. The EPM was surrounded by an opaque curtain to avoid disturbance by the observer. Before each trial, the maze was cleaned with

water containing a low concentration of a detergent. Rats were placed individually in the centre square facing a closed arm and were allowed to explore the maze for five minutes. Behaviour was measured by means of a video camera mounted above the platform and scored by a trained observer pressing pre-set keys on a PC (Plus-maze version 2.0; Ernst Fricke). An open/closed-arm entry was defined as both fore-paws of the rat being on the respective arm of the EPM. The following parameters of anxiety-related behaviour were measured: the percentage of time spent on the open arms [time on open arms / (time on open arms + time in closed arms) × 100 %], the percentage of open arm entries [open arm entries / (open + closed arm entries) × 100 %], and the latency to the first open arm entry. The number of closed arm entries was used as measure of locomotor activity.

Surgery

LAB and HAB male rats were anesthetised with isoflurane (Forene®, Abbott GmbH & Co. KG, Wiesbaden, Germany), injected with 0.05 ml of an antibiotic substance (Tardomyocel®, Bayer Vital GmbH, Leverkusen, Germany) to prevent infections, and mounted on a stereotaxic frame. The microdialysis probes (self-made; molecular cut-off 18 kDa, for details see: Neumann *et al.*, 1993; Bosch *et al.*, 2005) were implanted stereotaxically into the mediolateral septum. The coordinates relative to bregma were: -0.2 mm caudal, +2.0 mm lateral to the midline, 6.0 mm beneath the surface of the skull; angle of 20 ° to avoid damage to the sagittal sinus; nose: -3.5 mm (Paxinos & Watson, 1998; see Fig. 15). It was previously demonstrated by Engelmann *et al.* (1992) that [^{3}H]AVP infused by retrodialysis in the mediolateral septum reaches the entire septal area. Moreover, two other studies have used the same coordinates and showed that manipulation of the septal AVP system by application of synthetic AVP or the V1a-A d(CH2)5Tyr(Me)AVP using retrodialysis improved social recognition and reduced swimming behaviour, respectively (Engelmann & Landgraf, 1994; Ebner *et al.*, 1999). The probes were flushed and filled with sterile Ringer's solution (pH 7.4,

B. Braun Melsungen AG, Melsungen, Germany), and were fixed to the skull with two jeweller's screws and dental cement (Kallocryl, Speiko-Dr. Speier GmbH, Muenster, Germany). Two approx. 5 cm long pieces of polyethylene tubing (PE 20, Karmann & Droll, Karlsfeld, Germany) filled with Ringer's solution were connected to the inflow and the outflow of the microdialysis probe and fixed with dental cement. One day after surgery, rats were familiarized with the experimental procedure to minimize non-specific stress responses during the experiment.

Experimental procedures

Arginine vasopressin release within the mediolateral septum during exposure to the resident-intruder test

Two days after surgery, the microdialysis probes of LAB (n = 8) and HAB (n = 8) resident rats were connected to a syringe mounted onto a microinfusion pump via polyethylene tubing and perfused with sterile Ringer's solution (3.3 µl/min, pH 7.4) starting at 11:00 a.m. for two hours before the start of the experiment to establish an equilibrium between inside and outside of the microdialysis membrane. Thereafter, five consecutive 30-minute dialysates were collected: samples 1 and 2 were taken under basal (undisturbed) conditions, sample 3 during exposure to the RI test, and samples 4 and 5 after exposure to the RI test, i.e. again under undisturbed conditions. The microdialysates were collected directly into Eppendorf tubes containing 10 µl 0.1 M HCl, immediately frozen on dry ice, and subsequently stored at -20°C until quantification of AVP by radioimmunoassay.

c-Fos immunoreactivity within the lateral septum in response to the resident-intruder test

To investigate whether the differences in aggression and in septal AVP release between LAB and HAB residents are accompanied by a difference in neuronal activation of the lateral septum, male LAB (n = 6) and HAB (n = 5) residents were perfused one hour after the onset

of the RI test (Veenema et al., 2007b), and brains were removed and processed for c-Fos immunoreactivity as described below.

Manipulation of the septal arginine vasopressin system

To study the causal involvement of reduced septal AVP release in the display of high aggression, LAB rats were retrodialysed with synthetic AVP. In contrast, HAB rats were retrodialysed with the selective AVP V1a-A $d(CH_2)_5Tyr(Me)AVP$ (courtesy of Dr. M. Manning, Toledo, OH, USA), as HAB rats tended to show an increase in AVP release during the RI test. Resident LAB (n = 17) and HAB (n = 19) rats were stereotaxically implanted with a microdialysis probe within the mediolateral septum and underwent initially the same procedure as described above. However, after the 2-hour perfusion period without sampling, microdialysis was continued with either Ringer's solution (controls, LAB: n = 9, HAB: n = 10), Ringer's solution containing 1 µg/ml synthetic AVP (only LAB: n = 8) or Ringer's solution containing 10 µg/ml of the V1a-A (only HAB: n = 9). During the 1-hour period of retrodialysis, a total amount of approx. 1 ng of synthetic AVP and approx. 10 ng of V1a-A was delivered locally into the mediolateral septum (Engelmann *et al.*, 1992) of LAB and HAB rats, respectively. Thirty minutes after the beginning of the retrodialysis procedure, the LAB and HAB residents were exposed to the RI test during ongoing dialysis and their behaviour was monitored. Thirty minutes after the onset of the RI test, the microdialysis probes were disconnected and the LAB and HAB males were carried in their home cages to an adjacent room for immediately testing for anxiety-related behaviour on the EPM.

Histology

At the end of the experiments, rats were decapitated under CO_2 anaesthesia, brains were removed, quickly frozen in pre-chilled *n*-methylbutane on dry ice, and stored at -20°C. To histologically verify the placement of the probe in the mediolateral septum, brains were cut

into 40-µm coronal cryostat sections and stained with cresyl violet. Only those rats with correct probe placements (see Fig. 15) were included in the statistical analysis resulting in the number of rats indicated in parentheses.

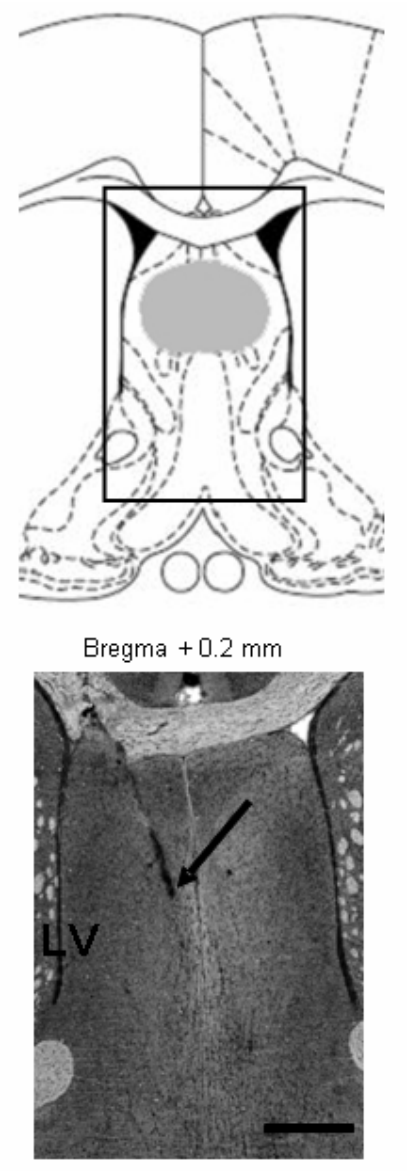

Fig. 15: Schematic drawing of the mediolateral septum [adapted from Paxinos and Watson (1998); the grey area indicates the respective target region] and a representative enlargement of the microphotograph of a Cresyl-stained coronal section of the rat brain after removal of the microdialysis probe (bottom; arrow indicates the placement of the tip of the microdialysis probe). LV = lateral ventricle; Scale bar = 1 mm.

Radioimmunoassay of arginine vasopressin

AVP content was measured in lyophilised dialysates by a highly sensitive and selective radioimmunoassay (detection limit: 0.03 pg per sample; cross-reactivity of the antiserum with other related peptides, including oxytocin, was less than 0.7 %; for a more detailed

description see Landgraf et al. 1995a). To eliminate interassay variation, all samples to be compared were measured in the same assay.

c-Fos immunocytochemistry within the lateral septum

c-Fos immunoreactivity is a widely used marker of neuronal activity (Sagar *et al.*, 1988) and was applied to compare the neuronal activity within the lateral septum between male LAB and HAB rats exposed to the RI test. One hour after the RI test, the resident rats were deeply anaesthetised with pentobarbital (0.1 ml/100 g, i.p.; Sigma) and perfused intracardially with 150 ml ice-cold phosphate-buffered saline followed by 300 ml 4 % paraformaldehyde in ice-cold phosphate-buffer (pH 7.4). Brains were removed, post-fixed overnight in 4 % paraformaldehyde and immersed in 30 % sucrose for cryoprotection for three days. Immunocytochemistry was performed on coronal, cryostat-cut, free floating slices (50 µm). Incubation with anti-c-Fos rabbit antiserum (Oncogene; Cambridge, MA, USA) was followed by enhancement of the signal by binding of a secondary antibody and detection using the conventional avidin-biotin complex peroxidase reaction with diaminobenzidine as chromogen (vectastain ABC kit and DAB chromogen purchased from Alexis; Gruenberg, Germany). The dilution of the anti-c-Fos antiserum was 1:10,000. For quantification of c-Fos-positive cells, the number of stained neurons in the lateral septum was counted by two investigators in a blinded manner for each group on at least two sections from each rat brain. Sections were taken from the same level of the septal area according to the rat brain atlas of Paxinos and Watson (1998).

Statistics

All statistical analyses were performed using the software package SPSS (version 13). Behavioural and c-Fos data were analysed using one way ANOVA. AVP release was analysed using ANOVA for repeated measures (factor line × factor time). ANOVA was

followed by LSD *post hoc* test, when appropriate. Data are presented as means + SEM. Statistical significance was set at $p < 0.05$.

Results

Aggressive behaviour of LAB and HAB residents in the early dark phase

Confirming and extending recent findings in the early light phase (Veenema et al., 2007b), LAB and HAB residents showed a significant difference in intermale aggression during exposure to the RI test in the early dark phase. In detail, LAB males showed more aggressive behaviour than HAB males ($p < 0.05$; Fig. 16A); specifically, LAB males displayed more offensive upright posture ($p < 0.05$) and more lateral threat ($p = 0.051$) than HAB males (Fig. 16A). Furthermore, LAB males showed a reduced amount of social investigation ($p < 0.05$; Fig. 16A).

Arginine vasopressin release within the mediolateral septum during exposure to the resident-intruder test

Aggressive behaviour during ongoing microdialysis

Behavioural data from the RI test performed during ongoing microdialysis within the mediolateral septum confirmed the higher level of aggression of LAB rats (total aggression: $p < 0.01$; lateral threat: $p < 0.01$; offensive upright: $p < 0.05$; attack latency: $p < 0.01$; number of attacks: $p < 0.01$; Fig. 16B). Likewise, LAB males showed a reduced amount of social investigation than HAB males ($p < 0.05$; Fig. 16B).

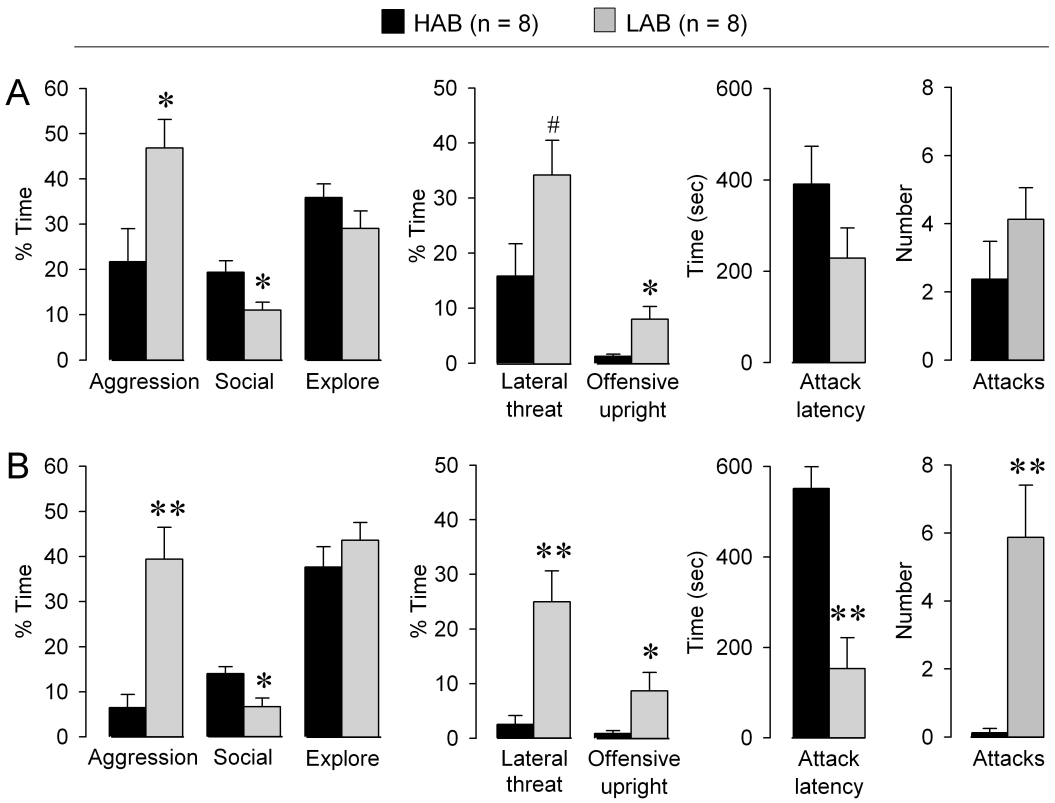

Fig. 16: Behaviour of HAB and LAB rats during the 10-minute RI test one day before surgery **(A)** and during ongoing microdialysis **(B)**. Aggression, social investigation and exploration are calculated as percentage of total time. As parameters of aggressive behaviour, the duration of lateral threat and offensive upright (calculated as percentage of total time), the attack latency and the number of attacks are shown. Data are presented as means + SEM. ** $p < 0.01$ versus HAB, * $p < 0.05$ versus HAB, # $p = 0.051$ versus HAB.

It should be noted, however, that by comparing the behavioural profiles of LAB and HAB residents before and after surgery, HAB rats showed a lower level of aggressive behaviour after surgery, i.e. under ongoing microdialysis (total aggression, $p < 0.05$; lateral threat, $p < 0.05$; attacks, $p < 0.05$; attack latency, $p = 0.051$). In contrast, LAB rats did not show a significant change in aggressive behaviour after surgery. Social investigation was reduced in both LAB ($p = 0.057$) and HAB ($p < 0.05$) rats, while exploration was higher in LAB rats ($p < 0.01$) after surgery (Fig. 16).

Arginine vasopressin release within the mediolateral septum

Exposure to the RI test induced a line-dependent change in AVP release within the mediolateral septum (factor line × time; $F_{(4,48)} = 3.58$; $p < 0.05$). In detail, LAB rats showed a significant decrease in AVP release during the RI test exposure ($p < 0.05$ versus samples 1, 2 and 5; Fig. 17A). In contrast, local AVP release in HAB males rather tended to increase during the RI test exposure (sample 3) which became significantly different compared with sample 4 after termination of the RI test ($p < 0.05$, Fig. 17A). Accordingly, AVP release during exposure to the RI test was significantly lower in LAB compared with HAB rats ($p < 0.01$; Fig. 17A). No line difference was found for absolute AVP content under basal conditions (average of samples 1 and 2: LAB, 26.2 ± 5.2 pg; HAB, 27.1 ± 5.3 pg).

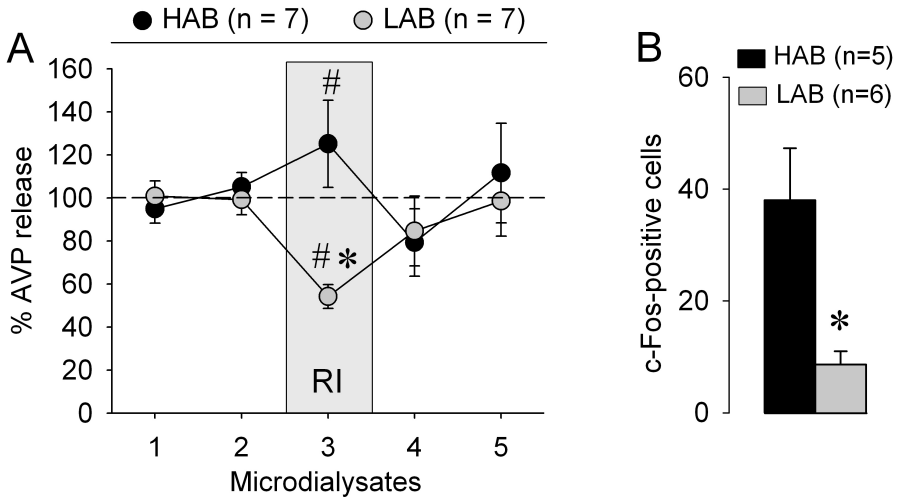

Fig. 17: (A) AVP release within the mediolateral septum of HAB and LAB residents under basal conditions and during and after exposure to the RI test given as percentage of baseline. Dialysate no. 1 and 2 were taken under basal conditions, no. 3 included exposure to the RI test, and no. 4 and 5 were taken after exposure to the RI test under undisturbed conditions. **(B)** Number of c-Fos immuno-positive cells in the lateral septum one hour after the onset of the RI test. Data are presented as means ± SEM. * $p < 0.05$ versus HAB, # $p < 0.05$ versus sample 1, 2 and 5 (LAB) or versus sample 3 (HAB).

c-Fos immunoreactivity within the lateral septum in response to the RI test

The number of c-Fos-positive cells in the lateral septum was significantly lower in LAB compared with HAB rats as assessed one hour after exposure to the RI test ($p < 0.05$; Fig. 17B).

Manipulation of the septal arginine vasopressin system in LAB rats

Aggressive behaviour

As LAB rats showed a significant decrease in septal AVP release during the display of aggressive behaviour, LAB rats were retrodialysed with synthetic AVP to investigate the effects of locally elevated AVP concentrations in the lateral septum on aggressive behaviour. Local treatment of LAB residents with synthetic AVP into the septum did not significantly affect any measure of aggressive behaviour or social investigation during the RI test (Fig. 18A).

Anxiety-related behaviour

Retrodialysis of AVP into the mediolateral septum of LAB rats increased anxiety-related behaviour on the EPM. Compared with Ringer-perfused rats, AVP treated LAB rats showed fewer open arm entries (percentage of entries; $p < 0.05$) and spent less time on the open arms (percentage of entries; $p < 0.005$) (Fig. 18B). There was also a tendency towards a longer latency to enter an open arm of the EPM in AVP-treated LAB rats ($p = 0.058$), whereas general locomotion (number of closed arm entries) was unchanged (Fig. 18B).

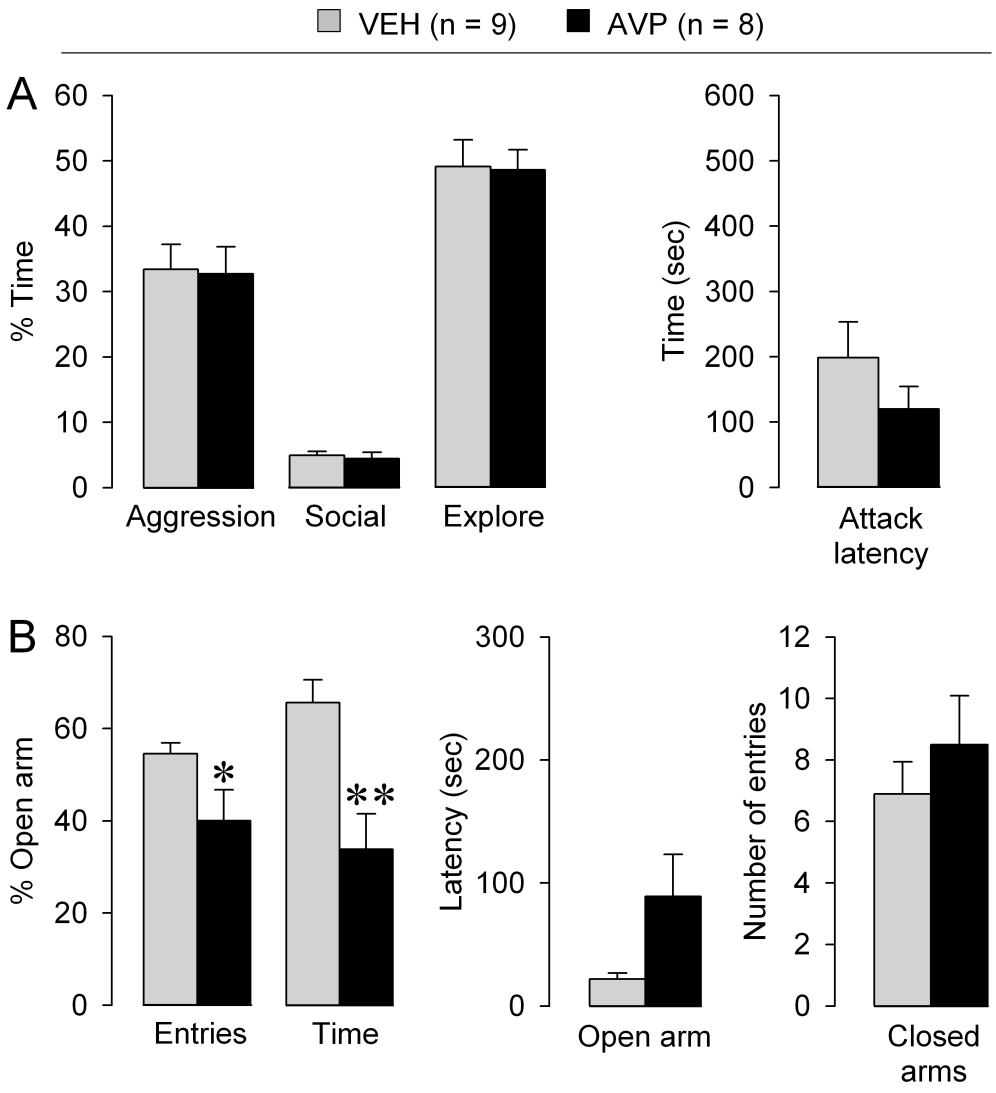

Fig. 18: Behavioural consequences of retrodialysis within the mediolateral septum of LAB males perfused with either Ringer's solution (VEH) or Ringer's solution containing synthetic AVP (1µg/ml) during the exposure to the RI test **(A)** or on the EPM **(B)**. Data are presented as means + SEM. * $p < 0.05$ versus VEH, ** $p < 0.005$ versus VEH.

Manipulation of the septal arginine vasopressin system in HAB rats

Aggressive behaviour

As septal AVP release in HAB residents tended to increase during exposure to the RI test, HAB rats were retrodialysed with a selective V1a-A to assess the effects of local receptor blockade within the lateral septum on aggressive behaviour. Local V1a-A treatment had no

significant effect on any measure of aggressive behaviour during the RI test, but reduced non-aggressive social behaviour ($p < 0.05$; Fig. 19A).

Anxiety-related behaviour

Application of the V1a-A into the mediolateral septum of HAB rats did not change any measure of anxiety-related behaviour nor general locomotion on the EPM (Fig. 19B).

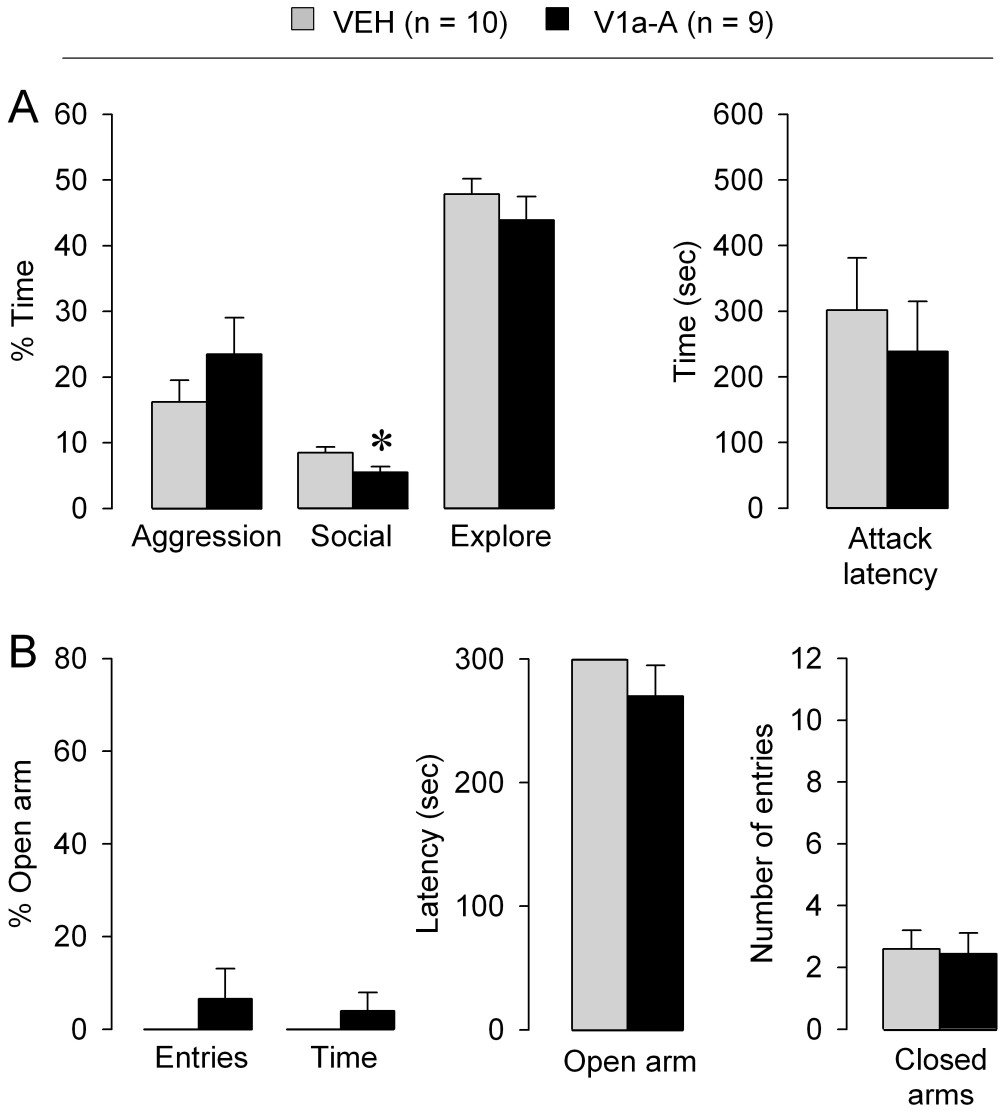

Fig. 19: Behavioural consequences of retrodialysis within the mediolateral septum of HAB males perfused with either Ringer's solution (VEH) or Ringer's solution containing a V1a-A (10µg/ml) during exposure to the RI test **(A)** or on the EPM **(B)**. Data are presented as means + SEM. * $p < 0.05$ versus VEH.

Discussion

The present study demonstrates for the first time the dynamics of intracerebral AVP release patterns during the display of intermale aggression in adult rats. Local AVP release within the mediolateral septum was bidirectionally altered depending on the level of aggression during the RI test. Thus, a high level of aggressive behaviour, as displayed by male LAB residents, was associated with a significant decrease in septal AVP release. In contrast, the low level of aggression of HAB residents was rather associated with an increase in septal AVP release. Pharmacological manipulation of the septal AVP system, by local application of either synthetic AVP in LAB rats or a selective V1a-A in HAB rats, did not affect the level of aggressive behaviour. However, application of AVP into the septum enhanced anxiety-related behaviour on the EPM in LAB rats, while local application of the V1a-A reduced social investigation during the RI test in HAB rats, demonstrating that the behavioural profile can specifically be altered using retrodialysis.

Recently, we found that LAB and HAB male rats differ in their level of intermale aggression during the early light phase, with the LAB residents being more aggressive (Veenema et al., 2007b). Here, we extend this finding by showing a similar line difference in aggression during the early dark phase. Thus, irrespective of the time of day, a low level of innate anxiety, as seen in LAB rats, is accompanied by a higher level of aggressive behaviour. Importantly, the line difference in aggression was still present after surgery and under ongoing microdialysis. It should be noted that the level of aggressive behaviour in HAB rats was lower under ongoing microdialysis than during the initial RI test. In contrast, the level of aggressive behaviour in LAB males was similar during the two RI tests. Whether the change in aggression in HAB rats was due to repeated testing, surgery or the microdialysis procedure is not clear at present.

AVP has been implicated in aggression in several species, including rats (Koolhaas *et al.*, 1990; Everts *et al.*, 1997), mice (Compaan *et al.*, 1993; Bester-Meredith *et al.*, 2005),

hamsters (Albers *et al.*, 1992; Ferris *et al.*, 1997), birds (Goodson *et al.*, 2006) and humans (Coccaro *et al.*, 1998; Thompson *et al.*, 2004). Especially, extrahypothalamic AVP neurons originating in the medial amygdala and the BNST and projecting to the lateral septum (De Vries & Buijs, 1983; Caffe *et al.*, 1987) seem to be involved in the regulation of aggressive behaviours in rodents (Irvin *et al.*, 1990; Koolhaas *et al.*, 1990; Koolhaas *et al.*, 1991; Compaan *et al.*, 1993; Ferris & Delville, 1994; Everts *et al.*, 1997). However, in most of the studies either exogenous AVP was applied or immunocytochemistry for the detection of AVP protein was used. Neither of these techniques provides an accurate indication of local AVP release patterns, for example in response to a certain stimulus (Landgraf & Neumann, 2004). Here, we show by *in vivo* monitoring of AVP release within the mediolateral septum that a high level of aggression, as displayed by LAB residents, is accompanied by a significant decrease in local AVP release. In contrast, HAB residents showed a tendency towards an increase in AVP release during the RI test. As a result, septal AVP release was significantly lower in LAB compared with HAB residents in response to the RI test.

Neuronal activation of the lateral septum, as assessed by the number of c-Fos-positive cells, was significantly lower in the high-aggressive LAB compared with the low-aggressive HAB residents one hour after exposure to the RI test. Likewise, high-aggressive wild house mice show a reduced neuronal activation of the lateral septum in response to the RI test compared with low-aggressive wild house mice (Haller *et al.*, 2006). In support, neuronal activation within the lateral septum is lower in dominant aggressive male hamsters compared with subordinates (Kollack-Walker *et al.*, 1997). These studies suggest that low activation of the septum is a prerequisite for the display of aggressive behaviour. As the lateral septum has been implicated in the modulation of anxiety (Sheehan *et al.*, 2004) as well as social recognition (Engelmann *et al.*, 1996), we speculate that a low activity of the lateral septum might promote aggressive behaviour by reducing anxiety and/or altering social recognition abilities. The reduction of AVP release into the extracellular fluid of the mediolateral septum

as seen in LAB residents might be one of several components affecting septal activity in response to the RI test. Clearly, further research is necessary to investigate the precise role of septal activity in aggressive behaviour and whether there is an interplay between aggression, anxiety and social recognition abilities, Particularly in LAB rats, it might be of interest to investigate whether low anxiety, low social investigation behaviour, and low septal activity are associated with alterations in olfactory communication, which might play a role in the display of high aggression.

To investigate the behavioural relevance of the dynamic changes in septally released AVP in intermale aggression, the extracellular AVP concentration in the mediolateral septum was enhanced by local application of synthetic AVP in high-aggressive LAB rats via reverse microdialysis. On the other hand, blockade of local V1a receptors by a specific V1a-A in low-aggressive HAB rats should reveal the function of endogenous AVP within this brain region in the regulation of aggression. Contrary to our expectations, manipulation of the septal AVP system in either direction did not alter the level of aggressive behaviour during the RI test. Thus, it seems less likely that, at least in LAB and HAB rats, AVP in the septum is directly involved in the regulation of aggressive behaviour. Experiments performed in NAB rats or other rodents may help to further clarify the role of the septal AVP system in intermale aggression.

The fact that we were unable to observe changes in aggressive behaviour after pharmacological manipulation of the septal AVP system is unlikely to be due to an ineffective low dose of locally applied AVP or V1a-A. Synthetic AVP significantly enhanced anxiety-related behaviour on the EPM in LAB rats, thereby confirming local anxiogenic effects of AVP (Landgraf *et al.*, 1995a; Millan, 2003). Likewise, application of the V1a-A into the septum significantly reduced social investigation behaviour in HAB rats during the RI test. Local treatment with the V1a-A did not reduce the anxiety level of HAB rats on the EPM. Similarly, administration of the same V1a-A via retrodialysis into the PVN only tended to

reduce anxiety-related behaviour of HAB rats on the EPM (Wigger *et al.*, 2004), demonstrating the robust anxious phenotype of the HAB rats.

Considering previous findings, the lack of an effect of local application of synthetic AVP or the V1a-A into the septum on aggressive behaviour in the present study is difficult to explain. However, the following features of the septal AVP system might be taken into account. Firstly, in male rats, the AVP system originating in the BNST and medial amygdala and projecting to the septum is highly responsive to gonadal steroids, as castration strongly reduces septal AVP fibre density (De Vries *et al.*, 1985). Castrated rats also showed a reduction in aggressive behaviour, which could be reversed by microinjection of AVP into the lateral septum (Koolhaas *et al.*, 1991). Similar to castrated rats, the lateral septum of intact male golden hamsters is almost devoid of AVP-immunoreactive fibres (Ferris & Delville, 1994; Ferris *et al.*, 1995) and microinjections of AVP into the lateral septum of golden hamsters induced high levels of flank marking (Irvin *et al.*, 1990). The results of these rat and hamster studies may indicate that administration of AVP into the septum enhances aggression/flank marking only under conditions of a chronic hypo-active state of the septal AVP system.

Secondly, the main function of the lateral septum is to integrate sensory stimuli and to transmit this information to brain regions that are more directly involved in the behavioural output, such as the hypothalamus (Sheehan *et al.*, 2004). This modulatory role of the lateral septum is well characterised in hamsters, where animals with a lesioned septum showed increased flank marking behaviour during stimulation of the anterior hypothalamus, while hamsters with a lesioned anterior hypothalamus did not show enhanced flank marking when the septum was stimulated (Ferris, 1992). Therefore, it is likely that manipulation of the septal AVP system might have been insufficient in altering the activity of other more downstream brain regions, including hypothalamic areas, and hence, had no effect on aggressive behaviour in LAB or HAB rats.

Finally, instead of directly influencing aggression, septal AVP might predominantly affect other aspects of social behaviour and anxiety. The important role of septal AVP in anxiety is demonstrated by the significant anxiogenic effect of local AVP application in LAB rats exposed to the EPM. Whether septal AVP induces similar anxiogenic effects in a social context like the RI test is unclear at present. Moreover, septal AVP plays an essential role in social discrimination abilities (for review see Engelmann *et al.*, 1996). Infusion of an antisense oligodeoxynucleotide to the AVP-V1-receptor mRNA into the lateral septum of male rats reduced social memory ability (Landgraf *et al.*, 1995a). Conversely, overexpression of septal V1a receptors by use of an adenoviral vector increased social memory ability and social interaction in male rats (Landgraf *et al.*, 2003). Interestingly, HAB rats search for more social contact in a novel environment (Ohl *et al.*, 2001) and show more social investigation behaviour during the RI test (Veenema et al., 2007b, present study Fig. 2) than LAB rats. This higher level of social investigation was found to be reduced after local administration of V1a-A into the septum of HAB rats. These results substantiate the significant involvement of septal AVP in various aspects of non-aggressive social behaviour. Further detailed analysis using different strains and/or species is required to reveal the precise role of AVP in the septum, as well as in other brain regions, in the regulation of aggression and its link to other social behaviours and anxiety.

In conclusion, opposite AVP release patterns within the mediolateral septum during the display of aggression were found to accompany differences in aggression in LAB and HAB residents. Despite a significant reduction in septal AVP release in high-aggressive LAB males and a tendency towards an increase in low-aggressive HAB males, pharmacological manipulation of the septal AVP system did not affect aggressive behaviour. Instead, locally applied AVP enhanced anxiety-related behaviour in LAB rats, and local V1a-A treatment reduced social investigation in HAB rats. Thus, these results suggest that AVP released within the lateral septum has no direct effect on aggression, but rather influences social and anxiety-

related behaviours, which may – in interplay with other regulators – indirectly affect aggressive behaviour.

Chapter 5

Distinct vasopressin release patterns within the lateral septum and the bed nucleus of the stria terminalis during the display of intermale aggression

[adapted from: Veenema AH, Beiderbeck DI, Neumann ID; Distinct vasopressin release patterns within the lateral septum and the bed nucleus of the stria terminalis during the display of intermale aggression; In preparation]

Abstract

AVP has been implicated in intermale aggression, but little is known about AVP release patterns within distinct brain regions during the display of intermale aggression and, in turn, its behavioural consequences. We used intracerebral microdialysis to monitor the *in vivo* AVP release within the mediolateral septum and the dorsal part of the BNST of adult male Wistar rats exposed to the 10-minute RI test. Within the septum, a significant increase in AVP release was found in rats during the display of a substantial amount of aggressive behaviour during the RI test. In contrast, in non-aggressive rats, AVP release within the septum remained unchanged during the RI test. Pharmacological manipulation of the septal AVP system by local application of either synthetic AVP to non-aggressive rats or a specific AVP V1a-A [d(CH_2)$_5$Tyr(Me)AVP] to aggressive rats, did not change their respective aggression level in any direction, but application of AVP exerted an anxiogenic effect. Contrary to AVP release within the septum, AVP release within the BNST was significantly increased in non-aggressive rats compared with aggressive rats during the RI test. This release pattern appears to be stimulus-specific as both non-aggressive and aggressive rats showed a similar rise in local AVP release when exposed to forced swimming (ten minutes, 19°C). Moreover, bilateral application of synthetic AVP into the BNST of aggressive rats via reverse microdialysis significantly reduced the level of aggression, whereas anxiety-related behaviour on the EPM remained unchanged. Thus, intermale aggression is associated with distinct AVP release patterns within the septum and BNST. Our data point towards a direct involvement of AVP released within the BNST in the regulation of aggression, whereas AVP released in the septum rather occurs as a consequence of the display of aggression and may influence context-relevant behaviours like anxiety and social memory.

Introduction

The neuropeptide AVP is implicated in the regulation of intermale aggression in several mammalian species, including rats, mice, hamsters and humans (Ferris, 1992; Coccaro *et al.*, 1998; Koolhaas *et al.*, 1998; Campbell, 2008). Especially extrahypothalamic AVP neurons originating in the BNST and the medial amygdala and projecting to the lateral septum (De Vries & Buijs, 1983) seem to play an important role in aggressive behaviour (Irvin *et al.*, 1990; Koolhaas *et al.*, 1991; Hines *et al.*, 1992; Compaan *et al.*, 1993; Ferris & Delville, 1994; Everts *et al.*, 1997; Bester-Meredith *et al.*, 1999). Several of these studies reported profound differences in AVP-immunoreactive staining in the BNST and lateral septum between low- and high-aggressive rodent species, however, the direction of the correlation between AVP and aggression differed between studies. One explanation for these discrepancies might be the fact that AVP immunocytochemical staining rather provides a static picture of intracellular neuropeptide availability and does not reflect the dynamics of local AVP release during the display of high or low aggression (for review see: Landgraf & Neumann, 2004).

Using intracerebral microdialysis, we recently demonstrated that differences in intermale aggression are accompanied by opposite AVP release patterns within the lateral septum in rats bred for low or high anxiety-related behaviour (Beiderbeck et al., 2007). LAB residents are generally more aggressive than HAB residents during exposure to the RI test, as indicated by a shorter attack latency and a higher number of attacks (Beiderbeck et al., 2007; Veenema et al., 2007b). The display of substantial aggressive behaviour of LAB residents was accompanied by a significant decrease in septal AVP release during the RI test, whereas the low-aggressive HAB rats rather show an increase in septal AVP release (Beiderbeck *et al.*, 2007). This suggests that AVP release within the septum is negatively associated with the level of aggression. However, these findings might be unique to these particular rat lines

selectively bred for differences in innate anxiety for more than a decade (Landgraf & Wigger, 2002).

In the present study, we measured the *in vivo* release of AVP within the lateral septum of male NAB rats during exposure to the RI test. Additionally, we included the BNST, which is also part of the extrahypothalamic AVP system implicated in aggressive behaviour. To reveal the causal involvement of local AVP release patterns in the regulation of aggression, rats received a local application of either synthetic AVP or a selective AVP V1a-A [d(CH_2)$_5$Tyr(Me)AVP] within the septum or the BNST via retrodialysis during ongoing behavioural testing.

Materials and Methods

Animals

Experiments were carried out on male Wistar rats (Charles River Laboratories, Sulzfeld, Germany). Rats were housed in groups of 4-5 under standard laboratory conditions (12:12 h light/dark cycle with lights on at 6:00 a.m., 21 ± 1°C, 60 ± 5 % humidity, standard rat chow and water *ad libitum*). The experiments were approved by the Committee on Animal Health and Care of the Government of Oberpfalz and are in accordance with the *Guide for the Care and Use of Laboratory Animals* by the National Institute of Health.

Resident-intruder test

Two weeks before the start of the experiments, the 12:12 light/dark cycle was switched to lights off at 13:00 p.m., and each experimental male rat was housed in an observational cage (40 × 24 × 35 cm) together with a female Wistar rat to stimulate territorial behaviour. Male rats underwent the RI test at the age of 14-16 weeks (bodyweight: 350-450 g). The RI test was

carried out during the beginning of the dark phase (between 13:00 and 15:00 p.m.), and the experimental resident male was exposed in its home cage to a slightly smaller (20 – 50 g lighter) unfamiliar male Wistar rat for ten minutes. The tests were videotaped and the behavioural scoring was done using Eventlog (version 1.0, October 1986, R. Hedersen) by a researcher blinded to the treatment condition. The following parameters related to male aggression were scored (according to Beiderbeck et al., 2007): attack latency time, number of attacks, and the duration of lateral threat, clinch, offensive upright and keep down. The latter four behavioural parameters were summarised as total aggressive behaviour. Furthermore, social behaviour (consisting of investigating opponent, anogenital sniffing), exploration and self grooming were scored.

Elevated plus-maze

To quantify the effects of pharmacological manipulation of the AVP system on anxiety-related behaviour, male resident rats were tested on the EPM. The EPM consisted of two opposing open (50 × 10 cm) and two opposing closed (50 × 10 × 40 cm) arms, connected by a central area (10 × 10 cm). A raised edge (0.5 cm) on the open arms provided additional grip for the rats. The apparatus was made of dark grey plastics and was elevated to a height of 80 cm above the floor. Before each trial, the maze was cleaned with water containing a low concentration of a detergent. Rats were placed individually in the central area facing a closed arm and were allowed to explore the maze for five minutes. Behaviour was measured by means of a video camera mounted above the platform and scored by a trained observer pressing preset keys on a PC (Plus-maze version 2.0; E. Fricke). The following parameters of anxiety-related behaviour were measured: the percentage of time spent on the open arms [100 × time on open arms ⁄ (time on open arms + time in closed arms)] and the percentage of open

arm entries [100 × open arm entries / (open + closed arm entries)]. The number of closed arm entries was used as a measure of locomotor activity.

Surgery

Male rats were anaesthetized with isoflurane (Forene®, Abbott GmbH & Co. KG, Wiesbaden, Germany), injected with 0.05 ml of an antibiotic substance (Tardomyocel®, Bayer Vital GmbH, Leverkusen, Germany) to prevent infections, and mounted on a stereotaxic frame. The microdialysis probes (self made, molecular cut-off 18 kDa, for details see Neumann *et al.*, 1993) were implanted stereotaxically within the lateral septum (coordinates relative to bregma: -0.2 mm caudal, +2.5 mm lateral to the midline, 6.0 mm beneath the surface of the skull; angle of 20° to avoid damage to the sagittal sinus; nose: -3.5 mm; Paxinos & Watson, 1998) or the dorsal part of the BNST (-0.2 mm caudal, +2.0 mm lateral, 6.0 mm deep) (see Fig. 20). For retrodialysis experiments, unilateral implantations within the mediolateral septum (+2.0 mm lateral) or bilateral implantations within the BNST were performed. The probes were flushed and filled with sterile Ringer's solution (pH 7.4, B. Braun Melsungen AG, Melsungen, Germany), and were fixed to the skull with two jeweller's screws and dental cement (Kallocryl, Speiko-Dr. Speier GmbH, Muenster, Germany). Two approx. 5 cm long pieces of polyethylene tubing (PE 20, Karmann & Droll, Karlsfeld, Germany) filled with Ringer's solution were connected to the inflow and the outflow of the microdialysis probe and fixed with dental cement. One day after surgery, rats were familiarized with the experimental procedure to minimize non-specific stress responses during the experiment.

Experimental procedures

Arginine vasopressin release within the septum or bed nucleus of the stria terminalis during exposure to the resident-intruder test

Two days after surgery, the microdialysis probes of male resident rats (septum: n = 14; BNST, n = 13) were connected to a syringe mounted onto a microinfusion pump via polyethylene tubing and perfused with sterile Ringer's solution (3.3 µl/min, pH 7.4) starting at 11:00 a.m. for two hours before the start of the experiment to establish an equilibrium between inside and outside of the microdialysis membrane. Thereafter, five consecutive 30-minute dialysates were collected: samples 1 and 2 were taken under undisturbed (basal) conditions, sample 3 included the exposure to the RI test, and samples 4 and 5 after exposure to the RI test, i.e. again under undisturbed conditions. The microdialysates were collected directly into Eppendorf tubes containing 10 µl 0.1 M HCl, immediately frozen on dry ice, and subsequently stored at -20°C until quantification of AVP by radioimmunoassay.

To verify aggression-specific AVP release patterns, rats with a microdialysis probe within the BNST were exposed to forced swimming one day after exposure to the RI test. Rats underwent a similar microdialysis procedure as descibed above, except that sample 3 included the exposure to ten minutes of forced swimming in which the rats were gently put in a clear plastic cylinder (30 cm in diameter, 80 cm in height) filled with tap water (19^{o}C, 60 cm deep). Thereafter, rats were towel dried for 20 seconds and returned to their home cage.

Pharmacological manipulation of arginine vasopressin activity within the septum or the bed nucleus of the stria terminalis

Four days before surgery, male rats (septum, n = 25, BNST, n = 27) were pre-tested for aggressive behaviour in the RI test and subsequently divided into aggressive (attack latency < 400 seconds; aggressive behaviour > 10 % of total time) and non-aggressive (attack latency > 550; aggressive behaviour < 3 % of total time) groups. The causal involvement of AVP

release patterns in the regulation of aggression was determined by local application of either synthetic AVP or the selective AVP V1a-A d(CH_2)$_5$Tyr(Me)AVP (courtesy of Dr. M. Manning, Toledo, OH, USA) into the mediolateral septum or BNST of resident male rats using retrodialysis. Rats underwent the same procedure as described for microdialysis, except that after the 2-hour perfusion period without sampling microdialysis was continued with either Ringer's solution (vehicle), Ringer's solution containing 1 µg/ml synthetic AVP, or Ringer's solution containing 10 µg/ml of the V1a-A. During the 1-hour period of retrodialysis, a total amount of approx. 1 ng of synthetic AVP or approx. 10 ng of V1a-A was delivered locally into the mediolateral septum or into each side of the BNST (Engelmann *et al.*, 1992). Thirty minutes after the beginning of the retrodialysis procedure, the male residents underwent the RI test. The behaviour of rats with a microdialysis probe placed into the mediolateral septum was monitored during ongoing dialysis. To avoid behavioural restrictions, rats with bilateral microdialysis probes placed within the BNST were shortly disconnected from the tubings during the RI test and reconnected directly after the RI test. Twenty minutes after the end of the RI test, the microdialysis probes were disconnected and the rats were carried in their home cages to an adjacent room for immediate testing for anxiety-related behaviour on the EPM.

Histology

At the end of the experiments, rats were decapitated under CO_2 anaesthesia, brains were removed, quickly frozen in pre-chilled *n*-methylbutane on dry ice, and stored at -20°C. To histologically verify the placement of the probe within the mediolateral septum or within the BNST, brains were cut into 40-µm coronal cryostat sections and stained with cresyl violet. Only those rats with correct probe placements (see Fig. 20) were included in the statistical analysis resulting in the number of rats indicated in parentheses in the figures.

Radioimmunoassay of arginine vasopressin

AVP content was measured in lyophilised dialysates by a highly sensitive and selective radioimmunoassay (detection limit: 0.03 pg per sample; cross-reactivity of the antiserum with other related peptides, including oxytocin, was less than 0.7 %) (for details see Landgraf *et al.*, 1995b). To eliminate interassay variation, all samples to be compared were measured in the same assay.

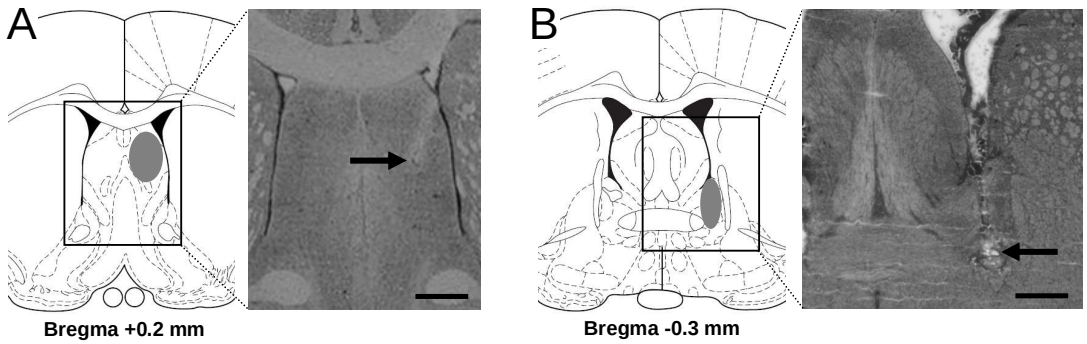

Fig. 20: Schematic drawings showing the location of the microdialysis probes within the lateral septum **(A)** or the BNST **(B)** (adapted from Paxinos & Watson, 1998) and the representative enlargement of the photomicrographs of a cresyl violet-stained coronal section of the rat brain after removal of the microdialysis probe. The arrows indicate the placement of the tip of the microdialysis probe. Scale bar = 1 mm.

Statistical analysis

Behaviour in the RI test and on the EPM was analysed with a one-way ANOVA. An ANOVA for repeated measures was used for analysing AVP release (factor time × factor aggression). When appropriate, ANOVA was followed by an LSD *post hoc* test. For all tests, the software package SPSS (version 16) was used. Data are presented as means ± SEM. Significance was accepted at $p < 0.05$.

Results

Arginine vasopressin release within the septum during exposure to the resident-intruder test

Based on their behaviour during the 10-minute RI test, male rats were classified as either aggressive (attack latency < 400 sec; aggressive behaviour > 10 % of total time) or non-aggressive (attack latency > 550; aggressive behaviour < 3 % of total time) (Fig. 21).

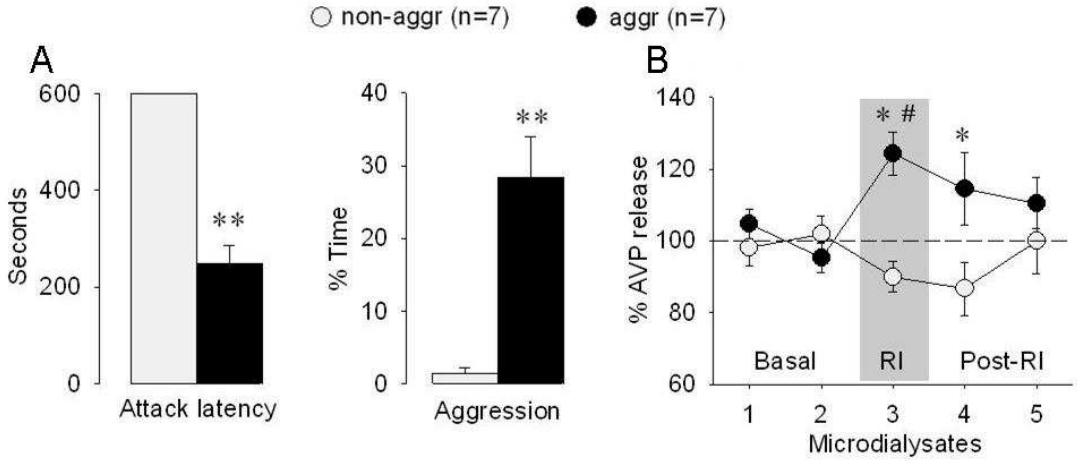

Fig. 21: AVP content in 30-minute microdialysates sampled within the lateral septum of non-aggressive and aggressive male rats exposed to the RI test. Male rats were divided into non-aggressive and aggressive groups, based on their attack latency time and the duration of aggressive behaviour **(A)** during the RI test under ongoing microdialysis. **(B)** AVP release within the lateral septum of non-aggressive and aggressive male Wistar rats under basal conditions and during and after exposure to the RI test given as percentage of baseline. Dialysate no. 1 and 2 were taken under basal conditions, no. 3 included exposure to the RI test, and no. 4 and 5 were taken after exposure to the RI test under undisturbed conditions. Data are presented as means ± SEM. * $p < 0.05$, ** $p < 0.001$ versus non-aggressive residents, $^{\#}$ $p < 0.05$ versus samples 1 and 2, ANOVA for repeated measures followed by LSD *post hoc* test.

Exposure to the RI test induced an aggression-dependent change in AVP release within the lateral septum (aggression × time: $F_{(4,48)} = 3.51$; $p < 0.05$). Aggressive male rats showed a significant increase in AVP release during the RI test ($p < 0.05$ versus baseline samples 1 and 2) (Fig. 21). In contrast, local AVP release in non-aggressive males during the RI test did not differ from baseline samples (Fig. 21). Accordingly, AVP release in the mediolateral septum

was significantly higher in aggressive versus non-aggressive rats during the RI test ($p < 0.005$) (Fig. 21). No difference was found between non-aggressive and aggressive rats for absolute AVP content under basal conditions (average of samples 1 and 2: non-aggressive rats, 7.3 ± 2.7 pg; aggressive rats, 5.4 ± 1.8 pg).

Pharmacological manipulation of arginine vasopressin activity in the lateral septum

Effects on aggressive behaviour in the resident-intruder test

To study the causal involvement of the AVP release within the septum in the regulation of aggression, another set of non-aggressive rats received either vehicle or synthetic AVP and another set of aggressive rats received either vehicle or the V1a-A into the mediolateral septum via retrodialysis. Local application of either synthetic AVP or the V1a-A did not alter any parameter of aggressive behaviour (Fig. 22) or any other behaviour in the RI test (data not shown).

Effects on anxiety-related behaviour on the elevated plus-maze

Application of synthetic AVP into the mediolateral septum of non-aggressive rats reduced the percentage of time on the open arms ($p < 0.05$) and the percentage entries into the open arms ($p < 0.05$) compared with vehicle-treated non-aggressive rats (Fig. 22A). Local application of the V1a-A into the mediolateral septum of aggressive rats did not change any behavioural parameter on the EPM (Fig. 22B).

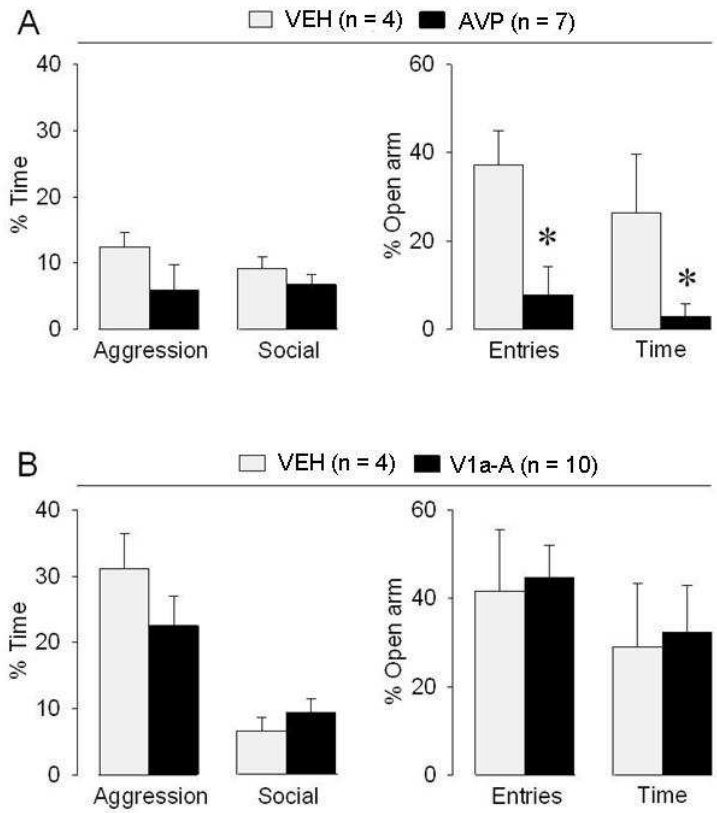

Fig. 22: Behavioural consequences of manipulation of the AVP system within the mediolateral septum via retrodialysis in RI test (left) and EPM (right) in **(A)** Non-aggressive rats perfused with either Ringer's solution (VEH) or Ringer's solution containing synthetic AVP (1 µg/ml), and **(B)** Aggressive rats perfused with either Ringer's solution (VEH) or Ringer's solution containing the V1a-A (10 µg/ml). Data are presented as means + SEM. * $p < 0.05$ vs. VEH, ANOVA.

Arginine vasopressin release within the bed nucleus of the stria terminalis during exposure to the resident-intruder test

Based on their behaviour during the 10-minute RI test, male rats were classified as either aggressive (attack latency < 400 sec; aggressive behaviour > 10 % of total time) or non-aggressive (attack latency > 550; aggressive behaviour < 3 % of total time) (Fig. 23).

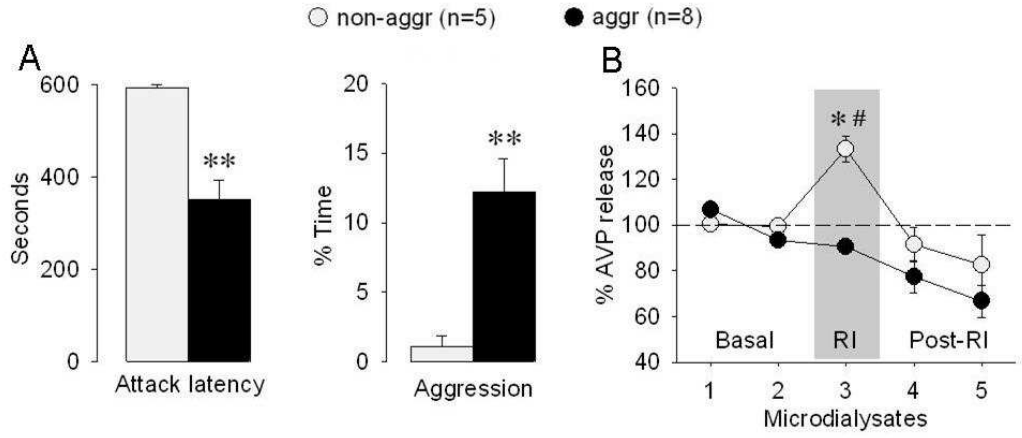

Fig. 23: AVP content in 30-minute microdialysates sampled within the BNST of non-aggressive and aggressive male rats exposed to the RI test. Male rats were divided into non-aggressive and aggressive groups, based on their attack latency time and the duration of aggressive behaviour **(A)** during the RI test under ongoing microdialysis. **(B)** AVP within the BNST of non-aggressive and aggressive male Wistar rats under basal conditions and during and after exposure to the RI test given as percentage of baseline. Dialysate no. 1 and 2 were taken under basal conditions, no. 3 included exposure to the RI test, and no. 4 and 5 were taken after exposure to the RI test under undisturbed conditions. Data are presented as means ± SEM. * $p < 0.05$ versus aggressive residents, ** $p < 0.005$ versus non-aggressive residents, $^{\#}$ $p < 0.05$ versus samples 1 and 2, ANOVA for repeated measures followed by LSD *post hoc* test.

Exposure to the RI test induced an aggression-dependent change in AVP release within the BNST (aggression × time: $F_{(4,44)} = 4.70$; $p < 0.005$). Non-aggressive males showed a significant increase in AVP release during the RI test ($p < 0.005$ versus baseline samples 1 and 2) (Fig. 23). In contrast, in aggressive males, local AVP release during the RI test did not differ from baseline samples (Fig. 23). Accordingly, AVP release during exposure to the RI test was significantly higher in non-aggressive versus aggressive rats ($p < 0.001$) (Fig. 23). No difference was found between non-aggressive and aggressive rats for absolute AVP content under basal conditions (average of samples 1 and 2: non-aggressive rats, 41.8 ± 3.1 pg; aggressive rats, 57.5 ± 3.6 pg).

To investigate the behavioural specificity of AVP release patterns in the BNST, non-aggressive and aggressive rats were exposed to ten minutes of forced swimming one day after exposure to the RI test. Non-aggressive and aggressive rats showed a similar increase in AVP

release within the BNST during forced swimming compared with respective baseline samples ($F_{(4,44)} = 4.26$, $p < 0.01$) (Tab. 5). *Post hoc* testing did not reveal any further significance.

Tab. 5: AVP release within the BNST of non-aggressive (n = 5) and aggressive (n = 8) male rats under basal conditions and during and after exposure to ten minutes of forced swimming (FS) given as percentage of baseline.

	Basal 1	Basal 2	FS	post-FS 1	post-FS 2
Non-aggr rats	109 ± 6.8	90.8 ± 6.8	119 ± 21	104 ± 9.0	90.7 ± 14
Aggr rats	109 ± 4.4	90.2 ± 4.4	130 ± 7.2	106 ± 13	80.1 ± 7.8

Data are presented as means ± SEM.

Pharmacological manipulation of arginine vasopressin activity within the bed nucleus of the stria terminalis

Effects on aggressive behaviour in the resident-intruder test

Local application of the V1a-A into the BNST of non-aggressive rats did not affect any parameter of aggressive behaviour (Fig. 24A) or any other behaviour in the RI test. In contrast, local application of AVP within the BNST of aggressive rats significantly reduced the level of intermale aggressive behaviour ($p < 0.05$) (Fig. 24B). There were no effects of local AVP on attack latency, number of attacks, or on non-aggressive behaviours.

Effects on anxiety-related behaviour on the elevated plus-maze

Local application of either the V1a-A or synthetic AVP into the BNST of non-aggressive and aggressive male rats, respectively, did not significantly change any behavioural parameter on the EPM (Fig. 24).

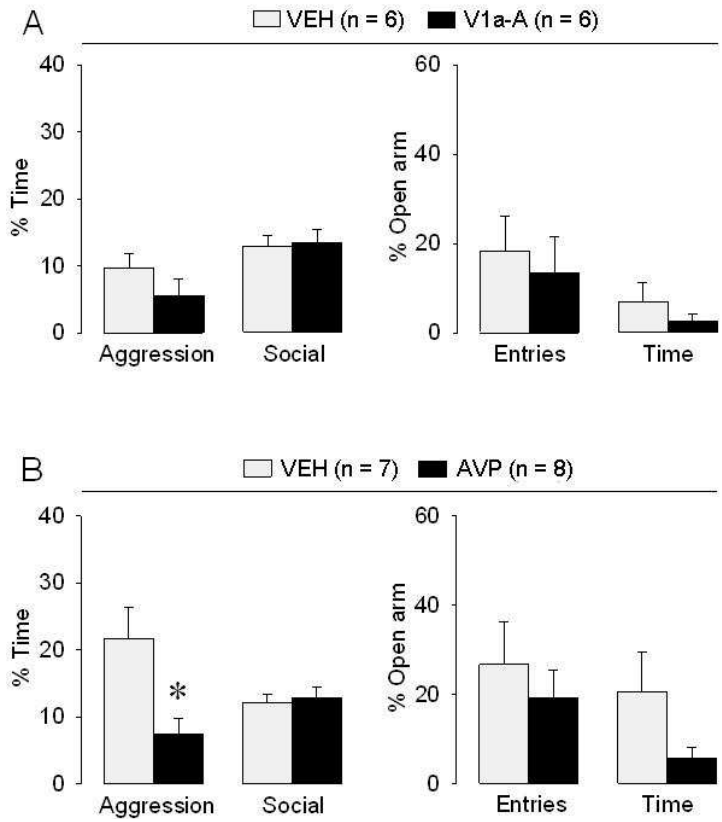

Fig. 24: Behavioural consequences of manipulation of the AVP system within the bed nucleus of the stria terminalis in RI test (left) and EPM (right) in **(A)** Non-aggressive rats perfused with either Ringer's solution (VEH) or Ringer's solution containing the V1a-A (10 µg/ml), and **(B)** Aggressive male rats perfused with either Ringer's solution (VEH) or Ringer's solution containing synthetic AVP (1 µg/ml). Data are presented as means + SEM. * $p < 0.05$ vs. VEH, ANOVA.

Discussion

In the present study, distinct local AVP response patterns were found within the lateral septum and the BNST during the display of intermale aggression in adult rats. Aggressive rats showed an increase in AVP release within the lateral septum, whereas non-aggressive rats showed an increase in AVP release within the BNST when being exposed to the RI test. Administration of synthetic AVP or a specific V1a-A into the mediolateral septum did not alter aggressive behaviours, whereas administration of synthetic AVP into the BNST of aggressive rats reduced the level of aggression. Furthermore, synthetic AVP administered into

the septum, but not into the BNST, significantly increased anxiety-related behaviour. These data suggest that AVP released within the BNST modulates the display of aggression, whereas AVP release within the lateral septum may facilitate aggression-related behaviours like for example anxiety.

Role of arginine vasopressin release within the lateral septum in intermale aggression

The suggested role for AVP neurotransmission within the lateral septum in the regulation of intermale aggression is mainly based on three findings. First, AVP fibre density in the lateral septum is under strong influence of gonadal steroids and is approx. twice as high in male compared with female rats (De Vries *et al.*, 1981; De Vries & Buijs, 1983; de Vries *et al.*, 1984; De Vries *et al.*, 1985). It has therefore been suggested that AVP within the septum might underlie male-specific behaviours, most notably aggressive behaviour. Second, several studies have reported large differences in AVP fibre density within the lateral septum between high- and low-aggressive males of different rodent species, suggesting a correlation between septal AVP and the display of aggression. Third, local application of AVP within the septum increased aggressive behaviour in otherwise low-aggressive castrated rats (Koolhaas *et al.*, 1991) and increased flank marking (a territorial/aggressive display) in golden hamsters (Irvin *et al.*, 1990). Although these data seem convincing in demonstrating a role for AVP within the septum in aggressive behaviour, there are some paradoxical findings, too. For example, the variation in septal AVP fibre density among males is found to be as large as the variation of septal AVP fibre density between males and females (Koolhaas *et al.*, 1991; de Vries, 2008). Moreover, some studies report that those males showing low AVP fibre density have the highest level of aggression. Lastly, results obtained by local application of AVP within the septum of castrated rats and male golden hamsters, both nearly lacking AVP-immunoreactive

fibres in the septum, may not give an accurate prediction about the endogenous function of AVP within the septum in the regulation of intermale aggression.

To clarify the role of AVP in the septum in the regulation of intermale aggression, we recently measured AVP release within the mediolateral septum of genetically selected Wistar rats showing different levels of intermale aggression. Here, high-aggressive rats showed a significant decrease in septal AVP release, while low-aggressive rats rather showed an increase in septal AVP release during exposure to the RI test (Beiderbeck et al., 2007). Surprisingly, pharmacological manipulation of AVP neurotransmission in the septum failed to change the level of aggression in high- as well as in low-aggressive rats (Beiderbeck et al., 2007). As these data were obtained from rats genetically bred for a difference in anxiety, the AVP release patterns might be unique to these LAB and HAB rat lines.

We now demonstrate that in Wistar rats, classified as either aggressive or non-aggressive, AVP release patterns within the septum are opposite to those found in LAB and HAB rats. Here, aggressive rats showed a significant increase in AVP release within the septum during exposure to the RI test compared with basal AVP release and compared with the AVP release in non-aggressive rats. Non-aggressive rats did not show a change in AVP release within the septum. These data suggest a positive correlation between septal AVP release and the display of aggression. However, similar to our previous findings in LAB and HAB rats, pharmacological manipulation of the AVP system within the septum did not change the level of aggressive behaviour in either aggressive or non-aggressive rats. Nevertheless, application of AVP into the septum of non-aggressive rats induced an increase in anxiety-related behaviour as measured on the EPM. The latter finding confirms other studies demonstrating an anxiogenic effect of AVP within the septum (Landgraf *et al.*, 1995a; Millan, 2003; Beiderbeck *et al.*, 2007). Therefore, we propose that the change in AVP release within the septum might be the subsequent result, rather than the cause, of the display of aggression.

Similarly, the septal AVP system has been implicated in the behavioural stress response as seen in the forced swim test (Ebner *et al.*, 1999). However, the septal AVP release is stressor-specific and does not respond to social defeat (Ebner *et al.*, 2000). Generally, the role for the lateral septum is at the present seen as a relay point in the neuronal circuits of complex social behaviours (Bielsky et al., 2005), and especially aggression, collecting and processing social information and rather indirectly regulating the behavioural outcome. Besides its action as fast neurotransmitter, AVP is known as a neuromodulator (Landgraf & Neumann, 2004). As such, alterations in AVP neurotransmission might have effects on a set of subsequent behaviours which are relevant in the context of aggression (Veenema & Neumann, 2008). These behaviours may include anxiety, as demonstrated in the present study, but may comprise social memory as well. Indeed, the septal AVP system seems to play a fundamental role in the processing of olfactory cues of conspecifics. Administration of synthetic AVP into the septum improves social memory, whereas administration of a V1a-A into the septum abolishes social memory in male rats (Engelmann & Landgraf, 1994; Engelmann *et al.*, 1994).

Role of arginine vasopressin release within the bed nucleus of the stria terminalis in intermale aggression

The BNST is a sexually dimorphic brain region (del Abril *et al.*, 1987; Han & De Vries, 2003) and is thought to be involved in the regulation of intermale aggression (Hines et al., 1992). The ventral part of the BNST contains AVP-expressing neurons, whereas the dorsal part of the BNST contains a high density of AVP V1a receptors (De Vries & Buijs, 1983; Caffe *et al.*, 1987). The presence of these receptors suggests that local AVP release could occur from somata and dendrites, as previously described for the magnocellular neurons of the hypothalamic PVN and supraoptic nucleus (Pow & Morris, 1989; Ludwig *et al.*, 2002;

Landgraf & Neumann, 2004). However, in contrast to these hypothalamic brain regions (Engelmann *et al.*, 1999; Ebner *et al.*, 2005), there is a sparse evidence for AVP release within the BNST.

Here, we report for the first time the *in vivo* release of AVP within the BNST of male rats. Importantly, non-aggressive rats showed an increase in local AVP release when being exposed to the RI test, whereas aggressive rats showed no change in AVP release within the BNST. This AVP release pattern seems to be associated with the level of aggression based on the following findings. First, both non-aggressive and aggressive rats showed a similar increase in AVP release when exposed to ten minutes of forced swimming. Second, bilateral administration of synthetic AVP into the BNST significantly reduced the level of intermale aggression in aggressive rats. Finally, bilateral administration of synthetic AVP into the BNST of aggressive rats did not result in a change in anxiety-related behaviour as measured on the EPM. Together, these findings suggest that AVP released within the dorsal part of the BNST is negatively associated with the display of intermale aggression in Wistar rats.

Our data are in line with some, but not all, studies on the role of AVP within the BNST in the regulation of aggression. For example, a decrease in intermale aggression in prairie voles is associated with an increase in AVP immunoreactivity in the dorsal BNST but a decrease in the ventral BNST (Frazier et al., 2006). Moreover, the higher level of aggressive behaviour in California mice compared with White-footed mice is accompanied by an increased level of AVP staining within the BNST (Bester-Meredith et al., 1999). Additionally, a decrease in RI aggression in cross-fostered California mice is associated with a decrease in ventral AVP immunoreactivity in the BNST (Bester-Meredith & Marler, 2001). Furthermore, an increase in neuronal activation in the BNST was found in hamsters and rats in response to aggression (Kollack-Walker & Newman, 1995; Delville *et al.*, 2000; Veening *et al.*, 2005) or aggressive

motivation (Ferris et al., 2008). Finally, injection of AVP into the BNST in hamsters leads to increased flank marking, which is part of aggressive behaviour in hamsters (Irvin et al., 1990). However, an increased neuronal activation does not have to be linked to an increase in AVP release and the AVP system within the BNST could exert species-specific influences on intermale aggressive behaviour.

In conclusion, we could further substantiate the role of AVP in the regulaton of aggression, demonstrating brain region-specific AVP release patterns during the RI test and a brain region-specific behavioural significance of such release. Our results suggest an indirect effect of septal AVP in the regulation of aggression. In contrast, AVP released within the BNST exerts a direct effect on aggressive behaviour. We propose that AVP might have brain region-specific effects on diverse behaviours by acting via two different modes of action. Exposure to the RI test may trigger the release of AVP within the BNST and result in a direct and acute effect on the level of aggression, whereas RI test-induced release of AVP may facilitate behavioural responses including, for example, anxiety and social memory at a later time point.

Chapter 6

General Discussion

Contents

1. Summary of results

Violent and escalated aggression, the resulting psychological and physical injuries as well as the costs induced thereby are a huge burden for the human society. A better understanding of the mechanisms underlying aggression is essential for novel therapy, treatment and prevention strategies. To this end, animal models are needed, but so far, suitable animal models for aggressive behaviours are scarce. Therefore, the major aim of the present thesis was to investigate, whether two rat lines bred for low or high anxiety-related behaviour can be used as an animal model for research on male aggressive behaviours.

LAB and HAB rats have been selectively bred for low and high anxiety-related behaviour since 1993. HAB rats show behavioural as well as neurobiological phenotypes that are related to some characteristics seen in patients suffering from depression and/or anxiety-related diseases and are therefore established as an animal model for these psychopathologies (Landgraf & Wigger, 2002; 2003; Wigger *et al.*, 2004). So far, LAB rats were exclusively used as an appropriate control, bred and housed under identical conditions. However, first observations of their social behaviour revealed that LAB rats tended to be more aggressive under basal conditions in group-housed rats as well as in the cage of an aggressive conspecific (Henniger *et al.*, 2000; Frank *et al.*, 2006). This was the basis for further experiments on social, and especially aggressive, behaviours in LAB rats. To provide insights into the stability of the behavioural phenotypes of LAB and HAB rat lines, chapter 2 investigated the time course of anxiety-related behaviour as measured on the EPM and of aggressive behaviour as measured during the RI test over a period of six years. Results show that the line difference in anxiety-related behaviour has been stable and robust over the 6-year observation period and that the aggressive behaviour has been unchanged over the years. Additionally, a detailed behavioural profile of the rat lines during the RI test was created in LAB, HAB and NAB rats (the commercially available Wistar rats from which LAB and HAB rats originate).

LAB rats were more aggressive than NAB rats, whereas HAB rats displayed an intermediate level of aggression. This was reflected by a longer duration of total aggressive behaviour, lateral threat and offensive upright in LAB compared with NAB rats. HAB rats displayed intermediate levels of total aggressive behaviour and the different elements of aggression. Moreover, almost 10 % of the LAB rats were highly aggressive, i.e. they showed aggressive behaviour for more than 55 % of the total time of the RI test. In contrast, none of the HAB or NAB rats showed such a high level of aggression. Finally, LAB, HAB and NAB rats were tested for abnormal forms of aggressive behaviour towards a male, a female and a narcotised male rat. Both LAB and HAB rats showed abnormal forms of aggressive behaviour, including attacks towards vulnerable body parts of the male intruder and attacks towards harmless opponents like a female or a narcotised male intruder. Taken together, both LAB and HAB rats showed a higher level and abnormal forms of aggressive behaviour compared with NAB rats.

A second aim of the thesis was to investigate neurobiological and neuroendocrine mechanisms involved in the regulation of aggressive and abnormal aggressive behaviour. A better understanding of the regulation of aggression is the basis for the development of drugs for the treatment of aggressive outbursts and violent behaviour in humans. Differences between aggressive and non-aggressive rats in endocrine, neurotransmitter or neuromodulator systems can be helpful to find targets for therapeutical drugs to reduce escalated aggressive and violent behaviours. In this thesis, I concentrated on the role of the serotonin system, the HPA axis and the AVP system, as these three systems are thought to be involved in the regulation of aggression.

Thus, in chapter 2, the role of the serotonin system in aggressive behaviour was studied in LAB rats, which were chosen, because they showed the highest level of aggression. A single

systemic injection of the preferential serotonin 1_A autoreceptor agonist S-15535, resulting in an acute decrease in serotonin release in brain regions receiving serotonergic projections (mainly originating from the dorsal raphe nuclei), selectively reduced the number of attacks towards a conscious or narcotised male intruder rat without affecting the total duration of aggressive behaviour displayed by the LAB resident rats. This suggests the involvement of the serotonin system in particular in the attack component of aggressive as well as abnormal aggressive behaviour in LAB rats.

In chapter 3, line differences in the reactivity of the HPA system in response to the RI test were investigated, as changes in the HPA system activity have been associated with enhanced aggression. Higher plasma ACTH concentrations were found in LAB compared with HAB rats in response to the RI test. In both LAB and HAB rats, exposure to the RI test induced a significant increase in plasma corticosterone concentrations, whereas in NAB rats no such increase in corticosterone was found.

Additionally, in chapter 3, the neuronal activation within selected brain regions upon exposure to the RI test was determined in LAB and HAB rats by measuring the expression of the immediate-early gene *c-fos*. LAB rats showed a higher neuronal activation of the parvocellular part of the PVN in response to the RI test. Furthermore, a tendency towards a higher activation in response to the RI test was found in the central and medial amygdala as well as in the hypothalamic attack area in LAB rats compared with HAB rats.

In chapters 4 and 5, the involvement of the brain AVP system in the regulation of aggressive behaviour was studied, measuring the in vivo AVP release within the lateral septum and the BNST during the RI test. A higher level of aggressive behaviour, as seen in LAB rats, was accompanied by a decrease of septal AVP release. In support, this was accompanied by a

lower neuronal activation within the septum in response to the RI test. In contrast, HAB rats showed an increase in local AVP release. Application of synthetic AVP into the lateral septum of LAB rats did not affect the level of aggressive behaviour, but resulted in an increase in anxiety as measured on the EPM. Moreover, blocking the V1a receptors by the selective AVP V1a-A d$(CH_2)_5$Tyr(Me)AVP in the septum of HAB rats did not affect aggressive behaviour, but reduced the duration of social investigation during the RI test.

In chapter 5, I extended the studies on the AVP-mediated regulaton of aggression by studying the involvement of the AVP system in the lateral septum and in the BNST in the regulation of aggressive behaviour in male NAB rats using microdialysis. NAB rats were chosen in order to be able to draw general conclusions on the role of AVP released within the septum or BNST on aggression also in non-selected rats purchased from Charles River Laboratories. In addition to the lateral septum, the BNST seems to be involved in the regulation of aggression. The BNST contains a high density of V1a receptors and sends AVPergic projections to the lateral septum. NAB rats were classified as either aggressive or non-aggressive according to their performance in the RI test. The aggressive rats showed a significant increase in AVP release within the septum in response to the RI test compared with non-aggressive rats, which showed no change in septal AVP release. Application of either the V1a-A into the lateral septum of aggressive rats, or synthetic AVP into the lateral septum of non-aggressive rats did not result in a change in aggressive behaviour. However, application of synthetic AVP into the septum of non-aggressive rats induced an increase in anxiety as measured on the EPM.
In another set of aggressive and non-aggressive male NAB rats, AVP release was measured in the BNST. In contrast to the AVP release patterns in the lateral septum, AVP release in response to the RI test remained unchanged in aggressive rats, whereas a significant increase in AVP release within the BNST was found in non-aggressive rats. Importantly, administration of synthetic AVP into the BNST of aggressive rats resulted in a significant

reduction in the level of aggressive behaviour. Taken together, intermale aggression was found to be associated with distinct AVP release patterns within the lateral septum and the BNST. Pharmacological manipulation of the AVP system in the BNST directly affected the display of aggressive behaviour, whereas no effect on aggression was found in the septum. I hypothesize that the observed change in AVP release within the septum during exposure to the RI test may rather be the consequence of the display of aggression and modulate behaviours associated with aggression, such as social investigation and anxiety.

2. Animal models for excessive and abnormal aggression

2.1 LAB rats

LAB rats have been selectively bred for low anxiety-related behaviour measured on the EPM since 1993 as described in chapter 2. The low anxiety profile was accompanied by a high expression of aggression in comparison with HAB and NAB rats, as reflected by higher levels of several elements of aggressive behaviour, including threat, lateral threat, and offensive upright (see Fig. 7). Among these, lateral threat was the most prominent element of aggression comprising more than half of the total aggression time. Importantly, lateral threat is considered the most important aggressive behaviour which often precedes an attack. Of all LAB rats tested for aggression during the last 5-year period, almost 80 % showed aggressive behaviour for more than 15 % during the 10-minute RI test and are categorised as medium-aggressive (see Fig. 8). Moreover, almost 10 % of LAB rats show aggressive behaviour for more than 55 % of the time and are considered highly aggressive. In contrast to LAB rats, more than half of the NAB rats showed less than 15 % aggressive behaviour and were classified as low-aggressive. Medium-aggressive individuals were found in NAB (45 %) as well as in HAB (80 %) rats, but highly aggressive individuals were exclusively found in LAB rats (see chapter 2).

LAB rats further showed forms of abnormal aggression as assessed in different tests (see chapter 2). During confrontation with an unknown, slightly smaller intruder male in the regular RI test, the majority of attacks are normally directed towards less vulnerable body parts, i.e. the back and the flanks of the intruder. Indeed, NAB rats directed more than 90 % of the attacks towards these body parts. In contrast, LAB rats directed more than 50 % of the attacks towards vulnerable body parts of the intruder, such as head, throat and belly (see Fig. 9B). In line with this, the latency to attack a vulnerable body part was significantly shorter in LAB rats (see Fig. 9A). Interestingly, HAB rats also showed a high number (50 %) of attacks towards vulnerable body parts (see Fig. 9B), but with a longer latency (see Fig. 9A). In another test, residents were confronted with a non-oestrus female intruder rat which should not elicit aggressive behaviour in males. Indeed, whereas HAB and NAB rarely attacked the female intruder, LAB rats attacked the female significantly faster and more often (see Fig. 9). In a third test, a narcotised male intruder was found to be attacked by LAB, HAB as well as by NAB rats, but LAB rats had the shortest attack latency (see Fig. 9A). Moreover, LAB rats showed a high number of attacks directed towards the head of the unconscious intruder (see Fig. 9B).

These results clearly indicate that LAB and, to a lesser extent, also HAB rats display not only high, but also abnormal forms of aggression. Haller and Kruk (2006) suggested the following criteria for abnormal aggression: (i) Mismatch between provocation and response, (ii) disregarding species-specific rules (e.g. attacking females, attacking vulnerable body parts), (iii) insensitivity towards the social signals of the opponent (e.g. ignoring submissiveness by continuing attacking). By showing intense attacks towards the head of a narcotised rat, LAB rats clearly showed a mismatch between provocation and response. An unconscious conspecific represents no threat for the resident's territory. The same is true for female intruders. Moreover, attacking females is additionally a sign for disregard of species-specific rules. There is no biological reason for a naïve male to attack a female rat, because it could

provide the possibility for reproductive success. Concerning the third criterion for abnormal aggression, data are missing for LAB rats. However, it seems that LAB rats continue with severe attacks even if the male intruder shows defensive/submissive behaviour, e.g., lying on its back. Additional screening of the behavioural patterns of LAB rats during the RI test would be needed to quantify attacks conducted after submissive signals of the opponent. Taken together, LAB rats show clear signs of abnormal aggression in different situations.

Besides the high level and abnormal forms of aggression, it is interesting to note that LAB rats also show a reduced level of non-aggressive social investigation during the RI test compared with HAB and NAB rats (see Figs. 7A, 12C, 16) indicating a general impairment of social interactions. In support, LAB males preferred to explore a novel environment in the modified hole board test, whereas HAB males preferred to stay close to their cage mates (Ohl et al., 2001). I suggest that these findings may indicate that social contact is stressful for LAB rats. This is supported by the observation that LAB rats generally show a higher HPA response when exposed to a social stimulus, i.e. both as a resident (see Fig. 14) or as intruder (Frank et al., 2006) in the RI test. Interestingly, in another animal model for high-aggressive behaviour, namely the SAL and LAL mice selected for short and long attack latency, there are also line differences in HPA axis responsiveness. The aggressive SAL mice show a lower corticosterone response to a non-social stimulus (Veenema et al., 2003b) comparable with LAB rats, whereas SAL mice also show a lower response to a social stressor compared with the less aggressive conspecifics (Veenema et al., 2005b). This is in line with the fact that exaggerated aggression in humans can be linked to both a state of hyper- and hypo-arousal, which is characterised amongst others by high and low levels of glucocorticoids, respectively (Haller & Kruk, 2006). These results suggest a different underlying mechanism for the high level of aggression shown in LAB rats and SAL mice. In LAB rats, the high amount of aggression could be linked to some kind of social anxiety or social phobia. The high amount of aggression and abnormal aggression displayed against a male, female or unconscious male

rat together with their low amount of non-aggressive social behaviour, such as approaching and investigating the conspecific, suggests LAB rats as an animal model for aggressive and abnormal aggressive behaviour as well as for antisocial behaviour.

2.2 HAB rats

HAB rats have been selectively bred for high anxiety-related behaviour (less than 10 % of time spent on the open arms of the EPM) since 1993 (see chapter 2). Interestingly, this selection based on anxiety led to a higher aggressive behaviour compared with NAB rats. HAB rats show a higher level of total aggression than NAB rats and they spend almost 50 % of the total aggression time with lateral threat, which is the most important display of aggression (see Fig. 7). Furthermore, almost 80 % of the HAB rats had a level of aggression between 15 and 55 % and were therefore categorized as medium-aggressive (see Fig. 8).

HAB rats further showed abnormal aggression, consisting of attacks towards vulnerable body parts of the intruder in the RI test. Compared with NAB rats (8.3 ± 5.3 %), HAB rats (47.5 ± 8.7 %) showed a higher percentage of attacks directed towards vulnerable body parts of the intruder (see Fig. 9B). Thus, HAB rats showed a higher level of aggression compared with NAB rats, and they additionally showed signs of abnormal aggressive behaviour. Therefore, they could be a suitable animal model for research on aggression, especially linked with a high level of trait anxiety.

2.3 Robustness of the behavioural profiles

Behavioural data of LAB, HAB and NAB rats have been collected and evaluated over six years. The bidirectional selective breeding resulted in a robust line difference in anxiety-related behaviour between low anxious LAB and high anxious HAB rats. Likewise, the level of aggressive behaviour shown during a standard RI test did not change over the years in HAB rats. However, LAB and NAB rats showed a reduction of aggression in 2005/2006. This

drop in aggressive behaviour in LAB rats could be explained by the fact that all LAB rats tested in the RI test in this time period have been tested during winter, where I found a significant decrease of aggression in dependence of season in LAB rats. This explanation is not applicable for NAB rats as they were tested in different seasons in the respective time period. Additionally, NAB rats did not show a seasonal effect on aggression. Although NAB rats are an outbred rat line, it cannot completely be excluded that there are natural fluctuations or even a drift in such a complex behaviour like aggression. Despite these influences, LAB rats are more aggressive than NAB rats, independent of season throughout the years. HAB rats mostly show an intermediate level of aggression below that of LAB rats (see Figs. 5B, 7, 12, 16, Tab. 3), but above that of NAB rats (see Fig. 7).

Behavioural data collected from different seasons revealed a seasonal effect in LAB rats for both anxiety-related and aggressive behaviour. Anxiety was found to be further reduced in LAB rats during summer, whereas aggressive behaviour is reduced during winter time. Neither HAB nor NAB rats showed influences of season on anxiety or aggression.

In contrast to HAB and NAB rats, the behavioural profile of LAB rats shows parallels with the profile of wild-type rats that were caught from the wild and are bred since several generations at the University of Groningen, The Netherlands. Both LAB and wild-type rats show a great variability in aggressive behaviour, including low- (<15 % aggression), medium- (between 15 and 55 % aggression) and high-aggressive (>55 % aggression) individuals. High-aggressive rats have not been found in the HAB or NAB rats tested, but they were existent in the LAB breeding line (see Fig. 8). However, the variability in the level of aggression is bigger in wild-type rats as they include rats showing more than 80 % aggression, which has not been seen in LAB rats. In contrast, the amount of rats showing low aggression is actually lower in LAB (13.2 %) compared with wild-type (30.4 %) rats. Furthermore, in the laboratory-bred wild-type rats, a reduced aggression during winter and summer has been found (de Boer et al., 2003). Although this second trough of aggression in summer was

missing in LAB rats, these results support the assumption of LAB rats being more "wild-type-like" than HAB or NAB rats. It is not yet clear, why these seasonal effects on anxiety and aggression are only seen in LAB, but not in HAB or NAB rats. Probably, olfactory stimuli lead to a seasonal change of circulating hormones exclusively in LAB rats resulting in reduced anxiety or aggression. Interestingly, a seasonal rhythm of aggression can also be found in species without a breeding season such as monkeys and, in part, also in humans (Michael & Zumpe, 1981; Maes *et al.*, 1993). Although seasonal rhythms of serotonin and androgens have often correlated with such rhythmic changes in aggression, the regulation must be more complex (Miczek et al., 2002). For example, increasing experience with aggressive encounters leads to a reduced influence of androgens on the display of aggression (Scott & Fredericson, 1951) and the correlation between aggression and androgens can disappear depending on the social context (Wallen, 1996). These data suggest that seasonal variations in circulating hormones together with the genetic background of the selectively bred LAB rats lead to seasonal behavioural changes.

In contrast to the seasonal influence on aggressive behaviour, there is not much known about the influence of seasons on anxiety. However, there are some studies that show seasonal influences on anxiety in mental disordered patients (Marriott *et al.*, 1994; de Graaf *et al.*, 2005; Ohtani *et al.*, 2006). To my knowledge, studies in laboratory animals dealing with this issue are lacking so far.

Taken together, the behavioural differences between LAB, HAB and NAB rats in anxiety-related behaviour and aggression are robust. Thus, both LAB and HAB rats can be applied as an interesting new animal model for the research on aggressive behaviour as well as on abnormal aggression, especially in the context of differing levels of innate anxiety.

3. Anxiety and aggression

Continuous breeding for low and high anxiety-related behaviour, respectively, resulted in a robust difference in this behavioural dimension accompanied by an elevated level of aggressive behaviour in both the LAB and HAB breeding line. Therefore, one cannot speak of a co-selection of high anxiety and low aggression and vice versa. Accordingly, statistical analysis revealed a U-shaped correlation between anxiety and aggression in LAB, HAB and NAB rats with LAB and HAB rats showing a high level of aggression accompanied by a low or high level of anxiety, respectively. NAB rats showed an intermediate level of anxiety as well as relatively low levels of aggression (see Fig. 5C). In chapter 3, a negative linear correlation between anxiety and intermale aggression has been described, but this was largely due to the large individual variance in both behaviours within the LAB rats. Additionally these data are not totally comparable. Data collected over the last years and presented in chapter 2 were obtained from untreated animals tested during the dark phase of the light/dark cycle, wherease those in the experiments of chapter 3 have been tested during the light phase using animals that were equipped with a jugular vein catheter. Importantly, also under these conditions, LAB rats showed the highest level of aggression, while NAB rats were the lowest aggressive line, HAB rats displayed an intermediate level of aggression, thus confirming the results presented in chapter 2.

Taken together, these results point against a direct linear correlation of high anxiety-related behaviour with low aggression and vice versa. In the literature, the findings concerning the correlation between anxiety and aggression are conflicting. Turku mice were selectively bred for low- and high-aggressive behaviour. Interestingly, in these mouse lines, selection for low aggression resulted in mice with high anxiety and vice versa (Nyberg *et al.*, 2003). In contrast, SAL and LAL mouse lines do not show differences in anxiety on the EPM (Veenema et al., 2003a) or in the light-dark box (Hogg *et al.*, 2000). Additionally, there is no correlation between anxiety and aggressive behaviour in wild-type rats kept and bred in the

laboratory (de Boer *et al.*, 2003). Thus, a robust correlation between low anxiety and high aggression or vice versa is generally lacking. However, it has to be considered that there might be different forms of anxiety. Most findings in the literature are based on the measurement of anxiety in a non-social context, such as the EPM or the light-dark box, which elicit a stronger stress response and a high level of anxiety-related in HAB compared with LAB rats (Landgraf et al., 1999). As shown in chapter 2 (Fig. 5C) this type of anxiety is not linked in a linear manner to aggressive behaviour. On the other hand, there is anxiety related to social contexts as seen in patients suffering from social anxiety disorder which is the most common anxiety disorder (Stein & Stein, 2008). A similar behavioural phenotype can experimentally be induced in mice. Repeatedly defeated mice develop a long-lasting aversion to social contact (Berton et al., 2006). Aggressive behaviour was not investigated in these mice, allowing no conclusion concerning the link of social anxiety and aggression. However, in a social preference test which consists of measuring the time the experimental animal spends in contact with a caged male rat versus the time the animal spends with an empty cage, LAB rats do not show social preference, whereas both HAB and NAB rats do (Lukas et al., 2008). Additionally, social stressors, such as the RI test either as resident (chapter 3) or as intruder (Frank et al., 2006), induced a higher stress response in LAB compared with HAB rats. Taken together, these findings could be interpreted as enheightened level of social anxiety in LAB rats. Therefore, I hypothesise that in the LAB and HAB rats the high level of aggression is linked to a high level of anxiety in a social and a non-social context, respectively. In contrast, in the highly aggressive SAL mice, a social stressor did not induce an elevated stress response as seen in LAB rats (Veenema et al., 2005b) indicating that social anxiety is probably not underlying the high levels of aggression displayed by SAL mice. Thus, a robust correlation between anxiety neither in a non-social nor in a social context and anxiety can be found.

To further investigate the relationship between anxiety and aggression, I manipulated the level of anxiety pharmacologically. Substances affecting the GABAergic system like pentylenetetrazole (PTZ) and diazepam (DIA) have been shown to increase (Anseloni & Brandao, 1997) or decrease (Liebsch et al., 1998a) anxiety-related behaviour. Here, PTZ and DIA were used to assess whether pharmacological manipulation of anxiety via $GABA_A$ receptors is accompanied by changes in the level of intermale aggression. Therefore, rats were injected with PTZ (25 mg/kg body weight dissolved in 0.9 % saline, i.p; Sigma) or DIA (2 mg/kg body weight dissolved in 0.9 % saline; i.p.; Sigma) and subjected to the RI test as resident 30 minutes later. As no further anxiolytic effect of DIA was found in LAB rats, they only received PTZ. Liebsch et al. (1998a) showed an increase in the percentage of time spent on the open arms of the EPM in LABs, but this could be due to different testing conditions (light/dark phase) and to the relatively low level of open arm exploration in control LAB rats (20 % time on open arms in comparision to 45 % in my experiment). Similarly, in HAB rats, treatment with the anxiogenic substance PTZ did not further increase their level of anxiety, which could be due to a ceiling-effect. Consequently, HAB rats were only treated with DIA. Groups of NAB rats, which display an intermediate level of anxiety-related behaviour, received either PTZ or DIA. All controls were given an injection with 0.9 % saline (vehicle; i.p.; 1 ml/kg body weight). In LAB rats, PTZ significantly reduced the level of aggression (ANOVA: $F_{(1,22)} = 35.7$; $p < 0.001$; Tab. 6), in particular the display of lateral threat ($F_{(1,22)} = 16.5$; $p < 0.01$), offensive upright ($F_{(1,22)} = 11.4$; $p < 0.01$), keep down ($F_{(1,22)} = 4.82$; $p < 0.01$) and threat ($F_{(1,22)} = 15.8$; $p < 0.01$). Additionally, the number of attacks decreased ($F_{(1,22)} = 16.0$; $p < 0.01$) and the attack latency was longer ($F_{(1,22)} = 13.3$; $p < 0.001$) after treatment with PTZ. It has to be mentioned that PTZ-treated LAB rats showed more immobility than vehicle-treated controls ($F_{(1,22)} = 5.39$; $p < 0.05$; Tab. 6).

Tab. 6: Behavioural parameters of male LAB and HAB rats during the RI test 30 minutes after an acute injection of PTZ (LAB), DIA (HAB), or vehicle (= VEH). Aggressive behaviour, social investigation, exploration and immobility were calculated as percentage of time.

	LAB		HAB	
	VEH (n = 11)	PTZ (n = 12)	VEH (n = 12)	DIA (n = 12)
Aggressive behaviour	20.8 ± 3.4	1.0 ± 0.5$^{\#}$	11.2 ± 3.5	19.2 ± 3.5
Attack latency (sec)	298 ± 57	556 ± 44*	404 ± 61	220 ± 53*
Social investigation	15.2 ± 3.0	7.2 ± 1.4*	18.0 ± 1.5	16.3 ± 1.4
Exploration	46.5 ± 2.7	68.8 ± 3.3$^{\#}$	47.9 ± 4.5	46.8 ± 2.3
Immobility	3.5 ± 0.7	9.3 ± 2.3*	3.0 ± 0.8	9.2 ± 2.5*

Data are presented as means ± SEM. ANOVA for treatment effects, * $p < 0.05$ versus respective vehicle, $^{\#}$ $p < 0.001$ versus respective vehicle.

These results could implicate that the reduction in aggressive behaviour after PTZ was due to a possible sedative drug effect. However, this is rather unlikely as LAB rats treated with PTZ also showed a significantly higher amount of explorative behaviour during the RI test compared with controls ($F_{(1,22)} = 27.1$; $p < 0.001$; Tab. 6). Together with the finding that PTZ-treated LAB rats show a lower level of social investigation ($F_{(1,22)} = 6.16$; $p < 0.05$; Tab. 6), this leads to the assumption that all forms of social behaviour (aggression, social investigation) have been reduced by PTZ. Indeed, LAB resident rats treated with PTZ tended to squeak and to avoid contact when the intruder rat approached. Singewald et al. (2003) showed that four different anxiogenics, including FG-7142 which acts on the GABAergic system, increase the neuronal activation in the central amygdala, BNST, lateral septum, PVN, and other brain regions. As the lateral septum is an important brain region for non-aggressive social behaviours (chapter 4, Dantzer *et al.*, 1988; Engelmann & Landgraf, 1994; Landgraf *et al.*, 1995a; Landgraf *et al.*, 2003), changes in neuronal activation could directly affect social approach or social investigation as seen in PTZ-treated LAB rats. On the other hand, DIA has been shown to increase social contact during the social interaction test in NAB rats, whereas

treatment with an anxiogenic substance (*meta*-chlorophenylpiperazine) resulted in a reduction of social interaction (Rex et al., 1996). This is in line with the reduced non-aggressive social behaviour seen in PTZ-treated LAB rats.

Activation of GABA receptors by DIA resulted in a significant increase in keep down (ANOVA: $F_{(1,23)} = 5.05$; $p < 0.05$) and a decrease in attack latency ($F_{(1,23)} = 5.21$; $p < 0.05$; Tab. 6) in HAB rats. The total level of aggressive behaviour was not influenced by DIA. Treatment with DIA induced an increase in immobility ($F_{(1,23)} = 5.63$; $p < 0.05$) and a decrease in mounting ($F_{(1,23)} = 5.00$; $p < 0.05$) and self grooming ($F_{(1,23)} = 8.91$; $p < 0.01$; Tab. 6). The reduction in self grooming suggests that DIA decreases the general arousal state in HAB rats, as self grooming is thought to reflect high arousal (van Erp *et al.*, 1994). Although increased immobility in DIA-treated HAB rats could reflect general sedation, the amount of exploratory behaviour was not affected. This finding together with the observation of partly increased aggressive behaviour after DIA-treatment speaks against a general sedative effect of DIA.

Tab. 7: Behavioural parameters of male NAB rats during the RI test 30 minutes after an acute injection of PTZ, DIA, or vehicle (0.9 % saline; VEH). Aggressive behaviour, social investigation, exploration and immobility were calculated as percentage of time.

	VEH (n = 8)	PTZ (n = 7)	DIA (n = 8)	$F_{(2,20)}/p$
Aggressive behaviour	6.3 ± 3.9	0.0 ± 0.0	0.9 ± 0.7	2.01/0.16
Attack latency (sec)	523 ± 51	600 ± 0	600 ± 0	2.16/0.14
Social investigation	18.2 ± 2.2	9.5 ± 1.7^{+}	18.3 ± 2.9	4.34/0.03
Exploration	51.8 ± 4.6	75.6 ± 2.2*	49.1 ± 2.2	18.3/<0.001
Immobility	8.2 ± 3.8	6.1 ± 1.3	12.6 ± 4.2	0.89/0.43

Data are presented as means ± SEM. ANOVA for treatment effects, * $p < 0.05$ versus vehicle, $^{+}$ $p = 0.055$ versus vehicle, Bonferroni *post hoc* test.

In NAB rats, application of neither PTZ nor DIA changed their aggressive behaviour (Tab. 7). Importantly, NAB rats showed an overall low level of aggression in this experiment, which could explain the absence of an effect of PTZ on aggression. Nevertheless, overall treatment effects were found for explorative behaviour (ANOVA: $F_{(2,20)} = 18.3$; $p < 0.001$) and for social investigation ($F_{(2,20)} = 4.34$; $p < 0.05$). Here, PTZ induced an increase in cage exploration (Bonferroni *post-hoc* test: $p < 0.001$; Tab. 7), but reduced social investigation ($p = 0.055$; Tab. 7). These findings are in line with those seen in LAB rats and with results obtained with another anxiogenic substance resulting in a decreased social investigation during the social investigation test (Rex et al., 1996).

In NAB rats, there was no effect of DIA in any of the behaviours measured. So far, pharmacological manipulation of anxiety has been shown in a non-social context as, for example, the EPM (Millan, 2003) or in the social interaction test (File & Seth, 2003). However, further studies are needed with varying doses or timings to extend the results of the present thesis dealing with the effects of DIA on the behaviour of NAB rats during the RI test. Taken together, pharmacologically altered anxiety is inversely linked to aggressive behaviour only in LAB and HAB rats. Increase of anxiety by PTZ led to reduced aggression and social investigation in LAB rats. Vice versa, reduction of anxiety by DIA resulted in a partly increase in aggressive behaviour in HAB rats.

4. Role of testosterone in aggression

To investigate line differences in testosterone levels in response to aggressive behaviour, LAB, HAB and NAB rats were implanted a jugular vein catheter and subjected to the RI test (see chapter 3). Basal samples taken under undisturbed conditions did not differ between the lines. Sixty minutes after the onset of the RI test, an increase in plasma testosterone was seen in LAB and NAB rats, whereas there was no change in HAB rats (see Fig. 14). Male aggression has been related to testosterone levels in several species, including humans

(Dijkstra *et al.*, 1992; Banks & Dabbs, 1996; Lucion *et al.*, 1996; Wingfield *et al.*, 2001). In rats, castration induces a reduction of aggressive behaviour which can be rescued by testosterone administration. Furthermore, it has been shown, that testosterone treatment results in a higher aggressiveness in rats – even towards females (McGinnis *et al.*, 2002; Farrell & McGinnis, 2004; Cunningham & McGinnis, 2007). However, the relationship between testosterone and aggression is not that clear. For example, in humans, aggression correlates with elevated levels of testosterone only in adolescent men (Mattsson *et al.*, 1980; Dabbs *et al.*, 1991), but not in prepubertal males (Susman *et al.*, 1987; Constantino *et al.*, 1993). Furthermore, in hamsters kept under short-day conditions, even a negative correlation between testosterone and aggressive behaviour has been shown (Jasnow *et al.*, 2000) and castration in rats does not lead to a complete loss of aggression as castrated resident rats are still able to defeat an intruder (Christie & Barfield, 1979). In the present study, both aggressive LAB rats as well as non-aggressive NAB rats showed an increased plasma testosterone level after being exposed to the RI test as resident. Therefore, a direct link between aggressive behaviour and the plasma testosterone level is unlikely.

5. Role of the hypothalamic-pituitary-adrenal axis in aggression

HPA axis responses in LAB, HAB and NAB male rats exposed to the RI test as resident were assessed using jugular vein catheterisation, blood sampling and subsequent quantification of the plasma ACTH and corticosterone levels by radioimmunoassay (see chapter 3). Basal ACTH concentrations did not differ between the lines, whereas basal levels of corticosterone were higher in LAB compared with HAB rats (see Fig. 14). Clinical studies showed a link between high basal cortisol levels and the level of aggression in adolescent males (van Bokhoven *et al.*, 2005b). However, there are also clinical and preclinical studies showing that low basal levels of glucocorticoids are linked with a high level of aggression (McBurnett *et al.*, 2000; Haller *et al.*, 2001; Shoal *et al.*, 2003; Haller *et al.*, 2004). Thus, alterations of basal

glucocorticoid levels in either direction can result in exaggerated aggressive behaviour. It has to be mentioned that basal differences in corticosterone levels were not found in other studies using LAB and HAB rats (Landgraf *et al.*, 1999; Frank *et al.*, 2006). This could be explained by differences in the experimental conditions, in particular the time point of blood sampling.

To assess the plasma ACTH and corticosterone levels in response to the RI, plasma samples were taken 15 minutes after the beginning of the RI test. A significantly higher rise of the plasma ACTH level was found in LAB compared with HAB rats in response to the RI test, whereas no rise was seen in NAB rats. There was no line difference in corticosterone levels in response to the RI test as LAB, HAB and NAB rats showed an increase. Thus, the display of aggressive behaviour was accompanied by an elevated HPA axis activity confirming former clinical studies (Gerra et al., 1997; McBurnett et al., 2005). Moreover, aggressive behaviour in rodents can be increased by acute glucocorticoid treatment (Kruk et al., 2004; Mikics et al., 2004). Finally, circadian rhythms of glucocorticoids and aggressive behaviour are linked resulting in a peak of glucocorticoids and aggression during the early dark phase (Haller et al., 2000b).

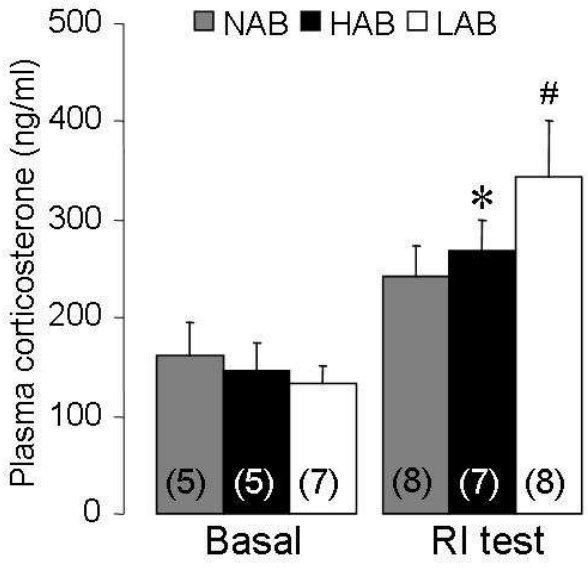

Fig. 25: Plasma corticosterone concentrations in male NAB, HAB and LAB rats measured in trunk blood of rats killed either under basal conditions (control group) or 30 minutes after the beginning of the RI test. * $p < 0.05$ versus respective control, $^{\#}$ $p < 0.001$ versus respective control, two way ANOVA (factor line × factor aggression) followed by Bonferroni *post hoc* test. Numbers in parenthesis indicate group size.

The results of the experiments in chapter 3 were obtained in rats tested during the light phase of the light/dark cycle. In order to increase the level of aggression, a similar experiment was performed in the dark phase showing no change in basal levels of corticosterone in trunk blood of rats killed either under basal (undisturbed) conditions or 20 minutes after the end of the RI test. Due to the diurnal rhythm, the corticosterone levels were found elevated in all three groups during the early dark phase compared with those from the light phase. The results showed that exposure to the RI test increases plasma corticosterone in HAB and LAB rats (Fig. 25). In contrast, there was only a tendency of an increase in NAB rats. Additionally, it has to be mentioned that in this particular set of rats, the difference in aggressive behaviour between the lines was not significant, but the trend towards an elevated aggression in LAB rats compared with NAB rats and an intermediate level in HAB rats was present. Thus, although basal line differences were not consistent between the studies, the display of aggressive behaviour was accompanied by an elevated HPA axis response, especially in LAB and HAB rats which showed higher levels of aggression compared with NAB rats.

To investigate the effect of pharmacological manipulation of the $GABA_A$ receptors on plasma corticosterone levels in response to the RI test, LAB, HAB and NAB rats treated with PTZ/DIA (like described above) were included in this experiment. A significant treatment effect was found in NAB rats (ANOVA: $F_{(2,19)} = 4.78$; $p < 0.05$) resulting in an elevated plasma corticosterone level in PTZ-treated rats compared to controls (Fig. 26). Similar results were found in LAB rats (Student's *t*-test: $p < 0.05$; Fig. 26). DIA did not alter plasma corticosterone levels in NAB or HAB rats that underwent the RI test (Fig. 26). Although an increase in plasma corticosterone was found in LAB rats in response to the display of aggressive behaviour, a further increase in corticosterone levels in PTZ-treated LAB rats did not lead to an increase in aggression. Thus, to a certain extent, an increase in HPA axis activity results in an increase of aggressive behaviour, but additional amounts of corticosterone like seen after treatment with PTZ in LAB and NAB rats, rather block

aggressive behaviour. These data suggest that the display of aggression in LAB rats requires a rise in corticosterone, and that a shift towards higher or lower levels lead to reduced aggressive behaviour. However, there are data in the literature showing no correlation between the HPA axis reactivity and aggression. For example, in wild-type rats, the intermediate-aggressive group of rats showed the highest level of corticosterone in response to a social stressor, whereas high- and low-aggressive rats did not differ in corticosterone response (Sgoifo et al., 1996). One has to take into account that these results were obtained when the experimental rat was placed as intruder into the home cage of an aggressive conspecific and are therefore not directly comparable to the corticosterone levels measured in this study which were assessed in response to the experimental rat being the resident in the RI test.

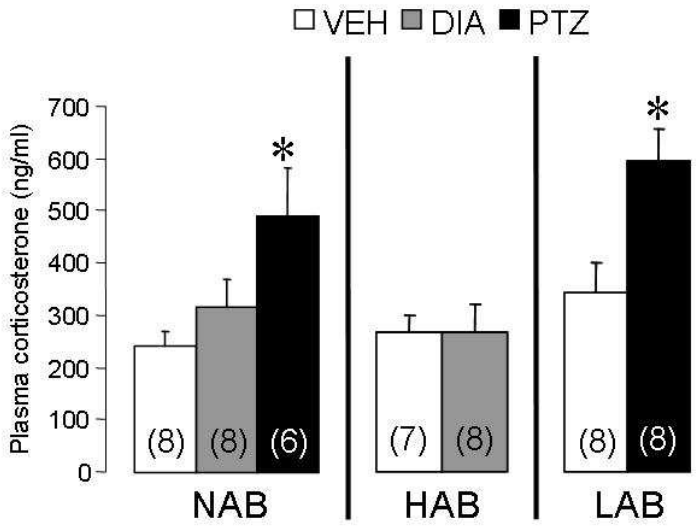

Fig. 26: Effects on plasma corticosterone concentrations of an acute injection of DIA or PTZ. Controls received vehicle (0.9 % saline; VEH). Rats were killed 30 minutes after the beginning of the RI test (= 60 minutes after injection), * $p < 0.05$ versus vehicle (VEH); NAB: ANOVA followed by Bonferroni *post hoc* test, HAB/LAB: Student's *t*-test. Numbers in parenthesis indicate group size.

Taken together, the higher amount of aggressive behaviour shown during the RI test in LAB and HAB rats is accompanied by an elevated HPA axis response compared with the low-aggressive NAB rats. Preclinical and clinical studies have shown that changes in the HPA axis can lead to exaggerated aggressiveness. Hyper-arousal-driven aggression, which is associated with high autonomic and HPA axis responses, can be seen in patients with mood disorders, such as depression (Mazur, 1994; Cohen *et al.*, 1996; Haller & Kruk, 2006). Furthermore, elevated aggressive behaviour in combination with a state of hyper-arousal has also been found in animals (Hayden-Hixson & Ferris, 1991; de Almeida & Miczek, 2002; Kruk *et al.*, 2004; Mikics *et al.*, 2004). In line with this, being exposed to a social stressor, such as the RI test either as resident (see chapter 3) or as intruder (Frank *et al.*, 2006), leads to a higher HPA axis response in LAB rats. Thus, the hyperreactivity of the HPA axis in LAB rats is dependent on the quality of stressor – being a non-social or a social stressor. When exposed to non-social stressors, such as a novel environment, LAB males show a lower HPA axis response compared with HAB rats (Landgraf *et al.*, 1999; Salome *et al.*, 2004; Neumann *et al.*, 2005b). In contrast, the high level of aggression seen in LAB rats is linked to a high HPA axis response.

Why does a further elevation of corticosterone by PTZ-treatment not lead to a further increase in aggressive behaviour in LAB rats? First, it is possible, that this is due to a ceiling-effect. When all receptors are occupied by corticosterone, no further activation of this pathway is possible. Corticosterone present in high concentrations could induce opposite effects by unspecific binding and activating alternative neuronal pathways within the brain. Second, the aggression-promoting effect of corticosterone induced by PTZ could be blocked by the simultaneous increase in anxiety. Although a certain amount of anxiety rather could induce aggression, I hypothesize that a very high level of panic-like anxiety does block any kind of non-aggressive social or aggressive behaviour as seen in PTZ-treated LAB rats during the RI test.

As shown in chapter 3 and figure 25, HAB rats also show an increase in plasma corticosterone in response to the RI test. However, treatment of HAB rats with DIA resulted in an increase in aggressive behaviour without affecting the corticosterone level.

Taken together, this leads to the conclusion that the HPA axis is partly involved in the regulation of aggression, but other neuronal systems might play a more important role depending on the breeding line or on the experimental conditions. Further research will be needed to gain insight in the complex interplay of aggression and corticosterone or the other levels of the transmitters of the HPA axis.

6. Brain regions activated by aggressive behaviour

The aggression-induced activation of different brain regions in LAB and HAB rats was investigated by means of c-Fos immunocytochemistry (see chapters 3 and 4). Neuronal activation was higher in the parvocellular part of the PVN in the high-aggressive LAB rats compared with the less-aggressive HAB rats (see Tab. 4). Additionally, there was a tendency towards a higher activation in the hypothalamic attack area and in the central and medial amygdala in LAB rats compared with HAB rats (see Tab. 4). In contrast, reduced neuronal activation was found in the lateral septum of the high-aggressive LAB compared with the less-aggressive HAB rats (see Fig. 17B).

An additional experiment was performed investigating the expression of three different immediate-early genes (*arc*, *c-fos*, *zif268*) in response to the RI test and under basal conditions (control) as this represents the first measurement of neuronal activation in brain slices. Therefore, LAB and NAB rats, which show high versus low aggression, were killed either under undisturbed conditions or 30 minutes after the onset of the RI test. The brains were taken out, immediately frozen in pre-chilled *n*-methylbutane on dry ice, and stored at -20°C. Cryo-cut 16-µm brain sections were used for *in situ* hybridisations. The hybridisation protocol was adopted from De Vries *et al.* (1994). The expression of three different

immediate-early genes – *arc, c-fos* and *zif268* – was investigated using highly specific ^{35}S-labelled oligonucleotide probes (*c-fos*: 5'-CAG-CGG-GAG-GAT-GAC-GCC-TCG-TAG-TCC-GCG-TTG-AAA-CCC-GAG-AAC-ATC-3', *arc*: 5'-CTT-GGT-TGC-CCA-TCC-TCA-CCT-GGC-ACC-CAA-GAC-TGG-TAT-TGC-TGA-3´, zif268: 5´-TTC-TCG-TTG-GTC-AGA-CCG-ATG-TCC-ATC-ACA-TTC-TCT-GTA-GCC-ATC-3´; MWG Biotech, Ebersberg, Germany) in a variety of brain regions (Tab. 8) that have been suggested to be implicated in the regulation of aggression. The display of aggressive behaviour induced differences in neuronal activation patterns in LAB compared with NAB resident rats (Tab. 8). Regions including the prelimbic cortex, the primary and secondary motor cortex, the nucleus accumbens core, the dorsal BNST, the CA3 region and the dentate gyrus of the hippocampus are activated in the aggressive LAB rats compared to the low-aggressive NAB rats as well as to basal conditions. This suggests that they are implicated in the regulation of the enheightened aggressive behaviour in LAB rats and is in line with previous findings showing a higher level of C-Fos protein in response to the display of aggression in the prelimbic cortex, nucleus accumbens, BNST, and hippocampus (Halasz *et al.*, 2002; de Boer *et al.*, 2003; Veening *et al.*, 2005; Halasz *et al.*, 2006). The higher neuronal activation in the motor cortex is probably based on the fact that LAB rats showed more aggression and accordingly were more active than NAB rats.

Importantly, the use of three different immediate-early genes revealed specific expression patterns within some of the brain regions investigated. Immediate-early genes are expressed immediately in response to extracellular stimuli and can affect signal transduction and transcription of genes. C-Fos was the first marker of neuronal activation used to study effects of diverse stimuli on living animals (Morgan et al., 1987; Sagar et al., 1988). The protein products of most immediate-early genes are transcription factors that exert a direct regulatory effect on transcription of other genes. In contrast, some immediate-early genes code for proteins that are involved in distinct cell functions via non-genomic pathways (Steward &

Worley, 2002). For example, *arc* (activity-regulated cytoskeleton-associated protein) encodes a protein that is associated to actin and involved in synaptic plasticity and learning (Steward *et al.*, 1998; Guzowski *et al.*, 2000; Guzowski *et al.*, 2001; Moga *et al.*, 2004). In contrast, *c-fos* and *zif268* (also known as *krox*-24, *egr*-1, *TIS* 8, *NGFI*-A or *zenk*) encode for transcription factors (Tischmeyer & Grimm, 1999). Similar to *arc*, *zif268* has been implicated in memory consolidation (Jones et al., 2001). A previous study comparing the expression patterns of *arc*, *c-fos* and *zif268* revealed that, while these immediate-early genes are predominantly co-localised, they display differences in activation following a spatial learning task, with *arc* being most responsive (Guzowski et al., 2001). The former is in line with the results in LAB rats, showing an increase in expression of all three immediate-early genes investigated in the prelimbic and orbitofrontal cortex. In contrast, there are brain regions like for example the cingulate cortex (*zif268*), the primary motor cortex (*arc*) or the dentate gyrus (*c-fos*), where a gene-specific activation was found. By using *in situ* hybridisation instead of immunocytochemistry and, additionally to *c-fos*, adding *arc* and *zif268* as further indicators of neuronal activation, I extended earlier studies on c-Fos immunocytochemistry in the context of aggression in rats and mice. Halasz et al. (2002; 2006) investigated c-Fos-immunoreactivity in untreated or glucocorticoid-deficient Wistar rats subjected to the RI test as residents. Haller et al. (2006) compared the neuronal activation of high-aggressive SAL and low-aggressive LAL mice, whereas Veening et al. (2005) looked for neuronal activation patterns in wild-type rats in response to an aggressive encounter.

Combining these studies with the results of the present thesis shows that especially the BNST showed a strong activation in response to aggressive behaviour across several experimental conditions in rats and mice. The c-Fos protein level in the BNST was increased in Wistar rats and wild-type rats in response to exposure to an intruder in the home cage of the experimental rat (Halasz et al., 2002; Veening et al., 2005). Similar results were found in mice selected for high and low levels of aggression with the aggressive SAL mice showing a higher neuronal

activation in the BNST compared with their low-aggressive conspecifics (Haller et al., 2006). Additionally, the LAB residents had a higher level of *c-fos* mRNA in the BNST than NAB residents (Tab. 8). There are also studies in hamsters that show neuronal activation in the BNST in response to aggression (Kollack-Walker & Newman, 1995; Delville *et al.*, 2000).

Tab. 8: Neuronal activation in different brain regions of LAB and NAB rats 20 minutes after exposure to the RI test. Neuronal activation was measured by quantifying the mRNA expression of the immediate-early genes *arc*, *c-fos* and *zif268*. Data were analysed by two way ANOVA (factor line × factor aggression), followed by Bonferroni *post-hoc* test when appropriate. ↑ increased neuronal activation

	LAB RI vs. basal				LAB RI vs. NAB RI		
	arc	*c-fos*	*zif268*		*arc*	*c-fos*	*zif268*
Prefrontal cortex							
Prelimbic cortex	↑	↑	↑				↑
Infralimbic cortex	↑	↑					
Ventral orbital cortex	↑	↑	↑				
Agranular insular cortex						↑	
Cingulate cortex I			↑				
Cingulate cortex II			↑				
Primary motor cortex	↑						↑
Secondary motor cortex	↑		↑				↑
Basal ganglia							
Caudate putamen dorsal		↑					
Nucleus accumbens core		↑				↑	↑
Nucleus accumbens shell						↑	↑
Septum dorsal						↑	
Septum ventral						↑	
BNST dorsal	↑				↑		
Medial preoptic area					↑		↑
Ventral pallidum					↑		
Hypothalamic regions							
Anterior hypothalamus		↑					
PVN		↑					
Hippocampus							
CA1						↑	
CA2						↑	
CA3		↑				↑	
Dentate gyrus		↑				↑	

In general, especially the AVP system within the BNST projecting to the lateral septum has been implicated in the regulation of aggressive behaviour (De Vries & Buijs, 1983; Irvin *et al.*, 1990; Koolhaas *et al.*, 1991; Hines *et al.*, 1992; Compaan *et al.*, 1993; Ferris & Delville, 1994; Bester-Meredith *et al.*, 1999; Everts & Koolhaas, 1999). Supporting this hypothesis, I could confirm a direct influence of the AVP system in the BNST on the level of aggressive behaviour shown during the RI test by using reverse microdialysis to pharmacologically manipulate the AVP system locally within the BNST (see chapter 5, and page 152ff).

Besides the BNST, the nucleus accumbens was also activated in LAB rats in response to the RI test both compared to basal conditions and low-aggressive NAB rats which is in line with earlier findings of an increase in neuronal activation in the nucleus accumbens after display of aggression (de Boer et al., 2003). Furthermore, a study by Ferrari et al. (2003) showed an increase in dopamine release within the nucleus accumbens in rats in anticipation of an aggressive encounter. The nucleus accumbens receives serotonergic and dopaminergic projections from the dorsal raphe nuclei and the ventral tegmental area, respectively (Azmitia & Segal, 1978; Molliver, 1987; Heimer *et al.*, 1991). An important role in motivation, reward, emotion and integration of complex behaviours has been suggested for the nucleus accumbens (Le Moal & Simon, 1991; Kirby *et al.*, 1997; Wise, 2002) and it has been suggested to be a key regulator of aggression and impulsive behaviour (Miczek *et al.*, 1994; Cardinal *et al.*, 2001). Could the high level of aggression in LAB rats be facilitated by an activated neuronal reward/motivation circuitry? To test this hypothesis, I investigated the influence of local injections of an anaesthetic (Lidocaine, Sigma) into the nucleus accumbens on intermale aggressive behaviour in LAB rats. Therefore, adult LAB rats were bilaterally implanted with guide cannulas directed at the nucleus accumbens [stereotaxic coordinates relative to bregma were: -1.7 mm caudal, ± 1.6 mm lateral to the midline, 4.6 mm beneath the surface of the skull; nose: -3.5 mm; according to: Paxinos and Watson (1998)]. The rats were handled several times before the start of the experiment to reduce stress effects. Two days after

surgery, the LAB rats were bilaterally injected with 1 µl of lidocaine (20 mg/ml) dissolved in 0.9 % saline and directly returned to their home cage. Control rats received 1 µl of 0.9 % saline. Five minutes after the injection, the experimental rats were confronted with a male intruder rat in their home cage for ten minutes.

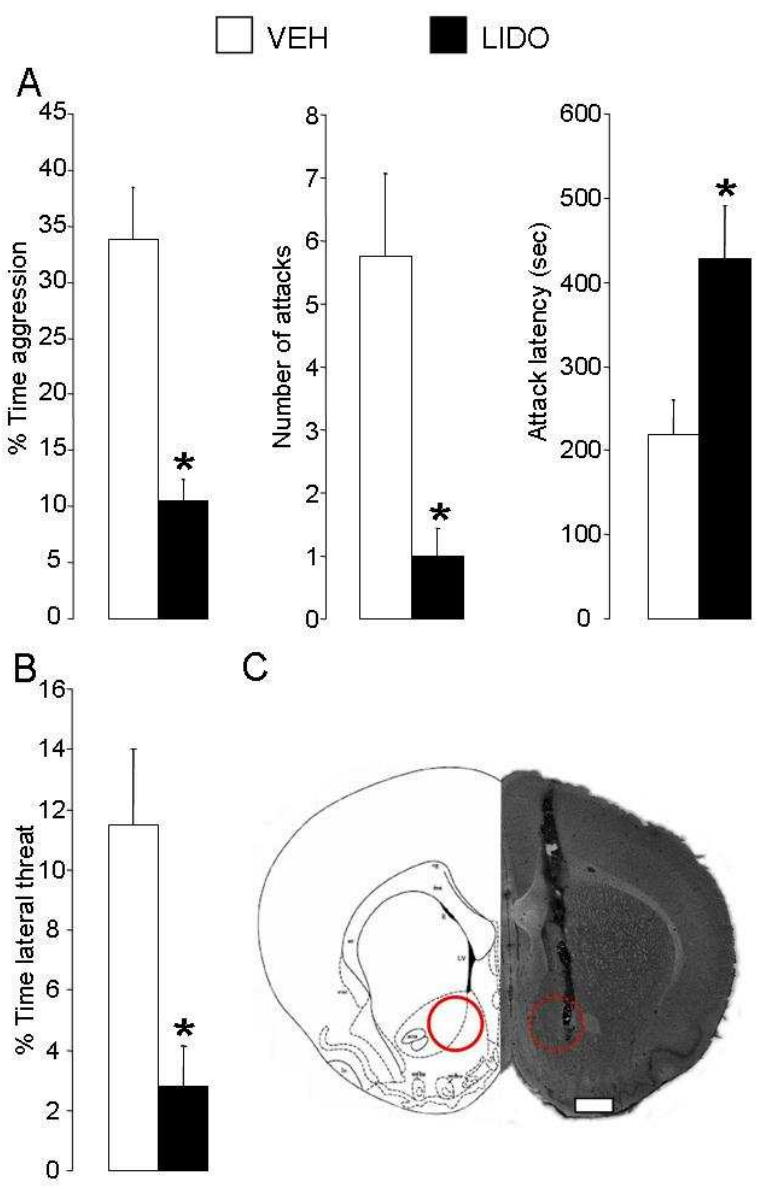

Fig. 27: Effects of bilateral inhibition of the nucleus accumbens on aggressive behaviour by local injection of lidocaine in LAB rats. Controls received vehicle (0.9 % saline; VEH) **(A)** Percentage of time of total aggressive behaviour during the RI test, number of attacks and attack latency time; **(B)** Percentage of time spent with lateral threat; **(C)** Schematic drawing of the nucleus accumbens [adapted from Paxinos and Watson (1998); the red circle indicates the respective target region] and a representative enlargement of the microphotograph of a Cresyl-stained coronal section of the rat brain after removal of the guide cannula; Scale bar = 1 mm; Data are presented as means + SEM; Student's *t*-test; * $p < 0.05$.

Behavioural scoring revealed a significant reduction of aggressive behaviour in the lidocaine-treated LAB rats (Fig. 27). Bilateral inhibition of the nucleus accumbens resulted in a decrease in aggressive behaviour (Student's *t*-test: $p < 0.005$), and in its elements threat ($p < 0.005$), lateral threat ($p < 0.05$), offensive upright ($p < 0.05$), and keep down ($p < 0.05$) as well as in a decrease in the number of attacks ($p < 0.01$). In addition, injection of lidocaine into the nucleus accumbens induced a longer attack latency ($p < 0.01$), and increased the amount of mounting (3.6 % ± 1.3 %) and self grooming (11.8 % ± 2.4 %) versus vehicle-treated LAB rats (mounting: 0.1 % ± 0.03 %; self grooming: 4.4 % ± 1.2 %). The latter could be due to a higher level of arousal as for example the dopaminergic system within the nucleus accumbens is implicated in general arousal (Paredes & Agmo, 2004). I suggest that increased mounting, similar to increased self grooming, constitutes a displacement activity to cope with the stressful situation.

Taken together, both the BNST and the nucleus accumbens seem to be important in regulating aggressive behaviour. Additionally, the patterns of the immediate-early gene activation in LAB and NAB rats suggests the prefrontal cortex for further study on the neuronal mechanisms underlying aggressive behaviour, as there was an increase in mRNA level across the different immediate-early genes in several parts of the prefrontal cortex. Prefrontal cortical brain regions like, for example, the prelimbic cortex have been shown to be activated in response to aggression in a previous study investigating c-Fos protein (Halasz et al., 2006). In humans, prefrontal deficits have been linked to violent behaviour suggesting an inhibitory role for these brain regions in aggression (Damasio *et al.*, 1994; Critchley *et al.*, 2000; Hawkins & Trobst, 2000; Bassarath, 2001; Blair, 2004). Animal studies supported these findings (de Bruin et al., 1983; De Bruin, 1990), but there were also studies showing an increased c-Fos activation within the prefrontal cortex of mice selected for a high level of aggressive behaviour (Halasz et al., 2006). Accordingly, increased activity in prefrontal regions can also be related to high aggression in humans (Harmon-Jones & Sigelman, 2001;

Dougherty *et al.*, 2004; Sander *et al.*, 2005) as, for example, the ventromedial prefrontal cortex is activated when the subject attacks an opponent in a video game (King et al., 2006). However, the same activation is seen in the context of compassionate behaviour in a video game (King et al., 2006). These conflicting data clearly implicate the prefrontal cortex in the regulation of emotion-related behaviours, and especially aggression, but the detailed underlying neuronal mechanisms need further clarification.

7. Role of arginine vasopressin release within septum and bed nucleus of the stria terminalis in aggression

AVP is thought to be an important regulator of aggressive behaviour in several species, including rodents and humans. The AVP system originating in the medial amygdala and projecting to the BNST and the lateral septum is androgen-dependent and more pronounced in males than in females. Castration leads to a decrease of intermale aggression in rats that is accompanied by a reduction in the number of AVP-immunoreactive cells within the BNST and the medial amygdala as well as in the density of AVP fibres within the lateral septum. (De Vries et al., 1992). Especially AVP within the septum and BNST has been implicated in the regulation of aggression. AVP-immunoreactive staining in the BNST and the number of AVP V1a receptors in the lateral septum were higher in males of the aggressive California mice compared with the less-aggressive white-footed mice (Bester-Meredith et al., 1999). Furthermore, a local injection of AVP into the lateral septum led to an increase in offensive aggression in castrated male rats (Koolhaas et al., 1991), and increased flank marking which is part of the aggressive behaviour in golden hamsters (Ferris & Delville, 1994). Contradictory results were obtained from studies in wild-type rats and wild house mice. Aggressive wild-type rats had a lower level of AVP and a lower AVP fibre density in the lateral septum than their less-aggressive conspecifics (Everts et al., 1997). Accordingly, high-

aggressive wild house mice had fewer AVP-immunoreactive cells in the BNST as well as a lower AVP-immunoreactive staining in the lateral septum compared with low-aggressive wild house mice (Compaan et al., 1993). Moreover, the mating-induced reduction in aggressive behaviour in male prairie voles is accompanied by a reduction in the density of AVP-immunoreactive fibres in the lateral septum (Bamshad et al., 1994; Insel et al., 1995). Taken together, data on the role of AVP within the septum and the BNST were inconsistent and mostly relied on indirect parameters measured, such as AVP fibre density or AVP-immunoreactive cells. Based on this information, no final conclusions about the amount of effectively released AVP in response to aggression could be drawn. Therefore, in the present thesis, the AVP release in response to the RI test was measured and depending on the results, the AVP system within the lateral septum (see Chapters 4 and 5) and BNST (see Chapter 5) was manipulated pharmacologically.

Both brain regions showed specific AVP release patterns in response to the RI test in aggressive versus low-/non-aggressive rats. Compared to the less-aggressive HAB rats showing rather an increase, LAB resident rats had a decrease in septal AVP release in response to the RI test (see Fig. 17A). In contrast, aggressive NAB rats (selected according to their attack latency time and the percentage of aggressive behaviour shown during an initial RI test) showed a higher AVP release in response to the RI test than non-aggressive NAB rats (see Fig. 21). Pharmacological manipulation of the septal AVP system by using reverse microdialysis of synthetic AVP in LAB (see Fig. 18A) and non-aggressive NAB (see Fig. 22A) rats or the V1a-A in HAB (see Fig. 19A) and aggressive NAB (see Fig. 22B) rats did not affect aggressive behaviour. These results implicate that the septal AVP system is not directly involved in the regulation of aggression in either of the lines. However, effects on anxiety-related and non-aggressive social behaviours were found. Increasing the amount of AVP in the septum resulted in a higher level of anxiety-related behaviour in both LAB (see Fig. 18B) and non-aggressive NAB (see Fig. 22A) rats, whereas blocking the AVP V1a

receptors by reverse microdialysis in HAB rats led to a decrease in social investigation (see Fig. 19A), thereby confirming the adequacy of the reverse microdialysis procedure. In a follow-up experiment, the AVP release within the BNST in response to the RI test was investigated in aggressive and non-aggressive NAB rats. Exposure to the RI test resulted in an increase in AVP release within the BNST in non-aggressive rats, whereas there was no increase in aggressive NAB rats (see Fig. 23). AVP release patterns measured one day later during forced swimming showed no differences between aggressive and non-aggressive NAB rats (see Tab. 5). Thus, the AVP release within the BNST is clearly context-specific. To assess the behavioural relevance of the AVP release within the BNST, reverse microdialysis of AVP in aggressive and of V1a-A in non-aggressive NAB rats was performed within the BNST. Increasing the level of AVP in the BNST in aggressive rats resulted in a decrease of total aggressive behaviour without affecting the attack latency, the number of attacks, non-aggressive social behaviour during the RI test or anxiety as measured on the EPM (see Fig. 24B). In contrast, blocking the AVP V1a receptors in non-aggressive NAB rats had no effect on either behaviour in the RI or EPM test (see Fig. 24A). Taken together, these data suggest that AVP release within the BNST is implicated in the regulation of aggression, whereas AVP released within the septum does not seem to exert direct effects on aggressive behaviour. However, indirect effects of AVP within the septum on anxiety-related or non-aggressive social behaviours could also influence aggressive behaviour.

Although distinct AVP release patterns were found in the lateral septum of aggressive versus non-aggressive rats, manipulation of the AVP system within this brain region did not affect aggressive behaviour suggesting no direct link between septal AVP and the display of aggression. The discrepancy between this result and the finding that local injections of AVP into the lateral septum facilitates aggressive behaviour in castrated male rats and in hamsters could be due to the relatively low amount of AVP fibres in the septum of hamsters and castrated rats (Koolhaas *et al.*, 1991; Ferris & Delville, 1994; Ferris *et al.*, 1995). I predict that

the AVP system in the lateral septum is only implicated in the regulation of aggressive behaviour in a state of a chronically low AVP system. Although the septal AVP release in response to the RI test was reduced in aggressive LAB rats compared with HAB rats, there was no difference between the lines under basal conditions (see Chapter 4). Similarly, basal AVP release within the septum did not differ between aggressive and non-aggressive NAB rats (see Chapter 5).

In contrast to septal AVP, our data show that the AVP system within the BNST exerts a direct effect on the display of aggressive behaviour. However, AVP released in response to the RI test within the lateral septum, which receives AVP projections from the BNST, is not directly implicated in the regulation of aggression. I hypothesise that changes in the release of septal AVP are rather the consequence than the cause of the display of aggressive behaviour. Aggression-induced changes in septal AVP release could then exert acute or even long-lasting effects on other, aggression-related behaviours, such as anxiety and non-aggressive social behaviours. As AVP within the septum is also released in response to forced swimming and is implicated in the behavioural response to this stressor (Ebner *et al.*, 1999), septal AVP could serve to cope with stressful situations. However, the septal AVP release is stressor-specific and was not elevated in response to social defeat (Ebner *et al.*, 2000).

Septal AVP is known to be implicated in anxiety-related and non-aggressive social behaviours (Landgraf *et al.*, 1995a; Engelmann *et al.*, 1996; Landgraf *et al.*, 2003; Millan, 2003) which was confirmed by the experiments of the present thesis (see Chapters 4 and 5). Several studies showed that septal AVP plays an important role in social memory. Tests such as the social recognition or social discrimination paradigm are used to investigate social memory in rodents. Decrease of the AVP V1a receptors by local infusion of an antisense oligodeoxynucleotide within the lateral septum recuced the social memory ability in rats (Landgraf et al., 1995a), whereas overexpression of AVP V1a receptors in the septum by means of an adenoviral vector increased social memory abilities as well as social interaction

in male rats (Landgraf et al., 2003). Repeated encounters of pairs of male hamsters showed a reduction in aggression over the time. This implicates that social memory enables the hamsters to remember their respective social status when exposed to the same opponent again (Ferris, 1992). Could the distinct release patterns of septal AVP between aggressive and non-aggressive rats be associated with differences in social memory abilities?

To test this hypothesis, NAB rats were subjected to the RI test to assess their level of aggression. Performance of the social discrimination test revealed, that both aggressive and intermediate-aggressive rats were able to discriminate between a novel and a known juvenile rat two hours after the first exposure, whereas non-aggressive rats were not (Fig. 28).

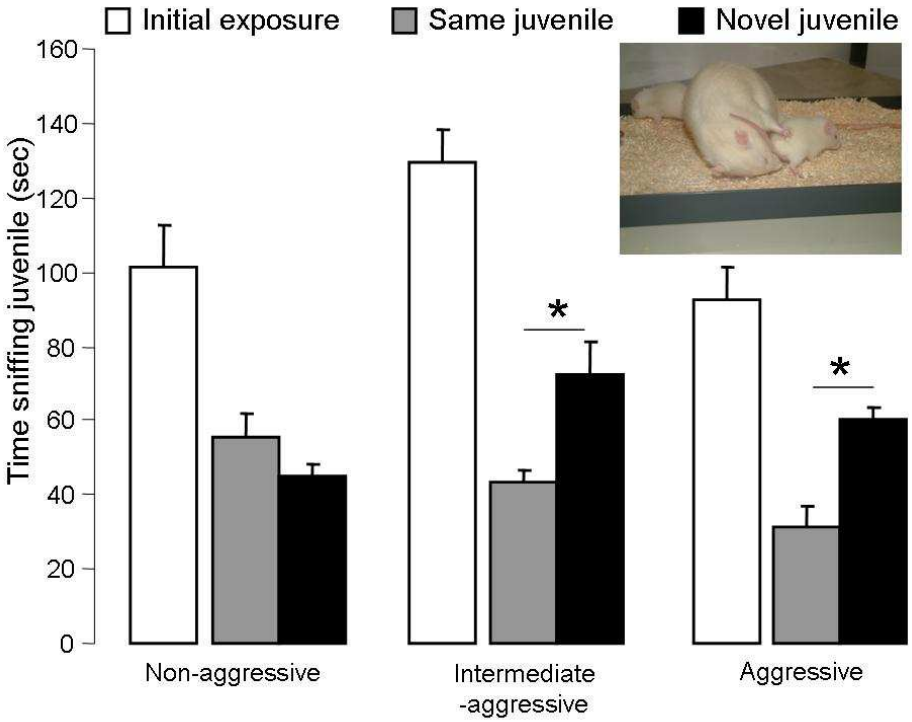

Fig. 28: Social discrimination with an interexposure interval of two hours performed in non-aggressive (n = 7), intermediate-aggressive (n = 8) and aggressive (n = 6) male NAB rats. The time male NAB rats spent sniffing the juvenile is shown. During the first exposure a single juvenile rat (3-4 weeks of age) was placed into the home cage of the adult male for four minutes. Two hours later, the same juvenile together with a novel juvenile was placed into the home cage of the NAB rat for four minutes. The time sniffing the same and the novel juvenile was measured. A longer sniffing-time for the novel juvenile indicates social memory. Student's *t*-test; * $p < 0.05$. Insertion: Adult male resident rat showing anogenital sniffing towards a juvenile rat

Together with the finding that aggressive NAB rats had a higher septal AVP release during the RI, this could indicate that the activated AVP system within the septum enhances social memory ability. However, it is not clear, whether aggressive NAB rats also show a higher septal AVP release when confronted with a juvenile rat that does not elicit aggressive behaviour. Therefore, future experiments using microdialysis could be used to measure the *in vivo* release of AVP within the lateral septum during social contact with a juvenile stimulus rat in aggressive and non-aggressive rats.

Taken together, the AVP system in the BNST seems to exert a direct effect on aggressive behaviour, whereas septal AVP is rather implicated in aggression-related behaviours such as anxiety-related behaviour and social memory, although detailed mechanisms need to be revealed.

8. Role of serotonin in aggression

For a long time, the serotonin-deficiency-theory of aggression has been dominant: a low level of serotonin in the brain leads to exaggerated aggressive behaviour. One of the basic studies in humans showed that excessive aggression and impulsive personality associated with reduced levels of the serotonin metabolite 5-hydroxyindoleacetic acid in the cerebrospinal fluid (Berman et al., 1997; Kavoussi et al., 1997). Further evidence derived by studies showing that substances pharmacologically activating serotonin $1_A/1_B$ receptors or antagonizing serotonin $2_{A/C}$ receptors are capable of reducing aggressive behaviour in various species, including humans (Lindgren & Kantak, 1987; Blanchard *et al.*, 1988; Coccaro *et al.*, 1990; Bell & Hobson, 1994; Olivier *et al.*, 1995; Joppa *et al.*, 1997; Sperry *et al.*, 2003; Olivier, 2004). However, it has to be mentioned, that in most cases this reduction of aggressive behaviour is accompanied by sedation or motor inactivity, which suggests a rather unspecific effect on aggression. Furthermore, both serotonin 1_A and 1_B receptors can be found

as inhibitory autoreceptors on serotonergic neurons on the soma and dendrites in the raphe nuclei and as inhibitory postsynaptic heteroreceptors in several brain regions such as the nucleus accumbens or the hippocampus (Pineyro & Blier, 1999). Therefore, the pathways via which serotonin agonists and antagonists exert their effects are not clear. An agonist acting on autoreceptors will lead to an inhibition of serotonin release, whereas acting on postsynaptic sites has the opposite effect.

To distinguish between activation and inactivation of the central serotonin system, a highly selective serotonin 1_A receptor agonist (S-15535 = 4-(benzodioxan-5-yl)1-(indan-2-yl)piperazine) was used. S-15535 acts as agonist on serotonin 1_A autoreceptors and, in part, also as antagonist at postsynaptic serotonin 1_A receptors (Millan et al., 1993). Administration of S-15535 resulted in a reduction of aggressive behaviour in male wild-type rats without having an effect on non-aggressive motor behaviours or inducing sedation (de Boer & Koolhaas, 2005). These results suggest a facilitating effect for acute serotonin neurotransmission on aggressive behaviour. Combined with earlier studies showing a link between low levels of serotonin and escalated and violent forms of aggression (Olivier *et al.*, 1995; Berman *et al.*, 1997; Millan *et al.*, 1997; de Boer *et al.*, 1999; 2000), these data suggest that prolonged low levels of serotonin facilitate abnormal aggression, whereas the actual display of adapted forms of aggression is linked to an acute release of brain serotonin. To asses the influence of the serotonin system on aggressive and abnormal aggressive behaviour in resident LAB rats, they were injected s.c. with S-15535 (4 mg/kg; kindly provided by Dr. S. F. de Boer) 30 minutes before being confronted with a male intruder rat placed into their home cage. Administration of S-15535 reduced the display of lateral threat and offensive upright as well as the number of attacks towards a male intruder rat. Three days later, the resident LAB rats were treated the same but were confronted with a narcotised male intruder 30 minutes after the injection. S-15535 also reduced the number of attacks towards a narcotised male intruder rat. Other elements of aggressive behaviour have rarely been shown

by resident LAB rats when a narcotised rat was placed into their home cage. The total amount of aggression was not changed after treatment with S-15535 neither towards a male nor towards a narcotised male intruder rat. These results show, that an acute reduction of the serotonin release in the brain in LAB rats selectively reduces the attack component of aggressive behaviour.

9. Concluding remarks

LAB rats are characterised by a high level of aggressive behaviour which is accompanied by a high HPA axis response and an enhanced neuronal activation of the PVN. This suggests that aggression in LAB rats is linked to an increased stress reactivity. In general, social interactions were more stressfull for LAB rats than for HAB rats, and LAB rats showed a reduced level of non-aggressive social behaviour, i.e. social investigation. Additionally, LAB rats met several criteria of abnormal aggressive behaviour by attacking vulnerable body parts of a smaller male intruder as well as by attacking females or especially the head of a narcotised male intruder. Taken together, LAB rats are an interesting animal model for antisocial behaviours, including high and abnormal forms of aggression combined with a low level of non-aggressive social behaviour.

HAB rats displayed an intermediate level of aggression which was accompanied by an elevated HPA axis response compared with NAB rats. Furthermore, HAB rats showed a high level of attacks directed to vulnerable body parts of a smaller intruder which is considered to be abnormal aggressive behaviour. Therefore HAB rats are a suitable animal model to study the underlying neurobiological mechanisms of high and abnormal aggression especially with regard to their background of high anxiety-related behaviour.

As both LAB and HAB rats showed a higher level of aggression than NAB rats accompanied by an either low or high level of anxiety measured on the EPM, no linear correlation between anxiety and aggression was found. Instead, a U-shaped correlation was found between anxiety

and aggression in LAB, HAB and NAB rats. However, the increase in HPA axis reactivity in response to social stressors combined with the low amount of social investigation seen in LAB rats suggests a high level of anxiety in response to social stressors. Thus, the high level of intermale aggression in LAB rats could be linked to a high level of social anxiety. Further studies are needed to gain more insight in the relationship between these complex social and anxiety-related behaviours and the underlying neurobiological mechanisms.

Specific AVP release patterns have been found in the lateral septum in response to the RI test in LAB, HAB and NAB rats, but pharmacological manipulation of the septal AVP system did not affect aggressive behaviour in either of the breeding lines. In contrast, anxiety-related behaviour and non-aggressive social behaviour were influenced by manipulations of the AVP system within the septum. These results suggest that the different AVP release patterns within the lateral septum rather are a consequence than the reason for the display of aggression. However, AVP released within the lateral septum modulates aggression-related behaviours, such as anxiety and social investigation, and can thereby indirectly influence aggressive behaviour.

The AVP release within the BNST showed an aggression-specific difference between aggressive and non-aggressive NAB rats. Furthermore, pharmacological manipulation of the AVP system within this brain region resulted in a direct effect on the display of aggression, implicating a direct regulatory effect of AVP released within the BNST.

A high level of aggression was linked to an elevated HPA axis response in LAB and HAB rats. However, the relationship between HPA axis response and aggression does not seem to be linear as treatment with an anxiogenic substance elevated neuronal activation within the PVN as well as plasma corticosterone levels, but simultaneously reduced aggression in LAB rats. The detailed mechanisms remain to be investigated.

Pharmacological manipulation of the serotonergic neurotransmission in LAB rats resulted in a decrease in the amount of attacks towards a male or a narcotised male intruder rat. As the total

level of aggression remained unchanged, serotonin specifically influences the attack component of aggressive behaviour in LAB rats.

In conclusion, the results of the present thesis show that both LAB and HAB male rats are a suitable animal model to extend the findings of the present thesis on the neurobiological mechanisms underlying high and abnormal aggression.

Addendum

Summary in German

Summary in German – Zusammenfassung auf Deutsch

Adaptive Formen von Aggressionsverhalten gehören zum normalen Verhaltensrepertoire der meisten Tierarten. Sie dienen dem Erwerb und der Verteidigung von Nahrung, Territorium sowie Fortpflanzungspartnern und unterliegen artspezifischen Regeln. Abnormale Aggression hingegen verstößt gegen diese Regeln und kann zu schlimmen Verletzungen zum Teil mit Todesfolge führen. In der menschlichen Gesellschaft äußern sich abnormale Formen von Aggression in gewalttätigen Übergriffen und eskalierenden Auseinandersetzungen und führen zu psychischen und physischen Verletzungen bei den Opfern. Zusätzlich entstehen dadurch Kosten für die Behandlung der Opfer. Folglich stellen abnormale Aggression und die daraus resultierenden Gewalttaten in vielerlei Hinsicht eine immense Belastung für die Gesellschaft dar. Eine intensive Erforschung der Mechanismen, die Aggressionsverhalten regulieren, ist unabdingbar für die Entwicklung neuer Therapieansätze, Behandlungsmethoden sowie vorbeugender Maßnahmen. Um dies bewerkstelligen zu können, werden zunächst adäquate Tiermodelle für die Erforschung der neurobiologischen und molekularen Regulation von Aggressionen benötigt, von denen es im Moment nur sehr wenige geeignete gibt. Das hauptsächliche Ziel der vorliegenden Arbeit war es daher, zwei Rattenlinien, die selektiv auf niedrige („*low anxiety-related behaviour*“ = LAB) bzw. hohe („*high anxiety-related behaviour*“ = HAB) Ängstlichkeit gezüchtet wurden, als Tiermodell zur Erforschung von männlichem Aggressionsverhalten zu etablieren.

LAB- und HAB-Ratten werden seit 1993 auf niedrige bzw. hohe Ängstlichkeit gezüchtet. HAB-Ratten zeigen sowohl neurobiologische Merkmale als auch Verhaltensmerkmale, die gleichermaßen bei Patienten beobachtet werden, die an Depressionen und/oder Angsterkrankungen leiden, und sind daher seit einigen Jahren ein etabliertes Tiermodell für diese Arten von psychischen Erkrankungen. Bislang wurden die LAB-Ratten ausschließlich

als Kontrollzuchtlinie verwendet, die unter identischen Bedingungen gezüchtet und gehalten wird. Erste Beobachtungen zeigten jedoch, dass LAB-Ratten aggressiver sind als HAB-Ratten, da es bei dieser Zuchtlinie in Gruppenhaltung unter basalen Bedingungen tendenziell zu mehr aggressiven Interaktionen zwischen den Käfigbewohnern kam als bei HAB-Ratten. Zudem reagierten LAB-Ratten auch aggressiver, wenn sie als Eindringling (= *intruder*) während eines sog. *resident-intruder* (RI) Tests in den Käfig einer größeren männlichen Ratte (= *resident*) gesetzt wurden. Dies stellte den Ausgangspunkt für die Erforschung des Sozial- und insbesondere des Aggressionsverhaltens von LAB-Ratten dar. Um eine Aussage über die Beständigkeit der Verhaltensmerkmale der LAB- und HAB-Ratten treffen zu können, wurde in Kapitel 2 ein Zeitverlauf über die letzten sechs Jahre sowohl des Angstverhaltens, das auf der sog. *Elevated plus-maze* (EPM) gemessen wird, sowie des Aggressionsverhaltens erstellt. Das Aggressionsverhalten wurde dabei mittels des RI Tests bestimmt, bei welchem die Versuchstiere als *resident* zehn Minuten lang mit einem kleineren unbekannten männlichen Eindringling in ihrem Territorium konfrontiert wurden. Die Ergebnisse zeigen, dass sich der Unterschied im Angstverhalten zwischen LAB- und HAB-Ratten als beständig erwies und dass sich auch das Ausmaß des Aggressionsverhaltens über die letzten Jahre nicht geändert hat. Zusätzlich wurde aus den gesammelten Daten der RI Tests sowohl für LAB- und HAB-Ratten als auch für unselektierte Wistar-Ratten („*non-selected anxiety-related behaviour*“ = NAB), die die Ausgangsrasse für die Zucht der LAB- und HAB-Ratten bildeten, ein detailliertes Verhaltensprofil angefertigt. LAB-Ratten erwiesen sich als aggressiver als NAB-Ratten, während HAB-Ratten bezüglich des Aggressionsverhaltens eine intermediäre Stellung einnehmen. Dies äußerte sich in einem insgesamt erhöhten Maß an Aggressionsverhalten, sowie gesteigertem seitlichen (*lateral threat*) und aufrechten (*offensive upright*) Drohen bei LAB-Ratten im Vergleich zu NAB-Ratten. HAB-Ratten zeigten jeweils mittlere Werte. Weiterhin wurde festgestellt, dass nahezu 10 % der LAB-Ratten sehr aggressiv waren, d.h. dass sie mehr als 55 % der gesamten Zeit des RI Tests aggressives Verhalten zeigten. Im

Gegensatz dazu wurde ein so hohes Maß an Aggression weder bei HAB- noch bei NAB-Ratten gefunden. Abschließend wurden LAB-, HAB- und NAB-Ratten in einer abgewandelten Form des RI Tests auf abnormale Aggression gegenüber einem männlichen, einem weiblichen sowie einem narkotisierten männlichen Eindringling getestet. Sowohl LAB- als auch HAB-Ratten zeigten abnormales Aggressionsverhalten, was sich in Angriffen auf verletzliche Körperteile des männlichen Gegners sowie in Angriffen gegenüber harmlosen Gegnern, wie z.B. einer weiblichen oder einer narkotisierten männlichen Ratte, widerspiegelte. Zusammenfassend bedeutet dies, dass sowohl LAB- als auch HAB-Ratten im Vergleich zu NAB-Ratten ein erhöhtes Ausmaß an Aggression und zudem auch abnormale Formen von Aggression zeigten.

Ein weiteres Ziel der vorliegenden Arbeit war die Untersuchung der neurobiologischen und neuroendokrinen Mechanismen, die Aggressionsverhalten und dessen abnormale Formen regulieren. Ein besseres Verständnis der Regulation von Aggression stellt die Basis für die Entwicklung von Medikamenten zur Behandlung von aggressiven Ausbrüchen bei Menschen dar. Unterschiede zwischen aggressiven und nicht-aggressiven Ratten bezüglich endokriner Systeme oder Neurotransmitter- bzw. Neuromodulator-Systeme bieten die Möglichkeit, Ansatzpunkten für therapeutische Substanzen zu finden, die für die Behandlung von Menschen mit eskalierendem Aggressionsverhalten eingesetzt werden können. In der vorliegenden Arbeit habe ich mich auf das Serotonin-System, die Hypothalamus-Hypophysen-Nebennierenrindenachse (*hypothalamic-pituitary-adrenal axis* = HPA-Achse) und das Arginin-Vasopressin (AVP)-System konzentriert, da Hinweise vorliegen, dass diese bei der Regulation von Aggression eine Rolle spielen.

In Kapitel 2 wurde daher die Rolle des Serotonin-Systems bei der Regulation von Aggressionsverhalten bei LAB-Ratten untersucht, da diese am meisten Aggressionsverhalten

und abnormale Aggression zeigten. Die Auswirkung einer einmaligen peripheren Injektion des vorrangig am Serotonin-1_A-Autorezeptor wirkenden Serotonin-1_A-Agonisten S-15535 auf Aggression und abnormale Aggression bei LAB-Ratten wurde untersucht. S-15535 führt zu einer akuten Reduktion der Serotonin-Freisetzung in Gehirnregionen mit serotonerger Innervierung v.a. aus den Raphe-Kernen führt. Die Behandlung mit S-15535 bewirkte eine selektive Reduktion der Angriffe sowohl auf eine männliche als auch auf eine narkotisierte männliche Ratte, ohne jedoch die gesamte Menge an Aggressionsverhalten zu beeinflussen. Dies weist auf eine Beteiligung des Serotonin-Systems vor allem an der Angriffs-Komponente des Aggressionsverhaltens bei normaler und abnormaler Aggression bei LAB-Ratten hin.

In Kapitel 3 wurde untersucht, ob es Unterschiede bezüglich der HPA-Achsen-Antwort auf den RI Test zwischen den Rattenlinien gibt, da Veränderungen des HPA-Systems mit erhöhter Aggression in Zusammenhang gebracht wurden. Eine durch den RI Test erhöhte Plasmakonzentration von adrenocorticotropem Hormon (ACTH) wurde in LAB-Ratten im Vergleich mit HAB-Ratten nachgewiesen. Sowohl bei LAB- als auch bei HAB-Ratten kam es durch den RI Test zu einem signifikanten Anstieg der Corticosteronkonzentration im Plasma, während dies bei NAB-Ratten nicht der Fall war.

Des Weiteren wurde in Kapitel 3 die durch den RI Test ausgelöste neuronale Aktivierung in Gehirnen von LAB- und HAB-Ratten bestimmt. Dazu wurde die Expression des Gens *c-fos* gemessen, welches zu den sog. *immediate-early genes* gehört. Diese Gene weisen in einer Zelle bereits kurz nach einem äußeren Stimulus eine erhöhte Aktivierung auf und lassen daher Rückschlüsse auf die Gehirnregionen zu, die eine Rolle bei der Reaktion auf bestimmte Stimuli und der Regulation des zugehörigen Verhaltens, wie z.B. in diesem Fall Aggressionsverhalten, spielen können. LAB-Ratten zeigten eine erhöhte Aktivierung im

hypothalamischen paraventrikulären Nucleus (PVN) und eine Tendenz zu einer höheren Aktivierung in der zentralen und medialen Amygdala sowie in der *hypothalamic attack area*.

In den Kapiteln 4 und 5 wurde der Einfluss des AVP-Systems auf das Aggressionsverhalten in zwei Gehirnregionen mit androgenabhängigen AVP-Fasern bzw. –Zellen untersucht, nämlich im lateralen Septum und im *bed nucleus of the stria terminalis* (BNST), die an der Regulation des Aggressionsverhaltens beteiligt sind. Das hohe Maß an Aggressionsverhalten bei LAB-Ratten ging einher mit einer erniedrigten Freisetzung von AVP sowie einer geringeren neuronalen Aktivierung im lateralen Septum. Im Gegensatz dazu zeigten HAB-Ratten eher einen Anstieg bezüglich der septalen AVP-Freisetzung. Eine Verabreichung von synthetischem AVP in das laterale Septum von LAB-Ratten, zeigte keinen Einfluss auf das Aggressionsverhalten, resultierte aber in einem gesteigerten Angstverhalten auf der EPM. Bei HAB-Ratten wurde das Aggressionsverhalten durch eine Blockierung der AVP-V1a-Rezeptoren mit Hilfe des selektiven AVP-V1a-Rezeptor-Antagonisten $d(CH_2)_5$Tyr(Me)AVP ebenfalls nicht beeinflusst, während sich jedoch die Zeit verringerte, die die Ratten mit nicht-aggressivem Sozialverhalten, d.h. mit dem Beschnüffeln der fremden Ratte, verbrachten.

In Kapitel 5 wurde der Einfluss des AVP-Systems im lateralen Septum sowie im BNST auf die Regulation von Aggressionsverhalten bei männlichen NAB-Ratten mithilfe von Mikrodialyse untersucht. Die Wahl fiel hierbei auf die nicht-selektiv gezüchtete, oft verwendete Wistar-Rattenlinie, um eine generellere Aussage über die Rolle des AVP-Systems in den beiden oben genannten Gehirnregionen treffen zu können. Die vasopressinergen Fasern, die ins laterale Septum ziehen, entstammen zum Teil dem BNST, in dem zudem eine große Anzahl an AVP-V1a-Rezeptoren lokalisiert ist. Hinweise aus verschiedenen Studien deuten auf eine regulatorische Rolle von AVP im BNST auf Aggression hin. Zunächst wurden männliche NAB-Ratten gemäß ihres Verhaltens im RI Test in aggressiv und nicht-

aggressiv eingeteilt. Anschließende Mikrodialyse-Versuche zeigten, dass aggressive Ratten einen signifikanten Anstieg der AVP-Freisetzung durch den RI Test im Vergleich zu nicht-aggressiven Ratten zeigten. Weder die Verabreichung des AVP-V1a-Rezeptor-Antagonisten ins laterale Septum aggressiver Ratten noch die des synthetischen AVPs ins laterale Septum nicht-aggressiver Ratten führte zu einer Veränderung im Aggressionsverhalten. Jedoch resultierte die Verabreichung von synthetischem AVP ins laterale Septum von nicht-aggressiven Ratten in einer Erhöhung der auf der EPM gemessenen Ängstlichkeit.

In einem weiteren Versuch wurde die AVP-Freisetzung im BNST von aggressiven und nicht-aggressiven NAB-Ratten während des RI Tests bestimmt. Im Gegensatz zu den Ergebnissen im lateralen Septum trat ein durch den RI Test verursachter, signifikanter Anstieg der AVP-Freisetzung im BNST bei nicht-aggressiven Ratten auf, während die lokale AVP-Freisetzung bei aggressiven Ratten in dieser Gehirnregion nicht durch den RI Test beeinflusst wurde. Interessanterweise führte die Verabreichung von synthetischem AVP in den BNST aggressiver Ratten zu einer signifikanten Reduktion des Aggressionsverhaltens. Zusammenfassend legen diese Ergebnise nahe, dass männliches Aggressionsverhalten mit deutlichen Unterschieden in der AVP-Freisetzung in beiden untersuchten Gehirnregionen zwischen aggressiven und wenig oder nicht-aggressiven Ratten einhergeht. Die pharmakologische Manipulation des AVP-Systems im BNST hatte einen direkten regulatorischen Einfluss auf das Aggressionsverhalten zur Folge, während kein Einfluss diesbezüglich im lateralen Septum festgestellt werden konnte. Daher scheint der beobachtete Unterschied in der AVP-Freisetzung im lateralen Septum während des Aggressionstests eher eine Folge des gezeigten Aggressionsverhaltens zu sein und Verhaltensweisen wie zum Beispiel soziales Erkundungsverhalten oder Angst zu beeinflussen, die im Zusammenhang mit Aggression stehen.

Zusammenfassend lässt sich sagen, dass LAB-Ratten ein hohes Maß an Aggressionsverhalten zeigten, das einherging mit einer erhöhten HPA-Achsen-Antwort sowie einer vermehrten neuronalen Aktivierung des PVNs. Dies lässt darauf schließen, dass Aggression bei LAB-Ratten mit einer erhöhten Stressantwort in Verbindung steht. Generell waren soziale Interaktionen bei LAB-Ratten mit einer höheren Stress-Achsen-Antwort verbunden als bei HAB-Ratten. Zudem zeigten LAB-Ratten ein geringeres Maß an nicht-aggressivem Sozialverhalten, d.h. soziales Erkundungsverhalten. Überdies erfüllen LAB-Ratten mehrere Kriterien abnormaler Aggression, da sie Angriffe auf verletzliche Körperteile eines kleineren Eindringlings zeigen, und Weibchen oder insbesondere den Kopfbereich eines narkotisierten männlichen Eindringlings angreifen. Daher stellen LAB-Ratten ein interessantes Tiermodell zur Erforschung von antisozialem Verhalten dar, das hohes sowie abnormes Aggressionsverhalten in Kombination mit einem geringen Maß an nicht-aggressivem Sozialverhalten einschließt.

HAB-Ratten wiesen ein intermediäres Maß an Aggressionsverhalten auf, das mit einer erhöhten HPA-Achsen-Antwort im Vergleich zu NAB-Ratten in Verbindung steht. Außerdem zeigten HAB-Ratten einen hohen Prozentsatz an Angriffen auf verletzliche Körperteile eines kleineren männlichen Eindringlings, was als abnormales Aggressionsverhalten eingestuft wird. Daher stellen HAB-Ratten ein adäquates Tiermodell dar, um die neurobiologischen Mechanismen zu erforschen, die erhöhtem und abnormalem Aggressionsverhalten insbesondere vor dem Hintergrund ihrer hohen angeborenen Ängstlichkeit zugrundeliegen.

Sowohl LAB- als auch HAB-Ratten waren aggressiver als NAB-Ratten, aber wiesen den durch die Selektion herausgezüchteten Unterschied im Angstverhalten auf der EPM auf. Daher wurde keine lineare Korrelation zwischen Angst und Aggression festgestellt. Stattdessen wiesen Angst und Aggression bei LAB-, HAB- und NAB-Ratten eine U-förmige Korrelation auf. Allerdings weist der in LAB-Ratten beobachtete Anstieg der durch soziale Stressoren ausgelöste HPA-Achsen-Aktivität in Kombination mit dem niedrigen Maß an

sozialem Erkundungsverhalten auf ein erhöhtes Maß an Angst vor sozialen Stressoren hin. Folglich könnte das hohe Maß an Aggressionsverhalten in LAB-Ratten mit einer erhöhten sozialen Angst in Verbindung stehen. Weiterführende Studien sind nötig, um einen tieferen Einblick in die Beziehung zwischen diesen komplexen Verhaltensweisen und den zugrunde liegenden neurobiologischen Mechanismen zu erhalten.

Spezifische Unterschiede in der AVP-Freisetzung im lateralen Septum durch den RI Test wurden bei LAB-, HAB- und NAB-Ratten festgestellt, jedoch zeigte eine pharmakologische Manipulation des AVP-Systems im lateralen Septum bei keiner der Rattenlinien einen Einfluss auf das Aggressionsverhalten. Im Gegensatz dazu konnten durch diese Behandlung sowohl das Angstverhalten als auch das nicht-aggressive Sozialverhalten beeinflusst werden. Diese Ergebnisse weisen darauf hin, dass die spezifischen Unterschiede in der AVP-Freisetzung im lateralen Septum eher die Folge von als die Ursache für Aggressionsverhalten sind. Dennoch könnte im lateralen Septum freigesetztes AVP durch indirekte Effekte auf Angst- oder nicht-aggressives Sozialverhalten Aggressionsverhalten beeinflussen.

Die AVP-Freisetzung im BNST wies spezifisch durch Aggressionsverhalten verursachte Unterschiede zwischen aggressiven und nicht-aggressiven NAB-Ratten auf. Darüberhinaus führte eine pharmakologische Manipulation des AVP-Systems im BNST zu einer direkten Beeinflussung des Aggressionsverhaltens, was auf eine direkte regulatorische Wirkung von AVP im BNST hinweist.

Ein hohes Ausmaß an Aggressionsverhalten stand im Zusammenhang mit einer erhöhten HPA-Achsen-Antwort bei LAB- und HAB-Ratten. Trotzdem besteht anscheinend keine lineare Beziehung zwischen HPA-Achse und Aggressionsverhalten, da eine Behandlung mit einer anxiogenen Substanz bei LAB-Ratten zu einer erhöhten neuronalen Aktivität im PVN sowie zu einer erhöhten Plasma-Corticosteronkonzentration führt, aber zugleich das

Aggressionsverhalten hemmte. Die genauen zugrunde liegenden Mechanismen sind noch zu erforschen.

Die pharmakologische Manipulation des Serotonin-Systems bei LAB-Ratten hatte eine Verringerung der Angriffe gegenüber einem männlichen oder einem narkotisierten männlichen Eindringling zur Folge. Da die Gesamtmenge des Aggressionsverhaltens unbeeinflusst blieb, wirkt das Serotonin-System bei LAB-Ratten spezifisch auf die Angriffs-Komponente des Aggressionsverhaltens.

Abschließend lässt sich feststellen, dass die Ergebnisse der vorliegenden Dissertation zeigen, dass sich sowohl LAB- als auch HAB-Ratten als Tiermodell eignen, um die in der vorliegenden Dissertation gewonnenen Ergebnisse bezüglich der neurobiologischen Mechanismen der Regulation von hohem und abnormalem Aggressionsverhalten zu erweitern.

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Acknowledgements

Acknowledgements - Danksagung

An erster Stelle möchte ich mich bei Frau Prof. Dr. Inga D. Neumann bedanken, die mir die Möglichkeit gegeben hat, die vorliegende Arbeit an ihrem Lehrstuhl durchzuführen. Die fachlichen Diskussionen mit ihr bezüglich künftiger Versuchsvorhaben, Interpretation von Daten oder Präsentation von Daten in schriftlicher oder mündlicher Form waren sehr interessant, sowie hilf- und lehrreich.

Mein Dank gilt insbesondere auch Frau Dr. Alexa H. Veenema, die mich auch zu ungewöhnlicheren Zeitpunkten experimentell unterstützt hat und die stets ein offenes Ohr für und Antworten auf Fragen aller Art hatte. Sie hat es verstanden, mich auch nach Tiefschlägen bezüglich Experimenten oder Manuskripten die Motivation nicht verlieren zu lassen. Herzlichen Dank dafür, Alexa. Von Dir habe ich sehr viel gelernt. (Sogar ein bisschen Niederländisch ☺; also: Lieve Alexa, van jou heb ik erg veel geleerd. Bedankt voor alles!)

Bedanken möchte ich mich auch bei Herrn Remco Bredewold für die tatkräftige Unterstützung bei der Durchführung unzähliger *in situ hyridisations* und vor allem auch für die unermüdliche Fehlersuche und –behebung, als diese eine Zeit lang nicht so gut funktionierten.

Bei Frau Gabriele Schindler, Frau Laura Traulsen und Frau Andrea Havasi bedanke ich mich für die Hilfe bei der Planung und Durchführung der LAB-/HAB-Rattenzucht, sowie zusammen mit Frau Luxiola Gonzalez und Herrn Rodrigue Maloumby für die unverzichtbare technische Unterstützung. Frau Gabriele Schindler war zudem eine sehr wichtige Stütze beim Schneiden und Färben von Rattengehirnen.

Cindy Baumgart und Michael Lukas danke ich für die Hilfe bei der Versuchsdurchführung. Außerdem möchte ich mich auch bei allen Praktikanden der Arbeitsgruppe bedanken, die bei Experimenten oder Vorbereitungen geholfen haben.

Ein herzliches Dankeschön auch an die gesamte Arbeitsgruppe für das positive Arbeitsklima sowie die vielfältige Unterstützung und Hilfe bei allen möglichen Problemen.

Bei Dr. David Slattery möchte ich mich für die Durchsicht der englischsprachigen Manuskripte sowie geduldige Beantwortung von Fragen, die die englische Aussprache oder Schreibweise betreffen, bedanken. Thank you very much!

Weiterhin gilt mein Dank Herrn Dr. József Halász für seine Hilfe bei der Auswertung der *attack target regions* bei den RI Tests, Herrn Prof. Dr. Rainer Landgraf sowie den Mitarbeitern seiner Arbeitsgruppe für die Unterstützung bei den Radioimmunoassays, und außerdem Herrn Dr. Morris Manning und Dr. Sietse F. De Boer für die Möglichkeit, den AVP-V1a-Rezeptor-Antagonist [d(CH_2)$_5$Tyr(Me)AVP] bzw. den Serotonin-1$_A$-Autorezeptor-Agonist (S-15535) in meinen Experimenten einzusetzen.

Mein besonderer Dank gilt natürlich meinen Eltern, die mir mein Studium ermöglicht haben. Vielen lieben Dank dafür und für das Verständnis für die stressige Lebensphase, die ich durchgemacht habe - auch an den Rest der Familie und an alle anderen (allen voran meine beste Freundin Stefanie Bach), die vor allem in der Endphase der Promotion unter der vielen „Keine-Zeit“ leiden mussten, die ich in den letzten Monaten hatte.

Abbreviations

Abbreviations

ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
AVP	Arginine vasopressin
BNST	Bed nucleus of the stria terminalis
CRH	Corticotropin releasing hormone
DIA	Diazepam
EPM	Elevated plus-maze
GABA	γ-aminobutyric acid
HAB	High anxiety-related behaviour
HPA	Hypothalamic-pituitary-adrenal
i.p.	Intraperitoneally
LAB	Low anxiety-related behaviour
LAL	Long attack latency
MAOA	Monoamine oxidase A
mRNA	Messenger ribonucleic acid
NAB	Non-selected Wistar
PTZ	Pentylenetetrazole
PVN	Paraventricular nucleus of the hypothalamus
RI	Resident-intruder
SAL	Short attack latency
s.c.	Subcutaneously
SEM	Standard error of the mean
V1a-A	Arginine vasopressin V1a receptor antagonist

Curriculum Vitae

List of publications

Curriculum Vitae

25. April 1980	Geboren als Tochter von Monika (geb. Lutz) und Johann Beiderbeck in Regensburg
Sept. 1986 - Juli 1990	Besuch der Von-der-Tann-Grundschule in Regensburg
Sept. 1990 – Juni 1999	Besuch des Werner-von-Siemens-Gymnasiums (mathematisch-naturwissenschaftlich) in Regensburg Hauptfächer: Biologie und Englisch
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Okt. 1999 – April 2005	Studiengang Biologie (Diplom) an der Universität Regensburg (Gesamtnote 1,1)
Juni 2004 – April 2005	Diplomarbeit mit dem Thema „Einfluss von Vasopressin und angeborener Ängstlichkeit auf Aggression bei männlichen Wistarratten (*Rattus norvegicus*)“ unter Anleitung von Frau Dr. Alexa Veenema am Lehrstuhl für Tierphysiologie und Neurobiologie von Frau Prof. Dr. Inga Neumann, Institut für Zoologie an der Universität Regensburg (Note 1,1)
Juni 2005 – jetzt	Doktorarbeit mit dem Thema „Underlying neurobiological mechanisms of high and abnormal aggression in male rats: link to trait anxiety” am Lehrstuhl für Tierphysiologie und Neurobiologie von Frau Prof. Dr. Inga Neumann, Institut für Zoologie an der Universität Regensburg

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Bosch O, Meddle S, Beiderbeck DI, Douglas AJ, Neumann ID, 2005. Brain oxytocin regulates maternal aggression. *J Neurosci.* 25, 6807-6815

Veenema AH, Torner L, Blume A, Beiderbeck DI, Neumann ID, 2007. Low inborn anxiety correlates with high intermale aggression: Link to ACTH response and neuronal activation of the hypothalamic paraventricular nucleus. *Horm Behav.* 51, 11-19

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Wiehager S, Beiderbeck DI, Gruber SH, El-Khoury A, Wamsteeker J, Neumann ID, Petersen A, Mathé AA. Increased levels of cocaine and amphetamine regulated transcript in two animal models of depression and anxiety. *Neurobiology of Desease,* accepted

Beiderbeck DI, Neumann ID, Veenema AH. Paradox of anxiety and aggression: both low and high trait anxiety are linked with high and abnormal forms of intermale aggression. In preparation

Veenema AH, Beiderbeck DI, Neumann ID. Distinct vasopressin release patterns within the lateral septum and the bed nucleus of the stria terminalis during the display of intermale aggression. In preparation

Author's Declaration - Eidesstattliche Erklärung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe des Literaturzitats gekennzeichnet.

Weitere Personen waren an der inhaltlich-materiellen Herstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich hierfür nicht die entgeltliche Hilfe eines Promotionsberaters oder anderer Personen in Anspruch genommen. Niemand hat von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Regensburg, den 09.02.2009

(Daniela Beiderbeck)