Neurobiological Mechanisms Involved in the Establishment and Maintenance of Dominance Hierarchies and its Modulation by Stress in Rats

THÈSE N^{O} 4892 (2011)

PRÉSENTÉE LE 27 JANVIER 2011

À LA FACULTÉ SCIENCES DE LA VIE
LABORATOIRE DE GÉNÉTIQUE COMPORTEMENTALE
PROGRAMME DOCTORAL EN NEUROSCIENCES

ÉCOLE POLYTECHNIQUE FÉDÉRALE DE LAUSANNE

POUR L'OBTENTION DU GRADE DE DOCTEUR ÈS SCIENCES

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Suisse
2011

Acknowledgments

I would like to thank the people that were around during the past four years that I spent in the Laboratory of Behavioral Genetics and that contributed to making these four years both an instructive and enjoyable period in my life.

First, I would like to thank Prof. Carmen Sandi for giving me the opportunity to do work on the exciting topic of social neuroscience in her group. I would like to thank her for her supervision, guidance and encouragement during the past four years.

I would like to thank all past and present lab members, especially Coralie Siegmund and Stamatina Tzanoulinou for their friendship, Maribel Cordero for all her help, the interesting discussions about the project and for always showing interest no matter how much work there was waiting to be done, Yannick Sevelinge(s) for teaching me how to do surgery, for helping me many, many times with my experiments and for making me laugh during the work we did together, and Angélique Vaucher for all her great help with my experiments.
I would also like to thank Reto Bisaz, Jorge Castro, Lisa Conboy, Martina Fantin, Emilien Gantelet, David Genoux, Jocelyn Grosse, Marianne Hald Larsen, Keerthana Iyer, Michael van der Kooij, Gedi Luksys, Cristina Marquez, Grégoire Parchet, Guillaume Poirier, Emil Polny, Clara Rossetti, Basira Salehi, Christina Schrick, Emilio Varea and Vandana Veenit for their help, for offering a nice working environment and for the great evenings spent outside EPFL.
I would like to thank Mark Fajans and Stefanie Klampfl for the work they have done for the work presented in this thesis while they were working as summer/master student in the group, and Manuel Bueno and Sylvain Lengacher for their advice on the real time PCR protocol.

I would like to thank my thesis committee Prof. Igor Branchi, Prof. Ruth Luthi-Carter, Prof. Ralf Schneggenburger and Dr. Ron Stoop for taking time to evaluate my work and for their helpful comments.

Special thanks to my friends in the Netherlands, Jacobien Buruma and Annet Zijlstra, and in Lausanne, Malik Benameur, Anika Ekrut and Leila Laredj, for all the nice times and all the lunch and coffee breaks that we spent together and for always being there for me whenever needed to share the good and bad things in life.

I would like to thank my parents, Gerrit and Ria Timmer, for all the love and support they have given me during all those years. They have always encouraged and supported me in the things I did and the decisions I took.

Last but not least, I would like to thank Dan Keller for all the love, encouragement and positive thoughts during the writing of my thesis, and for always being there for me.

Marjan

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Abstract

Stress can have a strong and long lasting effect on the establishment and maintenance of dominance hierarchies in rats. When two rats of a pair have not been stressed (non-stressed pair) before a first social encounter, they establish a dominance hierarchy, but the rank that is obtained during this first encounter is not maintained until a second encounter one week later. When one rat of the pair is stressed (stressed pair) before the first encounter, the stressed rat becomes subordinate during the first encounter and the hierarchy that is formed is long lasting and stable. In this thesis, the neurobiological mechanisms underlying the effects of stress on dominance hierarchies were studied.

In the first study, we investigated the role of increasing corticosterone levels before or just after a first encounter between two rats of a pair in the establishment and the long-term maintenance of a dominance hierarchy. We show that pre-social encounter corticosterone treatment does not affect the outcome of the hierarchy during a first encounter, but induces a long-term memory for the hierarchy when the corticosterone-injected rat becomes dominant during the encounter, but not when it becomes subordinate. Post-social encounter corticosterone leads to a long-term maintenance of the hierarchy only when the subordinate rat of the pair is injected with corticosterone. This corticosterone effect implicates glucocorticoids in the consolidation of the memory for a recently established hierarchy.

Next, we investigated the immediate and long-term changes in mRNA levels of receptors for oxytocin and vasopressin in medial amygdala and lateral septum. In subordinate rats of the stressed pairs, we found a downregulation in oxytocin receptor mRNA in medial amygdala at three hours after the first encounter, and a downregulation in vasopressin 1a receptor mRNA in lateral septum one week after the first encounter. We show that administration of an oxytocin antagonist in the medial amygdala of the subordinate rat immediately after a first encounter induces a long-term memory for the established hierarchy. Administration of a vasopressin 1a receptor antagonist in the lateral septum of the subordinate rat just before a second encounter mimics the effects of exposure to acute stress on the long-term establishment of a dominance hierarchy. These results suggest a role for oxytocin and vasopressin in the effects of stress on the long-term establishment of dominance hierarchies.

Finally, we investigated the long-term effects of acute stress and hierarchy formation on the establishment of a second hierarchy with an unfamiliar opponent. We show that the behavior of stressed subordinate rats towards an unfamiliar opponent depends on the previous status of the opponent: when paired with a previously dominant rat they become subordinate, whereas they become dominant when paired with a previously subordinate rat. Furthermore, we show that monoamine oxidase A and androgen receptor mRNA levels are downregulated in the lateral septum of stressed subordinate rats at one week after the first encounter; i.e., at the time when we have observed their flexible social behavior against unfamiliar opponents. The monoamine oxidase A inhibitor clorgyline was injected into the lateral septum to examine whether there was a causal relationship between changes in monoamine oxidase A mRNA levels and offensive behavior towards an unfamiliar subordinate opponent. However, our results did not support this hypothesis.

Taken together, we have shown that exposure to acute stress and a social encounter leads to changes in the mRNA levels of receptors for oxytocin, vasopressin and androgens and of monoamine oxidase A. These changes might underlie the effect of stress on the formation of a stable and long-lasting dominance hierarchy. Corticosterone plays a role in the long-term maintenance of the recently established hierarchy and hence it is an ideal candidate to mediate some of the observed neurobiological changes.

Key words: dominance hierarchy; aggression; memory; subordinate; corticosterone; oxytocin; vasopressin; monoamine oxidase A; receptors; medial amygdala; lateral septum; rat.

Résumé

Le stress peut avoir un effet important et durable sur l'établissement et le maintien de la mémoire des relations hiérarchique (dominance/soumission) chez les rats. En effet, la relation de dominance/soumission établie par deux rats non stressés lors d'une première rencontre sociale (paire non stressée) n'est pas retenue lors d'une deuxième rencontre une semaine plus tard. Cependant, si un des deux rats est stressé (chocs électriques aux pattes) immédiatement avant la première rencontre (paire stressée), le rat stressé devient soumis pendant la première rencontre. De plus, cette hiérarchie est durable et stable puisque elle est toujours présente une semaine plus tard. Le but de cette thèse a été d'étudier les mécanismes neurobiologiques impliqués dans l'effet du stress sur les relations de hiérarchie (dominance/soumission).

Dans une première étude, nous avons examiné le rôle d'une augmentation du niveau de corticostérone avant où immédiatement après une première rencontre entre deux rats dans l'établissement et le maintien d'une hiérarchie de dominance. Nous montrons qu'un traitement au corticostérone avant la première rencontre n'a pas d'effet sur le résultat de la mise en place de hiérarchie pendant la première rencontre, mais provoque une mémoire de cette hiérarchie uniquement lorsque le rat traité avec la corticostérone devient dominant. Le même traitement immédiatement après la rencontre provoque une mémoire de la hiérarchie seulement quand le rat subordonné est injecté avec de la corticostérone. Cet effet de la corticostérone montre donc que les glucocorticoïdes jouent un rôle crucial dans la consolidation de la mémoire d'une hiérarchie sociale récemment mise en place.

Nous avons ensuite étudié les changements immédiats et à long terme des niveaux d'ARNm des récepteurs à ocytocine et vasopressine dans l'amygdale médiane et le septum latéral. Nous avons montré i) trois heures après la première rencontre, une diminution des ARNm des récepteurs à ocytocine dans l'amygdale médiane des rats subordonnés des couples stressés et ii) une semaine après la première rencontre, une diminution des ARNm des récepteurs à vasopressine dans le septum latéral des rats subordonnés des couples stressés. Par ailleurs, nous montrons que l'administration d'un antagoniste aux récepteurs à ocytocine dans l'amygdale médiane d'un rat subordonné immédiatement après une première rencontre induit une mémoire de cette hiérarchie. De même, l'administration d'un antagoniste aux récepteurs à vasopressine 1a dans le septum latéral du rat subordonné juste avant une deuxième rencontre imite les effets d'une exposition à un stress aigu, c'est-à-dire que nous avons observé un maintien de la hiérarchie précédemment établie. Ces résultats suggèrent un rôle pour l'ocytocine et la vasopressine dans les effets du stress sur la mise en place à long terme des hiérarchies de dominance.

Enfin, nous avons étudié les effets à long terme d'un stress aigu sur la formation d'une deuxième hiérarchie avec un opposant inconnu. Nous montrons que le comportement des rats subordonnés suite à un stress envers un opposant inconnu dépend du statut antérieur de cet opposant : quand le rat stressé précédemment est mis en relation avec un opposant qui était dominant, il devient soumis. S'il est couplé avec un opposant qui était subordonné (sans être stressé), il devient dominant. De plus, nous montrons que les niveaux d'ARNm de la monoamine oxydase A et du récepteur androgène sont diminués dans le septum latéral des rats subordonnés et stressés une semaine après la première rencontre, à savoir, au moment où nous avons observé le comportement social flexible envers un opposant inconnu. Pour tester l'existence d'un lien causal entre les changements sur les niveaux de ARNm pour monoamine oxydase A et le comportement agressif vers un opposant inconnu qui était subordonné, nous avons injecté un inhibiteur de la monoamine oxydase A (clorgyline) dans le septum latéral, sans réussir à corroborer cette hypothèse.

Dans l’ensemble, nous avons montré qu’une exposition à stress aigu et une rencontre sociale entrainent des changements dans les niveaux d’ARNm pour les récepteurs à ocytocine, vasopressine et androgène et pour la monoamine oxydase A. Ces changements pourraient être à la base de l’effet du stress sur la formation d’une hiérarchie de dominance qui est stable et durable. La corticostérone joue un rôle dans le maintien à long terme d’une hiérarchie récemment établie et est donc un candidat idéal à la médiation des changements neurobiologiques observés.

Mots clés: hiérarchie de dominance, l’agression, la mémoire, soumis, la corticostérone, l’ocytocine, la vasopressine, la monoamine oxydase A, récepteurs, l’amygdale médiane, le septum latéral, rat.

List of abbreviations

5-HT	Serotonin
ACTH	Adrenocorticotropic hormone
AH	Anterior hypothalamus
AOB	Accessory olfactory bulb
AR	Androgen receptor
AVP	Arginine vasopressin
BNST	Bed nucleus of stria terminalis
CORT	Corticosterone
CRE	Cyclic AMP response element
CRF	Corticotropin releasing factor
CRH	Corticotropin releasing hormone
EPM	Elevated plus maze
FCT	Food competition test
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
HPA-axis	Hypothalamus-pituitary-adrenal axis
LS	Lateral septum
MAOA	Monoamine oxidase A
MeA	Medial amygdala
MPOA	Medial preoptic area
MR	Mineralocorticoid receptor
NCAM	Neural cell adhesion molecule
OT	Oxytocin
OTA	Oxytocin antagonist
OTR	Oxytocin receptor
Pns	Non-stressed pair
Ps	Stressed pair
PVN	Paraventricular nucleus
Rns	Non-stressed rat
Rs	Stressed rat
SON	Supraoptic nucleus
V1aR	Vasopressin 1a receptor
V1bR	Vasopressin 1b receptor
WCT	Water competition test

Chapter 1 - Introduction

Stress can have a strong and long lasting effect on the establishment of dominance hierarchies in rats and on the long-term memory that is formed for the established hierarchy. In our group, an animal model was developed that allows us to investigate the neurobiological mechanisms underlying these effects of stress on dominance hierarchies in adult male rats (Cordero and Sandi, 2007). The main characteristic of the model is that when both rats have not been stressed before the first encounter (the so called non-stressed pairs - Pns) a clear hierarchy is established during the first encounter, but the hierarchy is not stable over a long period of time. However, when one of the two rats of a pair is submitted to acute stress just before the first encounter (the stressed pairs - Ps), (1) the stressed rat usually becomes the subordinate individual in the first encounter and (2) the hierarchy that has been established during this first encounter is long lasting and stable, at least until a week after the first encounter.

The final goal is to use this model to progress on the understanding of the mechanisms involved in the development and long-term establishment of dominance hierarchies after exposure to stress, and to develop interventions that can reduce maladaptive or disproportional hierarchical relationships.

1.1 Stress

Animals maintain a certain dynamic equilibrium or homeostasis in their internal state. When an animal is exposed to stress, this homeostasis can be disturbed and physiological and behavioral adaptations are made to try to restore the homeostasis. When the exposure to stress lasts too long or is too severe, this can lead to deleterious effects on the health of the animal (McEwen, 2007).

Part of the stress response consists of a rapid activation of the sympathetic nervous system. This leads to the release of noradrenaline and adrenaline, which prepares the body for a ‘fight-flight’ response by increasing heart rate, blood pressure and blood glucose levels. Centrally, the hypothalamic-pituitary-adrenal (HPA) axis is activated. This leads to the release of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) from the paraventricular nucleus (PVN) of the hypothalamus. The release of CRH and AVP from the PVN is stimulated by limbic brain areas such as the amygdala, the hippocampus and the prefrontal cortex, which are activated by psychological stressors, and by brain-stem pathways, which are activated by sensory stimuli. CRH and AVP stimulate the release of

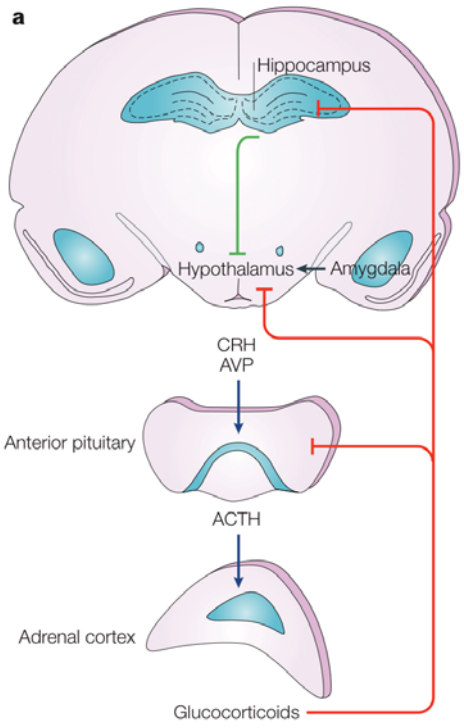

Figure 1. Exposure to stress leads to activation of the HPA axis: CRH and AVP are released from the hypothalamus, which stimulates release of ACTH from the pituitary gland into the peripheral blood stream. ACTH leads to glucocorticoid release from the adrenal glands. Glucocorticoids exert a negative feedback on the HPA axis through GRs in the hippocampus, hypothalamus and pituitary gland. *(Figure from Sandi, 2004)*

adrenocorticotropic hormone (ACTH) from the pituitary into the peripheral blood stream, which eventually leads to the release of glucocorticoids -corticosterone in rats and cortisol in humans- from the adrenal glands. These stress hormones reach their peak levels at 15-30 minutes after the onset of the stress response and return to normal levels after 60-90 minutes. Glucocorticoids control the activation of the HPA axis through negative feedback mechanisms at the level of the hypothalamus and the pituitary, but also at the level of the hippocampus, which has a high density of the intracellular glucocorticoid (GR) and mineralocorticoid receptors (MR) (Fig. 1) (reviewed by de Kloet et al., 2005; Sandi and Pinelo-Nava, 2007).

Glucocorticoids can rapidly access the brain due to their lipophilic nature. MRs are activated by a low concentration of glucocorticoids and play a role in the onset of the stress response. GRs are only activated by a high concentration of glucocorticoids, about 10 times as high as necessary for MR activation, and are important for the termination of the stress response and for recovery, i.e. through inhibition of CRH and AVP synthesis. GRs are also involved in memory formation, which allows for a more efficient stress response when the animal is re-exposed to the same situation. Besides in the hippocampus, a high density of GRs is found in the PVN and in limbic brain areas that modulate PVN activity. MRs are also

expressed in limbic brain areas such as amygdala and septum (de Kloet et al., 2005; Sandi and Pinelo-Nava, 2007).

After entering the cell, glucocorticoids bind to MRs and GRs that form homo- or heterodimers and that affect gene expression through their action as transcription factors, either by themselves, or through a stimulating or inhibiting interaction with other transcription factors, by binding to glucocorticoid response elements (GREs). GREs are sequences in the DNA that are located close to gene promoters, and binding of transcription factors to these sequences leads to stimulation or inhibition of expression of the gene that is controlled by the promoter. Glucocorticoids can affect gene expression for a wide number of genes (Morsink et al., 2007). The changes in gene expression can affect memory formation, but also lead to molecular changes in cell adhesion molecules (Sandi, 2004) and structural changes in dendritic structure and size (McEwen, 2005). Glucocorticoids can also induce fast non-genomic changes that act to bridge the time until the genomic changes have an effect, and possibly prepare the animal for the coming genomic changes. Non-genomic changes occur through binding of glucocorticoids to receptors on the cell membrane (Makara and Haller, 2001).

Stress has a U-shaped dose-response effect on learning and memory. Exposure to mild stress enhances memory formation, but exposure to excessive stress has negative effects on learning and memory formation (Joels, 2006; Sandi and Pinelo-Nava, 2007). The enhancement of learning and memory due to glucocorticoids has been extensively studied in a variety of learning tasks, including spatial learning (Oitzl and de Kloet, 1992; Sandi et al., 1997; Akirav et al., 2004; Brinks et al., 2009a; Conboy and Sandi, 2009), inhibitory avoidance (Sandi and Rose, 1994; Roozendaal and McGaugh, 1996; Sandi and Rose, 1997; Roozendaal et al., 2002) , fear conditioning (Cordero et al., 1998; Cordero and Sandi, 1998; Cordero et al., 2002), its reconsolidation (Tronel and Alberini, 2007) and extinction (Brinks et al., 2009b; Gourley et al., 2009), and object recognition in emotionally aroused, but not in non-aroused rats (Okuda et al., 2004; Roozendaal et al., 2006). This body of research has emphasized a role for glucocorticoids in the enhancement of memory consolidation of emotionally arousing experiences, while pointing at their negative effects on the retrieval of information or working memory (Roozendaal, 2000; Sandi and Pinelo-Nava, 2007; de Quervain et al., 2009). In our model, stress enhances the long-term memory that is formed for the established dominance hierarchy (Cordero and Sandi, 2007).

1.2 The formation of a dominance hierarchy

Animals that live in groups establish dominance hierarchies in which the dominant individual has priority access to limited resources such as water, food, females and space. The main function of the establishment of stable dominance hierarchies is to eliminate fighting within a group and thus to minimize energy costs (Van Kreveld, 1970). In laboratory rats that are kept together in a colony, a hierarchy usually develops in a few days and is stable for the time the group exists, as shown in the visible burrow system (Blanchard et al., 1988). Factors that determine which individual will become dominant can be divided in intrinsic factors, such as size, age, sex, strength, physiology and the level of aggressiveness (Parker, 1974; Chase et al., 2002) and extrinsic factors such as the winner and loser effect (reviewed by Chase et al., 1994). According to the winner effect, animals that have won an encounter are more likely to win future encounters, whereas according to the loser effect animals that have lost an encounter are more likely to lose future encounters. The animal model that was developed in our group, and that has been used throughout the studies described in the following chapters, shows that the extrinsic factor of acute stress can also affect the outcome of a dominance hierarchy (Cordero and Sandi, 2007). It has been shown before that dominance hierarchies and social defeat can be stressful (Blanchard et al., 1995; Meerlo et al., 1996), but the contribution of stress (either induced by the social interaction or administered exogenously) on the establishment of dominance hierarchies has not been widely studied yet.

In humans, sociological studies have shown that less egalitarian societies, as expressed in the relative difference in socioeconomic gradient, have more health problems such as high morbidity and high mortality, obesity, mental illness and homicide than more egalitarian societies (Wilkinson, 1999; Sapolsky, 2005; Wilkinson and Pickett, 2006, 2007). In many hierarchical groups, for example a class of school children, individuals in higher status positions suffer less from malaise (Wilkinson, 1999; Ostberg, 2003), but in some species being in possession of the dominant rank can be more stressful, depending on factors such as the stability of the hierarchy and resource inequity (Sapolsky, 2005). In rats, being subordinate is more stressful than being dominant (de Jong et al., 2005; Arakawa, 2006; Hoshaw et al., 2006). The fact that in many cases lower ranked individuals suffer more from malaise might be due to a prolonged activation of the HPA axis. Lower ranked individuals show social anxiety, which leads to this excessive HPA axis activation (Wilkinson, 1999).

It is important to understand the mechanisms underlying the establishment of unequal dominance hierarchies, since this initial establishment can affect the relationship between two individuals and between these individuals and others for a long time after an initial encounter,

and could eventually even lead to the development of psychopathologies.

1.3 Brain regions involved in social behavior

Social recognition and social memory are important for the expression of social behavior. Social recognition is the ability to recognize other individuals according to several classes, for example parent-offspring recognition, mate recognition and dominant-subordinate hierarchies. Social memory is the ability to remember previously learned information about other individuals, such as their hierarchical status. The formation of a social memory is thus important in the establishment of hierarchies. When animals are not in contact with each other after a first encounter, the social memory that is formed is usually not long-lasting, in the range of minutes to hours (Ferguson et al., 2002).

Social memory is different from other types of memory -like spatial memory, for example- and involves a specific neural circuit. In this neural circuit, information is mainly received in the form of pheromones, chemicals that transmit information about an individual and that are detected by the accessory olfactory bulb (AOB). The AOB projects to the medial nucleus of the amygdala (MeA), which then projects to the bed nucleus of stria terminalis (BNST) and the lateral septum (LS) (see Fig. 2). The LS has an output to the hippocampus (De Vries and Buijs, 1983; Caffe et al., 1987; Canteras et al., 1995; Calderazzo et al., 1996; Ferguson et al., 2002; Bielsky and Young, 2004). The important role of the hippocampus in

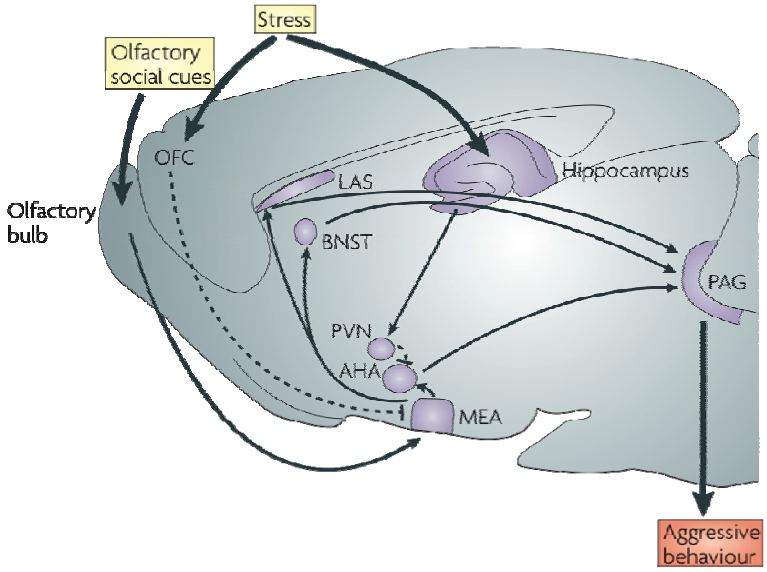

Figure 2. Olfactory information is processed in the olfactory bulb, which has an output to the medial amygdala (MEA). The medial amygdala has an output to both the lateral septum (LAS) and bed nucleus of stria terminalis (BNST). OFC = orbitofrontal cortex, AHA = anterior hypothalamic area, PVN = paraventricular nucleus, PAG = periaquaductal grey. *(Figure from Nelson and Trainor, 2007)*

learning and memory is well known; however, its involvement in social memory is not clear (Maaswinkel et al., 1996; Petrulis et al., 2000; Bannerman et al., 2001; Bannerman et al., 2002). Activation of MeA, BNST and LS was found after aggressive encounters in rats (Ferris et al., 2008, Martinez et al., 1998; Fekete et al., 2009), mice (Stork et al., 1997) and hamsters (Kollack-Walker and Newman, 1995; Kollack-Walker et al., 1997; Delville et al., 2000). In addition, exposure to the non-aggressive social investigation test increased c-fos activation in MeA, BNST and LS in mice (Ferguson et al., 2001). Since the MeA, BNST and LS are three brain regions that are involved in the regulation of social behavior (reviewed by Nelson and Trainor, 2007) and also play a role in the regulation of stress responses or are responsive to stress (Cullinan et al., 1993; Herman et al., 1994; Ebner et al., 2000; Neumann et al., 2000; Windle et al., 2004; Ebner et al., 2005; Choi et al., 2007; McElligott and Winder, 2009), they are the focus of the studies described in this thesis.

The MeA is the first of these three brain areas in the pathway that processes social information. The MeA shows c-fos activation after exposure to aggressive and non-aggressive social encounters in rats, mice and hamsters (Kollack-Walker and Newman 1995; Potegal et al., 1996; Kollack-Walker et al., 1997; Martinez et al., 1998; Delville et al., 2000; Ferris et al., 2008; Fekete et al., 2009). Lesions of the MeA lead to a reduction in social learning when the lesions are induced after an aggressive encounter (Vochteloo and Koolhaas, 1987) and to a reduction of conditioned avoidance of a dominant opponent after social defeat (Bolhuis et al., 1984; Luiten et al., 1985). In hamsters, inactivation of the MeA by administration of muscimol prevents the acquisition and expression of conditioned defeat (Markham and Huhman, 2008). This indicates that the MeA is essential for the formation of a memory for an opponent. Mice that are knockout for the neuropeptide oxytocin cannot form social memories, but this can be restored by specific infusions of oxytocin into the MeA (Ferguson et al., 2000; Ferguson et al., 2001). The MeA also plays a role in the stress response, since it can regulate HPA axis activity (Dayas et al., 1999; Mantella et al, 2004).

The LS has a regulatory function by integrating sensory stimuli and acting as a relay station to pass on information to brain areas that are involved in the execution of motivated behaviors, such as the hypothalamus. The LS has connections with brain areas that process affective information, such as the amygdala, the BNST and the hypothalamus, and with brain areas that process cognitive information, such as the hippocampus (reviewed by Sheehan et al., 2004). Plasticity of LS synapses is regulated by neuropeptides like AVP. The LS plays a role in the regulation of social behavior. Lesions of LS enhance aggression in rats, although they have no effect on intraspecific aggression (Albert and Chew, 1980; Albert and Walsh,

1982). Highly aggressive short attack latency (SAL) mice show a lower activation of septum than less aggressive low attack latency (LAL) mice, which might be related to the occurrence of violent attacks by SAL mice (Albert and Walsh, 1982; Haller et al., 2006). Subordinate hamsters show c-fos activation of LS after social defeat (Kollack-Walker et al., 1997). The LS is also involved in the regulation of the emotional response to social defeat stress, since administration of GR antagonists in LS prevents the usual increase in c-fos positive cells in hippocampus after social defeat (Calfa et al., 2007).

The BNST forms a relay station between the amygdala, the hippocampus and the prefrontal cortex on one side, and the PVN on the other side, and is involved in regulation of the HPA axis response to stress (Cullinan et al., 1993; Herman et al., 1994; Choi et al., 2007; McElligott and Winder, 2009). Stimulation of the BNST increases plasma corticosterone levels (Dunn, 1987), whereas lesions of BNST result in reduced CRH levels in the PVN and reduced ACTH and corticosterone plasma levels (Gray et al., 1993; Herman et al., 1994). At the behavioral level, BNST lesions enhance floating behavior in a forced swim test, suggesting that the BNST is involved in the modulation of coping behavior in response to stress (Schulz and Canbeyli, 2000). The BNST is also involved in the regulation of social behavior. Lesions of the BNST decrease aggression towards a male intruder in rats (Albert et al., 1989). The BNST is activated in subordinate hamsters after social defeat (Kollack-Walker et al., 1997). The role of the BNST in social behavior involves the neuropeptide AVP. Both positive (Bester-Meredith and Marler, 2001) and negative (Compaan et al., 1993; Everts et al., 1997; Veenema et al., 2010) correlations between AVP and aggression are found in BNST. Administration of AVP into the BNST inhibits aggression (Veenema et al., 2010).

1.4 Oxytocin and vasopressin

Oxytocin (OT) and AVP are two neuropeptides that are closely related and that are evolutionary conserved. Both neuropeptides play an important role in various social behaviors in many animal species. OT and AVP are nonapeptides with only two amino acids that differ between them (Cys-Tyr-**Ile**-Gln-Asn-Cys-Pro-**Leu**-GlyNH_2 for OT, and Cys-Tyr-**Phe**-Gln-Asn-Cys-Pro-**Arg**-GlyNH_2 for AVP, both with a sulphur bridge between the two cysteines, see Fig. 3). The neuropeptides are located on the same chromosome, probably due to gene duplication, with an intergene region of 3-12 kb in mouse, rat and human. AVP is oriented in the opposite transcriptional direction compared to OT. Both genes contain three exons and two introns. The exons code for the preprohormone that consists of a signal peptide, the nonapeptide and the first nine amino acid residues of the neurophysin hormone on exon 1, the

central part of neurophysin on exon 2 and the C-terminal part of neurophysin and a glycopeptide on exon 3. Neurophysin is a carrier protein that is transported in vesicles with OT or AVP and that plays a role in the targeting, packaging and storage of the neuropeptide before release into the blood stream (reviewed by Gimpl and Fahrenholz, 2001; Caldwell et al., 2008; Lee et al., 2009).

OT expression is regulated by estrogens and thyroid hormones through estrogen-response elements in the promoter of the OT gene (Richard and Zingg, 1990; Mohr and Schmitz, 1991), which also contains a cyclic AMP (cAMP) response element (CRE), suggesting that protein kinase A and C pathways play a role in OT gene expression too (Gimpl and Fahrenholz, 2001).

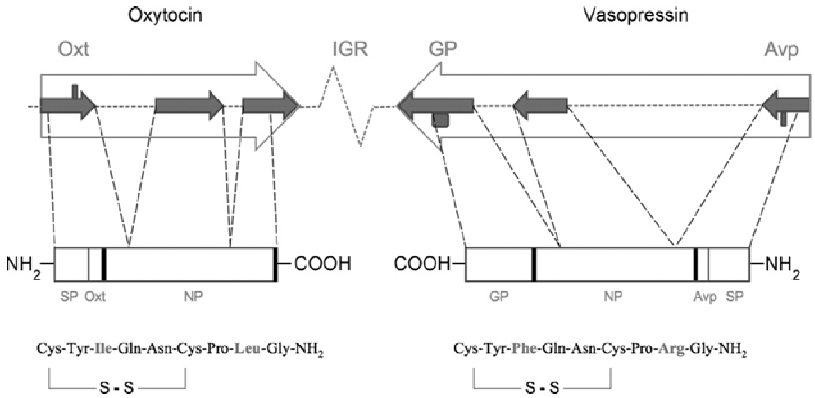

Figure 3. The nonapeptides oxytocin and vasopressin are located on the same chromosome with a small intergene region. OT and AVP only differ in two out of nine amino acids. The transcriptional direction of AVP is opposite to that of OT. (*Figure from Caldwell et al., 2008)*

Only one receptor type is known for OT, the OT receptor (OTR). OTRs belong to the class I G-protein coupled receptors that are coupled to phospholipase C through $G_{\alpha q11}$ and finally lead to the release of calcium, which can promote gene transcription and protein synthesis (Gimpl and Fahrenholz, 2001; Young and Gainer, 2003). Since OT can be released dendritically from neurons in the PVN and supraoptic nucleus (SON), the localization of OT release and OTR expression does not always match (Ludwig and Leng, 2006; Leng et al., 2008). OTR binding and mRNA levels in the rat brain are increased by estrogens and testosterone (Tribollet et al., 1990; Stevenson et al., 1994; Larcher et al., 1995; Breton and Zingg, 1997). In cell culture, OTRs are internalized within 5-10 minutes after stimulation with an OT agonist. OTRs are not recycled back to the cell surface (Gimpl and Fahrenholz, 2001).

For AVP, three receptor types have been found: the vasopressin receptor 1a (V1aR),

the vasopressin receptor 1b (V1bR) and the vasopressin receptor 2 (V2R). V1aR and V1bR are coupled to phospholipase C through $G_{\alpha q11}$, like the OTR. V2R is couple to G_s and acts through the cAMP system (Caldwell et al., 2008). In the current study, we will focus on the V1aR, since this receptor is expressed in the brain and has been involved in the regulation of social behavior.

OT and AVP are synthesized in the magnocellular neurons of the PVN and the SON of the hypothalamus. From here, the neuropeptides are transported to the posterior lobe of the pituitary gland (the neurohypophysis) and released as hormones into the blood stream where they exert peripheral effects, e.g. in milk ejection. Part of the OT and AVP that is synthesized in the PVN and SON is released centrally as neuropeptides through dendritic release. OT fiber endings have been found in various brain areas like MeA, LS, hippocampus, olfactory bulb, locus coeruleus and the raphe nucleus. Besides release from the hypothalamus, both OT and AVP are released locally from amygdala, BNST, septum, hippocampus and the olfactory bulb (Veinante and Freund-Mercier, 1997; Gimpl and Fahrenholz, 2001; Young and Gainer, 2003; Landgraf and Neumann, 2004). OTR and V1aR are expressed in MeA, BNST, LS and other brain areas (Kremarik, 1991; Yoshimura et al., 1993; Ostrowski et al., 1994; Veinante and Freund-Mercier, 1997; Ostrowski, 1998; Vaccari et al., 1998).

1.5 The role of OT and AVP in social behavior

OT and AVP and their receptors play an important role in the formation of social memory and are present in brain areas that are involved both in the formation of social memory and in the regulation of aggression (Gimpl and Fahrenholz, 2001). The roles of OT and AVP and their receptors in social behavior are largely conserved between species (Gimpl and Fahrenholz, 2001; Goodson and Bass, 2001).

Mice that are knockout for either OT or V1aR are not able to form a social memory (Ferguson et al., 2000; Ferguson et al., 2001; Bielsky et al., 2004). Administration of OT to OT knockout mice or re-expressing V1aR in mice lacking this receptor restores social memory (Ferguson et al., 2000; Bielsky et al., 2005). Substantial evidence indicates that administration of either OT or AVP facilitates the formation of social memories, whereas AVP antagonists prevent this (Dantzer et al., 1987; Le Moal et al., 1987; Popik and Van Ree, 1991; Engelmann and Landgraf, 1994; Engelmann et al., 1996; Popik et al., 1996; Landgraf et al., 2003). However, OT and AVP seem to have a different timing of expression and are thus likely to play a different role in the formation of social memories. Administration of OT to OT knockout mice is only effective in restoring social memory when this is done before a

social interaction (Ferguson et al., 2000; Ferguson et al., 2001), whereas re-expression of V1aR in V1aR knockout mice was shown to have an effect when this takes place after the social interaction (Bielsky et al., 2005). This suggests that OT may be critical for the encoding or initial processing of social memories, which probably occurs in the MeA, whereas AVP may be critical for the retention and recall of social memories, for which the LS and possibly also the hippocampus are important (Bielsky and Young, 2004).

1.6 The effect of stress on the establishment and maintenance of dominance hierarchies

As mentioned above, the contribution of stress to create social imbalance has been largely overlooked in studies of hierarchy formation. This occurs despite some suggestions that stress exposure can influence dominance-submissive relationships (Mikics et al., 2004). Since stress is a potent modulator of cognitive function and memory mechanisms (Sandi et al., 1997; Cordero et al., 1998; Cordero and Sandi, 1998; de Kloet et al., 1999; Roozendaal, 2002; Sandi, 2004), we hypothesized that stress can have important and long-lasting influences on social relationships by amplifying the memory for the social status that is established in a first encounter between conspecifics.

Stress and glucocorticoids affect aggressive and defensive behaviors. Treatment with glucocorticoids results in an increase in aggressive behavior of residents in a resident-intruder test (Haller et al., 1997; Mikics et al., 2004). Administration of the corticosterone synthesis inhibitor metyrapone leads to a decrease in aggressive behavior (Mikics et al., 2004). When a hierarchy has been established, corticosterone seems to have no effect on intra-colony aggression in both dominant and submissive rats (Mikics et al., 2007). This suggests that glucocorticoids have an effect particularly on the initial establishment of a dominance hierarchy.

1.7 Stress and its relation to OT and AVP

In rats, exposure to both emotional and physical stressors leads to OT and AVP release from the PVN and the SON of the hypothalamus (reviewed by Neumann, 2007). Exposure to social defeat increased OT release from the SON (Engelmann et al., 1999) and the LS (Ebner et al., 2000) and increased AVP release from the PVN (Wotjak et al., 1996). Forced swimming leads to an increase in OT and AVP release in the SON and the PVN (Wotjak et al., 1998).

OT on its turn affects the stress response. Intracerebral infusion of OT was found to attenuate the HPA axis response, as measured by the ACTH and corticosterone response to

stress and CRF mRNA expression in the PVN (Windle et al., 1997; Windle et al., 2004). Infusion of OT antagonists enhances ACTH levels in the blood (Neumann et al., 2000) and OT knockout mice show enhanced plasma corticosterone level (Mantella et al., 2004). Patchev et al. (1993) showed that glucocorticoids increase OT binding in the amygdala, LS and BNST. Increasing OT by intracerebral infusions leads to a more passive stress coping strategy and to inhibition of the HPA axis. This is thought to prevent an excessive stress response (Neumann et al., 2000; Windle et al., 2004; Ebner et al., 2005). V1aR binding in LS was also found to be regulated by glucocorticoids, with adrenalectomy decreasing V1aR binding and administration of dexamethasone restoring this effect (Watters et al., 1996). The hypothesis has been put forward that interactions between OT, AVP and glucocorticoids could provide a mechanism for dynamical changes in social behavior (Gimpl and Fahrenholz, 2001).

1.8 Monoamine oxidase A, serotonin, and social behavior

Monoamine oxidase A (MAOA) is a mitochondrial enzyme that is involved in the degradation of serotonin (5-HT), dopamine and noradrenaline. Mainly 5-HT has been shown to play a role in the regulation of aggression and to be related to the hierarchical status in several animal species (Kostowski et al., 1984; Blanchard et al., 1993; McKittrick et al., 1995; Ferris, 1996; Dhingra et al., 1997; Ferris et al., 1997; Larson and Summers, 2001).The activity of MAOA is related to aggression in animals and humans. MAOA knockout mice are more aggressive than their corresponding wild-type mice (Cases et al., 1995). In humans, several studies have related MAOA with increased aggression, including observations about (i) polymorphisms of the MAOA gene, with a low activity allele more expressed in highly aggressive individuals (McDermott et al., 2009); (ii) low activity of MAOA in depressed patients (Alia-Klein et al., 2008); and (iii) the lack of a functional MAOA gene linked to pathological aggression (Brunner et al., 1993).

1.9 Aim and goals of the study

The aim of the research presented in this thesis is to elucidate the neurobiological mechanisms underlying the effect of acute stress on the establishment and long-term maintenance of dominance hierarchies in adult male rats. Different aspects were studied in three sets of experiments.

The first question addressed was whether enhancing corticosterone could mimic the effects of acute stress on the establishment and maintenance of a dominance hierarchy. The

effect of increasing corticosterone levels around a first social encounter on the establishment of a hierarchy and the long-term memory for the established hierarchy was studied. One out of the two rats in a dyad was injected with corticosterone either just before (to evaluate effects on the establishment of a hierarchy and on the formation of a long-term memory) or immediately after (to test for effects on the long-term memory formation) a first encounter with an unfamiliar rat.

Next, we questioned whether OTR and V1aR play a role in the formation and long-term maintenance of a dominance hierarchy. The immediate and long-term changes in gene expression of OTR and V1aR were examined and antagonists of OTR and V1aR were used to mimic the effects of acute stress on hierarchy establishment.

Finally, we investigated the impact that exposure to acute stress and dominance hierarchy establishment could have on the formation of a second hierarchy with an unfamiliar opponent. Besides the behavior towards the unfamiliar opponent, we studied changes in gene expression levels of MAOA and the androgen receptor (AR) that could be involved in the neurobiological mechanisms underlying changes in behavior towards an unfamiliar opponent.

The following three chapters summarize the results that were found in the three sets of experiments. In addition, several experiments that were done at different phases of this study but not included in the former chapters are included in the Appendix. These experiments include the evaluation of (1) the effects of administration of the protein synthesis inhibitor anisomycin after the first encounter on memory formation, (2) the changes in *Otr* and *V1ar* mRNA levels in LS one week after administration of an OT antagonist in MeA, (3) a social preference test for a juvenile rat after exposure to acute stress, (4) the difference in offensive behavior during the first encounter between rats of non-stressed and stressed pairs, (5) mRNA levels of the glucocorticoid receptor *(Gr)* and neural cell adhesion molecule *(Ncam)* in MeA, LS and BNST, (6) mRNA levels of *Otr*, *V1ar*, *Ar*, *Maoa*, *Gr* and *Ncam* in hippocampus, (7) mRNA levels of *Otr* and *V1ar* in BNST and (8) plasma corticosterone levels at three hours and one week after the first encounter.

Chapter 2 - A role for glucocorticoids in the long-term establishment of a social hierarchy

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2.1 Abstract

Stress can affect the establishment and maintenance of social hierarchies. In the present study, we investigated the role of increasing corticosterone levels before or just after a first social encounter between two rats of a dyad in the establishment and the long-term maintenance of a social hierarchy. We show that pre-social encounter corticosterone treatment does not affect the outcome of the hierarchy during a first encounter, but induces a long-term memory for the hierarchy when the corticosterone-injected rat becomes dominant during the encounter, but not when it becomes subordinate. Post-social encounter corticosterone leads to a long-term maintenance of the hierarchy only when the subordinate rat of the dyad is injected with corticosterone. This corticosterone effect mimics previously reported actions of stress on the same model and, hence, implicates glucocorticoids in the consolidation of the memory for a recently established hierarchy.

Key words: corticosterone; social hierarchy; aggression; memory; subordinate; rat.

2.2 Introduction

Socially living animals establish dominance hierarchies, in which the dominant individual has priority access to limited resources such as water and food, space or females. Establishment of a hierarchy has the advantage of cutting down fighting within a group, eventually minimizing energy costs (Van Kreveld, 1970). In laboratory rats that are kept together in a colony, a hierarchy usually develops within a few days and is usually stable as long as the group keeps together (Blanchard et al., 1988).

In most cases, dominance hierarchies are the result of aggressive contests. We have recently shown that, in a dyadic contest between two male rats, stress experienced by one of the individuals just before their first encounter can determine the long-term establishment of a social hierarchy by influencing both the rank achieved during the social encounter and by facilitating a long-term memory for the achieved hierarchy (Cordero and Sandi, 2007). In non-stressed pairs, the social rank established during the first interaction is not maintained when animals are confronted one week later. The stress-induced potentiation of the hierarchy-linked recognition memory was blocked by injection of a protein synthesis inhibitor (Cordero and Sandi, 2007). Since glucocorticoids are main effectors of the activated stress system hypothalamus-pituitary-adrenocortical (HPA) axis and, due to their lipophilic nature, can readily enter the brain and affect neural function and cognition (de Kloet et al., 1999; de Kloet, 2000; de Kloet et al., 2005; Joels et al., 2006), we hypothesized that elevated glucocorticoid levels around the time of a first social encounter between two individuals could also affect the long-term establishment of social rank.

Support for this hypothesis comes from earlier work showing that both aggressive and defensive behaviors in rodents can be modulated by elevated corticosterone (Leshner et al., 1973; Leshner and Moyer, 1975; Leshner et al., 1975; Moyer and Leshner, 1976; Leshner and Politch, 1979; Haller et al., 1997; Haller et al., 2000a; Wood et al., 2003; Mikics et al., 2004). On its turn, inhibiting glucocorticoid function can, as well, have opposite modulatory actions in aggressive behaviors. Thus, treatment with the glucocorticoid synthesis inhibitor metyrapone or the mineralocorticoid receptor antagonist spironolactone was shown to decrease aggressive behaviors in resident-intruder tests (Haller et al., 2000b; Haller et al., 2004; Mikics et al., 2004), while glucocorticoid removal through adrenalectomy was found to lead to abnormal aggressive behavior (as indexed by increased attack/threat ratio) that could be restored with corticosterone injections (Haller et al., 2001). Importantly, once a hierarchy is established, corticosterone seems not to affect aggressive behavior in the colony, suggesting

that the initial establishment of a hierarchy –but not the stability of a well defined hierarchy- might be particularly sensitive to modulation by glucocorticoids (Mikics et al., 2007).

The long-term establishment of a social hierarchy involves at least the following two phases: (i) the determination of the social rank between the individuals involved in the specific social group, and (ii) the persistence of the acquired social hierarchy on encounters taking place at later time points. If we consider the phenomenon within the framework of memory, these two phases can be, respectively, considered equivalent to the 'acquisition' and 'consolidation' of newly acquired information (i.e., the new social rank). In this context, it is important to note that glucocorticoids have been extensively implicated in the modulation of memory in a variety of learning tasks, including spatial learning (Oitzl and de Kloet, 1992; Sandi et al., 1997; Oitzl et al., 2001; Akirav et al., 2004; Brinks et al., 2009a; Conboy and Sandi, 2009), inhibitory avoidance (Sandi and Rose, 1994; Roozendaal and McGaugh, 1996; Sandi and Rose, 1997; Roozendaal et al., 2002), fear conditioning (Cordero et al., 1998; Cordero and Sandi, 1998; Cordero et al., 2002), its reconsolidation (Tronel and Alberini, 2007) and extinction (Brinks et al., 2009b; Gourley et al., 2009), and object recognition in emotionally aroused, but not in non-aroused rats (Okuda et al., 2004; Roozendaal et al., 2006). This body of research has emphasized a role for glucocorticoids in the enhancement of memory consolidation of emotionally arousing experiences, while pointing at their negative effects on the retrieval of information or working memory (Roozendaal, 2000; Sandi and Pinelo-Nava, 2007; de Quervain et al., 2009).

Therefore, our aim here was to investigate the role of increasing corticosterone levels around the time of a first social interaction in the establishment and long-term maintenance of a dominance hierarchy. Using a rat model for the establishment of a social hierarchy that we recently developed (Cordero and Sandi, 2007), we studied the effects of injecting one, out of the two rats in a contest with corticosterone either just before (to evaluate effects on the establishment of a hierarchy and on the formation of a long-term memory) or immediately after (to test for effects on the long-term memory formation) a first encounter with an unfamiliar rat.

2.3 Materials and methods

Animals

Male Wistar rats (Charles River Laboratories, Lyon, France) weighing 250-275 grams at arrival were individually housed with *ad libitum* access to food and water. Animals were kept in a 12h light/dark cycle with lights on at 0700h, and a constant temperature of 22 ± 2 °C. Animals were left undisturbed for one week after arrival before starting the experiment. Rats were weighed once per week. Experiments were performed with approval of the Cantonal Veterinary Authorities (Vaud, Switzerland). Experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize animal suffering and to reduce the number of animals used.

All experiments took place between 0800h and 1400h, except for the water competition test that took place between 1400h and 1900h, after a water deprivation period of 6h (from 0800h until 1400h). All rats were handled for 2 min per day during three days before exposure to the elevated plus maze.

Elevated plus maze

Before the start of the experiment the 'elevated plus maze' (EPM) test was performed to measure anxiety-related behavior, in order to subsequently match animals in each dyad according to similar scores in this test (data not shown). The EPM consists of two opposing open arms (45x10 cm) and two opposing closed arms (45x10 cm with walls of 50 cm high) that extend from a central platform (10x10 cm), elevated 65 cm above the floor. The rats were placed individually on the central platform facing the same closed arm and were allowed to explore the EPM freely during 5 min. The behavior of each rat was video recorded and analyzed using a computerized tracking system (Ethovision 3.1.16, Noldus IT, the Netherlands). Time spent in the open and closed arms was measured.

Dyads and groups

Rats were distributed to dyads consisting of two rats that were matched for their body weight and levels of anxiety-related behavior (as indexed by the percent time spent in the open arms of the EPM). The rationale to pair animals for anxiety-related behavior is based on evidence indicating a link between anxiety trait and stress effects on social dominancy (Lucion and Vogel, 1994). Thus, the rats in each dyad were considered equal in their

probability to become dominant or subordinate during a first encounter. The fur of both rats in a dyad was marked on different body parts (i.e. neck and back) to help identifying the animals. Rats were habituated to marking during the three day habituation period that took place before the first social encounter. Dyads were divided randomly over the different groups. Each group consisted of 5-8 dyads.

Food and water competition test

In order to habituate animals to the rewarding food used in the 'food competition test' (FCT), they received eight Chocopop flakes (Kellogg's, Switzerland) in their homecages daily, during two days. The rats were habituated to the experimental setup for three days during which they were placed in a clean homecage for 20 min and in a food competition box for 10 min. The food competition box is a plastic box of 60x40 cm with walls of 32 cm high with a feeder containing eight Chocopops on one of the short sides. On day 1 of the experiment, the day after the last habituation day, the rats were submitted to a first encounter with an unfamiliar rat, consisting of a 'social interaction test' in a neutral homecage without food and water for 20 min, followed by a 'food competition test' (FCT) in which the rats had access to Chocopops during 10 min. After the tests each rat was returned to its homecage. The group without an encounter on day 1 was exposed to an extra day of habituation on that particular day.

The memory for the formed hierarchy was tested in a 'water competition test' (WCT) that was performed on day 8 after a preceding water deprivation period of 6 h for all animals included in the context. Rats were exposed to the same opponent as on day 1. The WCT was performed in a neutral homecage. After 2 min of habituation, a single bottle of water was presented and the behavior during the following 10 min was recorded. In addition, one experiment was performed to assess the rats' hierarchy on day 8 in a similar situation as in the first encounter (i.e., without former water deprivation and by testing their interactive behavior when exposed to a neutral, clean, homecage).

Behavior was video-recorded and scored blindly using The Observer (v.5.0.25, Noldus IT, the Netherlands). The duration and frequency of offensive and defensive behaviors was scored. Offensive behaviors were attacks (biting), keeping down (pushing the opponent to the floor), offensive upright (standing on the hind legs in upright position) and lateral threat (pushing or approaching the opponent showing its side with an arched back). Defensive behavior consisted of freezing (immobility), defensive upright (standing on the hind legs in response to offensive upright) and submissive posture (lying on the back). In the WCT the

time spent drinking was measured. The rat that showed most offensive behavior during each interaction was considered the dominant rat, the rat showing least offensive behavior and most defensive behavior the subordinate rat. Although, in our initial establishment of the model (Cordero and Sandi, 2007), the variable "percentage of passes over the feeder" in the FCT was validated as a good index to categorize animals' dominant–subordinate status, according to the scoring of animals' former behavior in the social interaction test, the results in this index were not informative when animals were exposed to different schemes of glucocorticoid manipulations in this study. Accordingly, instead of just presenting data based on the "percentage of passes over the feeder" index, the social hierarchy resulting from interactions on the first day was established according to the totality of behaviors scored throughout the whole 'social interaction test'.

Drugs

Corticosterone was injected IP as corticosterone-HBC (2-hydroxypropyl-β-cyclodextrin) complex (Sigma Chemical Co., Switzerland) at a dose of 5 mg/kg. This dose was selected because it was previously shown to mimic plasma steroid concentrations produced by substantial stress (Venero et al., 1996). The corticosterone-HBC complex was dissolved in saline. Saline was used as the vehicle. Rats were injected with corticosterone or vehicle either 5 min before the start of the first encounter or the extra habituation period on day 1, or immediately after the end of the FCT or the extra habituation period. The pre-social encounter corticosterone injections were given randomly to one rat of the dyad. For the post-social encounter injections either the dominant or the subordinate rat received the corticosterone injection.

Figure 1 gives an overview of the injection time for the different groups.

Statistics

The percentage of offensive behaviors within each dyad was calculated as the ratio between contestants' scoring in each confronted pair on the total of offensive behaviors scored across (i) the social interaction test (day 1) and (ii) the WCT (day 8). All results are expressed as the mean ± the standard error of the mean (S.E.M.). A paired two-tailed t-test was used to compare the percentage of offensive behaviors within a dyad of rats competing together. $P<0.05$ was considered significant.

The computer software SPSS for Windows (version 13.0) was used for statistical analysis.

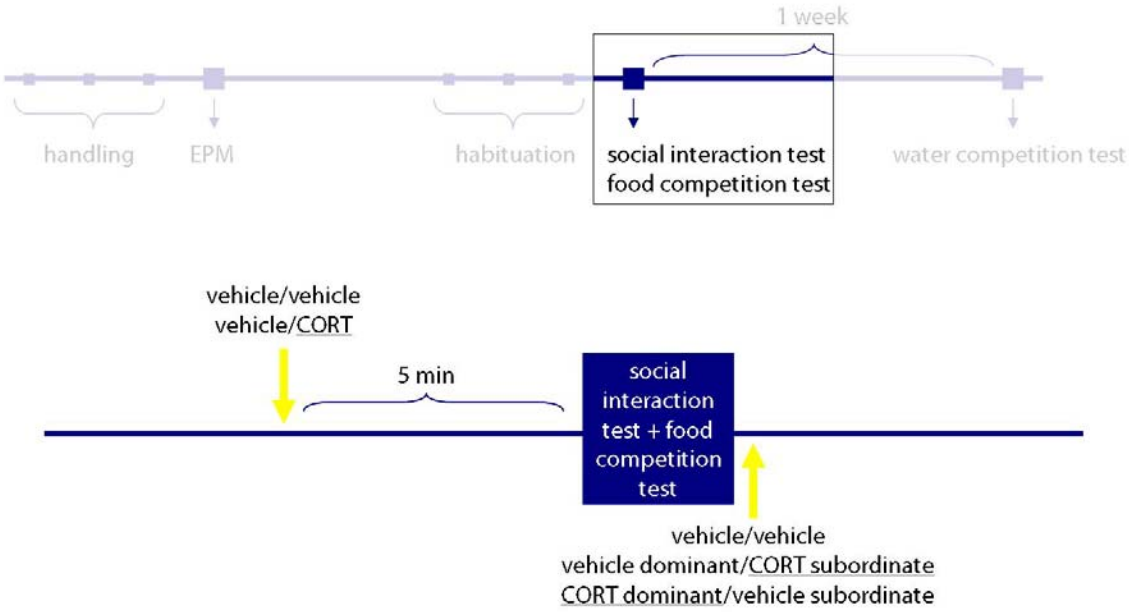

Figure 1. Overview of the experimental design. On the day of the first encounter the rats are injected with vehicle or corticosterone either 5 minutes before the start of a first encounter, or immediately after the first encounter. 'CORT' = corticosterone

2.4 Results

The effect of vehicle injections around a social encounter on the long-term maintenance of a social hierarchy

We have previously shown that, under the experimental conditions used in the current study, the social rank established by control pairs of male rats (not stressed or injected) through a first interaction and a food competition test on the first day of an encounter, is not maintained when the same animals are confronted 1 week later (Cordero and Sandi, 2007). Since the experimental design of the current study required to inject each animal of the dyad with either corticosterone or vehicle and either pre- or post-social encounter, we performed a first experiment to assess whether the injection procedure, by itself, could affect the long-term establishment of the hierarchy. As expected, vehicle-treated pairs formed a dominance hierarchy during the first encounter in which the dominant rat shows a higher percentage of offensive behaviors than the subordinate rat ($t = 3.646$, $df = 5$, $p<0.05$), but showed no maintenance of the hierarchy when evaluated 1 week later through the WCT: in some cases, the same hierarchy was found, in others the opposite hierarchy, leading on average to both animals in the pairs (former dominant and subordinate) showing similar levels of offensive behavior (n.s.; Fig. 2).

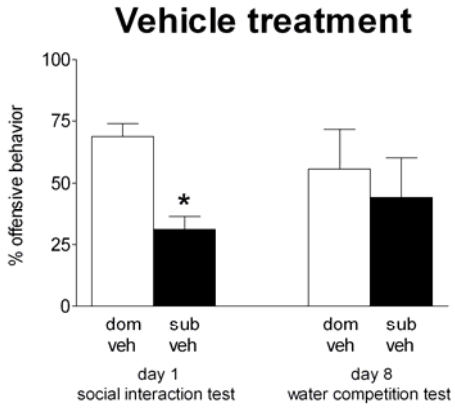

Figure 2. The percentage of offensive behaviors (mean ± S.E.M.) between two opponents shown in the social interaction test on day 1 and the water competition test on day 8 in the vehicle-treated group (n=6). The status 'dom' (dominant) or 'sub' (subordinate) refers to the status that was obtained during the social interaction test on day 1. 'veh' = vehicle. * $p<0.05$.

The effects of corticosterone treatment before a social encounter on the establishment and long-term maintenance of a social hierarchy

Pre-social encounter corticosterone treatment did not affect the establishment of the hierarchy during a first social encounter (Fig. 3A): five out of 12 rats with pre-social encounter corticosterone injections became dominant and seven out of 12 rats became subordinate (n.s.). Similarly, no evidence for an effect of corticosterone was observed when the same animals were further tested for their dominance hierarchy 7 days after the first encounter through the water competition test (n.s.; Fig 3A). Since some of the corticosterone-injected animals became dominant and others subordinate in the first interaction, we reasoned that corticosterone might have influenced the long-term establishment of the particular hierarchy established. Thus, we divided the results from this experiment into two categories depending on whether the pre-social encounter corticosterone-injected rat became (i) dominant or (ii) subordinate, and then analyzed the impact of each condition on the establishment of the long-term memory for the hierarchy. In the condition in which pre-social encounter corticosterone-injected rats became dominant (Fig. 3B), a hierarchy, in terms of differences in the percent of offensive behavior presented by both rats of the pair, was formed on day 1 ($t = -3.709$, 4 d.f., $p<0.05$). When evaluated 7 days afterwards through the WCT, the hierarchy was maintained ($t = -5.150$, $df = 4$, $p<0.01$). In the condition in which pre-social

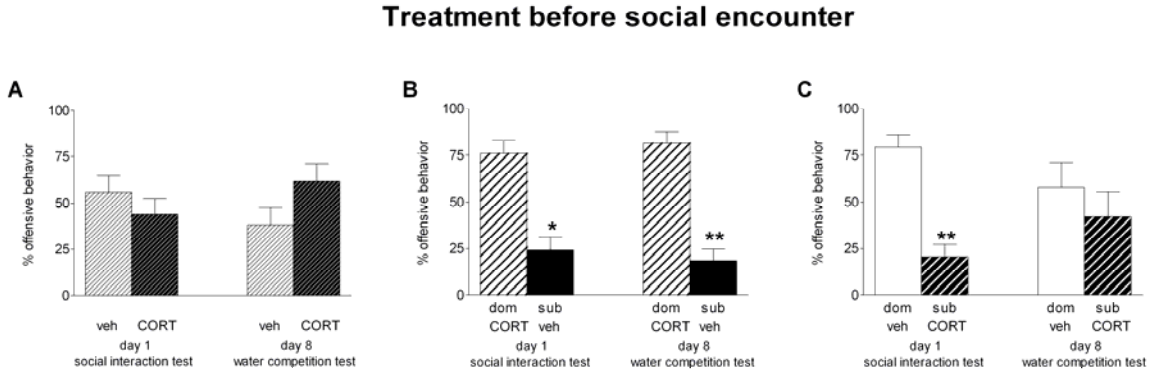

Figure 3. The percentage of total offensive behavior (mean ± S.E.M.) between two opponents shown in the social interaction test on day 1 and the water competition test on day 8 in the pre-social encounter injection groups. (A) The hierarchy of rats injected with vehicle or corticosterone before the first encounter (n=12). (B) Rats that became dominant after pre-social encounter corticosterone and their vehicle-treated subordinate opponents (n=5). (C) Rats that became subordinate after pre-social encounter corticosterone and their vehicle-treated dominant opponents (n=7). The status 'dom' (dominant) or 'sub' (subordinate) refers to the status that was obtained during the social interaction test on day 1. 'veh' = vehicle, 'CORT' = corticosterone. * $p<0.05$, ** $p<0.01$.

encounter corticosterone-injected rats became subordinate (Fig. 3C), a hierarchy was also evident during the first encounter ($t = 4.442$, $df = 6$, $p<0.01$), but no memory for the hierarchy was found in the WCT (n.s.).

The effect of corticosterone treatment after a social encounter on the long-term maintenance of a social hierarchy

We then investigated whether increasing corticosterone levels after a first social encounter in one of the two confronted individuals –either the dominant or the subordinate of the recently established hierarchy- would affect the long-term establishment of the social hierarchy. Thus, pairs of males were submitted to the first social encounter and one of them, half of the times either the dominant or the subordinate animal, was injected with corticosterone and the other with vehicle. When the dominant rat of the pair (first social encounter, $t = 3.363$, $df = 6$, $p<0.05$) received a corticosterone injection, no memory for the hierarchy was found on the WCT performed 7 days afterwards (n.s.; Fig. 4A). However, when the subordinate rat ($t = 5.342$, $df = 6$, $p<0.01$) was the one injected with corticosterone, evidence for the establishment of a long-term memory was observed, with corticosterone-injected rats behaving again as subordinates in the WCT ($t = 5.744$, $df = 6$, $p<0.01$, Fig. 4B).

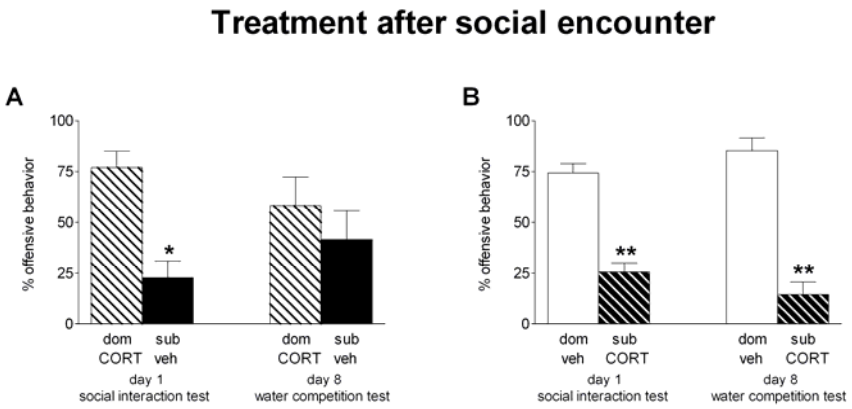

Figure 4. The percentage of total offensive behavior (mean ± S.E.M.) between two opponents shown in the social interaction test on day 1 and the water competition test on day 8 in the post-social encounter injection groups. (A) Post-social encounter corticosterone for dominant rat, post-social encounter vehicle for subordinate rat (n=7). (B) Post-social encounter vehicle for dominant rat, post-social encounter corticosterone for subordinate rat (n=7). The status 'dom' (dominant) or 'sub' (subordinate) refers to the status that was obtained during the social interaction test on day 1. 'veh' = vehicle, 'CORT' = corticosterone. * $p<0.05$, ** $p<0.01$.

The effect of corticosterone injections without exposure to a social encounter on the long-term maintenance of a social hierarchy

The results obtained under some of the conditions examined above imply that corticosterone injections around the time of the first encounter have an impact on the long-term maintenance of the hierarchy established on the day of the injection, as evaluated 1 week after the injection/encounter. However, the alternative possibility that corticosterone by itself -regardless of the associated social experience– has a long-term effect on the expression of the social hierarchy cannot be discarded. Therefore, we set an experiment to specifically test whether a corticosterone injection, given to one of the animals in a pair (while the other received a vehicle injection) 1 week before their first encounter would affect the emerging hierarchy at that later time point. As shown in Figure 5, administration of corticosterone without an associated social encounter on day 1 had no long-term effect on the hierarchy that was established during the WCT one week later (n.s.).

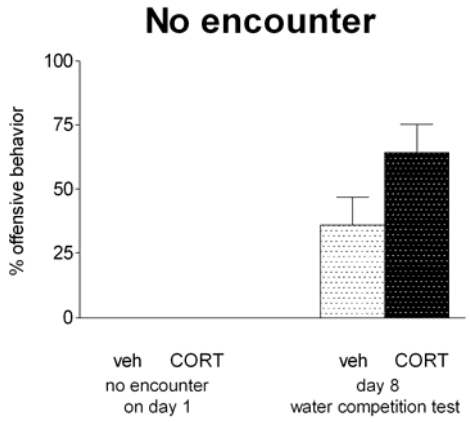

Figure 5. The percentage of total offensive behavior (mean ± S.E.M.) between two opponents shown in the water competition test on day 8 in the group that was not submitted to a social encounter on day 1, but only to injection of vehicle or corticosterone (n=7). 'veh' = vehicle, 'CORT' = corticosterone.

The effect of corticosterone injections administered after a first social encounter on the long-term maintenance of the acquired hierarchy as evaluated through a social interaction test

As shown in Figure 4B, administering corticosterone immediately after the first social encounter to the animal that had just become subordinate resulted in a long-term potentiation of the hierarchy, as evaluated on day 8. This is a particularly relevant finding in this study since it resembles previously reported effects of stress leading to a subordinate status and facilitating the long-term establishment of the social hierarchy (Cordero and Sandi, 2007). However, since the WCT delivered on day 8 differed from the initial social interaction test, it

could be argued that the novelty aspects (i.e., water deprivation for 6 h with the potential stress associated; novel situation) might have interfered with the expression of the hierarchy in the controls, the corticosterone-treated animals, or in both cases. To check for this possibility, we performed an additional experiment in which in one group both rats were injected with vehicle after the encounter, while in the other group the subordinate rat was injected post-encounter with corticosterone and the dominant rat with vehicle. Both the vehicle-injected group and the group with post-encounter corticosterone for the subordinate rat showed a clear hierarchy on day 1 ($t = 2.949$, $df = 7$, $p<0.05$ for the vehicle group, Fig. 6A; $t = 5.499$, $df = 7$, $p<0.001$ for the post-encounter corticosterone group, Fig. 6B). On day 8, the rats were submitted to a second social encounter test, without being submitted to any preceding water deprivation. The post-encounter vehicle-treated group showed no memory for the hierarchy (n.s.; Fig. 6A). The post-encounter corticosterone-treated group showed the same hierarchy as on day 1, with the corticosterone-injected subordinate rat displaying less offensive behavior than the vehicle-injected dominant rat ($t = 3.275$, $df = 7$, $p<0.05$, Fig. 6B).

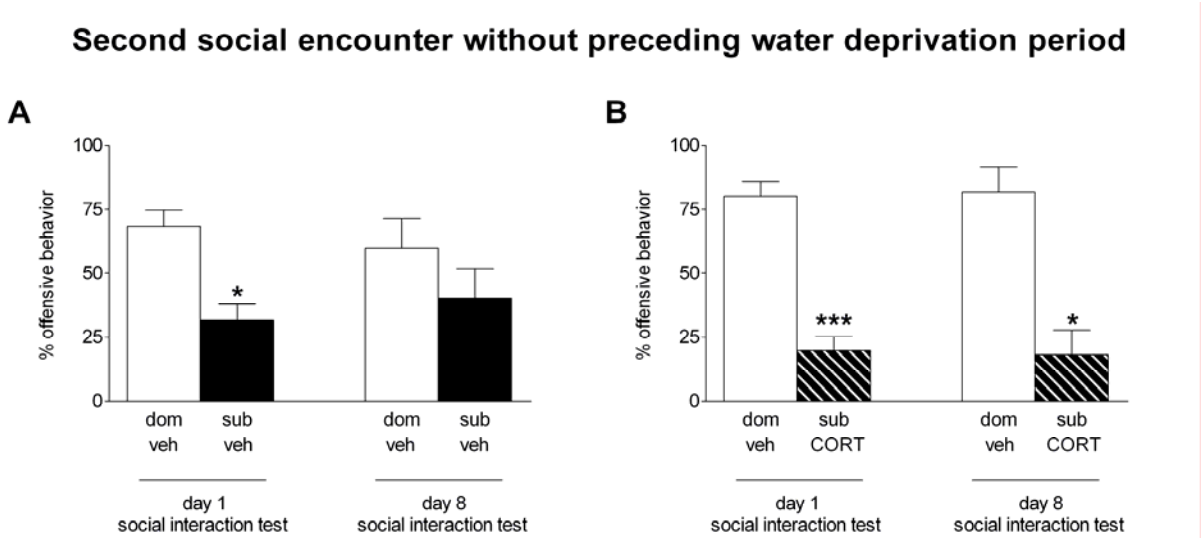

Figure 6. The percentage of total offensive behavior (mean ± S.E.M.) between two opponents shown in the first social interaction test on day 1 and the second social interaction test on day 8 without a preceding water deprivation period. (A) Rats that were treated with post-encounter vehicle injections (n=8). (B) Post-social encounter vehicle for the dominant rat, post-social encounter corticosterone for the subordinate rat (n=8). The status 'dom' (dominant) or 'sub' (subordinate) refers to the status that was obtained during the social interaction test on day 1. 'veh' = vehicle, 'CORT' = corticosterone. * $p<0.05$, *** $p<0.001$.

Analyses of the nature of the social interactions maintained during the first encounter in animals submitted to either pre- or post-social interaction treatments

Since corticosterone injections given before the first social encounter had an impact on the long-term establishment of the social hierarchy only when given to animals that became dominant, but not when applied to those that became subordinate, we hypothetized that the corticosterone treatment might have affected differently the social interaction in each of those two conditions. Therefore, we analyzed the nature of the social interactions maintained by the animals in those pairs and conditions in terms of the percent time spent in the characteristic offensive behaviors, as well as the interactions maintained by non-injected animals (i.e., those that among the whole study received post-social encounter injections, either saline or corticosterone). As can be observed in Figure 7, the nature of the social interactions maintained between the pairs in which corticosterone was injected before that particular encounter when the corticosterone-injected animal became dominant (Fig. 7A) was markedly different than the pattern of interaction observed under conditions in which the corticosterone-injected male became subordinate (Fig. 7B) or in which none of the animals of the pair had yet being injected (Fig. 7C). In both cases, when the corticosterone-treated rats became subordinate (Fig. 7B) or had not been injected (Fig. 7C), the subordinate rats displayed significantly lower lateral threats (corticosterone-injected: $t = 3.021$, $df = 6$, $p<0.05$; not-injected: $t = 5.696$, $df = 32$, $p<0.001$) and keeping down behaviors (corticosterone-injected: $t = 4.490$, $df = 6$, $p<0.01$; not-injected: $t = 4.978$, $df = 32$, $p<0.001$) than their dominant opponents, while the differences in attacks and offensive upright postures while observed in both conditions only reached significance for the untreated rats ($t = 4.268$, $df = 32$, $p<0.001$ for attacks and $t = 5.068$, $df = 32$, $p<0.001$ for offensive upright). On the contrary, when the corticosterone-treated rat became dominant, the interaction between the two animals in the pair did not differ in terms of attacks (n.s.) , lateral threats (n.s.) or offensive upright postures (n.s.). In this case, it was specifically the keeping down behavior what defined that the corticosterone-treated animal became dominant in the encounter ($t = 3.746$, $df = 4$, $p<0.05$, Fig. 7A).

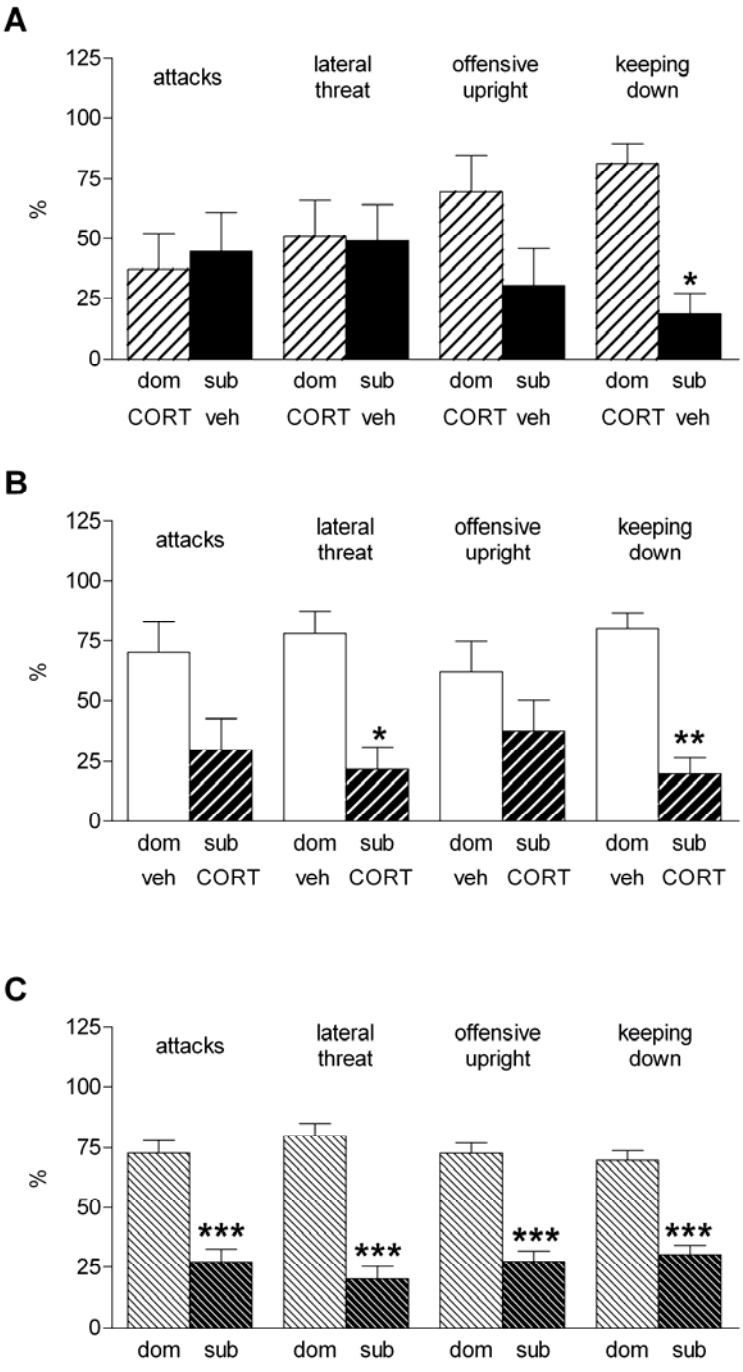

Figure 7. The percentage of the different offensive behaviors (attacks, lateral threat, offensive upright, keeping down; mean ± S.E.M.) between two opponents shown in the social interaction test on day 1. (A) The percentage of the different offensive behaviors in the pre-social encounter corticosterone group where the corticosterone-treated rat became dominant (n=5). (B) The percentage of the different offensive behaviors in the pre-social encounter corticosterone group where the corticosterone-treated rat became subordinate (n=7). (C) The percentage of the different offensive behaviors in the groups that received post-social encounter injections (vehicle-treated group and corticosterone-treated group, n=33). 'dom' = dominant, 'sub' = subordinate, 'veh' = vehicle, 'CORT' = corticosterone. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

2.5 Discussion

We have recently developed a model for the assessment of a long-term hierarchy between a pair of rats that, on their first encounter, have equal opportunities to become either dominant of subordinate. Under control conditions, the social rank established through a social interaction and food competition test on the first day, is not maintained when the same animals are confronted 1 week later. However, if one of the rats is stressed just before their first encounter, the dominance hierarchy developed on day 1 is still clearly observed one week later, with the stressed animal becoming subordinate in both social interactions. The potentiation of memory for the hierarchy by stress during the first encounter was shown to be blocked by injection of a protein synthesis inhibitor (Cordero and Sandi, 2007).

The stress hormone corticosterone has been implicated in the modulation of aggressive behavior and on the modulation of memory (see 2.1 Introduction). Thus, in this study, we evaluated whether enhancing corticosterone levels in one of the rats in each dyad either before or immediately after the first social confrontation would facilitate the maintenance of the hierarchy order established on that initial encounter when examined through a water competition test one week later. Since corticosterone treatments were given to either the dominant or the subordinate animal in each contest, we also evaluated whether corticosterone effects might depend on the social rank of the treated animal. Our results show that while enhancing corticosterone levels before the first social encounter does not determine the social order that is about to be established, such treatment can promote the long-term maintenance of the resulting hierarchy when the animal that becomes dominant is the one who had been injected with the hormone. Importantly, when the enhancement of corticosterone occurs after resolution of the contest, this treatment is effective to promote the long-term establishment of the hierarchy when received by the rat that had just become subordinate, but not by the dominant. Altogether, these results support a complex, but important role of corticosterone in the long-term establishment of social hierarchies.

First, we performed a control experiment to discard potential unspecific effects of the injection procedure, by itself, on the establishment and/or maintenance of the social hierarchy. By applying vehicle injections to each rat in the dyad either before or after the first social encounter, we confirmed that, as previously observed in untreated animals (Cordero and Sandi, 2007), the emergence of a social hierarchy was clear on day 1 (in terms of offensive behavior respectively displayed by animals that developed each social status) but it was not maintained when the same individuals were confronted one week later.

When examining the role of increased corticosterone levels in the establishment of an enduring hierarchy, the clearest results were those obtained when the hormone was applied after the social contest. Enhancing corticosterone levels after a first social encounter facilitated the transfer of the established social hierarchy to a future contest only when the hormone was given to the animal that has just become subordinate. No long-term impact was observed when the dominant animal was the one that received the treatment. The specificity of this effect to the linkage between corticosterone treatment and the emerging subordinate status was further confirmed by an experiment in which injecting corticosterone to one of the animals in each dyad (without any associated social experience on the same day) had no effect on the outcome of a water competition test given one week later. Moreover, it could be argued that stress associated with the water deprivation procedure (6 hours) required for the water competition test on day 8 might affect the internal milieu of the animals and consequently acting on state-dependent mechanisms that could, in turn, interfere with the expression of the formerly acquired hierarchy in controls and/or facilitate its expression by mimicking internal conditions of day 1 in formerly corticosterone-treated rats. However, results from a further experiment including a social interaction test on day 8 without former water deprivation did not support the state-dependent hypothesis. Instead, these results further confirmed that the potentiation of hierarchy expression by enhancing corticosterone levels in the subordinate animal after the social encounter is a robust phenomenon not only circumscribed to testing conditions involving acute stress.

This facilitation of the long-term expression of the hierarchy when subordinate rats receive corticosterone post-encounter is reminiscent of the effect induced by stressing one rat before the first social encounter; i.e., the stressed rat becomes subordinate and this manipulation leads to a long-term expression of the established hierarchy (Cordero and Sandi, 2007). Our current findings suggest that reinforcing the subordinate rat on its subordinate state is a sufficient condition to maintain the initially established hierarchy in future encounters. They also fit with the idea that, once a hierarchical relationship is established, the subordinate animal will play a central role in the maintenance of the hierarchy by showing submissive behavior presumably to avoid repeated physical exchanges with the dominant conspecifics (Summers et al., 2005). The fact that glucocorticoid effects were linked to keeping memory of a defeat, rather than of a victory, seems also congruent with the evolutionary reflection that remembering conditions leading to loss is particularly adaptive. Such an effect of glucocorticoids is in line with a wide body of evidence implicating post-training glucocorticoids in the consolidation of memories for a variety of learning types

(Oitzl and de Kloet, 1992; Sandi and Rose, 1994; Roozendaal and McGaugh, 1996; Sandi et al., 1997; Sandi and Rose, 1997; Cordero et al., 1998; Cordero and Sandi, 1998; Oitzl et al., 2001; Cordero et al., 2002; Roozendaal et al., 2002; Akirav et al., 2004; Okuda et al., 2004; Roozendaal et al., 2006; Tronel and Alberini, 2007; Brinks et al., 2009a; Brinks et al., 2009b; Conboy and Sandi, 2009; Gourley et al., 2009). Particularly relevant in this context are those studies in which a long-term memory was potentiated by injecting corticosterone immediately after training, including spatial learning acquired at a moderately stressful temperature (Sandi et al., 1997; Conboy and Sandi, 2009), fear conditioning involving low intensity shocks (Cordero and Sandi, 1998), inhibitory avoidance elicited through mild stressors (Sandi et al., 1995; Venero and Sandi, 1997), and object recognition (Okuda et al., 2004; Roozendaal et al., 2006). Interestingly, the corticosterone-induced facilitation of object recognition memory –a task which is clearly less stressful than the former three learning challenges- was only evident when corticosterone was administered to emotionally aroused, but not to non-aroused rats (Okuda et al. 2004; Roozendaal et al. 2006). It is tempting to compare these results with the differential effects of post-social encounter corticosterone injections found in our study for subordinate and dominant animals. Genomic effects have previously been described for delayed effects of both stress (Cordero and Sandi, 2007) and glucocorticoids (Mikics et al., 2004) in social interactions.

When corticosterone injections were given before the first social encounter, the treatment had no effect on the immediate establishment of the dominance hierarchy (about half of the injected rats became dominant and the other half subordinate), and it only led to the formation of a long-term memory for the hierarchy when the corticosterone-treated rat became the dominant rat of a dyad. Thus, the impact of this pre-social encounter hormonal manipulation greatly differs from our published model in which prior stress forces the resulting hierarchy, with the stressed male turning into the subordinate status in both the immediate and the delayed tests (Cordero and Sandi, 2007). This might be surprising according to earlier studies in diverse species reporting a link between increased glucocorticoid levels and increased aggressiveness (Brain and Evans, 1977; Heller, 1978; Hayden-Hixson and Ferris, 1991a, 1991b; Haller et al., 1997). However, a close examination of the literature reveals the existence of several studies in which increasing corticosterone levels was found to increase submissive behaviors (Leshner and Moyer, 1975; Leshner et al., 1975; Moyer and Leshner, 1976; Leshner and Politch, 1979; Leshner et al., 1980). The explanation for this apparent discrepancy seems to be explained by the fact that the effects of corticosterone treatment are context-dependent: the experimental conditions used in the

studies in which corticosterone increased aggressiveness elicited, by themselves, aggressive responses (i.e., confrontation with rather passive opponents); conversely, those conditions in which corticosterone increased submissiveness elicited, by themselves, aggressive responses (i.e., confrontation with rather aggressive opponents). These findings led to the hypothesis that corticosterone would act by increasing animals' arousal (Leshner et al., 1980) and, hence, its capability to recognizing the type of opponent it is exposed to. As a result, when exposed to an aggressive opponent, the increase in corticosterone would lead to an increase in submissive behavior. When exposed to a passive opponent, the increase in corticosterone would lead to an increase in aggressive behavior. In our experimental conditions, animals are matched for their body weight and anxiety levels with the goal of confronting two animals with equal opportunities to become either dominant or subordinate. Therefore, our findings that about 50% of the corticosterone-injected animals becomes dominant and the other 50% subordinate in this first encounter fit with the idea that enhanced corticosterone levels would facilitate the naturally emerging social responses. In this connection, Haller et al. (1997) have also proposed an interesting concept of context-specificity for corticosterone actions in aggressive behavior related to the prior history of social encounters of the treated animal.

These results also indicate 'immediate' effects of increasing corticosterone levels in social behaviors and fit with previously described rapid effects of glucocorticoids in behavior (Sandi et al., 1996a, 1996b; Mikics et al., 2005). In particular, they support a growing body of evidence indicating that social behaviors are susceptible to be influenced by rapid, non-genomic effects of increasing glucocorticoid levels (Haller et al., 1997; Makara and Haller, 2001; Mikics et al., 2004).

The fact that pre-social encounter corticosterone injections promoted a long-term hierarchy for the condition in which the corticosterone-treated was the dominant, rather than the subordinate, rat is somehow an unexpected finding. In both stress pre-social encounter (Cordero and Sandi, 2007) and corticosterone post-social encounter conditions (this study), long-term expression of the hierarchy is evident when the steroid-treated rat was the subordinate. Evaluation of the interaction profile in terms of the separate offensive behaviors displayed by each of the rats in the dyad (Fig. 7A) reveals a very different pattern to the one observed in control animals (Fig. 7C) or in the dyad in which the injected rat became subordinate (Fig. 7B). In contrast to control conditions, no differences were found in offensive behaviors such as attacks or lateral threats in the dyad in which the corticosterone-treated rat became dominant, with the dominance hierarchy being defined in this case by a very strong difference in keeping down behaviors. Although our data does not allow

excluding a direct effect of corticosterone in the rat that became dominant, it is tempting to speculate that this difference in the nature of the interaction might have provoked an alteration in the stress reactivity and/or perception of the interaction in the animal that became subordinate, eventually reinforcing the establishment of a long-term memory for the emerging social hierarchy. Since the consolidation of the endurance of the social hierarchy by stress was blocked by a protein synthesis inhibitor (Cordero and Sandi, 2007), one way to investigate whether a particular individual in the dyad keeps a long-term memory when corticosterone is given before the first social encounter could be by injecting a protein synthesis inhibitor to either the steroid- or the vehicle-treated rat. In any case, the open question still remains as to why pre-encounter corticosterone treatment in animals that became subordinate would not have as well facilitated a long-term establishment of the hierarchy, as one would have expected from the reported effects of stress (Cordero and Sandi, 2007).

It is important to note that the current findings refer to experimental conditions in which glucocorticoids are enhanced acutely. Chronic elevation of glucocorticoids have been shown to generally inhibit aggression (Summers et al., 2005), while chronic glucocorticoid deficiency to promoting abnormal, even pathological, aggression in rats (Haller et al., 2001; Haller et al., 2004; Haller and Kruk, 2006). Furthermore, it is important to consider that besides its direct effect, acute treatment with corticosterone can also have an effect on other parts of the HPA-axis through the negative feedback mechanism, whose potential effects on social behavior and long-term memory cannot be discarded.

In summary, the main finding of this study is that enhancing glucocorticoid levels in the rat that has just emerged as subordinate in a dyadic encounter facilitates the long-term establishment of the resulting hierarchy that –under control conditions- would not be kept, mimicking the effect of stressing one rat in the dyad just before the social confrontation (Cordero and Sandi, 2007). These findings support a role for glucocorticoids on the consolidation of memories for a recently established hierarchy and, hence, on the induction of neuroadaptive changes that mediate the “loser” effects. As opposed to the rapid effects of the steroid discussed above, genomic effects are expected to mediate these long-lasting changes. This study provides an ideal model to investigate the mechanisms whereby glucocorticoids potentiate the long-term establishment of emerging social hierarchies.

2.6 Acknowledgements

This work was supported by grants from the EU 7th (FP7- HEALTH-F2M-2007-201600; MemStick) FP and from the Swiss National Science Foundation (310000-120791) to C.S. The authors wish to thank to Maria Isabel Cordero, Mark Fajans, Grégoire Parchet and Angélique Vaucher for technical assistance.

2.7 References

Akirav I., Kozenicky M., Tal D., Sandi C. Venero C., Richter-Levin G. (2004). A facilitative role for corticosterone in the acquisition of a spatial task under moderate stress. Learn Mem 11:188-195.

Blanchard R.J., Flannelly K.J., Blanchard D.C. (1988). Life-span studies of dominance and aggression in established colonies of laboratory rats. Physiol Behav 43:1-7.

Brain P.F., Evans A.E. (1977). Acute influences of some ACTH-related peptides on fighting and adrenocortical activity in male laboratory mice. Pharmacology, biochemistry, and behavior 7:425-433.

Brinks V., Berger S., Gass P., de Kloet E.R., Oitzl M.S. (2009a). Mineralocorticoid receptors in control of emotional arousal and fear memory. Horm Behav 56:232-238.

Brinks V., de Kloet E.R., Oitzl M.S.. (2009b). Corticosterone facilitates extinction of fear memory in BALB/c mice but strengthens cue related fear in C57BL/6 mice. Exp Neurol 216:375-382.

Conboy L., Sandi C. (2009). Stress at learning facilitates memory formation by regulating AMPA receptor trafficking through a glucocorticoid action. Neuropsychopharmacology.

Cordero M.I., Kruyt N.D., Merino J.J., Sandi C. (2002). Glucocorticoid involvement in memory formation in a rat model for traumatic memory. Stress 5:73-79.

Cordero M.I., Merino J.J., Sandi C. (1998). Correlational relationship between shock intensity and corticosterone secretion on the establishment and subsequent expression of contextual fear conditioning. Behav Neurosci 112:885-891.

Cordero M.I., Sandi C. (1998). A role for brain glucocorticoid receptors in contextual fear conditioning: dependence upon training intensity. Brain Res 786:11-17.

Cordero M.I., Sandi C. (2007). Stress amplifies memory for social hierarchy. Frontiers in neuroscience 1:175-184.

de Kloet E.R. (2000). Stress in the brain. Eur J Neurosci 405:187-198.

de Kloet E.R., Joels M., Holsboer F. (2005). Stress and the brain: from adaptation to disease. Nat Rev Neurosci 6:463-475.

de Kloet E.R., Oitzl M.S., Joels M. (1999). Stress and cognition: are corticosteroids good or bad guys? Trends in Neurosci 22:422-426.

de Quervain D.J., Aerni A., Schelling G., Roozendaal B. (2009). Glucocorticoids and the regulation of memory in health and disease. Front Neuroendocrinol 30:358-370.

Gourley S.L., Kedves A.T., Olausson P., Taylor J.R. (2009). A history of corticosterone exposure regulates fear extinction and cortical NR2B, GluR2/3, and BDNF. Neuropsychopharmacology 34:707-716.

Haller J., Albert I., Makara G.B. (1997). Acute behavioural effects of corticosterone lack specificity but show marked context-dependency. J Neuroendocrinol 9:515-518.

Haller J., Halasz J., Mikics E., Kruk M.R. (2004). Chronic glucocorticoid deficiency-induced abnormal aggression, autonomic hypoarousal, and social deficit in rats. J Neuroendocrinol 16:550-557.

Haller J., Halasz J., Mikics E., Kruk M.R., Makara G.B. (2000a). Ultradian corticosterone rhythm and the propensity to behave aggressively in male rats. J Neuroendocrinol 12:937-940.

Haller J., Kruk M.R. (2006). Normal and abnormal aggression: human disorders and novel laboratory models. Neurosci Biobehav Rev 30:292-303.

Haller J., Millar S., van de Schraaf J., de Kloet R.E., Kruk M.R. (2000b). The active phase-related increase in corticosterone and aggression are linked. J Neuroendocrinol 12:431-436.
Haller J., van de Schraaf J., Kruk M.R. (2001). Deviant forms of aggression in glucocorticoid hyporeactive rats: a model for 'pathological' aggression? J Neuroendocrinol 13:102-107.
Hayden-Hixson D.M., Ferris C.F. (1991a). Cortisol exerts site-, context- and dose-dependent effects on agonistic responding in hamsters. J Neuroendocrinol 3:613-622.
Hayden-Hixson D.M., Ferris C.F. (1991b). Steroid-specific regulation of agonistic responding in the anterior hypothalamus of male hamsters. Physiol Behav 50:793-799.
Heller K.E. (1978). Role of corticosterone in the control of post-shock fighting behaviour in male laboratory mice. Behavioural Processes 3:211-222.
Joels M., Pu Z., Wiegert O., Oitzl M.S., Krugers H.J. (2006). Learning under stress: How does it work? Trends Cog Sci 10:152-158.
Leshner A.I., Korn S.J., Mixon J.F., Rosenthal C., Besser A.K. (1980). Effects of corticosterone on submissiveness in mice: some temporal and theoretical considerations. Physiol Behav 24:283-288.
Leshner A.I., Moyer J.A. (1975). Androgens and agonistic behavior in mice: relevance to aggression and irrelevance to avoidance-of-attack. Physiol Behav 15:695-699.
Leshner A.I., Moyer J.A., Walker W.A. (1975). Pituitary-adrenocortical activity and avoidance-of-attack in mice. Physiol Behav 15:689-693.
Leshner A.I., Politch J.A. (1979). Hormonal control of submissiveness in mice: irrelevance of the androgens and relevance of the pituitary-adrenal hormones. Physiol Behav 22:531-534.
Leshner A.I., Walker W.A., Johnson A.E., Kelling J.S., Kreisler S.J., Svare B.B. (1973). Pituitary adrenocortical activity and intermale aggressiveness in isolated mice. Physiol Behav 11:705-711.
Makara G.B., Haller J. (2001). Non-genomic effects of glucocorticoids in the neural system. Evidence, mechanisms and implications. Prog Neurobiol 65:367-390.
Mikics E., Barsy B., Barsvari B., Haller J. (2005). Behavioral specificity of non-genomic glucocorticoid effects in rats: effects on risk assessment in the elevated plus-maze and the open-field. Horm Behav 48:152-162.
Mikics E., Barsy B., Haller J. (2007). The effect glucocorticoids on aggressiveness in established colonies of rats. Psychoneuroendocrinology 32:160-170.
Mikics E., Kruk M.R., Haller J. (2004). Genomic and non-genomic effects of glucocorticoids on aggressive behavior in male rats. Psychoneuroendocrinology 29:618-635.
Moyer J.A., Leshner A.I. (1976). Pituitary-adrenal effects on avoidance-of-attack in mice: separation of the effects of ACTH and corticosterone. Physiol Behav 17:297-301.
Oitzl M.S., de Kloet E.R. (1992). Selective corticosteroid antagonists modulate specific aspects of spatial orientation learning. Behav Neurosci 106:62-71.
Oitzl M.S., Reichardt H.M., Joels M., de Kloet E.R. (2001). Point mutation in the mouse glucocorticoid receptor preventing DNA binding impairs spatial memory. Proc Natl Acad Sci U S A 98:12790-12795.
Okuda S., Roozendaal B., McGaugh J.L. (2004). Glucocorticoid effects on object recognition memory require training-associated emotional arousal. Proc Natl Acad Sci U S A 101:853-858.
Roozendaal B. (2000). 1999 Curt P. Richter award. Glucocorticoids and the regulation of memory consolidation. Psychoneuroendocrinology 25:213-238.

Roozendaal B., McGaugh J.L. (1996). Amygdaloid nuclei lesions differentially affect glucocorticoid-induced memory enhancement in an inhibitory avoidance task. Neurobiol Learn Mem 65:1-8.

Roozendaal B., Okuda S., Van der Zee E.A., McGaugh J.L. (2006). Glucocorticoid enhancement of memory requires arousal-induced noradrenergic activation in the basolateral amygdala. Proc Natl Acad Sci U S A 103:6741-6746.

Roozendaal B., Quirarte G.L., McGaugh J.L. (2002). Glucocorticoids interact with the basolateral amygdala beta-adrenoceptor--cAMP/cAMP/PKA system in influencing memory consolidation. Eur J Neurosci 15:553-560.

Sandi C., Loscertales M., Guaza C. (1997). Experience-dependent facilitating effect of corticosterone on spatial memory formation in the water maze. Eur J Neurosci 9:637-642.

Sandi C., Pinelo-Nava M.T. (2007). Stress and memory: behavioral effects and neurobiological mechanisms. Neural plasticity 2007:article ID 78970.

Sandi C., Rose S.P.R. (1994). Corticosterone enhances long-term retention in one-day-old chicks trained in a weak passive avoidance learning paradigm. Brain Res 647:106-112.

Sandi C., Rose S.P.R. (1997). Training-dependent biphasic effects of corticosterone in memory formation for a passive avoidance task in chicks. Psychopharmacology 133:152-160.

Sandi C., Rose S.P.R., Mileusnic R., Lancashire C. (1995). Corticosterone facilitates long-term memory formation via enhanced glycoprotein synthesis. Neuroscience 69:1087-1093.

Sandi C, Venero C, Guaza C. 1996a. Nitric oxide synthesis inhibitors prevent rapid behavioral effects of corticosterone in rats. Neuroendocrinology 63:446-453.

Sandi C., Venero C., Guaza C. (1996b). Novelty-related rapid locomotor effects of corticosterone in rats. Eur J Neurosci 8:794-800.

Summers C.H., Forster G.L., Korzan W.J., Watt M.J., Larson E.T., Overli O., Hoglund E., Ronan P.J., Summers T.R., Renner K.J., Greenberg N. (2005). Dynamics and mechanics of social rank reversal. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 191:241-252.

Tronel S., Alberini C.M. (2007). Persistent disruption of a traumatic memory by postretrieval inactivation of glucocorticoid receptors in the amygdala. Biol Psychiatry 62:33-39.

Van Kreveld D. (1970). A selective review of dominance-subordinate relations in animals. Genetics and Psychology Monographs 81:143-173.

Venero C., Guaza C., Sandi C. (1996). Regional and temporal modulation of brain glycoprotein synthesis by corticosterone. Neuroreport 7:2819-2822.

Venero C., Sandi C. (1997). Effects of NMDA and AMPA receptor antagonists on corticosterone facilitation of long-term memory in the chick. Eur J Neurosci 9:1923-1928.

Wood G.E., Young L.T., Reagan L.P., McEwen B.S. (2003). Acute and chronic restraint stress alter the incidence of social conflict in male rats. Horm Behav 43:205-213.

Chapter 3 - Evidence for a role of oxytocin and vasopressin 1a receptors in the long-term establishment of dominance hierarchies

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3.1 Abstract

Exposure to acute stress can affect the establishment and maintenance of dominance hierarchies. When two non-stressed rats are exposed to each other in a social encounter, they establish a hierarchy, but the rank obtained during the first encounter in not maintained when the rats are re-exposed to each other one week later. When one rat of a pair is stressed before a first encounter, the stressed rat becomes subordinate and the hierarchy that is formed is stable for at least one week. Oxytocin and vasopressin are two neuropeptides that play an important role in the regulation of social behavior. In the present study, we investigated the immediate and long-term changes in mRNA levels of receptors for oxytocin and vasopressin in medial amygdala and lateral septum. In the medial amygdala of subordinate rats of a stressed pair, we found a downregulation in oxytocin receptor mRNA at three hours after the first encounter. In the same group, a downregulation in the vasopressin 1a receptor was found in lateral septum one week after the first encounter. We show that administration of a selective oxytocin antagonist in the medial amygdala of the subordinate rat immediately after a first encounter induces a long-term memory for the established hierarchy. Administration of a selective vasopressin 1a receptor antagonist in the lateral septum of the subordinate rat just before a second encounter mimics the effects of exposure to acute stress on the long-term establishment of the dominance hierarchy. These results suggest a role for oxytocin and vasopressin in the long-term establishment of dominance hierarchies in adult male rats. The effects of acute stress on dominance hierarchy formation and maintenance might be mediated by changes in oxytocinergic and vasopressinergic pathways in brain areas that are involved in the regulation of social behavior.

Key words: oxytocin; vasopressin; receptors; dominance hierarchy; memory; subordinate; rat; medial amygdala, lateral septum.

3.2 Introduction

Most animals that live in groups establish dominance hierarchies in which the dominant animal has priority access to limited resources such as water, food, space and females. One of the main functions of establishing dominance hierarchies is to eliminate fighting in a group and thus to minimize energy costs (Van Kreveld, 1970). In laboratory rats that are kept in a colony a stable hierarchy usually develops within a few days (Blanchard et al., 1988).

Social recognition and social memory are critical for the establishment and maintenance of social relationships, including social attachment, sexual behavior, parental care and dominance hierarchies (Bielsky and Young, 2004). Social memory for offspring and mates is usually long-lasting for days, months or even years (Ferguson et al., 2002). However, recognition of an unfamiliar individual tends to be short-lasting, in the range of minutes to hours. Due to the importance of social recognition, the hypothesis has been put forward that specific pathways in the brain have developed for this purpose. Among the brain areas that have been involved in these pathways are the medial amygdala (MeA) and lateral septum (LS) (Ferguson et al., 2002; Bielsky and Young, 2004). Oxytocin (OT) and arginine vasopressin (AVP) are two neuropeptides that play an important role in the regulation of social behavior (reviewed by Veenema and Neumann, 2008). These neuropeptides, and their receptors oxytocin receptor (OTR) and vasopressin receptor 1a (V1aR) are expressed in the above mentioned brain areas that are involved in social behavior (Ostrowski et al., 1994; Ostrowski, 1998; Newman, 1999; Gimpl and Fahrenholz, 2001; Goodson, 2005).

Mice that are knockout for OT or V1aR are not able to form social memories (Ferguson et al., 2000; Ferguson et al., 2001; Bielsky et al., 2004; Crawley et al., 2007). Administration of OT to OT knockout mice before a social encounter or re-expression of V1aR in V1aR knockout mice restores the ability to form social memories (Ferguson et al., 2000; Bielsky et al., 2005). In rats, administration of AVP or of low but not high doses of OT facilitates social recognition (Popik and Van Ree, 1991; Popik and Vetulani, 1991; Popik et al., 1992; Benelli et al., 1995), whereas administration of OT or AVP antagonists blocks social recognition (Dantzer et al., 1987; Le Moal et al., 1987; Dantzer et al., 1988; Popik and Van Ree, 1991; Popik and Vetulani, 1991; Popik et al., 1992; Benelli et al., 1995; Landgraf et al., 1995; Engelmann et al., 1996; Popik et al., 1996; van Wimersma Greidanus and Maigret, 1996; Everts and Koolhaas, 1997; Engelmann et al., 1998; Everts and Koolhaas, 1999; Landgraf et al., 2003; Choleris et al., 2007). Besides its role in social recognition, AVP has

also been implicated in aggressive behavior (Ferris et al., 1984; Irvin et al., 1990; Winslow and Insel, 1993; Delville et al., 1996).

In rats, exposure to different types of emotional and physical stressors has been reported to lead to OT and AVP release from the paraventricular nucleus (PVN) and the supraoptic nucleus (SON) of the hypothalamus (reviewed by Neumann, 2007). Thus, exposure to a single social defeat was shown to increase OT release from the SON (Engelmann et al., 1999) and the LS (Ebner et al., 2000) and AVP release from the PVN (Wotjak et al., 1996). Increases in both OT and AVP release in the SON and the PVN were found after repeated exposure to forced swimming (Wotjak et al., 1998).

Interestingly, intracerebral infusion of OT, on its turn, was found to attenuate the response of the HPA axis, as measured by the ACTH and corticosterone response to stress and CRF mRNA expression in the PVN (Windle et al., 1997; Windle et al., 2004). Infusion of OT antagonists enhances ACTH levels in the blood (Neumann et al., 2000a) and OT knockout mice show enhanced plasma corticosterone level (Mantella et al., 2004). Patchev et al. (1993) showed that glucocorticoids increase OT binding in the amygdala, LS and bed nucleus of stria terminalis. Increasing OT by intracerebral infusions leads to a more passive stress coping strategy and to inhibition of the HPA axis. This is thought to prevent an excessive stress response (Neumann et al., 2000a; Windle et al., 2004; Ebner et al., 2005). V1aR binding in LS was also found to be regulated by glucocorticoids, with adrenalectomy decreasing V1aR binding and administration of dexamethasone restoring this effect (Watters et al., 1996). Interestingly, the hypothesis has been put forward that interactions between OT, AVP and glucocorticoids could provide a mechanism for dynamical changes in social behavior (Gimpl and Fahrenholz, 2001).

In an animal model that was developed in our group (Cordero and Sandi, 2007), we have shown that when one rat of a dyad is exposed to acute stress prior to a first encounter, this stressed rat becomes the subordinate individual in the first encounter, and the established dominance hierarchy is stable for at least one week. When two non-stressed rats are submitted to a social encounter, a hierarchy is formed during the first encounter, but the social rank obtained on the first day is not maintained during a second encounter one week later. In the current study, we investigated the role of OTR and V1aR in the stress facilitation of a long-term dominance hierarchy between two adult male rats. Specifically, we studied the immediate and long-term changes in gene expression of *Otr* and *V1aR* and the causal relationship between gene expression and behavior by using antagonists of OT and AVP. We

expect that both OT and AVP are causally involved in the long-term establishment of a dominance hierarchy, but that they may exert their effects in different brain areas.

3.3 Materials and methods

Animals

Male Wistar rats (Charles River Laboratories, Lyon, France) weighing 250-275 grams at arrival were individually housed with *ad libitum* access to food and water. Animals were kept in a 12h light/dark cycle with lights on at 0700h, and a constant temperature of 22 ± 2 °C. Animals were left undisturbed for one week after arrival before starting the experiment, except when surgery was performed, which was performed a few days after arrival. Rats were weighed once per week. Experiments were performed with approval of the Cantonal Veterinary Authorities (Vaud, Switzerland).

All experiments took place between 0800h and 1400h, except for the water competition test that took place between 1400h and 1900h, after a water deprivation period of 6h (from 0800h until 1400h). All rats were handled for 2 min per day during three days before exposure to the elevated plus maze (see below).

Surgery

Rats were anaesthetized with ketamine (70 mg/kg) and xylazine (6 mg/kg), administered by i.p. injection. 10 mm long stainless steel guide cannulae of 23 gauge (Plastics One, Roanoke, USA) were implanted bilaterally in the MeA at -2.50 mm anterior and ±3.3 mm lateral relative to Bregma and -7.9 mm ventral relative to dura (Paxinos and Watson, 1998) using a standard stereotaxic frame (Stoelting, USA). Bilateral 4 mm long stainless steel guide cannulae of 22 gauge with a distance of 1.5 mm between the guide cannulae (Plastics One, Roanoke, USA) were implanted in the LS at +0.20 mm anterior, ±0.75 mm lateral and -3.7 mm ventral relative to Bregma. Cannulae were fixed to the skull with dental acrylic cement (Kaladent, Switzerland) and anchored with Vetbond Tissue Adhesive glue (3M). Dummy cannulae (10mm for MeA, 4mm for LS, Plastics One, Roanoke, USA) were inserted into the cannulae to protect them from blocking. Rats received an injection with Antisedan (0.04 ml s.c., Pfizer, Switzerland) after the surgery to reverse the effects of the anesthetics. Paracetamol was added to the drinking water as analgesic in the days following surgery. The rats were allowed to recover for at least one week before the start of the experiment.

Drugs and infusion procedure

The selective OT antagonist (OTA) desGly-NH_2-d$(CH_2)_5$[D-Tyr^2,Thr^4]OVT was used for infusions into the MeA, the selective V1aR antagonist d$(CH_2)_5$[Tyr^2(Me)^2]AVP for

infusions into the LS (both antagonists were a generous gift of dr. M. Manning, Toledo, OH, USA). The OTA and V1aR antagonist were dissolved in saline. For MeA, internal injectors of 11 mm (extending 1 mm from the end of the guide cannula, 30 gauge, Plastics One, Roanoke, USA) were used to administer the drug. For LS, a bilateral internal injector of 6 mm (extending 2 mm from the end of the guide cannula, 28 gauge, Plastics One, Roanoke, USA) was used. The injectors were connected to 10 µl microsyringes (SGE Analytical Science, Australia) on an automated infusion pump (Harvard Apparatus, MA, USA) through polyethylene tubing. 1 µg of OTA in 1 µl of saline was infused into the MeA of each hemisphere at a rate of 0.5 µl/min. 10 ng of V1aR antagonist in 1 µl of saline was infused bilaterally into the LS at rate of 0.5 µl/min. After the infusion the injector was left in place for 1 min to allow diffusion of the drug. Saline was used as a vehicle. The OTA was infused

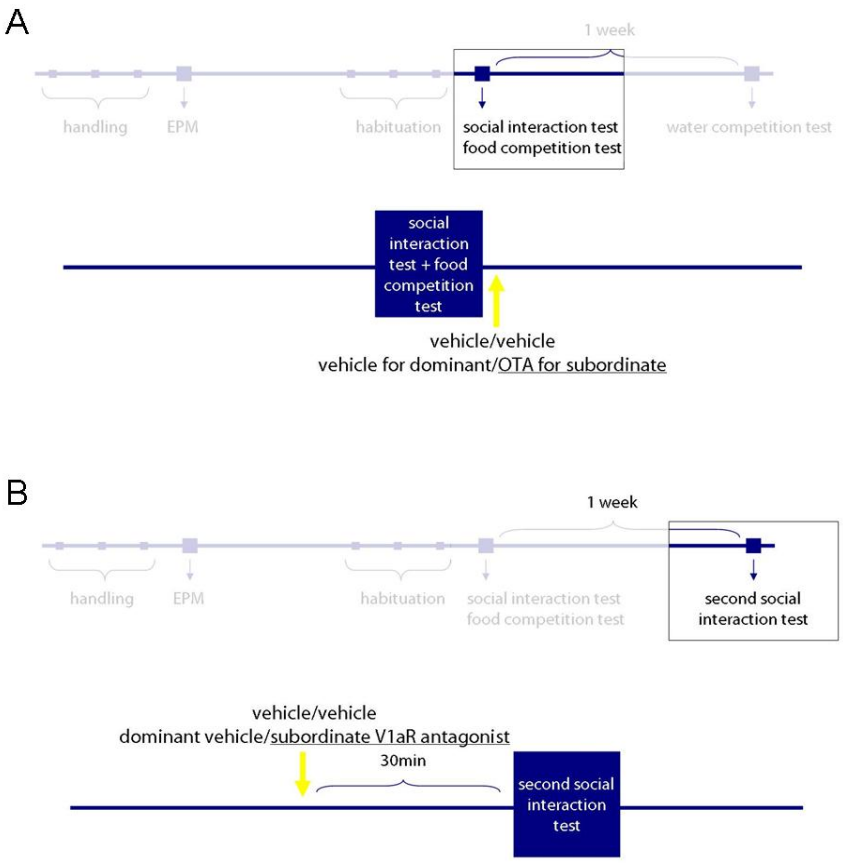

Figure 1. Overview of the experimental design. (A) In one experiment, both rats were infused with vehicle in the medial amygdala, or the dominant rat of the pair was infused with vehicle and the subordinate rat with OTA immediately after the first encounter on day 1. (B). In another experiment, both rats were infused with vehicle in the lateral septum 30 min before the second encounter, or the previously dominant rat was infused with vehicle and the previously subordinate rat with V1aR antagonist. 'OTA' = oxytocin antagonist

immediately after the encounter to the subordinate rat or after the last habituation session in the group that was not exposed to a social encounter on day 1. The V1aR antagonist was infused 30 min before the second encounter to the subordinate rat. The other rat of the pair and both rats of control pairs were infused with vehicle.

Elevated plus maze

Before the start of the experiment the 'elevated plus maze' (EPM) test was performed to measure anxiety-related behavior. The EPM consists of two opposing open arms (45x10 cm) and two opposing closed arms (45x10 cm with walls of 50 cm high) that extend from a central platform (10x10 cm), elevated 65 cm above the floor. The rats were placed individually on the central platform facing the same closed arm and were allowed to explore the EPM freely during 5 min. The behavior of each rat was video recorded and analyzed using a computerized tracking system (Ethovision 3.1.16, Noldus IT, the Netherlands). Time spent in the open and closed arms was measured.

Dyads and groups

Rats were distributed to dyads consisting of two rats that were matched for their body weight and anxiety level. The anxiety level was defined by the time spent in the open arms of the EPM. Thus, the rats in each dyad were considered equal in their probability to become dominant or subordinate during a first encounter. The fur of both rats in a dyad was marked on different body parts (i.e. neck and back) to help identifying the animals. Rats were habituated to marking during the three day habituation period that took place before the first social encounter. Dyads were divided randomly over the different groups. Each group consisted of 7-14 dyads.

Stress delivery – Contextual fear conditioning

In non-stressed pairs (Pns) none of the rats were stressed prior to the social encounter. In stressed pairs (Ps) one rat of the pair was stressed using a contextual fear conditioning paradigm just before the first social encounter. Fear conditioning was performed in an observation chamber of 30x37x25 cm (Panlab, Spain) made of black steel walls and a Plexiglas ceiling and door. The floor consisted of 20 steel rods connected to a shock source and solid-state scrambler for delivery of electrical shocks. The observation chamber was lit by a 20 W light bulb and there was a background noise of 68 dB. The protocol consisted of a habituation period of 3 min, followed by 3 footshocks of 1 mA lasting 1 sec each with an

intershock interval of 60 sec. 30 sec after the last shock the rat was taken out and immediately submitted to the first social encounter with an unfamiliar rat in an adjacent room.

Food and water competition test

In order to habituate animals to the rewarding food used in the 'food competition test' (FCT), they received eight Chocopop flakes (Kellogg's, Switzerland) in their homecages daily, during two days before the start of the habituation to the experimental setup. The rats were habituated to the experimental setup for three days during which they were first placed in a clean homecage for 20 min and then in a food competition box for 10 min. The food competition box is a plastic box of 60x40 cm with walls of 32 cm high with a feeder containing eight Chocopops on one of the short sides. On day 1 of the experiment, the day after the last habituation day, the rats were submitted to a first encounter with an unfamiliar rat, consisting of a 'social interaction test' in a neutral homecage without food and water for 20 min, followed by a 'food competition test' (FCT) in which the rats had to compete for Chocopops during 10 min. After the tests each rat was returned to its homecage. The group without an encounter on day 1 was exposed to an extra day of habituation.

The memory for the formed hierarchy was tested in a 'water competition test' (WCT) that was performed on day 8 after a preceding water deprivation period of 6h. Rats were exposed to the same opponent as on day 1. The WCT was performed in a neutral homecage. After 2 min of habituation, a single bottle of water was presented and the behavior during the following 10 min was recorded. In the experiment using the V1aR antagonist the second encounter was performed without a preceding water deprivation period and without presentation of the bottle of water to exclude any effects of the antagonist on vasopressinergic water regulation. Previous experiments have shown that the outcome of a second social encounter without water deprivation is the same as the outcome of a WCT (Timmer and Sandi, 2010).

Behavior was video-recorded and scored blindly using The Observer (v.5.0.25, Noldus IT, the Netherlands). The duration and frequency of offensive and defensive behaviors was scored. Offensive behaviors were attacks (biting), keeping down (pushing the opponent to the floor), offensive upright (standing on the hind legs in upright position) and lateral threat (pushing or approaching the opponent showing its side with an arched back). Defensive behavior consisted of freezing (immobility), defensive upright (standing on the hind legs in response to offensive upright) and submissive posture (lying on the back). In the FCT the number of consumed Chocopops and the number of passes over the feeder was counted and in

the WCT the time spent drinking was measured. The rat that showed most offensive behavior during the interaction was considered the dominant rat, the rat showing least offensive behavior and most defensive behavior the subordinate rat.

Two control groups were added for the gene expression experiments. The first control group was submitted to habituation, but not to a social encounter. The stressed control group was submitted to habituation and to foot shock stress (as described above), but not to a social encounter.

Sacrifice

For the gene expression experiments rats were sacrificed either on day 1, three hours after the end of the food competition test, or on day 8, one week after the social interaction and food competition test under basal conditions. The animals were decapitated, the brain was quickly removed and frozen in isopentane at a temperature between -50 and -40 °C and stored at -80 °C until further processing.

In experiments involving cannulation, in order to check the position of the cannulae in the OTA and V1aR antagonist experiments rats were injected i.p. with pentobarbital. Then, 1 µl of ink was subsequently infused into the MeA or LS to mark the position of drug delivery. The rats were decapitated and the brains removed, frozen in isopentane and stored at -80 °C until further processing. 40 µm thick coronal sections were stained with thionin to detect the cannula position.

Laser capture micro dissection

For the gene expression experiment, coronal sections of 20 µm were taken for 'laser capture micro dissection' (LCM). The sections were put on membrane covered slides (Palm Microlaser Technologies, Carl Zeiss, Germany) and stored at -20 °C covered with RNA*later* ICE (Ambion, TX, USA). Prior to LCM the sections were stained with the HistoGene LCM Frozen Section Staining kit following the protocol (Arcturus Bioscience, Switzerland). The MeA and the LS from the right hemisphere were dissected through LCM. The target tissue was catapulted into a PALM adhesive cap (Palm Microlaser Technologies, Carl Zeiss, Germany) and subsequently incubated in 100 µl of lysis buffer (RNAqueous Micro Kit, Ambion, TX, USA) for 30 min at 42 °C. After spinning down the tissue was stored at -80 °C until further processing.

RNA isolation

RNA was isolated from the tissue using the RNAqueous Micro Kit (Ambion, TX, USA). First, 3 µl of LCM additive was added. Subsequently 129 µl of 100% ethanol was added to the mixture. The lysate/ethanol mixture was loaded onto a Micro Filter Cartridge Assembly and centrifuged for 1 minute at 10000g. Afterward 180 µl of Wash Solution 1 was added and the mixture was centrifuged again for 1 min at 10000g. 180 µl of Wash Solution 2/3 was added and the mixture was centrifuged for 30 sec at 14000g. The flow-through was discarded and the Micro Filter Cartridge was centrifuged for another minute at 14000g. The Micro Filter Cartridge was transferred into a Micro Elution Tube and 8 µl of preheated Elution Solution was added. The mixture was left for 5 min at room temperature and subsequently centrifuged for 1 min at 14000g. Another 8 µl of Elution Solution was added and the mixture was centrifuged again at 14000g. The RNA was stored at -80 °C until further processing

Reverse transcription and preamplification

9.62 µl of the extracted RNA was converted into cDNA using Taqman Reverse Transcriptase Reagents (Applied Biosystems, CA, USA). 1.25 µl of random hexamers and 2.5 µl of RT 10x buffer were added to reach an end volume of 13.37 µl. The mixture was brought to 65 °C for 10 min followed by 10 min at room temperature in a Thermocycler (Primus 96, Peqlab, Germany). Following this step 5.5 µl of $MgCl_2$, 5 µl of dNTPs (10 mM), 0.5 µl of RNAse inhibitor and 0.63 µl of Multiscribe Reverse were added to each sample. Using the Thermocycler, the reverse transcription was executed for 10 min at 25 °C, 25 min at 48 °C and 5 min at 95 °C. The obtained cDNA was stored at -20 °C until further processing.

The cDNA was preamplified using Pre-amp MasterMix for TaqMan assays (Applied Biosystems, CA, USA). A pool of TaqMan Gene Expression Assays for the genes of interest (see ‘Real time PCR’) was made in ultrapure water (Gibco, NY, USA) at a concentration of 1% for each Assay. 25 µl of Taqman PreAmp MasterMix, 12.5 µl of the pooled Assay mix and 12.5 µl of cDNA were mixed for each sample. The preamplification was done by an enzyme activation period of 10 min at 95 °C, followed by 10 cycles of 15 sec at 95 °C and 4 min at 60 °C. After finishing the run the samples were immediately placed on ice and diluted 5x in ice-cold ultrapure water (Gibco, NY, USA). Samples were stored at -20 °C until further processing.

Quantitative real time PCR

Quantitative real time PCR was performed using TaqMan Gene Expression Assays for the genes for the oxytocin receptor (*Otr*) and the arginine vasopressin receptor 1A (*V1ar*) with the control genes ribosomal protein S18 (*Rps18*), ribosomal protein S29 (*Rps29*) and eukaryotic elongation factor 1 alpha 1 (*Eef1a1*) (see Table 1 for the used Gene Expression Assays, Applied Biosystems, CA, USA). First, a reaction mixture containing 4.5 µl preamplified cDNA, 0.5 µl 20x Taqman Gene Expression Assay for the genes of interest and 5 µl 2x PCR Master Mix (TaqMan Universal PCR master mix no AmpErase UNG, Applied Biosystems, CA, USA) per reaction of 10 µl was made. Triplicates of 10 µl of each reaction mixture were transferred to a well in a 384-well optical reaction plate (Applied Biosystems, CA, USA). The real time PCR was performed using 40 cycles of 15 sec at 95 °C and 1 min at 60 °C (7900HT, Applied Biosystems, CA, USA), preceded by 2 min at 50 °C and 10 min at 95 °C.

The software SDS (v.2.3, Applied Biosystems, CA, USA) was used for running and analyzing the quantitative real time PCR. Each target gene was compared to three internal control genes (*Rps18, Rps29, Eef1a1*) using the ΔΔCt method (described by Livak and Schmittgen, 2001). The results are expressed as fold change compared to the control groups.

Table 1. Overview of the used Gene Expression Assays (Applied Biosystems).

Gene	Gene Expression Assay
Oxytocin receptor (*Otr*)	Rn00563503_m1
Arginine vasopressin receptor 1a (*V1ar*)	Rn00583910_m1
Ribosomal protein S18 (*Rps18*)	Rn01428915_g1
Ribosomal protein S29 (*Rps29*)	Rn00820645_g1
Eukaryotic elongation factor 1a1 (*Eef1a1*)	Rn01639851_g1

Statistics

All results are expressed as the mean ± the standard error of the mean (S.E.M.).

For statistical analyses of mRNA expression levels, the fold change of the different groups compared to the non-stressed control group was calculated. One-way ANOVA was used to test statistical significance between the dominant and subordinate rats of Pns and Ps. *A priori* comparisons to test differences between two specific groups were established and evaluated through either unpaired or paired two-tailed Student t-tests. Paired t-tests were applied to compare mRNA expression levels of dominant and subordinate rats that competed

together, while unpaired t-tests were applied when comparisons involved animals that had not directly interacted. *A priori* comparisons for group comparisons were (1) the subordinate rat of Ps with the subordinate rat of Pns, (2) the subordinate rat of Ps with the dominant rat of Ps, (3) the dominant rat of Ps with the dominant rat of Pns, (4) the subordinate rat of Pns with the dominant rat of Pns, (5) the subordinate rat of Ps with the stressed control group and (6) each experimental group compared to non-stressed controls.

For the behavioral experiments, the percentage of offensive behaviors within each dyad was calculated. A paired two-tailed Student t-test was used to compare the percentage of offensive behaviors within a dyad of rats competing together.

The computer software SPSS for Windows (version 13.0) was used for statistical analysis. $P<0.05$ was considered significant.

3.4 Results

Exposure to acute stress induces a long-term memory for the established hierarchy

First, we evaluated whether exposure of one rat of a dyad to acute stress before a first encounter induces a long-term memory for the hierarchy that was established on day 1. Rats that have not been submitted to stress (Pns) form a hierarchy when exposed to a first social encounter on day 1 ($t = 7.844$, $df = 6$, $p<0.001$), but on day 8 the hierarchy is not maintained (Fig. 2A). When one rat of a dyad is exposed to acute stress (Ps), the stressed rat become subordinate during the first encounter, and one week later the same hierarchy is still observed ($t = 5.083$, $df = 5$, $p<0.01$ for day 1, $t = 3.109$, $df = 5$, $p<0.05$ for day 8, Fig. 2B). Therefore, under our experimental conditions, we have replicated previous observations (Cordero and Sandi, 2007) that under control conditions the social rank established through a first social interaction test between two males is not maintained when the animals are confronted one week later, whereas when one rat of the dyad is stressed before the first encounter, the dominance hierarchy established on day 1 is still clearly observed one week later, with the stressed rat becoming submissive.

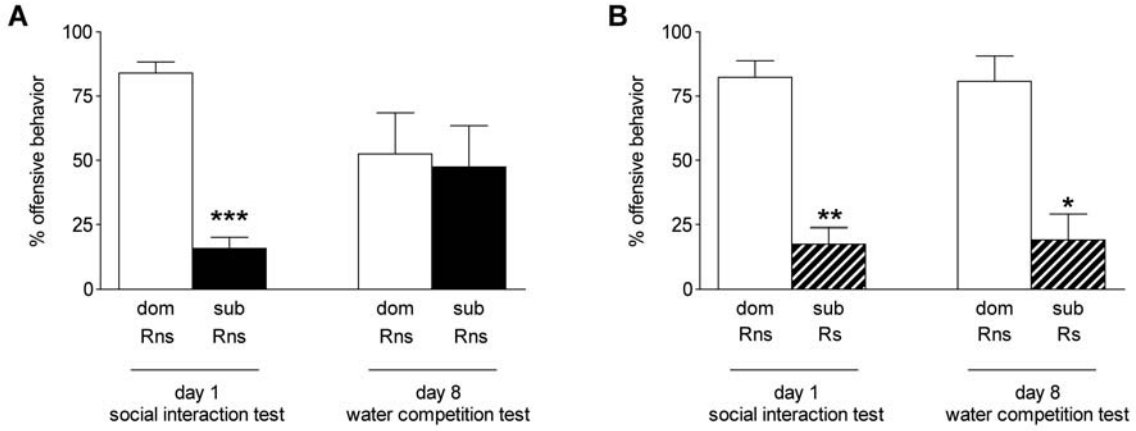

Figure 2. The percentage of total offensive behavior (mean ± S.E.M.) between two opponents shown in the social interaction test on day 1 and the water competition test on day 8. (A) The hierarchy of rats of non-stressed pairs (n=7). (B) The hierarchy of rats of the stressed pairs (n=6). The status 'dom' (dominant) or 'sub' (subordinate) refers to the status that was obtained during the social interaction test on day 1. 'Rns' = non-stressed rat, 'Rs' = stressed rat. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. the other rat of the dyad.

Changes in mRNA levels 3h and 1 week after a first encounter

The immediate (3h) and long term (1 week, under basal conditions) effects of stress and hierarchy formation on the levels of *Otr* and *V1ar* mRNA were studied in MeA and LS, brain areas that are important in the regulation of social behavior and stress responses.

The animals of Pns and Ps were submitted to a first encounter on day 1. The pairs formed a clear hierarchy on day 1 ($t = 5.527$, $df = 19$, $p<0.001$ for Pns, Fig. 3A; $t = 2.943$, $df = 19$, $p<0.01$, Fig. 3B). A part of the rats (from all groups: Pns, Ps, controls and stressed controls) was sacrificed three hours after the end of the first encounter to study the immediate changes in *Otr* and *V1ar* mRNA levels in MeA and LS. The other rats (again from all groups: Pns, Ps, controls and stressed controls) were sacrificed one week after the first encounter, under basal conditions, in order to study the long-term changes in *Otr* and *V1ar* mRNA levels that could underlie the long-term establishment of the dominance hierarchy.

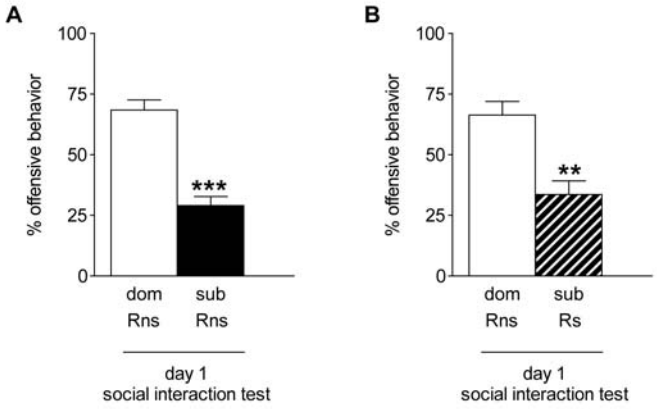

Figure 3. The percentage of total offensive behavior (mean ± S.E.M.) between two opponents shown in the social interaction test on day 1. (A) The hierarchy of rats of non-stressed pairs (n=20). (B) The hierarchy of rats of the stressed pairs (n=20). The status 'dom' (dominant) or 'sub' (subordinate) refers to the status that was obtained during the social interaction test on day 1. 'Rns' = non-stressed rat, 'Rs' = stressed rat. ** $p<0.01$, *** $p<0.001$ vs. the other rat of the dyad.

ANOVA for data from the dominant and subordinate rats of both Pns and Ps denotes a significant difference in *Otr* mRNA expression in MeA at 3h after the first encounter ($F_{3,48} = 2.807$, $p<0.05$). No significant differences are found in any of the other genes or time points in MeA and LS.

Student t-test indicates significantly different *Otr* mRNA levels in MeA of the subordinate rats of Ps at 3h, which is lower compared to the dominant rats of Ps and the

dominant and subordinate rats of Pns (Fig. 4A, Table 2). In LS, the dominant rats of Pns have lower *Otr* mRNA levels than the subordinate rats of Pns (Fig. 4B, Table 2).

After 1 week the subordinate rats of Ps show an increase in *Otr* mRNA in MeA compared to the control group (Fig. 4A, Table 2). In LS, a difference in *V1ar* mRNA is found in the same group, which is lower than controls and the dominant rats of both Pns and Ps (Fig. 5B, Table 3).

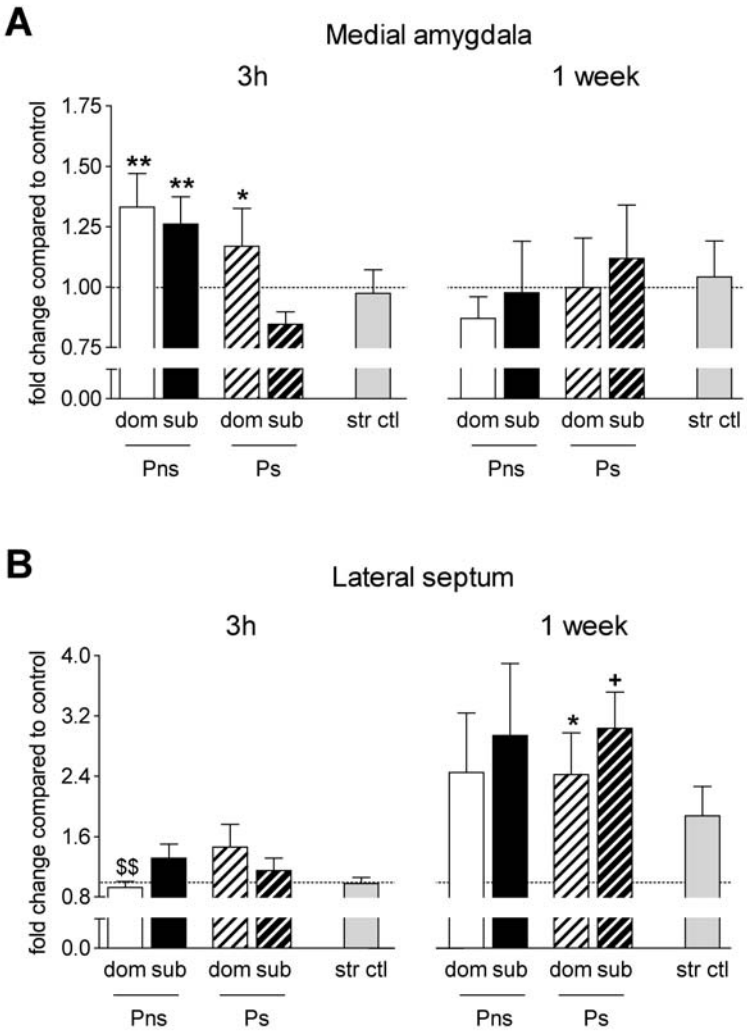

Figure 4. The expression of *Otr* mRNA in MeA and LS 3h and 1 week after the first encounter (mean ± S.E.M.). (A) *Otr* mRNA expression in MeA. (B) *Otr* mRNA expression in LS. 'str ctl' = stressed control group, 'dom' = dominant, 'sub' = subordinate, 'Pns' = non-stressed pair, 'Ps' = stressed pair. * $p<0.05$ vs. subordinate rats of Ps, ** $p<0.01$ vs. subordinate rats of Ps, + $p<0.05$ vs. controls, $$ $p<0.01$ vs. subordinate rats of Pns. For the 3h time point each group consists of 12-14 animals, for the 1 week time point of 6-8 animals.

Table 2. Overview of the outcome of paired and unpaired t-tests that compare the mRNA levels of *Otr* in MeA and LS. All comparisons are between the group mentioned in the top row and the group mentioned in the cell. 'Pns' = non-stressed pair, 'Ps' = stressed pair, 'dom' = dominant, 'sub' = subordinate, 'str control' = stressed control group.

		Compared to Ps sub	Compared to Pns dom	Compared to control
MeA OTR	3h	**Ps dom: t = 3.028, df = 10, p = 0.013 *** **Pns sub: t = 3.157, df = 22, p = 0.005 **** str control: t = 1.107, df = 22, p = 0.280	Ps dom: t = 0.775, df = 23, p = 0.446 Pns sub: t = 0.041, df = 12, p = 0.968	str control: t = 0.174, df = 24, p = 0.863 Pns dom: t = 1.843, df = 25, p = 0.077 Pns sub: t = 1.648, df = 24, p = 0.112 Ps dom: t = 0.892, df = 22, df = 0.382 Ps sub: t = 1.184, df = 22, p = 0.249
	1 week	Ps dom: t = 1.764, df = 7, p = 0.121 Pns sub: t = 0.361, df = 13, p = 0.724 str control: t = 0.157, df = 14, p = 0.877	Ps dom: t = 0.512, df = 12, p = 0.618 Pns sub: t = 0.775, df = 5, p = 0.473	str control: t = 0.175, df = 12, p = 0.864 Pns dom: t = 0.572, df = 11, p = 0.579 Pns sub: t = 0.083, df = 11, p = 0.935 Ps dom: t = 0.006, df = 13, p = 0.995 Ps sub: t = 0.296, df = 14, p = 0.771
LS OTR	3h	Ps dom: t = 2.032, df = 10, p = 0.070 Pns sub: t = 0.665, df = 21, p = 0.513 str control: t = 1.017, df = 22, p = 0.320	Ps dom: t = 1.830, df = 22, p = 0.081 **Pns sub: t = 3.765, df = 11, p = 0.003 ****	str control: t = 0.187, df = 24, p = 0.853 Pns dom: t = 0.635, df = 24, p = 0.531 Pns sub: t = 1.599, df = 23, p = 0.123 Ps dom: t = 1.562, df = 22, p = 0.133 Ps sub: t = 0.862, df = 22, p = 0.398
	1 week	**Ps dom: t = 2.798, df = 7, p = 0.027 *** Pns sub: t = 0.110, df = 13, p = 0.914 str control: t = 1.450, df = 13, p = 0.171	Ps dom: t = 0.028, df = 12, p = 0.978 Pns sub: t = 1.440, df = 5, p = 0.209	str control: t = 1.820, df = 10, p = 0.099 Pns dom: t = 1.732, df = 10, p = 0.114 Pns sub: t = 1.954, df = 10, p = 0.079 Ps dom: t = 2.073, df = 12, p = 0.060 **Ps sub: t = 2.941, df = 13, p = 0.011 ***

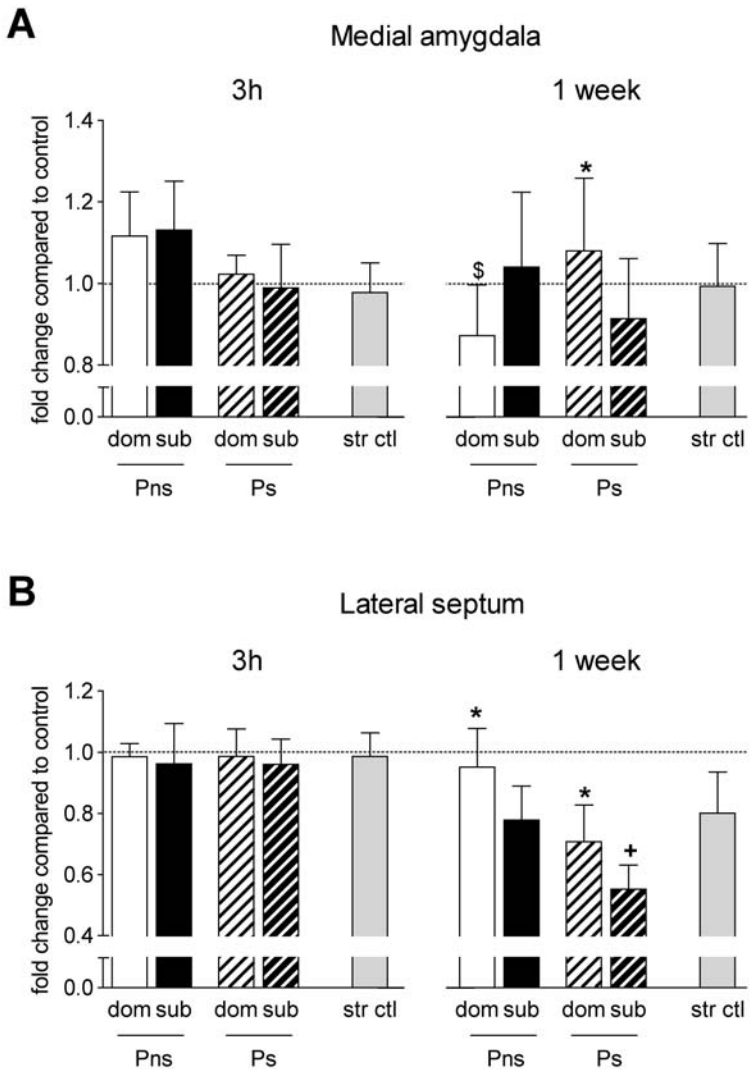

Figure 5. The expression of *V1ar* mRNA in MeA and LS 3h and 1 week after the first encounter (mean ± S.E.M.). (A) *V1ar* mRNA expression in MeA. (B) *V1ar* mRNA expression in LS. 'str' = stressed control group, 'dom' = dominant, 'sub' = subordinate, 'Pns' = non-stressed pair, 'Ps' = stressed pair. * $p<0.05$ vs. subordinate rats of Ps, + $p<0.05$ vs. controls, $ $p<0.05$ vs. subordinate rats of Pns. For the 3h time point each group consists of 12-14 animals, for the 1 week time point of 6-8 animals.

Table 3. Overview of the outcome of paired and unpaired t-tests that compare the mRNA levels of *V1aR* in MeA and LS. All comparisons are between the group mentioned in the top row and the group mentioned in the cell. 'Pns' = non-stressed pair, 'Ps' = stressed pair, 'dom' = dominant, 'sub' = subordinate, 'str control' = stressed control group.

		Compared to Ps sub	Compared to Pns dom	Compared to control
MeA V1aR	3h	Ps dom: t = 0.482, df = 11, p = 0.640 Pns sub: t = 0.878, df = 23, p = 0.389 str control: t = 0.088, df = 23, p = 0.931	Ps dom: t = 0.772, df = 23, p = 0.448 Pns sub: t = 0.436, df = 12 p = 0.671	str control: t = 0.201, df = 24, p = 0.842 Pns dom: t = 0.861, df = 24, p = 0.398 Pns sub: t = 0.908, df = 24, p = 0.373 Ps dom: t = 0.235, df = 23, p = 0.816 Ps sub: t = 0.081, df = 23, p = 0.936
	1 week	**Ps dom: t = 2.943, df = 7, p = 0.022 *** Pns sub: t = 0.663, df = 13, p = 0.519 str control: t = 0.560, df = 14, p = 0.584	Ps dom: t = 0.887, df = 12, p = 0.393 **Pns sub: t = 2.626, df = 5, p = 0.047 ***	str control: t = 0.033, df = 12, p = 0.974 Pns dom: t = 0.575, df = 11, p = 0.577 Pns sub: t = 0.158, df = 11, p = 0.877 Ps dom: t = 0.317, df = 13, p = 0.756 Ps sub: t = 0.491, df = 14, p = 0.631
LS V1aR	3h	Ps dom: t = 0.788, df = 10, p = 0.449 Pns sub: t = 0.007, df = 23, p = 0.994 str control: t = 0.226, df = 22, p = 0.823	Ps dom: t = 0.003, df = 23, p = 0.998 Pns sub: t = 0.266, df = 13, p = 0.794	str control: t = 0.117, df = 24, p = 0.908 Pns dom: t = 0.147, df = 25, p = 0.885 Pns sub: t = 0.235, df = 25, p = 0.816 Ps dom: t = 0.109, df = 22, p = 0.914 Ps sub: t = 0.318, df = 22, p = 0.754
	1 week	**Ps dom: t = 2.572, df = 7, p = 0.037 *** Pns sub: t = 1.641, df = 13, p = 0.125 str control: t = 1.596, df = 14, p = 0.133	Ps dom: t = 1.377, df = 12, p = 0.194 Pns sub: t = 2.290, df = 5, p = 0.071	str control: t = 0.819, df = 12, p = 0.429 Pns dom: t = 0.195, df = 11, p = 0.849 Pns sub: t = 0.913, df = 11, p = 0.381 Ps dom: t = 1.280, df = 13, p = 0.223 **Ps sub: t = 2.198, df = 14, p = 0.045 ***

Infusion of a selective OTA into the MeA

Since a downregulation in *Otr* mRNA was found in the MeA 3h after the first encounter in the stressed male that becomes subordinate, we reasoned that if this molecular change is involved in the long-term maintenance of the hierarchy, mimicking a reduction in OTR function in subordinate, but not formerly stressed males, would facilitate a long-term hierarchy also in that pair. Therefore, in this experiment, we administered a selective OTA

into the MeA of non-stressed subordinate rats immediately after the first encounter on day 1. The long-term establishment of the hierarchy was tested in a WCT one week after the first encounter.

In the non-stressed control group, both rats of the dyad were infused with vehicle into the MeA. A hierarchy was formed on day 1 ($t = 10.07$, $df = 7$, $p<0.001$), but no memory for the hierarchy was found 1 week later, as expected (Fig. 6A). When OTA was given to the subordinate rat immediately after the first encounter ($t = 3.339$, $df = 9$, $p<0.01$), the same hierarchy is found 1 week later ($t = 2.912$, $df = 9$, $p<0.05$, Fig. 6B), suggesting a role for OTR in MeA in the long-term establishment of the hierarchy. To check the effect of the OTA without the combination of exposure to a social experience, we infused OTA into the MeA of

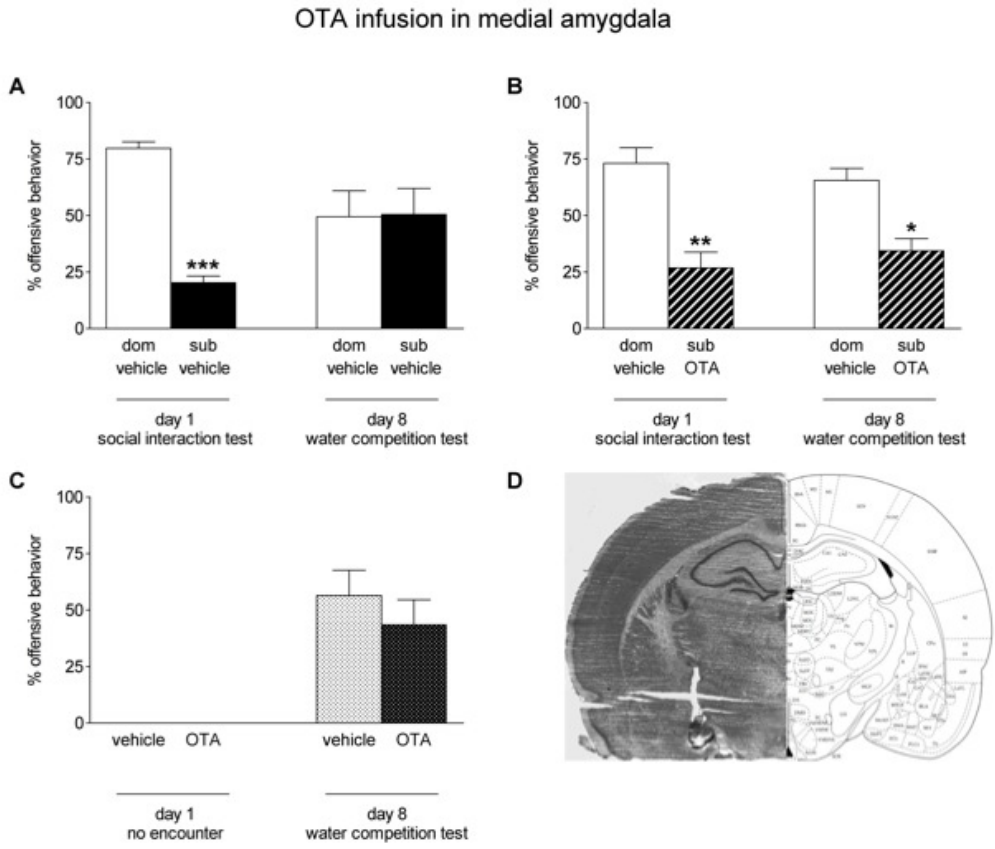

Figure 6. The percentage of total offensive behavior (mean ± S.E.M.) between two opponents shown in the social interaction test on day 1 and the water competition test on day 8. (A) The hierarchy of rats that were infused with vehicle (n=8). (B) Group in which the subordinate rat was infused with OTA immediately after the first encounter (n=10). (C) Rats that were infused with either vehicle or OTA on day 1 without exposure to an encounter until day 8 (n=7). (D) Representative photograph of the injection site in the MeA. The status 'dom' (dominant) or 'sub' (subordinate) refers to the status that was obtained during the social interaction test on day 1. 'OTA' = oxytocin antagonist. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. the other rat of the dyad.

one rat of each dyad and tested the effect on the establishment of a hierarchy one week later. No effect of OTA infusion on the establishment of a hierarchy during the WCT on day 8 was found (Fig. 6C). Fig. 6D shows a representative example of the injection site in the MeA.

Infusion of a selective V1aR antagonist into the LS

To test whether the effect of acute stress on the long-term hierarchy establishment can be mimicked by the administration of a V1aR antagonist, we infused the antagonist into the LS of non-stressed subordinate rats 30 minutes before the second encounter that took place one week after the first encounter. Both groups had established a clear hierarchy on day 1 ($t = 6.355$, $df = 9$, $p<0.001$ for the vehicle group, Fig. 7A; $t = 5.857$, $df = 6$, $p<0.01$ for the V1aR antagonist treated group, Fig. 7B; note that both groups were equivalent –untreated- at that

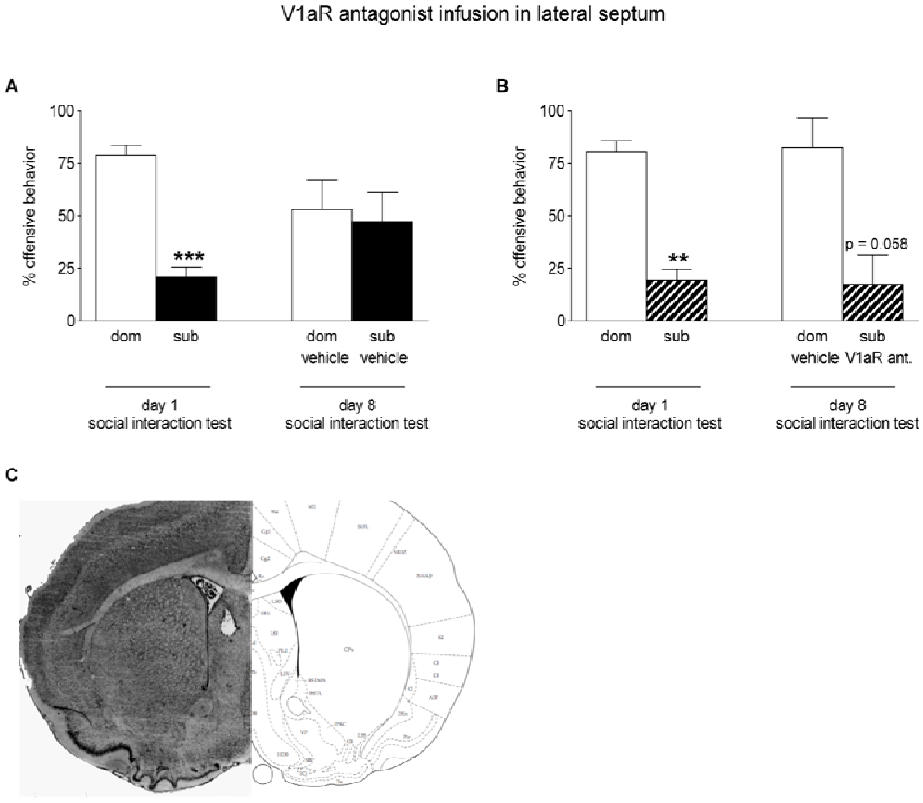

Figure 7. The percentage of total offensive behavior (mean ± S.E.M.) between two opponents shown in the social interaction test on day 1 and a second social interaction test on day 8. (A) The hierarchy of rats that were infused with vehicle 30min before the second encounter (n=10). (B) Group in which the subordinate rat was infused with V1aR antagonist 30min before the second encounter (n=7). (C) A representative picture of the placement of a cannula in lateral septum. The status 'dom' (dominant) or 'sub' (subordinate) refers to the status that was obtained during the social interaction test on day 1. 'V1aR ant.' = V1aR antagonist. ** $p<0.01$, ***$p<0.001$ vs. the other rat of the dyad.

time point). The vehicle-treated rats showed no memory for the hierarchy when they were tested one week after the first encounter (Fig. 7A). When the subordinate rat was infused with the V1aR antagonist before the second encounter there was a tendency for these rats to become subordinate ($t = 2.342$, $df = 6$, $p = 0.058$, Fig. 7B), which happened in 6 out of 7 pairs. A representative picture of LS cannulation is shown in Fig. 7C.

3.5 Discussion

In our recently developed model to study the establishment and maintenance of dominance hierarchies between two adult male rats, we have shown that two non-stressed rats (Pns) that have an equal opportunity to become dominant or subordinate establish a hierarchy during a first encounter, but this hierarchy is not maintained when the same pair is confronted again one week later. When one of the rats is stressed just before the first encounter (Ps), the stressed rat becomes the subordinate rat of the pair during the first encounter, and the formed hierarchy is stable and lasts for at least one week (Cordero and Sandi, 2007). Here, we have presented further evidence confirming this model and attempted to investigate neurobiological mechanisms involved in the long-term facilitation of the hierarchy by stress.

The neuropeptides OT and AVP play an important role in the regulation of social behavior and stress responses (Ferguson et al., 2000; Ferguson et al., 2001; Bielsky and Young, 2004; Crawley et al., 2007). In the current study we have examined the role of the neuropeptide receptors OTR and V1aR in the establishment and maintenance of dominance hierarchies that is induced by exposure to acute stress. We have studied immediate and long-term changes in mRNA levels of *Otr* and *V1ar* in MeA and LS, brain areas that are involved in social memory, aggressive behavior and regulation of the stress response. We have subsequently tested whether administration of antagonists of OTR in the MeA and of V1aR in the LS could mimic the effects of acute stress on the long-term establishment of a dominance hierarchy. Our results support the view that OTR in the MeA plays a role in the initial stages of the long-term establishment of the hierarchy in the subordinate rat of the pair, whereas V1aR in the LS plays a role at a later time point during re-exposure to the other rat of the dyad, possibly in the retrieval of the memory or in the regulation of aggressive behavior.

First, we performed an experiment in which the effect of acute stress on the establishment and maintenance of dominance hierarchies was studied. As before (Cordero and Sandi, 2007), we showed that rats of Pns establish a hierarchy on day 1, but that this hierarchy is not maintained in the water competition test one week after the first encounter. In Ps, the stressed rats became subordinate during the first encounter and in the water competition test on day 8 the same hierarchy as on day 1 was found.

Next, we performed an experiment to study potential changes in *Otr* and *V1ar* mRNA levels in the MeA and LS at different times after establishment of a dominance hierarchy under the influence of stress. In MeA, subordinate rats of Ps showed lower mRNA levels for the *Otr* than dominant rats of Ps and both (dominant and subordinate) rats of Pns at 3h after

the first encounter. After one week, the differences in *Otr* mRNA levels in MeA have disappeared. In LS, there were no clear differences in *Otr* mRNA levels between the groups, except for an increase in the level one week after the encounter in the subordinate rat of Ps compared to controls. No changes among the different groups and comparisons were observed in *V1ar* mRNA levels in the MeA, both at 3h and one week after the first encounter. In LS, the subordinate rats of Ps have lower *V1ar* mRNA levels compared to the control group and the dominant rats of both Pns and Ps. No changes were found at 3h after the first encounter.

The decrease in *Otr* mRNA in the subordinate rats of Ps 3h after the first encounter led to the question whether the long-term establishment of the hierarchy in Ps could be mimicked by administration of a selective OT antagonist (OTA) into the MeA of non-stressed subordinate rats immediately after the first encounter. In control animals that were infused with vehicle, the hierarchy that was established on day 1 was clear, but it was not maintained in a second encounter 1 week later. When the subordinate rat of the pair was infused with OTA immediately after the encounter the hierarchy found in the WCT after one week was the same as on day 1. Thus, mimicking the reduction of *Otr* mRNA in the MeA by administration of a selective OTA to non-stressed subordinate rats leads to a long-term establishment of a dominance hierarchy. To exclude non-specific effects of OTA treatment, a control experiment was performed in which one rat of each pair was infused with vehicle and the other with OTA on day 1, without exposure to an encounter. One week after the infusion the first encounter took place. There was no long-term effect of OTA administration on the outcome of this first encounter, which supports the view that the effect on long-term hierarchy establishment that was found for OTA administered to non-stressed subordinate rats is specific for the combination with the social encounter.

Finally, an experiment was performed to test whether the administration of a V1aR antagonist into the LS of the subordinate rats of Ps just before a second encounter can mimic the long-term maintenance of the dominance hierarchy as induced by exposure to acute stress. When the two rats of a non-stressed pair were infused with vehicle into the LS before the second encounter, no maintenance of the hierarchy as established on day 1 was found. When the subordinate rats of non-stressed pairs were infused with a selective V1aR antagonist into the LS 30 min before the second encounter, the subordinate V1aR-treated rats became again subordinate during this second encounter. This shows that mimicking the downregulation of V1aR in the LS by giving an antagonist of this receptor type at the time of a second encounter induces submission.

Systemic and central administrations of low doses of OT have been shown to facilitate social recognition of a juvenile rat by adult male rats, whereas systemic administration of high doses attenuates social recognition. This suggests that the effect of OT on social recognition in males follows an inverted U-shape response (Popik and Van Ree, 1991; Popik and Vetulani, 1991; Popik et al., 1992; Benelli et al., 1995). In female rats, OT infused i.c.v. has no effect on social recognition, although infusion of an OTA interferes with social recognition (Engelmann et al., 1998). Similarly, in female mice, administration of OTR antisense DNA into the MeA blocks social recognition (Choleris et al., 2007). In all these cases administration of OT or OTA took place before the social interaction. In the current experiment OTA was infused after the encounter and the effect was measured 1 week later. Thus, during the first social encounter OT signaling was unaffected by the treatment. The present finding of a downregulation in OTR mRNA levels in the MeA might be due to a high release of OT during the exposure to stress and/or the first encounter, which could enhance the social memory formation, although OT release is something that has not been measured in the current experiment. However, cell culture experiments have shown that exposure to OT results in a downregulation of OTR (Insel et al., 1992; Di Scala-Guenot and Strosser, 1995). The MeA is one of the first brain areas in the pathway of brain areas processing social information to become activated after exposure to a conspecific. OT in MeA has been shown to be essential for the processing or initial retention of social information by using OT knockout mice. These mice can form a spatial memory and have normal smell, but cannot form social memories. Infusion of OT into the MeA is sufficient to restore the social memory (Ferguson et al., 2000; Ferguson et al., 2001). OT knockout mice show normal social approach, social interaction, perception and recognition of social cues, which suggests that OT is required selectively for higher order processing of social memory formation (Crawley et al., 2007). Therefore, it is plausible to hypothesize that the stress-induced enhancement of a memory for the dominance hierarchy is linked to increased OT release in the MeA at the time of the first encounter, and to the subsequent observed downregulation in OTR expression, which, on its turn, might affect downstream targets such as the LS.

The role of AVP in social recognition has been more widely studied and has been more clearly established than the role of OT. In rats, systemic or central AVP injections enhance social recognition, as does administration of V1aR viral vectors in the LS (Le Moal et al., 1987; Dantzer et al., 1988; Popik et al., 1991; Popik and Van Ree, 1992; Engelmann and Landgraf, 1994). Administration of antagonists of AVP and V1aR and downregulation of V1aR in LS disrupt social recognition in rats (Dantzer et al., 1987; Dantzer et al., 1988;

Bluthe et al., 1990; Landgraf et al., 1995; van Wimersma Greidanus and Maigret, 1996; Everts and Koolhaas, 1997, 1999; Landgraf et al., 2003). Studies using V1aR knockout mice show that these mice have impaired social recognition (Bielsky et al., 2004). In mice, V1aR is specifically important in the LS for social recognition, not in the MeA. Increased levels of AVP release and V1aR activation may play a role in increasing the duration of social memories (Bielsky et al., 2005).

Besides its role in social recognition, the role of AVP in aggression has been clearly established. Central infusions of AVP facilitate aggressive behavior in rats and hamsters (Ferris et al., 1984; Irvin et al., 1990; Winslow and Insel, 1993; Delville et al., 1996). In hamsters, administration of V1aR antagonists blocks aggressive behavior when infused into the anterior hypothalamus (Ferris et al., 1985; Ferris and Potegal, 1988; Delville et al., 1996; Ferris et al., 2006). More specifically, V1aR antagonists infused in the anterior hypothalamus of the dominant hamster blocks the offensive behavior flank marking in the dominant hamster and increases flank marking in the untreated subordinate hamster, whereas administration of AVP in subordinates increases flank marking (Ferris et al., 1985). During a resident-intruder test, aggressive rats show an increased AVP release in the LS, with the level of aggression and AVP release in the LS being positively correlated (Veenema and Neumann, 2008; Veenema et al., 2010). After exposure to a resident-intruder test, a negative correlation between AVP content and fiber density in LS and intermale aggression was found in rats (Everts et al., 1997; Everts and Koolhaas, 1999). Exposure to a food competition test led to a decrease in V1aR binding in both dominant and subordinate rats in LS, but dominant rats showed a stronger decrease in V1aR binding than subordinate rats (Askew et al., 2006). Lesions of LS after a food competition test reversed the hierarchical status when the dominant rat was lesioned, but not when the subordinate rat was lesioned (Costanzo et al., 1977). Aggressive mice show a higher AVP innervation in the LS than non-aggressive mice (Compaan et al., 1993). V1aR knockout mice show normal aggressive behavior, whereas V1bR knockout mice show no offensive aggression at all (Wersinger et al., 2002; Wersinger et al., 2007a; Wersinger et al., 2007b).

Stress induces changes in both the oxytocinergic and vasopressinergic system, and OT on its turn regulates the HPA axis. Exposure to stressors such as forced swimming and social defeat leads to the release of OT and AVP from the SON and/or the PVN of the hypothalamus (Wotjak et al., 1996; Wotjak et al., 1998; Engelmann et al., 1999; Neumann, 2007), of OT in the LS (Ebner et al., 2000) and in the CeA (Ebner et al., 2005). Administration of OT leads to suppression of stress-induced HPA axis activity and activates downstream brain areas such as

LS (Windle et al., 1997; Windle et al., 2004). Exposure to restraint stress activates the MeA and OT neurons in the PVN. OT knockout female mice show a higher corticosterone release after exposure to stress and less stress-induced MeA activation (Mantella et al., 2004). The expression of OTR in the brain is upregulated by dexamethasone, glucocorticoids and after prolonged stress (Patchev et al., 1993; Liberzon and Young, 1997). V1aR is regulated by glucocorticoids too. Adrenalectomy reduces V1aR density in LS, which can be reversed by administration of dexamethasone. Glucocorticoids might facilitate memory formation and the retention of information by enhancing AVP transmission by increasing V1aR density (Watters et al., 1996). The increase in OT release in the amygdala leads to inhibition of the HPA axis and to a more passive coping style. This is thought to prevent an excessive stress response (Neumann et al., 2000b; Windle et al., 2004; Ebner et al., 2005). Interestingly, we have recently found evidence for a role of glucocorticoids in the long-term established of the social hierarchy in our model (Timmer and Sandi, 2010). A possible mechanism by which OT and AVP can have an effect on the long-term establishment of a dominance hierarchy might be through an effect of exposure to acute stress on the release of these neuropeptides from the hypothalamus and other brain areas. It has been shown that exposure to a variety of stressors induces OT and AVP release from the hypothalamus or OT release from LS (Wotjak et al., 1996; Engelmann et al., 1999; Ebner et al., 2000). An increase in OT release in the MeA of the subordinate rat of the stressed pair could play a role in the enhancement of a long-term memory for a dominance hierarchy. Since the MeA has a vasopressinergic output to the downstream area LS, changes in OT signaling in the MeA might lead to changes in V1aR mRNA levels in LS, which might play a role at a later time point in the effects of acute stress on hierarchy establishment.

OT release in the amygdala leads to a more passive coping style (Ebner et al., 2005), whereas AVP release in LS leads to a more active coping style (Ebner et al., 1999; Koolhaas et al., 2010). An active coping style is associated with a higher aggression level (reviewed by Koolhaas et al., 2010). It is therefore tempting to hypothesize that, in our model, exposure to acute stress might lead to a more passive coping style through an increased OT release in the amygdala and this, in turn, plays a role in the stressed rat becoming subordinate. Furthermore, on the long-term maintenance of the hierarchy, the lower V1aR mRNA expression in LS observed in the stress condition one week after the first encounter (or administration of a selective V1aR antagonist in subordinate, not formerly stressed rats) could lead to a decreased sensitivity to AVP release in LS.

In summary, we have shown changes in the oxytocinergic and vasopressinergic system that are possibly involved in the long-term establishment of a dominance hierarchy as facilitated by stress. We found a lower *Otr* mRNA level in the MeA of subordinate rats of stressed pairs at 3h after a first social encounter compared to the dominant rats of stressed pairs and both dominant and subordinate rats of non-stressed pairs. Administration of a selective OTA into the MeA of non-stressed subordinate rats immediately after a first encounter induced a long-term memory for the dominance hierarchy. Long-term changes that were found were a lower *V1aR* mRNA level in the LS of the subordinate rats of stressed pairs compared to controls and dominant rats of both non-stressed and stressed pairs, one week after the first encounter. Administration of a selective V1aR antagonist into the LS of the subordinate rats of non-stressed pairs mimics the effect of exposure to acute stress on the long-term establishment of a dominance hierarchy. These findings suggest that changes in oxytocinergic and vasopressinergic systems are involved in the immediate and long-term effects of exposure to acute stress on the maintenance of a dominance hierarchy.

3.6 Acknowledgements

This work was supported by grants from the EU 7th (FP7- HEALTH-F2M-2007-201600; MemStick) FP and from the Swiss National Science Foundation (310000-120791) to C.S. The authors wish to thank to Grégoire Parchet and Angélique Vaucher for technical assistance and Manuel Bueno and Sylvain Lengacher for their advise on the gene expression experiments.

3.7 References

Askew A., Gonzalez F.A., Stahl J.M., Karom M.C. (2006). Food competition and social experience effects on V1a receptor binding in the forebrain of male Long-Evans hooded rats. Horm Behav 49:328-336.

Benelli A., Bertolini A., Poggioli R., Menozzi B., Basaglia R., Arletti R. (1995). Polymodal dose-response curve for oxytocin in the social recognition test. Neuropeptides 28:251-255.

Bielsky I.F., Hu S.B., Ren X., Terwilliger E.F., Young L.J. (2005). The V1a vasopressin receptor is necessary and sufficient for normal social recognition: a gene replacement study. Neuron 47:503-513.

Bielsky I.F., Hu S.B., Szegda K.L., Westphal H., Young L.J. (2004). Profound impairment in social recognition and reduction in anxiety-like behavior in vasopressin V1a receptor knockout mice. Neuropsychopharmacology 29:483-493.

Bielsky I.F., Young L.J. (2004). Oxytocin, vasopressin, and social recognition in mammals. Peptides 25:1565-1574.

Blanchard R.J., Flannelly K.J., Blanchard D.C. (1988). Life-span studies of dominance and aggression in established colonies of laboratory rats. Physiol Behav 43:1-7.

Bluthe R.M., Schoenen J., Dantzer R. (1990). Androgen-dependent vasopressinergic neurons are involved in social recognition in rats. Brain Res 519:150-157.

Choleris E., Little S.R., Mong J.A., Puram S.V., Langer R., Pfaff D.W. (2007). Microparticle-based delivery of oxytocin receptor antisense DNA in the medial amygdala blocks social recognition in female mice. Proc Natl Acad Sci U S A 104:4670-4675.

Compaan J.C., Buijs R.M., Pool C.W., De Ruiter A.J., Koolhaas J.M. (1993). Differential lateral septal vasopressin innervation in aggressive and nonaggressive male mice. Brain research bulletin 30:1-6.

Cordero M.I., Sandi C. (2007). Stress amplifies memory for social hierarchy. Frontiers in neuroscience 1:175-184.

Costanzo D.J., Enloe L.J., Hothersall D. (1977). Effects of septal lesions on social dominance in rats. Behav Biol 20:454-462.

Crawley J.N., Chen T., Puri A., Washburn R., Sullivan T.L., Hill J.M., Young N.B., Nadler J.J., Moy S.S., Young L.J., Caldwell H.K., Young W.S. 3rd (2007). Social approach behaviors in oxytocin knockout mice: Comparison of two independent lines tested in different laboratory environments. Neuropeptides.

Dantzer R., Bluthe R.M., Koob G.F., Le Moal M. (1987). Modulation of social memory in male rats by neurohypophyseal peptides. Psychopharmacology 91:363-368.

Dantzer R., Koob G.F., Bluthe R.M., Le Moal M. (1988). Septal vasopressin modulates social memory in male rats. Brain Res 457:143-147.

Delville Y., Mansour K.M., Ferris C.F. (1996). Serotonin blocks vasopressin-facilitated offensive aggression: interactions within the ventrolateral hypothalamus of golden hamsters. Physiol Behav 59:813-816.

Di Scala-Guenot D., Strosser M.T. (1995). Downregulation of the oxytocin receptor on cultured astroglial cells. Am J Physiol 268:C413-418.

Ebner K., Bosch O.J., Kromer S.A., Singewald N., Neumann I.D. (2005). Release of oxytocin in the rat central amygdala modulates stress-coping behavior and the release of excitatory amino acids. Neuropsychopharmacology 30:223-230.

Ebner K., Wotjak C.T., Holsboer F., Landgraf R., Engelmann M. (1999). Vasopressin released within the septal brain area during swim stress modulates the behavioural stress response in rats. Eur J Neurosci 11:997-1002.

Ebner K., Wotjak C.T., Landgraf R., Engelmann M. (2000). A single social defeat experience selectively stimulates the release of oxytocin, but not vasopressin, within the septal brain area of male rats. Brain Res 872:87-92.

Engelmann M., Ebner K., Landgraf R., Holsboer F., Wotjak C.T. (1999). Emotional stress triggers intrahypothalamic but not peripheral release of oxytocin in male rats. Journal of Neuroendocrinology 11:867-872.

Engelmann M., Ebner K., Wotjak C.T., Landgraf R. (1998). Endogenous oxytocin is involved in short-term olfactory memory in female rats. Behav Brain Res 90:89-94.

Engelmann M., Landgraf R. (1994). Microdialysis administration of vasopressin into the septum improves social recognition in Brattleboro rats. Physiol Behav 55:145-149.

Engelmann M., Wotjak C.T., Neumann I.D., Ludwig M., Landgraf R. (1996). Behavioral consequences of intracerebral vasopressin and oxytocin: focus on learning and memory. Neurosci Biobehav Rev 20:341-358.

Everts H.G., De Ruiter A.J., Koolhaas J.M. (1997). Differential lateral septal vasopressin in wild-type rats: correlation with aggression. Horm Behav 31:136-144.

Everts H.G., Koolhaas J.M. (1997). Lateral septal vasopressin in rats: role in social and object recognition? Brain Res 760:1-7.

Everts H.G., Koolhaas J.M. (1999). Differential modulation of lateral septal vasopressin receptor blockade in spatial learning, social recognition, and anxiety-related behaviors in rats. Behav Brain Res 99:7-16.

Ferguson J.N., Aldag J.M., Insel T.R., Young L.J. (2001). Oxytocin in the medial amygdala is essential for social recognition in the mouse. J Neurosci 21:8278-8285.

Ferguson J.N., Young L.J., Hearn E.F., Matzuk M.M., Insel T.R., Winslow J.T. (2000). Social amnesia in mice lacking the oxytocin gene. Nature genetics 25:284-288.

Ferguson J.N., Young L.J., Insel T.R. (2002). The neuroendocrine basis of social recognition. Front Neuroendocrinol 23:200-224.

Ferris C.F., Albers H.E., Wesolowski S.M., Goldman B.D., Luman S.E. (1984). Vasopressin injected into the hypothalamus triggers a stereotypic behavior in golden hamsters. Science 224:521-523.

Ferris C.F., Lu S.F., Messenger T., Guillon C.D., Heindel N., Miller M., Koppel G., Robert Bruns F., Simon N.G. (2006). Orally active vasopressin V1a receptor antagonist, SRX251, selectively blocks aggressive behavior. Pharmacology, biochemistry, and behavior 83:169-174.

Ferris C.F., Pollock J., Albers H.E., Leeman S.E. (1985). Inhibition of flank-marking behavior in golden hamsters by microinjection of a vasopressin antagonist into the hypothalamus. Neuroscience letters 55:239-243.

Ferris C.F., Potegal M. (1988). Vasopressin receptor blockade in the anterior hypothalamus suppresses aggression in hamsters. Physiol Behav 44:235-239.

Gimpl G., Fahrenholz F. (2001). The oxytocin receptor system: structure, function, and regulation. Physiol Rev 81:629-683.

Goodson J.L. (2005). The vertebrate social behavior network: Evolutionary themes and variations. Horm Behav 48.

Insel T.R., Winslow J.T., Witt D.M. (1992). Homologous regulation of brain oxytocin receptors. Endocrinology 130:2602-2608.

Irvin R.W., Szot P., Dorsa D.M., Potegal M., Ferris C.F. (1990). Vasopressin in the septal area of the golden hamster controls scent marking and grooming. Physiol Behav 48:693-699.

Koolhaas J.M., de Boer S.F., Coppens C.M., Buwalda B. (2010). Neuroendocrinology of coping styles: towards understanding the biology of individual variation. Front Neuroendocrinol 31:307-321.

Landgraf R., Frank E., Aldag J.M., Neumann I.D., Sharer C.A., Ren X., Terwilliger E.F., Niwa M., Wigger A., Young L.J. (2003). Viral vector-mediated gene transfer of the vole V1a vasopressin receptor in the rat septum: improved social discrimination and active social behaviour. Eur J Neurosci 18:403-411.

Landgraf R., Gerstberger R., Montkowski A., Probst J.C., Wotjak C.T., Holsboer F., Engelmann M. (1995). V1 vasopressin receptor antisense oligodeoxynucleotide into septum reduces vasopressin binding, social discrimination abilities, and anxiety-related behavior in rats. J Neurosci 15:4250-4258.

Le Moal M., Dantzer R., Michaud B., Koob G.F. (1987). Centrally injected arginine vasopressin (AVP) facilitates social memory in rats. Neuroscience letters 77:353-359.

Liberzon I,. Young E.A. (1997). Effects of stress and glucocorticoids on CNS oxytocin receptor binding. Psychoneuroendocrinology 22:411-422.

Livak K.J., Schmittgen T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.

Mantella R.C., Vollmer R.R., Rinaman L., Li X., Amico J.A. (2004). Enhanced corticosterone concentrations and attenuated Fos expression in the medial amygdala of female oxytocin knockout mice exposed to psychogenic stress. Am J Physiol Regul Integr Comp Physiol 287:R1494-1504.

Neumann I.D. (2007). Stimuli and consequences of dendritic release of oxytocin within the brain. Biochem Soc Trans 35:1252-1257.

Neumann I.D., Kromer S.A., Toschi N., Ebner K. (2000a). Brain oxytocin inhibits the (re)activity of the hypothalamo-pituitary-adrenal axis in male rats: involvement of hypothalamic and limbic brain regions. Regulatory peptides 96:31-38.

Neumann I.D., Wigger A., Torner L., Holsboer F., Landgraf R. (2000b). Brain oxytocin inhibits basal and stress-induced activity of the hypothalamo-pituitary-adrenal axis in male and female rats: partial action within the paraventricular nucleus. J Neuroendocrinol 12:235-243.

Newman S.W. (1999). The medial extended amygdala in male reproductive behavior. A node in the mammalian social behavior network. Ann N Y Acad Sci 877:242-257.

Ostrowski N.L. (1998). Oxytocin receptor mRNA expression in rat brain: implications for behavioral integration and reproductive success. Psychoneuroendocrinology 23:989-1004.

Ostrowski N.L., Lolait S.J., Young W.S. 3rd. (1994). Cellular localization of vasopressin V1a receptor messenger ribonucleic acid in adult male rat brain, pineal, and brain vasculature. Endocrinology 135:1511-1528.

Patchev V.K., Schlosser S.F., Hassan A.H., Almeida O.F. (1993). Oxytocin binding sites in rat limbic and hypothalamic structures: site-specific modulation by adrenal and gonadal steroids. Neuroscience 57:537-543.

Paxinos G., Watson C. (1998). The rat brain in stereotaxic coordinates. Academic Press, New York.

Popik P., Van Ree J.M. (1991). Oxytocin but not vasopressin facilitates social recognition following injection into the medial preoptic area of the rat brain. Eur Neuropsychopharmacol 1:555-560.

Popik P., Van Ree J.M. (1992). Long-term facilitation of social recognition in rats by vasopressin related peptides: a structure-activity study. Life Sci 50:567-572.

Popik P., Vetulani J. (1991). Opposite action of oxytocin and its peptide antagonists on social memory in rats. Neuropeptides 18:23-27.

Popik P., Vetulani J., van Ree J.M. (1992). Low doses of oxytocin facilitate social recognition in rats. Psychopharmacology 106:71-74.

Popik P., Vetulani J., Van Ree J.M. (1996). Facilitation and attenuation of social recognition in rats by different oxytocin-related peptides. Eur J Pharmacol 308:113-116.
Popik P., Wolterink G., De Brabander H., van Ree J.M. (1991). Neuropeptides related to [Arg8]vasopressin facilitates social recognition in rats. Physiol Behav 49:1031-1035.
Timmer M., Sandi C. (2010). A role for glucocorticoids in the long-term establishment of a social hierarchy. Psychoneuroendocrinology, 35(10): 1543-1552.
Van Kreveld D. (1970). A selective review of dominance-subordinate relations in animals. Genetics and Psychology Monographs 81:143-173.
van Wimersma Greidanus T.B., Maigret C. (1996). The role of limbic vasopressin and oxytocin in social recognition. Brain Res 713:153-159.
Veenema A.H., Beiderbeck D.I., Lukas M., Neumann I.D. (2010). Distinct correlations of vasopressin release within the lateral septum and the bed nucleus of the stria terminalis with the display of intermale aggression. Horm Behav 58:273-281.
Veenema A.H., Neumann I.D. (2008). Central vasopressin and oxytocin release: regulation of complex social behaviours. Progress in brain research 170:261-276.
Watters J.J., Wilkinson C.W., Dorsa D.M. (1996). Glucocorticoid regulation of vasopressin V1a receptors in rat forebrain. Brain Res Mol Brain Res 38:276-284.
Wersinger S.R., Caldwell H.K., Christiansen M., Young W.S. 3rd. (2007a). Disruption of the vasopressin 1b receptor gene impairs the attack component of aggressive behavior in mice. Genes Brain Behav 6:653-660.
Wersinger S.R., Caldwell H.K., Martinez L., Gold P., Hu S.B., Young W.S. 3rd. (2007b). Vasopressin 1a receptor knockout mice have a subtle olfactory deficit but normal aggression. Genes Brain Behav 6:540-551.
Wersinger S.R., Ginns E.I., O'Carroll A.M., Lolait S.J., Young W.S. 3rd. (2002). Vasopressin V1b receptor knockout reduces aggressive behavior in male mice. Molecular psychiatry 7:975-984.
Windle R.J., Kershaw Y.M., Shanks N., Wood S.A., Lightman S.L., Ingram C.D. (2004). Oxytocin attenuates stress-induced c-fos mRNA expression in specific forebrain regions associated with modulation of hypothalamo-pituitary-adrenal activity. J Neurosci 24:2974-2982.
Windle R.J., Shanks N., Lightman S.L., Ingram C.D. (1997). Central oxytocin administration reduces stress-induced corticosterone release and anxiety behavior in rats. Endocrinology 138:2829-2834.
Winslow J.T., Insel T.R. (1993). Effects of central vasopressin administration to infant rats. Eur J Pharmacol 233:101-107.
Wotjak C.T., Ganster J., Kohl G., Holsboer F., Landgraf R., Engelmann M. (1998). Dissociated central and peripheral release of vasopressin, but not oxytocin, in response to repeated swim stress: new insights into the secretory capacities of peptidergic neurons. Neuroscience 85:1209-1222.
Wotjak C.T., Kubota M., Liebsch G., Montkowski A., Holsboer F., Neumann I.D., Landgraf R. (1996). Release of vasopressin within the rat paraventricular nucleus in response to emotional stress: a novel mechanism of regulating adrenocorticotropic hormone secretion? J Neurosci 16:7725-7732.

Chapter 4 - The long-term effects of acute stress and hierarchy establishment on aggressive behavior towards an unfamiliar opponent and on monoamine oxidase A and androgen receptor mRNA levels

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4.1 Abstract

Exposure to acute stress affects the long-term establishment of dominance hierarchies. In the present study, we investigated the long-term effect of acute stress and hierarchy establishment on the establishment of a second hierarchy with an unfamiliar opponent, one week after a first encounter. We show that the behavior of a stressed subordinate rat towards an unfamiliar opponent depends on the previous status of the opponent: when paired with a previously dominant rat it becomes subordinate, whereas it becomes dominant when paired with a previously subordinate rat. Furthermore, we show that monoamine oxidase A and androgen receptor mRNA levels are downregulated in the lateral septum of stressed subordinate rats at one week after the first encounter; i.e., at the time when we have observed their flexible social behavior against unfamiliar opponents. 15 μg of the monoamine oxidase A inhibitor clorgyline was injected into the lateral septum to examine whether there was a causal relationship between changes in monoamine oxidase A mRNA levels and aggressive behavior towards an unfamiliar subordinate opponent. However, our results did not support this hypothesis.

Key words: dominance hierarchy; aggression; monoamine oxidase a; subordinate; rat; clorgyline.

4.2 Introduction

Animals that live together in groups form dominance hierarchies to minimize the cost of fighting for resources, space and females (Van Kreveld, 1970). Factors that determine which individual becomes dominant can be divided into intrinsic factors, such as size, age, sex, strength, physiology and level of aggressiveness (Parker, 1974; Chase et al., 2002) and extrinsic factors, such as winner and loser effects (reviewed by Chase et al., 1994). According to the winner and loser effects, an animal that has won an encounter is more likely to win subsequent encounters, and an animal that has lost an encounter is more likely to lose subsequent encounters. Rats form almost linear dominance hierarchies in which each individual is dominant over all the rats with a lower rank and subordinate to all the rats with a higher rank (Berdoy et al., 1995).

Aggression is important in the establishment and maintenance of dominance hierarchies. Normal aggression is important for the survival of an individual, but excessive aggression or aggression that is expressed out of context is considered to be pathological (reviewed by Nelson and Trainor, 2007). The neural circuitry that has been involved in the regulation of aggression consists of the medial preoptic area (MPOA), lateral septum (LS), anterior hypothalamus (AH), ventromedial hypothalamus (VMH), periaquaductal grey (PAG), medial amygdala (MeA) and bed nucleus of stria terminalis (BNST) (Nelson and Trainor, 2007). Lesions of LS, BNST, AH and MeA have been shown to reduce intermale aggression (reviewed by Kruk, 1991). To ensure the success of living in groups, impulsivity and aggression need to be carefully modulated (Lesch and Merschdorf, 2000). The neurotransmitters serotonin (5-HT), dopamine and gamma-aminobutyric acid (GABA) play a role in the regulation of aggression (reviewed by de Almeida et al., 2005).

Monoamine oxidase A (MAOA) is a mitochondrial enzyme involved in the degradation of 5-HT, dopamine and noradrenaline. The activity of MAOA is related to aggression in animals and humans. MAOA knockout mice are more aggressive (Cases et al., 1995). In humans, studies on polymorphisms of MAOA show that the presence of a low activity allele of MAOA (McDermott et al., 2009), low activity of MAOA in depressed patients (Alia-Klein et al., 2008) or the lack of a functional MAOA gene (Brunner et al., 1993) is related to higher aggression.

Steroid hormones such as testosterone promote aggressiveness in LS, amygdala and the dorsal raphe nucleus (Simon et al., 1998). In subordinate rats living in a visible burrow system the level of testosterone is reduced (Blanchard et al., 1993, 1995; Hardy et al., 2002). Work in

mice indicated that androgens do not play a role in the regulation of submission (Leshner and Politch, 1979), but they were shown to affect the intensity of aggression (Leshner and Moyer, 1975). Although, in general, stress leads to a reduction in testosterone (Retana-Marquez et al., 2003; Dong et al., 2004), it was also shown that acute stress can lead to an increase in testosterone in the initial phases of acute stress, mainly during agonistic encounters (reviewed by Chichinadze and Chichinadze, 2008).

In an animal model developed in our group, exposure to acute stress was shown to affect the establishment and maintenance of a dominance hierarchy in male adult rats (Cordero and Sandi, 2007). More precisely, non-stressed pairs (Pns; consisting of two unstressed rats) establish a hierarchy during a first encounter, but do not form a long-term memory for the hierarchy, as tested in a water competition test one week after the first encounter. However, when one rat of a pair is stressed (Ps, stressed pair), the stressed rat usually becomes the subordinate rat of the pair and a long-term memory for the hierarchy is formed.

The aim of the current experiment was to investigate the long-term effect of stress and the rank obtained in a first encounter on aggressive behavior towards an unfamiliar rat one week after the first encounter, as well as potential neurobiological mechanisms underlying stress effects. For this purpose, we evaluated behavior of both the dominant and subordinate rat of Ps when exposed to an unfamiliar unstressed rat that had been either dominant or subordinate in a previous encounter, or naïve. Then, we examined potential changes in *Maoa* and androgen receptor *(Ar)* mRNA levels in several brain areas that are involved in social behaviors and aggression: MeA, LS and BNST. Finally, in order to test whether the observed changes in gene expression were functionally involved in regulation of the long-term effects of stress and a first encounter on aggressive behavior, we investigated the behavioral implications of injecting the MAOA inhibitor clorgyline into the LS of one rat on each pair before an encounter with an unfamiliar rat, one week after a first encounter.

4.3 Material and Methods

Animals

Male Wistar rats (Charles River Laboratories, Lyon, France) weighing 250-275 grams at arrival were individually housed with *ad libitum* access to food and water. Animals were kept in a 12h light/dark cycle with lights on at 0700h, and a constant temperature of 22 ± 2 °C. Animals were left undisturbed for one week after arrival before starting the experiment, except when surgery was performed, which was performed a few days after arrival. Rats were weighed once per week. Experiments were performed with approval of the Cantonal Veterinary Authorities (Vaud, Switzerland).

All experiments took place between 0800h and 1400h, except for the water competition test that took place between 1400h and 1900h, after a water deprivation period of 6h (from 0800h until 1400h). All rats were handled for 2 min per day during three days before exposure to the elevated plus maze (see below).

Surgery

Rats were anaesthetized with ketamine (70 mg/kg) and xylazine (6 mg/kg), administered by i.p. injection. Bilateral 4 mm long stainless steel guide cannulae of 22 gauge with a distance of 1.5 mm between the guide cannulae (Plastics One, Roanoke, USA) were implanted in the LS at +0.20 mm anterior, ±0.75 mm lateral and -3.7 mm ventral relative to Bregma (Paxinos and Watson, 1998) using a standard stereotaxic frame (Stoelting, USA). Cannulae were fixed to the skull with dental acrylic cement (Kaladent, Switzerland) and anchored with Vetbond Tissue Adhesive glue (3M). Dummy cannulae (10mm for MeA, 4mm for LS, Plastics One, Roanoke, USA) were inserted into the cannulae to protect them from blocking. Rats received an injection with Antisedan (0.04 ml s.c., Pfizer, Switzerland) after the surgery to reverse the effects of the anesthetics. Paracetamol was added to the drinking water as analgesic in the days following surgery. The rats were allowed to recover for at least one week before the start of the experiment.

Drugs and infusion procedure

Clorgyline (N-methyl-N-propargyl-3-3[2,4-dichlorophenoxy]propylamine hydrochloride, Sigma-Aldrich, Switzerland) was used for infusions into the LS. Clorgyline was dissolved in saline. A bilateral internal injector of 6 mm (extending 2 mm from the end of the guide cannula, 28 gauge, Plastics One, Roanoke, USA) was used. The injectors were

connected to 10 μl microsyringes (SGE Analytical Science, Australia) on an automated infusion pump (Harvard Apparatus, MA, USA) through polyethylene tubing. 15 μg of clorgyline in 1 μl of saline was infused into the LS of each hemisphere at a rate of 0.5 μl/min. After the infusion the injector was left in place for 1 min to allow diffusion of the drug. Saline was used as a vehicle. Clorgyline was infused 3h before the second encounter to the one rat of each pair. Rats of control pairs were infused with vehicle.

Elevated plus maze

Before the start of the experiment the 'elevated plus maze' (EPM) test was performed to measure anxiety-related behavior. The EPM consists of two opposing open arms (45x10 cm) and two opposing closed arms (45x10 cm with walls of 50 cm high) that extend from a central platform (10x10 cm), elevated 65 cm above the floor. The rats were placed individually on the central platform facing the same closed arm and were allowed to explore the EPM freely during 5 min. The behavior of each rat was video recorded and analyzed using a computerized tracking system (Ethovision 3.1.16, Noldus IT, the Netherlands). Time spent in the open and closed arms was measured.

Dyads and groups

Rats were distributed to dyads consisting of two rats that were matched for their body weight and anxiety level. The anxiety level was defined by the time spent in the open arms of the EPM. Thus, the rats in each dyad were considered equal in their probability to become dominant or subordinate during a first encounter. The fur of both rats in a dyad was marked on different body parts (i.e. neck and back) to help identifying the animals. Rats were habituated to marking during the three day habituation period that took place before the first social encounter. Dyads were divided randomly over the different groups. Each group consisted of 6-14 dyads.

Stress delivery – Contextual fear conditioning

In non-stressed pairs (Pns) none of the rats were stressed prior to the social encounter. In stressed pairs (Ps) one rat of the pair was stressed using a contextual fear conditioning paradigm just before the first social encounter. Fear conditioning was performed in an observation chamber of 30x37x25 cm (Panlab, Spain) made of black steel walls and a Plexiglas ceiling and door. The floor consisted of 20 steel rods connected to a shock source

and solid-state scrambler for delivery of electrical shocks. The observation chamber was lit by a 20 W light bulb and there was a background noise of 68 dB. The protocol consisted of a habituation period of 3 min, followed by 3 footshocks of 1 mA lasting 1 sec each with an intershock interval of 60 sec. 30 sec after the last shock the rat was taken out and immediately submitted to the first social encounter with an unfamiliar rat in an adjacent room.

Food and water competition test

In order to habituate animals to the rewarding food used in the 'food competition test' (FCT), they received eight Chocopop flakes (Kellogg's, Switzerland) in their homecages daily, during two days before the start of the habituation to the experimental setup. The rats were habituated to the experimental setup for three days during which they were first placed in a clean homecage for 20 min and then in a food competition box for 10 min. The food competition box is a plastic box of 60x40 cm with walls of 32 cm high with a feeder containing eight Chocopops on one of the short sides. On day 1 of the experiment, the day after the last habituation day, the rats were submitted to a first encounter with an unfamiliar rat, consisting of a 'social interaction test' in a neutral homecage without food and water for 20 min, followed by a 'food competition test' (FCT) in which the rats had to compete for Chocopops during 10 min. After the tests each rat was returned to its homecage. The group without an encounter on day 1 was exposed to an extra day of habituation.

The memory for the formed hierarchy or behavior towards an unfamiliar individual was tested in a 'water competition test' (WCT) that was performed on day 8 after a preceding water deprivation period of 6h. Rats were exposed to the same opponent as on day 1 or to an unfamiliar opponent that was equal in anxiety level and body weight. The WCT was performed in a neutral homecage. After 2 min of habituation, a single bottle of water was presented and the behavior during the following 10 min was recorded.

Behavior was video-recorded and scored blindly using The Observer (v.5.0.25, Noldus IT, the Netherlands). The duration and frequency of offensive and defensive behaviors was scored. Offensive behaviors were attacks (biting), keeping down (pushing the opponent to the floor), offensive upright (standing on the hind legs in upright position) and lateral threat (pushing or approaching the opponent showing its side with an arched back). Defensive behavior consisted of freezing (immobility), defensive upright (standing on the hind legs in response to offensive upright) and submissive posture (lying on the back). In the FCT the number of consumed Chocopops and the number of passes over the feeder was counted and in the WCT the time spent drinking was measured. The rat that showed most offensive behavior

during the interaction was considered the dominant rat, the rat showing least offensive behavior and most defensive behavior the subordinate rat.

Control groups for studying gene expression levels were submitted to habituation and exposure to the experimental setup on day 1, with or without exposure to stress (control group and stressed control group), but not to social interaction.

Sacrifice

For the gene expression experiments rats were sacrificed either on day 1, three hours after the end of the food competition test, or on day 8, one week after the social interaction and food competition test under basal conditions. The animals were decapitated, the brain was quickly removed and frozen in isopentane at a temperature between -50 and -40 °C and stored at -80 °C until further processing.

In experiments involving cannulation, in order to check the position of the cannulae rats were injected i.p. with pentobarbital. 1 µl of ink was subsequently infused into the LS to mark the position of drug delivery. The rats were decapitated and the brains removed, frozen in isopentane and stored at -80 °C until further processing. 40 µm thick coronal sections were stained with thionin to detect the cannula position.

Laser capture micro dissection

For the gene expression experiment, coronal sections of 20 µm were taken for 'laser capture micro dissection' (LCM). The sections were put on membrane covered slides (Palm Microlaser Technologies, Carl Zeiss, Germany) and stored at -20 °C covered with RNA*later* ICE (Ambion, TX, USA). Prior to LCM the sections were stained with the HistoGene LCM Frozen Section Staining kit following the protocol (Arcturus Bioscience, Switzerland). The MeA and the LS from the right hemisphere were dissected through LCM. The target tissue was catapulted into a PALM adhesive cap (Palm Microlaser Technologies, Carl Zeiss, Germany) and subsequently incubated in 100 µl of lysis buffer (RNAqueous Micro Kit, Ambion, TX, USA) for 30 min at 42 °C. After spinning down the tissue was stored at -80 °C until further processing.

RNA isolation

RNA was isolated from the tissue using the RNAqueous Micro Kit (Ambion, TX, USA). First, 3 µl of LCM additive was added. Subsequently 129 µl of 100% ethanol was

added to the mixture. The lysate/ethanol mixture was loaded onto a Micro Filter Cartridge Assembly and centrifuged for 1 minute at 10000g. Afterward 180 µl of Wash Solution 1 was added and the mixture was centrifuged again for 1 min at 10000g. 180 µl of Wash Solution 2/3 was added and the mixture was centrifuged for 30 sec at 14000g. The flow-through was discarded and the Micro Filter Cartridge was centrifuged for another minute at 14000g. The Micro Filter Cartridge was transferred into a Micro Elution Tube and 8 µl of preheated Elution Solution was added. The mixture was left for 5 min at room temperature and subsequently centrifuged for 1 min at 14000g. Another 8 µl of Elution Solution was added and the mixture was centrifuged again at 14000g. The RNA was stored at -80 °C until further processing

Reverse transcription and preamplification

9.62 µl of the extracted RNA was converted into cDNA using Taqman Reverse Transcriptase Reagents (Applied Biosystems, CA, USA). 1.25 µl of random hexamers and 2.5 µl of RT 10x buffer were added to reach an end volume of 13.37 µl. The mixture was brought to 65 °C for 10 min followed by 10 min at room temperature in a Thermocycler (Primus 96, Peqlab, Germany). Following this step 5.5 µl of $MgCl_2$, 5 µl of dNTPs (10 mM), 0.5 µl of RNAse inhibitor and 0.63 µl of Multiscribe Reverse were added to each sample. Using the Thermocycler, the reverse transcription was executed for 10 min at 25 °C, 25 min at 48 °C and 5 min at 95 °C. The obtained cDNA was stored at -20 °C until further processing.

The cDNA was preamplified using Pre-amp MasterMix for TaqMan assays (Applied Biosystems, CA, USA). A pool of TaqMan Gene Expression Assays for the genes of interest (see 'Real time PCR') was made in ultrapure water (Gibco, NY, USA) at a volume of 1% for each Assay. 25 µl of Taqman PreAmp MasterMix, 12.5 µl of the pooled Assay mix and 12.5 µl of cDNA were mixed for each sample. The preamplification was done by an enzyme activation period of 10 min at 95 °C, followed by 10 cycles of 15 sec at 95 °C and 4 min at 60 °C. After finishing the run the samples were immediately placed on ice and diluted 5x in ice-cold ultrapure water (Gibco, NY, USA). Samples were stored at -20 °C until further processing.

Quantitative real time PCR

Quantitative real time PCR was performed using TaqMan Gene Expression Assays for the genes for monoamine oxidase A (*Maoa*) and the androgen receptor (*Ar*) with the control

genes ribosomal protein S18 (*Rps18*), ribosomal protein S29 (*Rps29*) and eukaryotic elongation factor 1 alpha 1 (*Eef1a1*) (see Table 1 for the used Gene Expression Assays, Applied Biosystems, CA, USA). First, a reaction mixture containing 4.5 µl preamplified cDNA, 0.5 µl 20x Taqman Gene Expression Assay for the genes of interest and 5 µl 2x PCR Master Mix (TaqMan Universal PCR master mix no AmpErase UNG, Applied Biosystems, CA, USA) per reaction of 10 µl was made. Triplicates of 10 µl of each reaction mixture were transferred to a well in a 384-well optical reaction plate (Applied Biosystems, CA, USA). The real time PCR was performed using 40 cycles of 15 sec at 95 °C and 1 min at 60 °C (7900HT, Applied Biosystems, CA, USA), preceded by 2 min at 50 °C and 10 min at 95 °C.

The software SDS (v.2.3, Applied Biosystems, CA, USA) was used for running and analyzing the quantitative real time PCR. Each target gene was compared to three internal control genes (*Rps18, Rps29, Eef1a1*) using the ΔΔCt method (described by Livak and Schmittgen, 2001). The results are expressed as fold change compared to the control groups.

Table 1. Overview of the used Gene Expression Assays.

Gene	Gene Expression Assay
Monoamine oxidase A *(Maoa)*	Rn01430958_m1
Androgen receptor *(Ar)*	Rn00560747_m1
Ribosomal protein S18 *(Rps18)*	Rn01428915_g1
Ribosomal protein S29 *(Rps29)*	Rn00820645_g1
Eukaryotic elongation factor 1a1 *(Eef1a1)*	Rn01639851_g1

Statistics

All results are expressed as the mean ± the standard error of the mean (S.E.M.).

For statistical analyses of mRNA expression levels, the fold change of the different groups compared to the non-stressed control group was calculated. One-way ANOVA was used to test statistical significance between the dominant and subordinate rats of Pns and Ps. *A priori* comparisons to test differences between two specific groups were established and evaluated through either unpaired or paired two-tailed Student t-tests. Paired t-tests were applied to compare mRNA expression levels of dominant and subordinate rats that competed together, while unpaired t-tests were applied when comparisons involved animals that had not directly interacted. *A priori* comparisons for group comparisons were (1) the subordinate rat of Ps with the subordinate rat of Pns, (2) the subordinate rat of Ps with the dominant rat of Ps,

(3) the dominant rat of Ps with the dominant rat of Pns, (4) the subordinate rat of Pns with the dominant rat of Pns, (5) the subordinate rat of Ps with the stressed control group and (6) each experimental group compared to non-stressed controls.

For the behavioral experiments, the percentage of offensive behaviors within each dyad was calculated. A paired two-tailed Student t-test was used to compare the percentage of offensive behaviors within a dyad of rats competing together.

The computer software SPSS for Windows (version 13.0) was used for statistical analysis. $P<0.05$ was considered significant.

4.4 Results

How becoming subordinate following stress affects social behavior to unfamiliar individuals in future encounters

First, we performed an experiment in which non-stressed and stressed rats were confronted to each other and established a dominance hierarchy on day 1. One week after the first encounter, both rats were exposed to an unfamiliar non-stressed rat that was previously dominant or subordinate in a first encounter one week earlier, or naïve. We examined the offensive behavior towards an unfamiliar individual and tested whether the behavior was different according to the previous status of the unfamiliar rat.

The rats of pairs consisting of a non-stressed and a stressed rat (Ps) established a hierarchy on day 1 with the stressed rats becoming subordinate ($t = 3.210$, $df = 21$, $p<0.01$, Fig. 1A&B). When the previously dominant rat of Ps was paired with a previously dominant or subordinate rat of Pns, or a naïve rat, the previous status of these unfamiliar rats did not affect the outcome of the WCT on day 8 (Fig. 1A), with the percentage of offensive behaviors

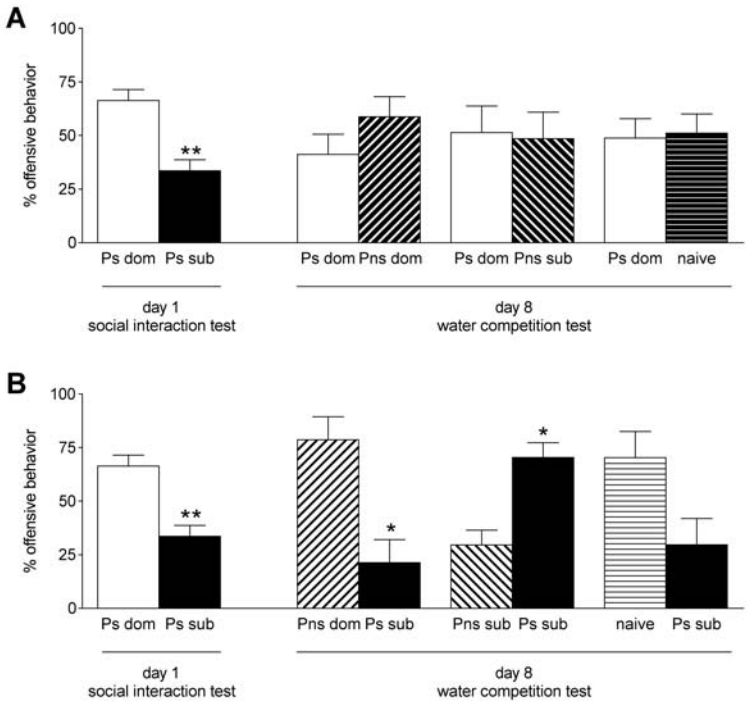

Figure 1. The percentage of total offensive behavior (mean ± S.E.M.) between two opponents shown in the social interaction test on day 1 and the water competition test on day 8. (A) The hierarchy of Ps on day 1 and of the subordinate rat of Ps when paired with a previously dominant (n=8), subordinate (n=8) or naïve (n=6) rat. (B) The hierarchy of Ps on day 1 and of the dominant rat of Ps when paired with a previously dominant (n=7), subordinate (n=9) or naïve (n=6) rat. The status 'dom' (dominant) or 'sub' (subordinate) refers to the status that was obtained during the social interaction test on day 1. * $p<0.05$, ** $p<0.01$ vs. the other rat of the pair.

in each animal in the dyad being fairly equivalent. However, when it was the stressed subordinate rat (Ps) that was exposed to an unfamiliar dominant, subordinate or naïve rat during the WCT, a different outcome was observed depending on the previous status of the unfamiliar rat. Thus, when the subordinate rat of Ps was paired with a previously dominant rat of Pns the subordinate rat of Ps became subordinate ($t = 2.663$, $df = 7$, $p<0.05$, Fig. 1B). When the subordinate rat of Ps was paired with a previously subordinate rat of Pns it became dominant during this second encounter ($t = -2.978$, $df = 7$, $p<0.05$, Fig. 1B). When paired with a naïve rat there was no significant difference ($t = 1.660$, $df = 5$, $p=0.1579$) between the offensive behavior of the subordinate rat of Ps and the naïve rat (Fig. 1B).

Changes in mRNA levels 3h and 1 week after a first encounter

Next, the immediate (3h) and long-term (1 week) changes in mRNA levels of monoamine oxidase A *(Maoa)* and the androgen receptor *(Ar)* after exposure to stress and/or a social encounter were studied in MeA, LS and BNST.

One-way ANOVA for data from the dominant and subordinate rats of both Pns and Ps indicates a lack of significant difference among dominant and subordinate rats of Pns and Ps for both *Maoa* and *Ar* in MeA, LS and BNST.

In MeA, the dominant rat of Ps shows a lower *Maoa* mRNA level than the subordinate rat 3h after the encounter, whereas in Pns the dominant rat shows a higher *Maoa* mRNA level than the subordinate rat (Table 2, Fig. 2A). The subordinate rats of Ps have a lower *Maoa* mRNA level in LS than the dominant rats of Ps 3h after the encounter (Table 2, Fig. 2B). In BNST, the dominant and subordinate rats of Pns, the subordinate rats of Ps and the stressed controls show an increase in *Maoa* mRNA compared to controls. Both in Pns and Ps the dominant rats have a lower *Maoa* mRNA levels than the subordinate rats of the same pair (Table 2, Fig. 2C).

One week after the first encounter there are no differences in *Maoa* mRNA in MeA (Fig. 2A) and BNST (Fig. 2C). In LS the subordinate rats of Ps show a decrease in *Maoa* mRNA compared to controls, stressed controls and the dominant rats of Ps (Table 2, Fig. 2B).

In summary, the main changes observed in the subordinate rats of Ps were found with regards to their dominant counterparts, and consisted of a decrease in *Maoa* mRNA in LS 3h and 1 week after the first encounter.

Table 2. Overview of the outcome of paired and unpaired t-tests that compare *Maoa* mRNA levels in MeA, LS and BNST. 'Pns dom' = dominant rat of Pns, 'Pns sub' = subordinate rat of Pns, 'Ps dom' = dominant rat of Ps, 'Ps sub' = subordinate rat of Ps, 'str ctl' = stressed control.

		Compared to Ps sub	Compared to Pns sub	Compared to control
MeA	3h	Ps dom: t = 2.231, df = 11, p<0.05	Pns dom: t = -2.501, df = 12, p<0.05	-
	1 week	-	-	-
LS	3h	Ps dom: t = 4.107, df = 11, p<0.01	-	-
	1 week	Pns dom: t = 3.155, df = 7, p<0.05 Str ctl: t = 2.797, df = 12, p<0.05	-	Ps sub: t = 2.149, df = 13, p = 0.05
BNST	3h	Ps dom: t = -3.567, df = 10, p<0.01	Pns dom: t = -4.245, df = 13, p<0.01	Pns dom: t = -2.554, df = 25, p<0.05 Pns sub: t = -3.116, df = 25, p<0.01 Ps sub: t = -2.570, df = 13, p<0.05 str ctl: t = -2.920, df = 25, p<0.01
	1 week	-	-	-

Three hours after the first encounter, the dominant rats of Pns have a lower *Ar* mRNA level in MeA than the subordinate rats of Pns (Table 3, Fig. 3A). In LS, no changes are found after 3h (Fig. 3B). The dominant and subordinate rats of Ps and the stressed controls show an increase in *Ar* mRNA compared to controls at 3h after the first encounter (Table 3, Fig. 3C).

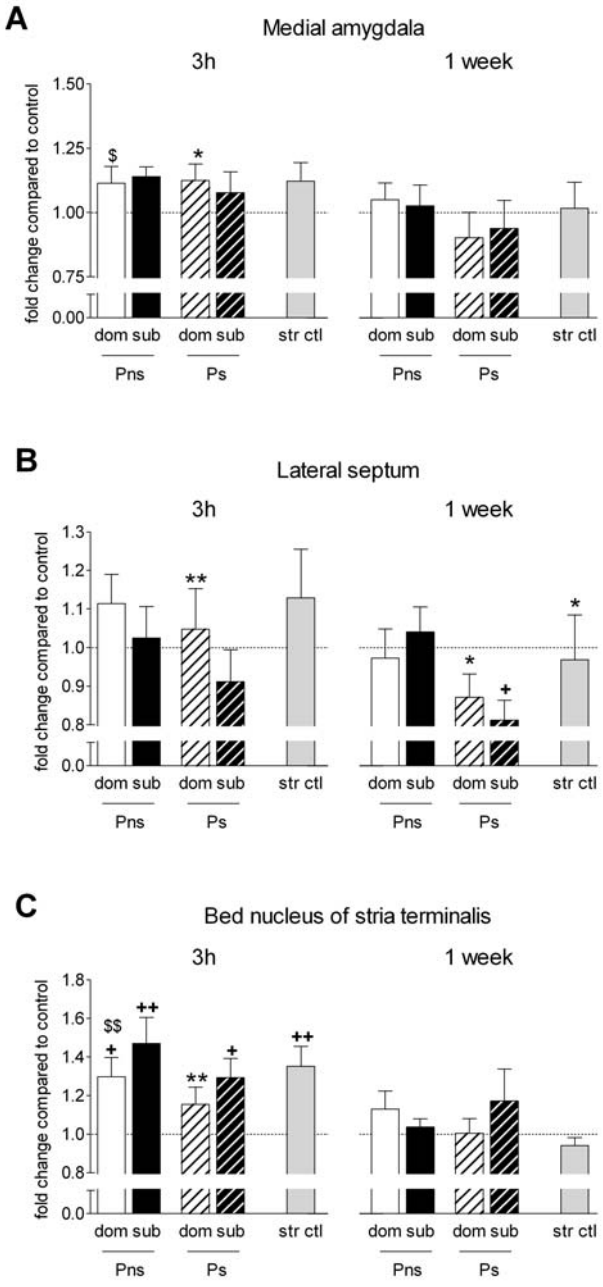

Figure 2. The expression of *Maoa* mRNA in MeA, LS and BNST 3h and 1 week after the first encounter (mean ± S.E.M.). (A) *Maoa* mRNA expression in MeA. (B) *Maoa* mRNA expression in LS. (C) *Maoa* mRNA expression in BNST. 'str ctl' = stressed control group, 'dom' = dominant, 'sub' = subordinate, 'Pns' = non-stressed pair, 'Ps' = stressed pair. * $p<0.05$ vs. subordinate rats of Ps, ** $p<0.01$ vs. subordinate rats of Ps, + $p<0.05$ vs. controls, ++ $p<0.01$ vs. controls, $< p0.05, between the dominant and subordinate rat of Pns, $$ $p<0.01$ between the dominant and subordinate rat of Pns. For the 3h time point each group consists of 12-14 animals, for the 1 week time point of 6-8 animals.

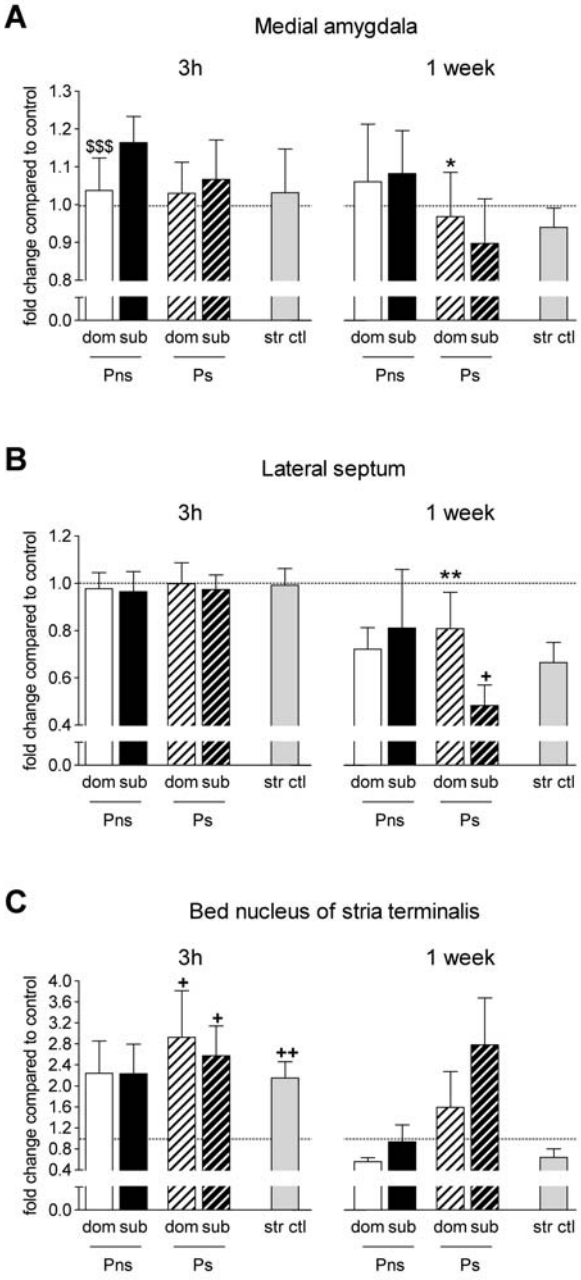

Figure 3. The expression of *Ar* mRNA in MeA, LS and BNST 3h and 1 week after the first encounter (mean ± S.E.M.). (A) *Ar* mRNA expression in MeA. (B) *Ar* mRNA expression in LS. (C) *Ar* mRNA expression in BNST. 'str ctl' = stressed control group, 'dom' = dominant, 'sub' = subordinate, 'Pns' = non-stressed pair, 'Ps' = stressed pair. * $p<0.05$ vs. subordinate rats of Ps, ** $p<0.01$ vs. subordinate rats of Ps, + $p<0.05$ vs. controls, ++ $p<0.01$ vs. controls, \$\$\$ $p<0.001$ between the dominant and subordinate rat of Pns. For the 3h time point each group consists of 12-14 animals, for the 1 week time point of 6-8 animals.

One week after the first encounter the dominant rats of Ps show a higher *Ar* mRNA level compared to the subordinate rat of Ps in both MeA (Table 3, Fig. 3A) and LS (Table 3, Fig. 3B). In LS, the subordinate rats of Ps show a significant decrease in *Ar* mRNA compared to controls (Table 3, Fig. 3B). No significant differences were found in BNST (Fig. 3C).

In summary, the main changes observed in the subordinate rats of Ps were found with respect to the dominant rat of Ps and consisted of a decrease in *Ar* mRNA in LS 1 week after the first encounter

Table 3. Overview of the outcome of paired and unpaired t-tests that compare *Ar* mRNA levels in MeA, LS and BNST. 'Pns dom' = dominant rat of Pns, 'Pns sub' = subordinate rat of Pns, 'Ps dom' = dominant rat of Ps, 'Ps sub' = subordinate rat of Ps, 'str ctl' = stressed control.

		Compared to Ps sub	Compared to Pns sub	Compared to control
MeA	3h	-	Pns dom: t = -5.273, df = 13, p<0.001	-
	1 week	Ps dom: t = 2.551, df = 7, p<0.05	-	-
LS	3h	-	-	-
	1 week	Ps dom: t = 4.470, df = 7, p<0.01	-	Ps sub: t = 2.576, df = 13, p<0.05
BNST	3h	-	-	Ps sub: t = -2.707, df = 21, p<0.05 Ps dom: t = -2.108, df = 22, p<0.05 Str ctl: t = -2.985, df = 24, p<0.01
	1 week	-	-	-

Infusion of an inhibitor of MAOA into the LS

Since the subordinate rats of Ps show a lower *Maoa* mRNA level compared to the dominant rats of Ps and controls 1 week after the first encounter, we next investigated whether this downregulation could be associated with the behavior displayed by these animals towards an unfamiliar subordinate individual as seen in Fig. 1B. Therefore we infused clorgyline, an inhibitor of MAOA, into the LS at 3h before the WCT in one rat of each pair of unfamiliar individuals.

First, to exclude an effect of infusion, we infused vehicle into the LS of a dominant rat that would be paired with an unfamiliar dominant rat, and of a subordinate rat that would be paired with an unfamiliar subordinate rat. On day 1, a clear hierarchy had been established in the original pairs ($t = 10.76$, $df = 35$, $p<0.001$, Fig. 4A-D). Both in pairs consisting of two unfamiliar dominant rats or of two unfamiliar subordinate rats infusion had no effect on the

outcome of the WCT (Fig. 4A&C). Next, we infused clorgyline into the LS of dominant rats that would be paired with an unfamiliar dominant rat and of subordinate rats that would be paired with an unfamiliar subordinate rat. Here as well infusion had no effect on the outcome of the WCT (Fig. 4B&D). Thus, infusion of an inhibitor of MAOA has no effect on the outcome of an encounter with an unfamiliar individual. Figure 4E shows a representative picture of the position of the LS cannula.

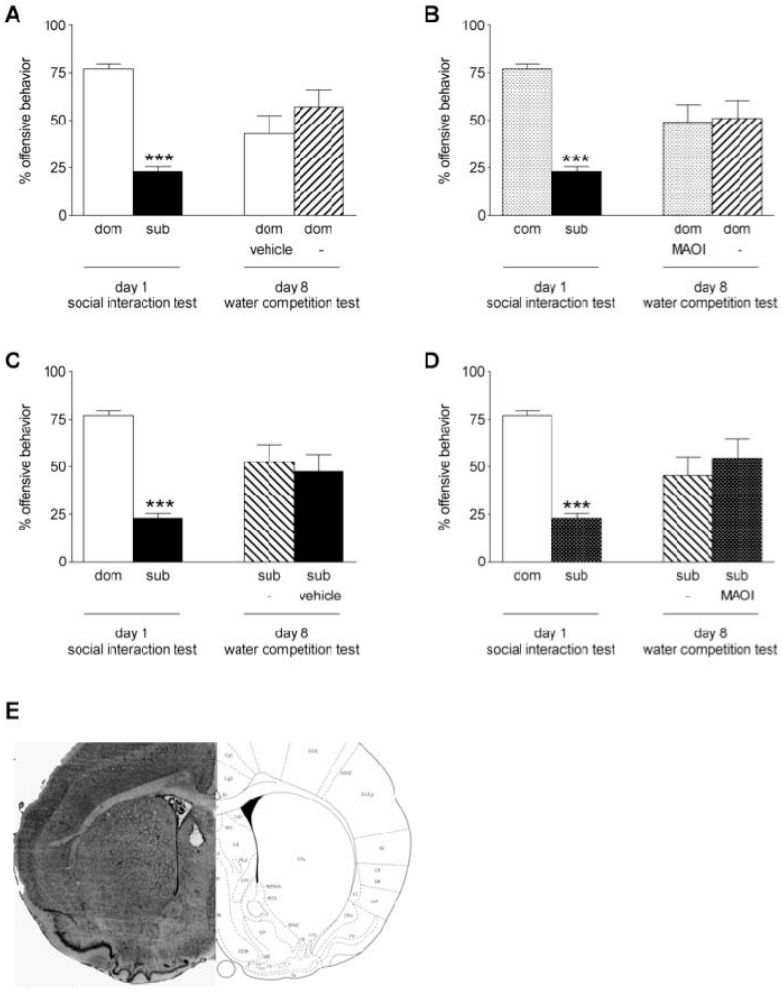

Figure 4. The percentage of total offensive behavior (mean ± S.E.M.) between two opponents shown in the social interaction test on day 1 and the water competition test on day 8. (A) The hierarchy between two previously dominant rats of which one was infused with vehicle (n=7). (B) The hierarchy between two previously dominant rats of which one was infused with clorgyline (n=7). The hierarchy between two previously subordinate rats of which one was infused with vehicle (n=7). (D) The hierarchy between two previously subordinate rats of which one was infused with clorgyline (n=9). (E) Representative picture of the position of the cannula in lateral septum. The status 'dom' (dominant) or 'sub' (subordinate) refers to the status that was obtained during the social interaction test on day 1. 'MAOI' = MAOA inhibitor (clorgyline). *** $p<0.001$ vs. the other rat of the pair.

4.5 Discussion

We used an animal model that has recently been developed in our group (Cordero and Sandi, 2007) to examine the establishment and maintenance of dominance hierarchies in rats. In this model, when two non-stressed rats (non-stressed pairs, Pns) are exposed to each other, a dominance hierarchy is established during the first encounter, but the rank obtained during the first encounter is not maintained until a second encounter one week later. Exposure to acute stress for one rat of a pair (stressed pairs, Ps) leads to induction of submissiveness during a first encounter, and to a long-term memory for the established hierarchy (Cordero and Sandi, 2007). In the current study, we investigated the long-term consequences of exposure to acute stress and the establishment of a dominance hierarchy on the establishment of a second hierarchy with an unfamiliar opponent. Therefore, in a WCT given one week after the first encounter, rats from Ps were exposed to an unfamiliar opponent that was previously dominant or subordinate, or naïve. We also investigated mRNA levels for the monoamine degradation enzyme MAOA and for the steroid receptor AR. Finally, we tested whether the changes that were found in *Maoa* mRNA levels could be linked with the observed effects of stress on aggressive behavior towards an unfamiliar subordinate opponent by administration of the MAOA inhibitor clorgyline.

First, we aimed to examine whether the aggressive behavior towards an unfamiliar rat was changed by prior exposure to acute stress linked to a first social encounter. We exposed rats from Ps to an unfamiliar partner during the WCT one week after the first encounter. This unfamiliar partner was previously dominant or subordinate in a Pns, or naïve. We showed that for the dominant rat of Ps the previous status of the unfamiliar rat does not affect the outcome of the WCT. When paired with either a previously dominant, subordinate or naïve rat, the dominant rats of Ps became dominant in about half of the cases, and subordinate in the other half. For the subordinate stressed rat of Ps the previous status of the unfamiliar individual did affect the outcome of the WCT: when paired with a previously dominant rat, the subordinate rats of Ps became subordinate. When paired with a previously subordinate rat, the subordinate rats of Ps became dominant. There was no significant effect of pairing with a naïve rat, although there seems to be a tendency for the subordinate rats of Ps to become subordinate. These results strongly suggest that stress linked to a first subordinate experience sensitizes animals to perceive the potential status of unfamiliar animals in future contests, facilitating the development of the winner and loser effects. Brown (1992) has shown that subordinate rats recognize dominant rats based on identity rather than status, since subordinate rats react

differently to the odor of familiar and unfamiliar dominant rats. Also in hamsters, defeated animals are capable to recognize individual winners rather than winners in general (Lai and Johnston, 2002; Petrulis et al., 2004). Conceivably, stress and social defeat affect the subordinate rats of Ps to develop a high sensitivity to signals that are sent out by their opponent and react accordingly to this, resulting in subordinate behavior when paired with a previously dominant rat, and dominant behavior when paired with a previously subordinate rat.

Next, we studied the levels of *Maoa* and *Ar* mRNA in MeA, BNST and LS, brain areas that are involved in the regulation of social behaviors such as aggression. For *Maoa* mRNA in MeA, the dominant rats of Pns have lower levels than the subordinate rats of Pns, whereas in Ps the subordinate rats have lower levels than dominant rats 3h after the first encounter. After one week the difference is no longer there. In LS, the subordinate rats of Ps have lower *Maoa* mRNA levels compared to dominant rats 3h after the first encounter, and lower levels than the dominant rats of Ps, controls and stressed controls one week after the first encounter. In BNST the dominant rats of both Pns and Ps have a lower *Maoa* mRNA level than the subordinate rats of the same pair. Compared to controls all groups show an increase in *Maoa* mRNA, except for the dominant rats of Ps. No differences are found in BNST one week after the first encounter. For *Ar* mRNA in MeA, 3h after the first encounter the dominant rats of Pns show a lower level than the subordinate rats, whereas after one week the dominant rats of Ps show a higher level than the subordinate rats. In LS, no change is found in any of the groups 3h after the encounter. However, after 1 week the subordinate rats of Ps show a decreased *Ar* mRNA level compared to controls and the dominant rats of Ps. In BNST an increase in *Ar* mRNA is found in both rats of Ps and in stressed controls compared to controls at 3h after the first encounter. There are no significant changes after 1 week.

To test whether the downregulation of *Maoa* mRNA in the LS of the subordinate rats of Ps one week after the first encounter can be linked to the fact that subordinate rats of Ps became dominant when paired with a previously subordinate rat, we infused an inhibitor of MAOA into the LS of the subordinate rats, before pairing them with a previously subordinate rat one week after the first encounter. There was no effect of administration of the MAOA inhibitor clorgyline into the LS on the outcome of the WCT with an unfamiliar opponent.

Dominance hierarchies are established to minimize the costs of fighting in a group (Van Kreveld, 1970). Intrinsic factors such as size, age, sex, strength, physiology and level of aggressiveness (Parker, 1974; Chase et al., 2002) and extrinsic factors such as winner and loser effects (Chase et al., 1994) determine which individual becomes dominant. Experiments

studying dominance hierarchies in cichlid fish suggest that both intrinsic and extrinsic factors play an important role in defining the outcome of a dominance hierarchy (Chase et al., 2002). Animals have different ways of estimating their chances of winning. According to the social cue hypothesis animals assess the strengths and weaknesses of their opponent before or during an encounter (Rutte et al., 2006). Since the behavior of the subordinate rats of Ps is dependent on the previous status of the opponent, this hypothesis might be able to explain our results. It is possible that the subordinate rats of Ps have undergone molecular changes in the brain that make these rats more sensitive to the signals of their opponent. We found a downregulation of several genes specifically in the LS of the subordinate rats of Ps, such as in *Maoa* and *Ar*, but also in *V1ar* (chapter 3) that might be involved in this enhanced sensitivity.

The monoamine degradation enzyme MAOA is linked to the regulation of aggression. MAOA knockout mice are more aggressive than wild-type mice (Cases et al., 1995). In humans, low activity of MAOA is correlated with aggression (Alia-Klein et al., 2008; McDermott et al., 2009). A Dutch family carrying a point mutation in the MAOA gene shows borderline mental retardation and impulsive aggression (Brunner et al., 1993). The subordinate rats of Ps in the current study have a decrease in *Maoa* mRNA one week after the encounter. This decrease in *Maoa* might play a role in the increased aggression towards an unfamiliar subordinate rat. One of the neurotransmitters that is degraded by MAOA is 5-HT, which been shown to play a role in the regulation of aggression and to be related to the hierarchical status in several animal species (Kostowski et al., 1984; Blanchard et al., 1993; McKittrick et al., 1995; Ferris, 1996; Dhingra et al., 1997; Ferris et al., 1997; Larson and Summers, 2001). These findings are in agreement with our result of a reduced *Maoa* mRNA level in the subordinate rats of Ps. However, no causal relationship of MAOA activity with the behavior towards an unfamiliar individual could be shown.

Besides monoamines such as 5-HT, steroid hormones such as testosterone could play a role in aggressive behavior. Steroid hormones promote aggressiveness in LS, amygdala and dorsal raphe nucleus (Simon et al., 1998). Subordinate rats in a visible burrow system have a reduced testosterone level (Blanchard et al., 1993; Blanchard et al., 1995; Hardy et al., 2002). Whereas chronic stress leads to a reduction in testosterone, acute stress can lead to an increase in testosterone in the initial phases of acute stress, mainly during agonistic encounters (Chichinadze and Chichinadze, 2008). Androgens are likely mediators of winner and loser effects (Rutte et al., 2006). In male cichlid fish, social dominance is associated with an increase in AR and estrogen receptor mRNA expression, which makes the dominant males more sensitive to sex steroids (Burmeister et al., 2007). The observed downregulation in *Ar*

mRNA in subordinate rats of Ps possibly also explains part of the behavioral effects that were found.

In summary, we have shown that the behavior of subordinate rats of Ps towards an unfamiliar opponent depends on the previously obtained status of the unfamiliar rat: when the opponent was previously dominant, the subordinate rats of Ps become subordinate, whereas they become dominant when the opponent was previously subordinate. For dominant rats of Ps the previous status of the unfamiliar opponent has no effect on the outcome of the hierarchy in the WCT. Furthermore, we have shown that the subordinate rats of Ps show a downregulation in *Maoa* mRNA in LS one week after the first encounter, as well as a downregulation in *Ar* mRNA. However, experiments using the MAOA inhibitor clorgyline did not mimic the long-term effect on offensive behavior towards an unfamiliar opponent, so a causal relationship between the observed behavior and the changes in *Maoa* mRNA levels has not been shown in this study.

4.6 Acknowledgements

This work was supported by grants from the EU 7th (FP7- HEALTH-F2M-2007-201600; MemStick) FP and from the Swiss National Science Foundation (310000-120791) to C.S. The authors wish to thank to Grégoire Parchet and Angélique Vaucher for technical assistance.

4.7 References

Alia-Klein N., Goldstein R.Z., Kriplani A., Logan J., Tomasi D., Williams B., Telang F., Shumay E., Biegon A., Craig I.W., Henn F., Wang G.J., Volkow N.D., Fowler J.S. (2008). Brain monoamine oxidase A activity predicts trait aggression. J Neurosci 28:5099-5104.

Berdoy M., Smith P., MacDonald D.W. (1995). Stability of social status in wild rats: age and the role of settled dominance. Behaviour 132:193-212.

Blanchard D.C., Sakai R.R., McEwen B., Weiss S.M., Blanchard R.J. (1993). Subordination stress: behavioral, brain, and neuroendocrine correlates. Behav Brain Res 58:113-121.

Blanchard D.C., Spencer R.L., Weiss S.M., Blanchard R.J., McEwen B., Sakai R.R. (1995). Visible burrow system as a model of chronic social stress: behavioral and neuroendocrine correlates. Psychoneuroendocrinology 20:117-134.

Brown R.E. (1992). Responses of dominant and subordinate male rats to the odors of male and female conspecifics. Aggressive behavior 18:129-138.

Brunner H.G., Nelen M., Breakefield X.O., Ropers H.H., van Oost B.A. (1993). Abnormal behavior associated with a point mutation in the structural gene for monoamine oxidase A. Science 262:578-580.

Burmeister S.S., Kailasanath V., Fernald R.D. (2007). Social dominance regulates androgen and estrogen receptor gene expression. Horm Behav 51:164-170.

Cases O., Seif I., Grimsby J., Gaspar P., Chen K., Pournin S., Muller U., Aguet M., Babinet C., Shih J.C., et al. (1995). Aggressive behavior and altered amounts of brain serotonin and norepinephrine in mice lacking MAOA. Science 268:1763-1766.

Chase I.D., Bartolomeo C., Dugatkin L.A. (1994). Aggressive interactions and inter-contest interval: how long do winners keep winning? Anim Behav 48:393-400.

Chase I.D., Tovey C., Spangler-Martin D., Manfredonia M. (2002). Individual differences versus social dynamics in the formation of animal dominance hierarchies. Proc Natl Acad Sci U S A 99:5744-5749.

Chichinadze K., Chichinadze N. (2008). Stress-induced increase of testosterone: contributions of social status and sympathetic reactivity. Physiol Behav 94:595-603.

Cordero M.I., Sandi C. (2007). Stress amplifies memory for social hierarchy. Frontiers in neuroscience 1:175-184.

de Almeida R.M., Ferrari P.F., Parmigiani S., Miczek K.A. (2005). Escalated aggressive behavior: dopamine, serotonin and GABA. Eur J Pharmacol 526:51-64.

Dhingra N.K., Raju T.R., Meti B.L. (1997). Selective reduction of monoamine oxidase A and B in the frontal cortex of subordinate rats. Brain Res 758:237-240.

Dong Q., Salva A., Sottas C.M., Niu E., Holmes M., Hardy M.P. (2004). Rapid glucocorticoid mediation of suppressed testosterone biosynthesis in male mice subjected to immobilization stress. Journal of andrology 25:973-981.

Ferris C.F. (1996). Serotonin diminishes aggression by suppressing the activity of the vasopressin system. Ann N Y Acad Sci 794:98-103.

Ferris C.F., Melloni R.H. Jr., Koppel G., Perry K.W., Fuller R.W., Delville Y. (1997). Vasopressin/serotonin interactions in the anterior hypothalamus control aggressive behavior in golden hamsters. J Neurosci 17:4331-4340.

Hardy M.P., Sottas C.M., Ge R., McKittrick C.R., Tamashiro K.L., McEwen B.S., Haider S.G., Markham C.M., Blanchard R.J., Blanchard D.C., Sakai R.R. (2002). Trends of reproductive hormones in male rats during psychosocial stress: role of glucocorticoid metabolism in behavioral dominance. Biol Reprod 67:1750-1755.

Kostowski W., Plewako M., Bidzinski A. (1984). Brain serotonergic neurons: their role in a form of dominance-subordination behavior in rats. Physiol Behav 33:365-371.
Kruk M.R. (1991). Ethology and pharmacology of hypothalamic aggression in the rat. Neurosci Biobehav Rev 15:527-538.
Lai W.S., Johnston R.E. (2002). Individual recognition after fighting by golden hamsters: a new method. Physiol Behav 76:225-239.
Larson E.T., Summers C.H. (2001). Serotonin reverses dominant social status. Behav Brain Res 121:95-102.
Lesch K.P., Merschdorf U. (2000). Impulsivity, aggression, and serotonin: a molecular psychobiological perspective. Behav Sci Law 18:581-604.
Leshner A.I., Moyer J.A. (1975). Androgens and agonistic behavior in mice: relevance to aggression and irrelevance to avoidance-of-attack. Physiol Behav 15:695-699.
Leshner A.I., Politch J.A. (1979). Hormonal control of submissiveness in mice: irrelevance of the androgens and relevance of the pituitary-adrenal hormones. Physiol Behav 22:531-534.
Livak K.J., Schmittgen T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.
McDermott R., Tingley D., Cowden J., Frazzetto G., Johnson D.D. (2009). Monoamine oxidase A gene (MAOA) predicts behavioral aggression following provocation. Proc Natl Acad Sci U S A 106:2118-2123.
McKittrick C.R., Blanchard D.C., Blanchard R.J., McEwen B.S., Sakai R.R. (1995). Serotonin receptor binding in a colony model of chronic social stress. Biol Psychiatry 37:383-393.
Nelson R.J., Trainor B.C. (2007). Neural mechanisms of aggression. Nat Rev Neurosci 8:536-546.
Parker G.A. (1974). Assessment strategy and the evolution of fighting behaviour. J Theor Biol 47:223-243.
Paxinos G., Watson C. (1998). The rat brain in stereotaxic coordinates. Academic Press, New York.
Petrulis A., Weidner M., Johnston R.E. (2004). Recognition of competitors by male golden hamsters. Physiol Behav 81:629-638.
Retana-Marquez S., Bonilla-Jaime H., Vazquez-Palacios G., Martinez-Garcia R., Velazquez-Moctezuma J. (2003). Changes in masculine sexual behavior, corticosterone and testosterone in response to acute and chronic stress in male rats. Horm Behav 44:327-337.
Rutte C., Taborsky M., Brinkhof M.W. (2006). What sets the odds of winning and losing? Trends Ecol Evol 21:16-21.
Simon N.G., Cologer-Clifford A., Lu S.F., McKenna S.E., Hu S. (1998). Testosterone and its metabolites modulate 5HT1A and 5HT1B agonist effects on intermale aggression. Neurosci Biobehav Rev 23:325-336.
Van Kreveld D. (1970). A selective review of dominance-subordinate relations in animals. Genetics and Psychology Monographs 81:143-173.

Chapter 5 - General discussion and future perspectives

In this thesis we studied the neurobiological mechanisms underlying the effects of acute stress on the establishment and maintenance of dominance hierarchies in adult rats. More specifically, we studied (1) the role of glucocorticoids in the long-term establishment of dominance hierarchies, (2) the role of oxytocin and vasopressin receptors in the long-term establishment of dominance hierarchies and (3) the long-term effects of acute stress and dominance hierarchy formation on a second hierarchy formation with an unfamiliar opponent and on possible neurobiological mechanisms underlying these effects.

5.1 The role of glucocorticoids in the long-term establishment of dominance hierarchies

When two non-stressed rats (non-stressed pair, Pns) of equal body weight and anxiety level are submitted to a social encounter, a dominance hierarchy is established, but when the same rats are re-exposed to each other one week later, the hierarchy that was formed during the first encounter is not maintained. Exposure to acute stress for one rat of the pair (stressed pair, Ps) before a first social encounter induces submissiveness in the stressed rat, and leads to a long-term memory maintenance for the established hierarchy (Cordero and Sandi, 2007).

To examine whether enhancing corticosterone levels plays a role in the initial establishment of a dominance hierarchy and in the long-term maintenance of the hierarchy, we injected one rat of each pair with corticosterone, either just before the first encounter, or immediately after the first encounter. We showed that enhancing corticosterone levels before the first encounter has no effect on the outcome of the hierarchy. Furthermore, we showed that when the rat that was treated with corticosterone before the first encounter becomes dominant, but not when it becomes subordinate, a long-term maintenance of the established hierarchy is induced. Third, we showed that when the subordinate rat, but not the dominant rat, is treated with corticosterone immediately after the first encounter, the long-term maintenance of the hierarchy is enhanced.

Corticosterone treatment had no effect on the outcome of the first encounter, in contrast to exposure to acute stress (Cordero and Sandi, 2007). Exposure to inescapable foot shock stress might lead to a wider array of physiological changes than only enhancing corticosterone levels, which could explain the discrepancy. Various studies show that increased glucocorticoid levels lead to an increase in aggression (Brain and Evans, 1977; Heller, 1978; Hayden-Hixson and Ferris, 1991; Haller et al., 1997), whereas other show that increasing corticosterone levels induces submissive behavior (Leshner and Moyer, 1975;

Leshner et al., 1975; Moyer and Leshner, 1976; Leshner and Politch, 1979; Leshner et al., 1980). It is possible that by increasing an animal's arousal (Leshner et al., 1980), corticosterone enhances the capability of recognizing whether the opponent is aggressive or passive, leading to an increase in subordinate or aggressive behavior depending on the opponent. Since there is an equal chance of meeting an opponent that is either aggressive or passive, this might explain why half of the pre-training corticosterone-treated rats respond by becoming dominant during the first encounter, and half by becoming subordinate.

It is unexpected that pre-training corticosterone injections only induce a long-term maintenance of the hierarchy when the corticosterone-treated rat becomes dominant, and not when it becomes subordinate. When one rat of a pair is submitted to acute stress in the form of foot shocks, the stressed rat becomes subordinate and a long-term memory is formed for the hierarchy (Cordero and Sandi, 2007), so we would have expected to see the same effect of corticosterone treatment. Based on the current data it is not clear why no long-term memory is kept for the hierarchy when the corticosterone-treated rat becomes subordinate.

Several studies using memory tests such as spatial learning (Sandi et al., 1997; Conboy and Sandi, 2009), fear conditioning (Cordero and Sandi, 1998), inhibitory avoidance (Sandi et al., 1995; Venero and Sandi, 1997) and object recognition (Okuda et al, 2004; Roozendaal et al., 2006) have shown that corticosterone potentiates long-term memory when injected after training. This is similar to our finding that corticosterone enhances the memory for the dominance hierarchy when injected in the subordinate rat after the encounter. Although we have not studied this in the current thesis, our hypothesis is that it is the subordinate rat of the stressed pair that keeps the memory for the hierarchy, and not the dominant rat. This fits with the finding that post-encounter corticosterone injections only induce a long-term memory when administered to the subordinate rat.

An interesting follow-up study would be to investigate in which brain region corticosterone exerts its effect. A candidate brain region is the amygdala, which has a feedback onto the HPA axis and plays a role in contextual fear conditioning and social memory. Experiments studying the effects of corticosterone infusion into various nuclei of the amygdala on the establishment and maintenance of dominance hierarchies are currently ongoing in our group.

5.2 The role of oxytocin and vasopressin receptors in the long-term maintenance of dominance hierarchies

Oxytocin (OT) and arginine vasopressin (AVP) are two neuropeptides that play a role in the regulation of social behavior, but also of the stress response (Ferguson et al., 2000; Ferguson et al., 2001; Bielsky et al., 2004; Crawley et al., 2007). Therefore, we hypothesized that changes in OT receptors (OTR) and AVP 1a receptors (V1aR) play an important role in the long-term maintenance of dominance hierarchies induced by exposure to acute stress.

We show that exposure to acute stress and a social encounter leads to a downregulation in *Otr* mRNA in the medial amygdala (MeA) of the subordinate rats of stressed pairs at three hours after the first encounter. On the long-term, one week after the encounter, we found a downregulation in *V1ar* mRNA in the lateral septum (LS) of the subordinate rats of stressed pairs. When non-stressed subordinate rats were infused with an OT antagonist immediately after the first encounter, the established hierarchy was stable when tested one week later, which shows that blockade of OTR in the subordinate rat after an encounter plays a role in inducing a long-term memory for the dominance hierarchy. Administration of a V1aR antagonist into the LS of non-stressed subordinate rats before the second encounter, one week after the first encounter, induced submissiveness in the subordinate rats, which shows that V1aR in LS might be involved in the effects of acute stress on the long-term establishment of the hierarchy.

These results suggest that OT in MeA and AVP in LS might play a role at different stages in the long-term establishment of a dominance hierarchy, at least with regards to stress influences on this phenomenon. Social information passes from the accessory olfactory bulb to the MeA, from where it is passed on to several downstream brain regions such as LS. OT knockout mice show no activation of MeA and cannot form a social memory when exposed to a conspecific. Administration of OT into the MeA in these mice is sufficient to restore social memory formation, but only when it is administered before the encounter (Ferguson et al., 2000; Ferguson et al., 2001). This shows that OT in the MeA is essential for the processing of social information. Furthermore, the amygdala has an output to the HPA axis and OT in the amygdala plays a role in the attenuation of the stress response, possibly to prevent excessive HPA axis activation (Neumann et al., 2000; Windle et al., 2004; Ebner et al., 2005). It is tempting to speculate that the downregulation in *Otr* mRNA in MeA observed in our study might be caused by a high OT release in MeA (Insel et al., 1992; Di Scala-Guenot and Strosser, 1995). In the future it would be interesting to investigate OT release in the MeA around the time of exposure to foot shocks and the first encounter using microdialysis.

The changes observed in our study in vasopressinergic neurotransmission in the LS seem to play a role at a later time point. Whether changes in *V1ar* mRNA in LS and blocking V1aR in LS by administration of an antagonist affects memory retrieval or reflects a change in social behavior cannot be concluded from the present data. Administration of a V1aR antagonist before a first encounter between two naïve rats might tell us whether the effect of the antagonist is specific for rats that were subordinate in a previous encounter, or whether it has a similar effect in naïve rats and thus induces submissive behavior rather than having an effect on the retrieval of a memory that has been formed for the hierarchy. Again, microdialysis would give us more information about AVP release in LS around the first and second encounter.

It would be useful in future studies to examine protein levels of the studied molecules using Western blots and to perform receptor-binding studies.

5.3 The effect of acute stress and hierarchy establishment on the establishment of a second dominance hierarchy with an unfamiliar opponent

When a previously subordinate rat from a stressed pair is exposed to an unfamiliar opponent in a water competition test one week after the first encounter, the outcome of the water competition test depends on the previous status of the unfamiliar opponent. When the opponent was dominant in a previous encounter, the stressed subordinate rat becomes subordinate in the water competition test. When the opponent was subordinate in a previous encounter, the stressed subordinate rat becomes dominant in the water competition test. When the dominant rat of a stressed pair is exposed to an unfamiliar opponent, the previous status of the opponent does not affect the outcome of the water competition test. In half of the cases the dominant rat of the stressed pair becomes dominant, in the other half subordinate. One week after the first encounter, subordinate rats of stressed pairs show a downregulation in monoamine oxidase A *(Maoa),* androgen receptor *(Ar)* and *V1ar* (see discussion above) mRNA in LS compared to the dominant rats of stressed pairs and to controls. When the MAOA inhibitor clorgyline was infused into the LS of an unstressed subordinate rat before exposure to an unfamiliar subordinate opponent, the treatment had no effect on the outcome of the water competition test.

To identify which molecular changes can explain why subordinate rats of stressed pairs become dominant when paired with an unfamiliar subordinate opponent, there are several protein candidates to be explored. In chapter 3, we have shown that subordinate rats of stressed pairs have a downregulation in *V1ar* mRNA in LS compared to the dominant rats of

stressed pairs and controls. AVP plays a role in social recognition and the regulation of aggression (reviewed by Veenema and Neumann, 2008). Therefore, changes in *V1ar* mRNA levels could, at least partly, explain the behavior that was observed in the current experiment. Administration of a V1aR antagonist in the LS of a subordinate rat before an encounter with an unfamiliar subordinate opponent could tell us whether V1aR is involved in the observed effect. Furthermore, we have shown a downregulation in *Ar* mRNA in subordinate rats of stressed pairs in LS at one week after the first encounter. This change could also play a role in the observed behavior, so experiments using androgen antagonists would be useful to examine the role of the AR in the observed effect.

5.4 The long-term effects of acute stress and hierarchy formation on social behavior

Depending on the hierarchical organization within a group, either being the dominant or subordinate individual can be more stressful (Sapolsky, 2005). In rats, social defeat or being the subordinate individual in a visible burrow system has many disadvantageous effects on health and well-being. Social defeat in a resident-intruder test leads to a reduced body weight, reduced heart rate, reduced body temperature, reduced activity, lower cellular immunocompetence, higher plasma corticosterone levels and augmented responses to stressors (de Jong et al., 2005; Arakawa, 2006). After repeated exposure to food competition tests, subordinate rats show a lower level of cell proliferation in the dentate gyrus and more depressive-like behavior in a forced swim test (Hoshaw et al., 2006). These long-term negative effects of losing an encounter have been suggested to show resemblance to depressive-like symptoms. It has been hypothesized that depression in humans might have evolved as an exaggerated and persistent display of subordinate signs, which could have the function to avoid participation in conflicts that seem unwinnable (Wilkinson, 1999). If something similar happens in the rats in the model that we use, this could mean that when the stressed subordinate rats are exposed to a familiar or an unfamiliar dominant rat, the submissive behavior that they show to avoid a potentially harmful conflict can be considered as a depressive-like symptom.

MAOA is involved in the degradation of serotonin (5-HT), noradrenaline and dopamine. 5-HT modulates emotional behavior such as anxiety, impulsivity and aggression and has been implicated in depression (Lesch and Meschdorf, 2000). MAOA knockout mice are more aggressive and have a higher 5-HT level than wild-type mice (Cases et al., 1995). In humans, low activity of MAOA or a mutation in the *Maoa* gene is correlated with aggression (Alia-Klein et al., 2008, McDermott et al., 2009; Brunner et al., 1993). The subordinate rats of

stressed pairs in the current study show a decrease in *Maoa* mRNA one week after the encounter. This decrease in *Maoa* might play a role in the fact that subordinate rats of stressed pairs become dominant when paired with an unfamiliar subordinate rat. A decrease in *Maoa* suggests a possible increase in 5-HT levels or a prolonged action of 5-HT, although this has not been shown in the current study. 5-HT has been shown to play a role in the hierarchical status in several animal species. In the visible burrow system, subordinate rats show an increased 5-HIAA/5-HT ratio and alterations in 5-HT_{1a}-receptor binding (Blanchard et al., 1993; McKittrick et al., 1995). Ranks in a water competition test can be reversed when the dominant rat is treated with drugs that stimulate 5-HT neurons or receptors, and when the subordinate rat is treated with drugs that block 5-HT neurons or receptors (Kostowski et al., 1984). In a worker-parasite paradigm subordinate rats have a lower MAOA enzyme activity than dominant rats (Dhingra et al., 1997). In lizards, dominant males show an increase in 5-HT in MeA 1h after the initiation of fighting, whereas subordinates show an increase starting only 24h after the initiation of fighting, with a peak level after one week. Selective serotonin reuptake inhibitors (SSRIs), resulting in a higher 5-HT level, reduced aggression in lizards and reversed dominance in half of the dyads (Larson and Summers, 2001). Highly aggressive SAL mice show lower 5-HT levels in the prefrontal cortex than less aggressive LAL mice after repeated exposure to resident intruder tests (Caramaschi et al., 2008). In hamsters, 5-HT decreases the level of aggression, possibly by interfering with the action of AVP that facilitates aggression (Ferris, 1996; Ferris et al., 1997). In monkeys, a lowered 5-HT functioning is related to lower ranks in social groups, probably due to less competent social behavior and greater impulsive aggression (reviewed in Lesch and Merschdorf, 2000). All these findings show that 5-HT plays an important role in the regulation of aggression and social ranks, and fit with our result of a reduced *Maoa* mRNA level in the subordinate rats of stressed pairs. Although no causal relationship of MAOA activity with the behavior towards an unfamiliar individual could be shown in the current study, it would be interesting to further explore changes in the 5-HT system in the rats exposed to our model.

5.5 Winner and loser effects

Dominance hierarchies are established to minimize the costs of fighting in a group (Van Kreveld, 1970). Intrinsic factors such as size, age, sex, strength, physiology and level of aggressiveness (Parker, 1974; Chase et al., 2002) and extrinsic factors such as winner and loser effects (Chase et al., 1994) determine which individual becomes dominant. Experiments studying dominance hierarchies in cichlid fish suggest that both intrinsic and extrinsic factors

play an important role in defining the outcome of a dominance hierarchy (Chase et al., 2002). In our model, the intrinsic factors body weight and anxiety level, and also age, are kept similar between the two rats of a dyad to exclude as many intrinsic factors as possible in determining the hierarchy. Dugatkin and Earley (2004) have described that when individual recognition occurs, winner and loser effects on hierarchy establishment disappear. Since individual recognition rather than recognition of a hierarchical status seems to occur in rats during aggressive encounters (Brown, 1992; Cordero and Sandi, 2007), this would mean that winner and loser effects cannot explain why subordinate rats of stressed pairs become dominant when paired with an unfamiliar previously subordinate rat. Animals have different ways of estimating their chances of winning. According to the social cue hypothesis animals assess the strengths and weaknesses of their opponent before or during an encounter (Rutte et al., 2006). Since the behavior of the subordinate rats of stressed pairs is dependent on the previous status of the opponent, the social cue hypothesis could be used to explain our results. Social cues used in this hypothesis could be signs of exhaustion or injury in losers, or chemical cues. It is possible that the subordinate rats of stressed pairs have undergone molecular changes in the brain that make these rats more sensitive to the signals of their opponents. We found a downregulation of several genes specifically in the LS of the subordinate rats of stressed pairs, such as in *Maoa* and *Ar*, but also in *V1ar* that might be involved in this enhanced sensitivity, which also fits with the proposed role of the LS in the regulation of social behavior.

5.6 General conclusion

We have shown that corticosterone has no effect on the initial outcome of an encounter between two unfamiliar opponents, but that corticosterone mimics the effects of acute stress on the long-term maintenance of a dominance hierarchy when administered pre-training to the dominant rat, or post-training to the subordinate rat. This suggests that corticosterone plays a role in the consolidation of the memory for a recently established hierarchy.

Furthermore, we have shown that the OTR in the MeA and in the V1aR in the LS play a role in the immediate and long-term effects of acute stress on the maintenance of a dominance hierarchy, since administration of OT antagonists to non-stressed subordinate rats after a first encounter induces a long-term memory for the hierarchy and administration of V1aR antagonists to non-stressed subordinate rats before a second encounter induces submissive behavior.

Finally, we have shown that a second hierarchy that is established when subordinate rats of Ps are paired with unfamiliar opponent depends on the previous status of the opponent, with subordinate rats becoming dominant when exposed to a previously subordinate opponent. Although changes in *Maoa* mRNA levels were found, using a MAOA inhibitor did not mimic the effects of acute stress on social behavior.

Taken together, we have shown that exposure to acute stress and a social encounter leads to a wide array of neurobiological changes in the receptors for the neuropeptides OT and AVP, the testosterone receptor AR and the monoamine degradation enzyme MAOA. These changes might underlie the formation of a long-term memory for an established hierarchy, the long-term effects of acute stress on the hierarchy, and long-term changes in offensive behavior towards an unfamiliar opponent. Since administration of corticosterone mimics the effects of acute stress on behavior, corticosterone might play a role in inducing the changes in neurobiological mechanisms that were investigated in this thesis.

Bibliography

Akirav I., Kozenicky M., Tal D., Sandi C., Venero C., Richter-Levin G. (2004). A facilitative role for corticosterone in the acquisition of a spatial task under moderate stress. Learn Mem 11:188-195.

Albert D.J., Chew G.L. (1980). The septal forebrain and the inhibitory modulation of attack and defense in the rat. A review. Behavioral and neural biology 30:357-388.

Albert D.J., Petrovic D.M., Walsh M.L., Jonik R.H. (1989). Medial accumbens lesions attenuate testosterone-dependent aggression in male rats. Physiol Behav 46:625-631.

Albert D.J., Walsh M.L. (1982). The inhibitory modulation of agonistic behavior in the rat brain: a review. Neurosci Biobehav Rev 6:125-143.

Alia-Klein N., Goldstein R.Z., Kriplani A., Logan J., Tomasi D., Williams B., Telang F., Shumay E., Biegon A., Craig I.W., Henn F., Wang G.J., Volkow N.D., Fowler J.S. (2008). Brain monoamine oxidase A activity predicts trait aggression. J Neurosci 28:5099-5104.

Arakawa H. (2006). Changes in the pattern of exploratory behavior are associated with the emergence of social dominance relationships in male rats. Dev Psychobiol 48:39-47.

Askew A., Gonzalez F.A., Stahl J.M., Karom M.C. (2006). Food competition and social experience effects on V1a receptor binding in the forebrain of male Long-Evans hooded rats. Horm Behav 49:328-336.

Bannerman D.M., Lemaire M., Beggs S., Rawlins J.N., Iversen S.D. (2001). Cytotoxic lesions of the hippocampus increase social investigation but do not impair social-recognition memory. Exp Brain Res 138:100-109.

Bannerman D.M., Lemaire M., Yee B.K., Iversen S.D., Oswald C.J., Good M.A., Rawlins J.N. (2002). Selective cytotoxic lesions of the retrohippocampal region produce a mild deficit in social recognition memory. Exp Brain Res 142:395-401.

Benelli A., Bertolini A., Poggioli R., Menozzi B., Basaglia R., Arletti R. (1995). Polymodal dose-response curve for oxytocin in the social recognition test. Neuropeptides 28:251-255.

Berdoy M., Smith P., MacDonald D.W. (1995). Stability of social status in wild rats: age and the role of settled dominance. Behaviour 132:193-212.

Bester-Meredith J.K., Marler C.A. (2001). Vasopressin and aggression in cross-fostered California mice (Peromyscus californicus) and white-footed mice (Peromyscus leucopus). Horm Behav 40:51-64.

Bielsky I.F., Hu S.B., Ren X., Terwilliger E.F., Young L.J. (2005). The V1a vasopressin receptor is necessary and sufficient for normal social recognition: a gene replacement study. Neuron 47:503-513.

Bielsky I.F., Hu S.B., Szegda K.L., Westphal H., Young L.J. (2004). Profound impairment in social recognition and reduction in anxiety-like behavior in vasopressin V1a receptor knockout mice. Neuropsychopharmacology 29:483-493.

Bielsky I.F., Young L.J. (2004). Oxytocin, vasopressin, and social recognition in mammals. Peptides 25:1565-1574.

Blanchard D.C., Sakai R.R., McEwen B., Weiss S.M., Blanchard R.J. (1993). Subordination stress: behavioral, brain, and neuroendocrine correlates. Behav Brain Res 58:113-121.

Blanchard D.C., Spencer R.L., Weiss S.M., Blanchard R.J., McEwen B., Sakai R.R. (1995). Visible burrow system as a model of chronic social stress: behavioral and neuroendocrine correlates. Psychoneuroendocrinology 20:117-134.

Blanchard R.J., Flannelly K.J., Blanchard D.C. (1988). Life-span studies of dominance and aggression in established colonies of laboratory rats. Physiol Behav 43:1-7.

Bluthe R.M., Schoenen J., Dantzer R. (1990). Androgen-dependent vasopressinergic neurons are involved in social recognition in rats. Brain Res 519:150-157.
Bolhuis J.J., Fitzgerald R.E., Dijk D.J., Koolhaas J.M. (1984). The corticomedial amygdala and learning in an agonistic situation in the rat. Physiol Behav 32:575-579.
Brain P.F., Evans A.E. (1977). Acute influences of some ACTH-related peptides on fighting and adrenocortical activity in male laboratory mice. Pharmacology, biochemistry, and behavior 7:425-433.
Breton C., Zingg H.H. (1997). Expression and region-specific regulation of the oxytocin receptor gene in rat brain. Endocrinology 138:1857-1862.
Brinks V., Berger S., Gass P., de Kloet E.R., Oitzl M.S. (2009). Mineralocorticoid receptors in control of emotional arousal and fear memory. Horm Behav 56:232-238.
Brinks V., de Kloet E.R., Oitzl M.S. (2009). Corticosterone facilitates extinction of fear memory in BALB/c mice but strengthens cue related fear in C57BL/6 mice. Exp Neurol 216:375-382.
Brown R.E. (1992). Responses of dominant and subordinate male rats to the odors of male and female conspecifics. Aggressive behavior 18:129-138.
Brunner H.G., Nelen M., Breakefield X.O., Ropers H.H., van Oost B.A. (1993). Abnormal behavior associated with a point mutation in the structural gene for monoamine oxidase A. Science 262:578-580.
Burmeister S.S., Kailasanath V., Fernald R.D. (2007). Social dominance regulates androgen and estrogen receptor gene expression. Horm Behav 51:164-170.
Caffe A.R., van Leeuwen F.W., Luiten P.G. (1987). Vasopressin cells in the medial amygdala of the rat project to the lateral septum and ventral hippocampus. The Journal of comparative neurology 261:237-252.
Calderazzo L., Cavalheiro E.A., Macchi G., Molinari M., Bentivoglio M. (1996). Branched connections to the septum and to the entorhinal cortex from the hippocampus, amygdala, and diencephalon in the rat. Brain research bulletin 40:245-251.
Caldwell H.K., Lee H.J., Macbeth A.H., Young W.S. 3rd (2008). Vasopressin: behavioral roles of an "original" neuropeptide. Prog Neurobiol 84:1-24.
Calfa G., Bussolino D., Molina V.A. (2007). Involvement of the lateral septum and the ventral Hippocampus in the emotional sequelae induced by social defeat: role of glucocorticoid receptors. Behav Brain Res 181:23-34.
Canteras N.S., Simerly R.B., Swanson L.W. (1995). Organization of projections from the medial nucleus of the amygdala: a PHAL study in the rat. The Journal of comparative neurology 360:213-245.
Caramaschi D., de Boer S.F., de Vries H., Koolhaas, J.M. (2008). Development of violence in mice through repeated victory along with changes in prefrontal cortex neurochemistry. Behav Brain Res 189:263-272.
Cases O., Seif I., Grimsby J., Gaspar P., Chen K., Pournin S., Muller U., Aguet M., Babinet C., Shih J.C., et al. (1995). Aggressive behavior and altered amounts of brain serotonin and norepinephrine in mice lacking MAOA. Science 268:1763-1766.
Chase I.D., Bartolomeo C., Dugatkin L.A. (1994). Aggressive interactions and inter-contest interval: how long do winners keep winning? Anim Behav 48:393-400.
Chase I.D., Tovey C., Spangler-Martin D., Manfredonia M. (2002). Individual differences versus social dynamics in the formation of animal dominance hierarchies. Proc Natl Acad Sci U S A 99:5744-5749.
Chichinadze K., Chichinadze N. (2008). Stress-induced increase of testosterone: contributions of social status and sympathetic reactivity. Physiol Behav 94:595-603.
Choi D.C., Furay A.R., Evanson N.K., Ostrander M.M., Ulrich-Lai Y.M., Herman J.P. (2007). Bed nucleus of the stria terminalis subregions differentially regulate

hypothalamic-pituitary-adrenal axis activity: implications for the integration of limbic inputs. J Neurosci 27:2025-2034.
Choleris E., Little S.R., Mong J.A., Puram S.V., Langer R., Pfaff D.W. (2007). Microparticle-based delivery of oxytocin receptor antisense DNA in the medial amygdala blocks social recognition in female mice. Proc Natl Acad Sci U S A 104:4670-4675.
Compaan J.C., Buijs R.M., Pool C.W., De Ruiter A.J., Koolhaas J.M. (1993). Differential lateral septal vasopressin innervation in aggressive and nonaggressive male mice. Brain research bulletin 30:1-6.
Conboy L., Sandi C. (2009). Stress at learning facilitates memory formation by regulating AMPA receptor trafficking through a glucocorticoid action. Neuropsychopharmacology.
Cordero M.I., Kruyt N.D., Merino J.J., Sandi C. (2002). Glucocorticoid involvement in memory formation in a rat model for traumatic memory. Stress 5:73-79.
Cordero M.I., Merino J.J., Sandi C. (1998). Correlational relationship between shock intensity and corticosterone secretion on the establishment and subsequent expression of contextual fear conditioning. Behav Neurosci 112:885-891.
Cordero M.I., Sandi C. (1998). A role for brain glucocorticoid receptors in contextual fear conditioning: dependence upon training intensity. Brain Res 786:11-17.
Cordero M.I., Sandi C. (2007). Stress amplifies memory for social hierarchy. Frontiers in neuroscience 1:175-184.
Costanzo D.J., Enloe L.J., Hothersall D. (1977). Effects of septal lesions on social dominance in rats. Behav Biol 20:454-462.
Crawley J.N., Chen T., Puri A., Washburn R., Sullivan T.L., Hill J.M., Young N.B., Nadler J.J., Moy S.S., Young L.J., Caldwell H.K., Young W.S. 3rd (2007). Social approach behaviors in oxytocin knockout mice: Comparison of two independent lines tested in different laboratory environments. Neuropeptides.
Cullinan W.E., Herman J.P., Watson S.J. (1993). Ventral subicular interaction with the hypothalamic paraventricular nucleus: evidence for a relay in the bed nucleus of the stria terminalis. The Journal of comparative neurology 332:1-20.
Dantzer R., Bluthe R.M., Koob G.F., Le Moal M. (1987). Modulation of social memory in male rats by neurohypophyseal peptides. Psychopharmacology 91:363-368.
Dantzer R., Koob G.F., Bluthe R.M., Le Moal M. (1988). Septal vasopressin modulates social memory in male rats. Brain Res 457:143-147.
Dayas C.V., Buller K.M., Day T.A. (1999). Neuroendocrine responses to an emotional stressor: evidence for involvement of the medial but not the central amygdala. Eur J Neurosci 11:2312-2322.
de Almeida R.M., Ferrari P.F., Parmigiani S., Miczek K.A. (2005). Escalated aggressive behavior: dopamine, serotonin and GABA. Eur J Pharmacol 526:51-64.
de Jong J.G., van der Vegt B.J., Buwalda B., Koolhaas J.M. (2005). Social environment determines the long-term effects of social defeat. Physiol Behav 84:87-95.
de Kloet E.R. (2000). Stress in the brain. Eur J Neurosci 405:187-198.
de Kloet E.R., Joels M., Holsboer F. (2005). Stress and the brain: from adaptation to disease. Nat Rev Neurosci 6:463-475.
de Kloet E.R., Oitzl M.S., Joels M. (1999). Stress and cognition: are corticosteroids good or bad guys? Trends in Neurosci 22:422-426.
de Quervain D.J., Aerni A., Schelling G., Roozendaal B. (2009). Glucocorticoids and the regulation of memory in health and disease. Front Neuroendocrinol 30:358-370.
De Vries G.J., Buijs R.M. (1983). The origin of the vasopressinergic and oxytocinergic innervation of the rat brain with special reference to the lateral septum. Brain Res 273:307-317.

Delville Y., De Vries G.J., Ferris C.F. (2000). Neural connections of the anterior hypothalamus and agonistic behavior in golden hamsters. Brain, behavior and evolution 55:53-76.
Delville Y., Mansour K.M., Ferris C.F. (1996). Serotonin blocks vasopressin-facilitated offensive aggression: interactions within the ventrolateral hypothalamus of golden hamsters. Physiol Behav 59:813-816.
Dhingra N.K., Raju T.R., Meti B.L. (1997). Selective reduction of monoamine oxidase A and B in the frontal cortex of subordinate rats. Brain Res 758:237-240.
Di Scala-Guenot D., Strosser M.T. (1995). Downregulation of the oxytocin receptor on cultured astroglial cells. Am J Physiol 268:C413-418.
Dong Q., Salva A., Sottas C.M., Niu E., Holmes M., Hardy M.P. (2004). Rapid glucocorticoid mediation of suppressed testosterone biosynthesis in male mice subjected to immobilization stress. Journal of andrology 25:973-981.
Dugatkin L.A., Earley R.L. (2004). Individual recognition, dominance hierarchies and winner and loser effects. Proc Biol Sci 271:1537-1540.
Dunn J.D. (1987). Plasma corticosterone responses to electrical stimulation of the bed nucleus of the stria terminalis. Brain Res 407:327-331.
Ebner K., Bosch O.J., Kromer S.A., Singewald N., Neumann I.D. (2005). Release of oxytocin in the rat central amygdala modulates stress-coping behavior and the release of excitatory amino acids. Neuropsychopharmacology 30:223-230.
Ebner K., Wotjak C.T., Landgraf R., Engelmann M. (2000). A single social defeat experience selectively stimulates the release of oxytocin, but not vasopressin, within the septal brain area of male rats. Brain Res 872:87-92.
Engelmann M., Ebner K., Landgraf R., Holsboer F., Wotjak C.T. (1999). Emotional stress triggers intrahypothalamic but not peripheral release of oxytocin in male rats. Journal of Neuroendocrinology 11:867-872.
Engelmann M., Ebner K., Wotjak C.T., Landgraf R. (1998). Endogenous oxytocin is involved in short-term olfactory memory in female rats. Behav Brain Res 90:89-94.
Engelmann M., Landgraf R. (1994). Microdialysis administration of vasopressin into the septum improves social recognition in Brattleboro rats. Physiol Behav 55:145-149.
Engelmann M., Wotjak C.T., Neumann I., Ludwig M., Landgraf R. (1996). Behavioral consequences of intracerebral vasopressin and oxytocin: focus on learning and memory. Neurosci Biobehav Rev 20:341-358.
Everts H.G., De Ruiter A.J., Koolhaas J.M. (1997). Differential lateral septal vasopressin in wild-type rats: correlation with aggression. Horm Behav 31:136-144.
Everts H.G., Koolhaas J.M. (1997). Lateral septal vasopressin in rats: role in social and object recognition? Brain Res 760:1-7.
Everts H.G., Koolhaas J.M. (1999). Differential modulation of lateral septal vasopressin receptor blockade in spatial learning, social recognition, and anxiety-related behaviors in rats. Behav Brain Res 99:7-16.
Fekete E.M., Zhao Y., Li C., Sabino V., Vale W.W., Zorrilla E.P. (2009). Social defeat stress activates medial amygdala cells that express type 2 corticotropin-releasing factor receptor mRNA. Neuroscience 162:5-13.
Ferguson J.N., Aldag J.M., Insel T.R., Young L.J. (2001). Oxytocin in the medial amygdala is essential for social recognition in the mouse. J Neurosci 21:8278-8285.
Ferguson J.N., Young L.J., Hearn E.F., Matzuk M.M., Insel T.R., Winslow J.T. (2000). Social amnesia in mice lacking the oxytocin gene. Nature genetics 25:284-288.
Ferguson J.N., Young L.J., Insel T.R. (2002). The neuroendocrine basis of social recognition. Front Neuroendocrinol 23:200-224.

Ferris C.F. (1996). Serotonin diminishes aggression by suppressing the activity of the vasopressin system. Ann N Y Acad Sci 794:98-103.
Ferris C.F., Albers H.E., Wesolowski S.M., Goldman B.D., Luman S.E. (1984). Vasopressin injected into the hypothalamus triggers a stereotypic behavior in golden hamsters. Science 224:521-523.
Ferris C.F., Lu S.F., Messenger T., Guillon C.D., Heindel N., Miller M., Koppel G., Robert Bruns F., Simon N.G. (2006). Orally active vasopressin V1a receptor antagonist, SRX251, selectively blocks aggressive behavior. Pharmacology, biochemistry, and behavior 83:169-174.
Ferris C.F., Melloni R.H. Jr., Koppel G., Perry K.W., Fuller R.W., Delville Y. (1997). Vasopressin/serotonin interactions in the anterior hypothalamus control aggressive behavior in golden hamsters. J Neurosci 17:4331-4340.
Ferris C.F., Pollock J., Albers H.E., Leeman S.E. (1985). Inhibition of flank-marking behavior in golden hamsters by microinjection of a vasopressin antagonist into the hypothalamus. Neuroscience letters 55:239-243.
Ferris C.F., Potegal M. (1988). Vasopressin receptor blockade in the anterior hypothalamus suppresses aggression in hamsters. Physiol Behav 44:235-239.
Ferris C.F., Stolberg T., Kulkarni P., Murugavel M., Blanchard R., Blanchard D.C., Febo M., Brevard M., Simon N.G. (2008). Imaging the neural circuitry and chemical control of aggressive motivation. BMC neuroscience 9:111.
Gimpl G., Fahrenholz F. (2001). The oxytocin receptor system: structure, function, and regulation. Physiol Rev 81:629-683.
Goodson J.L., Bass A.H. (2001). Social behavior functions and related anatomical characteristics of vasotocin/vasopressin systems in vertebrates. Brain Res Brain Res Rev 35:246-265.
Gourley S.L., Kedves A.T., Olausson P., Taylor J.R. (2009). A history of corticosterone exposure regulates fear extinction and cortical NR2B, GluR2/3, and BDNF. Neuropsychopharmacology 34:707-716.
Gray T.S., Piechowski R.A., Yracheta J.M., Rittenhouse P.A., Bethea C.L., Van de Kar L.D. (1993). Ibotenic acid lesions in the bed nucleus of the stria terminalis attenuate conditioned stress-induced increases in prolactin, ACTH and corticosterone. Neuroendocrinology 57:517-524.
Haller J., Albert I., Makara G.B. (1997). Acute behavioural effects of corticosterone lack specificity but show marked context-dependency. J Neuroendocrinol 9:515-518.
Haller J., Halasz J., Mikics E., Kruk M.R. (2004). Chronic glucocorticoid deficiency-induced abnormal aggression, autonomic hypoarousal, and social deficit in rats. J Neuroendocrinol 16:550-557.
Haller J., Halasz J., Mikics E., Kruk M.R., Makara G.B. (2000). Ultradian corticosterone rhythm and the propensity to behave aggressively in male rats. J Neuroendocrinol 12:937-940.
Haller J., Kruk M.R. (2006). Normal and abnormal aggression: human disorders and novel laboratory models. Neurosci Biobehav Rev 30:292-303.
Haller J., Millar S., van de Schraaf J., de Kloet R.E., Kruk M.R. (2000). The active phase-related increase in corticosterone and aggression are linked. J Neuroendocrinol 12:431-436.
Haller J., Toth M., Halasz J., De Boer S.F. (2006). Patterns of violent aggression-induced brain c-fos expression in male mice selected for aggressiveness. Physiol Behav 88:173-182.

Haller J., van de Schraaf J., Kruk M.R. (2001). Deviant forms of aggression in glucocorticoid hyporeactive rats: a model for 'pathological' aggression? J Neuroendocrinol 13:102-107.

Hardy M.P., Sottas C.M., Ge R., McKittrick C.R., Tamashiro K.L., McEwen B.S., Haider S.G., Markham C.M., Blanchard R.J., Blanchard D.C., Sakai R.R. (2002). Trends of reproductive hormones in male rats during psychosocial stress: role of glucocorticoid metabolism in behavioral dominance. Biol Reprod 67:1750-1755.

Hayden-Hixson D.M., Ferris C.F. (1991). Cortisol exerts site-, context- and dose-dependent effects on agonistic responding in hamsters. J Neuroendocrinol 3:613-622.

Hayden-Hixson D.M., Ferris C.F. (1991). Steroid-specific regulation of agonistic responding in the anterior hypothalamus of male hamsters. Physiol Behav 50:793-799.

Heller K.E. (1978). Role of corticosterone in the control of post-shock fighting behaviour in male laboratory mice. Behavioural Processes 3:211-222.

Herman J.P., Cullinan W.E., Watson S.J. (1994). Involvement of the bed nucleus of the stria terminalis in tonic regulation of paraventricular hypothalamic CRH and AVP mRNA expression. J Neuroendocrinol 6:433-442.

Hoshaw B.A., Evans J.C., Mueller B., Valentino R.J., Lucki I. (2006). Social competition in rats: cell proliferation and behavior. Behav Brain Res 175:343-351.

Insel T.R., Winslow J.T., Witt D.M. (1992). Homologous regulation of brain oxytocin receptors. Endocrinology 130:2602-2608.

Irvin R.W., Szot P., Dorsa D.M., Potegal M., Ferris C.F. (1990). Vasopressin in the septal area of the golden hamster controls scent marking and grooming. Physiol Behav 48:693-699.

Joels M. (2006). Corticosteroid effects in the brain: U-shape it. Trends in pharmacological sciences 27:244-250.

Joels M., Pu Z., Wiegert O., Oitzl M.S., Krugers H.J. (2006). Learning under stress: How does it work? Trends Cog Sci 10:152-158.

Kollack-Walker S., Newman S.W. (1995). Mating and agonistic behavior produce different patterns of Fos immunolabeling in the male Syrian hamster brain. Neuroscience 66:721-736.

Kollack-Walker S., Watson S.J., Akil H. (1997). Social stress in hamsters: defeat activates specific neurocircuits within the brain. J Neurosci 17:8842-8855.

Koolhaas J.M., de Boer S.F., Coppens C.M., Buwalda B. (2010). Neuroendocrinology of coping styles: towards understanding the biology of individual variation. Front Neuroendocrinol 31:307-321.

Kostowski W., Plewako M., Bidzinski A. (1984). Brain serotonergic neurons: their role in a form of dominance-subordination behavior in rats. Physiol Behav 33:365-371.

Kremarik M.J., Stoeckel, M.E. (1991). Autoradiographic detection of oxytocin- and vasopressin-binding sites in various subnuclei of the bed nucleus of the stria terminalis in the rat. Effects on functional and experimental sexual steroid variations. Journal of Neuroendocrinology 3.

Kruk M.R. (1991). Ethology and pharmacology of hypothalamic aggression in the rat. Neurosci Biobehav Rev 15:527-538.

Lai W.S., Johnston R.E. (2002). Individual recognition after fighting by golden hamsters: a new method. Physiol Behav 76:225-239.

Landgraf R., Frank E., Aldag J.M., Neumann I.D., Sharer C.A., Ren X., Terwilliger E.F., Niwa M., Wigger A., Young L.J. (2003). Viral vector-mediated gene transfer of the vole V1a vasopressin receptor in the rat septum: improved social discrimination and active social behaviour. Eur J Neurosci 18:403-411.

Landgraf R., Gerstberger R., Montkowski A., Probst J.C., Wotjak C.T., Holsboer F., Engelmann M. (1995). V1 vasopressin receptor antisense oligodeoxynucleotide into septum reduces vasopressin binding, social discrimination abilities, and anxiety-related behavior in rats. J Neurosci 15:4250-4258.

Landgraf R., Neumann I.D. (2004). Vasopressin and oxytocin release within the brain: a dynamic concept of multiple and variable modes of neuropeptide communication. Front Neuroendocrinol 25:150-176.

Larcher A., Neculcea J., Chu K., Zingg H.H. (1995). Effects of retinoic acid and estrogens on oxytocin gene expression in the rat uterus: in vitro and in vivo studies. Mol Cell Endocrinol 114:69-76.

Larson E.T., Summers C.H. (2001). Serotonin reverses dominant social status. Behav Brain Res 121:95-102.

Le Moal M., Dantzer R., Michaud B., Koob G.F. (1987). Centrally injected arginine vasopressin (AVP) facilitates social memory in rats. Neuroscience letters 77:353-359.

Lee H.J., Macbeth A.H., Pagani J.H., Young W.S. 3rd (2009). Oxytocin: the great facilitator of life. Prog Neurobiol 88:127-151.

Leng G., Caquineau C., Ludwig M. (2008). Priming in oxytocin cells and in gonadotrophs. Neurochem Res 33:668-677.

Lesch K.P., Merschdorf U. (2000). Impulsivity, aggression, and serotonin: a molecular psychobiological perspective. Behav Sci Law 18:581-604.

Leshner A.I., Korn S.J., Mixon J.F., Rosenthal C., Besser A.K. (1980). Effects of corticosterone on submissiveness in mice: some temporal and theoretical considerations. Physiol Behav 24:283-288.

Leshner A.I., Moyer J.A. (1975). Androgens and agonistic behavior in mice: relevance to aggression and irrelevance to avoidance-of-attack. Physiol Behav 15:695-699.

Leshner A.I., Moyer J.A., Walker W.A. (1975). Pituitary-adrenocortical activity and avoidance-of-attack in mice. Physiol Behav 15:689-693.

Leshner A.I., Politch J.A. (1979). Hormonal control of submissiveness in mice: irrelevance of the androgens and relevance of the pituitary-adrenal hormones. Physiol Behav 22:531-534.

Leshner A.I., Walker W.A., Johnson A.E., Kelling J.S., Kreisler S.J., Svare B.B. (1973). Pituitary adrenocortical activity and intermale aggressiveness in isolated mice. Physiol Behav 11:705-711.

Liberzon I., Young E.A. (1997). Effects of stress and glucocorticoids on CNS oxytocin receptor binding. Psychoneuroendocrinology 22:411-422.

Livak K.J., Schmittgen T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.

Lucion A., Vogel W.H. (1994). Effects of stress on defensive aggression and dominance in a water competition test. Integr Physiol Behav Sci 29:415-422.

Ludwig M., Leng G. (2006). Dendritic peptide release and peptide-dependent behaviours. Nat Rev Neurosci 7:126-136.

Luiten P.G., Koolhaas J.M., de Boer S., Koopmans S.J. (1985). The cortico-medial amygdala in the central nervous system organization of agonistic behavior. Brain Res 332:283-297.

Maaswinkel H., Baars A.M., Gispen W.H., Spruijt B.M. (1996). Roles of the basolateral amygdala and hippocampus in social recognition in rats. Physiol Behav 60:55-63.

Makara G.B., Haller J. (2001). Non-genomic effects of glucocorticoids in the neural system. Evidence, mechanisms and implications. Prog Neurobiol 65:367-390.

Mantella R.C., Vollmer R.R., Rinaman L., Li X., Amico J.A. (2004). Enhanced corticosterone concentrations and attenuated Fos expression in the medial amygdala of female

oxytocin knockout mice exposed to psychogenic stress. Am J Physiol Regul Integr Comp Physiol 287:R1494-1504.
Markham C.M., Huhman K.L. (2008). Is the medial amygdala part of the neural circuit modulating conditioned defeat in Syrian hamsters? Learn Mem 15:6-12.
Martinez M., Phillips P.J., Herbert J. (1998). Adaptation in patterns of c-fos expression in the brain associated with exposure to either single or repeated social stress in male rats. Eur J Neurosci 10:20-33.
McDermott R., Tingley D., Cowden J., Frazzetto G., Johnson D.D. (2009). Monoamine oxidase A gene (MAOA) predicts behavioral aggression following provocation. Proc Natl Acad Sci U S A 106:2118-2123.
McElligott Z.A., Winder D.G. (2009). Modulation of glutamatergic synaptic transmission in the bed nucleus of the stria terminalis. Prog Neuropsychopharmacol Biol Psychiatry 33:1329-1335.
McEwen B.S. (2005). Glucocorticoids, depression, and mood disorders: structural remodeling in the brain. Metabolism 54:20-23.
McEwen B.S. (2007). Physiology and neurobiology of stress and adaptation: central role of the brain. Physiol Rev 87:873-904.
McKittrick C.R., Blanchard D.C., Blanchard R.J., McEwen B.S., Sakai R.R. (1995). Serotonin receptor binding in a colony model of chronic social stress. Biol Psychiatry 37:383-393.
Meerlo P., Overkamp G.J., Daan S., Van Den Hoofdakker R.H., Koolhaas J.M. (1996). Changes in Behaviour and Body Weight Following a Single or Double Social Defeat in Rats. Stress 1:21-32.
Mikics E., Barsy B., Barsvari B., Haller J. (2005). Behavioral specificity of non-genomic glucocorticoid effects in rats: effects on risk assessment in the elevated plus-maze and the open-field. Horm Behav 48:152-162.
Mikics E., Barsy B., Haller J. (2007). The effect glucocorticoids on aggressiveness in established colonies of rats. Psychoneuroendocrinology 32:160-170.
Mikics E., Kruk M.R., Haller J. (2004). Genomic and non-genomic effects of glucocorticoids on aggressive behavior in male rats. Psychoneuroendocrinology 29:618-635.
Mohr E., Schmitz E. (1991). Functional characterization of estrogen and glucocorticoid responsive elements in the rat oxytocin gene. Brain Res Mol Brain Res 9:293-298.
Morsink M.C., Van Gemert N.G., Steenbergen P.J., Joels M., De Kloet E.R., Datson N.A. (2007). Rapid glucocorticoid effects on the expression of hippocampal neurotransmission-related genes. Brain Res 1150:14-20.
Moyer J.A., Leshner A.I. (1976). Pituitary-adrenal effects on avoidance-of-attack in mice: separation of the effects of ACTH and corticosterone. Physiol Behav 17:297-301.
Nelson R.J., Trainor B.C. (2007). Neural mechanisms of aggression. Nat Rev Neurosci 8:536-546.
Neumann I.D. (2007). Stimuli and consequences of dendritic release of oxytocin within the brain. Biochem Soc Trans 35:1252-1257.
Neumann I.D., Kromer S.A., Toschi N., Ebner K. (2000). Brain oxytocin inhibits the (re)activity of the hypothalamo-pituitary-adrenal axis in male rats: involvement of hypothalamic and limbic brain regions. Regulatory peptides 96:31-38.
Neumann I.D., Wigger A., Torner L., Holsboer F., Landgraf R. (2000). Brain oxytocin inhibits basal and stress-induced activity of the hypothalamo-pituitary-adrenal axis in male and female rats: partial action within the paraventricular nucleus. J Neuroendocrinol 12:235-243.
Newman S.W. (1999). The medial extended amygdala in male reproductive behavior. A node in the mammalian social behavior network. Ann N Y Acad Sci 877:242-257.

Oitzl M.S., de Kloet E.R. (1992). Selective corticosteroid antagonists modulate specific aspects of spatial orientation learning. Behav Neurosci 106:62-71.
Oitzl M.S., Reichardt H.M., Joels M., de Kloet E.R. (2001). Point mutation in the mouse glucocorticoid receptor preventing DNA binding impairs spatial memory. Proc Natl Acad Sci U S A 98:12790-12795.
Okuda S., Roozendaal B., McGaugh J.L. (2004). Glucocorticoid effects on object recognition memory require training-associated emotional arousal. Proc Natl Acad Sci U S A 101:853-858.
Ostberg V. (2003). Children in classrooms: peer status, status distribution and mental well-being. Social science & medicine (1982) 56:17-29.
Ostrowski N.L. (1998). Oxytocin receptor mRNA expression in rat brain: implications for behavioral integration and reproductive success. Psychoneuroendocrinology 23:989-1004.
Ostrowski N.L., Lolait S.J., Young W.S. 3rd (1994). Cellular localization of vasopressin V1a receptor messenger ribonucleic acid in adult male rat brain, pineal, and brain vasculature. Endocrinology 135:1511-1528.
Parker G.A. (1974). Assessment strategy and the evolution of fighting behaviour. J Theor Biol 47:223-243.
Patchev V.K., Schlosser S.F., Hassan A.H., Almeida O.F. (1993). Oxytocin binding sites in rat limbic and hypothalamic structures: site-specific modulation by adrenal and gonadal steroids. Neuroscience 57:537-543.
Paxinos G., Watson C. (1998). The rat brain in stereotaxic coordinates. Academic Press, New York.
Petrulis A., Peng M., Johnston R.E. (2000). The role of the hippocampal system in social odor discrimination and scent-marking in female golden hamsters (Mesocricetus auratus). Behav Neurosci 114:184-195.
Petrulis A., Weidner M., Johnston R. (2004). Recognition of competitors by male golden hamsters. Physiol Behav 81:629-638.
Popik P., Van Ree J.M. (1991). Oxytocin but not vasopressin facilitates social recognition following injection into the medial preoptic area of the rat brain. Eur Neuropsychopharmacol 1:555-560.
Popik P., Vetulani J. (1991). Opposite action of oxytocin and its peptide antagonists on social memory in rats. Neuropeptides 18:23-27.
Popik P., Vetulani J., van Ree J.M. (1992). Low doses of oxytocin facilitate social recognition in rats. Psychopharmacology 106:71-74.
Popik P., Vetulani J., Van Ree J.M. (1996). Facilitation and attenuation of social recognition in rats by different oxytocin-related peptides. Eur J Pharmacol 308:113-116.
Potegal M., Ferris C.F., Hebert M., Meyerhoff J., Skaredoff L. (1996). Attack priming in female Syrian golden hamsters is associated with a c-fos-coupled process within the corticomedial amygdala. Neuroscience 75:869-880.
Retana-Marquez S., Bonilla-Jaime H., Vazquez-Palacios G., Martinez-Garcia R., Velazquez-Moctezuma J. (2003). Changes in masculine sexual behavior, corticosterone and testosterone in response to acute and chronic stress in male rats. Horm Behav 44:327-337.
Richard, S., Zingg H.H. (1990). The human oxytocin gene promoter is regulated by estrogens. J Biol Chem 265(11):6098-6103.
Roozendaal B. (2000). 1999 Curt P. Richter award. Glucocorticoids and the regulation of memory consolidation. Psychoneuroendocrinology 25:213-238.
Roozendaal B. (2002). Stress and memory: opposing effects of glucocorticoids on memory consolidation and memory retrieval. Neurobiol Learn Mem 78:578-595.

Roozendaal B., McGaugh J.L. (1996). Amygdaloid nuclei lesions differentially affect glucocorticoid-induced memory enhancement in an inhibitory avoidance task. Neurobiol Learn Mem 65:1-8.
Roozendaal B., Okuda S., Van der Zee E.A., McGaugh J.L. (2006). Glucocorticoid enhancement of memory requires arousal-induced noradrenergic activation in the basolateral amygdala. Proc Natl Acad Sci U S A 103:6741-6746.
Roozendaal B., Quirarte G.L., McGaugh J.L. (2002). Glucocorticoids interact with the basolateral amygdala beta-adrenoceptor--cAMP/cAMP/PKA system in influencing memory consolidation. Eur J Neurosci 15:553-560.
Rutte C., Taborsky M., Brinkhof M.W. (2006). What sets the odds of winning and losing? Trends Ecol Evol 21:16-21.
Sandi C. (2004). Stress, cognitive impairment and cell adhesion molecules. Nat Rev Neurosci 5:917-930.
Sandi C., Loscertales M., Guaza C. (1997). Experience-dependent facilitating effect of corticosterone on spatial memory formation in the water maze. Eur J Neurosci 9:637-642.
Sandi C., Pinelo-Nava M.T. (2007). Stress and memory: behavioral effects and neurobiological mechanisms. Neural plasticity 2007:article ID 78970.
Sandi C., Rose S.P.R. (1994). Corticosterone enhances long-term retention in one-day-old chicks trained in a weak passive avoidance learning paradigm. Brain Res 647:106-112.
Sandi C., Rose S.P.R. (1997). Training-dependent biphasic effects of corticosterone in memory formation for a passive avoidance task in chicks. Psychopharmacology 133:152-160.
Sandi C., Rose S.P.R., Mileusnic R., Lancashire C. (1995). Corticosterone facilitates long-term memory formation via enhanced glycoprotein synthesis. Neuroscience 69:1087-1093.
Sandi C., Venero C., Guaza C. (1996). Nitric oxide synthesis inhibitors prevent rapid behavioral effects of corticosterone in rats. Neuroendocrinology 63:446-453.
Sandi C., Venero C., Guaza C. (1996). Novelty-related rapid locomotor effects of corticosterone in rats. Eur J Neurosci 8:794-800.
Sapolsky R.M. (2005). The influence of social hierarchy on primate health. Science 308:648-652.
Schulz D., Canbeyli R.S. (2000). Lesion of the bed nucleus of the stria terminalis enhances learned despair. Brain research bulletin 52:83-87.
Sheehan T.P., Chambers R.A., Russell D.S. (2004). Regulation of affect by the lateral septum: implications for neuropsychiatry. Brain Res Brain Res Rev 46:71-117.
Simon N.G., Cologer-Clifford A., Lu S.F., McKenna S.E., Hu S. (1998). Testosterone and its metabolites modulate 5HT1A and 5HT1B agonist effects on intermale aggression. Neurosci Biobehav Rev 23:325-336.
Stevenson K.R., Riley P.R., Stewart H.J., Flint A.P., Wathes D.C. (1994). Localization of oxytocin receptor mRNA in the ovine uterus during the oestrous cycle and early pregnancy. J Mol Endocrinol 12:93-105.
Stork O., Welzl H., Cremer H., Schachner M. (1997). Increased intermale aggression and neuroendocrine response in mice deficient for the neural cell adhesion molecule (NCAM). Eur J Neurosci 9:1117-1125.
Summers C.H., Forster G.L., Korzan W.J., Watt M.J., Larson E.T., Overli O., Hoglund E., Ronan P.J., Summers T.R., Renner K.J., Greenberg N. (2005). Dynamics and mechanics of social rank reversal. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 191:241-252.

Timmer M., Sandi C. (2010). A role for glucocorticoids in the long-term establishment of a social hierarchy. Psychoneuroendocrinology, 35(10): 1543-1552.
Tribollet E., Audigier S., Dubois-Dauphin M., Dreifuss J.J. (1990). Gonadal steroids regulate oxytocin receptors but not vasopressin receptors in the brain of male and female rats. An autoradiographical study. Brain Res 511:129-140.
Tronel S., Alberini C.M. (2007). Persistent disruption of a traumatic memory by postretrieval inactivation of glucocorticoid receptors in the amygdala. Biol Psychiatry 62:33-39.
Vaccari C., Lolait S.J., Ostrowski N.L. (1998). Comparative distribution of vasopressin V1b and oxytocin receptor messenger ribonucleic acids in brain. Endocrinology 139:5015-5033.
Van Kreveld D. (1970). A selective review of dominance-subordinate relations in animals. Genetics and Psychology Monographs 81:143-173.
van Wimersma Greidanus T.B., Maigret C. (1996). The role of limbic vasopressin and oxytocin in social recognition. Brain Res 713:153-159.
Veenema A.H., Beiderbeck D.I., Lukas M., Neumann I.D. (2010). Distinct correlations of vasopressin release within the lateral septum and the bed nucleus of the stria terminalis with the display of intermale aggression. Horm Behav 58:273-281.
Veenema A.H., Neumann I.D. (2008). Central vasopressin and oxytocin release: regulation of complex social behaviours. Progress in brain research 170:261-276.
Veinante P., Freund-Mercier M.J. (1997). Distribution of oxytocin- and vasopressin-binding sites in the rat extended amygdala: a histoautoradiographic study. The Journal of comparative neurology 383:305-325.
Venero C., Guaza C., Sandi C. (1996). Regional and temporal modulation of brain glycoprotein synthesis by corticosterone. Neuroreport 7:2819-2822.
Venero C, Sandi C. 1997. Effects of NMDA and AMPA receptor antagonists on corticosterone facilitation of long-term memory in the chick. Eur J Neurosci 9:1923-1928.
Vochteloo J.D., Koolhaas J.M. (1987). Medial amygdala lesions in male rats reduce aggressive behavior: interference with experience. Physiol Behav 41:99-102.
Watters J.J., Wilkinson C.W., Dorsa D.M. (1996). Glucocorticoid regulation of vasopressin V1a receptors in rat forebrain. Brain Res Mol Brain Res 38:276-284.
Wersinger S.R., Caldwell H.K., Christiansen M., Young W.S. 3rd (2007). Disruption of the vasopressin 1b receptor gene impairs the attack component of aggressive behavior in mice. Genes Brain Behav 6:653-660.
Wersinger S.R., Caldwell H.K., Martinez L., Gold P., Hu S.B., Young W.S. 3rd (2007). Vasopressin 1a receptor knockout mice have a subtle olfactory deficit but normal aggression. Genes Brain Behav 6:540-551.
Wersinger S.R., Ginns E.I., O'Carroll A.M., Lolait S.J., Young W.S. 3rd (2002). Vasopressin V1b receptor knockout reduces aggressive behavior in male mice. Molecular psychiatry 7:975-984.
Wilkinson R.G. (1999). Health, hierarchy, and social anxiety. Ann N Y Acad Sci 896:48-63.
Wilkinson R.G., Pickett K.E. (2006). Income inequality and population health: a review and explanation of the evidence. Social science & medicine (1982) 62:1768-1784.
Wilkinson R.G., Pickett K.E. (2007). The problems of relative deprivation: why some societies do better than others. Social science & medicine (1982) 65:1965-1978.
Windle R.J., Kershaw Y.M., Shanks N., Wood S.A., Lightman S.L., Ingram C.D. (2004). Oxytocin attenuates stress-induced c-fos mRNA expression in specific forebrain regions associated with modulation of hypothalamo-pituitary-adrenal activity. J Neurosci 24:2974-2982.

Windle R.J., Shanks N., Lightman S.L., Ingram C.D. (1997). Central oxytocin administration reduces stress-induced corticosterone release and anxiety behavior in rats. Endocrinology 138:2829-2834.

Winslow J.T., Insel T.R. (1993). Effects of central vasopressin administration to infant rats. Eur J Pharmacol 233:101-107.

Wood G.E., Young L.T., Reagan L.P., McEwen B.S. (2003). Acute and chronic restraint stress alter the incidence of social conflict in male rats. Horm Behav 43:205-213.

Wotjak C.T., Ganster J., Kohl G., Holsboer F., Landgraf R., Engelmann M. (1998). Dissociated central and peripheral release of vasopressin, but not oxytocin, in response to repeated swim stress: new insights into the secretory capacities of peptidergic neurons. Neuroscience 85:1209-1222.

Wotjak C.T., Kubota M., Liebsch G., Montkowski A., Holsboer F., Neumann I., Landgraf R. (1996). Release of vasopressin within the rat paraventricular nucleus in response to emotional stress: a novel mechanism of regulating adrenocorticotropic hormone secretion? J Neurosci 16:7725-7732.

Yoshimura R., Kiyama H., Kimura T., Araki T., Maeno H., Tanizawa O., Tohyama M. (1993). Localization of oxytocin receptor messenger ribonucleic acid in the rat brain. Endocrinology 133(3):1239-1246.

Young W.S. 3rd, Gainer H. (2003). Transgenesis and the study of expression, cellular targeting and function of oxytocin, vasopressin and their receptors. Neuroendocrinology 78:185-203.

Appendix

Chapter 2, 3 and 4 described the main results of this thesis research. In addition, several experiments that were done at different phases of this study but not included in the former chapters are included in this Appendix. These experiments include the evaluation of (1) the effects of administration of the protein synthesis inhibitor anisomycin after the first encounter on memory formation, (2) the changes in *Otr* and *V1ar* mRNA levels in LS one week after administration of an OT antagonist in MeA, (3) a social preference test for a juvenile rat after exposure to acute stress, (4) the difference in offensive behavior during the first encounter between rats of non-stressed and stressed pairs, (5) mRNA levels of the glucocorticoid receptor *(Gr)* and neural cell adhesion molecule *(Ncam)* in MeA, LS and BNST, (6) mRNA levels of *Otr*, *V1ar*, *Ar*, *Maoa*, *Gr* and *Ncam* in hippocampus, (7) mRNA levels of *Otr* and *V1ar* in BNST and (8) plasma corticosterone levels at three hours and one week after the first encounter.

Materials and methods

Animals

Male Wistar rats (Charles River Laboratories, Lyon, France) weighing 250-275 grams at arrival were individually housed with *ad libitum* access to food and water. Animals were kept in a 12h light/dark cycle with lights on at 0700h, and a constant temperature of 22 ± 2 °C. Animals were left undisturbed for one week after arrival before starting the experiment, except when surgery was performed, which was performed a few days after arrival. Rats were weighed once per week. Experiments were performed with approval of the Cantonal Veterinary Authorities (Vaud, Switzerland).

All experiments took place between 0800h and 1400h, except for the water competition test that took place between 1400h and 1900h, after a water deprivation period of 6h (from 0800h until 1400h). All rats were handled for 2 min per day during three days before exposure to the elevated plus maze (see below).

Surgery

Rats were anaesthetized with ketamine (70 mg/kg) and xylazine (6 mg/kg), administered by i.p. injection. 10 mm long stainless steel guide cannulae of 23 gauge (Plastics One, Roanoke, USA) were implanted bilaterally in the MeA at -2.50 mm anterior and ±3.3 mm lateral relative to Bregma and -7.9 mm ventral relative to dura (Paxinos and Watson, 1998) using a standard stereotaxic frame (Stoelting, USA). Cannulae were fixed to the skull with dental acrylic cement (Kaladent, Switzerland) and anchored with Vetbond Tissue Adhesive glue (3M). Dummy cannulae (10mm, Plastics One, Roanoke, USA) were inserted into the cannulae to protect them from blocking. Rats received an injection with Antisedan (0.04 ml s.c., Pfizer, Switzerland) after the surgery to reverse the effects of the anesthetics. Paracetamol was added to the drinking water as analgesic in the days following surgery. The rats were allowed to recover for at least one week before the start of the experiment.

Drugs and infusion procedure

The selective OT antagonist (OTA) desGly-NH_2-d$(CH_2)_5$[D-Tyr^2,Thr^4]OVT was used for infusions into the MeA (the OTA was a generous gift of dr. M. Manning, Toledo, OH, USA). The OTA was dissolved in saline. Internal injectors of 11 mm (extending 1 mm from the end of the guide cannula, 30 gauge, Plastics One, Roanoke, USA) were used to administer the drug. The injectors were connected to 10 µl microsyringes (SGE Analytical Science,

Australia) on an automated infusion pump (Harvard Apparatus, MA, USA) through polyethylene tubing. 1 μg of OTA in 1 μl of saline was infused into the MeA of each hemisphere at a rate of 0.5 μl/min. After the infusion the injector was left in place for 1 min to allow diffusion of the drug. Saline was used as a vehicle. The OTA was infused immediately after the encounter to the subordinate rat. The other rat of the pair and both rats of control pairs were infused with vehicle.

The protein synthesis inhibitor anisomycin was injected IP (Sigma Chemical Co., Switzerland) at a dose of 150 mg/kg. The dose of 150 mg/kg was used in previous experiments in our group (Cordero and Sandi, 2007). Anisomycin was dissolved in saline by adding 1 N HCl. The pH was adjusted to 7.4 by adding NaOH. One rat of each pair (either the dominant or subordinate rat) was injected with anisomycin immediately after the end of the first encounter. The other rat of the pair was injected with vehicle. In control pairs both rats were injected with vehicle.

Elevated plus maze

Before the start of the experiment the 'elevated plus maze' (EPM) test was performed to measure anxiety-related behavior. The EPM consists of two opposing open arms (45x10 cm) and two opposing closed arms (45x10 cm with walls of 50 cm high) that extend from a central platform (10x10 cm), elevated 65 cm above the floor. The rats were placed individually on the central platform facing the same closed arm and were allowed to explore the EPM freely during 5 min. The behavior of each rat was video recorded and analyzed using a computerized tracking system (Ethovision 3.1.16, Noldus IT, the Netherlands). Time spent in the open and closed arms was measured.

Dyads and groups

Rats were distributed to dyads consisting of two rats that were matched for their body weight and anxiety level. The anxiety level was defined by the time spent in the open arms of the EPM. Thus, the rats in each dyad were considered equal in their probability to become dominant or subordinate during a first encounter. The fur of both rats in a dyad was marked on different body parts (i.e. neck and back) to help identifying the animals. Rats were habituated to marking during the three day habituation period that took place before the first social encounter. Dyads were divided randomly over the different groups. Each group consisted of 7-14 dyads.

Stress delivery – Contextual fear conditioning

In non-stressed pairs (Pns) none of the rats were stressed prior to the social encounter. In stressed pairs (Ps) one rat of the pair was stressed using a contextual fear conditioning paradigm just before the first social encounter. Fear conditioning was performed in an observation chamber of 30x37x25 cm (Panlab, Spain) made of black steel walls and a Plexiglas ceiling and door. The floor consisted of 20 steel rods connected to a shock source and solid-state scrambler for delivery of electrical shocks. The observation chamber was lit by a 20 W light bulb and there was a background noise of 68 dB. The protocol consisted of a habituation period of 3 min, followed by 3 footshocks of 1 mA lasting 1 sec each with an intershock interval of 60 sec. 30 sec after the last shock the rat was taken out and immediately submitted to the first social encounter with an unfamiliar rat in an adjacent room.

Food and water competition test

In order to habituate animals to the rewarding food used in the 'food competition test' (FCT), they received eight Chocopop flakes (Kellogg's, Switzerland) in their homecages daily, during two days before the start of the habituation to the experimental setup. The rats were habituated to the experimental setup for three days during which they were first placed in a clean homecage for 20 min and then in a food competition box for 10 min. The food competition box is a plastic box of 60x40 cm with walls of 32 cm high with a feeder containing eight Chocopops on one of the short sides. On day 1 of the experiment, the day after the last habituation day, the rats were submitted to a first encounter with an unfamiliar rat, consisting of a 'social interaction test' in a neutral homecage without food and water for 20 min, followed by a 'food competition test' (FCT) in which the rats had to compete for Chocopops during 10 min. After the tests each rat was returned to its homecage. The group without an encounter on day 1 was exposed to an extra day of habituation.

The memory for the formed hierarchy was tested in a 'water competition test' (WCT) that was performed on day 8 after a preceding water deprivation period of 6h. Rats were exposed to the same opponent as on day 1. The WCT was performed in a neutral homecage. After 2 min of habituation, a single bottle of water was presented and the behavior during the following 10 min was recorded.

Behavior was video-recorded and scored blindly using The Observer (v.5.0.25, Noldus IT, the Netherlands). The duration and frequency of offensive and defensive behaviors was scored. Offensive behaviors were attacks (biting), keeping down (pushing the opponent to the floor), offensive upright (standing on the hind legs in upright position) and lateral threat

(pushing or approaching the opponent showing its side with an arched back). Defensive behavior consisted of freezing (immobility), defensive upright (standing on the hind legs in response to offensive upright) and submissive posture (lying on the back). In the FCT the number of consumed Chocopops and the number of passes over the feeder was counted and in the WCT the time spent drinking was measured. The rat that showed most offensive behavior during the interaction was considered the dominant rat, the rat showing least offensive behavior and most defensive behavior the subordinate rat.

Control groups for studying gene expression levels were submitted to habituation and exposure to the experimental setup on day 1, with or without exposure to stress (control group and stressed control group), but not to social interaction.

Social preference test

The social preference test was carried out in a rectangular, three-chambered arena made out of grey opaque plastic. The two outer compartments were 30 x 35 cm and the center compartment 20 x 35 cm. Retractable doorways separated the compartments from each other. The walls were 35 cm high. In each outer compartment a Plexiglas tube with perforations was placed. During the test, the rats were exposed to an object inside the tube in one compartment and to a juvenile rat of 43 days old in the tube in the opposite compartment. After 2 minutes of habituation in the center compartment, the rats were allowed to explore the arena during 20 minutes. The time spent actively exploring the juvenile and the object was measured.

Sacrifice

For the gene expression experiments rats were sacrificed either on day 1, three hours after the end of the food competition test, or on day 8, one week after the social interaction and food competition test under basal conditions. The animals were decapitated, the brain was quickly removed and frozen in isopentane at a temperature between -50 and -40 °C and stored at -80 °C until further processing.

Trunk blood was taken in tubes containing 100µl heparine. Blood samples were centrifuged and the plasma was stored at -20 °C. Corticosterone levels were measured using enzyme-linked immunosorbent assays (ELISA) (Assay Design, Ann Arbor, MI, USA).

In experiments involving cannulation, in order to check the position of the cannulae rats were injected i.p. with pentobarbital. 1 µl of ink was subsequently infused into the MeA to mark the position of drug delivery. The rats were decapitated and the brains removed, frozen

in isopentane and stored at -80 °C until further processing. 40 μm thick coronal sections were stained with thionin to detect the cannula position.

Laser capture micro dissection

For the gene expression experiment, coronal sections of 20 μm were taken for 'laser capture micro dissection' (LCM). The sections were put on membrane covered slides (Palm Microlaser Technologies, Carl Zeiss, Germany) and stored at -20 °C covered with RNA*later* ICE (Ambion, TX, USA). Prior to LCM the sections were stained with the HistoGene LCM Frozen Section Staining kit following the protocol (Arcturus Bioscience, Switzerland). The MeA and the LS from the right hemisphere were dissected through LCM. The target tissue was catapulted into a PALM adhesive cap (Palm Microlaser Technologies, Carl Zeiss, Germany) and subsequently incubated in 100 μl of lysis buffer (RNAqueous Micro Kit, Ambion, TX, USA) for 30 min at 42 °C. After spinning down the tissue was stored at -80 °C until further processing.

RNA isolation

RNA was isolated from the tissue using the RNAqueous Micro Kit (Ambion, TX, USA). First, 3 μl of LCM additive was added. Subsequently 129 μl of 100% ethanol was added to the mixture. The lysate/ethanol mixture was loaded onto a Micro Filter Cartridge Assembly and centrifuged for 1 minute at 10000g. Afterward 180 μl of Wash Solution 1 was added and the mixture was centrifuged again for 1 min at 10000g. 180 μl of Wash Solution 2/3 was added and the mixture was centrifuged for 30 sec at 14000g. The flow-through was discarded and the Micro Filter Cartridge was centrifuged for another minute at 14000g. The Micro Filter Cartridge was transferred into a Micro Elution Tube and 8 μl of preheated Elution Solution was added. The mixture was left for 5 min at room temperature and subsequently centrifuged for 1 min at 14000g. Another 8 μl of Elution Solution was added and the mixture was centrifuged again at 14000g. The RNA was stored at -80 °C until further processing

Reverse transcription and preamplification

9.62 μl of the extracted RNA was converted into cDNA using Taqman Reverse Transcriptase Reagents (Applied Biosystems, CA, USA). 1.25 μl of random hexamers and 2.5 μl of RT 10x buffer were added to reach an end volume of 13.37 μl. The mixture was brought

to 65 °C for 10 min followed by 10 min at room temperature in a Thermocycler (Primus 96, Peqlab, Germany). Following this step 5.5 µl of $MgCl_2$, 5 µl of dNTPs (10 mM), 0.5 µl of RNAse inhibitor and 0.63 µl of Multiscribe Reverse were added to each sample. Using the Thermocycler, the reverse transcription was executed for 10 min at 25 °C, 25 min at 48 °C and 5 min at 95 °C. The obtained cDNA was stored at -20 °C until further processing.

The cDNA was preamplified using Pre-amp MasterMix for TaqMan assays (Applied Biosystems, CA, USA). A pool of TaqMan Gene Expression Assays for the genes of interest (see 'Real time PCR') was made in ultrapure water (Gibco, NY, USA) at a volume of 1% for each Assay. 25 µl of Taqman PreAmp MasterMix, 12.5 µl of the pooled Assay mix and 12.5 µl of cDNA were mixed for each sample. The preamplification was done by an enzyme activation period of 10 min at 95 °C, followed by 10 cycles of 15 sec at 95 °C and 4 min at 60 °C. After finishing the run the samples were immediately placed on ice and diluted 5x in ice-cold ultrapure water (Gibco, NY, USA). Samples were stored at -20 °C until further processing.

Quantitative real time PCR

Quantitative real time PCR was performed using TaqMan Gene Expression Assays for the genes for the oxytocin receptor (*Otr*), the arginine vasopressin receptor 1A (*V1ar*), monoamine oxidase A *(Maoa)*, androgen receptor *(Ar)*, glucocorticoid receptor *(Gr)* and neural cell adhesion molecule *(Ncam)* with the control genes ribosomal protein S18 (*Rps18*), ribosomal protein S29 (*Rps29*) and eukaryotic elongation factor 1 alpha 1 (*Eef1a1*) (see Table I for the used Gene Expression Assays, Applied Biosystems, CA, USA). First, a reaction mixture containing 4.5 µl preamplified cDNA, 0.5 µl 20x Taqman Gene Expression Assay for the genes of interest and 5 µl 2x PCR Master Mix (TaqMan Universal PCR master mix no AmpErase UNG, Applied Biosystems, CA, USA) per reaction of 10 µl was made. Triplicates of 10 µl of each reaction mixture were transferred to a well in a 384-well optical reaction plate (Applied Biosystems, CA, USA). The real time PCR was performed using 40 cycles of 15 sec at 95 °C and 1 min at 60 °C (7900HT, Applied Biosystems, CA, USA), preceded by 2 min at 50 °C and 10 min at 95 °C.

The software SDS (v.2.3, Applied Biosystems, CA, USA) was used for running and analyzing the quantitative real time PCR. Each target gene was compared to three internal control genes (*Rps18, Rps29, Eef1a1*) using the ΔΔCt method (described by Livak and Schmittgen, 2001). The results are expressed as fold change compared to the control groups.

Table I. Overview of the used Gene Expression Assays (Applied Biosystems).

Gene	Gene Expression Assay
Oxytocin receptor *(Otr)*	Rn00563503_m1
Arginine vasopressin receptor 1a *(V1ar)*	Rn00583910_m1
Monoamine oxidase A *(Maoa)*	Rn01430958_m1
Androgen receptor *(Ar)*	Rn00560747_m1
Glucocorticoid receptor *(Gr)*	Rn00560747_m1
Neural cell adhesion molecule *(Ncam)*	Rn01418541_m1
Ribosomal protein S18 (*Rps18*)	Rn01428915_g1
Ribosomal protein S29 (*Rps29*)	Rn00820645_g1
Eukaryotic elongation factor 1a1 (*Eef1a1*)	Rn01639851_g1

Statistics

All results are expressed as the mean ± the standard error of the mean (S.E.M.).

For statistical analyses of mRNA expression levels, the fold change of the different groups compared to the non-stressed control group was calculated. One-way ANOVA was used to test statistical significance between the dominant and subordinate rats of Pns and Ps. *A priori* comparisons to test differences between two specific groups were established and evaluated through either unpaired or paired two-tailed Student t-tests. Paired t-tests were applied to compare mRNA expression levels of dominant and subordinate rats that competed together, while unpaired t-tests were applied when comparisons involved animals that had not directly interacted. *A priori* comparisons for group comparisons were (1) the subordinate rat of Ps with the subordinate rat of Pns, (2) the subordinate rat of Ps with the dominant rat of Ps, (3) the dominant rat of Ps with the dominant rat of Pns, (4) the subordinate rat of Pns with the dominant rat of Pns, (5) the subordinate rat of Ps with the stressed control group and (6) each experimental group compared to non-stressed controls. The same comparisons were made for corticosterone levels.

For the behavioral experiments, the percentage of offensive behaviors within each dyad was calculated. A paired two-tailed Student t-test was used to compare the percentage of offensive behaviors within a dyad of rats competing together.

The computer software SPSS for Windows (version 13.0) was used for statistical analysis. $P<0.05$ was considered significant.

Results

The effect of the protein synthesis inhibitor anisomycin on the memory for a social hierarchy

We have previously shown that when protein synthesis is inhibited by administration of anisomycin to both the dominant and subordinate rat of Ps, no memory for the social hierarchy formed on day 1 is found on day 8 (Cordero and Sandi, 2007). In the present experiment only one of the two rats received a post-encounter anisomycin injection: in half of the pairs the non-stressed dominant rat and in the other half the stressed subordinate rat. The other rat of the pair was injected with vehicle. In the control group both rats were injected with vehicle. All three groups form a hierarchy on day 1 ($t = 3.819$, $df = 7$, $p<0.01$ for the control group, Fig. IA; $t = 4.823$, $df = 7$, $p<0.01$ for the group in which the non-stressed dominant rat of Ps was treated with anisomycin, Fig. IB; $t = 2.548$, $df = 7$, $p<0.05$ for the group in which the stressed subordinate rat of Ps was treated with anisomycin, Fig. IC). After one week the same hierarchy was found in the control group ($t = 2.327$, $df = 7$, $p\leq0.05$, Fig. IA), which corresponds with previous findings. When the non-stressed dominant rat was injected with anisomycin no memory for the hierarchy was found (n.s., Fig. IB). However, when the stressed subordinate rat was treated with anisomycin the hierarchy found on day 8 was the same as on day 1 ($t = 3.113$, $df = 7$, $p<0.05$, Fig. IC).

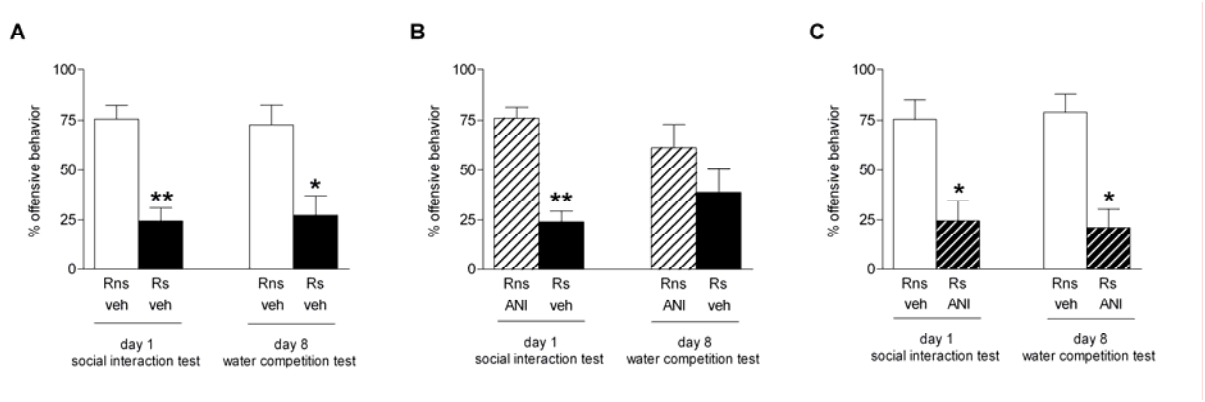

Figure I. The percentage of total offensive behavior (mean ± S.E.M.) between two opponents shown in the social interaction test on day 1 and the water competition test on day 8 after treatment with anisomycin or vehicle. (A) The hierarchy of rats injected with vehicle after the first encounter (n=8). (B) Anisomycin treatment of the non-stressed dominant rat of Ps after the first encounter (n=8) (C) Anisomycin treatment of the stressed subordinate rat of Ps after the first encounter (n=8). The status 'dom' (dominant) or 'sub' (subordinate) refers to the status that was obtained during the social interaction test on day 1. 'Rns' = non-stressed rat, 'Rs' = stressed rat, 'veh' = vehicle, 'ANI' = anisomycin. * $p\leq0.05$, ** $p<0.01$ vs. the other rat of the pair.

The effect of OTA infusion on OTR and V1aR mRNA levels in LS 1 week after the first encounter

Since administration of OTA into the MeA has the same long-term effects on the behavior in the WCT as administration of foot shocks in the stressed rat of Ps, we wanted to know whether administration of OTA in the MeA also affects the long-term effects on gene expression in a similar way. To study this, we administered vehicle or OTA to rats that were not exposed to an encounter and served as controls (data not shown), and OTA immediately after the first encounter to the subordinate rat of Pns and vehicle to the dominant rat of Pns. 1 week later the rats were sacrificed under basal conditions and the mRNA levels for OTR and V1aR in LS were measured. There is no significant change in the expression of OTR in LS (Fig. IIA). In the V1aR a downregulation was found in the V1aR of the subordinate OTA-treated rat compared to the dominant vehicle treated rat ($t = 2.564$, $df = 8$, $p<0.05$, Fig. IIB), which is similar to the changes in V1aR expression in LS found in the subordinate rat of Ps 1 week after the first encounter (Chapter 3).

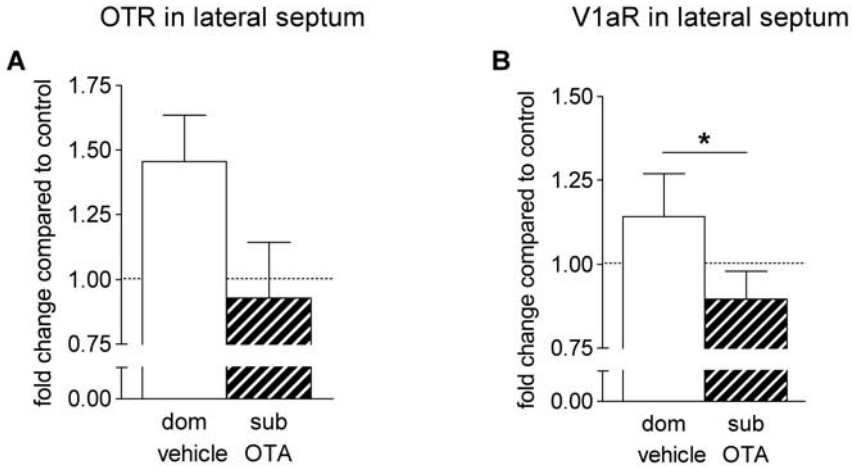

Figure II. The expression of *Otr* and *V1ar* mRNA in LS 1 week after the first encounter and vehicle or OTA administration (mean ± S.E.M.). (A) *Otr* mRNA expression in LS (n=9). (B) *V1ar* mRNA expression in LS (n=9). 'OTA' = oxytocin antagonist, 'dom' = dominant, 'sub' = subordinate. * $p<0.05$ vs. the subordinate rat.

Social preference for a juvenile after exposure to acute stress

To test whether acute stress leads to social avoidance, we submitted the rats to a social preference test with an unfamiliar juvenile in one compartment and an object in the opposite compartment. In both the control and stress group the rats spend more time exploring the juvenile than the object (data not shown). The stressed rats spend a higher percentage of exploration in the juvenile compartment than the non-stressed rats ($t = 2.932$, $df = 11$, $p<0.05$, Fig. III). This shows that, at least in a non-aggressive setting, acute foot shock stress does not lead to avoidance of social interaction or a decrease in social interest.

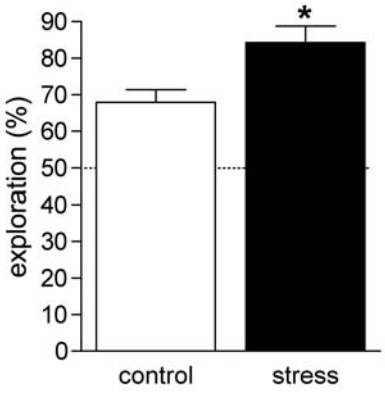

Figure III. Percentage of exploration of an unfamiliar juvenile rat. * $p<0.05$ vs. control.

Differences between Pns and Ps in the first encounter

In the current experiment we wanted to investigate whether there is a difference in the pattern of offensive behavior between Pns and Ps during the first encounter. Therefore, the time spent on offensive behavior was examined per minute of the encounter (Fig. IVA&B).

In Pns an effect of both time and status are found (time: $F_{19,817} = 3.377$, $p<0.001$; status $F_{1,43} = 88.867$, $p<0.001$; interaction time x status $F_{19,817} = 2.309$, $p<0.01$). Significant differences between the offensive behavior shown by the dominant and subordinate rat are found at minute 5, 6, 9, 10, 11, 13, 15 and 16 (Fig. IVA).

In Ps also an effect of time and status are found (time: $F_{19,798} = 2.967$, $p<0.001$; status $F_{1,42} = 56.522$, $p<0.001$; interaction time x status $F_{19,798} = 1.315$ $p=0.165$). The difference between the dominant and subordinate rat is less clear than in Pns, with only significant differences at minute 6, 9, 10 and 16 (Fig. IVB). The stressed rat of the Ps seems to show a higher level of offensive behavior around 7 and 13 minutes after the start of the encounter compared to the subordinate rats of Pns (Fig. V).

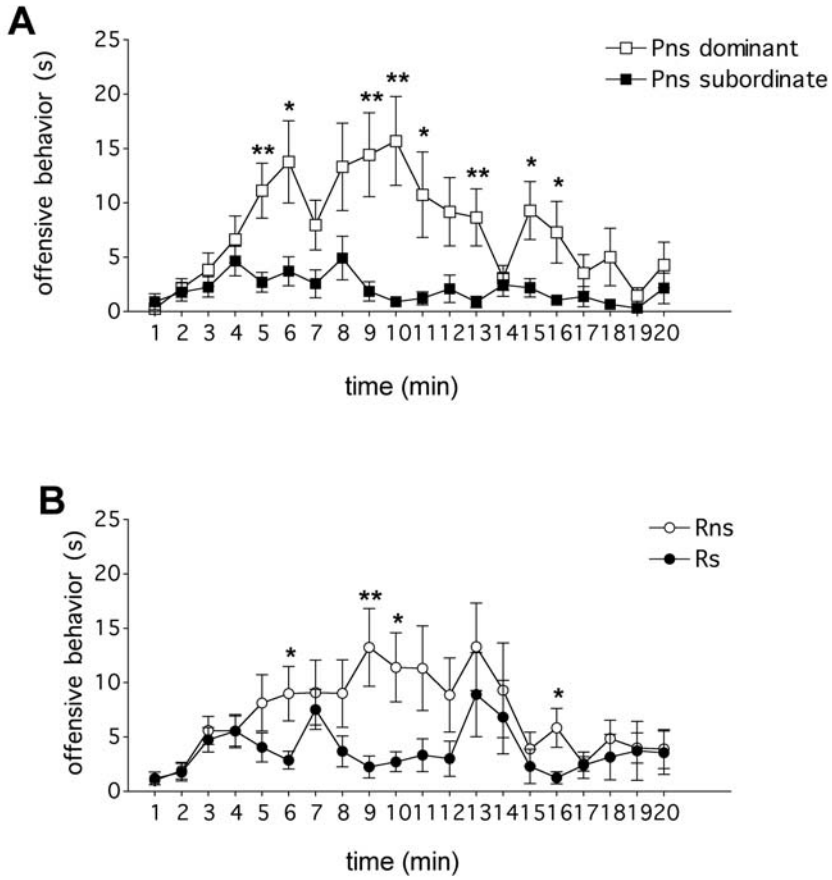

Figure IV. The time of total offensive behavior (mean ± S.E.M.) between two opponents shown in the social interaction test on day 1. (A) The offensive behavior during the 20 minutes of the first encounter in Pns. (B) The offensive behavior during the 20 minutes of the first encounter in Ps. 'Rns' = non-stressed rat, 'Rs' = stressed rat. * $p<0.05$, ** $p<0.01$ vs. the other rat of the pair.

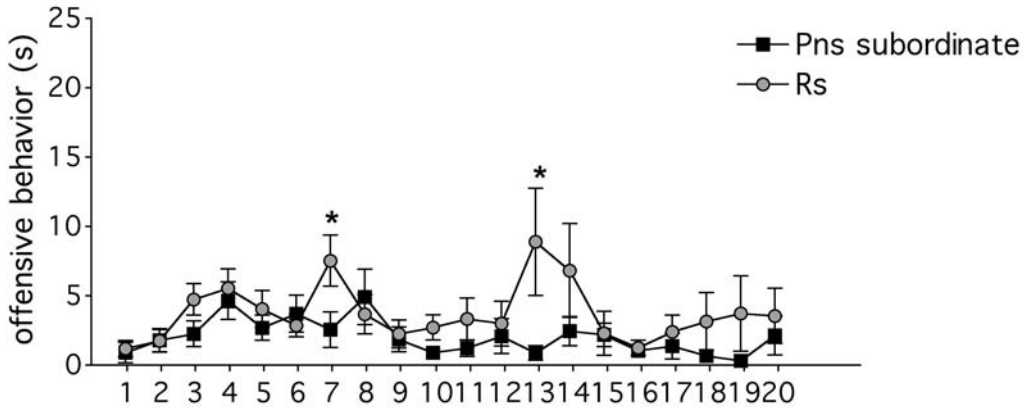

Figure V. The time of total offensive behavior (mean ± S.E.M.) of the subordinate rats of Pns and Ps in the social interaction test on day 1. 'Rs' = stressed rat. * $p<0.05$ vs. the subordinate rats of Pns.

GR and NCAM gene expression in MeA, BNST and LS

The immediate (3h) and long-term (1 week) changes in mRNA levels of *Gr* and *NCAM* after exposure to stress and/or a social encounter were studied in MeA, BNST and LS.

No changes are found in *Gr* mRNA in MeA 3h or 1 week after the first encounter. In LS, 3h after the first encounter the dominant rats of non-stressed pairs show a lower *Gr* mRNA level than the subordinate rats of non-stressed pairs, whereas the dominant rats of stressed pairs show a higher *Gr* mRNA level than the subordinate rats of stressed pairs. In BNST, the dominant rats of both non-stressed and stressed pairs show a lower *Gr* mRNA levels than the subordinate rats of the same pairs (Table IV, Fig. VI).

In MeA, no differences are found in *Ncam* mRNA at 3h or 1 week after the encounter. In LS, the dominant rats of non-stressed pairs have a lower *Ncam* level than the subordinate rats of non-stressed pairs one week after the encounter. In stressed pairs, the dominant rats have a higher *Ncam* mRNA level than subordinate rats. The subordinate rats of non-stressed pairs have a higher *Ncam* mRNA level than the subordinate rats of stressed pairs. 3h after the encounter the dominant rats of non-stressed pairs have a decrease in *Ncam* mRNA in BNST compared to the subordinate rats of non-stressed pairs. One week after the encounter the subordinate rats of non-stressed pairs have a higher *Ncam* mRNA level in BNST than dominant rats of non-stressed pairs and subordinate rats of stressed pairs (Table IV, Fig. VI).

Table II. Overview of the significant differences in paired and unpaired t-tests that compare *Gr* and *NCAM* mRNA levels in MeA, LS and BNST. 'Pns dom' = dominant rat of Pns, 'Pns sub' = subordinate rat of Pns, 'Ps dom' = dominant rat of Ps, 'Ps sub' = subordinate rat of Ps, 'str ctl' = stressed control.

		Compared to Ps sub	Compared to Pns sub	Compared to control
MeA GR	3h	-	-	-
	1 week	-	-	-
LS GR	3h	Ps dom: t = 3.863, df = 5, p<0.05	Pns dom: t = 2.910, df = 5, p<0.05	-
	1 week	-	-	-
BNST GR	3h	Ps dom: t = 3.515, df = 5, p<0.05	Pns dom: t = 6.747, df = 5, p<0.01	-
	1 week	-	-	-
MeA NCAM	3h	-	-	-
	1 week	-	-	-
LS NCAM	3h	-	-	-
	1 week	Pns sub: t = 2.608, df = 11, p<0.05 Ps dom: t = 3.096, df = 5, p<0.05	Pns dom: T = 3.786, df = 5, p<0.05	-
BNST NCAM	3h	-	Pns dom: t = 2.566, df = 5, p<0.05	-
	1 week	Pns sub: t = 2.553, df = 11, p<0.05	Pns dom: t = 5.072, df = 5, p<0.01	-

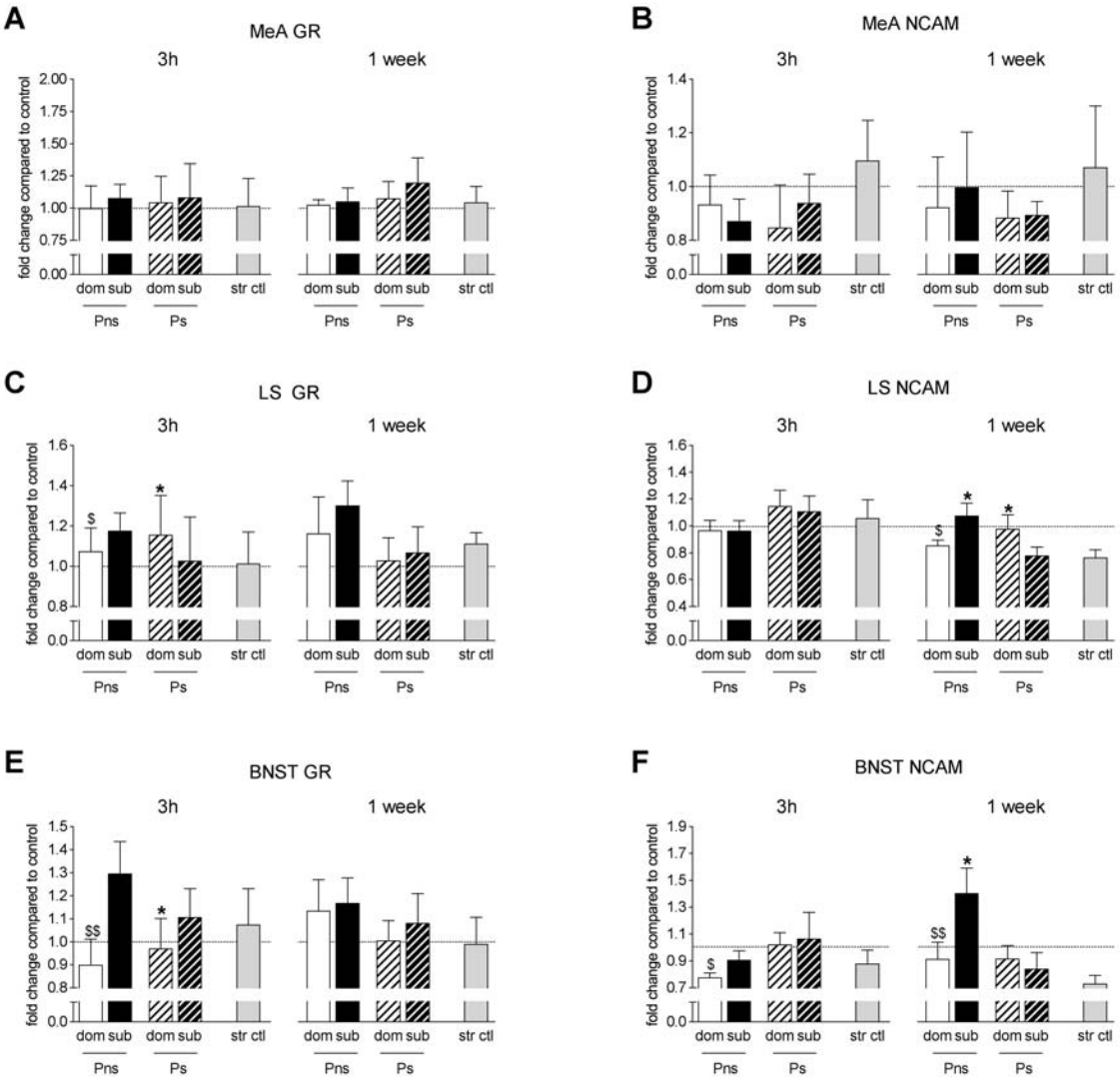

Figure VI. The expression of *Gr* and *Ncam* mRNA in MeA, LS and BNST at 3h and 1 week after the first encounter (mean ± S.E.M.). (A) *Gr* mRNA expression in MeA. (B) *Ncam* mRNA expression in MeA. (C) *Gr* mRNA expression in LS. (D) *Ncam* mRNA expression in LS. (E) *Gr* mRNA expression in BNST. (F) *Ncam* mRNA expression in BNST. 'str ctl' = stressed control group, 'dom' = dominant, 'sub' = subordinate, 'Pns' = non-stressed pair, 'Ps' = stressed pair. * $p<0.05$ vs. subordinate rat of Ps, $ $p<0.05$ vs. subordinate rat of Pns, $$ $p<0.01$ vs. subordinate rat of Pns Each group consists of 5-14 animals.

Gene expression hippocampus

The immediate (3h) and long-term (1 week) changes in mRNA levels of *Otr, V1ar, Maoa, Ar, Gr* and *NCAM* after exposure to stress and/or a social encounter were studied in hippocampus.

One-way ANOVA between the dominant and subordinate rats of both Pns and Ps indicates a lack of significant difference among dominant and subordinate rats of Pns and Ps for all genes in hippocampus.

3h after the first encounter the stressed control group has a lower *V1ar* mRNA level than the subordinate rats of Ps and the dominant rats of the non-stressed pair (Pns) have a lower level than the subordinate rats of the same pair 3h after the first encounter (Table III, Fig. VII). For *Ar* and *Gr*, the dominant rats of the non-stressed pair (Pns) have lower levels than the subordinate rats of the same pair (Table III, Fig. VII). No changes are found in *Otr, Maoa and NCAM* mRNA 3h after the first encounter (Table III, Fig. VII).

1 week after the encounter there are no changes in *Otr* and *V1ar* mRNA levels (Table III, Fig. VII). In *Maoa*, the dominant rats of the stressed pair (Ps) have a higher level than the subordinate rats 1 week after the first encounter (Table III, Fig. VII). The dominant rats of Ps have a higher *Ar* and *Gr* mRNA level than the subordinate rats of Ps (Table III, Fig. VII). Dominant rats of Ps have a lower *Ncam* mRNA level than subordinate rats of Ps, and stressed controls have a lower level than controls (Table III, Fig. VII).

Table III. Overview of the significant differences in paired and unpaired t-tests that compare *Otr, V1ar, Maoa, Ar, Gr* and *NCAM* mRNA levels in hippocampus. 'Pns dom' = dominant rat of Pns, 'Pns sub' = subordinate rat of Pns, 'Ps dom' = dominant rat of Ps, 'Ps sub' = subordinate rat of Ps, 'str ctl' = stressed control.

		Compared to Ps sub	Compared to Pns sub	Compared to control
OTR	3h	-	-	-
	1 week	-	-	-
V1aR	3h	Str ctl: t = -2.580, df = 9, p<0.05	Pns dom: t = -2.794, df = 5, p<0.05	-
	1 week	-	-	-
MAOA	3h	-	-	-
	1 week	Ps dom: t = 4.194, df = 6, p<0.01	-	-
AR	3h	-	Pns dom: t = -12.277, df = 4, p<0.001	-
	1 week	Ps dom: t = 6.325, df = 6, p=0.001	-	-
GR	3h	-	Pns dom: t = -12.004, df = 4, p<0.001	-
	1 week	Ps dom: t = 3.137, df = 6, p<0.05	-	-
NCAM	3h	-	-	-
	1 week	Ps dom: t = -2.631, df = 6, p<0.05	-	Str ctl: t = 2.782, df = 12, p<0.05

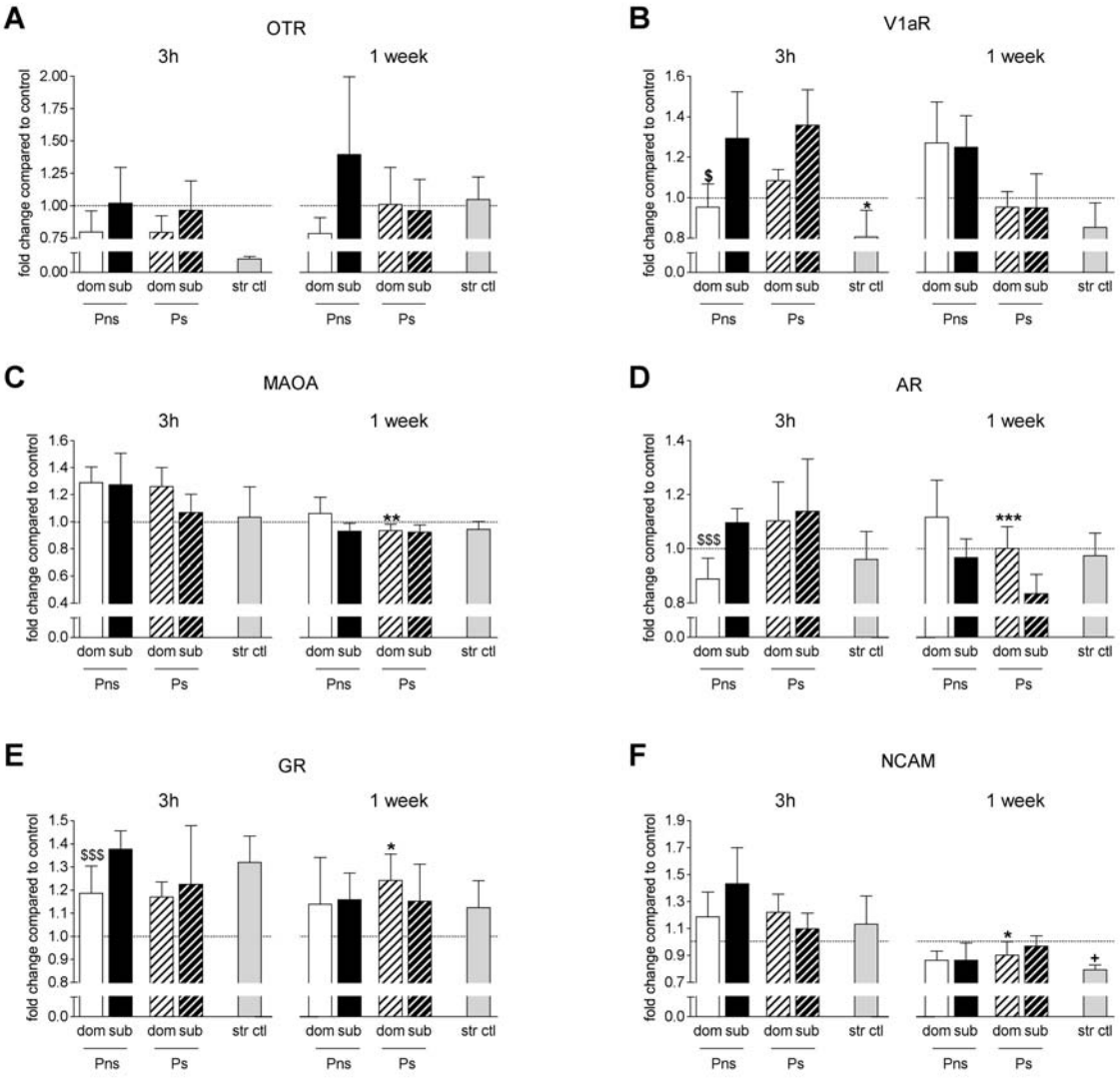

Figure VII. The expression of *Otr*, *V1ar*, *Maoa*, *Ar*, *Gr* and *Ncam* mRNA in hippocampus at 3h and 1 week after the first encounter (mean ± S.E.M.). (A) *Otr* mRNA expression. (B) *V1ar* mRNA expression. (C) *Maoa* mRNA expression. (D) *Ar* mRNA expression. (E) *Gr* mRNA expression. (F) *Ncam* mRNA expression. 'str ctl' = stressed control group, 'dom' = dominant, 'sub' = subordinate, 'Pns' = non-stressed pair, 'Ps' = stressed pair. * $p<0.05$ vs. subordinate rats of Ps, ** $p<0.01$ vs. subordinate rat of Ps, *** $p<0.001$ vs. subordinate rats of Ps, + $p<0.05$ vs. controls, $ $p<0.05$ vs. subordinate rats of Pns $$$ $p<0.001$ vs. subordinate rats of Pns. Each group consists of 5-8 animals.

OTR and V1aR gene expression in BNST

The immediate (3h) and long-term (1 week) changes in mRNA levels of *Otr* and *V1ar* after exposure to stress and/or a social encounter were studied in BNST.

For OTR, 3h after the first encounter the dominant rats of the stressed pair show an increase compared to controls (Table IV, Fig. VIII). After 1 week the dominant rats of the stressed pair have a lower *Otr* mRNA level than subordinate rats of the stressed pair (Table IV, Fig. VIII).

Dominant rats of non-stressed pairs show a lower *V1ar* mRNA level than the subordinate rats of non-stressed pairs 3h after the first encounter (Table IV, Fig. VIII). After 1 week, the subordinate rats of non-stressed pairs have a lower *V1ar* mRNA level than subordinate rats of stressed pairs (Table IV, Fig. VIII).

Table IV. Overview of the significant differences in paired and unpaired t-tests that compare *Otr* and *V1ar*, mRNA levels in BNST. 'Pns dom' = dominant rat of Pns, 'Pns sub' = subordinate rat of Pns, 'Ps dom' = dominant rat of Ps, 'Ps sub' = subordinate rat of Ps.

		Compared to Ps sub	Compared to Pns sub	Compared to control
OTR	3h	-	-	Ps dom: $t = 2.246$, $df = 23$, $p<0.05$
	1 week	Ps dom: $t = 3.194$, $df = 7$, $p<0.05$	-	-
V1aR	3h	-	Pns dom: $t = 4.006$, $df = 12$, $p<0.01$	-
	1 week	Pns sub: $t = 2.284$, $df = 12$, $p<0.05$	-	-

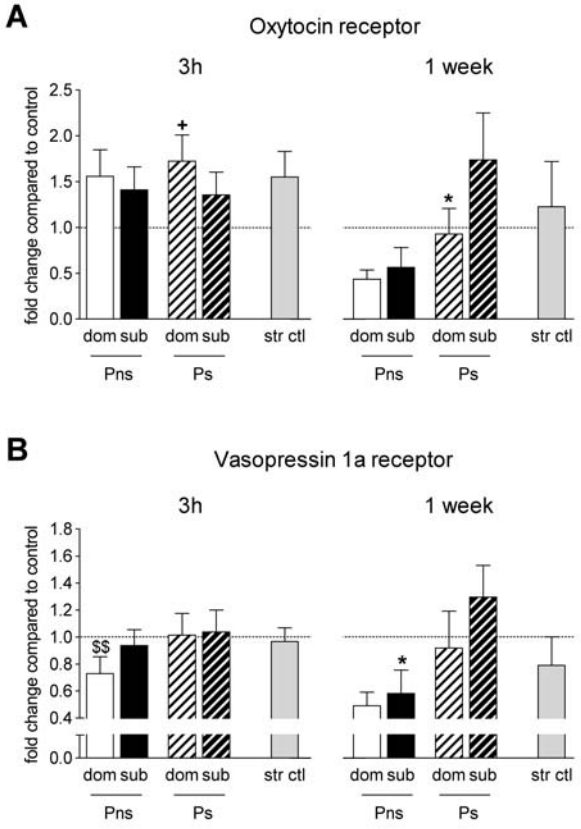

Figure VIII. The expression of *Otr* and *V1ar* mRNA in BNST at 3h and 1 week after the first encounter (mean ± S.E.M.). (A) *Otr* mRNA expression. (B) *V1ar* mRNA expression. 'str ctl' = stressed control group, 'dom' = dominant, 'sub' = subordinate, 'Pns' = non-stressed pair, 'Ps' = stressed pair. * $p<0.05$ vs. subordinate rats of Ps, + $p<0.05$ vs .controls, $$ $p<0.01$ vs. subordinate rats of Pns. Each group consists of 5-14 animals.

Corticosterone levels

The immediate (3h) and long-term (1 week) changes in corticosterone levels after exposure to stress and/or a social encounter were measured (Fig. IX).

One-way ANOVA between all six groups shows an effect 3h after the first encounter ($F_{5,79}$ = 2.471, $p<0.05$), but not one week after the first encounter. Student t-test indicates a significant difference between the subordinate rats of the stressed pair (Ps) and the stressed control group (t = 2.850, df = 24, $p<0.01$). After one week there is a tendency for a difference between the dominant and subordinate rats of the stressed pair (t = 2.215, df = 7, p = 0.0624).

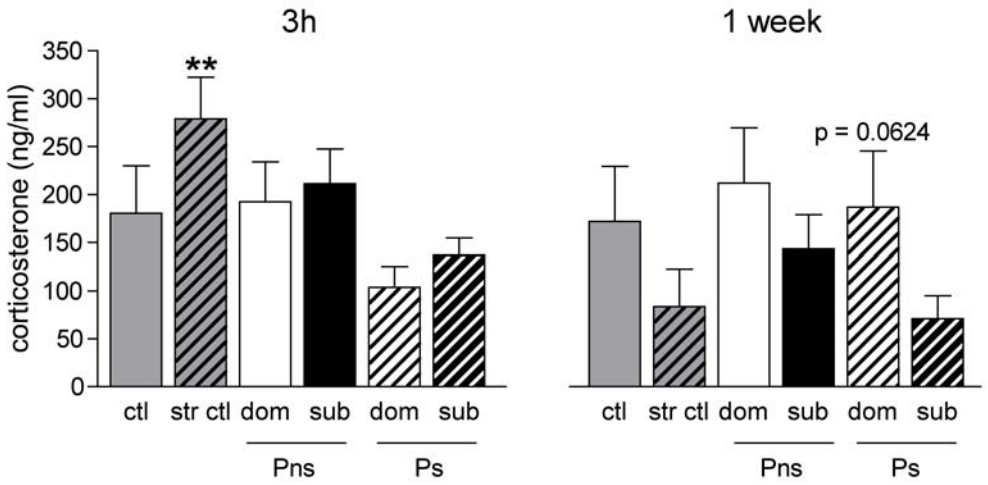

Figure IX. Corticosterone levels at 3h and 1 week after the first encounter. 'Pns' = non-stressed pair, 'Ps' = stressed pair, 'ctl' = control group, 'str ctl' = stressed control group, 'dom' = dominant, 'sub' = subordinate. The status 'dom' and 'sub' refers to the status obtained on day 1. ** $p<0.01$ vs. the subordinate rats of Ps.

Curriculum vitae

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Education

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2001 – 2006:	**M.Sc. in Biology, specialization in Behavioral and Neurosciences** University of Groningen, the Netherlands
1995 – 2001:	**Secondary school** Leeuwarden, the Netherlands

Research projects

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2005 – 2006:	Internship in Behavioral Neuroscience, University of Aberdeen, United Kingdom Topic: 'The involvement of various neurotransmitter systems in the effects of whole body vibration on cognition and motor function in mice'
2005:	Internship in Developmental Genetics, University of Groningen Topic: 'Implication of UTF1 in the transition between self-renewal and differentiation in embryonic stem cells'
2004 – 2005:	Internship in Molecular Neuroscience, University of Groningen Topic: Changes in hippocampal protein kinase A during the formation and modification of spatial memory'

Publications

Timmer, M. and Sandi, C. (2010). A role for glucocorticoids in the long-term establishment of a social hierarchy. Psychoneuroendocrinology 35(10): 1543-1552.

Havekes, R., **Timmer, M.** and Van der Zee, E.A. (2007). Regional differences in hippocampal PKA immunoreactivity after training and reversal training in a spatial Y-maze task. Hippocampus 17(5): 338-348.

Conference proceedings

Timmer, M., Sevelinge, Y., Cordero, M.I. & Sandi, C. (2010). Evidence for a role of oxytocin receptor in the medial amygdala in the long-term memory of a social hierarchy. 7th Forum of European Neuroscience (FENS), Amsterdam, the Netherlands (Poster).

Timmer, M., Cordero, M.I., Sevelinge, Y. & Sandi, C. (2009). Downregulation of oxytocin receptor mRNA in medial amygdala plays a role in the long-term formation of a social hierarchy in rats. 8th World Congress on Neurohypophyseal Hormones (WCNH), Kitakyushu, Japan (Poster).

Timmer, M. & Sandi, C. (2009). Acute stress has a long-lasting effect on offensive behaviour in a dominance hierarchy test and leads to a downregulation of monoamine oxidase A expression in lateral septum. EPFL Latsis Symposium 'Violence', Lausanne, Switzerland (Poster).

Timmer, M., Cordero, M.I. & Sandi, C. (2008). The effect of stress on oxytocin and vasopressin receptors gene expression in a social memory test. 5th Annual Lemanic Neuroscience Meeting, Les Diablerets, Switzerland (Poster).

Timmer, M., Cordero, M.I. & Sandi, C. (2008). The effect of stress on oxytocin and vasopressin receptors gene expression in a social memory test. 6th Forum of European Neuroscience (FENS), Geneve, Switzerland (Poster).

Timmer, M., Cordero, M.I. & Sandi, C. (2007). The effect of stress on oxytocin and vasopressin gene expression in a social memory test. 7th World Congress on Neurohypophyseal Hormones (WCNH), Regensburg, Germany (Poster).

Timmer, M., Cordero, M.I. & Sandi, C. (2007). The effect of stress on oxytocin and vasopressin gene expression in a social memory test. 4th Annual Lemanic Neuroscience Meeting, Les Diablerets, Switzerland (Poster).

Languages

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- French: fair (level B1/B2)
- German: basic knowledge