Effects of chronic psychosocial stress on HPA axis functionality in male C57BL/6 mice and the impact of trait anxiety on the individual stress vulnerability

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CHAPTER 1

GENERAL INTRODUCTION

1 Stress

In 1865 the French physiologist Claude Bernard (1813 – 1878) was the first one describing the importance of a stable internal equilibrium of an organism compared to the fluctuating external environment. This idea was rendered more precisely over half a century later by Walter Cannon (1871 – 1945) defining the internal equilibrium and its maintenance within an adequate range as "homeostasis" (Cannon, 1929). Disruption of this homeostasis, e.g. through exposure to cold, traumatic pain or emotional distress, activates the adrenal medulla and the sympathetic nervous system (SNS), coined together the "sympathoadrenal" system. This promotes the "fight or flight" response which restores the internal milieu. It was in 1936 when Hans Selye, with his groundbreaking paper "A syndrome produced by diverse nocuous agents", defined "stress" as "the non-specific response of the body to any physical demand" (Selye, 1936a). He also established the term "stressor". Stressors, which can be physical or chemical in nature, cause the physiological stress response (Selye, 1975).

The modern concept of stress research entitles stressors as consciously or unconsciously sensed threat to homeostasis (McEwen and Stellar, 1993; Goldstein and Kopin, 2007). The specificity of the stress response is dependent on the particular type of stressor, the way in which the organism perceives the stressor, and the personal ability to cope with it (Goldstein and Kopin, 2007).

1.1 The Stress System

Following stressor exposure the two main stress response systems, the SNS and the hypothalamic-pituitary-adrenal (HPA) axis, get activated. Both systems, differing in their time course and in their effector hormones, coordinate the restoration of the homeostasis of the organism following stressor exposure, a processed called allostasis (Sterling et al., 1988). Moreover, also behavioural responses to a stressful experience take place, like increased arousal, alertness, heightened attention, anxiety or aggression, while feeding or sexual behaviour

are suppressed (Stratakis and Chrousos, 1995; Herman and Cullinan, 1997; McEwen and Wingfield, 2003). In the following sections the SNS and the HPA axis are discussed in detail, with the main focus directed on the latter one.

1.1.2 Sympathetic nervous system (SNS)

In response to stressful stimuli, the SNS, together with the parasympathetic and enteric nervous system forming the autonomic nervous system, gets rapidly activated and controls cardiovascular, gastrointestinal, respiratory and other somatic systems (Chrousos, 1998). The control station of the SNS is the locus coeruleus (LC), located in the brain stem and containing a cluster of norepinephrine (NE)-containing neurons. The LC-NE system innervates nearly the entire central nervous system (CNS) (Berridge and Waterhouse, 2003) and gets activated by diverse stressors, e.g. shock, hypotension, swim and social stressors (Valentino and Van Bockstaele, 2008), probably mediated by the afferents of the paraventricular nucleus (PVN) which innervate dendrites in the LC (Reyes et al., 2005). The LC also projects to sympathetic preganglionic neurons, emphasizing its role in regulating the SNS (Valentino and Van Bockstaele, 2008).

The cell bodies of the preganglionic neurons originate in the intermediolateral column (lateral horn) at the thoracic and lumbar region (“thoracolumbar system”) of the spinal cord. These sympathetic preganglionic neurons synapse with postganglionic neurons located in ganglia which lie on each side of the spinal cord, the sympathetic trunk. The postganglionic neurons innervate the peripheral organs, like e.g. heart and blood vessels leading amongst others to vasoconstriction and increase in the heart rate (Abboud, 2010) (see Fig. 1). Sympathetic preganglionic neurons also can directly innervate the adrenal medulla, for stimulating the release of catecholamines from chromaffin cells which are embryologically and anatomically homologous to the sympathetic ganglia. As the adrenal medulla consists of postganglionic neurons which originate from the SNS and release their neurotransmitters directly into the blood stream, the adrenal functions as an endocrine gland (Elenkov et al., 2000). While

the transmitter of the preganglionic neurons is acetylcholine, the end organs get stimulated by catecholamines secreted by the postsynaptic sympathetic fibers, thereby, NE/noradrenaline represents the major neurotransmitter (Holgert et al., 1998). In contrast, the adrenal gland secretes mainly epinephrine/adrenaline and only to a lesser extent NE following sympathetic stimulation (Holgert et al., 1998). The catecholamines bind to α- and β-adrenoreceptors (α_1, α_2, β_1, β_2, β_3) which belong to the family of seven-transmembrane domain receptors, also known as G-protein coupled receptors (GPCR) (Minneman et al., 1981). The catecholamines released in response to activation of the SNS prepare the organism for the "fight or flight" response. The major effects are increase in heart and breathing rate, blood pressure, attention and mobilization of energy while e.g. digestion is reduced.

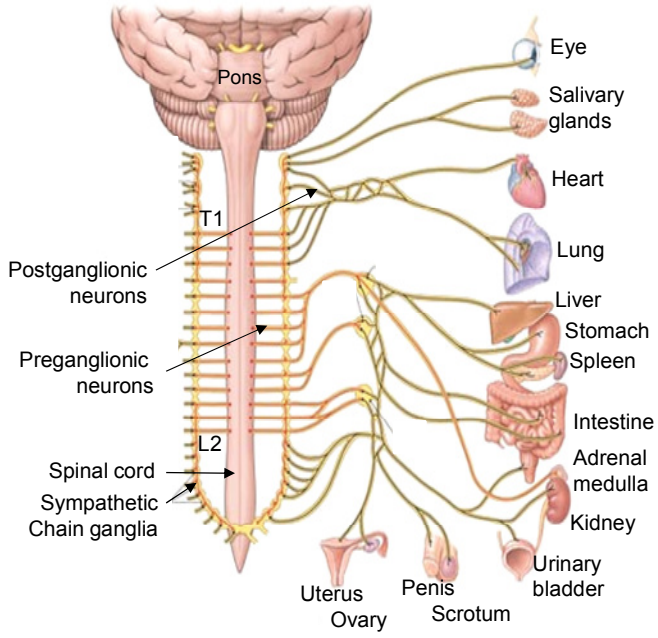

Figure 1: Schematic representation of the sympathetic nervous system (SNS). The preganglionic neurons of the SNS start from the first thoracic segment (T1) and extend to the second lumbar segment (L2) of the spinal cord. Their synapses with the postsynaptic neurons lie in small sympathetic ganglia, forming a chain near the spinal cord, called the sympathetic trunk. The postganglionic neurons run from the ganglia to the target organs. [taken and adapted from: wps.aw.com/bc_martini_ fap_9_oa/185/47590/12183 208.cw/-/12183 232/ index.html]

1.2.2 Hypothalamic-Pituitary-Adrenal (HPA) axis

Along with the SNS, the HPA axis gets, albeit in a delayed manner, activated in response to stressor exposure. In the following section the components of the HPA axis, i.e. hypothalamus, pituitary, and adrenal, are discussed in detail (see Fig. 2).

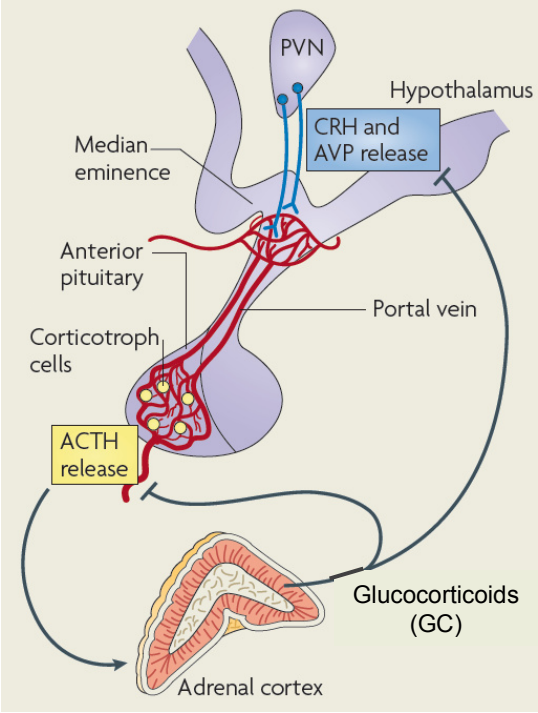

Figure 2: Schematic illustration of the HPA axis. Corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) are released from parvocellular neurons of the paraventricular nucleus (PVN) of the hypothalamus. Neurons of the PVN reach the median eminence and release CRH and AVP into the portal blood system. They trigger the release of adrenocorticotropic hormone (ACTH) from corticotroph cells of the anterior pituitary into the blood stream. ACTH in turn binds to its receptor in the adrenal cortex, stimulating the synthesis and release of glucocorticoids (GC, cortisol in humans, corticosterone in rats and mice) which negatively signal back to pituitary and PVN. [taken and adapted from (Lightman and Conway-Campbell, 2010)]

The Paraventricular nucleus of the Hypothalamus

The activation of the HPA axis starts in a distinct set of neurons located in the parvocellular PVN, one nucleus of the hypothalamus (Whitnall, 1993). These neurons produce the main adrenocorticotropic hormone (ACTH) secretagogues, corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) (Sawchenko et al., 1984; Whitnall et al., 1985; Aguilera, 1994; Herman and Cullinan, 1997). The PVN gets excitatory input from ascending brainstem systems, circumventricular organs and hypothalamic-basal forebrain pathways. In detail, catecholaminergic neurons from the lower brainstem, mainly activated in response to systemic stress, activate the PVN (Sawchenko and Swanson, 1982; Ziegler and Herman, 2002). Innervation from the circumventricular organs, activated during osmotic stress, is angiotensinergic and stimulates release and synthesis of CRH (Kovacs and Sawchenko, 1993; Ziegler and Herman, 2002).

The majority of projections to the PVN originates from the dorsomedial nucleus of the hypothalamus, medial preoptic area, anterioventral third ventricular region and ventral medial, posterior medial and intermediate divisions of the bed nucleus of the stria terminalis (BNST) (Ziegler and Herman, 2002). These local projections can be either excitatory or inhibitory in nature whereby most of them are *gamma*-aminobutyric acid (GABA)ergic and, therefore, inhibit the PVN and in turn HPA axis activity (see also Sec. 3.5). Limbic activation of the HPA axis is associated with the amygdala, from where the information is relayed via catecholaminergic pathways in the brainsteam or via the lateral BNST to the PVN (Herman and Cullinan, 1997; Ziegler and Herman, 2002).

The PVN is comprised of magnocellular and parvocellular neurons, whereupon HPA axis activation is supposed to be mainly regulated by the latter one (see Fig. 3). Both cell types can be differentiated according to their localization, their cell size, and their synthesized peptides (Swanson and Kuypers, 1980; van den Pol, 1982; Ma et al., 1999). The CRH-containing axons of the parvocellular neurons, whereby about 50% of the neurosecretory cells coexpress AVP (Sawchenko et al., 1984; Whitnall et al., 1985; Aguilera, 1994) project to the external zone of the median eminence, located at the bottom of the brain and release CRH and AVP

in the pituitary portal circulation from where they can directly target the anterior pituitary (Gillies et al., 1982; Rivier and Vale, 1983). While AVP alone is a weak ACTH secretagogue (Vale et al., 1983) it plays a role in regulating the HPA axis during acute and chronic stress (see also Sec. 4).

Magnocellular neurons of the PVN and also of the supraoptic nucleus (SON) express the neuropeptides Oxytocin (OXT) and AVP (Brownstein et al., 1980) and either project to the posterior pituitary from where the neurohormones are directly released into the blood stream or they can also project to limbic regions via their axon collaterals (Neumann and Landgraf, 2012). OXT is mainly associated with parturition and lactation, but it also exhibits anxiolytic properties and is implicated in the stress response (Cook, 1997; Hashiguchi et al., 1997; Neumann et al., 2000; Landgraf and Neumann, 2004; Waldherr and Neumann, 2007; Neumann and Landgraf, 2012). OXT release was demonstrated in response to various acute stressors, like shaker stress (Nishioka et al., 1998), forced swim (FS) (Wotjak et al., 1998) or Morris water maze testing (Engelmann et al., 2006). However social defeat (SD) elicited no increased OXT release in the PVN and SON (Wotjak et al., 1996), indicating stressor-dependend differences in the OXT release. HPA axis activation in response to an acute stressor was shown to be suppressed by OXT (Neumann et al., 2000; Windle et al., 2004). Magnocellular AVP, also known as antidiuretic hormone, controls water conservation in the kidney and is released upon osmotic stimulation (Leng et al., 1999; Stricker and Sved, 2002) and also regulates vasoconstriction (Altura and Altura, 1984).

Autonomic parvocellular neurons which express CRH and to a lesser extent other neuropeptides project to the brainstem and spinal cord (Swanson and Kuypers, 1980) and are involved in the regulation of the sympathoadrenal system (Aguilera and Liu, 2012). CRH is also present in other brain regions, mainly in limbic structures, like the BNST, the central amygdala (CeA), LC, cerebral cortex, cerebellum and the dorsal root neurons of the spinal cord (Sawchenko and Swanson, 1985). CRH in these brain regions acts as a neurotransmitter and is implicated in the behavioural response to stress.

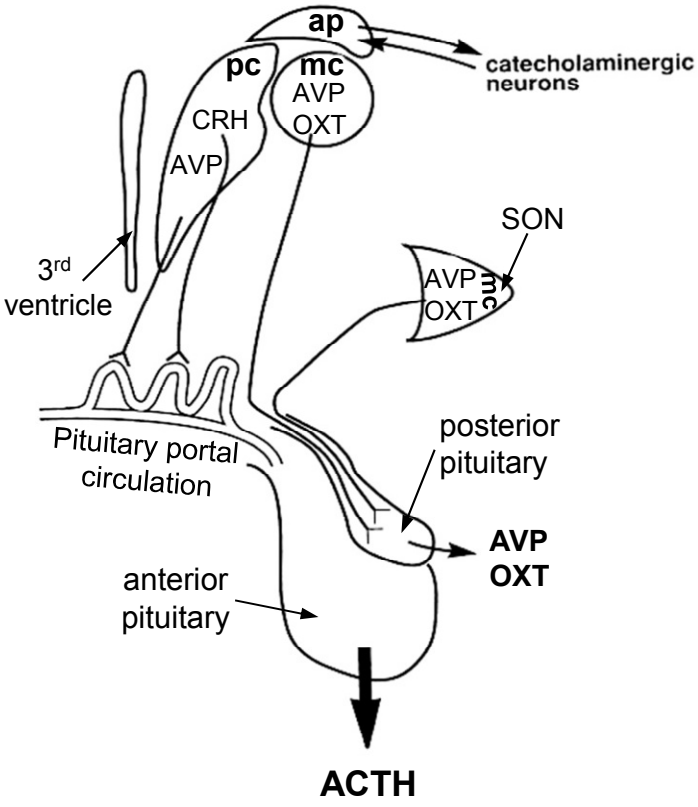

Figure 3: Schematic illustration of the different subregions of the PVN. The parvocellular division (pc) contains neurons expressing corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP). Both are released in the pituitary portal circulation, stimulating the release and synthesis of adrenocorticotropic hormone (ACTH) in the anterior pituitary. The magnocellular division (mc) of the PVN and of the supraoptic nucleus (SON) contain AVP and oxytocin (OXT) expressing neurons which project to the posterior pituitary and mediate the release of AVP and OXT in the peripheral circulation. The autonomic parvocellular neurons (ap) contain mainly CRH and to a lesser extent other neuropeptides and project to the brainstem and spinal cord. [taken and adapted from (Aguilera and Liu, 2012)]

The Pituitary

The pituitary gland, situated at the basis of the brain, is comprised of the anterior pituitary, consisting of the anterior and intermediate lobes and the neuropituitary also known as posterior lobe or posterior pituitary. While anterior and intermediate lobes consist of oral ectoderm, the posterior pituitary originates from neural tissue, i.e. from the infundibulum (Kelberman et al., 2009) (see Fig. 4).

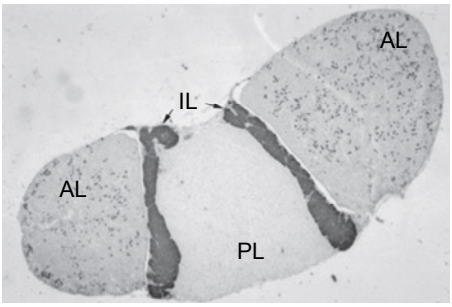

Figure 4: Rat pituitary section stained with antibody against γ-melanocyte stimulating hormone. The staining reveals the three regions of the pituitary; the anterior lobe/anterior pituitary, the intermediate lobe and the posterior lobe/posterior pituitary or neuropituitary. PL, posterior lobe, AL, anterior lobe; IL, intermediate lobe [taken and adapted from (Bicknell, 2008)]

The anterior pituitary contains five major cell types: the corticotrophs, thyrotropes, gonadotropes, somatotropes and lactotropes, producing different types of hormones which regulate adrenal function, thyroid function, reproduction, growth, and lactation, respectively. Corticotroph cells, comprising about 10 – 20 % of the total cell population of the anterior pituitary, produce ACTH and constitute the prinicipal site for HPA axis regulation (Yeung et al., 2006). The posterior lobe receives input from the axon terminals of the magnocelluar neurons of the PVN and also of the SON (Kelberman et al., 2009). In contrast, the anterior pituitary receives the endocrine information from parvocellular neurons via the portal blood (see also Fig. 3).

CRH released from these parvocellular neurons binds to the membrane-bound CRH receptor 1 (CRH-R1), the main subtype in pituitary corticotroph cells (Chalmers et al., 1996). CRH receptors, the CRH-R1 and the CRH receptor 2 (CRH-R2), are members of the GPCR and are coupled to adenylatcyclase which stimulates cyclic adenosine monophosphate (cAMP) synthesis and proteinkinase A (PKA) activity. CRH can bind to both receptor types but shows a higher affinity for the CRH-R1. The CRH-R1 is not only expressed in the pituitary but also widely distributed in the brain, especially in the cerebral cortex, cerebellum, amygdala, hippocampus, LC, and in the olfactory bulb (Grigoriadis et al., 1996). In the periphery it is amongst others localized in the adrenal, heart, spleen, colon, skin, testis, and placenta (Hillhouse and Grammatopoulos, 2006). In contrast, the CRH-R2 is widely expressed in the peripheral blood vessels, skeletal, smooth, and cardiac muscles but only in distinct areas of the brain, like lateral septal

nuclei, ventromedial nucleus of the hypothalamus, PVN, and BNST (Grigoriadis et al., 1996; Hillhouse and Grammatopoulos, 2006).

A binding protein for CRH (CRH-BP), located at different sites of the CNS, modulates CRH actions and probably acts as a negative regulator of CRH signalling (Thomson, 1998; Seasholtz et al., 2001). Moreover, some CRH-like peptides, the urocortins (urocortin 1, urocortin 2, urocortin 3), are distributed throughout stress-sensitive central and peripheral structures like the pituitary, gastrointestinal tract, testis, immune tissue, kidney, heart, adrenal, peripheral blood cells, muscle, and skin (Aguilera et al., 2004). While urocortin 1 binds to CRH-R1 and CRH-R2, Urocortin 2 and 3 selectively activate CRH-R2 (Dautzenberg and Hauger, 2002). The distribution pattern of the urocortins suggests a role in coordinating the stress response (Aguilera et al., 2004; Jamieson et al., 2006).

CRH administration was shown to increase gene transcription of pro-opiomelanocortin (POMC), the precursor of ACTH, in the anterior and intermediate lobe in *in vivo* as well as in *in vitro* studies (Affolter and Reisine, 1985; Lundblad and Roberts, 1988; Autelitano et al., 1990). After CRH binding to its receptor, cAMP and PKA activate via both calcium (Ca^{2+})- dependent and independent transduction signals the mitogen-activated protein kinase (MAPK) pathway which in turn activates Nur77 and Nurr1, two transcription factors (TF) involved in regulation of POMC expression (Murphy and Conneely, 1997; Kovalovsky et al., 2002).

As already mentioned before, POMC is only a precursor protein, from which different end products can be generated by endoproteolytic cleavage through proproteins and prohormone converting enzymes in a tissue specific manner (Marcinkiewicz et al., 1993). POMC is produced in corticotroph cells of the anterior pituitary and in melanotroph cells of the intermediate lobe (Bicknell, 2008). In the anterior pituitary, POMC, in a first step, is processed to pro-µ-melanocyte stimulating hormone, ACTH, and β-Lipotrophin. A main proportion of β-Lipotrophin is then further cleaved to β-endorphin. In the intermediate lobe POMC is cleaved to corticotrophin-like intermediate peptide, α- melanocyte

stimulating hormone, μ-Lipotrophin, and β–Endorphin whereby these products can be further processed (Bicknell, 2008).

In addition to its role in regulating POMC expression, CRH binding to CRH-R1 leads, as described above, to the activation of the PKA, which in turn - via a so far unknown pathway - opens voltage-dependent Ca^{2+} channels (VDCC), leading to increased influx of extracellular Ca^{2+} and eliciting the release of ACTH vesicles into the blood stream (Won et al., 1990; Kuryshev et al., 1996). Moreover, extracellular Ca^{2+} influx, via binding to a ryanodine receptor, enhances intracellular Ca^{2+} release which further promotes ACTH release (Yamamori et al., 2004) (see Fig. 5).

AVP receptors also belong to the family of GPCR and are divided into three major subtypes: AVP receptor 1a (AVPR-1a), AVP receptor 1b (AVPR-1b or V3), and V2. While the V2 receptor, which regulates the water resorption in the kidney, is coupled to Gs signalling via adenylate cyclase, the AVPR-1a, localized in smooth muscle cells and in the liver, and the AVPR-1b, localized in corticotroph cells, are coupled to Gq and signal via phospholipase C (PLC) (Aguilera and Rabadan-Diehl, 2000a).

While AVP alone is a weak stimulus for ACTH secretion, together with CRH it acts in a synergistic way on the corticotroph cells (Gillies et al., 1982; Antoni, 1993; Aguilera, 1994; Lightman, 2008). Activated PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 promotes the release of Ca^{2+} from IP_3 – sensitive intracellular stores (Tse and Lee, 1998) while DAG, in addition, activates proteinkinase C (PKC) which stimulates influx of extracellular Ca^{2+} via L-type Ca^{2+} channels (Won et al., 1990) (see Fig. 5). The synergistic effect of AVP and CRH on ACTH release seems to be mediated by the activation of PKC which contributes to the CRH-stimulated cAMP production by inhibiting the activity of phosphodiesterases leading in turn to a reduced degradation of cAMP (Abou-Samra et al., 1987). Interestingly, there are also studies describing heterodimers of CRH-R1 and AVPR-1b, probably contributing to the synergistic effect of CRH and AVP (Young et al., 2007) (see Fig. 5).

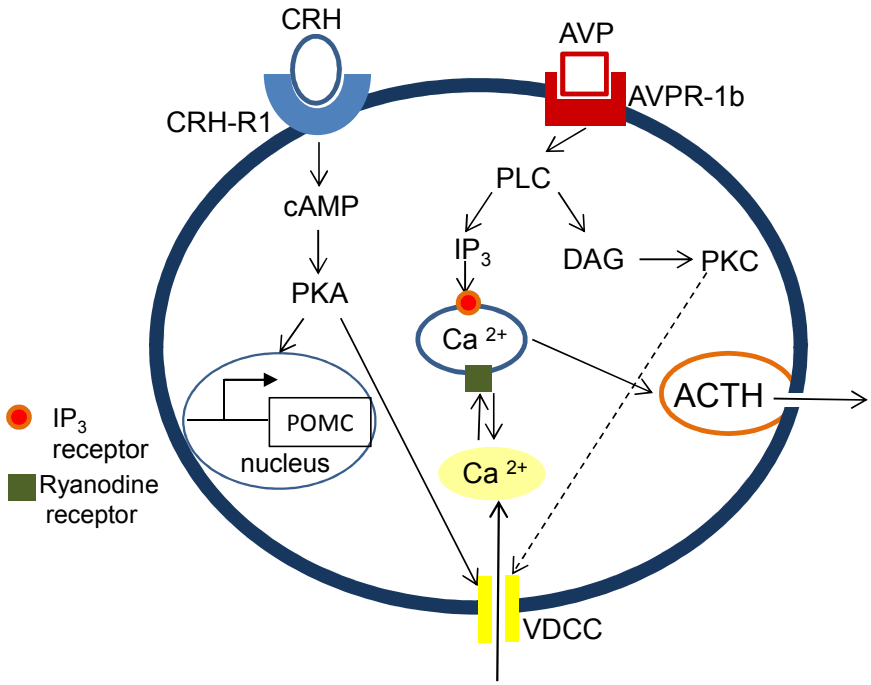

Figure 5: Schematic illustration of the intracellular pathways involved in corticotropin releasing hormone (CRH)- and arginine vasopressin (AVP)-mediated ACTH release in pituitary corticotroph cells. CRH binds to the CRH receptor 1 (CRH-R1), activates proteinkinase A (PKA) via cyclic AMP (cAMP) and stimulates pro-opiomelanocortin (POMC) gene transcription or triggers the influx of Ca^{2+} via voltage-gated channels (VDCC). Ca^{2+} that entered the cell via the VDCC can in turn stimulate the release of intracellular Ca^{2+} via binding to the ryanodine receptor. AVP, by binding to the AVP receptor 1b (AVPR-1b) activates phospholipase C (PLC) and via inositol 1,4,5-trisphosphate (IP3) promotes intracellular Ca^{2+} release. Diacylglycerol (DAG) can activate proteinkinase C (PKC) which can act synergistically with CRH to increase intracellular Ca^{2+}. Ca^{2+} is of importance for the fusion of the ACTH vesicles with the plasma membrane of the cell. [adapted from (Yamamori et al., 2004)]

Adrenal gland

ACTH released from the pituitary into the blood stream can bind to the melanocortin-2-receptor (Mc2r) at the adrenal cortex and triggers the synthesis and release of glucocorticoids (GC, cortisol in humans, corticosterone (CORT) in rats and mice) (Elias and Clark, 2000). The adrenal is composed of the adrenal cortex, producing primarily steroid hormones, and the adrenal medulla,

composed of mainly chromaffin cells, modified postganglionic sympathetic neurons, secreting primarily the catecholamines ephinephrine/adrenaline and NE/noradrenaline upon stimulation by the sympathetic preganglionic neurons (Diaz-Flores et al., 2008) (see also Sec. 2.1.2). In detail, the adrenal cortex consists of three layers. The innermost layer, the zona reticularis produces GC and in some species like in humans also androgens, estrogens and progestins. The outermost layer, the zona glomerulosa, synthesizes and secretes mineralocorticoids (aldosterone) for controlling the salt and water balance in the kidney. The medial layer, the zona fasciculata, synthesizes the main amount of GC (Rosol et al., 2001). GC secretion is mainly stimulated by ACTH, but also other hormones produced by the medulla, like adrenaline, have a stimulating effect (Bremner et al., 1996).

The Mc2r belongs to the family of melanocortin receptors (Mc1r to Mc5r), members of the GPCR, and stimulates the activation of adenylatcyclase and the production of cAMP. Within minutes, the protein expression of the steroid acute regulatory protein - expressed in all steroid hormone-producing cells (adrenal, gonads) - is increased which stimulates steroidogenesis (Cherradi and Capponi, 1998; Miller, 2008). Mc2r mRNA expression was also shown to be increased following stimulation with ACTH in human as well as in mouse adrenocortical cells *in vitro* (Mountjoy et al., 1994).

GC, mainly released from the zona fasciculata and zona reticularis, belong to the family of steroid hormones. Because of their common origin and their similar effectiveness with the mineralocorticoids they are denotet corticosteroids. While in humans the cortisol is the main corticosteroid (Nishida et al., 1977), in mice and rats it is CORT. GC can exert diverse effects on the whole body. For instance they play a major role in energy mobilization by increasing the blood glucose levels, they are implicated in modulation of the immune systems as well as in the regulation of cardiovascular functions (Sapolsky et al., 2000). Moreover, GC play a central role in the basal HPA axis activity as well as in the termination of the stress response by exerting a negative feedback at different levels of the HPA axis (see also Sec. 3.5).

1.2 Acute vs. chronic/repeated stress

The term “stress“ is mostly used in a negative sense, whereby it is often buried in oblivion, that the acute stress response constitutes one of the major survival mechanisms of an organism. The synchronal interplay between all physiological systems, resulting in coordinated activation of cardiovascular, locomotor, neuroendocrine and also immune system facilitates the “fight or flight” reaction (Dhabhar, 2009). Sterling and Eyer (1988), therefore, coined the term “allostasis”, meaning that the body returns to homeostasis by active processes. To achieve this allostasis, the stress hormones, mainly catecholamines and GC from the adrenal medulla and the adrenal cortex, respectively, are released (McEwen, 1998b).

Stress is composed of a series of events, starting with a stimulus (stressor) that is perceived by the brain (stress perception), disrupts the homeostasis and in turn activates physiological systems in the body (stress response) to restore the physiological balance (Dhabhar, 2009). The acute stress response is beneficial and adaptive for the body, contributes to the well-being of an organism, promotes survival, and increases its biological fitness. In contrast, repeated or chronic stressor exposure can have deleterious effects, promoting the development of somatic as well as affective disorders (McEwen, 1998a; Chrousos, 2009). McEwen and Stellar in 1993 introduced the term “allostatic load“ describing the long lasting burden and negative consequences of prolonged activation of the stress system (McEwen and Stellar, 1993).

Important criteria for distinguishing between acute and chronic stress are duration and intensity of the stressor. While acute stress lasts from minutes to hours, chronic stress occurs from days to month (Dhabhar, 2000). The intensity may be estimated by measurement of stress hormone levels, GC and catecholamines, and of heart rate and blood pressure (Dhabhar, 2000).

An acute stressor elicits a peak in blood GC levels within 15 – 30 min and a decline to basal levels 60 – 120 min later (Paskitti et al., 2000; de Kloet et al., 2005). In contrast, mice exposed to chronic SD over a period of 3 weeks (Keeney et al., 2006; Hartmann et al., 2012a) or exposed to chronic subordination in the

visible burrow system for 14 days (Albeck et al., 1997) showed sustained elevated plasma CORT levels compared with control mice. This prolonged release of GC can have deleterious effects on the organism, e.g. the damage of the hippocampus (McEwen, 1998a) and inhibition of the immune function (Dhabhar, 2000). Nevertheless, there are also chronic stress paradigms resulting in unchanged or even decreased plasma GC levels. For instance, in rats, repeated exposure to noise (4 h/day for 3 weeks) did not affect basal plasma CORT levels (Armario et al., 1986), whereby chronic social isolation for 3 weeks resulted in reduced basal plasma CORT levels compared with controls (Adzic et al., 2009; Djordjevic et al., 2012).

Chronic stress paradigms can be further divided in homotypic (same stimulus) or heterotypic (different stimuli) stressors whereby homotypic stressor exposure commonly results in adaptation of the stress response (Bartolomucci, 2007; Wood et al., 2010). As the homeostasis of an organism is important to guarantee survival, such adaptational mechanisms are indispensable to cope with repeated internal as well as external stimuli or stressors which are not life threatening. However, despite adapting to repeated innocuous stimuli, it is important for an organism to respond to a novel and perhaps dangerous challenge, in an adequate manner. Nevertheless the mechanisms underlying this adaptation and sensitization process are only poorly understood (Aguilera, 1994). The adaptation of the ACTH response, in-depth described in a review of Aguilera (1994), during chronic stress involves a bulk of mechanisms, with the regulation of the ACTH secretagogues CRH and AVP as well as their receptors on the pituitary, and the GC feedback playing a major role (Aguilera, 1994).

Three different patterns, characterized by the degree of adaptation to the persistent stressor and the magnitude of response to a novel, heterotypic superimposed stressor, are described. The first one is characterized by a desensitization of the ACTH response to a repeated/chronic stressor (physical or psychological stressor like repeated immobilization) and a hyperresponsiveness to the novel stressor (see Fig. 6 top). In the second pattern, no desensitization of the ACTH release (repeated painful stimuli like ip hypertonic saline injection) but

a hyperresponsiveness to the novel stimulus is observed (see Fig. 6 bottom). The third pattern is characterized of a small and transient increase in plasma ACTH (e.g. prolonged osmotic stimulation during water deprivation) and a hyporesponsivenss to the novel stressor (Aguilera, 1994).

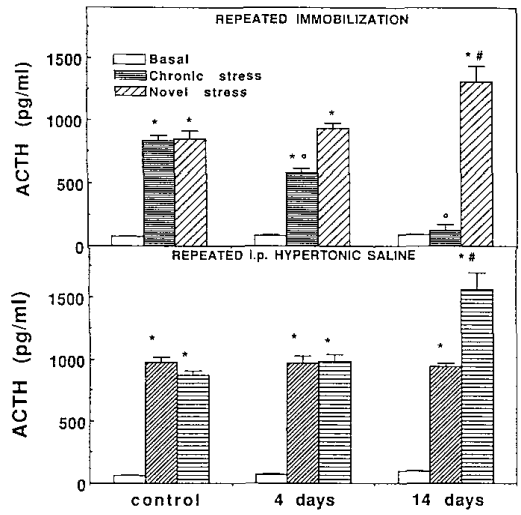

Figure 6: Plasma adrenocorticotropic hormone (ACTH) release during adaptation to repeated/chronic stressor exposure. Shown are plasma ACTH levels in controls and rats subjected to repeated immobilization (2 h/day, upper panel) or ip injection of hypertonic saline (5 ml, lower panel) for 14 days under basal conditions and following exposure to an acute novel stressor (ip hypertonic saline injection in the upper group and 30 min immobilization in the lower group). [Taken and adapted from (Aguilera, 1994)]

According to Aguilera and colleagues, AVP, under basal conditions only a weak stimulator of the ACTH release, becomes more and more attention as an important factor in the process of adaptation to repeated/chronic stressors and in the hyperresponsiveness to a novel heterotypic stressor, mediated by its synergistic effect on CRH-mediated ACTH secretion (Aguilera, 1994; Aguilera and Rabadan-Diehl, 2000a). Interestingly, following repeated immobilization, a significant increase in CRH neurons coexpressing AVP (from 50 % in controls to 90 % in chronically stressed animals) (Bartanusz et al., 1993) and also an increase in AVP mRNA (Ma and Lightman, 1998; Ma and Aguilera, 1999) has been described. This indicates that stress paradigms associated with a hyperresponsiveness to a novel heterotypic stressor (repeated immobilization,

repeated hypertonic saline injection) increase AVP expression while prolonged osmotic stress elicited no enhanced AVP activation in parvocellular neurons (Aguilera, 1994).

Also regulation of CRH mRNA expression depends on the type and duration of the homotypic stressor (Aguilera, 1998; Aguilera and Rabadan-Diehl, 2000a). In stress paradigms associated with sustained ACTH responses, like foot shock (Imaki et al., 1991; Sawchenko et al., 1993) or ip hypertonic saline injection (Ma and Aguilera, 1999), CRH mRNA levels are elevated. In contrast, following repeated restraint stress (Ma and Lightman, 1998; Ma et al., 1999) or colony-housing (De Goeij et al., 1992), associated with desensitization of the ACTH response, CRH levels are unchanged.

As indicated above, the expression of AVP increases in chronic stress paradigms associated with hyperresponsiveness to a novel heterotypic stressor but not during osmotic stimulation (Aguilera, 1994; Aguilera and Rabadan-Diehl, 2000a). Besides changes in PVN AVP expression, thereby, regulation of pituitary AVPR-1b expression seems to play a major role, as there is a good correlation between receptor content in the pituitary and corticotroph ACTH secretion in response to a superimposed heterotypic stressor (Aguilera, 1994; Aguilera et al., 1994; Aguilera and Rabadan-Diehl, 2000b). Under chronic stress conditions associated with reduced ACTH release in response to a novel stressor (chronic osmotic stimulation), AVP receptors decrease, while stress paradigms with enhanced ACTH response to a novel stressor (repeated immobilization, repeated hypertonic saline injection) result in receptor up-regulation (Aguilera and Rabadan-Diehl, 2000b), whereby mRNA not always correlates with AVP binding (Rabadan-Diehl et al., 1995; Rabadan-Diehl et al., 1997). The increase in AVPR-1b expression seems to be mediated by AVP as well as by other neuropeptides in the pituitary portal blood and also by GC. While increased AVP mRNA expression in the PVN positively correlated with the number of AVP-R1b receptors and ACTH response to a novel stressor following chronic stress (Aguilera, 1994), GC were found to reduce AVPR-1b binding despite increasing its mRNA levels as well as the coupling of the receptor to PLC. The increased

coupling of the AVPR-1b to PLC facilitates the corticotroph response to AVP in spite of eleveated GC levels (Rabadan-Diehl and Aguilera, 1998; Aguilera and Rabadan-Diehl, 2000b).

Given that in response to many chronic stress paradigms AVP, in contrast to CRH expression, is increased, this suggested for a long time that AVP plays a primary role in adaptation of the HPA axis response to chronic stimulation (de Goeij et al., 1991; Ma et al., 1997). However, studies conducted in AVP deficient Brattleboro rats or following pharmacological blockade of AVP receptors refuted the hypothesis of AVP becoming the main regulator during chronic stressor exposure. Administration of an AVPR-1b antagonist significantly reduced the response to acute restraint stress or lipopolysaccharide stimulation in rats (Spiga et al., 2009). AVP deficient Brattleboro rats showed a normal response to most acute stressors and only a slight reduction in ACTH release during repeated restraint stress (Zelena et al., 2004). Moreover, administration of a non-selective V1 receptor antagonist was not able to block the hyperresponsiveness of repeatedly restraint rats to an acute stressor, indicating that AVPR-1b up-regulation, described for this stress paradigm, is not required for the sensitization of the ACTH response (Chen et al., 2008). Overall, these studies suggest that AVP is important for the full ACTH response in some acute stress paradigms, but it is not necessary for the ACTH hyperresponsiveness to a superimposed acute heterotypic stressor, indicating that AVP might play an alternative role in the process of stress adaptation (Aguilera et al., 2008). A role for AVP in stimulating proliferation of corticotroph cells has been shown in the murine corticotroph tumor cell line AtT20 (van Wijk et al., 1995), during GC deficiency and chronic stress (Subburaju and Aguilera, 2007).

In chronic stress paradigms associated with ACTH hyperresponsivenss to a heterotypic stressor and also in chronic stress models associated with maintained ACTH release (repeated hypertonic saline injection) CRH-R1 gets down-regulated and desensitized, indicated by a reduced cAMP and ACTH response to CRH (Kiss and Aguilera, 1993; Aguilera, 1994). However, for the CRH-R1 it could be demonstrated that the receptor number in the pituitary does

not always correlate with the pituitary responsiveness. Thus, a decreased CRH binding following chronic homotypic stressor exposure (e.g. repeated restraint) resulted in increased POMC mRNA expression (Aguilera et al., 2001). In addition, there is no correlation between CRH-R1 binding and CRH-R1 mRNA levels, suggesting that CRH-R1 down-regulation seems to be mediated by changes in the translation efficiency and/or receptor internalization or desensitization (Aguilera et al., 2001). Down-regulation of CRH-R1 binding can be ascribed to CRH and was shown to be facilitated by concomitant administration of AVP (Hauger and Aguilera, 1993). In addition, administration of GC decrease CRH binding *in vivo* and *in vitro* (Childs et al., 1986; Schwartz et al., 1986; Hauger et al., 1987) while CRH-R1 mRNA levels decreased only transiently (Rabadan-Diehl et al., 1996; Iredale and Duman, 1997), suggesting an inhibition of the CRH-R1 by GC at the post-transcriptional level. Overall the studies indicate, that i) the number of CRH-R1 is not indicative for the corticotroph responsiveness, as a small number of receptors is sufficient for a full ACTH response and that ii) post-transcriptional mechanisms play the major role in the regulation of its expression (Aguilera et al., 2004).

1.3 Psychosocial stress

Selye`s proposed concept of the general adaptation syndrome only referred to physical stressors like cold exposure or surgical injury (Selye, 1936b). Nevertheless, it became more and more evident, that the type of stressor, e.g. psychological, social or physical in nature, determines the consequences on behaviour and physiology. While water deprivation produced a duration-dependent anxiolytic effect on the elevated plus-maze (EPM), restraint stress (1 h) had an opposite effect even though plasma CORT levels were increased in both groups (McBlane and Handley, 1994). These differences in stress responsiveness clearly indicate that stress research should focus on those types of stressors that reflect the natural condition a mammalian species, especially humans, are normally exposed to, in order to find treatment strategies for e.g. stress-related disorders. In humans, the most naturalistic type of stressor every

individual is exposed to is of social and/or psychological nature (Brown and Prudo, 1981; Bartolomucci et al., 2005). Psychological stress can be caused by environmental demands that are thought to exceed the individual's capability to deal with, implying the cognitive appraisal of the stimulus (Sapolsky, 2005; Cohen et al., 2007). Social stress is mainly the result of alterations in the social life of an individual, concerning the relationship and interaction between individuals including e.g. disputes over resources or the social rank (Blanchard et al., 2001). Given that chronic psychosocial stressors are acknowledged risk factors for the development of stress-related disorders in humans (Hemingway and Marmot, 1999; Wahrendorf et al., 2012), animal models mimicking this chronic psychosocial burden are important and powerful tools for studying the physiological, neuroendocrine and immunological mechanisms that underlie the development of these diseases.

The chronic subordinate colony housing (CSC, 19 days) paradigm constitutes one of these appropriate animal models as it induces chronic psychosocial stress in male mice. Thereby four experimental mice are housed together with a larger dominant male mouse for 19 consecutive days, resulting in a number of physiological, neuroendocrine, immunological and also behavioural alterations. CSC mice develop somatic as well as affective disorders (Reber et al., 2007; Slattery et al., 2012; Uschold-Schmidt et al., 2012) and display typical signs of chronic stress, like adrenal hypertrophy and thymus involution (Reber et al., 2007; Uschold-Schmidt et al., 2012). Moreover, following CSC exposure, mice exhibit basal hypocorticism in the evening and show a GC resistance in different cells in the periphery (Reber et al., 2007). But, interestingly, in response to an acute heterotypic stressor exposure (5 min elevated platform (EPF)), they show an exaggerated CORT release (Uschold-Schmidt et al., 2012). This indicates an adaptation to the chronic stressor exposure and a concomitant sensitization of the HPA axis in response to an acute challenge, at least at the level of the adrenal glands. Whether or not changes at higher HPA axis levels, for instance at the pituitary, are also contributing to these adaptation/sensitization processes have not been analysed in detail yet.

2 GC Signalling

Upon binding of ACTH to the adrenal cortex GCs are released into the blood stream to exert a variety of effects, which are determined by a number of factors, like the availability of the corticosteroids in the cell as well as the type of receptor.

2.1 Corticosteroid availability

The activity of the HPA axis, i.e. ACTH and GC release, follows a circadian rhythm. Peak cortisol levels are secreted at the end of the resting period, i.e. in the morning. In contrast, nocturnal animals like rodents show the CORT peak at the end of the afternoon immediately before the start of the dark phase (Young et al., 2004; Lightman et al., 2008). Coordination of this rhythmic release pattern is orchestrated by the suprachiasmatic nucleus of the hypothalamus. This nucleus controls on the one hand CRH and AVP release (Reppert and Weaver, 2002) and on the other hand, via the autonomic nervous system, the adrenal gland`s sensitivity to ACTH (Ulrich-Lai et al., 2006), preparing the organism for the increased metabolic demand during the activity phase.

Not only the release of GC from the adrenal glands is of importance but also the availability in the target tissue is regulated by multiple factors. Corticosteroids can be bound to specific carrier proteins, e.g. the corticosteroid-binding globulin (CBG) or albumin and they are also subjected to enzymatic conversions, both influencing the availability and, thus, bioactivity of these hormones.

CBG is a glycoprotein, synthesized in the liver, to which under normal conditions about 80 – 90 % of circulating corticosteroids are bound. 10 – 15 % are bound to albumin, resulting in only about 5 - 10 % of free corticosteroids (Cizza and Rother, 2012). As only free corticosteroids are able to enter the target cells, the binding proteins constitute an important mechanism to protect, particularly the brain, from the dangerous effects of high corticosteroid levels. Regulation of CBG levels enables a fast mechanism of adaptation of the corticosteroid levels to different situations, e.g. ultradian or circadian variations, stress (Qian et al., 2011) or disease. Levels of circulating CBG rise in response to an acute stressor in

order to restrain the CORT rise (Qian et al., 2011) while chronic stressor exposure evoques a decrease in CBG leading to an increase in the free corticosteroid levels (Stefanski, 2000; Henley and Lightman, 2011)

Entering the brain involves the passage of the blood-brain barrier, composed of specialized endothelial cells, protecting the brain from probably damaging compounds and maintaining the neuronal homeostasis. While the small and highly lipophilic CORT can pass the blood-brain barrier via diffusion, the penetration of cortisol as well as of synthetic GC, e.g. dexamethasone (Dex), is limited as they are substrates for the multidrug resistance (mdr) 1a P-glycoprotein, located in the membrane of endothelial but also of other peripheral cells (Meijer et al., 1998; Karssen et al., 2001). In the cell, tissue specific enzymes, the most common one is the 11ß-hydroxysteroid dehydrogenase (11ß-HSD), regulate the interconversion of active GC (cortisol, CORT) and their inert 11-keto forms (cortisone, 11-dehydrocorticosterone). 11ß-HSD type 1 is expressed in the liver, adipose tissue, bone, lung and pituitary and in the CNS, especially in the hippocampus, cerebellum and neocortex, catalizing the conversion of the 11-keto in its active form (Harris et al., 2001; Cooper and Stewart, 2009). The type 1 form of this enzyme, therefore, plays a major role in regulating GC levels in the brain under basal and stress conditions (Harris et al., 2001). 11ß-HSD type 2 is mainly expressed in mineralocorticoid target tissue, like kidney, colon, and salivary glands, converting GC in their inactive form, rendering them unable to bind to the mineralocorticoid receptor (MR) and, therefore, enabling aldosterone to exert its effects by binding to the MR (Cooper and Stewart, 2009).

2.2 Corticosteroid receptor types in the brain

The actions of the corticosteroids are mediated by two types of receptors; namely the MR and the glucocorticoid receptor (GR). Their names already implicate the processes they are involved in, mineral balance (Yang and Young, 2009) and gluconeogenesis (Revollo and Cidlowski, 2009), respectively. Binding studies revealed, that the MR binds GC with a 10-fold higher affinity (K_D ~ 0.5 nM) than

GR (K_D ~ 5 nM) (Reul and de Kloet, 1985; de Kloet et al., 1998). MR are mainly distributed in limbic brain structures with the highest density in the hippocampus and the lateral septum, while low expression levels are found in the amygdala, hypothalamus and also outside of the brain in the pituitary (Moguilewsky and Raynaud, 1980; Reul and de Kloet, 1985). GR are distributed all over the brain, e.g. in the lateral septum, hippocampus, nucleus tractus solitarii, LC, amygdala, and PVN (Reul and de Kloet, 1985). According to the binding studies, low freely circulating GC levels in the brain during the diurnal through (0.5 – 1 nM) already occupy most of the MR (Reul et al., 2000), whereas GR become additionally occupied at higher CORT levels, i.e. at the circadian peak and during stress (Reul and de Kloet, 1985). As under most circumstances the MR is already occupied, it is hypothesized that for the MR its protein expression while for the GR the ligand concentration constitute the most important regulators (de Kloet et al., 2000). For the GR, two main isoforms, the GRα and the shorter form, the GRß, are described which differ in their carboxy termini. While GRα binds GC and mediates a variety of GC effects, the GRβ is unable to bind GC and is, therefore, transcriptionally inactive and, in addition, can inhibit the transcriptional activity of GRα (Oakley et al., 1999; Revollo and Cidlowski, 2009). For the MR four isoforms exist, with the MRα and MRß representing the two main types which show different tissue-specific expression patterns (Zennaro et al., 1995; Zennaro et al., 1997).

GR and MR are members of an evolutionary conserved family of nuclear receptors which are composed of three functional domains. The N-terminal transactivation domain with an activation function motif (AF-1) for the interaction with the transcriptional machinery and/or TF, the central DNA-binding domain (DBD) containing two zinc fingers for interaction with the DNA and receptor dimerization, and the C-terminal ligand-binding domain, containing a ligand-binding motif, a nuclear localization signal (NLS) and a further activation motif (AF-2) for interaction with other TF (Giguere et al., 1986; Bamberger et al., 1996; Revollo and Cidlowski, 2009) (see Fig. 7).

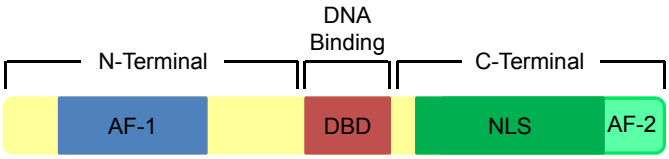

Figure 7: Schematic illustration of the protein structure of the human GR. The gene is composed of a N-Terminal region, containing an activation function (AF-1) motif, a DNA binding domain (DBD), a C-Terminal region, containing a nuclear localization signal (NLS) and another activation motif (AF-2). [adapted from (Revollo and Cidlowski, 2009)]

The properties of the different domains already give insight into the functions of these receptors. The unactivated receptor is bound to a multiprotein complex consisting of molecular chaperones, the heat shock proteins (hsp90 and hsp70), which serve the proper folding of peptides and proteins in the cytoplasm. Other co-chaperones, like the acidic protein p23 and immunophilins that bind via their tetratricopeptide repeat (TPR) domain to a TPR domain on the hsp90 complement the complex (Tai et al., 1992; Hutchison et al., 1993; Pratt and Toft, 1997). Immunophilins, disposing peptidylproly-isomerase activity, bind immunosuppressive drugs, like FK506, rapamycin and cyclosporine A. Some of these participate in the corticosteroid receptor-hsp90 complex, e.g. FK506-binding protein 51 (FKBP51) and FK506-binding protein 52 (FKBP52). Given that both bind to the same site on the hsp90 independent heterocomplexes can be created (Pratt and Toft, 1997). The whole complex around the GR regulates the receptor folding, maturation, activation, and also trafficking into the nucleus (Grad and Picard, 2007). Upon ligand binding primarily a conformational change of the receptor takes place, exposing two NLS which promote the translocation into the nucleus (Picard and Yamamoto, 1987). The receptors then either form dimers which bind to the DNA on so called GC-responsive elements (GRE) enabling the transcription of different target genes or they can also act as monomers. The general opinion was that the GR has to dissociate from the hsp90 chaperone complex in order to promote translocation (de Kloet et al., 1998). Nevertheless, pharmacological inhibition of hsp90 has been shown to lead to a delay in nuclear translocation of the GR-ligand complex (Czar et al., 1997). Therefore, the translocation concept gets renewed by conveying the immunophilins FKBP51

and FKBP52 an important role in hormone-bound receptor trafficking (Galigniana et al., 2010a). It suggests that FKBP51, bound to hsp90, has to be exchanged by FKBP52 which binds to dynein, enabling the translocation to the nucleus via microtubules (Davies et al., 2002; Galigniana et al., 2010b) (see Fig. 8).

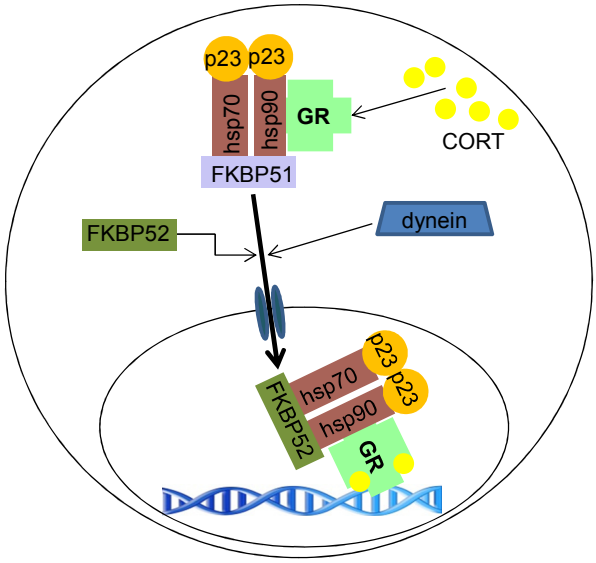

Figure 8: Schematic illustration of the concept of glucocorticoid receptor (GR) nuclear translocation. In the cytoplasm, the GR is associated with heatshock proteins (hsp 90 and hsp70), two acidic proteins (p23) and one immunophilin, the FK506-binding protein 51 (FKBP51). Once CORT binds to the GR receptor complex FKBP51 is exchanged against FKBP52. In turn, dynein can bind and the receptor complex translocates into the nucleus through the nuclear pore where it can bind to the DNA. To simplify the illustration only a monomer of the receptor complex is depicted. [adapted from (Binder, 2009)]

FKBP51 and FKBP52

Since their discovery, the FK506 binding proteins gained more and more attention as they represent important regulators of GR (Denny et al., 2000) and also MR (Gallo et al., 2007). As already mentioned before, FKBP51 binds to hsp90 via a TPR-domain and lowers the receptors affinity for its ligand (Wochnik et al., 2005). The significance for FKBP51 was discovered in New World Monkey, a monkey line characterized by extremely high cortisol levels compared with other monkeys or humans. But, surprisingly, they show no behavioural or

physiological signs known to be linked with hypercorticism, as the target organs exhibit GC resistance mediated by an overexpression of FKBP51 (Denny et al., 2000). In addition, *in vitro* studies demonstrated that increased FKBP51 levels not only reduce the affinity of the receptor for its ligand but also reduce subsequent nuclear translocation (Wochnik et al., 2005). Interestingly, GC induce FKBP51 transcription via GR which in turn restrains GR functionality, forming part of a short negative feedback loop regulating GR activity (Vermeer et al., 2003).

Scharf and colleagues (2011) could show that FKBP51 is not only expressed in peripheral tissues but also in GR-expressing brain regions, like hippocampus, amygdala, and PVN. Stressor exposure was shown to induce FKBP51 expression whereby the basal expression pattern influenced the extent to which its expression was induced. Regions containing low levels of FKBP51 under basal conditions showed a higher induction compared with regions showing higher basal expression levels. An evidence for FKBP51 regulating GR sensitivity and that high levels result in lower GR responsiveness which suggests the use of baseline FKBP51 levels as marker for GR sensitivity (Binder, 2009; Scharf et al., 2011). Genetic polymorphisms in the gene encoding for FKBP51, associated with an increased FKBP51 expression, lead to decreased negative feedback regulation of the stress hormone system and, therefore, increase the risk for development of psychiatric disorders, like major depression, bipolar disorder, and post-traumatic stress disorder (Binder, 2009).

In opposite to FKBP51, FKBP52 is a positive regulator for trafficking of GR and MR into the nucleus via interaction with dynein (Silverstein et al., 1999; Galigniana et al., 2010b). FKBP52 also stimulates GR-mediated gene transcription (Ning and Sanchez, 1993) but not the transcriptional activity of the MR (Gallo et al., 2007).

2.3 Transcriptional Regulation

Upon translocation into the nucleus, MR and GR can modulate gene transcription by binding to a GRE or by interaction with other TF (Beato and Sanchez-Pacheco, 1996). GR and MR have a nearly identical DBD recognizing the GRE

on the DNA in the proximity of gene promoters where they can bind either as homo- (Karst et al., 2000) or heterodimers (Liu et al., 1995; Ou et al., 2001). It is hypothesized that GR are more potent transcription activators than MR and that heterodimers have different functions compared to homodimers (de Kloet et al., 2000). Hence, according to the GC concentrations, three different dimer conformations are possible, that can bind to different DNA targets and regulate a variety of target genes in a distinct manner (Kellendonk et al., 2002). Heterodimers are observered predominantely at higher concentrations, i.e. under conditions of stressful events (Nishi and Kawata, 2007). Nevertheless not much is known about the exact differences between the binding of hetero- or homodimers to the promoter sequence of different genes. Upon binding to the GRE, a number of co-factors are recruited to the receptor complex and interaction with the general transcription machinery takes place. Transcription of target genes is then either enhanced, by binding to positive GRE, a process called transactivation, or repressed, by binding to negative GRE. Negative GRE are less commonly observed, but some are part of the negative feedback process of the HPA axis (see also Sec. 3.5) or of the immune system (Zhang et al., 1997; Datson et al., 2008).

GR can also interact as monomer with other TF, e.g. activator protein 1, cAMP response element binding protein, or nuclear factor-kappa B, that are activated by other signalling pathways in response to inflammation or immune activation. Normally, GR repress the effects of these TF on gene transcription (transrepression), accounting for the inhibitory effects of GR on the immune system (de Kloet et al., 1998; Datson et al., 2008) (see Fig. 9). The transcriptional response is further regulated by phosphorylations of the receptor by cell-specific kinases (e.g. MAPK, cyclin-dependent kinase CDK, glycogen synthase kinase GSK-3) which can modulate the receptor activity (Galliher-Beckley and Cidlowski, 2009).

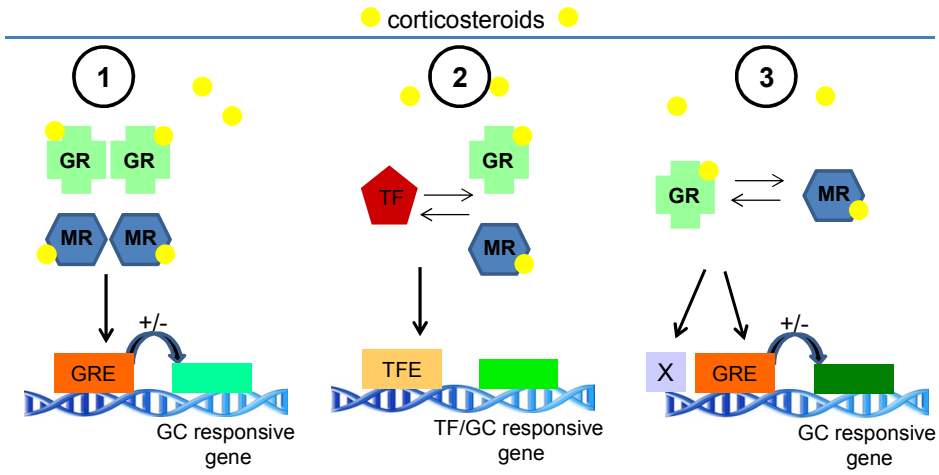

Figure 9: Molecular mechanisms of glucocorticoid actions on gene expression. (1) Homodimerization: Upon binding of corticosteroids, mineralocorticoid receptor (MR) or glucocorticoid receptor (GR) can translocate into the nucleus, form homodimers and bind to specific elements on the DNA, the GC-responsive elements (GRE), in the promoter region of GC-responsive genes and increase (transactivation) or decrease their transcription. (2) Transrepression: Activated GR can interact with transcription factors (TF) by direct protein-protein interaction, inhibiting the binding of the TF to the TF elements (TFE), resulting in a down-regulation of TF and/or GC-responsive genes. (3) Heterodimerization: Activated MR and GR can build heterodimers and bind to GRE or yet unknown DNA elements (indicated by X), affecting expression of GC-responsive genes. [adapted from (de Kloet et al., 1998)]

The transcriptional response mediated by the GR is highly dynamic, displaying different expression waves over the time. In hippocampal slices, administration of 100 nM CORT down-regulated a majority of genes after 1 h, up-regulated some genes after 3 h and following 5 h of stimulation, gene transcription was nearly back to baseline (Morsink et al., 2006).

GC regulate a wide number of genes involved in cellular processes like energy metabolism (e.g. glycolysis and gluconeognesis), signal transduction, regulation of neurotransmitters and their receptors, e.g. AVP-R1a, OXT receptor and in neuronal plasticity, e.g. the expression of the clock gene period 1 (Per1). Importantly, they are also implicated in the regulation of the HPA axis and in corticosteroid signalling itself (Datson et al., 2008; Lightman and Conway-Campbell, 2010).

2.4 Non-genomic pathways

GC-mediated effects, as described above, are mostly slow in onset. The translocation of the receptor to the nucleus (10 – 30 min) and the transcription (5 – 120 min) are time consuming steps, indicating that it takes a delay of at least 15 min or often hours until physiological responses occur (de Kloet et al., 1999; Haller et al., 2008). Therefore, rapid GC-mediated effects, seen after seconds to minutes (Di et al., 2003; Karst et al., 2005) cannot be ascribed to the classical effects at the transcriptional level while slow effects cannot automatically be ascribed to a genomic effect (Haller et al., 2008). Already 30 years ago, binding of steroids at the synaptic membrane was found (Towle and Sze, 1983) revealing early evidence that membrane-bound receptors may, most likely in addition to the cytoplasmic GR, play an important role in mediating these non-genomic GC effects. To support the concept of membrane-bound GR or MR, there are some criteria that have to be fulfilled. First of all, CORT applied in the intracellular compartment may not exert the rapid non-genomic effects. CORT or Dex conjucated to bovine serum albumin (BSA), which is, therefore, unable to enter the cell, must exert the same effects as the unbound homolog (Groeneweg et al., 2012). The most compelling evidence for the presence of MR and GR at the membrane was done by demonstrating their localization via electron microscopy (Johnson et al., 2005; Prager et al., 2010). Nevertheless, as some of the GC-mediated effects cannot be blocked with common MR and GR antagonists, a novel membrane-associated receptor seems to be implicated, from whom not much is known to date. GPCR are one of the most promising candidates, as blockade of these receptor types prevents some of the MR and GR independent GC effects (Di et al., 2003; Tasker et al., 2006).

2.5 Negative feedback effects of GC

Much of the attention drawn towards GC effects has focused on the termination of the HPA axis stress response, whereby the major sites for this negative feedback inhibition are the anterior pituitary gland (Miller et al., 1992), the

hippocampus, the PVN (Kovacs and Makara, 1988; Feldman and Weidenfeld, 1999; Weiser et al., 2011), and to a lesser extent also the amygdala (Reul and de Kloet, 1985) and the medial prefrontal cortex (PFC) (Diorio et al., 1993; Hill et al., 2011) (see Fig. 10). As depicted in the previous sections, GC can exert a variety of effects, mediated either via genomic or non-genomic signalling. In the following section I will focus on the rapid, non-genomic and the delayed, genomic effects of GC signalling in different brain regions and in the pituitary involved in the negative feedback response.

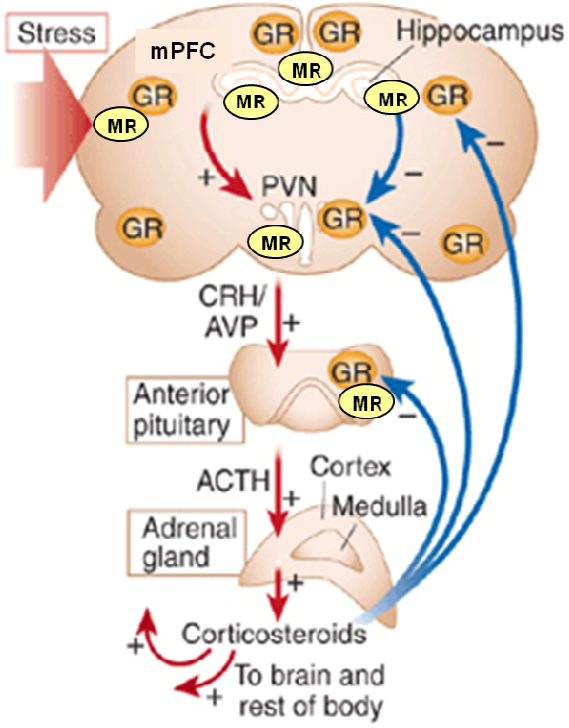

Figure 10: Schematic illustration of the HPA axis including sites of negative feedback inhibition. Activation of the HPA axis by stressful stimuli activates the paraventricular nucleus (PVN). As a consequence corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) which stimulate the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary and in turn the release of corticosteroids from the cortex of the adrenal gland (red arrows). Released corticosteroids can bind to the glucocorticoid receptor (GR) and mineralocorticoid receptors (MR), located in the brain (hippocampus, medial prefrontal cortex (mPFC), PVN) and in the pituitary and trigger the negative feedback response (blue arrows). [taken and adapted from (Akil, 2005)]

Hippocampus

The hippocampus, also known as the hippocampal formation, forms a C-shaped structure, located dorsal and posterior to the septal nuclei and running to the temporal cortex. The hippocampal formation compromises four different structures; the dentate gyrus (DG), the Ammon`s horn (cornu Ammonis, CA1, CA2, CA3, CA4), the subicular complex, composed of subiculum, pre-subiculum and parasubiculum and the entorhinal cortex (EC), divided in medial and lateral subdivisions. All of these structures are interconnected via intrinsic structures (Amaral and Witter, 1989).

The most important afferences of the hippocampus attain via the EC, which is connected to various parts of the cerebral cortex. Fibers of the perforant path start in the EC and travel to the DG, from where granule cells pass on the information via mossy fibers to the CA3 region. Shaffer collaterals, the axon collaterals of CA3 pyramidal cells, project to the CA1 region which gives the input to the subiculum (Sub). The Sub, representing the main output of the hippocampus, projects back to the EC. The EC can also directly project to the CA3, CA1 and Sub (Amaral and Witter, 1989; Braak et al., 1996) (see Fig. 11).

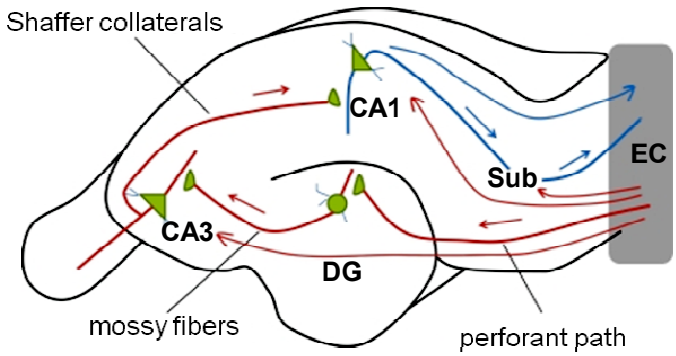

Figure 11: Schematic illustration of the neuronal circuitry in the hippocampal formation. The entorhinal cortex (EC) projects via the perforant path to the dentate gyrus (DG) which in turn via mossy fibers provides input to the CA3 region, from where Shaffer collaterals project to the CA1 region. CA1 sends in turn projections to the subiculum (Sub). [adapted from (Zhang and Zhu, 2011)]

The inhibitory influence of the hippocampus on the HPA axis activity was demonstrated by numerous lesion and stimulation studies. In rodents, hippocampal damage increased PVN CRH mRNA expression (Herman et al., 1989; Herman et al., 1992) and ACTH release (Knigge and Hays, 1963). In turn, in humans (Rubin et al., 1966) as well as in rodents (Feldman and Weidenfeld, 2001), stimulation studies revealed an inhibitory role of the hippocampus on HPA axis activity. Nevertheless, not the whole hippocampal formation is involved in the feedback, but rather the Sub, the major output of the HC. The Sub projects to many areas, e.g. to limbic cortices, nucleus accumbens, lateral septum, BNST, preoptic area and hypothalamus. Thereby especially the ventral Sub represents the major innervation of the hypothalamic regions. As the ventral Sub signals via the excitatory transmitter glutamate (Glu), and almost no direct connection to the PVN exists, an inhibitory relay must be available, whereat the posterior/lateral BNST was identified, projecting GABAergic neurons to the PVN (Forray and Gysling, 2004; Herman and Mueller, 2006).

Rapid activation of CA1 hippocampal pyramidal cells within minutes (< 5 min) after CORT administration, promotes miniature excitatory postsynaptic currents (mEPSC) and, therefore, increases Glu release probability, mediated by the MR localized on the presynaptic membrane which signals via ERK1/2 signalling pathway (Karst et al., 2005; Olijslagers et al., 2008). Moreover, at the postsynaptic membrane, activation of membrane-bound MR coupled to a G-protein, leads to a more depolarized potentials of the activation curve, increasing the likelihood for a postsynaptic action potential to occur (Olijslagers et al., 2008). Interestingly, this membrane-bound MR has a 10 fold lower affinity to CORT compared with the cytoplasmic MR, giving new insight in the role of the MR in HPA axis activity, as during low levels of circulating CORT, the cytoplasmic MR normally is already fully occupied (Karst et al., 2005). The nearly constant occupancy of the cytoplasmic MR is thought to contribute to the maintenance of the basal HPA axis activity and to determine the threshold or sensitivity for the stress response (de Kloet et al., 1998; Joels, 2007).

When CORT concentrations rise, also GR gets occupied, suppressing the hippocampal output which results in a disinhibition of the PVN (de Kloet et al., 1998). These effects are generally delayed and genomic in nature. Ca^{2+} current amplitude gets enhanced several hours after CORT administration or stressor exposure, mediated by protein synthesis and DNA-binding of GR homodimers (Karst et al., 2000) which increase the number of L-type Ca^{2+} channels (Chameau et al., 2007). This supports Ca^{2+} dependent K^+ conductances, leading to hyperpolarization and reduced spikes.

Taken together, MR and GR in the hippocampus mediate opposite effects, while MR, especially the membrane-bound MR, activation maintains hippocampal output and, therefore, inhibits PVN activity, GR activation decreases hippoampal output, which in turn disinhibits the PVN (see Fig. 12). This contrary effectiveness of the two receptors, underlines the importance of the proper balance between MR and GR (de Kloet et al., 1998).

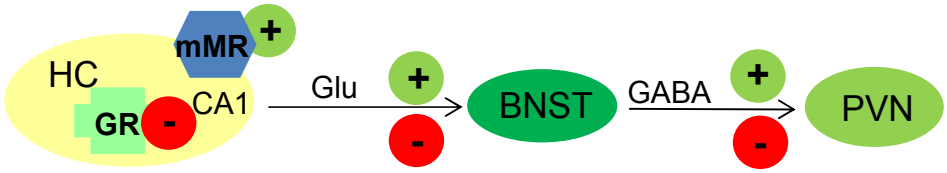

Figure 12: Schematic illustration of the mechanisms of negative feedback inhibition in the CA1 region of the hippocampus (HC). Within minutes, membrane-bound MR (mMR) get activated and enhance the release of glutamate (Glu). Activated GABAergic neurons in the posterior/lateral BNST in turn inhibit the PVN. In contrast, activation of cytoplasmic GR leads within hours to a decreased Glu release and in turn decreased GABA release from the posterior/lateral BNST and disinhibits the PVN.

PVN

The PVN not only gets inhibited or activated by the posterior/lateral BNST via hippocampus and medial PFC, but is itself subjected to the negative feedback regulation. Administration of CORT or Dex on parvocellular neurons of the PVN, including those producing CRH, decreased the release probability of Glu-containing vesicles from presynaptic Glu neurons within 3 to 5 min, mediated by

a membrane-specific effect (Di et al., 2003). Further experiments revealed an involvement of a postsynaptic G-protein coupled GR located on parvocellular neurons, which gets activated by GC. This is dependent on the $G_{\alpha}s$ subunit, mediating via cAMP-PKA pathway the release of endocannabinoids which act as retrograde messengers on cannabinoid receptor 1 on presynaptic neurons, suppressing the Glu release from excitatory synaptic terminals. Thus, the frequency of mEPSC is suppressed in the PVN (Di et al., 2003). On magnocellular PVN neurons, similar effects were found, acting via the same signalling pathways (Di et al., 2005). In these cells, GC cause, in addition, an increase in GABA signalling, via the $G_{\beta}\mu$ subunit. Thereby, the activity of the nitric oxid synthase is increased leading to the release of nitric oxid which acts on inhibitory synaptic terminals leading to the release of GABA. Inhibition of Glu and concomitant increase in GABA release results in an overall rapid inhibition of the PVN mediated by GC (Di et al., 2003; Di et al., 2009).

One of the most important targets for negative feedback regulation of the HPA axis is the CRH gene in the parvocellular neurons of the PVN (Imaki et al., 1995). A nGRE located on the CRH promoter, enables GR binding and in turn decreases transcription of CRH in a genomic fashion (Malkoski et al., 1997). Moreover, a nGRE was also found in the gene for AVP leading to a inhibition of AVP gene expression in parvocellular neurons of the PVN following stimulation with GC (Kim et al., 2001; Kuwahara et al., 2003).

Pituitary

Fast as well as delayed effects of GC have been observed at the level of the pituitary, where GR and also to a lesser extent MR are expressed (de Kloet et al., 2000). Fast negative feedback occurs already within 5 – 15 min after administration of CORT or of a GR agonist, inhibiting CRH-induced ACTH release independent of the cytoplasmic GR and *de novo* mRNA synthesis (Hinz and Hirschelmann, 2000). This rapid, non genomic feedback is mainly regulated by annexin 1 (ANXA1), also known as lipocortin 1, a member of the annexins, a class of proteins that are able to bind Ca^{2+} and negatively charged phospholipids.

ANXA1 is expressed in many cell types involved in inflammation, but also in the neuroendocrine system, like anterior pituitary and hypothalamus (Buckingham and Flower, 1997). Within the cell, it is mainly localized in the cytoplasm, but also on the external surface of the cell membrane attached to binding proteins. Immunohistochemical (IHC) analysis revealed that in the pituitary ANXA1 is not located in the endocrine cells but in the surrounding folliculostellate cells, therefore, acting mainly as a paracrine agent on the ACTH release (Traverso et al., 1999). GC influence the subcellular localization of ANXA1, mediating its exportation from the cytoplasm to the outer surface of the plasma membrane where it is retained through a Ca^{2+} dependent mechanism (Philip et al., 1997). ANXA1 can then bind to a member of the *N*-formyl peptide receptors on the corticotroph cells (John et al., 2007). This is assumed to – via a couple of so far unknown mediators – finally cause actin polymerization preventing in turn vesicle fusion with the membrane and, therefore, ACTH release (John et al., 2007).

While mediation of the transfer of ANXA1 to the cell membrane appears within 15 min of GC administration, GCs also induce the synthesis of ANXA1 in a delayed manner (< 2 h) in order to replenish the protein stores in the cytoplasm (Philip et al., 1997).

Another genomic effect exerted by GC in the pituitary constitutes the inhibition of POMC transcription by binding to a nGRE in the POMC gene promoter (Drouin et al., 1989; Drouin et al., 1993). GC also decrease CRH binding, whereas the mRNA expression of the CRH-R1 is only transiently decreased, favouring a post-transcriptional mechanism of GC action (Aguilera et al., 2004).

Other brain regions (PFC, Amygdala)

The role of the medial PFC (cingulate gyrus) in regulating the stress response is very complex, as the different subdivision (mainly prelimbic (PL) and infralimbic (InL) subdivision) exert divergent effects. Furthermore, high GR and very low MR expression indicate that medial PFC is only responsive to high GC concentrations (Ulrich-Lai and Herman, 2009). Fibers from the InL and to a minor division of the PL innervate predominantely GABAergic relay stations that project

to the PVN, including amongst others the BNST, lateral hypothalamus and nucleus of the solitary tract. Moreover InL and PL also project to the amygdala, raphe nucleus and, thereby, modulate the HPA axis activation mediated by these brain structures (Herman et al., 2003). Both, the PL and InL, are involved in the termination of the HPA axis activation following an acute stressor. In detail they are implicated in the response to acute psychogenic (restraint) (Diorio et al., 1993; McKlveen et al., 2013) but, at least the PL PFC, not to physical stress (Jones et al., 2011). However, they have distinct effects in terms of chronic stress (chronic variable stress) (McKlveen et al., 2013). While lesion of the InL PFC leads to HPA axis hyperresponsiveness in chronically stressed rats exposed to an acute heterotypic stressor, the PL is only involved in regulating the basal HPA axis activity under chronic stress conditions. This is indicated by increased CORT levels during chronic variable stress following knock-down of the GR in the InL PFC (McKlveen et al., 2013). Interestingly, knock-down of the GR in the InL PFC was shown to be responsible for the depressive-like behaviour the mice showed following exposure to the chronic variable stress (McKlveen et al., 2013).

Contrary to the hippocampus and the medial PFC, stimulation of the amgydala promotes (Redgate and Fahringer, 1973; Vouimba and Richter-Levin, 2013) while lesions reduce the CORT release in response to stressful stimuli (Knigge, 1961; Feldman and Conforti, 1981). Thereby, the CeA was found to be activated by homeostatic disruption and systemic stressors but not by psychogenic stressors, pointing towards a role in autonomic rather than in the HPA axis response to stress (Ulrich-Lai and Herman, 2009). In detail, Dayas and colleagues could show that physical stressors, like immune challenge and haemorrhage, preferentially activate CeA, while psychological stressors, like restraint or noise, preferentially activate medial amgydala (MeA) (Dayas et al., 2001). CeA and MeA project to similar relay stations like the medial PFC, but in contrast, the upstream neurons release GABA, indicating that activation of the PVN mediated via these amygdala structures is accomplished via disinhibition (Ulrich-Lai and Herman, 2009).

3 Chronic stress and GC signalling

HPA axis dysregulation

Maintenance of the homeostasis, which is challenged by different internal and external stressors, is fundamental for the well-being of an organism. This is mainly achieved by activation of both, the SNS and the HPA axis, which regulate a variety of physiological and behavioural responses. A proper functionality of the HPA axis, therefore, is essential for survival and for adaptation to stressors while dysregulation of the HPA axis, as a result of chronic activation, is implicated in the pathogenesis of stress-related disorders. For instance, affective disorders like major depressive disorder, psychiatric and anxiety disorders, burn out, chronic fatigue syndrome, panic disorders as well as somatic disorders like inflammatory bowel disease, cardiovascular disorders, and cancerogenesis (Gold et al., 1988; Chrousos and Gold, 1992; Levenstein et al., 1994; Heim et al., 2000; Mawdsley and Rampton, 2006; Chrousos, 2009; Dhabhar, 2009; Yehuda and Seckl, 2011). The exact mechanisms underlying the development of these stress-related disorders are not known, partly due to the lack of appropriate animal models. Nevertheless, studies of the last years focused more and more on a reduced and/or insufficient GC signalling following chronic psychosocial stress (Heim et al., 2000).

Decreased GC signalling can be either the result of a decreased hormone bioavailability, called hypocorticism, or of GC resistance in the target cells, or the combination of both (Raison and Miller, 2003). Hypocorticism was e.g. described in patients suffering from posttraumatic stress disorders (PTSD) while depressed patients displayed increased cortisol levels (Yehuda et al., 1996). However, despite the elevated cortisol levels, patients suffering from depression showed signs of reduced GC signalling as a consequence of GC insensitivity mediated by decreased GR expression and/or functionality (Holsboer, 1983; Pariante and Miller, 2001). Also the development of proinflammatory and autoimmune diseases and a decreased efficacy of wound healing are associated with a

decreased GC signalling and/or GC resistance mediated by prolonged increased GC levels (Dhabhar, 2009).

As GR and MR are the main mediators of the GC effects, either during hyper- or hyposecretion, alterations in the functionality or expression can underlie the pathology of stress-related diseases.

Alterations of the MR and GR expression following chronic stress

Dysregulations and dysfunctions of the corticosteroid receptors result in an altered negative feedback response and are implicated in the development of a variety of stress-related psychiatric disorders, like major depression, PTSD, and anxiety disorders (Chrousos and Gold, 1992; Pariante and Lightman, 2008). Hyperactivity of the HPA axis, mediated by a reduced negative feedback response, is one of the major findings in humans suffering from major depression and is mainly mediated by a diminished GR expression and/or functionality (Carroll et al., 1981; Holsboer, 1983; Pariante and Miller, 2001). In contrast, patients suffering from PTSD show an enhanced negative feedback sensitivity of the HPA axis and an increased number of lymphocyte GR (Yehuda et al., 1993; Yehuda et al., 1995; Yehuda, 1998).

Alterations of the gene and protein expression of GR and MR can have widespread effects on the stress response. In a receptor binding study using labeled Dex or aldosterone, Spencer and colleagues could show that adrenalectomy (ADX) in rats resulted in an increased GR binding in the brain (hippocampus, cortex, septum, hypothalamus) while MR binding was unaffected. Replacement with low doses of CORT blocked this effect while high doses of CORT resulted in a decreased MR and GR binding in the above mentioned brain regions in ADX rats, suggesting that expression of these receptors is subjected to the regulation by GC. Therefore, GC can regulate the extent of their actions not only by mediating the negative feedback inhibition but also by regulating their own receptor expression. Interestingly, receptors in the hippocampus were thereby shown to be extremely sensitive to these manipulations, while the pituitary was unaffected by ADX and CORT replacement (Spencer et al., 1991).

Regulation of MR and GR expression following chronic stressor exposure was overall found to be inconsistent. Some studies reported no alterations of the corticosteroid receptors (mRNA, binding, protein) in the hippocampus (Young et al., 1990; Djordjevic et al., 2009; Noguchi et al., 2010), in the pituitary (Sapolsky et al., 1984; Makino et al., 1995), and in the hypothalamus (Sapolsky et al., 1984) following chronic stress. In contrast, other studies reported a down-regulation of GR mRNA, binding and nuclear translocation in the hippocampus (Chao et al., 1993; Makino et al., 1995; Kitraki et al., 1999; Mizoguchi et al., 2001; Noguchi et al., 2010) and PVN (Makino et al., 1995; Sterlemann et al., 2008) as well as of MR mRNA in the hippocampus (Chao et al., 1993; Sterlemann et al., 2008) following chronic stress. Nevertheless, there are also studies reporting increased levels of GR protein in the PFC (Djordjevic et al., 2009) and increased nuclear translocation of the pituitary GR (Noguchi et al., 2010).

The different results clearly indicate that analysis of the receptor expression is not sufficient for analysis of the negative feedback and underlines the importance of the measurement of the receptor functionality. One possibility constitutes the testing of the inhibitory effectiveness of Dex *in vivo* on HPA axis activity/reactivity. As Dex is a synthetic steroid and as such only poorly passes the blood brain barrier - it is a ligand for the mdr 1a P-glycoprotein (see also Sec. 3.1) (Meijer et al., 1998) - this method specifically determines the feedback response at the level of the pituitary (Cole et al., 2000). Chronic stress paradigms resulting in HPA axis hyper-activity are often associated with a decreased negative feedback inhibition, shown by the failure of Dex to suppress basal and/or stress- or CRH-induced CORT levels, due to comprised GR functionality (Sapolsky et al., 1984; Mizoguchi et al., 2001). Another possibility would be to analyse the expression of GC-responsive genes in response to CORT or Dex stimulation *in vitro* to gain more insight into the transcriptional activity of the receptor.

4 Risk factors influencing the individual stress vulnerability

Stress is an acknowledged risk factor for the development of a variety of stress-related disorders like cardiovascular and inflammatory as well as psychiatric diseases like anxiety- or depression-related disorders in humans as well as in animals (McEwen, 2003; Dimsdale, 2008; Chrousos, 2009; Dhabhar, 2009). Interestingly, the effects of chronic stress also vary between individuals, with some being highly susceptible, while others being resilient to its negative consequences, a phenomenon found in humans as well as in rodents (Albeck et al., 1997; Feder et al., 2009; Stiller et al., 2011; Castro et al., 2012) (see Fig. 13A). In contrast to susceptible individuals, resilient ones are able to adapt to adversity, trauma, tragedy or other sources of stress (Yehuda et al., 2006; Franklin et al., 2012). These differences in stress vulnerability are supposed to depend on the interplay between genetic predispositions (DeRijk and de Kloet, 2005; Ising et al., 2008) and environmental factors like e.g. adverse events during early life, both affecting the adult stress response (Aisa et al., 2007; Eiland and McEwen, 2012) (see Fig. 13B). As the underlying mechanism are only poorly understood so far, the identification of behavioural and physiological characteristics involved in these differences in stress vulnerability is fundamental for the understanding of the development of stress-related disorders.

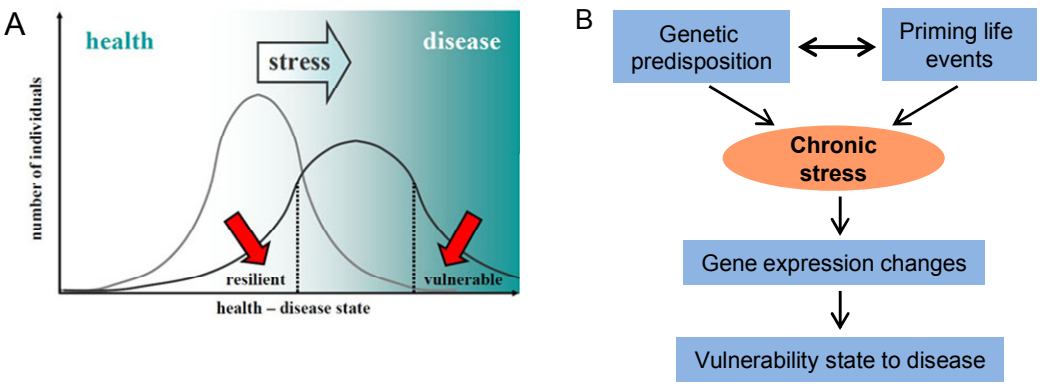

Figure 13: (A) Exposure of healthy individuals to chronic stress results in a shift of the Gaussian distribution from health to disease. Interestingly, there are some individuals which remain resilient (left part of the curve), while some are more vulnerable (right part

of the curve). [taken from http://www.mpipsykl.mpg.de/en/research/groups/ schmidt_m/misc/ schmidt _ m _00_01.jpg] **(B)** Response to stressors in adulthood is dependent on the genetic predisposition as well as on the individual`s history, influenced mainly by early life events. These two components can also interact with each other by mean that the genetic background has also an influence on the early life events. All these factors shift the individual stress reactivity, either in direction of resilience or susceptibility. [adapted from (de Kloet et al., 2005)]

Environmental factors

Stability of a child`s social environment during early life constitutes the most important factor concerning life-long health (McEwen, 2003). Early life stress, including child maltreatment, sexual abuse, loss of a parent (Agid et al., 2000) or living with mentally ill parent (Goodman and Gotlib, 1999), represents a major risk factor for the development of mental disorders like anxiety, major depression and PTSD (Famularo et al., 1992; Famularo et al., 1993; Pelcovitz et al., 1994; Heim and Nemeroff, 2001; Heim et al., 2009) but also inflammatory disorders during adulthood (Danese et al., 2007). The manifestation of these diseases is thereby often related to acute negative life events or persistent stressor exposure later in life (Heim and Nemeroff, 2001).

In rodents, pre- as well as postnatal stress, which occur in a period that is essential for the development of the pups, have profound consequences on stress vulnerability or resilience later in life. Maternal care was shown to play an important role, as high levels of maternal behaviour, like licking and grooming and nursing, have a beneficial effect on the offspring. In adulthood, the offspring showed a reduced ACTH and CORT responsiveness to an acute stressor exposure and enhanced GR-mediated feedback (Liu et al., 1997). Increased GR expression in the offspring of high licking and grooming mothers was mediated via an epigenetic mechanism, in detail by lowering the methylation of the GR promoter sequence in the hippocampus leading to an increased receptor expression (Zhang et al., 2013). In turn, the offspring of low locking and grooming mothers displayed reduced hippocampal GR expression (Meaney and Szyf, 2005). Also maternal separation (MS), i.e. separation of the pups from their

mother (3 h per day, postnatal day 1 – 14 or 3 – 10), results in negative behavioural and neuroendocrine alterations, like increased anxiety or enhanced ACTH release following acute stressor exposure (Wigger and Neumann, 1999; Veenema et al., 2006). It further was shown to reduce hippocampal neurogenesis (Lajud et al., 2012). Given that aversive experiences early in life can have profound consequences on stress-related parameters, it is likely that there is an interplay between early life stress and stressor exposure in adulthood. Interestingly, in a recently published study of our lab, it was demonstrated that MS mice showed an increased susceptibility to the consequences of chronic psychosocial stress (CSC, 19 days) in adulthood (see also Sec. 5) (Veenema et al., 2008).

Genetic predisposition

In addition to the environmental component, the genetic predisposition strongly determines the individual stress susceptibility and, therefore, also the development of stress-related disorders. E.g. genetic polymorphisms in the GR, MR or FKBP51 have been shown to increase the risk for the development of stress-related diseases (DeRijk and de Kloet, 2005; Ising et al., 2008). Nevertheless, due to ethical limitations in humans, studies related to genetic effects have to be conducted in appropriate animal models. Here, different approaches are possible for studying the gene and environment interaction. Modifying the genome of an animal, i.e. creating knock-out or transgenic animals makes it possible to study the influence of selected candidate genes. Mice are, therefore, preferred as the knock-out can technically be applied more easily than in rats or other species. One example would be the use of FKBP51 knock-out mice for studying the vulnerability to chronic stressor exposure (Hartmann et al., 2012b). A further possibility is to select a subpopulation within a large cohort of animals, according to behavioural or neuroendocrine parameters. E.g. following the recovery period of chronic social instability, animals were divided into two groups, one that recovered and one that was still affected from the chronic stress effects (Schmidt et al., 2010a). Also following chronic SD, two groups could be

separated – the susceptible and unsusceptible – characterized by the resistance to defeat-induced avoidance (Krishnan et al., 2007). These subpopulations would rather reflect a selected phenotype than only one single gene which helps to identify novel targets. Another advantage of this selection is the fact that effects that would normally not be detected in a large cohort can be detected by selection of extremes (Scharf and Schmidt, 2012). Another approach is to use a subpopulation generated by selective breeding. After breeding for several generations, the different phenotypes take more shape and allow the investigation of the underlying molecular mechanism.

The last approach was used to selectively and bidirectionally breed Wistar rats or CD1 mice, respectively, to select for high (HAB) and low (LAB) anxiety-related behaviour on the EPM whereby in this thesis mice were used exclusively (Landgraf and Wigger, 2002; Landgraf and Wigger, 2003; Krömer et al., 2005). Both mouse lines significantly differ from non-selected “normal” CD1 (mNAB) mice, with mHAB spending less than 10 % and mLAB more than 50 % time on the open arm of the EPM (Krömer et al., 2005). These behavioural differences reached a maximum after approximately nine generations (Landgraf et al., 2007). Not only on the EPM, but also in the dark-light avoidance test (Krömer et al., 2005) and in the open arm testing (Muigg et al., 2009) the extremes in trait anxiety were confirmed. Moreover, risk assessment during open arm exposure (Muigg et al., 2009) as well as during dark-light avoidance testing (Krömer et al., 2005) was higher in mLAB compared with mHAB mice. Already in the pups, the lower trait anxiety of the mLAB mice was evident, as mLAB pups showed less ultrasound vocalization compared with mHAB pups (Krömer et al., 2005). Moreover, tail-suspension and FS testing revealed differences between the two mouse lines with mLAB mice displaying a more active coping style compared with mHAB mice which showed signs of depressive-like behaviour. In contrast to the anxiety-related behaviour, depressive-like behaviour was comparable between mHAB and mNAB mice (Krömer et al., 2005; Muigg et al., 2009). Not only behavioural, but also distinct neuroendocrine and genetic differences are found in the two breeding lines. Basal plasma CORT levels were similar in both

groups, while in response to acute immobilization (15 min), mLAB mice showed an increased CORT response compared with mHAB mice (Gonik et al., 2012). Nevertheless, synthetic fox fecal odor resulted in a similar CORT response in mHAB and mLAB mice (Sotnikov et al., 2011), indicating that the acute stress responsiveness clearly seems to be stressor-dependent.

Interestingly, these differences in mHAB and mLAB mice can be ascribed to a single nucleotide polymorphism (SNP) in the AVP gene. AVP is involved in anxiety- and depression-like behaviour (Landgraf et al., 2007) hence a deficit in the synthesis and processing of this gene in mLAB mice results in the hypo-anxious phenotype (Kessler et al., 2007). Interestingly, in rats, a SNP in the promoter region of the AVP gene results in the high anxious phenotype, as this contributes to an increased AVP activity (Landgraf and Wigger, 2003; Murgatroyd et al., 2004). The SNP in the mLAB mice moreover induces signs of central diabetes insipidus (Kessler et al., 2007). Regarding the influences of the genetic predisposition, in this case the trait anxiety of the mice, on the stress vulnerability, there is an increasing knowledge about the pronounced differences in acute stress responsiveness, while there is not much known about the response to chronic stressor exposure in this breeding line. Therefore, it would be of major interest to investigate the influence of the trait anxiety of these mice on the susceptibility to chronic psychosocial stress. According to the fact, that chronic stress is known to increase the state anxiety (Hata et al., 2001; Krishnan et al., 2007; Reber et al., 2007; Schmidt et al., 2007; Slattery et al., 2012), one can speculate that the low anxiety phenotype might be stress protective, making the mLAB mice less vulnerable to the behavioural, physiological, neuroendocrine and immunological consequences of CSC compared with mHAB and mNAB mice. In contrast, mHAB mice might be more stress vulnerable compared with mNAB mice.

5 Effects of chronic subordinate colony housing (CSC) on physiological, neuroendocrine, immunological and behavioural parameters

CSC paradigm

The CSC paradigm, established by Reber and colleagues (2007), was used as an appropriate and pre-clinically validated model for chronic psychosocial stress in male C57BL/6 mice. Four experimental mice are housed together with a larger and more aggressive male mouse (resident), which is supposed to occupy the dominant position, for 19 consecutive days. Once per week, the resident is exchanged to avoid any habituation (see Fig. 14).

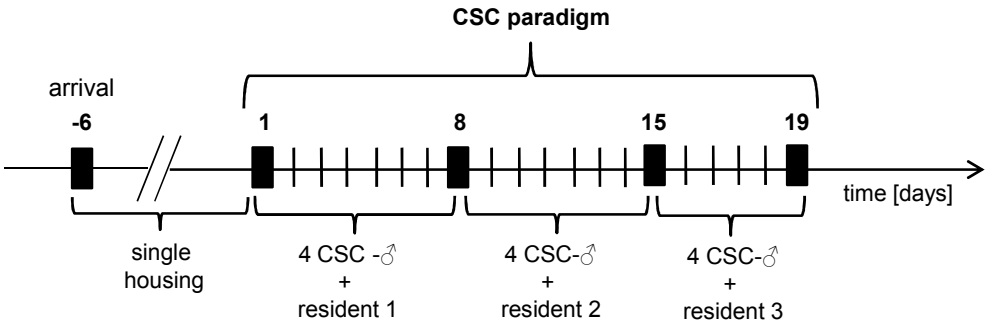

Figure 14: Schematic illustration of the time course of the chronic subordinate colony housing (CSC) paradigm. After arrival, all experimental mice are singly housed for one week before they are randomly assigned to the single-housed control (SHC) or the CSC group. In order to induce a chronic stressful situation, four male mice are housed together with a larger, dominant male mouse (resident) for 19 consecutive days. The resident (resident 1) is exchanged on days 8 (resident 2) and 15 (resident 3) by a new resident in order to avoid any habituation

When the CSC paradigm starts, it has to be ensured that the four experimental mice are in a subordinate while the resident adopts the dominant position. Therefore, behavioural observations during the first hour after setting up the colony are done, whereby the resident normally shows offensive behaviours, like chasing, mounting and attacking towards the four experimental mice. In contrast,

the four CSC mice display a defensive behaviour characterized by flight and defensive upright (Reber and Neumann, 2008a). The experimental mice are then exposed to this chronic subordination for 19 consecutive days, while the control mice are single-housed (SHC) during this time. According to other studies (Bartolomucci et al., 2003; Gasparotto et al., 2005) single housing was convincingly demonstrated to be the adequate control group for the CSC paradigm, as group housing itself was shown to be stressful for the mice (Singewald et al., 2009). Compared to the SHC mice, CSC mice displayed typical signs of chronic stress, like physiological, neuroendocrine, immunological and behavioural alterations (Reber et al., 2007; Reber and Neumann, 2008a; Singewald et al., 2009; Slattery et al., 2012; Uschold-Schmidt et al., 2012) (see Fig. 15 A and B).

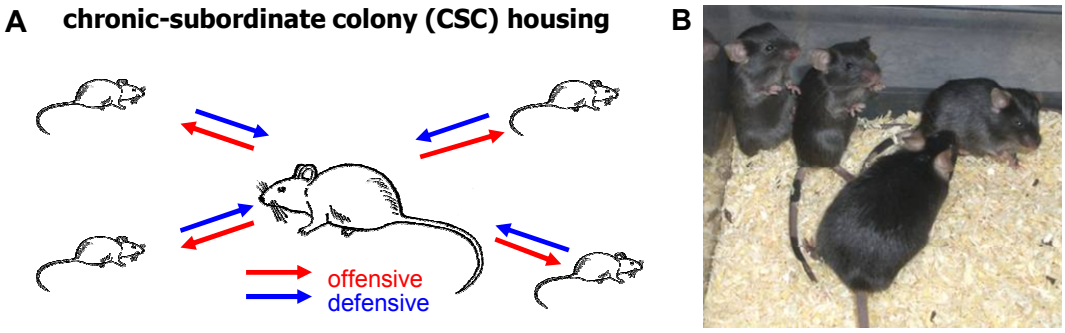

Figure 15: Schematic illustration (**A**) and a representative image (**B**) of the CSC paradigm. Three of the four experimental mice sit in one corner and display defensive behaviour (defensive upright posture: the two subordinate mice in the left corner) while one mouse shows flight (one subordinate on the right). The resident in the middle shows offensive behaviour and attacks the mouse that tries to flee. [taken from http://epub.uni-regensburg.de/10558/1/Version_Final_Offiziell.pdf]

CSC-induced physiological and neuroendocrine alterations

Analysis of physiological parameters revealed a reduced body weight gain and relative thymus weight following CSC exposure. Moreover, absolute adrenal weight and also pituitary weight, which was only measured 8 days following termination of the CSC exposure, was increased (see Fig. 16).

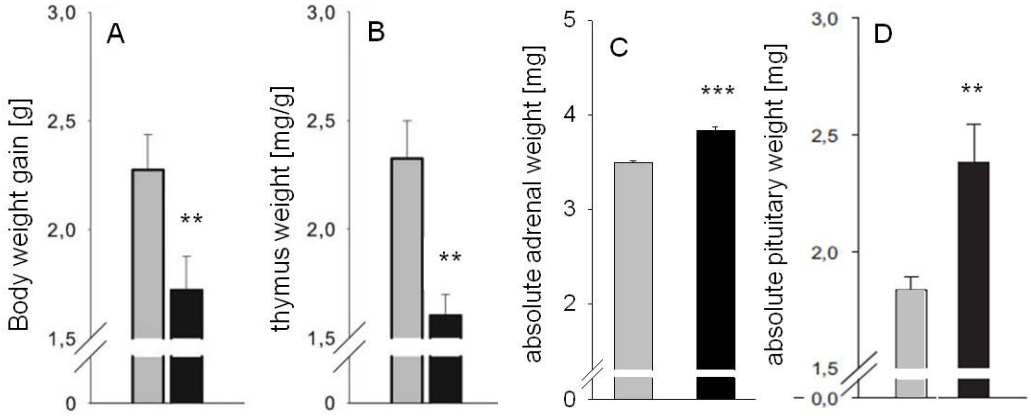

Figure 16: 19 days of CSC result in a decreased body weight gain **(A)**, decreased relative thymus weight **(B)**, and increased absolute adrenal weight **(C)** compared with SHC mice. In addition, 8 days following termination of the CSC paradigm, absolute pituitary weight was increased **(D)** compared with SHC mice. ■ SHC; ■ CSC. Data represent mean + SEM; **, $P < 0.01$; ***, $P < 0.001$ *vs.* respective SHC mice. [adapted from (Reber et al., 2007; Slattery et al., 2012; Uschold-Schmidt et al., 2012)]

Measurement of neuroendocrine parameters also revealed alterations in HPA axis functionality. Despite the increased adrenal weight, plasma morning CORT levels were unchanged, while plasma evening CORT levels were even decreased in CSC compared with SHC mice, indicating that CSC mice are not able to mount the circadian rise in plasma CORT (Reber et al., 2007). Stimulation of adrenal explants *in vitro* further revealed a decreased ACTH responsiveness in CSC compared with SHC mice further supporting the concept of an adrenal dysfunction (Reber et al., 2007; Uschold-Schmidt et al., 2012). Nevertheless, exposure to an acute heterotypic stressor (5 min EPM) resulted in an increased CORT release in CSC mice that even exceeded those of SHC mice, despite similar plasma ACTH levels. This indicates an increased capability to produce and secrete CORT upon an acute challenge. Overall this indicates that the

unchanged basal plasma CORT concentrations in the morning represent a mechanism of adaption protecting the body from increased GC levels during chronic stressor exposure while a sensitization process enables the organism to respond to a new challenge with an adequate GC response (Uschold-Schmidt et al., 2012) (see Fig. 17).

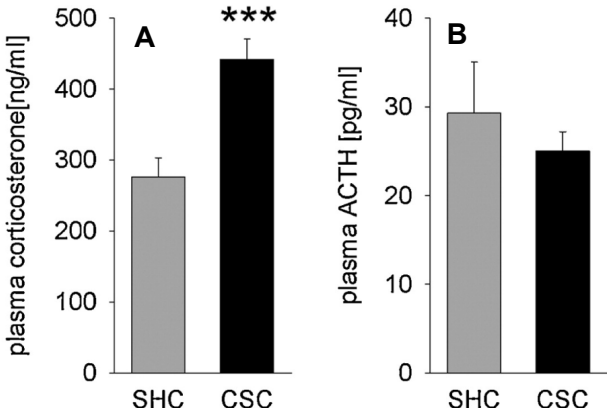

Figure 17: On day 20 of CSC, CSC and SHC mice were exposed to the EPF (5 min). Plasma CORT levels determined 5 min after termination of EPF exposure were increased in CSC compared with SHC mice **(A)** while plasma ACTH levels were unchanged **(B)**. ■ SHC; ■ CSC. Data represent mean + SEM. ***, $P < 0.001$ *vs.* respective SHC mice. [adapted from (Uschold-Schmidt et al., 2012)]

Furthermore, CSC mice display signs of GC resistance in peripheral cells, shown by a reduced sensitivity to different doses of GC in isolated splenocytes stimulated with lipopolysaccharides as well as in Th2 cells from peripheral lymph nodes (Reber et al., 2007; Schmidt et al., 2010b).

Analysis of CRH and AVP revealed an unchanged CRH mRNA and a decreased AVP mRNA expression in the PVN of CSC compared with SHC mice (Reber and Neumann, 2008a). Moreover, c-Fos expression in the PVN was similar under basal but higher under acute stress conditions in SHC compared with CSC mice (Singewald et al., 2009).

CSC-induced immunological and behavioural alterations

Concerning the immune system, anti-CD3 stimulated mesenteric lymph node cells (mesLNC) secreted increased amounts of interferon γ (IFN-y) following CSC exposure. In addition, the histological damage score, characterized by increased epithelial damage and leukocyte infiltration, was increased, pointing towards the development of a mild colonic inflammation (Reber et al., 2007). Not only the risk for development of a spontaneous colitis was enhanced, CSC mice also developed a more severe chemically-induced colitis following treatment with dextran sulfate sodium (DSS) (Reber et al., 2007; Veenema et al., 2008).

Moreover, CSC mice displayed a profound increase in anxiety-related behaviour indicated by spending less time in the bright zone of the light dark box (Reber and Neumann, 2008a), on the open arm of the EPM (Reber et al., 2007; Slattery et al., 2012), in the outer zone of the EPF (Uschold-Schmidt et al., 2012) and by spending less time exploring a novel object compared with SHC mice (Veenema et al., 2008). The exact mechanisms underlying this anxiogenic effect of CSC are not unraveled yet, but there might be an influence of altered neuronal activity patterns in brain regions involved in emotionality, like PVN, parts of the lateral septum, the dorsal CA3 region in the hippocampus and different subregions of the nucleus accumbens (Singewald et al., 2009). Interestingly, CSC mice showed no signs of depressive-like behaviour indicated by a comparable time spent immobile in the tail suspension and FS test between SHC and CSC mice. In addition, anhedonic-like behaviour, assessed in the saccharine preference test, was not affected by CSC (Slattery et al., 2012).

Influence of adverse early life events on the CSC-induced alterations

Interestingly, early life stress (MS, 3 h/day, day 1 - 14) aggravated the consequences of CSC exposure in adulthood. CSC mice previously exposed to MS showed a decreased GC secretion even during the nadir of the HPA axis acitivity, despite adrenal hypertrophy. While plasma ACTH levels were unchanged, CRH mRNA expression in the PVN was increased in MS CSC compared with SHC mice, indicating a pituitary insufficiency in addition the the

insufficiency of the adrenal glands. Moreover, MS increased the vulnerability to DSS-induced colitis, shown by a higher histological damage score and an increased IFN-γ of mesLNC in CSC compared with mice (Veenema et al., 2008). Overall, the findings indicate a profound influence of MS early in life on the consequences of CSC in adulthood, especially aggravating the effects on HPA axis functionality and on the immune system.

Taken together, CSC promotes a number of physiological, neuroendocrine, immunological and behavioural alterations. Basal evening hypocorticism and GC-resistance in the periphery indicate an impaired GC signalling. The increased anxiety as well as the increased inflammation supports the use of the CSC paradigm as a model for studying the mechanisms underlying the development of stress-related somatic as well as affective disorders. And, interestingly, the CSC paradigm represents an adequate model to study the influences of both, the environment (early life stress) and the genetic predisposition (by the use of selectively bred mice) on the development of stress-related disorders.

6 Aim and outline of the present thesis

Given the outlined informations in the sections above, the aims of my thesis were:

1) To assess whether changes at the pituitary or PVN level are contributing to the changes in HPA axis functionality following CSC
2) To investigate if CSC leads to alterations of the negative feedback inhibition at the level of the pituitary, PVN, hippocampus and PFC
3) To determine if the genetic predisposition, in detail the trait anxiety of an animal, influences the vulnerability to the behavioural, physiological, neuroendocrine and immunological consequences of CSC

The first aim of my thesis was to test the hypothesis that pituitary mechanisms are involved in the observed HPA axis adaptation/sensitization processes seen following CSC exposure, given that most of the studies so far focused only on the adrenals. Moreover, the increased pituitary weight in CSC compared with SHC mice 8 d after termination of CSC exposure already indicates that alterations at the level of the pituitary are not unlikely to occur. In addition, it can be hypothesized that the decreased AVP mRNA expression in the PVN following CSC might be mediated by an increased mRNA turn over due to an increased protein expression.

Therefore, basal as well as acute stress-induced (6-min FS) plasma ACTH concentrations were analysed. Furthermore, pituitary weight, the number of pituitary and ACTH positive cells as well as POMC expression was measured in SHC and CSC mice. Moreover, the receptors of AVP and CRH, the main ACTH secretagogues, were investigated by analysis of AVPR-1b and CRH-R1 mRNA and protein expression. To address CSC-induced changes on the AVP system in the PVN, an IHC staining was established, in order to assess the number of AVP positive magnocellular and parvocellular PVN neurons. In addition, the number of

OXT positive PVN neurons was analysed, given that ACTH secretion can, amongst CRH, AVP and other factors, also be stimulated by this neuropeptide.

The aim of the second part of this thesis was the analysis of the negative feedback inhibition at the level of the pituitary and at various brain sites, i.e. the hippocampus, PFC and PVN. Due to the GC resistance found in some peripheral cells following CSC exposure, one can speculate that also the negative feedback inhibition might be impaired. In the pituitary, the expression mRNA and/or protein expression of MR, GR and FKBP51 was assessed. To study the functionality of the GR, a dexamethasone suppression test (DST) was performed, whereby the *in vivo* response to Dex injection and subsequent acute heterotypic stressor (6-min FS) exposure following 19 days of CSC was measured. Moreover, the translocation of the GR into the nucleus upon acute stressor exposure (6-min FS) was determined.
At the different brain sites mentioned above, the mRNA and/or protein expression of MR, GR and FKBP51 (only in the hippocampus and PFC) was analysed. Moreover, at the level of the hippocampus, the functionality of the GR was assessed by stimulating isolated hippocampal cells *in vitro* with different CORT concentrations followed by measurement of the expression pattern of a GC-responsive gene (Per1) as well as of the cell viability in response to 24 h CORT stimulation.

The third part of the thesis mainly dealed with the influence of the genetic predisposition, in detail the trait anxiety of an animal, on the vulnerability to the consequences of CSC with respect to somatic and affective pathologies. Therefore, mice selectively bred for high (mHAB) and low (mLAB) anxiety-related behaviour and non-selected normal CD1 (mNAB) mice were exposed to the CSC paradigm and behavioural, physiological, neuroendocrine and immunological parameters were analysed. The hypothesis was that the low innate anxiety in mLAB mice might constitute a kind of stress-protective factor, as it is known from many studies that chronic stress has an anxiogenic effect. In turn, the high innate

anxiety might probably increase the vulnerable to the consequences of CSC compared mNAB mice. Therefore, mice of all three lines were either exposed to 19 days of CSC or single-housed for control and were subsequently tested on the EPM (on day 19 of CSC) to determine the breeding line-specific differences in anxiety-related behaviour. In addition, on day 20 of CSC body weight gain, adrenal and spleen weight, plasma CORT and ACTH concentrations and *in vitro* adrenal CORT secretion in response to ACTH were determined. Moreover, IFN-γ secretion from isolated mesLNC in response to anti-CD3 stimulation and the histological damage score of the colon were assessed. Finally, the influence of the genotype as well as of CSC on the GR, MR and FKBP51 protein expression was determined in the pituitary.

CHAPTER 2

MATERIAL AND METHODS

1 Material

1.1 Antibodies for protein, immunohistochemical and immunological analysis

1.1.1 Primary Antibodies

DENOTATION	COMPANY	SPECIES RAISED IN
GR (M-20) sc-1004	Santa Cruz Biotechnology, Inc., Heidelberg, Germany	rabbit polyclonal
MCR (H-300) sc-11412	Santa Cruz Biotechnology, Inc., Heidelberg, Germany	rabbit polyclonal
AVP Receptor V3 (D-20) sc-18105	Santa Cruz Biotechnology, Inc., Heidelberg, Germany	goat polyclonal
CRF-R1 (V-14) sc-12381	Santa Cruz Biotechnology, Inc., Heidelberg, Germany	goat polyclonal
POMC (FL-267) sc-20148	Santa Cruz Biotechnology, Inc., Heidelberg, Germany	rabbit polyclonal
FKBP51 (F-14) sc-11518	Santa Cruz Biotechnology, Inc., Heidelberg, Germany	goat polyclonal
ß-Tubulin #2146	Cell Signaling Technology, GmbH, Frankfurt am Main, Germany	rabbit polyclonal
TATA binding Protein ab63766	Abcam, Cambridge, UK	rabbit polyclonal
Anti-ACTH ab74976	Abcam, Cambridge, UK	rabbit polyclonal
AVP P41	Dr. Gainer, NIH, USA	mouse polyclonal
OXT P38	Dr. Gainer, NIH, USA	mouse polyclonal
aCD3 – raised against CD3 epsilon chain	provided by Prof. Dr. Werner Falk, Department of Internal Medicine, University Hospital Regensburg	hamster monoclonal

1.1.2 Secondary antibodies

DENOTATION	COMPANY	SPECIES RAISED IN
Anti goat (HRP-linked) sc-2020	Santa Cruz Biotechnology, Inc., Heidelberg, Germany	Donkey
Anti rabbit (HRP-linked) #7074	Cell Signaling Technology, GmbH, Frankfurt am Main, Germany	Goat
Anti mouse (biotinylated) BA-9200	Vector Laboratories, Burlingname, USA	Goat

1.2 Oligonucleotides

The following oligonucleotides were used as primers for qPCR amplifications. All primers were designed with the help of Primer Express 2.0 (Applied Biosystems, Forster City, CA, USA and purchased from Metabion (Martinsried, Germany).

GENE		5` - 3`SEQUENCE
GR	Fwd	CGGGACCACCTCCCAAA
	Rev	CCCCATAATGGCATCCCGAA
	Probe	TCTGCCTGGTGTGCTCC
MR	Fwd	GGACCAAATTACCCTCATCCA
	Rev	GTAT GTTTGTACGATCTCCAACTCAAG
	Probe	ATTCTTGGATGTGTCTATCATC
CRH-R1	Fwd	GTGACAGCCGCCTACAACTACTT
	Rev	GTAGCAGCCCTCACCGAACA
	Probe	CACGTAACCAACTTCTTCT
AVPR-1b	Fwd	GCTCACAGCTTGCTATGGCCTCAT
	Rev	CCAGC CTGT GTCTTGACTTTCAG
	Probe	TGCCACGAGATCTACAAGAAC
POMC	Fwd	CGGTGAAGGTGTACCCCAACGT
	Rev	GGACCTGCTCCA AGCCTAATGGCC
	Probe	AGGCCTTTCCCCTAGAGTTCA
Per1	Fwd	GTGCGCACGTAAGGGAACTG
	Rev	CCCATGCCATGTCCATACC
	Probe	TCTCCACGCTGGTGTT

1.3 RNA isolation and quantitative real-time PCR (qPCR)

PRODUCT	COMPANY
RNA later	Applied Biosystems, Forster City, CA, USA
RNeasy Mini Kit	Qiagen, Hilden, Germany
QIAshredder	Qiagen, Hilden, Germany
Reverse Transcription System	Promega, Mannheim, Germany
RNase-Free DNase Set	Qiagen, Hilden, Germany
Quant-iT™ RiboGreen® RNA Assay Kit	Molecular Probes, Eugene, Oregon, USA
Affinity Scribt™ Multiple Temperature cDNA Synthesis Kit	Stratagene, Agilent Technologies Company, USA
Rodent GAPDH Control Reagents	Applied Biosystems, USA
Brilliant II QPCR Master Mix with High Rox	Stratagene, Agilent Technologies Company, USA
Brilliant III Ultra-Fast QPCR Master Mix	Stratagene, Agilent Technologies Company, USA

1.4 Protein Extraction and Western Blotting

PRODUCT	COMPANY
Complete mini protease inhibitor	Roche Applied Science, Mannheim, Germany
Bicinchoninic Acid Protein Assay Kit	Thermo Scientific, Rockford, USA
Enhanced Chemiluminescence Western Blotting Detection Reagent	GE Healthcare, Freiburg, Germany
PageRuler™ Prestained Protein Ladder Plus	Fermentas, Thermo Scientific, Rockford, USA
Re-Blot Plus Mild/Strong Solution	Millipore, Temecula, USA
Nitrocellulose membrane, 0.45 µm	Bio-Rad, München, Germany

1.5 Immunohistochemical analysis

PRODUCT	COMPANY
Streptavidin-biotin-peroxidase-complex Vectastain ABC Kit Elite (PK-6100)	Vector Laboratories, Burlingname, USA
DAB Peroxidase Substrate Kit (SK-4100)	Vector Laboratories, Burlingname, USA
Vector Nova Red Peroxidase Substrate Kit (SK-4800)	Vector Laboratories, Burlingname, USA
Vectashield Mounting Medium (H-1400)	Vector Laboratories, Burlingname, USA

1.6 Neuroendocrine analysis

PRODUCT	COMPANY
ACTH Elisa Kit	IBL International, Hamburg, Germany
CORT Elisa Kit	IBL International, Hamburg, Germany
EDTA tubes	Sarstedt, Nürnbrecht, Germany

1.7 Immunological analysis

PRODUCT	COMPANY
Cell strainer 70 µm	Beckton Dickinson Biosciences, Heidelberg, Germany
Fetal bovine serum (FBS)	PAA laboratories,
IFN-y ELISA Kit	Biolegend, San Diego, USA
Penicillin	PAA Laboratories GmbH, Cölbe, Germany
Streptomycin	PAA Laboratories GmbH, Cölbe, Germany
RPMI 1640	Sigma-Aldrich, Deisenhofen, Germany

PRODUCT	COMPANY
Embedding cassettes	Simport Plastics Ltd., Bernard-Pilon, Beloeil QC, Canada
Eosin Y solution	Sigma-Aldrich, Deisenhofen, Germany
Harris haematoxylin	Sigma-Aldrich, Deisenhofen, Germany
Microscope slides	Gerhard Menzel GmbH, Braunschweig, Germany
Mounting solution, Roti Histokitt II	Carl Roth, Karlsruhe, Germany

1.8 *In vitro* studies

PRODUCT	COMPANY
Hippocampal cell isolation and stimulation	
DMEM, high glucose, pyruvate, no glutamine #21969	Invitrogen GmbH, Karlsruhe, Germany
10 x Trypsin solution	Sigma-Aldrich, Deisenhofen, Germany
Deoxyribonuclease (DNaseI)	Sigma-Aldrich, Deisenhofen, Germany
Fetal bovine serum (FBS)	PAA Laboratories GmbH, Cölbe, Germany
Hank`s balanced salt solution (HBSS) without Ca, Mg, phenol red	Invitrogen GmbH, Karlsruhe, Germany
Corticosterone minimum 92%	Sigma-Aldrich, Deisenhofen, Germany
CellTiter96 Aqueous One Solution Cell Proliferation Assay	Promega GmbH, Mannheim, Germany
Adrenal *in vitro* stimulation	
DMEM/F-12, L-Glutamine, no Hepes buffer or phenol red	Invitrogen GmbH, Karlsruhe, Germany
Adrenocorticotropic hormone Fragment 1-2	Sigma-Aldrich, Deisenhofen, Germany
Bovine serum albumin (BSA)	Sigma-Aldrich, Deisenhofen, Germany

1.9 Chemicals, Enzymes, Reagents and Equipment

Chemicals, enzymes and reagents used in this thesis were obtained from BioRad (München, Germany), Beckman Coulter (Krefeld, Germany), Biomol (Hamburg, Germany), BMG Labtech (Offenbach, Germany), Braun (Melsungen, Germany), Carl Roth GmbH (Karlsruhe, Germany), Merck (Darmstadt, Germany), Sigma-Aldrich (Deisenhofen, Germany). Cell culture materials, general plastic material and other equipment were purchased from Beckman Coulter (Krefeld, Germany), Eppendorf AG (Hamburg, Germany), BD Biosciences (Heidelberg, Germany), GE Healthcare (München, Germany), Gibco® Life Technologies GmbH (Darmstadt, Germany), Leica Microsystems (Wetzlar, Germany), Sarstedt (Nürnbrecht, Germany), Thermo Scientific (Rockford, USA) and VWR GmbH (Darmstadt, Germany).

1.10 Software

SOFTWARE	PURPOSE	SOURCE
Endnote 9.0	Reference Management	Thomson
Leica QWin V3	analysis of IHC staining	Leica Microsystems
SPSS Statistics 21.0	Statistical analysis	IBM
Microsoft Office XP 2007	Text processing	Microsoft
Image Lab 4.1	Western Blot analysis	BioRad Laboratories
SigmaPlot 11	Graph processing	Systat Software
SPSS Statistics 21.0	Statistical analysis	IBM

2 Methods

2.1 Animal models

2.1.1 Mice

Male C57BL/6 mice (Charles River, Sulzfeld, Germany) weighing 19 - 22 g (experimental mice) were individually housed in standard polycarbonate mouse cages (16 x 22 x 14 cm) for one week before the CSC paradigm started.

For studying the influence of the trait anxiety on the consequences of chronic psychosocial stress, adult male CD1 mice selectively inbred for high (mHAB) and low (mLAB) anxiety-related behaviour and non-selected normal CD1 (mNAB) mice (22 - 37 g body weight; bred at the Max Planck Institute of Psychiatry in Munich, Germany) were used as experimental mice. Importantly, differences in trait anxiety of these mice were not verified by testing those mice on the EPM prior to CSC exposure, as usually performed (Sotnikov et al., 2011). This is based on evidence showing that repeated (all mice are tested on the EPM on day 19 of CSC) EPM testing should be avoided as prior experience with this test can influence performance during retesting (File et al., 1993; Holmes and Rodgers, 1998; Roy et al., 2009).

The male offspring (weighing 30 - 35 g) of mHAB female mice (kindly provided by Prof. Dr. R. Landgraf, Max Planck Institute of Psychiatry in Munich) and C57BL/6 male mice (Charles River, Sulzfeld, Germany) were used as dominant animals.

All mice were kept under standard laboratory conditions (12 h light/dark cycle, lights on at 0600 h, 22°C, 60 % humidity) and had free access to tap water and standard mouse diet. All experimental protocols were approved by the Committee on Animal Health and Care of the local government, and conformed to international guidelines on the ethical use of animals. All efforts were made to minimize the number of animals used and their suffering.

2.1.2 Chronic subordinate colony housing (CSC)

The CSC paradigm was conducted as described previously (Reber et al., 2007; Reber and Neumann, 2008a; Reber et al., 2008b; Uschold-Schmidt et al., 2012; Füchsl et al., 2013). Briefly, one week after arrival, experimental mice were weighed and in a weight-matched manner assigned to the SHC or CSC group. Four experimental CSC mice, either four C57BL/6 mice or four mice of the same genotype, were housed together with a dominant male mouse for 19 consecutive days in order to induce a chronic stressful situation. During the first hour after putting the mice together, the behaviour was observed to guarantee that a hierarchical structure, with the resident obtaining the dominant and experimental mice the subordinate position, was established (Reber and Neumann, 2008a). Before the CSC procedure, the future dominant males were tested for their aggressive behaviour. Males that started to injure their opponents by harmful bites were not used. To avoid habituation, each dominant male was replaced by a novel dominant male at days 8 and 15. SHC mice remained undisturbed in their home cage except for change of bedding once a week. In a previous study it was convincingly demonstrated in our lab that single housing is the adequate control group for the CSC paradigm, as group housing itself was shown to be stressful for the mice (Singewald et al., 2009).

2.1.3 Acute heterotypic stressor exposure - Forced swim (FS)

The FS exposure on day 20 was used as acute heterotypic stressor and was conducted as described previously (Nyuyki et al., 2012; Slattery et al., 2012). Briefly, the FS tank consisted of an open top cylinder (25 cm height, 13 cm diameter) filled with tap water (21 $\pm$ 1°C) to a depth of about 13 cm. Mice were immersed into the water tank for 6 min and decapitated 10 min folling termination of FS exposure (under CO_2 anaesthesia). Trunk blood was collected and stored (see Sec. 2.3) and pituitaries were removed (see Sec 2.5.1). The water was changed after every animal.

2.2 Elevated plus-maze (EPM)

To assess the genotype-specific effects of CSC on anxiety-related behaviour, SHC and CSC mHAB, mNAB, and mLAB mice were transported to the EPM room on day 18. The next day (day 19 of CSC exposure), all mice were tested on the EPM between 0800 and 1100 h for 5 min as previously described (Reber et al., 2007). The EPM consisted of two open (6 x 30 cm) and two closed (6 x 30 x 17 cm) arms radiating from a central platform (6 x 6 cm) forming a plus shaped platform elevated 130 cm above the floor. To avoid falling, the edges of the open arms were 0.3 cm in height. Each mouse was placed on the central platform facing a closed arm. The maze was cleaned thoroughly before each test. A computer setup calculated the percentage of time spent on and the percentage of entries into the open arms of the EPM and the number of full entries. The number of closed arm entries was taken as measurement of locomotion. Afterwards, CSC mice were put back in their respective CSC colony and SHC mice were kept singly.

2.3 Trunk blood sampling and analysis of plasma ACTH and CORT

On day 20 SHC and CSC mice were rapidly killed by decapitation within 3 min after entering the animal room between 0800 and 1000 h. Therefore, all four CSC mice were simultaneously taken out and were rapidly killed under CO_2 anaesthesia within 3 min. Trunk blood was collected in EDTA-coated tubes (Sarstedt, Nürnbrecht, Germany) on ice and centrifuged at 4°C (5000 rpm, 10 min). Plasma samples were stored at -20°C until ACTH and/or CORT concentrations were assessed.

Plasma samples were analysed using a commercially available ELISA kit for ACTH (analytical sensitivity 0.22 pg/ml, intra-assay and inter-assay coefficients of variation $\leq$ 7.1%) and/or for CORT (analytical sensitivity < 0.564 ng/ml, intra-assay and inter-assay coefficients of variation $\leq$ 6.35%, IBL International, Hamburg, Germany). When applicable, plasma CORT:ACTH ratio (ACTH and CORT concentrations were applied in ng/ml) was calculated for each animal.

2.4 Dexamethasone suppression test (DST)

On day 20 SHC and CSC mice received an ip injection of either water-soluble Dex phosphate (dexamethasone 21-phosphate disodium salt, Sigma-Aldrich Deisenhofen, Germany) solved in sterile 0.9 % saline (3 µg/100 g body weight in 100 µl) or 0.9 % saline between 0700 and 0800 h and afterwards were returned to their home cage (SHC) or CSC cage, respectively. The dose of Dex was chosen according to another study conducted in mice (Ridder et al., 2005). Four hours following the injection, SHC and CSC were subjected to an acute heterotypic stressor (FS, 6 min, conducted as described in 2.1.3). 10 min following termination of FS exposure, mice were decapitated and trunk blood was collected and stored for the analysis of ACTH concentrations.

2.5 Preparation of organ tissue

2.5.1 Removal of different brain regions

The brain was taken out of the cranium, whereby the *dura mater* was removed to avoid any damage of the brain.

The PFC was obtained, by cutting off about 2 - 3 mm of the frontal part of the brain and was frozen in liquid nitrogen for protein analysis.

For withdrawal of the hippocampus, the two hemispheres were separated by a median cut and the hippocampus was exposed by applying tweezers and a razor blade. After that, the left and right hippocampus were dissected, pooled, weighed and either frozen in liquid nitrogen (protein analysis), stored in RNA later (mRNA analysis) or stored in HBSS on ice (hippocampal cell isolation).

For removal of the PVN, brains were taken out, snap-frozen in isopentane and stored at -80°C (protein analysis) or immersed in fixative (IHC analysis, see Sec. 2.8.2). For protein analysis, 200 µm cryo-sections were cut using a cryostat (at -20°C) and the PVN was punched out according to the mouse brain atlas (Paxinos and Franklin, 2001) and stored at -80°C until further preparation.

2.5.2 Determination of pituitary weight and pituitary cell number

After decapitation on day 20 the pituitary of each mouse was removed, weighed and either frozen in liquid nitrogen (protein analysis), stored in RNA later (mRNA analysis), stored in Hank`s balanced salt solution (HBSS) on ice (pituitary cell number) or embedded in protective freezing medium (IHC analysis)

For determination of the pituitary cell number, the whole pituitary was enzymatically digested in 2 ml HBSS (Life Technologies, Inc., Grand Island, NY, USA) containing Trypsin (Sigma-Aldrich, Deisenhofen, Germany) for 20 min at 37°C (95 % O_2, 5 % CO_2). Digestion was stopped by adding HBSS containing 10 % FCS. After two washing steps with HBSS, the pituitary was triturated with a pasteur pipette in 1 ml Dulbecco`s Modified Eagle Medium (DMEM/F-12, Life Technologies, Inc., Grand Island, NY, USA). After centrifugation (20°C, 10 min, 1500 rpm), cells were resuspended in DMEM/F-12 and the number of isolated cells per pituitary was assessed using a Cell Viability Analyzer (Vi-Cell™ XR, Beckman Coulter, Krefeld, Germany).

2.5.3 Determination of body weight gain, adrenal and spleen weight

On day 20 mice were weighed immediately before decapitation to assess the effects of CSC on body weight gain. Afterwards, the spleen and left and right adrenal glands of each animal were removed, pruned from fat and weighed separately. In addition, the sum of left and right absolute adrenal weights was calculated for each mouse. Values represent absolute weight (in mg). Until all mice were killed and adrenals removed, the latter were stored in ice-cold DMEM/F-12 containing 0.1 % BSA.

2.6 Molecular Techniques - quantitative real-time PCR (qPCR) using Taqman technology

mRNA in the tissue was quantified by use of quantitative real-time PCR (qRT-PCR), using TaqMan™ PCR (Applied Biosystems, Forster City, CA, USA). In addition to the common primer pair, a TaqMan probe containing a fluorophore at the 5`-end of the oligonucleotide probe and a quencher at the 3` -end is used. In this case, 6-carboxyfluorescein (FAM) was used as fluorophor and 6-carboxytetra-methylrhodamine (TAMRA) was used as quencher. As long as the probe is intact, the quencher represses the fluorescence signal of the fluorophor. When primer and probe bind to specific regions on the cDNA, the Taq-polymerase extends the primer and synthesizes the strand, whereby the 5` to 3`exonuclease activity of the polymerase degrades the probe, which leads to the release of the fluorophore. The fluorescence can subsequently be detected and is proportional to the released amount of fluorophor and in turn to the amount of PCR product.

RNA isolation

Tissue used for mRNA analysis was stored in RNA later (Applied Biosystems, Forster City, CA, USA) until extraction of the RNA. Therefore, the RNeasy Mini Kit (Qiagen, Hilden, Germany) was used and the amount of RNA was measured by a nanophotometer (Implen, München, Germany).

Reverse transcription into cDNA and semiquantitative RT-PCR

1000 ng/ml RNA was then reverse transcribed into first-strand cDNA by the use of the Reverse Transcription System (Promega, Mannheim, Germany). Expression levels of desired genes were quantified by TaqMan-qPCR (ABI PRISM 7900HT Sequence Detection System; Applied Biosystems, Foster City, CA, USA) as single-tube reactions (20 µl) in 384-well plates. The sequences of the primers and probes are depicted in Sec. 1.2. Glyceraldehyde-3-phosphatedehydrogenase (GAPDH)-Mix (Applied Biosystems, Foster City, CA, USA) served as housekeeping or reference gene.

TAQMAN-QPCR:		CYCLING:		
1 µl	cDNA	50°C	2 min	
1 µl (18 µM)	Fwd primer	95°C	10 min	
1 µl (18 µM)	Rev primer	95°C	15 s	40 repeats
1 µl (5 µM)	Probe	60°C	1 min	
10 µl	Brilliant II QPCR Master Mix with High Rox			
1 µl	GAPDH mix			
Ad 20 µl	sterile H_2O			

Average expression value of three individual measures normalized to GAPDH mRNA expression was quantified for each mouse.

For measurement of Per1 hnRNA expression in hippocampal cells a different TaqMan-qPCR protocol was applied:

TAQMAN-QPCR:		CYCLING:		
2 µl	cDNA	95°C	3 min	
0.3 µl (150 nM)	Fwd primer	95°C	15 s	40 repeats
0.3 µl (150 nM)	Rev primer	60°C	30 s	
0.35 µl (350 nM)	Probe			
10 µl	Brilliant III Ultra-Fast QPCR Mastermix			
0.6 µl	GAPDH mix			
Ad 20 µl	sterile H_2O			

2.7 Protein Techniques

2.7.1 Preparation of total cell lysates

In general, tissue used for protein analysis was rapidly removed and immediately frozen in liquid nitrogen and stored at -80°C until further preparation. All following analysis steps were carried out on ice to avoid any protein degradation. For preparation of total cell lysates an EDTA lysis buffer supplemented with complete mini protease inhibitor was used. Initially, tissue was homogenized with a Dounce homogenisator in an appropriate volume of EDTA lysis buffer. Samples were allowed to rotate at 4°C for 1h and subsequently cellular debris were pelleted by centrifugation (13.000 rpm, 30 min, 4°C; Eppendorf 5415R) and the total protein concentration was determined by using a commercial kit (see Sec. 2.7.3).

EDTA lysis buffer	
0.5 mM	EDTA
250 mM	NaCl
50 mM	HEPES
0.5 %	Igepal (v/v)

2.7.2 Preparation of fractionated cell lysates

For preparation of cytoplasmic and nuclear protein extract, the protein extraction was done according to the method of Kitchener and colleagues (Kitchener et al., 2004). All procedures were performed on ice or at 4°C. The tissue was homogenized using a Dounce homogenisator in an appropriate volume of S1 buffer supplemented with 0.5 mM dithiothreitol (DTT), 0.2 mM sodium orthovanadate (Na_3VO_4), 2 mM sodium fluoride (NaF) and subsequently centrifuged (7.000 rpm, 10 min, 4°C; Eppendorf 5415R). For the cytoplasmic fraction, the supernatant was transferred to a new reaction tube and centrifuged again (13.000 rpm, 30 min, 4°C, Eppendorf 5415R) and the final supernatant was stored at -80°C.

The remaining pellet was resuspended in an appropriate volume of S1 buffer supplemented with 0.5 mM DTT, 0.2 mM Na_3VO_4, 2 mM NaF and passed through a 25 gauge needle for 10 times using a 1 ml syringe to ensure complete disruption of all plasma membranes. The suspension was centrifuged (5.000 rpm, 10 min, 4°C, Eppendorf 5415R), the S/N was discarded and the pellet was gently resuspended in S1 buffer. After one more centrifugation step (5.000 rpm, 10 min, 4°C, Eppendorf 5415R), the pellet was washed 2 times with S1 buffer. In a next step, the nuclear proteins were extracted in 1.2 pellet volume of cold S2 buffer supplemented with 0.5 mM DTT, 0.2 mM Na_3VO_4, 2 mM NaF. After 1 h incubation on ice and subsequent vortexing, the sample was centrifuged and the protein concentration of the supernatant was determined (see also Sec. 2.7.3).

S1 buffer	
10 mM	Hepes
10 mM	KCl
1.5 mM	$MgCl_2$
0.1 mM	EDTA

S2 buffer	
10 mM	Hepes
400 mM	NaCl
1.5 mM	$MgCl_2$
0.1 mM	EDTA

2.7.3 Quantitative Determination of protein concentrations

Protein concentrations in the samples of the total and fractionated cell lysates were determined by using a commercial kit (Bicinchoninic Acid (BCA) Protein Assay Kit, Thermo Scientific, Rockford, USA). The assay is based on the the reduction of Cu^{2+} to Cu^{+} by the peptide bonds of proteins. Thereby the amount of reduction is proportional to the amount of protein present in the sample. The BCA in the kit forms a purple-blue complex with Cu^{+} and the absorbance can be measured at 562 nm. 5 µl of sample was, therefore, mixed with 200 µl of the BCA working reagent, incubated for 30 min at 37°C and the absorbance measured in an ELISA plate reader (Fluostar Optima, BMG Labtech, Offenburg, Germany). The originated BSA standard curve (0 – 2 µg/µl) was used to calculate the protein concentration of each sample.

2.7.4 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein lysates were subjected to electrophoresis on sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) to separate them according to their molecular weights. 10 % Polyacrylamide gels were prepared using the BioRad system, consisting of a stacking and a resolving gel. Equal amounts of protein lysates were mixed with denaturating buffer according to Laemmli (Laemmli, 1970), boiled for 5 min at 99°C in a thermoblock (Thermomixer Comfort, Eppendorf) and subjected to electrophoresis. PageRuler Prestained Protein Ladder Plus (Fermentas, Thermo Scientific, Rockford, USA) was used to determine the molecular weight of the proteins. The electrophoresis procedure was performed with Mini-PROTEAN® Tetra Cell in electrophoresis buffer at 110 V.

4 x Resolving gel buffer, pH 8.8	
1.5 M	Tris
10 %	SDS (w/v)

4 x Stacking gel buffer, pH 6.8	
0.5 M	1 M Tris
10 %	SDS (w/v)

Stacking gel (5 %)	
670 µl	30 % Acrylamide
1 ml	4 x stacking gel buffer
2.3 ml	H_2O bidest.
30 µl	APS
5 µl	TEMED

Resolving gel (10 %)	
4 ml	30 % Acrylamide
3 ml	4 x resolving gel buffer
5 ml	H_2O bidest.
90 µl	APS
9 µl	TEMED

4 x Loading dye	
2.4 ml	1 M Tris pH 6.8
0.8 g	SDS
4 µl	Glycerol
1 ml	ß-Mercaptoethanol
2.8 ml	H_2O bidest.
0.01%	Bromphenol blue

Electrophoresis buffer	
25 mM	Tris
86 mM	Glycin
3.5 mM	SDS
ad 1 l H_2O bidest.	

2.7.5 Western Blottting

Transfer of the proteins onto nitrocellulose membranes

For immunoblotting, the separated proteins were transferred onto nitrocellulose membrane (BioRad, Munich, Germany) using Trans-Blot® Electrophoretic Transfer Cell (BioRad, München, Germany). Therefore, two fiber pads, two whatman papers, gel, nitrocellulose membrane, two whatman papers and two fiber pads were assembled in a gel holder cassette. The electrophoretic transfer was performed in a blotting chamber filled with transfer buffer at 100 V for 1h. Afterwards, nitrocellulose membranes were incubated in blocking solution (see Table 1) on an orbital shaker, to saturate unspecific antibody binding sites on the nitrocellulose membrane.

Transfer buffer (pH 8.3)	
25 mM	Tris
192 mM	Glycin
20 %	Methanol (v/v)

Antibody staining

After blocking of the membrane, the blocking solution was discarded and the membrane was incubated with the primary antibody. The kind of blocking solution as well as the incubation time and dilution of the primary antibodies were determined for each antibody individually to ensure the optimal conditions (see Table 1). Following the incubation with the primary antibody, the nitrocellulose membrane was washed three times for 15 min with TBS-T or PBS-T respectively and incubated for 30 – 60 min with a horseradish-peroxidase (HRP)-conjugated secondary antibody at RT (see Table 1). Afterwards, the membrane was again washed three times for 15 min with TBS-T or PBS-T.

Protein visualization using Enhanced Chemiluminescence.

Immunoreactive bands were visualized by incubating the membrane with Enhanced Chemiluminescent Western Blotting detection reagent (ECL, GE Healtcare, Freiburg, Germany) according to the manufacturer`s instructions and using a Molecular Imager® ChemiDoc™ XRS+ system (Bio-Rad Laboratories, München, Germany). Semiquantitative densitometric analysis of the signals was performed using Image Lab™ Software (Bio-Rad Laboratories, München, Germany). Protein expression for each mouse was normalized to ß-Tubulin (whole cell lysate and cytoplasmic fraction) or TATA binding protein (nuclear fraction) expression and averaged per group.

Stripping of Western Blot membranes

For detection of various proteins on one membrane, each membrane was stripped, i.e. the antibody used before was effectively removed, using Re-Blot Plus Mild Antibody Stripping Solution (Millipore GmbH, Schwalbach, Germany). Following incubation with blocking solution (2 x 5 min), the nitrocellulose membrane was incubated with another antibody. The stripping procedure was mainly used to detect the housekeeping gene ß-Tubulin which was used as loading control for whole cell lysates and cytoplasmic fraction, whereby TATA binding protein was used as loading control for the nuclear fraction.

10 x TBS (pH 7.4)	
500 mM	Tris
1.5 M	NaCl

TBS-T	
0.1 %	Tween (v/v) in TBS

PBS (pH 7.4)	
140 mM	NaCl
3 mM	KCl
1.5 mM	KH_2PO_4
4 mM	Na_2HPO_4

PBS-T	
0.1 %	Tween (v/v) in PBS

PROTEIN OF INTEREST	BLOCKING PROTOCOL	PRIMARY ANTIBODY PROTOCOL	SECONDARY ANTIBODY PROTOCOL	SIZE [KDA]
CRH-R1	5 % BSA/TBS-T 1 h at RT	1/200 in 5 % BSA/TBS-T o/n at 4°C	Anti goat, 1/8500 in 3%BSA/TBS-T 30 min	~48
AVPR-1b	5 % MP/TBS-T 2h at RT	1/400 in 5 % MP/TBS-T o/n at 4°C	Anti goat, 1/1500 in TBS-T 30 min at RT	~ 45 - 50
GR	5 % MP/TBS-T 1h at RT	1/700 in 5 % MP/TBS-T for cytoplasm, 1/300 in 5 % MP/TBS-T for nucleus, o/n 4°C	Anti rabbit, 1/1000 in TBS-T 1 h at RT	~ 86
MR	5 % MP/PBS-T o/n at 4°C	1/200 in 5 % MP/PBS-T 2 h at RT	Anti rabbit, 1/2000 in PBS-T 1 h at RT	~107
FKBP51	5 % MP/TBS-T 1h at RT	1/500 in 5 % MP/TBS-T o/n at 4°C	Anti goat, 1/6000 in 2.5 % MP/TBS-T 30 min at RT	~ 51
POMC	3 % MP/TBS-T 1h at RT	1/700 in 3 % MP/TBS-T o/n at 4°C	Anti rabbit, 1/2000 in TBS-T 30 min at RT	~ 27
ß-Tubulin	5 % MP/TBS-T, 1h at RT or o/n at 4°C	1/1000 in 5 % MP/TBS-T, 1h at RT or o/n at 4°C	Anti rabbit, 1/1000 in TBS-T 30 min at RT	~ 50
TATA binding protein	5 % BSA/TBS-T 1 h at RT	1/200 in 5 % BSA/TBS-T, o/n at 4°C	Anti rabbit, 1/1000 in TBS-T 30 min at RT	~ 38

Table 1: Incubation protocols for every individual antibody. Blocking solution contained either milk powder (MP) or bovine serum albumin (BSA) in TBS-T or PBS-T respectively. Blocking or antibody staining was either performed at room temperature (RT) or at 4°C overnight (o/n).

2.8 Immunohistochemistry

2.8.1 ACTH immunostaining in pituitary cryo-sections

Cryo-sectioning of pituitary tissue

After removal on day 20 of CSC pituitaries were weighed and embedded in protective freezing medium (Tissue-Tek, Sakura Finetek Europe, Zoeterwoude, The Netherlands) and stored at -80°C. Subsequently, one series of five 5-µm cryo-sections were cut using a cryostat (at -20°C) and then thaw-mounted onto pre-coated slides (SuperFrost Plus, Menzel-Gläser, Braunschweig, Germany).

ACTH immunostaining in pituitary cryo-sections

Frozen sections were fixed with ice-cold acetone for 10 min, air-dried for 20 min and washed 2 times with PBS for 10 min. IHC slices were then blocked with PBS/10 % normal goat serum (NGS, Biozol Diagnostica, Eching, Germany) for 20 min followed by incubation with rabbit anti-ACTH antibody (1:200, Abcam, Cambridge, UK) in PBS/0.1% NGS overnight at 4°C. After washing 3 times with PBS for 5 min, IHC slices were incubated with the respective biotinylated anti-rabbit secondary antibody (1:500, Vector Laboratories, Loerrach, Germany) for 30 min at RT and afterwards washed 3 times with PBS for 5 min. Respective positive cells were visualized by the use of Vectastain ABC Kit followed by Vector NovaRed Substrate Kit (Vector Laboratories, Loerrach, Germany). Finally, sections were mounted with Vectashield Mounting Medium (Vector Laboratories, Loerrach, Germany) and covered with a glass cover slip. Per slide, the area positively stained for ACTH and the visible area of the anterior pituitary were quantified in digitized images of three to six sections using Leica QWin V3 (Leica Microsystems, Wetzlar, Germany). Positively stained pituitary tissue was expressed in percent of total anterior pituitary tissue. Furthermore, the number of ACTH positive pituitary cells [n] was counted in the same three to six digitized images and averaged per animal to provide individual means.

2.8.2 AVP and OXT immunostaining in brain cryo-sections

Cryo-sectioning of brain tissue

Immediately after decapitation the brains were collected and immersed for 24 h in fixative consisting of 4 % paraformaldehyde in PBS (pH 7.4). Afterwards they were cryo-protected in 30 % sucrose in PBS, snap-frozen in isopentane and stored at -80°C. Serial sections (20 µm) containing the PVN, according to the mouse brain atlas (Paxinos and Franklin, 2001), were cut using a cryostat (at -20°C) for AVP immunostaining and put in PBS containing 0.01 % sodium azide for free floating staining.

AVP and OXT immunostaining in brain cryo-sections

Free floating sections were incubated in PBS containing 10 % NGS (Biozol Diagnostica, Eching, Germany) and 0.3 % Triton-X (PBS/Triton) for 2 h at RT to block unspecific binding. This was followed by incubation with primary antibody against AVP or OXT (1:400, p41 (AVP), p38 (OXT) mouse monoclonal, which were a generous gift of Dr. Gainer, NIH, USA) in PBS/Triton/1 % NGS overnight at 4°C. Afterwards, sections were washed 3 times in PBS/Triton for 5 min and incubated with the respective biotinylated goat anti-mouse antibody (1:300, Vector Laboratories, Loerrach, Germany) for 2 h at RT and washed again 2 times with PBS/Triton for 5 min. Respective positive cells were visualized by the use of a Vectastain ABC Kit followed by DAB Peroxidase Substrate Kit (Vector Laboratories, Loerrach, Germany). Finally, the free floating sections were put on pre-coated slides (SuperFrost Plus, Menzel-Gläser, Braunschweig, Germany), mounted with Vectashield Mounting Medium (Vector Laboratories, Loerrach, Germany) and covered with a glass cover slip. Counting of positive cells was performed in digitized images using Leica QWin V3 (Leica Microsystems, Wetzlar, Germany). For analysis of AVP positive neurons, parvocellular neurons in the PVN were differentiated histologically from magnocellular neurons according to their smaller size and their lower level of AVP expression (Agid et al., 1999). According to the literature (Kadar et al., 2010), the size of magnocellular neurons was approximately set to > 14 µm and for parvocellular

neurons < 14 µm. For each animal, five to six sections, containing the regions rostral, rostral-medial, medial-caudal and caudal, were counted bilaterally. Numbers of parvocellular and magnocellular AVP positive neurons were counted within each region (1 – 2 sections per region) of the PVN and the average value for each mouse was calculated to provide individual means. The analysis for the number of OXT positive cells was done accordingly to the AVP analysis, whereby no differentiation between magno- and parvocellular neurons was performed. Omission of the primary antibody resulted in no immunoreactivity, confirming specificity of the antibody used. The characteristics of the AVP and OXT antibody are further described in the original publication (Whitnall et al., 1985).

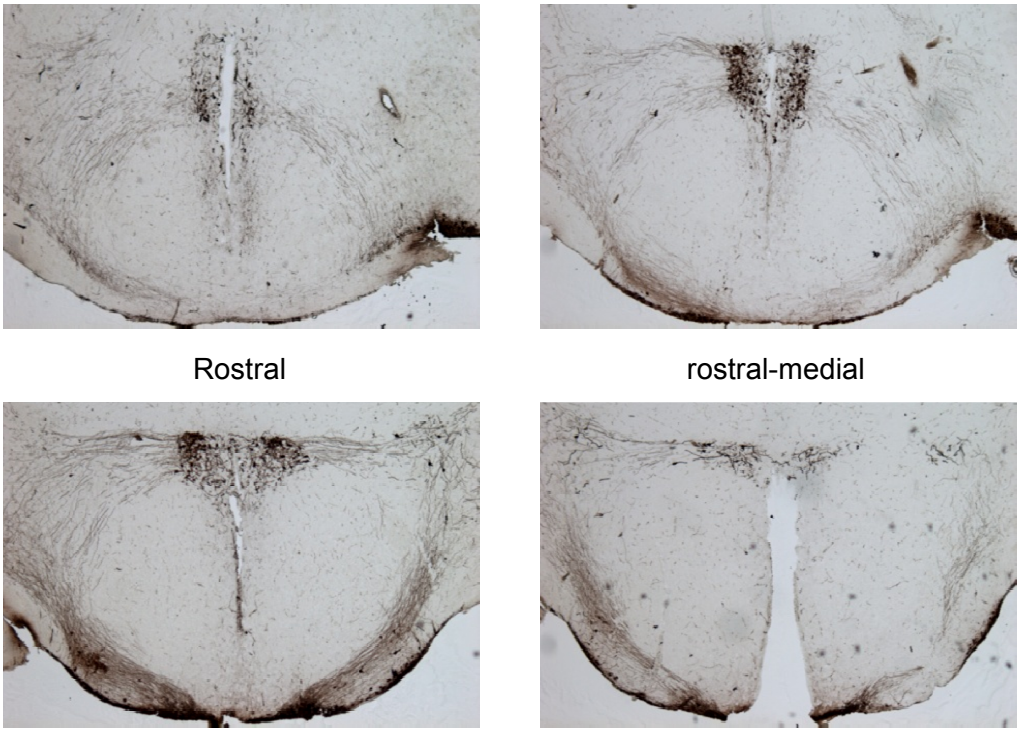

Figure 18: Representative images of mouse brain slices stained for AVP in the PVN. The images represent the four different regions, i.e. rostral, rostral-medial, medial-caudal and caudal which were analysed for AVP and OXT protein expression.

2.9 *In vitro* Techniques

2.9.1 ACTH stimulation of adrenal explants *in vitro*

ACTH stimulation was performed as described previously (Uschold-Schmidt et al., 2012). Briefly, the adrenal were cut into two halves, each containing cortical and medullary tissue. The halves were then weighed and pre-incubated in 200 µl DMEM/F-12 containing 0.1 % BSA for 4 h (37°C, 95 % O_2, 5 % CO_2) before any further treatment. Culture medium was then replaced and each half of one adrenal was incubated with either medium containing saline (basal) or medium containing ACTH (pharmacological dose of 100 nM) for 6 h at 37°C (95 % O_2, 5 % CO_2). Afterwards, supernatants were carefully removed and stored at -20°C until analysed using a commercially available ELISA for CORT (IBL International, Hamburg, Germany). CORT concentrations were calculated in relation to the weight of the respective adrenal explants (i.e. relative CORT secretion). To illustrate the *in vitro* adrenal CORT secretion in relation to the whole organism, relative CORT secretion from left and right adrenal gland of each mouse was summed up (total CORT secretion).

2.9.2 Hippocampal cell isolation und stimulation

Hippocampal cell isolation

For the hippocampal cell stimulation, left and right hippocampi were dissected, pooled and collected in 1.8 ml ice-chilled HBSS (Invitrogen GmbH, Karlsruhe, Germany). Thereafter 200 µl of Trypsin stock solution (Sigma- Aldrich, Deisenhofen, Germany) was added and the hippocampi were incubated for 20 min at 37°C/5% CO_2. Next, 50 µl of 10 mg/ml Deoxyribonuclease (Sigma-Aldrich, Deisenhofen, Germany) was added for 30 s in order to break down DNA and to avoid clumping of tissue during the subsequent trituration. Afterwards the tissue was washed once with HBSS/10% fetal bovine serum (FBS, PAA Laboratories GmbH, Cölbe, Germany) to stop the digestion and twice with HBSS. After discarding the S/N the tissue was homogenized with a plastic pasteur-pipette in 1 ml of DMEM (Invitrogen GmbH, Karlsruhe, Germany) and centrifuged

at 1000 rpm for 10 min at 20°C. The S/N was again discarded and the pellet was resuspended in 1 ml DMEM and the cell number was determined using a cell viability analyser (Vi-Cell XR, Beckmann Coulter, Krefeld, Germany). Afterwards the cell suspension was again centrifuged at 1000 rpm for 10 min at 20°C and the pellet was resuspended in the appropriate volume of DMEM/0.1 % BSA for the determination of the cell viability after 24 h and in DMEM without BSA for the measurement of the Per1 hnRNA expression. As BSA is necessary for cell survival but also contains small amounts of GC, it was not used for the 30 min stimulation (for Per1 hnRNA) but was included in the 24 h stimulation (cell viability measurement). All work was done under the clean bench.

Hippocampal cell viability measurement

2 x 10^5 hippocampal cells were plated onto a Poly-L-Lysine (Sigma Aldrich, Deisenhofen, Germany) coated 96-well plate and incubated with different CORT concentrations (0.1 µM, 1 µM, 10 µM, 100 µM, 1000 µM) (Sigma Aldrich, Deisenhofen, Germany) or vehicle for 24 h at 37°C/5 % CO_2. CORT was first dissolved in Ethanol (EtOH, 60 % of the total volume) and then further diluted to the appropriate concentration with saline. Vehicle consisted of saline containing comparable amounts of EtOH as used in the respective CORT stimulation condition. The cell viability of the hippocampal cells was determined 24 h later with a commercial available colorimetric assay (Cell Titer AQ_{ueous} One Solution Cell Proliferation Assay Promega, Mannheim, Germany). Thereby a yellow solution, containing a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazoli um, inner salt (MTS)] and an electron coupling reagent [phenazine ethosulfate (PES)], was added to the samples. Metabolic active cells produce $NADH^+$, which converts the tetrazolium compound into Formazan, a soluble red dye. The cell viability can be measured via the absorbance of Formazan in a photometer at a wavelength of 485 nm. The $NADH^+$ amount produced by the cells thereby correlates with the cell viability. In this case, 20 µl of MTS were added to the cell solution and incubated for 2 h at 37°C/5% CO_2. Afterwards the absorbance was measured with an ELISA plate

reader (BMG Labtech GmbH, Ortenberg, Germany) whereby all measurements were done in duplicates. To account for differences in background activity, the absorbance of wells containing only medium and vehicle or CORT was subtracted from the absorbance of wells containing cells and the corresponding treatment.

Hippocampal gene expression in response to CORT treatment

For measurement of the CORT-induced expression of Per1 hnRNA, 7 x 10^5 hippocampal cells were plated onto a Poly-L-Lysine (Sigma Aldrich, Deisenhofen, Germany) coated 96-well plate and incubated for 1 h (37°C, 95 % O_2, 5 % CO_2), to allow seeding of the cells in the wells. Afterwards the cells were incubated with 0.1 µM or 100 µM CORT or vehicle for 30 min (37°C, 95 % O_2, 5 % CO_2). CORT was first dissolved in EtOH (60% of the total volume) and then further diluted to the appropriate concentration with saline. Vehicle consisted of saline containing comparable amounts of EtOH as used in the respective CORT stimulation condition. A 30 min stimulation was chosen given that at this time point a maximal induction of hnRNA after an applied CORT pulse was demonstrated (Conway-Campbell et al., 2010). Thereafter the samples were centrifuged for 8 min at 800 rpm, the S/N was discarded and the pellet was resuspended in 150 µl of RLT buffer (Qiagen, Hilden, Germany) containing 1% beta-mercaptoethanol. The cell solution was stored at -80 °C until RNA extraction and qPCR were performed.

2.10 Analysis of immunological parameters

2.10.1 Determination of the histological damage score of the colon

In order to assess the histological damage score of the colon, the colon was removed and mechanically cleaned of faeces. Afterwards, 1 cm of the distal third was cut longitudinally, laid on a filter paper and fixed in 10 % formalin overnight. The next day the fixed tissue was embedded in paraffin and cut longitudinally. Three 3 µm sections were taken at 100 µm distance, stained with haematoxylin-eosin stained distance and were evaluated by histological scoring performed by

an investigator blind to treatment. For statistics, each individual score represents the mean of the three sections.

Haematoxylin-eosin staining:

Xylol	5 min	Eosin	30 s – 2 min
Xylol	5 min	dest H_2O	Rinsing
100 % ethanol	1 min	70% ethanol	1 min
100 % ethanol	1 min	70% ethanol	1 min
90 % ethanol	1 min	90% ethanol	1 min
70% ethanol	1 min	100% ethanol	1 min
dest H_2O	1 min	100% ethanol	1 min
Haematoxylin	10 – 15 min	Xylol	5 min
Hot water	3 min	Xylol	5 min
Cold water	3 min		

Histology was scored as follows (Obermeier et al., 2003; Reber et al., 2007):

Epithelium. 0, normal morphology; 1, loss of goblet cells; 2, loss of goblet cells in large areas; 3, loss of crypts and 4, loss of crypts in large areas
Infiltration. 0, no infiltration; 1, infiltrate around crypt basis; 2, infiltrate reaching to lamina muscularis mucosae; 3, extensive infiltration reaching the L. muscularis mucosae and thickening of the mucosa with abundant oedema; 4, infiltration of the L. submucosa.
The total histological score of each mouse represents the sum of the epithelium and infiltration score and ranges from 0 to 8.

2.10.2 Isolation and incubation of mesenteric lymph node cells

To determine the effects of CSC on the IFN-γ secretion of anti-CD3-stimulated mesLNC, mesLN (pooled from all mice per experimental group) were pruned from fat and collected on ice in RPMI medium. Lymph nodes were mechanically disrupted and filtered through a cell strainer (70 µm nylon, Falcon; Becton Dickinson Biosciences, Heidelberg, Germany). The cell suspension was centrifuged (1000 rpm, 15 min, 4°C, Allegra X-12R, Beckman Coulter), the

supernatant was discarded and cells were washed three times in RPMI medium. After the last washing step, the cells were resuspended in 5 ml RPMI medium and the cell number was assessed using a Cell Viability Analyser (Vi-Cell™ XR, Beckman Coulter, Krefeld, Germany) and adjusted to a concentration of 10^6 cells/ml.

One day before the cell stimulation, a 96-well plate had to be precoated with 2.5 µg/ml anti-CD3 antibody. Shortly before the cells were plated, the antibody was discarded and the wells were washed two times with PBS. Then 2 x 10^5 (200 µl) mesLNC were transferred to wells of the precoated 96-well plate. Eight wells were transferred with the respective number of cells per experimental group. After incubation for 24 h (37°C, 95 % O_2, 5 % CO_2), IFN-γ levels were measured in the supernatants of four wells per experimental group by ELISA (Biolegend, San Diego, CA, USA) according to the manufacturer and averaged per group. The results are depicted as individual values per treatment group for each of the two experiments and the respective mean.

RPMI Medium (1640)	
10 %	FBS (v/v)
100 u/ml	Penicillin
100 µg/ml	Streptomycin
3 x 10^{-5} M	Mercaptoethanol

2.11 Statistics

For statistical comparisons, the software package SPSS statistics (version 21.0) was used. Data of two experimental groups (SHC versus CSC) were analysed using the parametric Student's *t*-test. The number of AVP and OXT positive neurons (factor region and factor CSC), plasma ACTH levels following DST (factor CSC and factor stimulus), hippocampal cell viability and stimulation of Per1 hnRNA expression in hippocampal cells *in vitro* (factor CSC and factor CORT) were compared using a two-way analysis of variance (ANOVA) followed

by a *post hoc* Bonferroni test when appropriate. All parameters depending on two factors (factor CSC and factor genotype) were analysed by using a two-way ANOVA, followed by a *post hoc* Bonferroni test when appropriate. *In vitro* adrenal CORT secretion was compared by three-way ANOVA (factor genotype, factor CSC, factor ACTH), followed by a *post hoc* Bonferroni test when appropriate. Data represent the mean + SEM. Significance was taken at $P < 0.05$. For determination of IFN-γ secretion by mesLNC no SEM was calculated and no statistical analysis was performed, due to the low number of data pools (two pools per treatment group).

CHAPTER 3

RESULTS

1 Effects of 19 days of CSC on different parameters of the pituitary

1.1 Basal and acute heterotypic stress-induced plasma ACTH levels

First of all, basal plasma ACTH levels on day 20 of CSC were assessed. Moreover, another set of animals was exposed to an acute heterotypic stressor, in this case 6-min FS, on day 20 of CSC and trunk blood was collected 10 min following termination of the acute stressor exposure.

Compared with SHC mice, basal morning plasma ACTH concentrations were significantly increased in CSC mice (P = 0.001; Fig. 19A), as were plasma ACTH concentrations 10 min following termination of FS exposure (6 min) (P = 0.030; Fig. 19B).

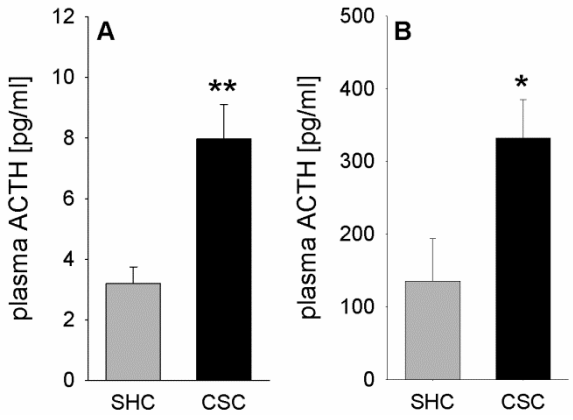

Figure 19: Effects of CSC on the basal morning and forced swim (FS)-induced plasma ACTH concentrations in trunk blood.

Following decapitation on day 20 of CSC between 0800 and 1000 h, basal morning plasma ACTH [pg/ml] concentrations were determined in trunk blood of SHC (n = 42) and CSC (n = 61) mice (A). Another set of SHC (n = 7) and CSC (n = 6) mice was exposed to 6 min forced swim (FS) on day 20 of CSC and decapitated 10 min following FS exposure whereby trunk blood was collected for determination of plasma ACTH [pg/ml] concentrations (B). ■ SHC; ■ CSC. Data represent the mean + SEM. * represent P < 0.05, ** represent P < 0.01 *vs.* respective SHC mice. [adapted from (Füchsl et al., **B** *submitted*)]

1.2 Absolute pituitary weight and total pituitary cell number

The increased pituitary weight found on day 28 (Slattery et al., 2012), i.e. 8 days following termination of the CSC paradigm, already indicated that the pituitary might be implicated in alterations of HPA axis functionality. Nevertheless, to confirm this result also on day 20 of CSC, pituitary weight, as well as the number of pituitary cells was determined immediately after 19 days of CSC.

Absolute pituitary weight was significantly increased (P = 0.001, Fig. 20A) on day 20 of CSC, supporting the finding of the previous study. Given that also the number of isolated pituitary cells was significantly increased (P = 0.030; Fig. 20B) in CSC compared with SHC mice, indicates that the increased pituitary weight is mediated by hyperplasia.

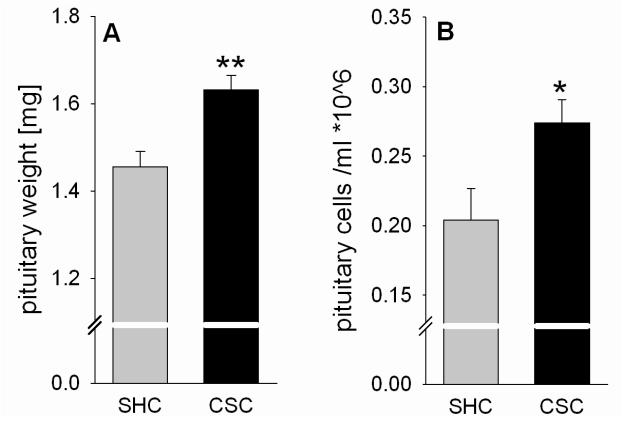

Figure 20: Effects of CSC on pituitary weight and pituitary cell number.

Following decapitation on day 20 of CSC the pituitaries of SHC (n = 42) and CSC (n = 61) mice were removed and absolute pituitary weight [mg] was determined (A). The pituitary cells of one set of SHC (n = 6) and CSC (n = 14) mice were isolated and subsequently quantified (per ml) by means of a Cell Viability Analyser (B). ■ SHC; ■ CSC. Data represent the mean + SEM. * represent P < 0.05, ** represent P < 0.01 *vs.* respective SHC mice. [adapted from (Füchsl et al., **B** *submitted*)]

1.3 ACTH staining in the pituitary

As only corticotroph cells are implicated in the regulation of the ACTH release, an IHC staining for ACTH in the pituitary was established in order to measure the percentage of ACTH positive tissue as well as the number of ACTH positive cells in the anterior pituitary.

A statistically significant increase in the percentage (in relation to anterior pituitary tissue) of ACTH positive tissue (P = 0.019; Fig. 21A/C), as well as in the number of ACTH positive cells (P = 0.009; Fig. 21B/C) was found in CSC compared with SHC mice. This finding indicates that the newly built pituitary cells determined in Sec. 1.2 are corticotroph cells.

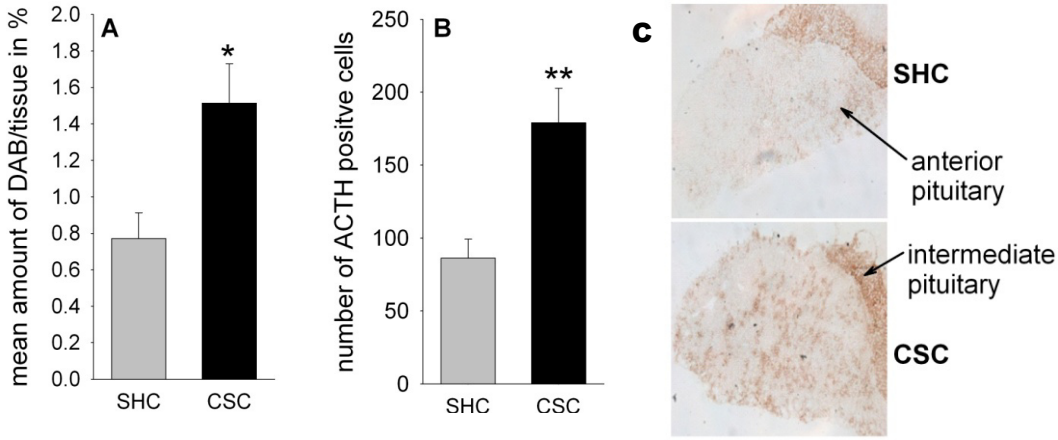

Figure 21: Effects of CSC on the percentage of ACTH positive tissue and on the number of ACTH positive cells.

Following decapitation on day 20 of CSC the pituitaries of SHC (n = 6) and CSC (n = 6) mice were removed and cut on a cryostat and subsequently one series of cry-sections was stained with an antibody against ACTH. Per animal, three to six different sections were analysed and averaged for the percentage of ACTH positive anterior pituitary tissue in relation to the total tissue (C). Moreover, the number of ACTH positive pituitary cells [n] was counted in the same three to six digitized images and averaged per animal to provide individual means. (D). ■ SHC; ■ CSC. Data represent the mean + SEM. * represent $P < 0.05$, ** represent $P < 0.01$ *vs.* respective SHC mice. (C) Representative images of cryo-sections stained with antibody against ACTH depicting anterior and intermediate pituitary. [adapted from (Füchsl et al., **B** *submitted*)]

1.4 POMC mRNA and protein expression

To get a hint if the newly built corticotroph cells are really functional and, therefore, able to produce ACTH, in a next step the mRNA and protein expression of the precursor of ACTH, namely POMC, was analysed.

Statistical analysis revealed no differences between CSC and SHC mice regarding POMC mRNA (Fig. 22A) and protein (Fig. 22B/C) expression.

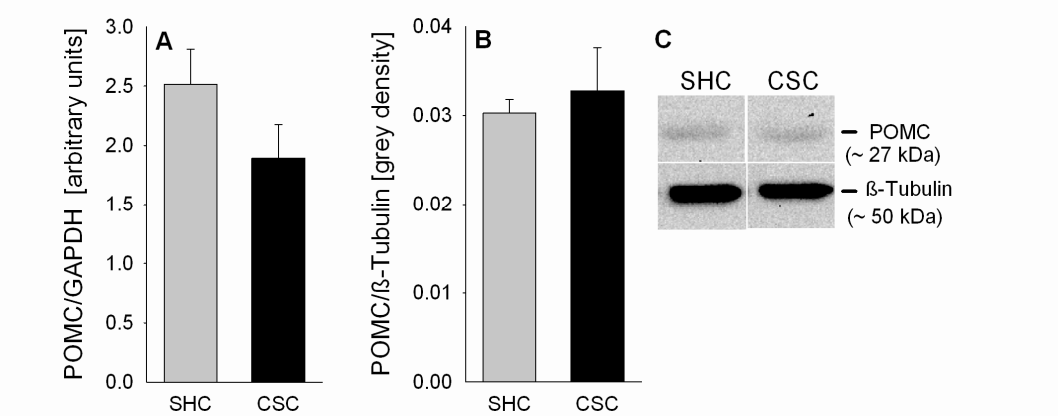

Figure 22: Effects of CSC on POMC mRNA and protein expression in the pituitary.
Following decapitation on day 20 pituitaries of both SHC and CSC mice were removed. RNA of SHC (n = 7) and CSC (n = 6) mice was extracted and reverse transcribed into cDNA for determination of mRNA expression [arbitrary units] for POMC via qPCR using TaqMan technology normalized to the mRNA expression of the housekeeping gene GAPDH (A). Protein was extracted from the pituitaries for determination of POMC expression [grey density] normalized to the loading control ß-Tubulin in SHC (n = 13) and CSC (n = 13) mice (B/C) ▒ SHC; ■ CSC. Data represent the mean + SEM. * represents $P < 0.05$ vs. respective SHC mice. (C) Representative images of bands detected for POMC (~ 27 kDa), and respective loading control ß-Tubulin (~ 50 kDa) are shown for SHC and CSC mice. [adapted from (Füchsl et al., **B** *submitted*)]

1.5 CRH-R1 and AVPR-1b mRNA and protein expression

Another important regulatory mechanism for the ACTH release at the level of the pituitary constitutes the expression of the CRH-R1 and AVPR-1b. Hence the expression pattern of both receptors was analysed, whereby also mRNA as well as protein was measured as it is known from the literature that both do not always correlate.

While mRNA expression of CRH-R1 was comparable (Fig. 23A), protein expression was significantly reduced in CSC compared with SHC mice (P = 0.029; Fig.23B/C). Both, mRNA (Fig 23D) and protein expression (Fig. 23E/F) expression of the AVPR-1b were not affected by CSC. The two bands detected for the AVPR-1b (Fig. 23F) represent the glycosylated and unglycosylated form of the receptor.

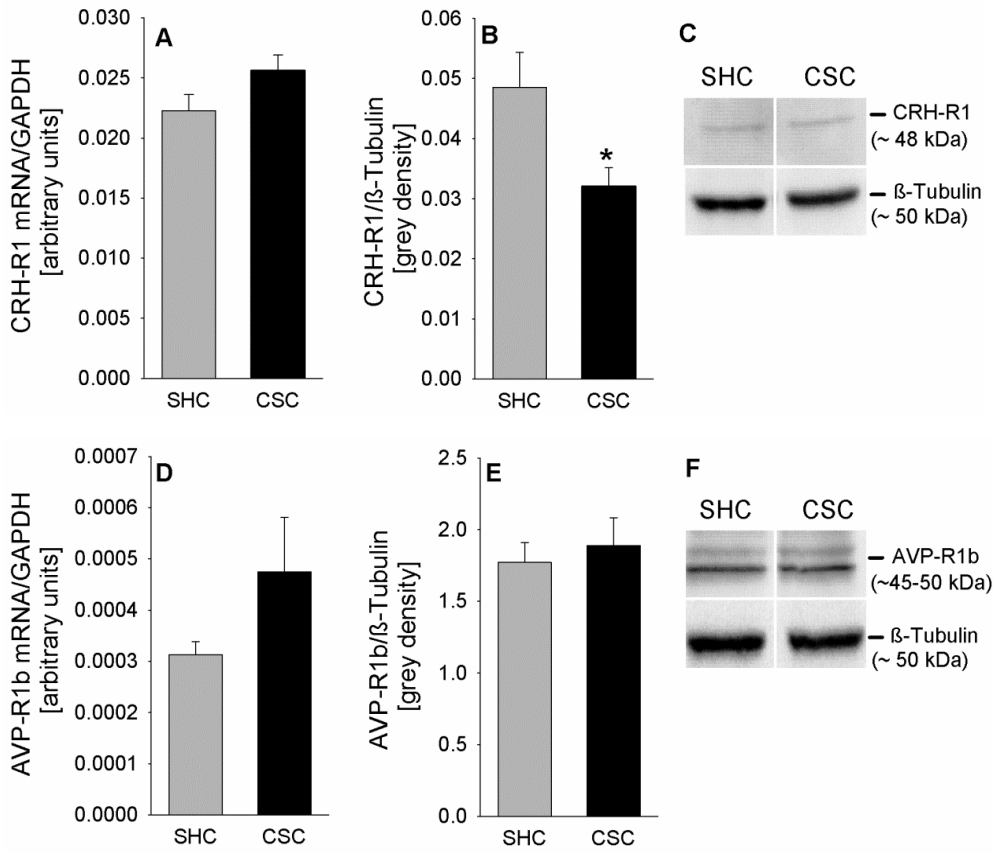

Figure 23: Effects of CSC on CRH-R1 and AVP-R1b mRNA and protein expression in the pituitary.

Following decapitation on day 20 pituitaries of both SHC and CSC mice were removed. RNA of SHC (n = 7) and CSC (n = 6) mice was extracted and reverse transcribed into cDNA for determination of mRNA expression [arbitrary units] for CRH-R1 or AVP-R1b via qPCR using TaqMan technology normalized to the mRNA expression of the housekeeping gene GAPDH (A/D). Protein was extracted from the pituitaries for determination of of CRH-R1 (SHC: n = 9, CSC: n = 12; B/C) and AVPR-1b (SHC: n = 8, CSC: n = 20; E/F) protein expression [grey density] normalized to the loading control ß-Tubulin. ■ SHC; ■ CSC. Data represent the mean + SEM. * represents $P < 0.05$ vs. respective SHC mice. Representative images of bands detected for CRH-R1 (~ 48 kDa; C) and AVP-R1b (~ 45 - 50 kDa; F) and respective loading control ß-Tubulin (~ 50 kDa; C/F) are shown for SHC and CSC mice. [adapted from (Füchsl et al., **B** *submitted*)]

2 Effects of 19 days of CSC on AVP and OXT expression in the PVN

In a previous study, AVP and OXT mRNA (Reber and Neumann, 2008a) expression were already analysed, whereby the former one was found to be decreased while the latter one was unchanged following CSC exposure. To further extent the knockwledge about the expression of these two neuropeptides, an IHC staining was established in order to measure also the protein expression of AVP and OXT in the PVN, whereby the whole PVN was cut from rostral to caudal, in order to loose no informations, as there might probably be differences in the different layers of the PVN. The PVN slices were thereby assigned to four different regions (rostral, rostral-medial, medial-caudal, caudal) (see Fig.18).

2.1 Number of AVP positive neurons in the PVN

Two-way ANOVA considering the factors region (rostral, rostral-medial, medial-caudal, caudal) as well as factor CSC only revealed significant effects for factor region in parvocellular ($F_{1,55}$ = 19.886; P < 0.001; Fig. 24A) and magnocellular ($F_{1,55}$ = 64.861; P < 0.001; Fig. 24B) AVP positive neurons. In detail, the number of AVP positive parvocellular and magnocellular neurons was significantly increased within the rostral-medial part of the PVN compared to the rostral (parvocellular: P = 0.037; magnocellular: P < 0.001), medial-caudal (parvocellular: P = 0.008; magnocellular: P < 0.001) and caudal part (parvocellular and magnocellular: P < 0.001).

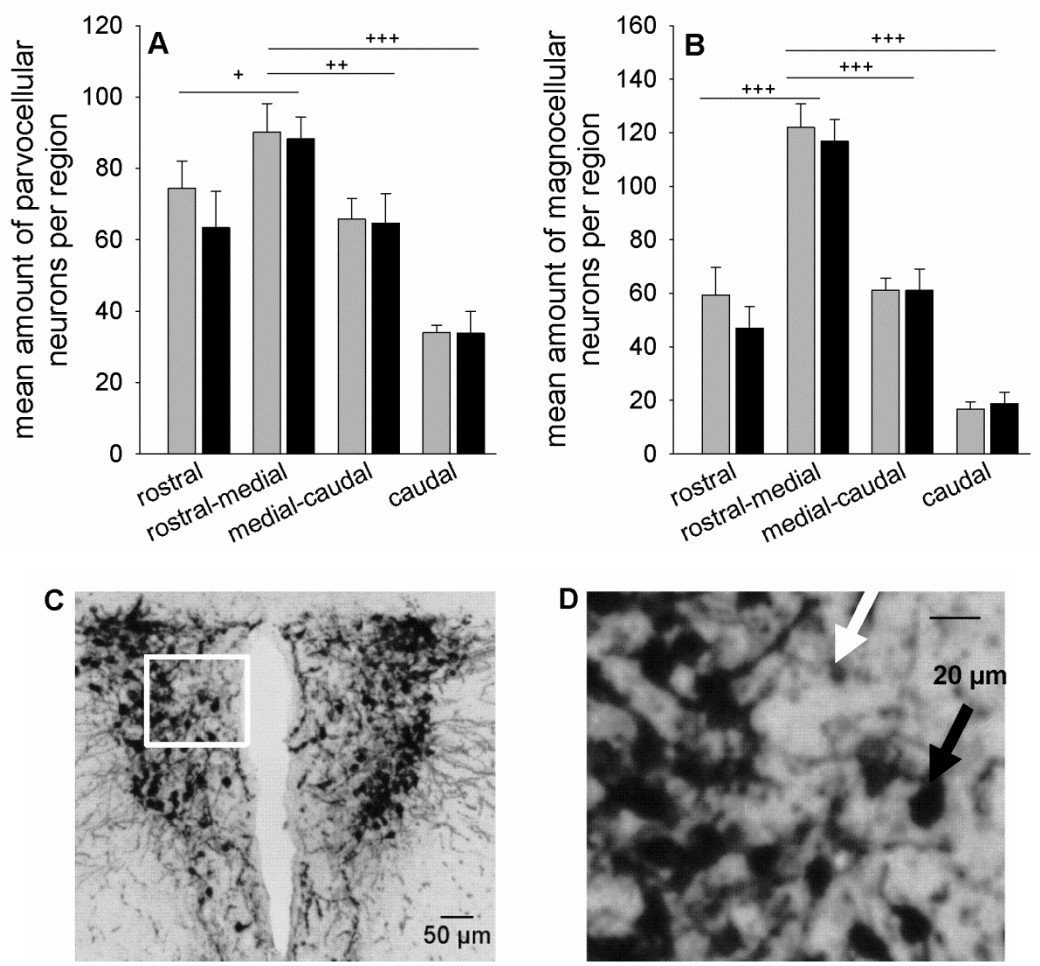

Figure 24: Effects of CSC on the number of AVP positive neurons in the PVN.

Following decapitation on day 20 of CSC brains were removed and immersed in fixative. One series of cryo-sections (5 – 7 sections per animal), containing the regions rostral, rostral-medial, medial-caudal and caudal was stained for AVP employing a free-floating approach. Number of parvocellular (A) and magnocellular (B) AVP positive neurons were counted within each region (1 – 2 sections per region) of the PVN and averaged per animal for SHC (n = 8 per region) and CSC (n = 7 – 8 per region). ■ SHC; ■ CSC. Data represent the mean + SEM. $^{+}$ represent $P < 0.05$, $^{++}$ represent $P < 0.01$, $^{+++}$ represent $P < 0.001$ vs. rostral-medial region. (C) Representative image of cry-section stained for AVP in the PVN (magnification: 5 x; scale bar 50 µm). (D) Higher magnification (400 x) of the magnocellular (black arrow) and parvocellular (white arrow) AVP positive neurons depicted in the white frame of Figure 4D (scale bar 20 µm). [adapted from (Füchsl et al., **B** *submitted*)]

2.2 Number of OXT positive neurons in the PVN

Two-way ANOVA considering the factors region (rostral, rostral-medial, medial-caudal, caudal) as well as factor CSC only revealed significant effects for factor region ($F_{1,54}$ = 28.019; P < 0.001; Fig. 25A). In detail, the number of OXT positive neurons was significantly increased within the rostral part of the PVN compared to the rostral-medial (P = 0.014.), medial-caudal (P < 0.001) and caudal part (P < 0.001).

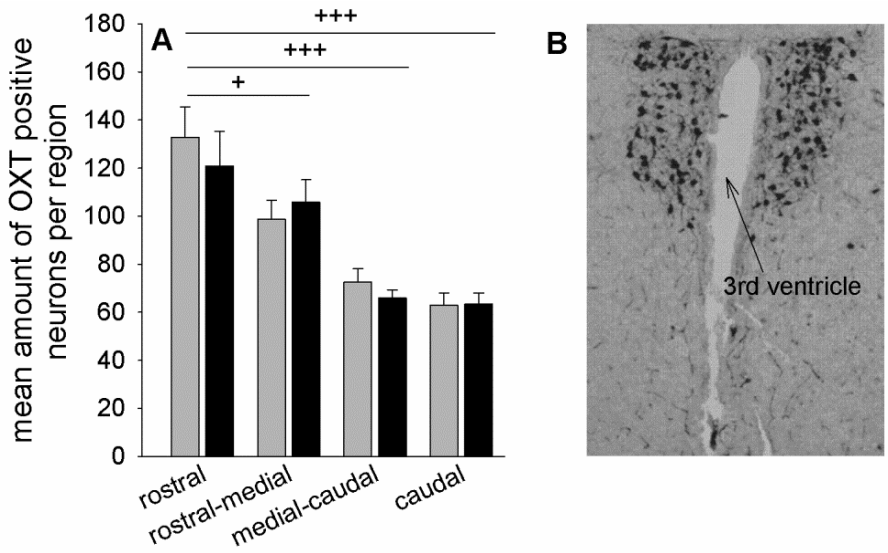

Figure 25: Effects of CSC on the number of OXT positive neurons in the PVN.
Following decapitation on day 20 of CSC brains were removed and immersed in fixative. One series of cryo-sections (5 – 7 sections per animal), containing the regions rostral, rostral-medial, medial-caudal and caudal was stained for OXT employing a free-floating approach. Number of OXT positive neurons (A) were counted within each region (1 – 2 sections per region) of the PVN and averaged per animal for SHC (n = 7 - 8 per region) and CSC (n = 7 – 8 per region). ■ SHC; ■ CSC. Data represent the mean + SEM. $^{+}$ represent P < 0.05, $^{++}$ represent P < 0.01, $^{+++}$ represent P < 0.001 vs. rostral region. (B) Representative image of cry-section stained for OXT in the PVN (magnification: 5 x).

3 Effects of 19 days of CSC on the negative feedback regulation at different levels

Negative feedback inhibition plays an important role in regulating ACTH release from the HPA axis, whereby the pituitary, hippocampus, PVN, and PFC are mainly involved. To determine if there are alterations in the negative feedback following 19 days of CSC, GR and MR expression was analysed in the pituitary, hippocampus, PVN and PFC. In addition, a DST was performed, to specifically test the feedback of the pituitary under *in vivo* conditions. At the level of the hippocampus, first of all the hippocampus weight as well as the number of isolated hippocampal cells was determined, as this might be sensitive to the effects of chronic stress. In addition, hippocampal cells were isolated and the *in vitro* ability of CORT to induce hnRNA of a GC-responsive gene (Per1) and the cell viability of isolated hippocampal cells in response to 24 h of CORT stimulation *in vitro* were analysed. Furthermore, the expression of FKBP51, involved in the regulation of GR sensitivity and/or functionality was measured in the pituitary, hippocampus and PFC. Due to methological problems, GR expression in the nucleus in response to an acute stressor (6-min FS), as indicator for the translocation efficiency of the receptor could only be measured in the pituitary and not in the various brain regions.

3.1 Analysis of the negative feedback at the level of the pituitary

3.1.1 GR and MR mRNA and protein expression

Statistical analysis revealed that under basal conditions GR mRNA (P = 0.01; Fig. 26A) as well as cytoplasmic protein expression (P = 0.011; Fig. 26B/C) were decreased in CSC compared with SHC mice. Moreover, while MR mRNA expression was comparable between CSC and SHC mice (Fig. 26D) MR protein expression (P = 0.014; Fig. 26E/F) was increased following CSC.

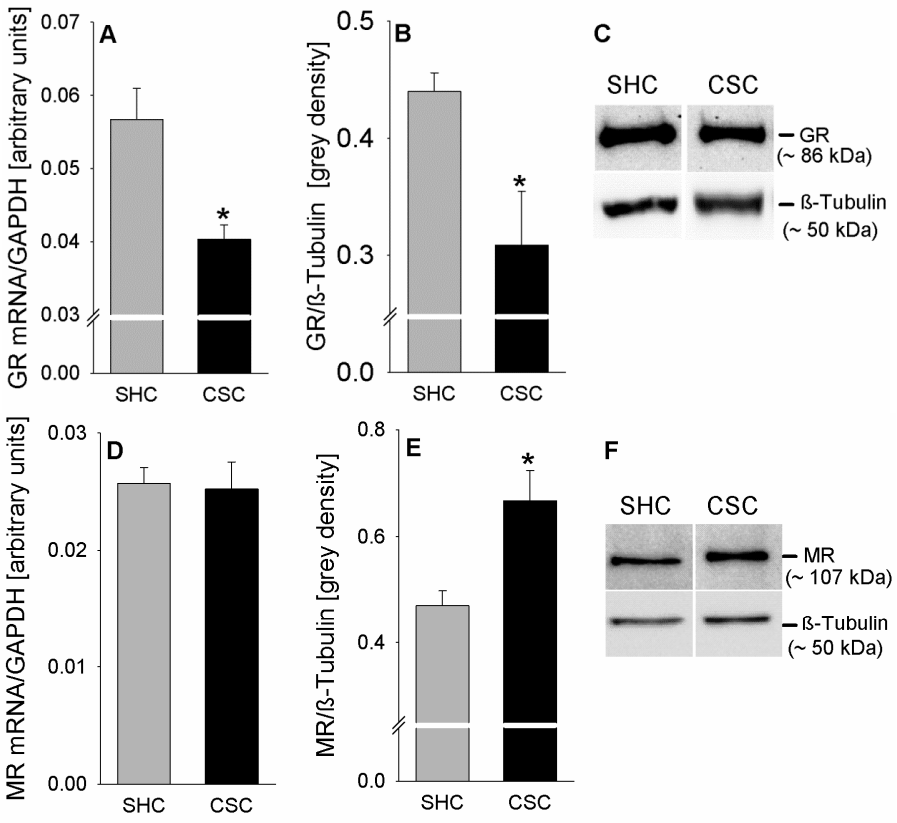

Figure 26: Effects of CSC on GR and MR mRNA and protein expression in the pituitary.

Following decapitation on day 20 pituitaries of both SHC and CSC mice were removed. Afterwards, either RNA of SHC (n = 6) and CSC (n = 5) mice was extracted and reverse transcribed into cDNA for determination of GR and MR expression [arbitrary units] via qPCR using TaqMan technology normalized to the mRNA expression of the housekeeping gene GAPDH (A/D) or protein was extracted from the pituitaries of SHC (GR: n = 11; MR: n = 8) and CSC (GR: n = 10; MR: n = 7) mice for determination of GR and MR protein expression [grey density] normalized to the loading control ß-Tubulin (B/E). ■ SHC; ■ CSC. Data represent the mean + SEM. * represent $P < 0.05$ vs. respective SHC mice. Representative images of bands detected for GR (~ 86 kDa; C) or MR (~ 107 kDa; F) and respective loading control ß-Tubulin (~ 50 kDa; C/F) are shown for SHC and CSC mice. [adapted from (Füchsl et al., **B** *submitted*)]

3.1.2 Dexamethasone suppression test (DST)

To check if the reduced GR expression in the pituitary results in a diminished *in vivo* feedback inhibition, SHC and CSC were injected with Dex or vehicle. Four hours later they were exposed to 6-min FS and 10 min following termination of FS exposure, trunk blood was collected for plasma ACTH analysis.

Plasma ACTH release during FS was dependent on both factor CSC ($F_{1,42}$ = 7.18; P = 0.010) and factor treatment ($F_{1,42}$ = 29.74; P < 0.001; Fig. 27). *Post hoc* analysis revealed an increased ACTH response to FS in vehicle-injected CSC compared with respective SHC mice (P = 0.002), an effect that was absent in Dex-blocked mice. Moreover, FS-induced ACTH release was significantly lower in Dex-blocked SHC (P = 0.018) and CSC (P < 0.001) mice compared with respective vehicle-injected mice.

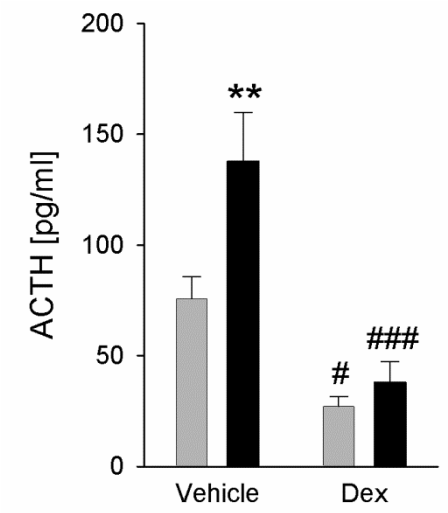

Figure 27: Effects of CSC on the plasma ACTH concentrations in trunk blood following DST.

On day 20 of CSC, between 0700 and 0800 h, SHC and CSC mice were injected with vehicle or Dex (30 µg/kg, ip). Four hours later all animals were exposed to forced swim for 6 min. 10 min after termination of the acute stressor exposure all mice were decapitated and trunk blood was collected for determination of plasma ACTH [pg/ml] (SHC: n = 11; CSC: n= 12). ▒ SHC; ■ CSC. Data represent the mean + SEM. ** represent P < 0.01 *vs.* respective SHC mice; # represent P < 0.05, ### represent P < 0.001 *vs.* respective vehicle group. [adapted from (Füchsl et al., **B** *submitted*)]

3.1.3 FKBP51 protein expression

Pituitary FKBP51 protein expression was significantly increased in CSC compared with SHC mice (*P* = 0.006; Fig. 28A/B).

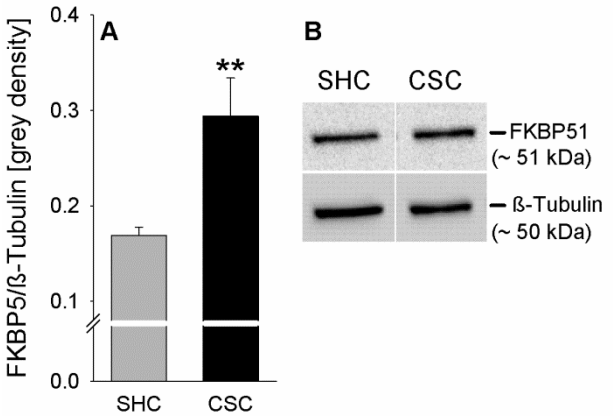

Figure 28: Effects of CSC on FKBP51 protein expression in the pituitary.
Following decapitation on day 20 of CSC pituitaries of both SHC (n = 13) and CSC (n = 13) mice were removed for protein extraction and subsequent determination of FKBP51 protein expression [grey density] normalized to the loading control ß-Tubulin (A). ■ SHC; ■ CSC. Data represent the mean + SEM. ** represents *P* < 0.01 vs. respective SHC mice. (B) Representative images of bands detected for FKBP51 (~51 kDa) and the loading control ß-Tubulin (~ 50 kDa) are shown for SHC and CSC mice. [adapted from (Füchsl et al., **B** *submitted*)]

3.1.4 GR protein expression in the nuclear fraction

For measurement of the GR protein expression in the nucleus, SHC and CSC mice were exposed to 6-min FS and 10 min following termination of the acute stressor exposure, mice were decapitated and pituitaries were removed.

Pituitary GR expression in the nucleus after 6-min FS exposure was similar in SHC and CSC mice (Fig. 29A/B).

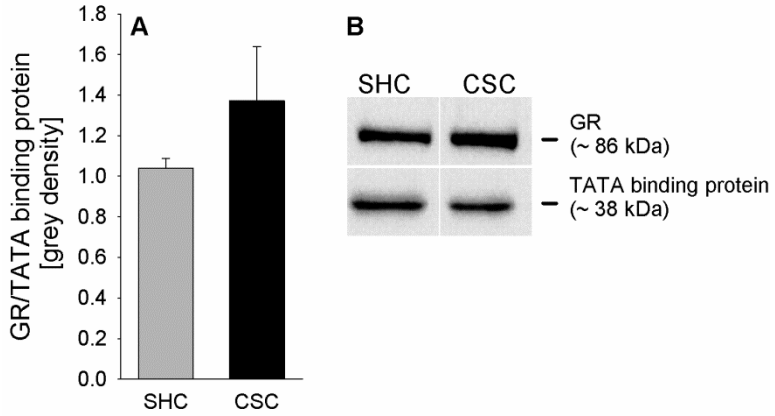

Figure 29: Effects of CSC GR nuclear protein expression in the pituitary following acute stress (6-min FS).

On day 20, SHC and CSC mice were exposed to 6 min FS and decapitated 10 min following FS exposure and pituitaries were removed. Afterwards nuclear protein was extracted from the pituitaries of SHC (n = 5) and CSC (n = 5) mice for determination of nuclear GR protein expression [grey density] normalized to the loading control TATA binding protein (A/B). ■ SHC; ■ CSC. Data represent the mean + SEM. Representative images of bands detected for nuclear GR (~ 86 kDa) and the loading control TATA binding protein (~36 kDa) are shown for SHC and CSC mice (B).

3.2 Analysis of the negative feedback at the level of the hippocampus

3.2.1 Absolute hippocampus weight and number of hippocampal cells

Statistical analysis revealed that CSC had no effect on the absolute hippocampus weight [mg] (Fig. 30A) as well as on the number of isolated hippocampal cells (Fig. 30B).

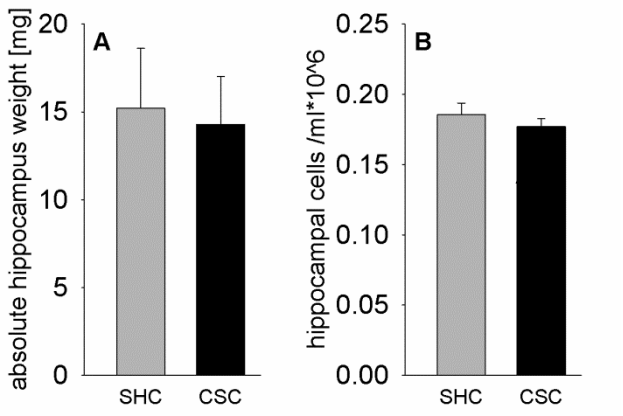

Figure 30: Effects of CSC on hippocampus weight and on hippocampal cell number.

Following decapitation on day 20 of CSC the left and right hippocampus of SHC (n = 20) and CSC (n = 27) mice were removed and weighed. Depicted is the absolute weight [mg] of the left and right hippocampus (sum of both, A). The hippocampal cells of SHC (n = 33) and CSC (n = 34) mice were isolated and subsequently quantified (per ml) by means of a Cell Viability Analyser (B). ■ SHC; ■ CSC. Data represent the mean + SEM.

3.2.2 GR and MR mRNA and protein expression

Statistical analysis revealed a decrease in GR mRNA (P = 0.011; Fig. 31A) and protein (P = 0.023; Fig 31B/C) expression in the hippocampus of CSC compared with SHC mice. In contrast, MR mRNA (Fig. 31D) and protein expression (Fig 31E/F) were not affected by CSC exposure.

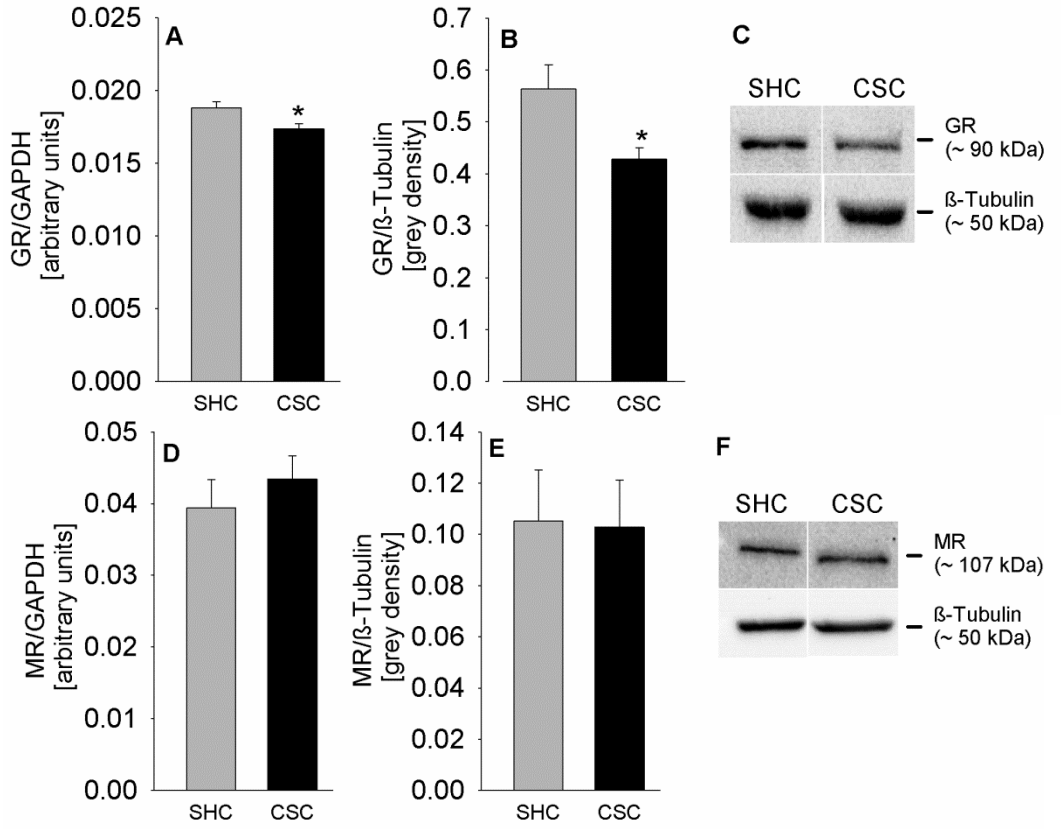

Figure 31: Effects of CSC on hippocampal GR and MR mRNA and protein expression.

Following decapitation on day 20 hippocampi of both SHC and CSC mice were removed. Afterwards, either RNA (A, D) of SHC (n = 13) and CSC (n = 19) mice was extracted and reverse transcribed into cDNA for determination of GR and MR mRNA expression [arbitrary units] via qPCR using TaqMan technology normalized to the mRNA expression of the housekeeping gene GAPDH (A/D) or protein (B, C, E, F) was extracted from the hippocampi of SHC (GR: n = 9; MR: n = 6) and CSC (GR: n = 7; MR: n = 6) mice for determination of GR (B/C) and MR (E/F) protein expression [grey density] normalized to the loading control ß-Tubulin ■ SHC; ■ CSC. Data represent the mean + SEM. * represent $P < 0.05$ vs. respective SHC mice. Representative images of bands detected for GR (~ 86 kDa; C) or MR (~ 107 kDa; F) and respective loading control ß-Tubulin (~ 50 kDa; C/F) are shown for SHC and CSC mice.

3.2.3 FKBP51 protein expression

Statistical analysis revealed that FKBP51 protein expression in the hippocampus (Fig. 32A/B) was not affected in CSC mice.

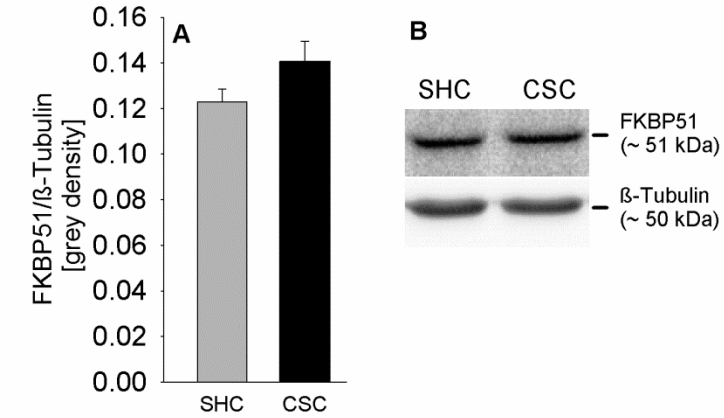

Figure 32: Effects of CSC on FKBP51 protein expression in the hippocampus.
Following decapitation on day 20 hippocampi of both SHC (n = 8) and CSC (n = 8) mice were removed and protein was extracted for determination of FKBP51 protein expression [grey density] normalized to the loading control ß-Tubulin (A/B). ■ SHC; ■ CSC. Data represent the mean + SEM. (B) Representative images of bands detected for FKBP51 (~ 51 kDa) and the loading control ß-Tubulin (~ 50 kDa) are shown for SHC and CSC mice.

3.2.4 Per1 hnRNA expression in hippocampal cells *in vitro*

In vitro stimulation of hippocampal cells with two different CORT concentrations in order to stimulate Per1 hnRNA expression revealed a significant effect of both factor CSC ($F_{1,50}$ = 9.34; P = 0.004) and factor CORT ($F_{2,50}$ = 16.67; P < 0.001; Fig. 33). *Post hoc* analysis indicated a significant increase of Per1 hnRNA in response to 0.1 µM (P = 0.023) and 100 µM (P < 0.001) CORT compared with basal conditions in CSC mice. Hippocampal cells of SHC mice showed a significant increase of Per1 hnRNA expression in response to 100 µM (P = 0.049) and a trend towards an increase in response to 0.1 µM (P = 0.063) CORT compared with basal conditions. Moreover, Per1 hnRNA expression was higher in hippocampal cells from CSC compared with SHC mice when stimulated with 100 µM CORT (P = 0.004).

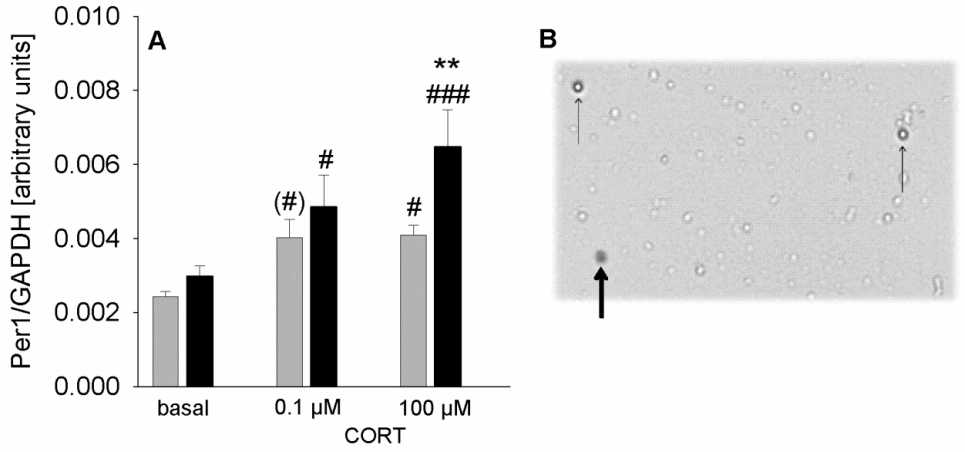

Figure 33: Effects of CSC on CORT-mediated increase in Per1 hnRNA expression in hippocampal cells *in vitro.*

Following decapitation on day 20 hippocampi of SHC and CSC mice were removed and hippocampal cells were isolated. 7 x 10^5 hippocampal cells were stimulated with vehicle (basal, SHC and CSC: n = 14), 0.1 µM (SHC and CSC: n = 7) or 100 µM CORT (SHC and CSC: n = 7) for 30 min. Afterwards, RNA of the cells was extracted and reverse transcribed into cDNA for determination of Per1 hnRNA [arbitrary units] via qPCR using TaqMan technology normalized to the mRNA expression of the housekeeping gene GAPDH (A). ■ SHC; ■ CSC. Data represent the mean + SEM. ** represent $P < 0.01$ vs. respective SHC mice; # represent $P < 0.05$; ### represent $P < 0.001$ vs. respective basal; (#) represent a trend vs. respective basal. **B** Representative image of the Cell Viability analyser showing living hippocampal cells (thin black arrow) and a dead cell (bold black arrow).

3.2.5 Hippocampal cell viability in response to corticosterone (CORT) stimulation

Hippocampal cell viability in response to 24 h *in vitro* stimulation with different CORT concentrations was dependent on the interaction of factors CSC and CORT ($F_{5,223} = 3.35$; $P = 0.006$; Fig. 34). *Post hoc* analysis revealed that in CSC mice 1 µM and 10 µM CORT and in SHC mice 100 µM CORT increase the cell viability compared with respective basal conditions. The highest CORT concentration (1000 µM) decreased cell viability in hippocampal cells of CSC mice compared with basal ($P = 0.007$) and also compared with those of respective SHC mice ($P = 0.005$).

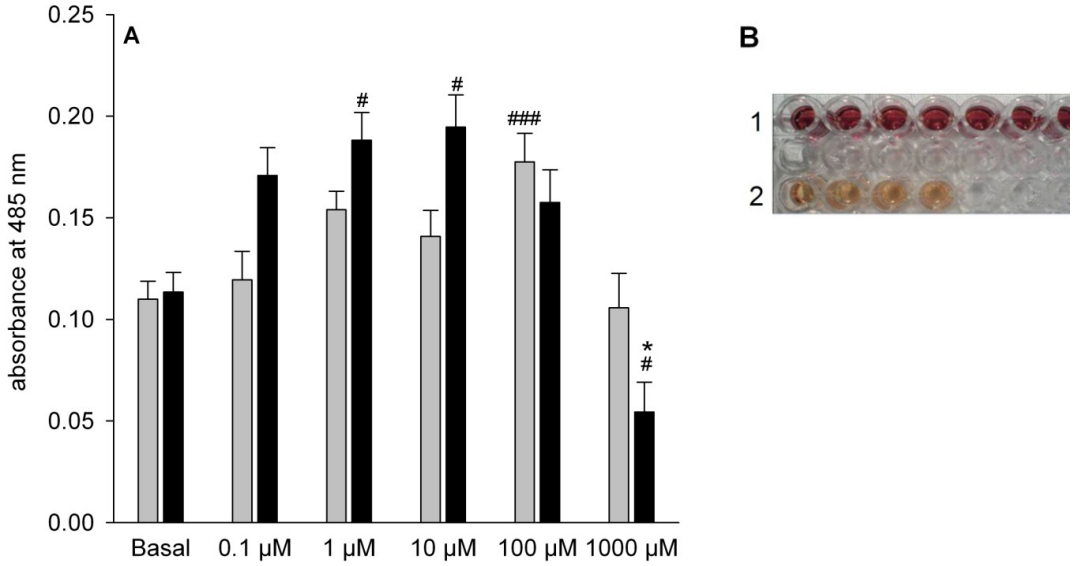

Figure 34: Effects of CSC on CORT-induced changes in hippocampal cell viability *in vitro.*

Following decapitation on day 20 hippocampi of SHC and CSC mice were removed and cells were isolated. 2 x 10^5 hippocampal cells were stimulated with vehicle (basal: SHC: n = 36; CSC: n = 36) or different CORT concentrations (0.1 µM (SHC: n = 10; CSC: n = 9); 1 µM (SHC: n = 10; CSC: n = 9); 10 µM (SHC: n = 10; CSC: n = 9); 100 µM (SHC: n = 26; CSC: n = 27); 1000 µM (SHC: n = 26; CSC: n = 27)] over24 h and subsequently cell viability [absorbance] was assessed via a commercially available cell proliferation assay (A). ■ SHC; ■ CSC. Data represent the mean + SEM. * represent $P < 0.05$ vs. respective SHC mice; # represent $P < 0.05$; ### represent $P < 0.001$ vs. respective basal. **B** Representative image of wells containing substrate of the proliferation assay and cells (red, 1) or substrate of the proliferation assay without cells (only medium; yellow, 2).

3.3 Analysis of the negative feedback at the level of the PVN

Statistical analysis revealed that GR (Fig. 35A/C) as well as MR (Fig. 35B/D) protein expression in the PVN were not changed following CSC exposure.

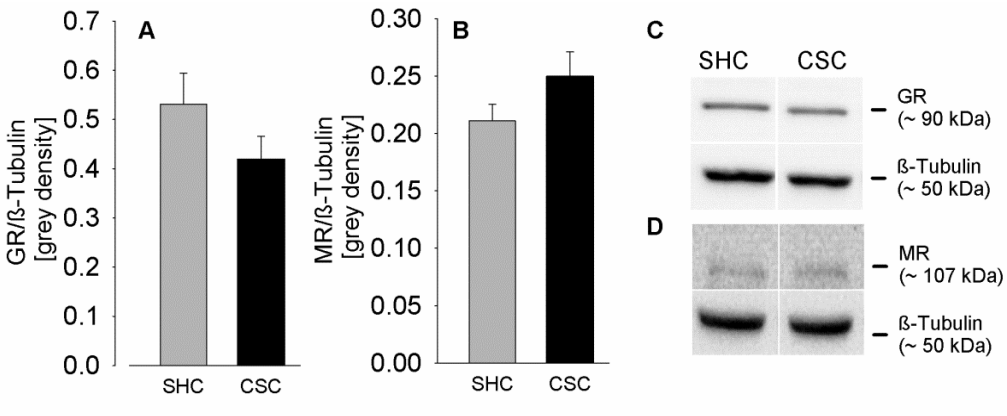

Figure 35: Effects of CSC on GR and MR protein expression in the PVN.

Following decapitation on day 20 brains of both SHC and CSC mice were removed and PVN tissue was punched out. Afterwards, protein was extracted from PVN punches for determination of GR (SHC: n= 11; CSC: n= 11; A/C) and MR (SHC: n= 7, CSC: n = 10; B/D) protein expression [grey density] normalized to the loading control ß-Tubulin. ■ SHC; ■ CSC. Data represent the mean + SEM. Representative images of bands detected for GR (~ 86 kDa; C) and MR (~ 107 kDa; D) and the loading control ß-Tubulin (~ 50 kDa C/D) are shown for SHC and CSC mice.

3.4 Analysis of the negative feedback at the level of the PFC

3.4.1 GR and MR protein expression

Statistical analysis revealed GR (*P* = 0.031; Fig. 36A/C) as well as MR (*P* = 0.003; Fig. 36B/D) protein expression in the PFC were increased in CSC compared with SHC mice.

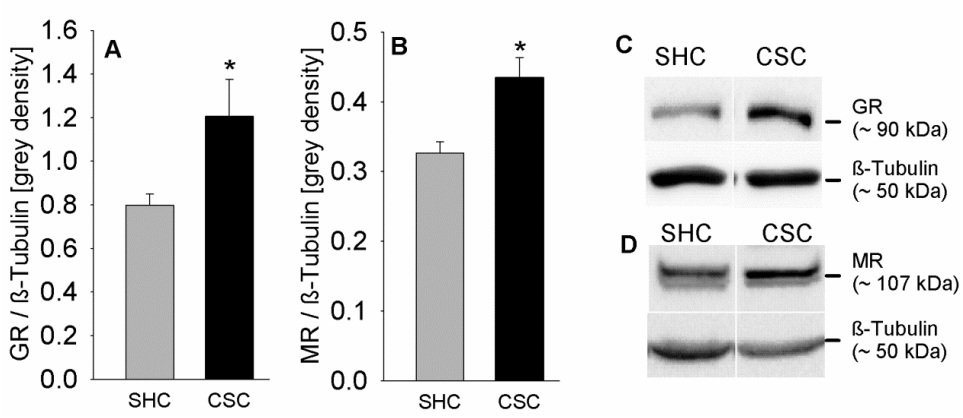

Figure 36: Effects of CSC on GR and MR protein expression in the PFC.

Following decapitation on day 20 brains were removed and the PFC was dissected of both SHC and CSC mice. Afterwards, protein was extracted for determination of GR (SHC and CSC: n = 18; A/C) and MR (SHC and CSC: n = 17; B/D) protein expression [grey density] normalized to the loading control ß-Tubulin. ■ SHC; ■ CSC. Data represent the mean + SEM. * represent *P* < 0.05, ** represent *P* < 0.01 vs. respective SHC mice Representative images of bands detected for GR (~ 86 kDa; C), MR (~ 107 kDa, D) and the loading control ß-Tubulin (~ 50 kDa C/D) are shown for SHC and CSC mice.

3.4.2 FKBP51 protein expression

Statistical analysis revealed that FKBP51 protein expression in the PFC was increased in CSC compared with SHC mice (*P* = 0.015; Fig. 37).

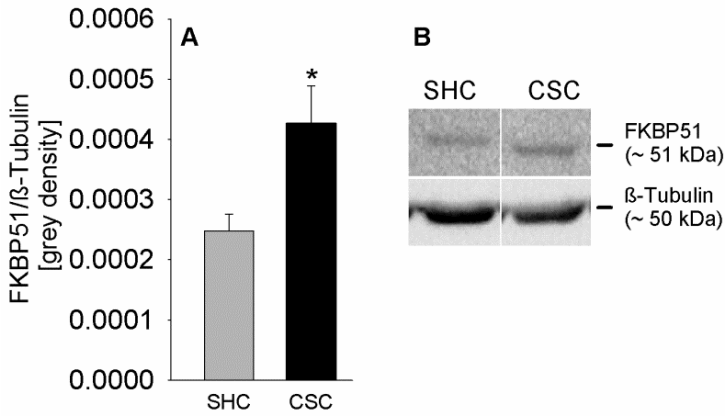

Figure 37: Effects of CSC on FKBP51 protein expression in the PFC.

Following decapitation on day 20 brains were removed and the PFC was dissected of both SHC (n = 16) and CSC (n = 17) mice. Afterwards, protein was extracted for determination of FKBPy51 protein expression [grey density] normalized to the loading control ß-Tubulin (A/B). ■ SHC; ■ CSC. Data represent the mean + SEM. * represent *P* < 0.05 vs. respective SHC mice. (B) Representative images of bands detected for FKBP51 (~ 51 kDa) and the loading control ß-Tubulin (~ 50 kDa) are shown for SHC and CSC mice.

4 Influence of trait anxiety on the consequences of 19 days of CSC

To assess whether the genetic predisposition of high versus low anxiety-related behaviour determines the vulnerability to the consequences of chronic psychosocial stress, mHAB, mNAB and mLAB mice were either exposed to 19 days of CSC or kept as control (SHC) mice. First of all, SHC and CSC mice of all three genotypes were tested on the EPM (on day 19 of CSC) to determine the breeding line-specific as well as the chronic stress-induced consequences on anxiety-related behaviour. The next day, effects of CSC on physiological, neuroendocrine and immunological parameters were determined under basal conditions. This included assessement of the body weight gain as well as adrenal and spleen weight, measurement of plasma ACTH and CORT concentrations, *in vitro* adrenal CORT secretion in response to ACTH, GR, MR and FKBP51 protein expression in the pituitary, IFN-γ secretion from isolated mesLNC in response to anti-CD3 stimulation, and the histological damage score of the colon.

4.1 Anxiety-related behaviour on the EPM

The percentage of time spent on the open arm of the EPM was found to be dependent on the interaction of factors genotype and CSC ($F_{2,46}$ = 14.75; P < 0.001; Fig. 38A). *Post hoc* analysis revealed an effect of CSC on anxiety-related behaviour only in mNAB mice which spent significantly less time on the open arm compared with respective SHC mNAB mice (P < 0.001). In contrast, CSC exposure did not alter anxiety-related behaviour in the mHAB and mLAB groups. Line-dependent differences in anxiety could be confirmed, as both SHC and CSC mice in the mLAB group spent significantly more time on the open arm compared to respective mice in the mNAB (P < 0.001) and mHAB groups (P < 0.001), and mNAB SHC mice further spent significantly more time on the open arm compared to the respective mice in the mHAB group (P < 0.001).

The percentage of entries into the open arm was found to be dependent on the interaction of factors genotype and CSC ($F_{2,46}$ = 3.82; *P* = 0.029; Fig. 38B). *Post hoc* analysis revealed that CSC mice showed a decreased percentage of open arm entries compared with SHC mice in the mNAB (*P* = 0.045), but not in the mHAB and mLAB groups. Moreover, CSC mice in the mLAB group showed a significantly higher percentage of open arm entries compared with respective mice in the mHAB (*P* < 0.001) and mNAB groups (*P* < 0.001). Line-dependent differences were confirmed as SHC mice of the mNAB and mLAB group showed a higher percentage of open arm entries compared to respective mice in the mHAB group (*P* < 0.001).

Full entries into the open arms as additional indicative of anxiety-related behaviour were also found to be dependent on the interaction of factors genotype and CSC ($F_{2,46}$ = 3.80; *P* = 0.030; Fig. 38C). *Post hoc* analysis revealed that CSC mice showed less full entries compared with SHC mice in the mNAB (*P* = 0.004), but not in the mHAB and mLAB group. Again, line-dependent differences were confirmed as both SHC and CSC mice in the mLAB group showed more full entries compared to respective mice in the mNAB (SHC: *P* = 0.010; CSC: *P* < 0.001) and mHAB (*P* < 0.001) groups, and mNAB SHC mice showed a higher number of full entries compared to mHAB SHC mice (*P* = 0.002).

The number of entries into the closed arms, indicative of locomotor activity, was not affected by CSC exposure (Fig. 38D). However, a genotype effect ($F_{2,46}$ = 5.45; *P* = 0.007) was found, with SHC mice of the mLAB group showing less closed arm entries compared to respective mice of the mHAB group (*P* = 0.002).

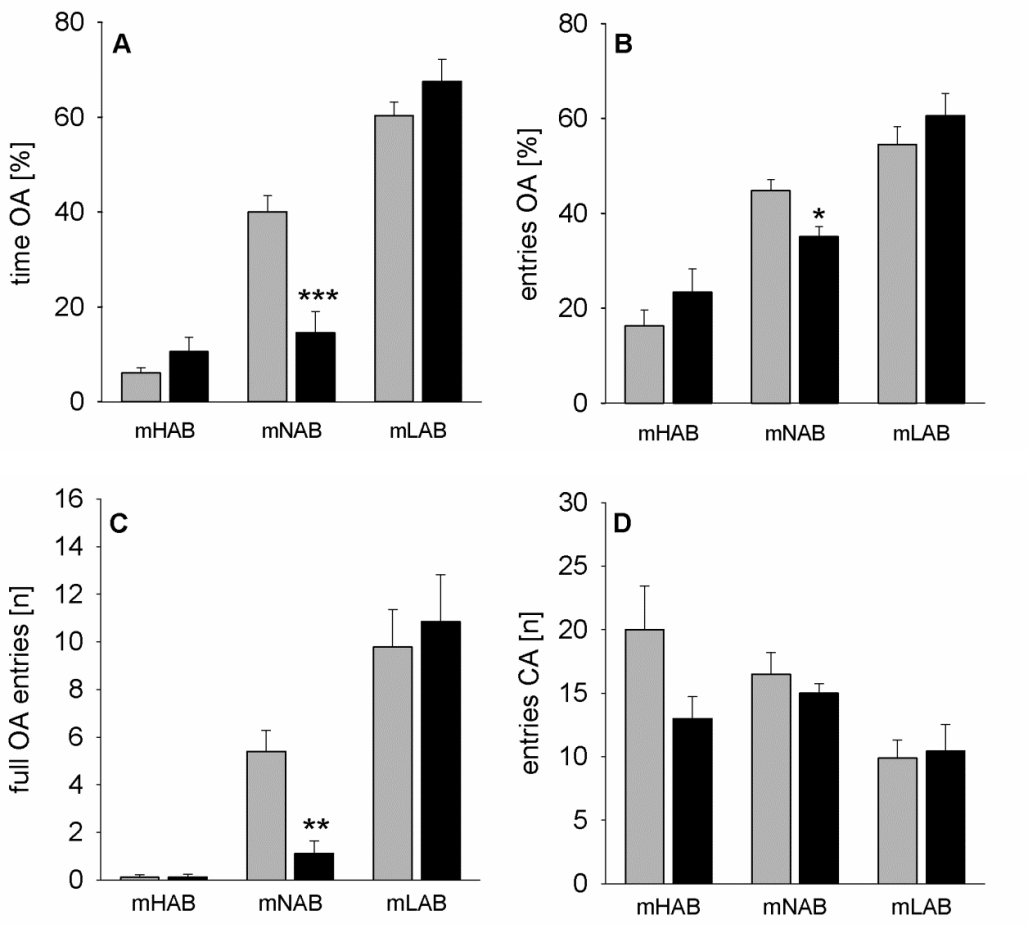

Figure 38: Genotype specific effects of CSC exposure on anxiety-related behaviour. To assess chronic psychosocial stress effects on anxiety-related behaviour, SHC (mHAB (n = 9); mNAB (n = 10); mLAB (n = 9)) and CSC (mHAB (n = 8); mNAB (n = 9); mLAB (n = 7)) were exposed to the elevated plus-maze (EPM) on day 19 of CSC. Data show the % of time on open arms (OA; A), the % of entries on OA (B), the number of full OA entries [n] (C) and the number of entries into closed arms (CA) (D). Symbols indicating significant differences are only shown for effects between SHC and CSC. For significant within-group differences see detailed information given in the results. ■ SHC; ■ CSC. Data represent the mean + SEM. * represent $P < 0.05$, ** represent $P < 0.01$, *** represent $P < 0.001$ *vs.* respective SHC mice. [adapted from (Füchsl et al., **A** *submitted*)]

4.2 Assessement of physiological parameters

Body weight gain

The body weight gain during CSC was also dependent on the interaction of factors genotype and CSC ($F_{2,46}$ = 4.25; *P* = 0.02; Fig. 39A). *Post hoc* analysis revealed that mLAB CSC mice gained significantly more body weight compared with respective SHC mice (*P* = 0.005), as well as compared with CSC mice in the mHAB (*P* = 0.006) and mNAB (*P* = 0.034) groups.

Absolute adrenal weight

Two-way ANOVA revealed that absolute adrenal weight was dependent on factor CSC ($F_{1,45}$ = 9.27; *P* = 0.004) and factor genotype (genotype: $F_{2,45}$ = 37.01; *P* < 0.001; Fig. 39B). In detail, CSC caused an increase in absolute adrenal weight in the mNAB (*P* = 0.043) and mHAB (*P* = 0.007), but not mLAB group when compared with respective SHC mice. With respect to line-difference, adrenal weight of both SHC and CSC mNAB mice was higher compared with respective mHAB (SHC and CSC: *P* < 0.001) and mLAB (SHC: *P* = 0.005; CSC: *P* < 0.001) mice and adrenal weight of mLAB SHC mice was increased compared with respective mHAB mice (*P* = 0.008).

Absolute spleen weight

Absolute spleen weight was found to be dependent on the interaction of factors CSC and genotype ($F_{2,20}$ = 3.67; *P* = 0.044; Fig. 39C). *Post hoc* analysis revealed that CSC significantly (mNAB: *P* < 0.001) or by trend (mHAB: *P* = 0.059) increased absolute spleen weight in the mNAB and mHAB, but not in mLAB, groups when compared with respective SHC mice (*P* < 0.001). Moreover, absolute spleen weight in mLAB SHC was higher than in respective mHAB (*P* < 0.001) and mNAB (*P* < 0.001) mice as was spleen weight in mLAB CSC compared with mHAB CSC mice (*P* = 0.011) and, by trend, with mNAB CSC mice (*P* = 0.051).

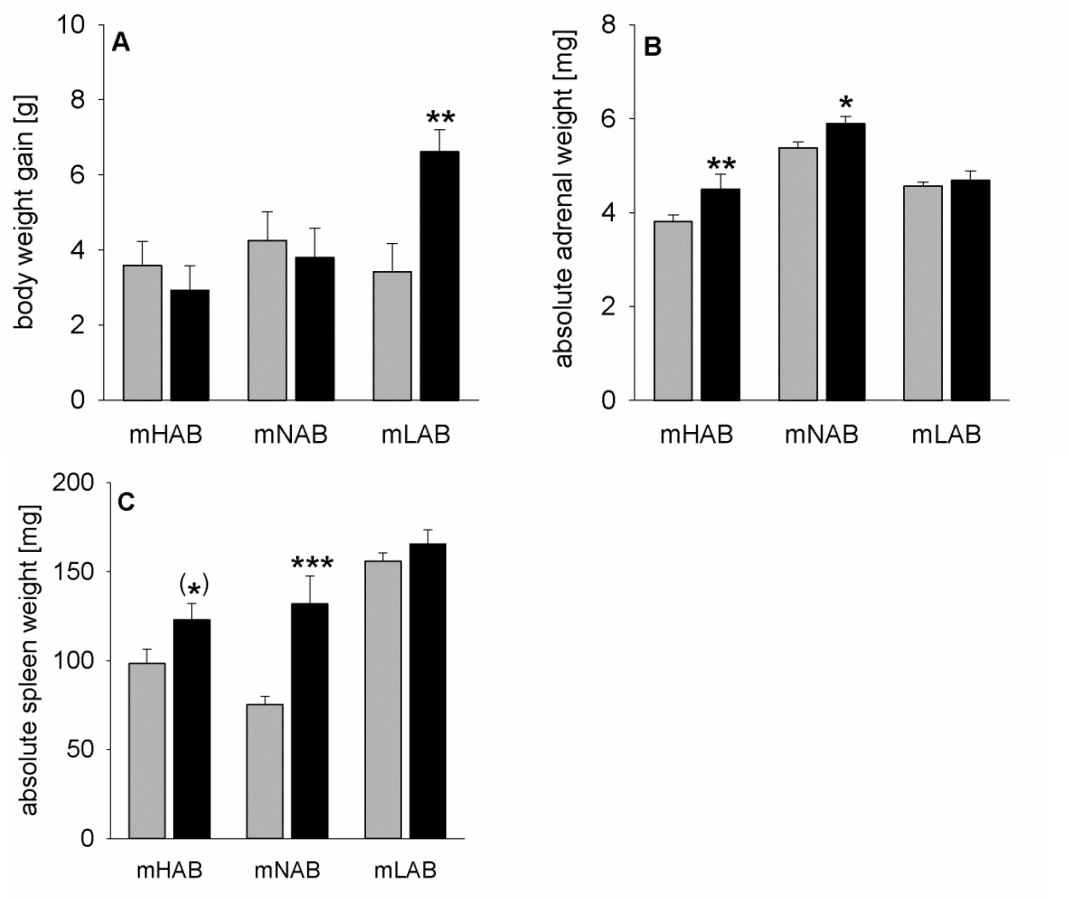

Figure 39: Genotype specific effects of CSC exposure on body weight gain, adrenal and spleen weight.

Before being killed by decapitation on day 20 of CSC between 0800 and 1000 h, body weight was assessed in SHC (mHAB (n = 10); mNAB (n = 10); mLAB (n = 9) and CSC (mHAB (n = 7)); mNAB (n = 9); mLAB (n = 7)) mice and body weight gain between day 1 and 20 was calculated (A). Afterwards, left and right adrenal glands were removed, pruned of fat and weight separately. Depicted is the absolute weight [mg] of the left and right adrenal glands (sum of both) of SHC (mHAB (n = 10); mNAB (n = 9); mLAB (n = 9)) and CSC (mHAB (n = 8); mNAB (n = 8); mLAB (n = 7)) mice (B). Spleens of SHC (mHAB (n = 5); mNAB (n = 5); mLAB (n = 4)) and CSC (mHAB (n = 4); mNAB (n = 4); mLAB (n = 4)) mice were also removed, pruned of fat and weighed (C). Symbols indicating significant differences are only shown for effects between SHC and CSC. For significant within-group differences see detailed information given in the results. ■ SHC; ■ CSC. Data represent the mean + SEM. * represent $P < 0.05$, ** represent $P < 0.01$, *** represent $P < 0.001$*vs.* respective SHC mice, (*) represent a trend *vs.* respective SHC mice. [adapted from (Füchsl et al., **A** *submitted*)]

4.3 Assessement of neuroendocrine parameters

4.3.1 Plasma CORT and ACTH concentrations

Plasma ACTH concentrations

Basal plasma ACTH concentrations were only found to be dependent on factor CSC ($F_{1,45}$ = 8.57; *P* = 0.005; Fig. 40A) with CSC mHAB mice showing increased plasma ACTH concentrations compared with respective SHC (*P* = 0.003) mice. CSC exposure did not alter plasma ACTH in mNAB and mLAB mice.

Plasma CORT concentrations

Plasma morning CORT concentrations were also dependent on the interaction of factors genotype and CSC ($F_{2,43}$ = 6.04; *P* = 0.005; Fig. 40B). *Post hoc* analysis revealed that CSC only reduced plasma CORT concentrations in mNAB mice (*P* = 0.001 versus SHC), but not in the mHAB and mLAB groups. Moreover, plasma CORT concentrations of mNAB SHC mice were higher compared with those of SHC mice in the mHAB (*P* < 0.001) and mLAB (*P* = 0.001) groups, but no significant difference was found between mHAB and mLAB SHC mice.

Plasma CORT:ACTH ratio

Statistical analysis revealed that the plasma CORT:ACTH ratio was only dependent on factor CSC ($F_{1,42}$ = 9.53; *P* = 0.004; Fig. 40C) with CSC compared with SHC mice showing a significantly (mNAB: *P* = 0.027) or by trend (mHAB: *P* = 0.061) reduced plasma CORT:ACTH ratio in the mHAB and mNAB, but not in the mLAB, groups.

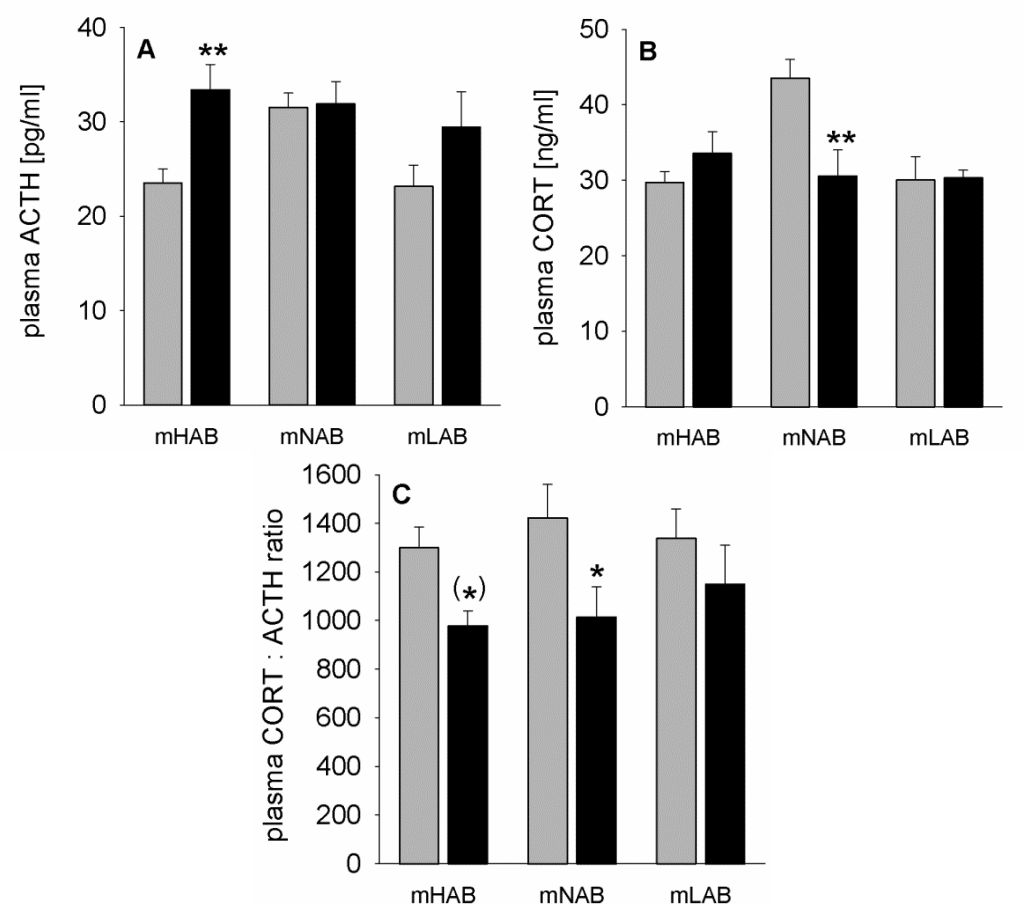

Figure 40: Genotype specific effects of CSC exposure on basal morning plasma ACTH and CORT concentrations in trunk blood.

Following decapitation on day 20 of CSC between 0800 and 1000 h, basal morning plasma ACTH [pg/ml] (A) and plasma CORT [ng/ml] (B) concentrations were determined in trunk blood of SHC (ACTH: mHAB (n = 10); mNAB (n = 10); mLAB (n = 9); CORT: mHAB (n = 10); mNAB (n = 9); mLAB (n = 9)) and CSC (ACTH: mHAB (n = 8); mNAB (n = 7); mLAB (n = 7); CORT: mHAB (n = 7); mNAB (n = 7); mLAB (n = 7)) mice in the mHAB, mNAB and mLAB groups. Moreover, the plasma CORT : ACTH ratio was calculated for each mouse and averaged per treatment group (SHC: mHAB (n = 10); mNAB (n = 9); mLAB (n = 9); CSC: mHAB (n = 7); mNAB (n = 6); mLAB (n = 7); C). Symbols indicating significant differences are only shown for effects between SHC and CSC. For significant within-group differences see detailed information given in the results. ■ SHC; ■ CSC. Data represent the mean + SEM. * represent $P < 0.05$, ** represent $P < 0.01$ *vs.* respective SHC mice, (*) represent a trend *vs.* respective SHC mice. [adapted from (Füchsl et al., **A** *submitted*)]

4.3.2 Adrenal *in vitro* ACTH responsiveness

In vitro CORT secretion during the stimulation of adrenal explants with ACTH (100 nM) was dependent on the interaction of factors genotype and ACTH ($F_{2,40}$ = 16.33; $P < 0.001$; Fig. 41). *Post hoc* analysis revealed that in response to ACTH, a significant increase in CORT release was found in SHC mice of all three genotypes (mNAB: $P = 0.001$; mHAB: $P = 0.028$; mLAB: $P < 0.001$) when compared with basal conditions. In contrast, ACTH-induced increase in CORT secretion in CSC mice was seen only in the mLAB ($P < 0.001$ versus basal), but not in the mHAB and mNAB group. Moreover, ACTH-stimulated CORT secretion was significantly higher in both SHC and CSC mice of the mLAB group compared with respective mice of both the mHAB (SHC and CSC: $P < 0.001$) and mNAB (SHC: $P = 0.046$; CSC: $P < 0.001$) group.

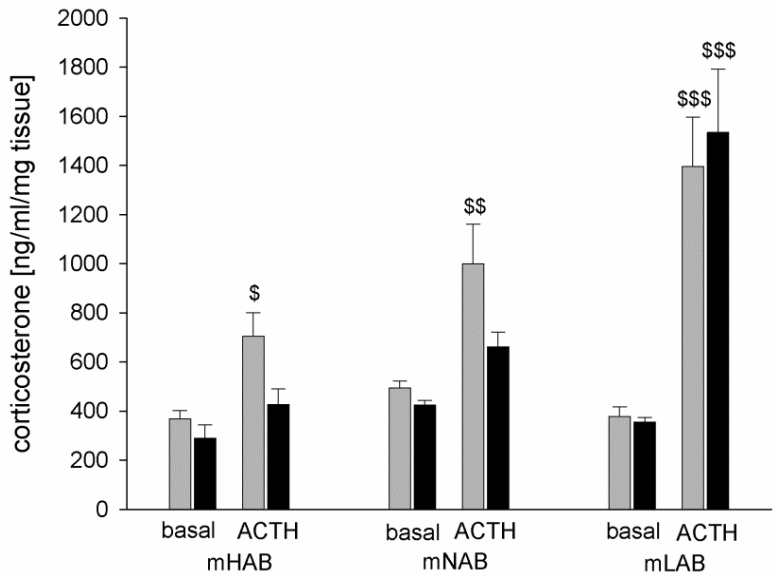

Figure 41: Genotype specific effects of CSC exposure on *in vitro* adrenal CORT secretion in response to ACTH.

Following decapitation on day 20 of CSC between 0800 and 1000 h, left and right adrenal glands were removed, pruned of fat and weighed separately. Afterwards, left and right adrenal glands were cut into two halves each. These halves of SHC (mHAB, mNAB: n = 5; mLAB: n = 4) and CSC (n = 4) mice were weighed again and incubated with medium containing either saline (basal) or 100 nM ACTH for 6 h. Afterwards, CORT concentrations [ng/ml/mg tissue] were determined in the supernatants. Symbols

indicating significant differences are only shown for effects between basal and ACTH values. For significant within-group differences see detailed information given in the results. ▒ SHC; ■ CSC. Data represent the mean + SEM. $^{\$}$ represent $P < 0.05$, $^{\$\$}$ represent $P < 0.01$, $^{\$\$\$}$ represent $P < 0.001$ *vs.* respective basal values. [adapted from (Füchsl et al., **A** *submitted*)]

4.4 Assessement of immunological parameters

IFN-γ secretion from isolated and anti-CD3-stimulated mesenteric lymph node cells (mesLNC)

To determine genotype-specific effects of CSC on the IFN-γ secretion from anti-CD3-stimulated mesLNC, mesLN from all 4 – 5 mice of each treatment group per experiment were pooled before isolation of mesLNC. IFN-γ concentrations of each single pool (2 pools per treatment group, as the experiment has been repeated) as well as the respective mean (both in pg/ml) are depicted in Figure 42A. Although proper statistical analysis has not been performed due to the low number of pools per treatment group (n = 2), our data indicate that CSC caused an increase in IFN-γ secretion from isolated and anti-CD3-stimulated mesLNC compared with SHC mice in the mHAB (SHC: 80 $\pm$ 20 pg/ml; CSC: 2071 $\pm$ 1375 pg/ml) and NAB (SHC: 178 $\pm$ 31 pg/ml; CSC: 743 $\pm$ 164 pg/ml), but not the mLAB (SHC: 96 $\pm$ 22 pg/ml; CSC: 122 $\pm$ 5 pg/ml), group.

Histological damage score of the colon

The histological damage score was neither affected by factor CSC nor by factor genotype (Fig. 42B).

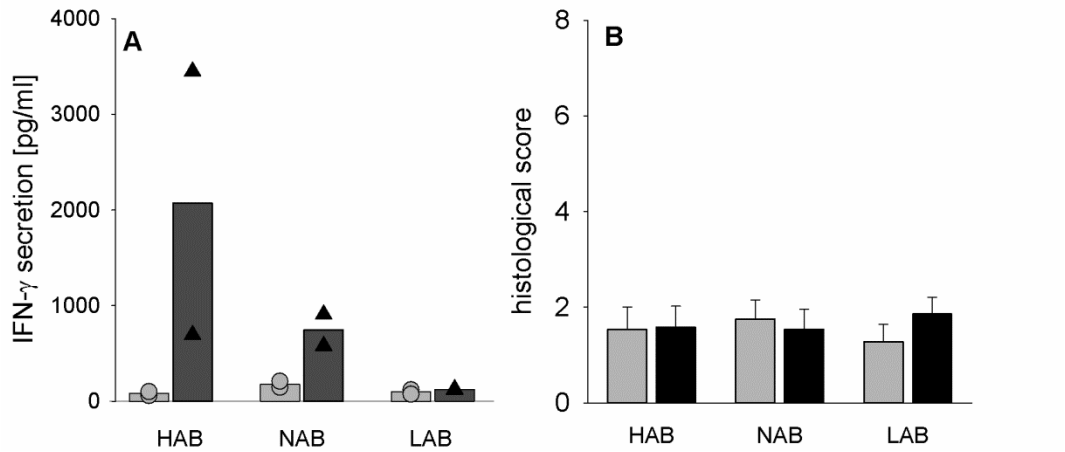

Figure 42: Genotype specific effects of CSC exposure on the anti-CD3-stimulated IFN-γ secretion from isolated mesenteric lymph node cells and on the histological damage score.

In each experiment (experiment 1 and 2) mice were decapitated on day 20 of CSC between 0800 and 1000 h. Afterwards, mesenteric lymph node cells were isolated and pooled per treatment group (one pool per experiment) for assessment of anti-CD3-induced IFN-γ secretion [pg/ml] (A). The respective means per treatment group are shown by bars; circles (SHC) and triangles (CSC) represent single pools of each experiment. Furthermore, the colon of each SHC (mHAB (n = 10); mNAB (n = 10); mLAB (n = 9)) and CSC (mHAB (n = 8); mNAB (n = 9); mLAB (n = 7)) mouse of experiment 1 and 2 was removed, mechanically cleaned, rinsed, embedded in paraffin, and cut in 3 µm sections on a microtome. Afterwards all sections were stained with haematoxylin-eosin and histological scoring was performed (B). ■ SHC; ■ CSC. Data represent the mean + SEM. [adapted from (Füchsl et al., **A** *submitted*)]

4.5 GR, MR and FKBP51 protein expression in the pituitary

GR (Fig. 43A) and MR (Fig. 43B) protein expression in the pituitary following CSC exposure were neither dependent of the factor genotype nor on the factor CSC. Only FKBP51 protein expression was dependent on the factor genotype ($F_{2,20}$ = 4.14; *P* = 0.031; Fig. C). *Post hoc* analysis revealed that in mNAB CSC mice, FKBP51 was significantly higher expressed compared with respective mHAB mice (*P* = 0.033).

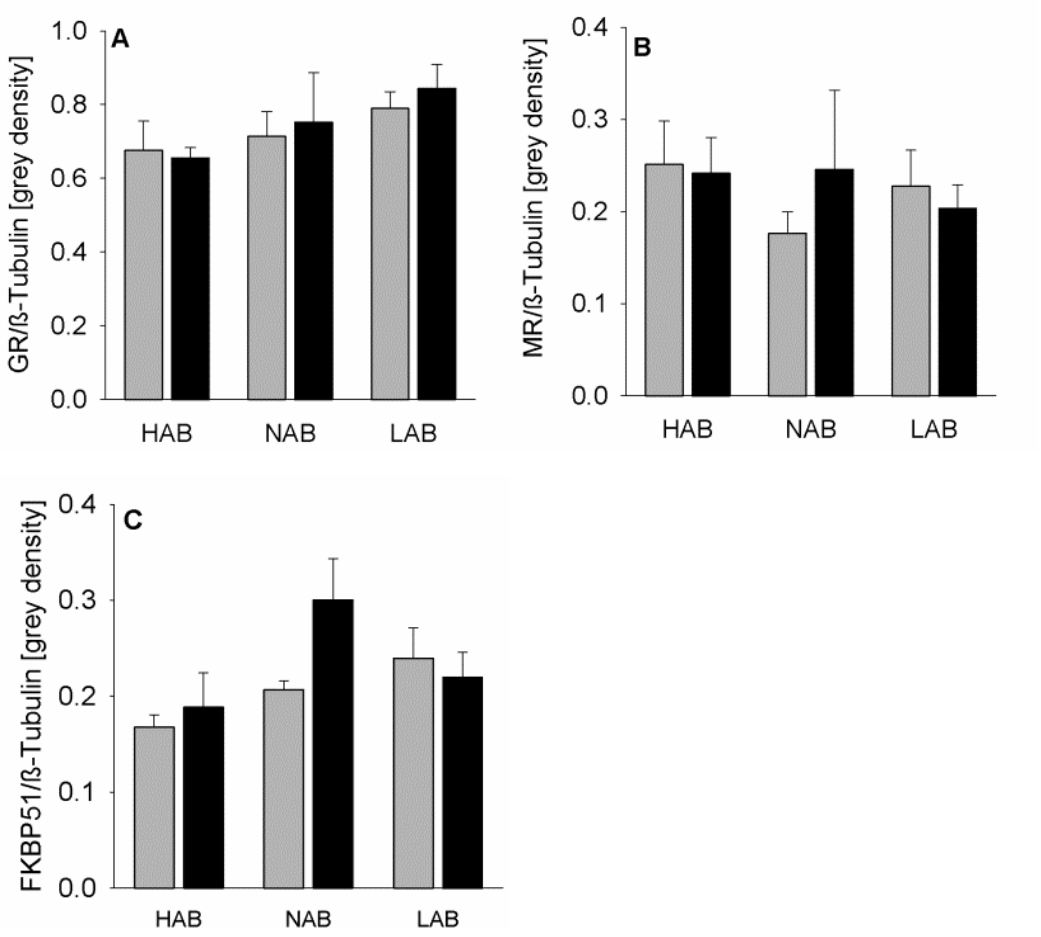

Figure 43: Genotype specific effects of CSC exposure on GR, MR and FKBP51 protein expression in the pituitary.

Following decapitation on day 20 of CSC pituitaries of both SHC and CSC mice were removed for protein extraction and subsequent determination of GR (SHC: n = 5, CSC: n = 4; A), MR (SHC: mNAB and mLAB (n = 5), mHAB (n = 4), CSC: n = 4; B) and FKBP51 (SHC: n = 5, CSC: n = 4; C) protein expression [grey density] normalized to the loading control ß-Tubulin. ■ SHC; ■ CSC. Data represent the mean + SEM.

CHAPTER 4

DISCUSSION

1 Effects of CSC on HPA axis functionality – a closer look at the level of the pituitary and PVN

1.1 Do adaptations at the level of the pituitary contribute to changes in HPA axis functionality following CSC?

The acute stress response, i.e. the activation of the SNS and the HPA axis, represents an adaptive and beneficial mechanism, promoting the survival of an organism. In contrast, prolonged activation of these stress systems under conditions of chronic stressor exposure can have deleterious effects on the body, contributing to the development of somatic as well as affective disorders (McEwen, 1998a; Chrousos and Kino, 2009). Therefore, to protect the organism from the consequences of prolonged or repeated stimulation with the same (homotypic) stressor, which is not life threatening, a process called adaptation is of major importance. This was shown in human (Kirschbaum et al., 1995; Schommer et al., 2003) as well as in animal studies (De Boer et al., 1988; Dobrakovova et al., 1993). However a process called sensitization enables the organism to respond to a subsequent acute heterotypic and probably dangerous stressor with an enhanced HPA axis response (Aguilera, 1994).

Such processes of adaptation and sensitization were also shown to occur in mice exposed to chronic psychosocial stress induced by the CSC paradigm. Thereby, especially the adrenal glands seem to play a major role (Uschold-Schmidt et al., 2012; Füchsl et al., 2013). Nevertheless, given that also other levels of the HPA axis might be involved in the prozess of adaptation/sensitization, the first aim of my thesis was to test the hypothesis that also pituitary mechanisms are involved in the observed HPA axis processes seen following 19 days of CSC.

In contrast to a desensitized HPA axis response to many repeated or chronic physiological and psychological stress paradigms (Hauger et al., 1990; Aguilera, 1994; Zelena et al., 2003), mice exposed to 19 days of CSC displayed increased basal plasma ACTH concentrations in the morning, even though plasma CORT levels were unchanged at that time of the day (Reber et al., 2007; Uschold-Schmidt et al., 2012), which gives further insight into the adrenal functionality and

ACTH sensitivity. Under *in vitro* conditions, enlarged adrenals showed a reduced responsiveness to ACTH stimulation following CSC exposure (Reber et al., 2007; Uschold-Schmidt et al., 2012). This could now also be demonstrated under *in vivo* conditions, as increased plasma ACTH levels were not paralleled by enhanced CORT levels. This adrenal ACTH insensitivity during/following CSC exposure might be beneficial in terms of protecting the body from increased CORT levels one would normally expect regarding the increased adrenal weight and ACTH levels. Interestingly, in a recently published study, me and colleagues could show that hyperplasia of adrenal cortical cells goes along with an enhanced availability and also capability to mobilize cholesterol in CSC mice (Füchsl et al., 2013), demonstrating that adrenals are still functional and, in case of normal ACTH responsiveness, probably would produce and secret more CORT than normally needed, resulting in hypercorticism.

In addition, ACTH release in response to a subsequent heterotypic stressor (6-min of FS) was exaggerated in CSC compared with SHC mice, indicating not only a hyper-activity under basal but also a hyper-reactivity of the pituitary following acute stressor exposure. This facilitation of the ACTH response to this novel superimposed stressor is in line with other chronic stress studies (Hauger et al., 1990; Bhatnagar and Vining, 2003) but in contrast to our own recent data showing increased plasma CORT and similar plasma ACTH levels 5 min following termination of a 5-min EPF exposure (Uschold-Schmidt et al., 2012). This might be due to the fact that the type of acute stressor as well as the temporal study design differed between the two studies. Given that plasma ACTH levels following FS were 5 – 10 times higher compared with those following EPF exposure clearly indicates that FS represents a much more severe stressor. The second main difference is the time span that elapsed between acute stressor exposure and plasma sampling. While mice in the Uschold-Schmidt study (2012) were killed only 5 min after termination of the EPF exposure, in the present study 10 min elapsed. The comparison of both studies suggests that the increased plasma ACTH response to an acute heterotypic stressor following CSC exposure needs a certain time and/or stressor intensity to develop. Unfortunately, due to

methological limitations, repeated assessement of plasma ACTH levels following acute stressor exposure in order to get more information about the ACTH release over the time is not possible in mice.

Overall, measurement of plasma CORT and ACTH levels under chronic and subsequent heterotypic stressor exposure in previous studies and the present thesis indicate that the adaptation to the chronic stressor is mainly achieved at the level of the adrenal gland (Uschold-Schmidt et al., 2012). This mechanism protects the body from high CORT levels one would normally expect regarding the pituitary hyper-activity and adrenal enlargement. Moreover, the process of facilitation/sensitization of the HPA axis response to an acute heterotypic stressor seems to be realized by the interplay of adrenal (Uschold-Schmidt et al., 2012; Füchsl et al., 2013) and pituitary mechanisms.

One intention of the present thesis was, therefore, to unravel the mechanisms underlying the increased pituitary activity under basal as well as re-activity following acute heterotypic stressor exposure in CSC compared with SHC mice.

1.2 Are increased plasma ACTH levels mediated by the pituitary?

The increased pituitary weight on day 28 of CSC, i.e. 8 days after termination of the CSC exposure (Slattery et al., 2012) gives a first hint how the increased plasma ACTH levels in CSC mice might be mediated. Also on day 20 of CSC I was able to confirm the enlargement of the pituitary which is already an important finding *per se*, demonstrating that the CSC-induced effects are reliable and long-lasting. An increased number of isolated pituitary cells and, most importantly, corticotroph pituitary cells in CSC compared with SHC mice indicates that the increase in the pituitary weight is mediated by hyperplasia of ACTH producing cells. The latter was indicated by an increased percentage of ACTH positive pituitary tissue as well as number of ACTH positive cells in CSC compared with SHC mice. This finding is of importance, given that the pituitary consists of five major cell types (Yeung et al., 2006) in the anterior pituitary, whereby the corticotroph cells are characterized by the production and secretion of ACTH. There are only a few studies examining alterations of the pituitary morphology

following chronic stress or long-term GC administration. Chronic mild (placing rats in a plastic holder) or severe (restraining rats with limbs extended) immobilization (5 h/day, 7 days) resulted in an increase in the relative pituitary weight as well as in the number of ACTH-immunoreactive cells (Kapitonova et al., 2010). Also ADX caused a sustained proliferation of corticotroph cells, probably mediated by AVP (Subburaju and Aguilera, 2007) while Dex administration in pregnant and lactating rats reduced the number of corticotroph cells in the offspring (Theogaraj et al., 2005). Nevertheless the increased number of ACTH positive cells in CSC compared with SHC mice together with unchanged relative (per mg pituitary tissue) POMC mRNA and protein expression strongly suggest that the newly formed corticotroph cells are fully functional and, therefore, mediate the increased basal and acute stressor-induced ACTH secretion in CSC mice.

Together, these findings, i.e. the increased pituitary weight mediated by hyperplasia of corticotroph cells, suggest that the increased plasma ACTH levels in CSC mice might be the result of changes at the level of the pituitary.

Nevertheless, one cannot exclude that the increased pituitary activity and re-activity might in addition be mediated by an increased stimulatory input from the PVN, acting on AVPR-1b and CRH-R1, the two main ACTH secretagogue receptors. Unaffected CRH-R1 mRNA and decreased protein expression in CSC compared with SHC mice are in line with the described discrepancy between CRH-R1 mRNA and CRH binding (Aguilera et al., 2004) and also with the decreased expression found in chronic stress paradigms associated with an hyperresponsiveness to an acute heterotypic stressor (Kiss and Aguilera, 1993; Aguilera, 1994). These stress paradigms are also characterized by increased AVPR-1b expression, mediated by AVP, GC or other neuropeptides in the pituitary portal blood (Aguilera, 1994; Aguilera and Rabadan-Diehl, 2000b). However, this could not be confirmed following CSC exposure given that AVPR-1b mRNA as well as protein expression were not affected. This might probably be due to the different type of stressors used or to differences in the release pattern of the factors mediating this increased expression.

Importantly, while the relationship between AVPR-1b expression and corticotroph responsiveness is supposed to correlate, this seems not to be the case for the CRH-R1 (Aguilera, 1994; Aguilera et al., 1994; Nikodemova et al., 2002). In chronically restraint rats, despite down-regulated CRH-R1 expression in the pituitary, the *in vitro* response to CRH was only partially diminished, while the ACTH release *in vivo* was not constrained at all (Hauger et al., 1988). Transferred to the results of the present thesis, this suggests that despite a decreased CRH-R1 protein expression, the response to CRH should be unchanged in CSC mice, which might also be the case in respone to AVP regarding the unchanged AVPR-1b mRNA and protein expression following CSC exposure. Together, these findings indicate that an increased receptor-mediated stimulation of pituitary cells seems, at the first glance, not to be responsible for the increased plasma ACTH levels following CSC exposure.

However, the data of the relative receptor expression might also implicate, that the newly formed corticotroph cells preferentially express the AVPR-1b and not the CRH-R1 indicating a shift in the sensitivity from CRH to AVP following CSC exposure. This would be in line with other studies, assigning AVP an important role during chronic or prolonged stressor exposure (Ma et al., 1999). Nevertheless, to really confirm this hypothesis, further studies are needed. First of all, the ACTH release in pituitary explants or in isolated pituitary cells in response to different doses of CRH, AVP and the combination of both must be quantified *in vitro*. Moreover, the signalling pathways of both receptors should be checked by measurement of cAMP or IP3 release in response to CRH or AVP stimulation, respectively. In addition an IHC staining for the co-expression of proliferation markers with ACTH, AVPR-1b and CRH-R1 would give further insights into the exact receptor expression pattern.

1.3 Is there an influence of the PVN on the increased plasma ACTH secretion?

Not only changes in receptor expression of the CRH-R1 and AVPR-1b might be implicated in an enhanced pituitary activity but also an increased stimulatory input due to an exaggerated production and/or release of the ACTH secretagogues. Based on the fact that the analysis of the AVPR-1b already suggested a more prominent role for AVP than for CRH on the ACTH secretion pattern following CSC, I decided to focus on the quantification of the number of AVP positive neurons in the PVN via IHC.

Further support for the analysis of PVN AVP protein is provided by previous data, revealing a decrease in AVP mRNA expression in the PVN following CSC exposure (Reber and Neumann, 2008a). At least in the SON, a reduction in the mRNA level was shown to be the result of an increased mRNA turn over, resulting in increased AVP protein synthesis (Yoshii et al., 2008). Moreover, AVP has been shown to exert a mitogenic effect in a number of cell types, amongst others corticotroph cells (van Wijk et al., 1995; Aguilera et al., 2008), indicating that AVP might be implicated in mediating the increased number of corticotroph cells following CSC

For the analysis of AVP protein expression, the PVN was divided into four different layers, namely the rostral, rostral-medial, medial-caudal and caudal part of the PVN (see also Fig. 18). Moreover, the analysis included the discrimination between magno- and parvocellular AVP positive neurons in the PVN, as magnocellular neurons are thought to be mainly involved in osmotic functions (Cunningham and Sawchenko, 1991), while parvocellular neurons are responsible for mediating the stress-induced effects (Rivier and Vale, 1983). This was done according to the literature (Ma and Aguilera, 1999; Kadar et al., 2010), whereby the criteria were staining intensity, localization pattern within the PVN and size. Magnocellular neurons were characterized by their larger size (~ > 14 µm) and their higher AVP expression compared with parvocellular neurons.

However, analysis of the magno- and parvocellular AVP containing neurons revealed no differences between SHC and CSC mice in any of the measured

regions, refuting the hypothesis of an increased involvement of AVP itself. The finding is at first glance also in contrast to other chronic or repeated stress studies, reporting an increase in AVP mRNA and/or protein synthesis and release (Ma and Lightman, 1998; Ma and Aguilera, 1999). Nevertheless, the experimental approach and the way in which the analysis of the AVP positive PVN neurons was performed seem to be in accordance with other studies. In detail, according to my analysis, it was evident that AVP neurons are mainly localized in the mid part of the PVN, which is also described in another study conducted in mice (Kadar et al., 2010).

With respect to CRH, only mRNA data from previous studies are available, revealing no differences between SHC and CSC mice (Reber et al., 2007) and, therefore, indicate most likely no involvement of CRH in the increased ACTH release following CSC.

Overall, the available data of CRH and AVP indicate that these neuropeptides are not involved in the enhanced pituitary activity and reactivity. However, to further reveal the role of the two peptides, secretion of AVP and CRH has to be measured by analysis of the peptide stores in the zona externa of the median eminence in future studies. Therefore, animals are administered a low, nontoxic amount of colchicine, which disrupts the axonal transport of the secretagogues from their production (PVN) to their release site (median eminence). Consequently the amount of peptide in the median eminence can be measured (Berkenbosch et al., 1989; De Goeij et al., 1992; Romero et al., 1993).

Not only CRH and AVP, but also other hypothalamic factors, like OXT, can stimulate the ACTH release. OXT, at physiological concentrations stimulated ACTH release via mobilization of Ca^{2+} in pituitary cells *in vitro* (Link et al., 1992). Given that OXT receptors are only rarely expressed on corticotroph cells, the effect of OXT on ACTH secretion was shown to be mediated via the AVPR-1b (Schlosser et al., 1994). Regarding the CSC paradigm, in a previous study OXT mRNA expression was found to be unchanged at all time points measured during as well as at the end of the CSC (Reber and Neumann, 2008a). This was further supported by the finding of the present study indicating also no differences in the

number of OXT positive neurons in the PVN following CSC exposure. This finding indicates that an increased expression of OXT cannot account for the enhanced ACTH secretion following CSC exposure. Expression and release of OXT in response to chronic stress is only poorly investigated. Following chronic homotypic stressor exposure (restraint stress), an increase in the number of OXT-positive magnocellular neurons in the PVN (Zheng et al., 2010; Yoshimoto et al., 2012) and an increase in central OXT release was found (Babygirija et al., 2012). OXT release was demonstrated in response to various acute stressors, like shaker stress (Nishioka et al., 1998) or FS (Wotjak et al., 1998) or Morris water maze testing (Engelmann et al., 2006), while SD elicited no increased OXT release in the PVN and SON (Wotjak et al., 1996).

2 Effects of CSC on the negative feedback inhibition at different levels

Negative feedback inhibition of the HPA axis is another important regulatory mechanism, essential for the termination of the stress response. Alterations or especially a down-regulation of the negative feedback might be implicated in the increased basal as well as acute stress-induced plasma ACTH levels following CSC exposure and was, therefore, another key aspect of my thesis.

The feedback, mediated via MR and GR, takes place at four major sites, i.e. in the pituitary, PVN, hippocampus, and PFC. As following CSC exposure a decreased GC sensitivity in peripheral cells, namely in splenocytes (Reber et al., 2007) and in T helper 2 cells from peripheral lymph nodes (Schmidt et al., 2010b) was found, alterations in the negative feedback inhibition are not unlikely to occur. In addition, an increased pituitary weight in patients suffering from depression and psychosis (Garner et al., 2005; Pariante et al., 2005), is often associated with a decreased negative feedback inhibition which might also underlie Addison`s Disease, a disorder characterized by hyperplasia of corticotroph cells and adrenal insufficiency (McNicol and Carbajo-Perez, 1999). Therefore, I analysed, MR and GR mRNA and/or protein expression and to some extent also GR functionality at the different sites involved in the HPA axis feedback response.

2.1. Effects of CSC on MR, GR and FKBP51 expression in different brain regions

Hippocampus

Chronic stressor exposure, like repeated restraint stress is associated with neuronal cell damage, indicated by neuronal atrophy and decreased dendritic branches in the hippocampus (Watanabe et al., 1992). Whether this neuronal atrophy also results in changes in the hippocampus weight or volume is only poorly investigated. One study could show via magnetic resonance imaging, that hippocampal volume in repeatedly restraint rats was decreased (Lee et al.,

2009). Following CSC exposure, I could not detect any alterations in hippocampus weight as well as in the number of isolated hippocampal cells, whereby future studies are needed to clarify if other morphological changes, like loss of dendritic branching or if impairments in learning or memory took place.

A decreased GR mRNA and protein expression in the hippocampus at first glance indicated an increased negative feedback function. A down-regulation of GR mRNA and protein in the hippocampus is well known in response to stimulation by its own ligand, as well as in response to chronic stressor exposure (Spencer et al., 1991; Makino et al., 1995; Kitraki et al., 1999). Since plasma CORT levels were only elevated on day 2 of the CSC procedure and returned to basal levels afterwards (Reber et al., 2007) CORT levels alone cannot be ascribed to this effect. This would support a study showing that GC are causative for a first decrease of the GR, but their availability is not necessary for a sustained down-regulation of the receptor (Kitraki et al., 1999). Therefore, also other factors, like neurotransmitters or catecholamines are discussed (Herman, 1993; Tritos et al., 1999).

While chronic SD exposure resulted in an increased FKBP51 expression in the hippocampus (Wagner et al., 2012) no changes in the FKBP51 expression were found following CSC exposure. However, it is not easy to draw conclusions from the FKBP51 expression data. The unchanged FKBP51 expression together with the decreased GR expression might on the one hand indicate a reduced GR sensitivity and/or functionality. On the other hand, an increased GR functionality and, therefore, transcriptional activity might be implicated in the sustained FKBP51 expression despite reduced receptor levels. The latter hypothesis would be supported by other studies showing that repeated stressor exposure did not affect GR affinity for GC despite a decreased receptor expression in the hippocampus (Sapolsky et al., 1984). Therefore, to examine potential functional alterations of the hippocampal GR *in vitro*, I analysed its ability to induce gene expression of a GC-responsive gene in isolated hippocampal cells and the hippocampal cell viability in response to CORT stimulation. Activation of GR in hippocampal slices with 100 nM CORT (~ 34 ng/ml CORT), a concentration

measured following acute FS exposure in the hippocampus of rats (Droste et al., 2008), resulted in different transcriptional responses over time (Morsink et al., 2006). Importantly, the hnRNA of the Per1 gene has been shown to be up-regulated in the hippocampus 30 min after administration of CORT in rats (Conway-Campbell et al., 2010; Lightman and Conway-Campbell, 2010). In response to 0.1 µM (100 nM) CORT, a concentration were binding to the GR occurs (Karst and Joels, 2005), hippocampal cells of CSC mice showed a significant increase in Per1 hnRNA expression compared with vehicle stimulated cells, while only a trend towards an increase was found in SHC mice. Application of an extremely high CORT concentration (100 µM) increased Per1 hnRNA expression in SHC and to a much greater extent in CSC mice. Overall these data indicate that despite the decrease in hippocampal GR expression in CSC mice the receptor seems to even show an enhanced sensitivity and/or functionality regarding GC-mediated gene transcription.

Prolonged exposure of hippocampal cells to GCs leads to neuronal damages, an effect that can also be mimicked in primary hippocampal cells *in vitro*. Thereby, an impaired energy metabolism (de Leon et al., 1997) or increased Glu release, leading to excitotoxicity (Dragunow et al., 1985; Huxtable, 1989), are discussed as underlying mechanisms. In the present study I used CORT concentrations ranging from 0.1 to 1000 µM. Already the lower concentrations (1 and 10 µM) induced an increase in cell viability in CSC mice, indicating a stimulatory effect on cell proliferation. An effect seen in SHC mice only with a concentration of 100 µM, indicating that hippocampal cells of CSC mice are more sensitive towards lower CORT concentrations than those of SHC mice. In contrast, the extremely high concentration of 1000 µM CORT diminished cell viability in CSC compared with SHC mice and, therefore, induced hippocampal cell death, a phenomenon already described above, indicating again a higher sensitivity of the GR towards CORT in CSC mice. The results of the CSC mice are also in accordance to the literature, where GCs are shown to regulate neuronal processes in an U-shaped manner, i.e. low levels of CORT increase while high levels decrease neuronal excitability (Joëls and de Kloet, 1992; Kellendonk et al., 2002; Joëls, 2006).

Taken together, despite decreased GR expression in the hippocampus, analysis of GR-mediated Per1 gene expression and cell viability measurement in response to CORT stimulation clearly points towards an unaltered or even enhanced sensitivity and/or functionality of the GR, which might probably also underlie the unchanged FKBP51 expression. Nevertheless, future studies are needed to unravel the factors mediating the increased GR activity. Thereby, one possibility would be to analyse the phosphorylation pattern of the GR which plays an important role in its transcriptional activity (Galliher-Beckley and Cidlowski, 2009).

Owing to the high occurrence of MR in the hippocampus, the balance between MR and GR is of major importance in this brain region. Thus, the unchanged MR together with the decreased GR expression indicate that the balance is shifted towards the MR. Talking about MR and GR in the hippocampus, one has to keep in mind, that there are remarkable differences between these two receptors in coordinating the negative feedback response. Activation of the MR, whereby the rapid effects are mediated via the membrane-bound MR, maintains hippocampal output, resulting in an activation of the posterior/lateral BNST which in turn inhibits the PVN. In contrast, activated cytoplasmic GR decreases, in a delayed manner, hippocampal output, resulting in a disinhibiton of the PVN, a discrepancy which underlines the importance of the proper balance between MR and GR (de Kloet et al., 1998). In CSC mice the balance between both receptors is shifted towards an increased impact of MR-mediated effects in the hippocampus, favouring an increased inhibitory influence on the PVN. But, as the *in vitro* studies measuring GR functionality revealed even an increased functionalit, it makes it hard to clearly interpret the overall influence of the hippocampal receptors on the negative feedback regulation in CSC mice. Therefore, further studies are needed applying MR and GR antagonists to differentiate the influence of each receptor. This is also of importance for unraveling the functionality of the MR given the possibility that despite the unchanged expression its functionality is altered.

Interestingly, the basal mRNA expression pattern clearly shows that MR mRNA is at least 2 times higher expressed compared with GR mRNA, while the protein expression is regulated the other way round. This finding is in accordance to the literature, describing a 1.5 to 5 fold higher abundance of MR compared to GR mRNA in the rat hippocampus and a binding capacity 2 to 3 times higher for GR compared to MR (Herman, 1993). This discrepancy implicates a shorter half-life and an increased transcription rate of the MR to keep the protein expression at a stable level (Herman, 1993).

Prefrontal Cortex

In the PFC, protein expression of GR, MR and FKBP51 was increased in CSC compared with SHC mice. Regarding GR expression, the result is in line to what was found in chronically isolated rats (Djordjevic et al., 2009; Djordjevic et al., 2010) and is suggested to be mediated by an enhanced phosphorylation at serine 232 of the GR gene. This leads to an increased transcriptional activity mediated by the GR itself in the absence of high CORT concentrations (Adzic et al., 2009). Nevertheless, one has to keep in mind, that regulation of HPA axis activity during/following chronic stress is differentially regulated in the PFC, whereby the PL and the InL exert distinct functions (Jones et al., 2011; McKlveen et al., 2013). Given that in the present thesis Western Blot analysis was used for GR measurement, it was not possible to differentiate between PL and InL PFC. Following acute stressor exposure, the GR in the PL as well as in the InL PFC is implicated in the inhibition of the ACTH and CORT response (Jones et al., 2011; McKlveen et al., 2013). Loss of the receptor in the InL PFC leads to increased CORT release in response to an acute heterotypic stressor in rats that underwent chronic stressor exposure. Moreover, the GR in the InL PFC seems to be involved not only in the physical but also in the emotional response to chronic stress, as a decrease of the GR in this locus seems to be responsible for the depressive-like behaviour observed in rats exposed to chronic variable stress (McKlveen et al., 2013). Given that CSC mice show no signs of depressive-like behaviour (Slattery et al., 2012) this might probably or at least in part, be due to

the increased GR expression mediated by the InL PFC. However, according to the study of McKlveen and colleagues (2013), an increased GR expression in the InL PFC decreases the CORT respone to an acute stressor in chronically stressed rats. This is clearly in contrast to what we see in CSC mice following 5-min of EPF exposure (Uschold-Schmidt et al., 2012), but might be due to the different kind of stressors and the temporally study design used. During chronic stressor exposure, the PL PFC is only implicated in regulating the basal morning CORT release (McKlveen et al., 2013). As plasma morning CORT levels are unchanged and plasma evening levels are decreased following CSC exposure (Reber et al., 2007; Uschold-Schmidt et al., 2012), this might be due to an increased GR-mediated effect of the PL region.

Importantly, with regard to the differences in GR expression and functionality in the hippocampus, it might also be possible that the increased GR expression is accompanied by an altered or even reduced functionality. Therefore, future studies are needed to reveal first of all via IHC if the GR is increased in both divisions and secondly, if the functionality of the GR in the PFC is altered.

Interestingly, in a recent study it could be shown that CRH neurons located in the PFC colocalize with GR which can be recruited to the CRH promoter and regulate CRH expression (Meng et al., 2011). Hence the increased GR levels in the PFC of CSC mice could probably contribute to an altered CRH expression which might be implicated in the increased anxiety-related behaviour displayed by CSC mice (Reber et al., 2007; Slattery et al., 2012; Uschold-Schmidt et al., 2012).

The role of the MR in the PFC in regulating HPA axis feedback inhibition is not as pronounced as that of the GR and to my knockwledge no studies are available examing its role following chronic stressor exposure. Forebrain-specific knock-down of the MR had no influence on basal as well as peak response to acute stress (Berger et al., 2006). Moreover, inhibition of the acute stress response under conditions of increased MR expression in the forebrain was only found in female mice (Rozeboom et al., 2007). Interestingly, forebrain MR over-expression reduced GR expression in the hippocampus (Rozeboom et al., 2007) a finding

which could also account for the decreased GR expression found in the hippocampus of CSC mice.

The mechanisms underlying the enhanced expression of FKBP51 in CSC compared with SHC mice are again not easy to unravel. Increased FKBP51 expression might implicate a reduced GR sensitivity and/or functionality, thereby, the increase in GR might represent a kind of compensatory mechanism. Another possibility might be that increased GR expression can increase the transcription of FKBP51. However, as CORT levels are unchanged following CSC exposure, this is only possible when GR sensitivity and/or functionality is concomitantly increased. This clearly demonstrates the necessity of future studies examining the functionality of the GR in the PFC.

PVN

The PVN is indirectly modulated by mainly GABAergic input from upstream corticolimbic structures, like the PFC and hippocampus, but it also expresses GR as well as MR itself. Protein expression of both receptors was not altered following CSC exposure. Chronic unpredictable stress and repeated immobilization elicited a decrease in GR (Herman et al., 1995; Makino et al., 1995) and an increase in MR mRNA (Makino et al., 1995) expression in the PVN. In contrast, chronic water immersion stress for 4 weeks had no influence on the GR expression in the hypothalamus (Mizoguchi et al., 2003). The inconsistent effects observed might be due to the kind of stressor applied and whether the whole hypothalamus or only the PVN was analysed. Nevertheless, to really confirm that negative feedback at the level of the PVN is unchanged, which is indicated at least by means of the receptor expression data, future studies are needed analysing the functionality of both receptors in the PVN.

Fornix transection, i.e. transection of the major path between hippocampus and hypothalamus, was shown to increase CRH and, interestingly, also GR and MR mRNA expression in the parvocellular PVN, indicating that also the expression of these two receptors in the PVN is regulated by the hippocampus (Han et al., 2007). This would support the finding of an unaltered hippocampal input to the

PVN following CSC exposure, as expression of MR and GR was unchanged. FKBP51 expression was not measured in the PVN, as the punches contained only small amounts of protein only sufficient for measurement of the receptors.

2.2 Effects of CSC on the negative feedback at the level of the pituitary

Less feedback inhibition at the level of the pituitary was at the first glance indicated by the decreased GR mRNA and protein expression in CSC mice, shown in other studies to result in an increased release of ACTH vesicles in the periphery (Philip et al., 1997; John et al., 2007) and increased POMC expression (Drouin et al., 1989; Drouin et al., 1993). As, at least relative POMC protein expression was unchanged, this was the first hint pointing towards an altered GR functionality in the pituitary. Therefore, to test if the feedback response under *in vivo* conditions is really compromised, a DST was performed. Dex (3 µg/100 g body weight) or vehicle (Saline) was injected ip in the morning and four hours later, mice were exposed to 6-min FS and blood was collected 10 min following termination of the acute heterotypic stressor exposure, to analyse if Dex is able to block the stress-induced increase in plasma ACTH. In contrast to what one would expect regarding the GR expression data, Dex was able to block the acute stress-induced rise in plasma ACTH in SHC as well as in CSC mice to a comparable or even increased extent. This indicates that despite the decreased GR expression, the functionality under *in vivo* conditions is not compromised, but even seems to be enhanced following CSC exposure.

Importantly, as described in Sec. 1.1, in un-injected mice, exposure to FS resulted in a significantly more pronounced rise in plasma ACTH in vehicle-injected CSC compared with SHC mice. This is of importance and indicates that the injection *per se* is not likely to mask possible effects in this approach.

Together with the decreased GR expression but unaffected Dex suppression I found an increased FKBP51 protein expression. Given that an ultra-short feedback loop increases FKBP51 expression via GC, an increased expression of this protein in CSC mice might be taken as a marker for increased ligand binding

of the GR and subsequent nuclear translocation and DNA binding in CSC compared with SHC mice (Binder, 2009). To my knowledge, there are no studies investigating the effects of chronic stress on FKBP51 expression in the pituitary, only in the hippocampus it was found to be increased following chronic SD stress (Wagner et al., 2012). The increased FKBP51 expression was paralleld by an increased GR translocation as well as an increased expression of a GR-sensitive gene (Wagner et al., 2012). Consequently, under *in vivo* conditions it is hard to disentangle whether increased FKBP51 is the consequence of an increased GR sensitivity and if consequently the enhanced FKBP51 expression in turn decreases GR sensitivity.

To clarify whether the decrease in relative GR expression is compensated by an increased GR translocation, I measured the GR protein expression in the nucleus following acute stressor exposure (6-min FS). Given the fact that there was no difference between SHC and CSC mice, this indicates that there is no increased GR translocation in CSC mice. Nevertheless, as only a low number of mice was tested, the measurement of the translocation has to be repeated as it seems that there might be a slight increase. Moreover, it underlines the importance of measuring further parameters, like phosphorylation of the GR or binding capability of CORT which might also be implicated in an increased GR functionality.

Moreover, to really disentangle the influence of FKBP51, it would also be of importance to analyse the antagonist of FKBP51, namely the FKBP52, which is essential for trafficking of the corticosteroid receptors into the nucleus. This would give further insight into the functionality of the receptors.

Nevertheless, the decreased GR together with the increased FKBP51 expression and the result of the DST strongly supports the hypothesis of an increased GR sensitivity and/or functionality. This is also in accordance to the finding at the level of the hippocampus, supporting the concept that the receptor expression is not a prerequisite for its functionality.

Another mechanism for compensating the decreased GR expression was shown in mice overexpressing MR in the hippocampus. Under stressful conditions, MR

was, thereby, able to partly reconstitute negative feedback despite GR deficiency (Harris et al., 2013). This might also be the case following CSC exposure, as MR protein expression in the pituitary was increased in CSC compared with SHC mice despite unchanged mRNA expression. The discrepancy between mRNA and protein expression would also be in line with the increased transcription rate described for the MR (Herman, 1993). However, to unravel the role of the increased MR expression, the DST has to be performed along with a MR and GR antagonist, to identify the role of each receptor in the negative feedback regulation at the level of the pituitary.

2.3. Summary of the negative feedback analysis

Overall, the analysis of the corticosteroid receptors revealed that the expression of GR as well as MR is differentially regulated in all four regions analysed. MR protein expression was up-regulated in the pituitary and PFC und unchanged in the hippocampus and PVN. GR protein expression was decreased in the pituitary and hippocampus, up-regulated in the PFC and unchanged in the PVN. These findings clearly indicate that the expression of both receptors is region-specific regulated. Moreover, I could show in my thesis that at least the GR expression in the hippocampus and pituitary itself is no criterion for its functionality. This is an important finding *per se*, suggesting that alterations of the receptor expression might dissemble an altered signalling.

For getting more informations about MR and GR functionality, measurement of the translocation would help to get more information about their functionality as it was shown for the pituitary. Unfortunately, due to methological problems, measurement of GR in the nucleus is not possible in all three brain regions, as the nuclear extraction resulted in contamination of the nuclear fraction with the cytoplasmic marker ß-Tubulin. Another important factor constitutes the phosphorylation of the GR, which was found to play an important role, especially under conditions of low CORT levels (Adzic et al., 2009). In mice, phosphorylation at Ser220 of the GR gene enhances its transcriptional activity, given that the interaction of the GR with GRE on the promoter of different genes

is increased while phosphorylation at Ser212 decreases its binding to GRE (Galliher-Beckley and Cidlowski, 2009). Moreover, studies using MR and/or GR antagonist would help to unravel the influence of each receptor in regulating GC-mediated effects, given the fact that an increased MR expression in the pituitary might probably rescue the reduced GR expression. Also measurement of the expression of GC-regulated genes, as performed in hippocampal cells, would give an insight into the transcriptional activity of the corticosteroid receptors.

Taken together, given that negative feedback inhibition at the level of the might hippocampus and pituitary even denote a tendency towards an increase this overall indicates that the increased plasma ACTH concentrations in CSC compared with SHC mice are not due to an impaired negative feedback inhibition which is in contrast to the GC resistance found in the periphery. This further supports the hypothesis that the increased number of corticotroph cells is mainly responsible for the pituitary hyper-activity under basal and hyper-reactivity following exposure to a superimposed acute stressor exposure.

3 CSC and the influence of trait anxiety on the individual stress vulnerability

In 1977, Zubin and Spring defined their model of “stress and vulnerability” in mental disorders. They outlined in detail that the ability to deal with stress, i.e. the vulnerability, varies within individuals. While people with low vulnerability need longer time or stressor intensity to become distressed, others with high vulnerability already suffer in response to low amounts of stress and develop serious mental disorders (Zubin and Spring, 1977). Therefore, it is of major importance to unravel the mechanisms underlying the vulnerability to stress and the development of stress-related disorders, whereby genetic as well as environmental factors, like the life history of an individual, are implicated (Kendler and Eaves, 1986).

Polymorphisms, altering the individual vulnerability to acute or chronic stressor exposure have been previously described, e.g. for FKBP51, involved in the regulation of the GR sensitivity and translocation efficiency (Binder, 2009; Klengel et al., 2013) or for the GR itself (Wüst et al., 2004). Polymorphisms leading to over-expression of FKBP51 increased recovery period following an acute social stressor exposure in humans (Trier Social Stress Test) (Ising et al., 2008), while chronic SD in FKBP51 knock-out mice produced a less stress vulnerable phenotype (Hartmann et al., 2012b). Also behavioural traits, like anxiety and temperament are associated with the development of stress-induced depression and enhanced GC reactivity (Sandi et al., 2008; Tyrka et al., 2008; Sandi and Richter-Levin, 2009). In high trait anxiety animals, the amygdaloid CRH system was more vulnerable following exposure to chronic unpredictable stress, indicating an increased vulnerability to develop psychiatric alterations associated with chronic stress (Sandi et al., 2008).

3.1 Effects of CSC on behavioural, physiological, neuroendocrine and immunological parameters

To assess the influence of genetic differences, in this case the trait anxiety of an individual, to the effects of 19 days of CSC, I used the well characterized mouse line bred for high (mHAB) and low (mLAB) anxiety-related behaviour, and non-seleceted normal CD1 (mNAB) mice. Thereby, all results obtained following CSC exposure were compared with results from previous studies conducted in C57BL/6 mice to validate the chronic stress procedure.

Behavioural parameters

On the EPM, breeding-line specific differences in innate anxiety between mNAB, mHAB and mLAB described in the literature (Krömer et al., 2005; Muigg et al., 2009) were confirmed with mHAB mice exhibiting an increased anxiety-like behaviour compared with mLAB mice, while mNAB mice displayed an intermediate behaviour phenotype. The anxiogenic effect of CSC in C57BL/6 mice (Reber et al., 2007; Slattery et al., 2012; Uschold-Schmidt et al., 2012) was confirmed in the mNAB, but neither in the mHAB nor in the mLAB group. At least in the mHAB group the lack of any detectable effect might be due to a ceiling effect given that already the mHAB SHC mice showed a high level of anxiety, e.g. displayed by spending only 6 % time on the open arm. To really confirm the ceiling effect, in future studies a different anxiety test, like light-dark box, should be used or the conditions of the EPM should be altered by making it less aversive for instance by changing the light conditions.

Importantly, locomotion measured by the number of closed arm entries was not altered by the CSC procedure in any of the genotype. Only mLAB SHC mice generally moved less than respective mHAB mice, an effect already reported before (Krömer et al., 2005). This might be explained by the fact that mLAB mice in general spent more than 50% of their time on the open arm and, therefore, consequently showed fewer closed arm entries compared with mHAB mice. This finding supports the hypothesis that the number of closed arm entries does not simple reflect locomotor activity, but also reflects anxiety as well (File, 2001).

However, detecting no anxiogenic effect in mLAB mice indicates, and that is a very exciting finding, resilience to the effects of CSC with respect to the anxiety-related behaviour. Thereby, one can also exclude that these mice are extremely robust and resistant to stressful life events or to any kind of manipulation, given that in rLAB rats it was shown that local application (via retrodialysis in the septum) of the anxiogenic neuropeptide AVP was able to increase anxiety-related behaviour in this breeding line (Beiderbeck et al., 2007).

Physiological parameters

To validate the chronic psychosocial stress paradigm as well as to examine genotype specific differences, physiological parameters known to be altered in C57BL/6 mice following CSC or following chronic stressor exposure in general were assessed.

Body weight gain was not affected in mNAB and mHAB mice, but was increased in mLAB CSC mice, compared with respective SHC mice. Nevertheless, as the chronic stress effect on this parameter was already shown to be inconsistent in C57BL/6 mice following CSC exposure, revealing a decrease (Reber et al., 2007; Veenema et al., 2008) but also no change (Slattery et al., 2012), as well as in other chronic stress paradigms (Bartolomucci et al., 2005; Schmidt et al., 2010a; Bartlang et al., 2012), this clearly indicates that body weight gain is no reliable indicator for chronic stress. Body weight gain is regulated by behavioural and neuroendocrine processes and is mainly determined by the input (food intake) and output (e.g. locomotion) (Bartolomucci et al., 2005). Therefore, analysis of the eating behaviour and/or locomotor activity would be necessary to unravel at least the mechanism underlying the increased body weight gain in CSC mLAB mice. However, the comparable body weight gain between mHAB and mNAB mice and the difference in the mLAB mice following CSC exposure again supports the concept of stress-resilience in the mLAB group.

One of the most reliable physiological parameter with respect to chronic stress is adrenal hypertrophy, shown following chronic stressor exposure in general (Retana-Marquez et al., 2003; Schmidt et al., 2007) and, importantly, also

following CSC exposure in C57BL/6 mice (Reber et al., 2007; Uschold-Schmidt et al., 2012; Füchsl et al., 2013). mNAB as well as mHAB mice displayed an increased adrenal weight following CSC, confirming on the one hand the reliability of CSC as a chronic psychosocial stress model and on the other hand the determination of adrenal weight as an reliable parameter for chronic stress. As only mLAB mice of the CSC group showed no adrenal hypertrophy, this further demonstrates that mice with a low anxious phenotype are resilient to the chronic stress effects.

Splenomegaly, reported in other chronic stress paradigms, like e.g. the social disruption paradigm in male mice (C57BL/6 and Balb/c) (Avitsur et al., 2003; Merlot et al., 2004) was found following CSC exposure in mNAB and at least by trend in mHAB mice, whereby this effect was not detected in mLAB mice, emphasizing again the reduced stress vulnerability in mLAB mice. Even though, spleen weight has never been assessed in C57BL/6 mice exposed to CSC, the studies mentioned above already indicate that splenomegaly is not mouse-line specific and, therefore, probably reflects a general consequence of 19 days of CSC.

Neuroendocrine parameters

Analysis of plasma CORT and ACTH levels is of major importance for analysis of basal HPA axis activity following chronic stressor exposure. In CSC mice of the mNAB and mLAB group, plasma ACTH concentrations in the morning were similar, while they were significantly increased in the mHAB group compared with respective SHC mice. An effect also found in C57BL/6 mice following CSC, where this is probably mediated by an increased number of corticotroph cells (see also Sec. 1.1). Nevertheless, future studies are needed to check if in mHAB CSC mice increased plasma ACTH levels are mediated by the same mechanism like in C57BL/6 mice, or if e.g. AVP plays a role in this process. In mHAB, mLAB and mNAB mice anxiety-related behaviour suggests to be positively correlated with the AVP expression (Landgraf et al., 2007). As under conditions of chronic stress AVP, in addition to CRH, is supposed to play a major role in stimulating

ACTH release from the pituitary (Ma et al., 1999), it is likely that following CSC in mHAB mice, the increased plasma ACTH release is driven by a stronger input of AVP due to their high anxiety level. Therefore, AVP has to be measured via IHC in all three breeding lines following CSC exposure. Similar basal ACTH concentrations in mHAB and mLAB SHC mice are at least in accordance to studies conducted in rats (Frank et al., 2006).

Neither in the mHAB nor in the mLAB group plasma CORT concentrations were affected by CSC, but, interestingly, mNAB mice of the CSC group even showed hypocorticism in the morning compared with respective SHC mice. An effect only described for plasma morning CORT concentrations in C57BL/6 previously exposed to MS early in life (Veenema et al., 2008) and for plasma evening concentrations in C57BL/6 mice, while plasma morning concentrations were similar in SHC and CSC mice of this strain (Reber et al., 2007; Uschold-Schmidt et al., 2012). This finding clearly shows that the genetic background, as mNAB are CD1 mice, as well as early life events (Veenema et al., 2008) can increase the vulnerability to the consequences of chronic psychosocial stress, in this case increasing the negative effects on HPA axis functionality.

Regarding line differences, mNAB SHC mice displayed significantly higher plasma morning CORT concentrations compared with respective mHAB and mLAB mice, which is most likely due to the higher adrenal weight this group shows. Similar basal CORT levels in mHAB and mLAB SHC mice are in accordance to other studies carried out in mice (Gonik et al., 2012) and rats (Liebsch et al., 1998; Frank et al., 2006) of this breeding line, even though adrenal weight of animals of the LAB line is higher in mice and lower in rats (Salome et al., 2006) compared with respective HAB animals. This might, at least in the rats, be due to a similar adrenal CORT content in the two lines (Salome et al., 2006). Nevertheless, this has to be further analysed in mice by measurement of the adrenal CORT content or of the capability to produce CORT. *In vitro* adrenal CORT secretion under basal conditions was similar in SHC mice of all three genotypes but is not in discrepancy to the above mentioned higher plasma CORT levels in mNAB mice, as *in vitro* data are expressed per mg of adrenal

tissue and adrenal weight is higher in mNAB SHC compared with mHAB and mLAB of the respective group.

In vitro responsiveness of the adrenal gland in response to ACTH stimulation (100 nM) revealed a complete loss of the CORT secretion in mNAB and mHAB CSC compared with respective SHC mice. In contrast, C57BL/6 mice only showed a partially attenuated ACTH-stimulated CORT secretion following CSC exposure (Reber et al., 2007; Uschold-Schmidt et al., 2012). This further indicates an increased vulnerability of HPA axis functionality of mice of the CD1 compared with the C57BL/6 strain.

At the first glance, basal plasma CORT:ACTH ratios which were similar in SHC mice of all breeding lines are in contrast to the increased adrenal *in vitro* ACTH responsiveness found in mLAB SHC mice. Nevertheless, this result is in line with the increased CORT response reported in mLAB compared with mHAB mice following acute restraint stress (15 min) (Gonik et al., 2012). Thus, measurement of the response to an acute stressor is a more reliable parameter for interpretation of the *in vitro* results obtained by stimulation of adrenals with ACTH instead of the basal plasma CORT:ACTH ratio.

The stimulation data together with the CORT:ACTH ratio strongly support a decreased ACTH responsiveness in CSC mice of the mNAB and mHAB line. This seems to be mediated by a reduced capability to produce CORT in the mNAB mice, given that plasma ACTH concentrations were unchanged and the adrenal weight was even increased. In the CSC mHAB group, the reduced *in vitro* ACTH responsiveness was paralleled by increased plasma ACTH concentrations together with an increased adrenal weight which did not result in enhanced plasma CORT concentrations. Nevertheless, it has to be determined in future studies if there is a real insufficiency of the adrenal glands or if this represents an adaptation to protect the organism from increased CORT levels, as the latter seems to be the case in C57BL/6 mice exposed to CSC (Uschold-Schmidt et al., 2012). However, assumed that this constitutes a mechanism of adaptation, then in mNAB CSC mice this adaptation was too intense causing even hypocorticism.

However, mLAB CSC mice did not show any of these alterations at the level of the adrenal gland, supporting againg their stress resilience.

Immunological parameters

CSC has repeatedly been shown to represent an adequate and pre-clinically validated model for chronic psychosocial stress, as it, amongst others, promotes the development of somatic disorders, as for instance spontaneous colitis (Reber et al., 2007; Reber et al., 2011; Reber, 2012). Measurement of the IFN-γ secretion of anti-CD3 stimulated isolated mesLNC revealed an increased secretion of this pro-inflammatory cytokine in CSC mice of the mHAB and mNAB group, which is in accordance to C57BL/6 mice (Reber et al., 2007; Reber et al., 2011), but not in the mLAB group. Nevertheless, regarding the histological damage score, which was increased in C57BL/6 mice following CSC (Reber et al., 2007; Reber et al., 2011), no alterations in any of the breeding lines were found, indicating that mice of the CD1 strain seem to be more resilient to the immunological, while they were shown to be more vulnerable to neuroendocrine alterations. Development of spontaneous colitis in C57BL/6 mice is influenced by various factors, like changes in immune competence, epithelial barrier function, and bacterial translocation during the initial phase of CSC exposure (Reber et al., 2011), indicating that further studies are needed to unravel possible differences between the CD1 and C57BL6 mouse lines following CSC exposure.

GR, MR and FKBP51 protein expression in the pituitary

As already mentioned at the beginning of Sec. 2, polymorphisms in the GR or in FKBP51 are known to influence the individual vulnerability to chronic stressor exposure (Wüst et al., 2004; Binder, 2009). Therefore, and because of the fact that alterations in MR, GR and FKBP51 expression have been revealed in this study in C57BL/6 mice (see also Chapter 3, Sec. 3), I also analysed the expression of these three proteins in the pituitary of mHAB, mLAB and mNAB mice following CSC exposure. In contrast to what was found in C57BL/6 mice, no alterations of GR, MR and FKBP51 were found in the pituitary following CSC

exposure. One possibility for this discrepancy reagarding the results found in C57BL/6 mice might be the fact that only a low amount of mice (4 -5 per breeding line) were analysed, which is also reflected in the high standard deviation. The only difference that was found was a higher FKBP51 expression in CSC mice of the mNAB compared with the respective mHAB group. However, to really disentangle the significance of this result, future studies are needed examining a higher amount of animals.

Conclusion

Overall, the results of the behavioural, physiological, neuroendocrine and immunological parameters clearly indicate, that the individual genetic predisposition has a strong impact on the vulnerability to chronic psychosocial stressor exposure with mLAB mice being resilient to the maladaptive effects of CSC (see Table 2). Moreover, it became evident that the effects of CSC might be mouse strain-dependent.

Mice of the low anxious phenotype showed no signs of somatic and affective maladaptations following CSC exposure. Therefore, the use of this breeding line enables the study of gene and environment interactions with respect to the development of stress-related disorders and makes it possible to analyse the mechanisms underlying the genetic predisposition of stress resilience. Against my expectation, mHAB showed no higher vulnerability to the effects of chronic stress compared with mNAB mice, although other studies demonstrated an increased chronic stress burden in high anxious mice (Sandi et al., 2008; Dhabhar et al., 2012). Nevertheless, this might be due to a kind of ceiling effect, as 19 days of CSC might constitute a too strong or intensive stressor. Thus, usage of a milder chronic psychosocial stress paradigm might enable the detection of possible differences between chronically stressed mNAB and mHAB mice. Moreover, as already mentioned before, it would be of interest to find out if mLAB mice are resilient to chronic stress *per se* or if they show signs of chronic stress-induced alterations when they are subjected to other chronic stress

paradigms, like chronic SD (Wagner et al., 2011) or exposure to an unstable social and hierarchical situation for several weeks (Schmidt et al., 2010a).

CSC READOUT PARAMETER	mHAB	mNAB	mLAB	C57BL/6
Body weight gain	↔	↔	↑	↓ ↔
Absolute adrenal weight	↑	↑	↔	↑
Absolute spleen weight	**(↑)**	↑	↔	
Basal plasma CORT (morning)	↔	↓	↔	↔
Basal plasma ACTH	↑	↔	↔	↑
Plasma CORT:ACTH ratio	**(↓)**	↓	↔	↓
In vitro adrenal ACTH responsiveness	↓	↓	↔	↓
Proinflammatory cytokine secretion from mesLNC	↑	↑	↔	↑
Histological damage score (colon)	↔	↔	↔	↑
Anxiety-like behaviour	↔	↑	↔	↑
GR pituitary	↔	↔	↔	↓
MR pituitary	↔	↔	↔	↑
FKBP51 pituitary	↔	↔	↔	↑

Table 2: Comparison of the effects of 19 days of CSC in mice selectively and bidirectionally bred for high (mHAB) and low (mLAB) anxiety-related behaviour, in non-selected normal (mNAB) and in C57BL/6 mice on behavioural, physiological, neuroendocrine and immunological parameters. CSC exposure results either in an increase (↑) or decrease (↓) of the examined parameter or had no effect (↔). Arrows in parentheses indicate an increase or decrease by trend. [Data are taken from (Reber et al., 2007; Reber et al., 2011; Slattery et al., 2012; Uschold-Schmidt et al., 2012), Chapter 3, Sec. 4]

3.2 Why are mice of the low-anxious phenotype resilient to the consequences of CSC?

One of the main questions arising from the study conducted in this breeding line is why mLAB mice are resilient to the consequences of chronic psychosocial stress. Thereby the SNP in the AVP gene - leading to a decreased synthesis and release of the anxiogenic neuropeptide - is mainly responsible for the low anxiety this breeding line shows (Bunck et al., 2009). But whether this is the only factor contributing to their stress resilience is still unknown, given that also other genes have been identified that seem to parallel the trait anxiety, including Glyoxylase-I (Krömer et al., 2005), enolase phosphatase (Ditzen et al., 2010) and TMEM123d (Erhardt et al., 2011).

In brattleboro rats, the lack of AVP only partly prevented the consequences of chronic stressor exposure, e.g. the increased basal CORT levels, but had no influence on the development of depressive-like changes (Varga et al., 2011) or on physiological alterations, like adrenal hypertrophy or thymus involution (Zelena et al., 2004). These studies indicate that lack of AVP alone cannot protect against all chronic stress-induced effects and, therefore, also other factors, like the genes mentioned above must be involved.

Nevertheless, to test if in mLAB mice, the SNP in the AVP gene is the main mediator of the stress resilience, one possibility would be to implant osmotic minipumps continuously delivering AVP in the brain during the CSC exposure and check for all parameters assessed before. Moreover, the reduced AVP expression might be compensated by increased CRH and/or OXT expression under basal as well as following chronic stressor exposure and has, therefore, to be analysed. At least under basal conditions, OXT mRNA expression in the PVN was shown to be similar in mHAB, mNAB and mLAB mice (Bunck et al., 2009).

Not only the anxiety trait itself might explain their stress reactivity, but also other personality traits, like aggression, exploration or activity. Amongst AVP itself, their low anxious phenotype might impact their coping strategy, i.e. their behaviour in response to the resident during the CSC procedure, even though at the start of the CSC paradigm, mice of all three lines were in the subordinate position. One

recent study from Castro and colleagues (2012) investigated the influence of the combination of the traits anxiety and exploration on the effects of chronic unpredictable stressor exposure. Thereby, animals with low anxiety and low exploration seemed to be resistant to behavioural alterations while highly anxious animals showed more depressive-like alterations. In combination with low exploration, highly anxious animals showed also a higher stress vulnerability demonstrated by increased physiological (higher CORT levels in response to acute stress) and neurobiological responses (increased pERK1/2 activation in amygdala and hippocampus) following chronic stress (Castro et al., 2012).

Thus, analysis of the behaviour of the experimental mice of the different breeding lines during CSC exposure would give further insight into the mechanisms of stress vulnerability. Proactive, i.e. more competitive rats have been shown to display higher autonomic stress responses, like increased blood pressure and increased catecholamine release compared with more reactive ones (Koolhaas et al., 1997). Rats displaying a more reactive and non-aggressive coping style showed higher basal plasma CORT levels as well as increased reactivity of plasma CORT and ACTH in response to an acute stressor (Veenema et al., 2003; Veenema et al., 2004). Overall this indicates that proactive coping results in alterations of the SNS, while reactive coping implicates higher HPA axis activity and reactivity (Koolhaas et al., 1999; Koolhaas et al., 2007). At least in rats it was shown that during SD rHAB exhibit a more passive strategy, demonstrated by increased freezing, while rLAB showed a more active behaviour (Frank et al., 2006). Moreover, inter-male aggression was higher in rLAB compared with rNAB, while rHAB showed an intermediate aggressive behaviour (Veenema et al., 2007; Beiderbeck et al., 2011). Transferred to the mice this would confirm that in mLAB mice no alterations of HPA axis functionality have been observed following CSC exposure while mHAB mice showed e.g. increased plasma ACTH levels. Moreover, high aggressive individuals also displayed increased GC levels and/or responses (Gerra et al., 1997; van Bokhoven et al., 2005), which would be in line with the increased adrenal ACTH responsiveness *in vitro* observed in mLAB mice.

Nevertheless, to really confirm these hypothesis, aggressive behaviour or behaviour in general during the CSC paradigm must be analysed in future studies. Moreover, not only parameters concerning HPA axis activation, but also parameters like catecholamine release, revealing alterations of the SNS, have to be assessed. According to the review of Koolhaas and colleagues (1999) and the aggression data obtained from the rHAB and rLAB rats, one would expect an increased activity of the SNS in mLAB mice following CSC exposure concomitant with no alterations in HPA axis activity as I already revealed in this study.

4. Summary and concluding remarks

In the present thesis, I provided further evidence for the alterations of HPA axis functionality following chronic stressor exposure as well as for the individual variations concerning the vulnerability to the CSC-induced effects.

I demonstrated that CSC increased plasma ACTH levels under basal conditions as well as in response to an acute heterotypic stressor, whereby this was mainly mediated by an increased number of pituitary corticotroph cells. As increased basal ACTH levels argue against an adaptation of the HPA axis in response to repeated stressor exposure, which was found at the level of the adrenal gland, this might constitute a kind of mal-adaptation at the level of the pituitary. Increased plasma ACTH levels in response to an acute heterotypic stressor are in line with the sensitization process described in the literature following chronic stressor exposure. Therefore, while pituitary mechanisms are not involved in the process of adaptation, I was able to demonstrate that the combination of pituitary and adrenal mechanisms is of importance to fulfill the sensitization process to a superimposed acute heterotypic stressor following CSC exposure.

In addition, analysis of the negative feedback inhibition at different levels of the HPA axis revealed a site-specific regulation of the corticosteroid receptors following CSC exposure. Thereby, GR expression was decreased in the hippocampus and pituitary, unchanged in the PVN and increased in the PFC. MR expression was increased in the pituitary and PFC and unchanged in the hippocampus and PVN. At the level of the pituitary the DST revealed a completely functional or even increased negative feedback inhibition despite decreased GR expression. The same phenomenon, i.e. decreased receptor expression concomitant with increased functionality, was also shown in the hippocampus. These results clearly demonstrate the importance of measuring the signalling of the receptor, e.g. by analysis of the phosphorylation pattern or of the nuclear translocation.

Both, the influence of life history of an individual, in detail the impact of early life stress analysed in the previous study (Veenema et al., 2008) and genetic factors, i.e. the trait anxiety of an individual analysed in the present study, extent the

knowledge of gene and environment interactions influencing the vulnerability to chronic psychosocial stress. I could clearly demonstrate that mice of the low anxious phenotype were resilient to the effects of 19 days of CSC regarding all parameters analysed. They neither showed behavioural, physiological, neuroendocrine nor immunological alterations, indicating that the chronic exposure to the resident had no effect in mLAB mice. Therefore, it is of major importance to unravel the underlying mechanism – it is very likely due to the low AVP expression – to find a treatment strategy for humans, protecting them from the negative consequences of chronic stress. Moreover the results clearly indicate that the analysis of chronic stress effects in a large cohort of animals should at least implicate the analysis of the trait anxiety of the animals and/or the stress coping, proactive or reactive, which might even differ in an inbred colony of mice. Selecting animals that differ extremely in their behaviour might give further insight into the individual variability in the vulnerability to the consequences of chronic stress. Importantly, these informations would help to promote the development of personalized medicine, given that the molecular mechanisms mediating the development of stress-related disorders probably varie between individuals.

Addendum

Summary in German - Deutsche Zusammenfassung

Der Begriff „Stress“ weckt meist nur negative Assoziationen, wobei oft in Vergessenheit gerät, dass die akute Stressantwort einen der fundamentalsten Überlebensmechanismen der Natur darstellt. Die Aktivierung des Kardiovaskulären-, des Bewegungs- und des Neuroendokrinensystems ermöglicht die sogenannte „Kampf oder Flucht“ Reaktion. Wenn es jedoch häufiger zu Stress kommt oder der Organismus die dauerhaften Belastungen und Anforderungen nicht mehr bewältigen kann, so spricht man von chronischem Stress. Chronischer Stress, beim Menschen vor allem ausgelöst durch psychosoziale Stressoren, verändert das physiologische Gleichgewicht, was unter bestimmten Umständen die Entwicklung von affektiven sowie auch körperlichen Erkrankungen begünstigen kann. Beispiele hierfür sind chronisch entzündliche Krankheiten des Verdauungstrakts, Erkrankungen des Herz-Kreislaufsystems, sowie Angst- und Depressionserkrankungen. Um die Homöostase des Körpers nach Stresseinwirkung wiederherstellen zu können werden Stresshormone, Katecholamine und Glucocorticoide (GC) ausgeschüttet. Da die Ausschüttung von GC über einen längeren Zeitraum negative Auswirkungen auf den Organismus hat, ist es besonders wichtig, dass eine Adaptation auf den chronischen Stressor erfolgt. Darüberhinaus gibt es einen Prozess der Sensitivierung, der es einem chronisch gestressten Organismus ermöglicht, auf eine neue, möglicherweise noch schwerwiegendere, Herausforderung adäquat reagieren zu können. Die Mechanismen der Adaptation der Hypothalamus-Hypophysen-Nebennierenrinden (Hypothalamic-pituitary-adrenal (HPA))-Achse sind vor allem auf Ebene der Hypophyse bekannt, wobei es bei chronischer Stressexposition zu einer Verringerung der Freisetzung des adrenocorticotropen Hormons (ACTH) kommt.

Interessanterweise zeigen jedoch nicht alle Individuen die gleiche Vulnerabilität gegenüber den durch chronischen Stress verursachten Folgen. Während die einen stark anfällig für Stress sind, sind die anderen deutlich belastbarer oder

auch resistent gegenüber chronischem Stress. Eine wichtige Rolle spielen dabei sowohl die genetische Prädisposition als auch Umweltfaktoren (z.B. Ereignisse in der Kindheit und frühen Jugend). Ein wichtiges Ziel der heutigen Stressforschung ist daher die Untersuchung der Mechanismen, die dieser unterschiedlichen Stressvulnerabilität zu Grunde liegen, um die Pathologie von stressbedingten Krankheiten verstehen zu können. Ein geeigneter Ansatz, um die Mechanismen von stressbedingten Krankheiten im Allgemeinen und die Vulnerabilität im Speziellen zu untersuchen, ist die Verwendung geeigneter Tiermodelle. In der vorliegenden Arbeit wurde hierfür das Modell der chronisch-subordinierten Koloniehaltung (chronic subordinate colony housing (CSC), 19 Tage), ein etabliertes und präklinsch-validiertes Modell um chronisch psychosozialen Stress in männlichen Mäusen hervorzurufen, verwendet. Hierbei werden die Versuchsmäuse, vier männliche, gleichaltrige C57BL/6 Mäuse, zusammen mit einer älteren, schwereren und aggressiveren männlichen Maus, auch Resident genannt, für 19 aufeinanderfolgende Tage in einer Kolonie gehalten. In dieser CSC-Kolonie übernimmt der Resident die dominante Rolle, während die Versuchsmäuse die subordinierte Position einnehmen. In zahlreichen Publikationen konnte bereits gezeigt werden, dass CSC-Exposition sowohl affektive als auch körperliche Erkrankungen der Mäuse zur Folge hat. CSC Mäuse zeigten dabei eine erhöhte Ängstlichkeit auf der *elevated plus-maze* (EPM) im Vergleich zu den ungestressten Kontrolltieren (single-housed control (SHC)). Auch kam es zu immunologischen Veränderungen, die sich in einer Verkleinerung des Thymus, sowie einer spontanen Entzündung des Colons äußerten. Die zugrundeliegenden Mechanismen sind dabei noch nicht eindeutig verstanden, jedoch zeigten die Versuchstiere Veränderungen der HPA-Achsen Funktionalität. Plasma Corticosteron (CORT)-Konzentrationen am Morgen waren am Ende der CSC-Exposition im Vergleich zu den SHC Tieren unverändert, während die Werte am Abend in den CSC Tieren niedriger waren. Dies bedeutet Hypocortizismus in der aktiven Phase der CSC Mäuse. Da auch Nebennieren, deren Gewicht erhöht war, *in vitro* eine verringerte CORT-Freisetzung nach Stimulation mit ACTH aufwiesen, deutete dies auf eine Nebenniereninsuffizienz

in den CSC Tieren hin. Jedoch waren CSC Mäuse in der Lage auf auf einen akuten heterotypischen, also unbekannten, Stressor (5 min erhöhte Plattform Exposition), mit einer erhöhten CORT-Ausschüttung, trotz unveränderter ACTH Konzentrationen, reagieren zu können. Dieses Ergebnis und die Tatsache, dass die CORT Synthese in CSC Tieren nicht beeinträchtigt war, waren ein Beweis für die Funktionalität der Nebennieren.

Die bisherigen Ergebnisse deuten daher darauf hin, dass auf Ebene der Nebenniere eine Adaptation stattfindet, welche die CSC Mäuse einerseits vor dauerhaft erhöhten Plasma CORT-Konzentrationen schützt. Andererseits ermöglicht ein Prozess der Sensitivierung eine erhöhte HPA-Achsenreaktivität auf einen akuten heterotypischen Stressor zeigen zu können. Diese Mechanismen der Adaptation und Sensitivierung wurden in anderen Studien hauptsächlich auf Ebene der Hypophyse gezeigt und nicht wie in den CSC Tieren auf Ebene der Nebenniere. Dennoch kann man vermuten, dass aufgrund des erhöhten Hypophysengewichts, welches 8 Tage nach Beendigung der CSC Exposition gefunden wurde und aufgrund der Tatsache, dass keine basalen ACTH Plasma Daten vorhanden sind, auch Hypophsenmechanismen an diesen Prozessen beteiligt sind.

Der Fokus des ersten Teils der Arbeit lag daher auf der Untersuchung unterschiedlicher Parameter der HPA-Achse nach CSC Exposition, wobei ein besonderer Fokus auf Hypophyse und PVN gelegt wurde. Die Analyse der basalen Plasma ACTH Konzentrationen am Morgen zeigte eine erhöhte Freisetzung in CSC gegenüber SHC Tieren. Darüberhinaus kam es auch nach einer akuten heterotypischen Stressorexposition (6 min *forced swim* (FS)) zu einer erhöhten ACTH Freisetzung in CSC Tieren. Auf den ersten Blick ist dies gegensätzlich zum Ergebnis der Uschold-Schmidt Studie 2012 in der unveränderte ACTH Werte als Antwort auf einen akuten heterotypischen Stressor gezeigt wurden. Dies ist jedoch auf die Art des Stressors und auf Unterschiede im zeitlichen Ablauf zurückzuführen. Anhand der absoluten Plasma ACTH Werte ist ersichtlich, dass FS einen wesentlich stärkeren Stressor als Exposition auf einer erhöhten Plattform darstellt. Außerdem betrug die

Zeitspanne zwischen Beendigung des akuten Stressors und der Tötung der Tiere in der vorliegenden Studie 10 min und in der vorherigen Studie 5 min. Diese Ergebnisse zeigen, dass die erhöhte ACTH Antwort auf einen akuten heterotypischen Stressor eine bestimmte Zeit oder Stressorintensität benötigt, um sich zu entwickeln. Im Allgemeinen bedeutet dies, dass der Mechanismus der Adaptation hauptsächlich auf Ebene der Nebenniere vermittelt wird, während Nebennieren- und Hypophysenmechanismen kombiniert werden um die Sensitivierung auf den akuten heterotypischen Stressor zu realisieren.

Als wahrscheinlichste Ursache für die Hypophysenhyperaktivität und –reaktivität in den CSC Tieren kommt das erhöhte Hypophysengewicht, das auch für Tag 20 bestätigt werden konnte und die erhöhte Anzahl an corticotrophen, dass heißt ACTH-produzierenden, Zellen in der Hypopyhse, in Frage. Eine unveränderte relative mRNA- und Proteinexpression von Pro-Opiomelanocortin, dem Vorläufer von ACTH, zeigte darüberhinaus, dass die neu gebildeten Zellen funktionsfähig sind. Da jedoch nicht ausgeschlossen werden kann, dass auch noch andere Mechanismen für die erhöhte ACTH-Freisetzung verantwortlich sind, wurde zuerst die Expression der Rezeptoren für Corticotropin-releasing Hormon (CRH) und Arginin-Vasopressin (AVP), die massgeblichen Einfluss auf die ACTH Freisetzung haben, untersucht. Eine unveränderte CRH-R1 mRNA- aber erniedrigte Proteinexpression in den CSC Tieren bestätigten die Ergebnisse anderer Studien, wobei auch gezeigt werden konnte, dass eine erniedrigte CRH-R1 nicht zu einer erniedrigten ACTH Freisetzung führte. Zusammen mit der unveränderten AVPR-1b mRNA- und Proteinexpression, deutet dies in den CSC Tieren darauf hin, dass beide Rezeptoren nicht an der erhöhten ACTH Sekretion beteiligt sind. Die erniedrigte CRH-R1 und die unveränderte AVPR-1b Expression lassen weiter vermuten, dass die neu gebildeten Hypophysenzellen sensitiver auf AVP als auf CRH reagieren. Dies muss jedoch unter anderem noch in Stimulationsversuchen gezeigt werden, wobei Hypophysen *in vitro* mit AVP, CRH und einer Kombination aus Beiden stimuliert und die ACTH Antwort gemessen werden soll.

Da zahlreiche Studien AVP eine wichtige Rolle in der Regulation der ACTH Freisetzung während chronischem oder länger anhaltenden Stress zuschreiben und aufgrund der Tatsache, dass momentan nur mRNA Daten vorhanden sind, wurde die Anzahl der parvo- und magnozellulären AVP-positiven Neurone im Nucleus paraventricularis (PVN) mittels immunohistochemischer Färbung gemessen, wobei jedoch kein Unterschied zwischen SHC und CSC Tieren gefunden wurde. Da nicht nur CRH und AVP die Freisetzung von ACTH stimulieren können, sondern beispielsweise auch Oxytocin (OXT), wurde auch die Anzahl der OXT-positiven Neurone im PVN von SHC und CSC Tieren gemessen, was wiederum keine Unterschiede lieferte.
Alles in allem konnte im ersten Teil der Arbeit nachgewiesen werden, dass CSC Exposition zu einer Hypophysenhyperaktivität und –reaktivität führt. Dabei deutete eine erhöhte Anzahl an corticotrophen Hypophysenzellen, zusammen mit unveränderter AVPR-1b und erniedrigter CRH-R1 Proteinexpression darauf hin, dass dies durch die neu gebildeten corticotrophen Zellen, die vermutlich sensitiver gegenüber AVP als CRH sind, vermittelt wird. Darüberhinaus konnte gezeigt werden, dass Veränderungen der AVP und/oder OXT Expression keinen Einfluss auf die Reaktivität der Hypophse nach CSC Exposition haben.

Der zweite Teil der Arbeit fokussierte sich auf die negative Feedbackinhibierung der HPA-Achse, hauptsächlich vermittelt durch Glucocorticoid- (GR) und Mineralocorticoidrezeptoren (MR), in der Hypophyse, im Hippocampus, im präfrontalen Cortex und im PVN.
Im Hippocampus war die GR mRNA- und Proteinexpression reduziert, während die MR mRNA- und Proteinexpression als auch die Proteinexpression des FK506-bindenden Proteins 51 (FKBP51), das in der Regulation der Sensitivität und Funktionalität der Corticosteroidrezeptoren involviert ist, nach CSC Exposition unverändert waren. Jedoch konnte *in vitro* in isolierten hippocampalen Zellen gezeigt werden, dass die Funktionalität des GR nicht eingeschränkt, sondern gar erhöht war. Sowohl die CORT-induzierte hnRNA Expression des Period1 Gens in isolierten hippocampalen Zellen als auch die hippocampale

Zellviabilität *in vitro* nach 24 Stunden CORT-Stimulation war in CSC Tieren höher als in SHC Tieren.

Im präfrontalen Cortex war die Proteinexpression von GR, MR und FKBP51 erhöht, während im PVN keine Unterschiede zwischen SHC und CSC Tieren im Hinblick auf GR und MR Proteinexpression gefunden werden konnten.

Auf Ebene der Hypophyse deutete eine reduzierte GR mRNA- und Proteinexpression auf eine verringerte negative Feedbackinhibierung hin. Jedoch zeigte das Ergebnis des Dexamethason (Dex) Unterdrückungstest eindeutig, dass das Feedback auf Ebene der Hypophyse unverändert, wenn nicht sogar erhöht war. Dabei wurden SHC und CSC Tiere am Morgen des Tag 20 ip mit Dex beziehungsweise Vehikel injiziert und 4 Stunden später einem akutem Stressor (6-min FS) ausgesetzt. 10 min nach Beendigung des Stressors wurden die Tiere getötet und Plasma ACTH Werte analysiert. Sowohl in SHC als auch in CSC Tieren war Dex in der Lage den stress-induzierten ACTH Anstieg zu blocken, was auch hier daraufhindeutet, dass der GR, trotz verringerter Expression, funktional ist. Dies wurde auch durch Messung der nukleären GR Expression nach akutem Stress und die erhöhte FKBP51 Expression, die wahrscheinlich auf eine erhöhte transkriptionelle Aktivität des GR vermittelt wird, bestätigt. Möglicherweise spielt dabei aber auch die erhöhte MR Proteinexpression und deren mögliche kompensatorische Wirkung eine wichtige Rolle, was in zukünftigen Studien noch genauer untersucht werden muss.

Zusammenfassend lässt sich feststellen, dass MR und GR Expression in der Hypophyse und den verschiedenen Gehirnregionen unterschiedlich reguliert werden. Darüberhinaus konnte, sowohl in der Hypophyse als auch im Hippocampus gezeigt werden, dass eine reduzierte Anzahl an GR nicht unbedingt eine erniedrigte Funktionalität zur Folge hat. Dieses Ergebnis verdeutlicht, dass allein die Messung der Rezeptorexpression keine Rückschlüsse auf die Funktionalität zulässt und unterstreicht die Wichtigkeit der Analyse von Phosphorylierungen und/oder Expression von GC-regulierten Genen nach Stimulation mit CORT oder Dex.

Die Stressvulnerabilität gegenüber den negativen Konsequenzen von Stress variiert zwischen den Individuen, wobei negative Lebensereignisse zusammen mit genetischen Faktoren eine wichtige Rolle spielen. In Übereinstimmung damit konnte in einer vorherigen Studie bereits gezeigt werden, dass die Konsequenzen von CSC in adulten Mäusen verstärkt werden können, wenn diese in ihrer frühen Lebensphase wiederholter mütterlicher Trennung ausgesetzt wurden. Der dritte Teil der Thesis hatte daher zum Ziel, den Einfluss der genetischen Prädisposition, in diesem Fall die angeborene Ängstlichkeit, auf die Auswirkungen von chronisch psychosozialem Stress zu untersuchen.

Dafür wurden CD1 Mäuse verwendet, die selektiv auf niedrige („low anxiety-related behaviour“ = mLAB) beziehungsweise hohe („high anxiety-related behaviour“ = mHAB) Ängstlichkeit gezüchtet wurden und nicht-selektierte normale CD1 Mäuse („normal anxiety-related behaviour“ = mNAB). Auf der EPM und in der Licht-Dunkel Box zeigten mLAB ein niedriges, mHAB ein hohes und mNAB ein mittleres Angstverhalten. Nicht nur die angeborene Angst ist unterschiedlich zwischen mHAB und mLAB Mäusen, auch die Reaktion auf einen akuten Stressor war in mLAB höher als in mHAB Mäusen, obwohl die basalen CORT Werte in beiden Gruppen ähnlich waren. Da es jedoch noch keine Studien gibt, welche die Reaktion auf chronischen Stress in diesen Zuchtlinien untersucht haben, wurden in dieser Arbeit die verhaltensrelevanten, physiologischen, neuroendokrinen und immunologischen Folgen nach CSC Exposition im Hinblick auf die genetische Prädisposition untersucht. Die Hypothese dabei war, dass die geringere Ängstlichkeit der mLAB Mäuse einen protektiven Effekt auf die Konsequenzen von chronischen Stress im Vergleich zu mNAB Mäusen hat, da aus zahlreichen Studien bekannt ist, dass chronische Stressexposition zu einer erhöhten Ängstlichkeit führt. Folglich müssten mHAB Mäuse stärker von den CSC-induzierten Effekten betroffen sein. In Übereinstimmung mit den bekannten Effekten von CSC in C57BL/6 Mäusen zeigten sowohl mHAB als auch mNAB CSC Mäuse ein erhöhtes Nebennierengewicht, eine erniedrigte Sensitivität der Nebennieren gegenüber ACTH *in vitro*, eine niedrigeres Plasma CORT:ACTH Verhältnis und eine erhöhte IFN-γ Freisetzung von isolierten mesenterialen

Lymphknotenzellen als Antwort auf eine anti-CD3 Stimulation im Vergleich zu SHC Tieren der gleichen Linie. Nichtsdestotrotz war der CSC-induzierte anxiogene Effekt nur bei den mNAB Tieren jedoch nicht bei den mHAB Tieren sichtbar. Dies ist höchstwahrscheinlich darauf zurückzuführen, dass mHAB Tiere bereits vor CSC-Exposition eine sehr hohe Ängstlichkeit aufwiesen, z.B. verbrachten sie weniger als 6 % der Zeit auf dem offenen Arm der EPM, die durch CSC-Exposition nicht weiter gesteigert werden konnte. Das interessanteste Ergebnis dieser Studie ist aber die Tatsache, dass mLAB Mäuse resistent gegenüber chronischem Stress waren, da alle analysierten Parameter nach CSC unverändert waren. Auch konnte gezeigt werden, dass mHAB Mäuse nicht anfälliger gegenüber den CSC-induzieren Effekten als mNAB Mäuse sind, was eventuell an der Intensität des Stressors liegen könnte, die eine Unterscheidung zwischen beiden Gruppen nicht möglich machte.

Zusammenfassend lässt sich feststellen, dass die genetische Prädisposition, in diesem Fall die angeborene Ängstlichkeit, die Vulnerabilität gegenüber chronisch psychosozialem Stress beeinflusst, wobei ein wenig ängstlicher Phänotyp Stressresistenz sowohl gegenüber affektiven als auch somatischen Folgen von CSC vermittelt. Die Kombination aus Mäusen mit angeborener extrem niedriger Ängstlichkeit und dem CSC Paradigma stellt daher ein wichtiges Werkzeug dar, um sowohl die molekularen Mechanismen die der Interaktion aus Genen und Umwelt zugrundeliegen als auch die Stressresistenz im Speziellen zu untersuchen.

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Abbreviations

ACTH	Adrenocorticotropic Hormone
ADX	Adrenalectomy
ANOVA	Analysis of Variance
ANXA1	Annexin 1
AVP	Arginine Vasopressin
AVPR-1a	Arginine Vasopressin Receptor 1a
AVPR-1b	Arginine Vasopressin Receptor 1b
BNST	Bed Nucleus of the Stria Terminalis
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium
cAMP	cyclic Adenosine Monophosphate
cDNA	complementary Deoxyribonucleic Acid
CBG	Corticosteroid-binding Globulin
CeA	Central Amygdala
CNS	Central Nervous System
CORT	Corticosterone
CRH	Corticotropin Releasing Hormone
CRH-BP	Corticotropin Releasing Hormone Binding Protein
CRH-R1	Corticotropin Releasing Hormone Receptor 1
CRH-R2	Corticotropin Releasing Hormone Receptor 2
CSC	Chronic subordinate Colony housing
DAG	Diacylglycerol
DBD	DNA-binding domain
Dex	Dexamethasone
DG	Dentate Gyrus
DMEM	Dulbecco`s Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DSS	Dextran Sulfate Sodium
DST	Dexamethasone Suppression Test
DTT	Dithiothreitol
EC	Entorhinal Cortex
EDTA	Ethylendiamintetraacetic Acid
ELISA	Enzyme Linked Immunoabsorbent Assay

EPF	Elevated Platform
EPM	Elevated Plus-Maze
FKBP51	FK506-binding protein 51
FKBP52	FK506-binding protein 52
FS	Forced Swim
GABA	*gamma*-aminobutyric acid
GAPDH	Glyceralaldehyd-3-Phosphatdehydrogenase
GC	Glucocorticoid
Glu	Glutamate
GPCR	G-Protein coupled Receptor
GR	Glucocorticoid Receptor
GRE	Glucocorticoid-responsive element
h	Hour
HAB	High Anxiety-related Behaviour
HBSS	Hank`s Balanced Salt Solution
HPA	Hypothalamic-Pituitary-Adrenal axis
HSP	Heatshock Protein
IFN-γ	Interferon γ
IHC	Immunohistochemical
InL	Infralimibic
Ip	Intraperitoneal
IP_3	Inositol 1,4,5-trisphosphate
k_d	Dissociation constant
kDa	kilo Dalton
LAB	Low Anxiety-related Behaviour
LC	Locus Coeruleus
MAPK	Mitogen-activated Protein Kinase
Mc	Magnocellular
Mc2r	Melanocortin-2-receptor
Mdr	Multidrug resistance
MeA	Medial Amygdala
mesLNC	mesenteric Lymph Node Cells
mEPSC	miniature Excitatory Postsynaptic Current
min	Minute
mM	Millimolar

MP	Milk Powder
mRNA	messenger Ribonucleic Acid
MR	Mineralocorticoid Receptor
MS	Maternal Separation
Na_3VO_4	Sodium Orthovanadate
NaF	Sodium Fluoride
NAB	Normal Anxiety-related Behaviour; non-selected normal CD1
NE	Norephinephrine, Noradrenaline
NGS	Normal Goat Serum
NLS	Nuclear Localization Signal
nM	Nanomolar
OA	Open arm
o/n	Overnight
OXT	Oxytocin
PBS	Phosphate-Buffered Saline
Pc	Parvocellular
Per1	Period 1
PFC	Prefrontal Cortex
PIP_2	Phosphatidylinositol 4,5-Bisphosphate
qRT-PCR	quantitative real-time Polymerase Chain Reaction
PKA	Proteinkinase A
PKC	Proteinkinase C
PL	Prelimbic
PLC	Phospholipase C
POMC	Pro-opiomelanocortin
PTSD	Posttraumatic Stress Disorder
PVN	Paraventricular Nucleus
RNA	Ribonucleic Acid
RT	Room Temperature
SD	Social Defeat
SHC	Single-housed Control
S/N	Supernatant
SNP	Single Nucleotide Polymorphism
SNS	Sympathetic Nervous System
SON	Supraoptic Nucleus

Sub	Subiculum
TBS	Tris-buffered Saline
TF	Transcription Factor
TPR	Tetratricopeptide Repeat
VDCC	Voltage-dependent Calcium Channels
11ß-HSD	11ß-Hydroxysteroid-Dehydrogenase

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

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

Regensburg, 04.10.2013 _____

Ort, Datum Andrea Füchsl