Chronic psychosocial stress and HPA axis functionality in male C57BL/6 mice – a closer look at the adrenal level

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Prof. Dr. rer. nat. Inga D. Neumann

Für meine Familie

Content

Chapter 1

General Introduction

Content

1 Introduction

Chronic stress is omnipresent in our modern society and thought to be the greatest health risk of the 21st century. Thereby, it is especially chronic psychosocial stress which represents a major burden for the individual by posing a high risk factor for the development of a variety of somatic as well as affective disorders. Inflammatory bowel disease (IBD) (Duffy et al., 1991; Bennett et al., 1998b; Bitton et al., 2003), cardiovascular diseases (Dimsdale, 2008), chronic fatigue syndrome (Heim et al., 2009), but also anxiety- and depression-related disorders (Agid et al., 1999; Heim and Nemeroff, 2001; Amat et al., 2005) are only a few to name.

Despite a great interest of research in this field, the mechanisms behind chronic stress-induced pathology are still poorly understood. Nevertheless, the predominant concept of the last decades of a chronic stress-induced hypothalamic-pituitary-adrenal (HPA) axis hyperactivity is challenged and opposite alterations are discussed (Heim et al., 2000a). In this context one mechanism gets more and more interesting, namely a decrease in glucocorticoid (GC) signalling. The latter can be characterized either by a reduced hormone bioavailability, the so called hypocorticism, by GC resistance of target cells, or by a combination of both (Raison and Miller, 2003). Importantly, there is evidence from human studies for a chronic stress-induced decrease in GC signalling and also for a reduced GC signalling in patients suffering from stress-related disorders like burn-out or chronic fatigue syndrome on the other hand (Heim et al., 2000a). However, the detailed mechanism underlying chronic stress-induced decrease in GC signalling and its consequences on the development of somatic and affective disorders are largely unknown.

One possibility to gain more insight into this mechanism is the use of appropriate animal models. The chronic subordinate colony housing (CSC), which was validated as an adequate and clinical relevant model of chronic psychosocial stress in male mice, seems to

represent such a model (Reber et al., 2007). Beside GC resistance of target cells, CSC mice are also characterized by basal evening hypocorticism, which seems to be mediated by chronic stress-induced alterations mainly at the adrenal level (for details see section 6 of the general introduction). Thus, the present thesis focuses on the effects of CSC on the functionality of the adrenal gland to gain more insight into the mechanisms underlying chronic stress-induced hypocorticism.

2 Stress

In the nineteenth century the French physiologist Claude Bernard (1865) postulated that the ability of an organism to maintain a constant internal environment is essential for life, independent of the external environment. Later on, this constant internal equilibrium of an organism was coined as "homeostasis" by the American physiologist Walter Bradford Cannon (Cannon, 1929a, b, 1939). Furthermore, Cannon also described the disruption of this internal equilibrium by different threats, leading to the activation of the sympathoadrenal system to restore homeostasis, but he never used the term "stress" (Goldstein and Kopin, 2007). The first and most generic definition of stress was given by the endocrinologist Hans Selye. He defined that "stress is the nonspecific response of the body to any demand" (Selye, 1936a). Later on, it was also Hans Selye who distinguished between the terms "stress" and "stressor". Thus, stressors were defined as specific challenges that cause, after being perceived, the physiological stress response (Selye, 1975). More than half a century passed before Selye´s doctrine of non-specificity underwent experimental testing, which failed to confirm it (Pacak et al., 1998b). Therefore, modern concepts explain stressors rather as a consciously or unconsciously sensed threat to

homeostasis. Moreover, the stress response has a degree of specificity depending on the particular stressor, the organism's perception of the stressor and the ability to cope with it (McEwen and Stellar, 1993; Goldstein and Kopin, 2007).

2.1 The stress systems

During exposure to stressful stimuli threatening homeostasis, two main systems are activated: The sympathetic nervous system (SNS) and the HPA axis. Activation of these systems, with their central control stations located in the locus coeruleus (LC) of the brain stem and in the paraventricular nucleus (PVN) of the hypothalamus (Kyrou and Tsigos, 2007), lead to time-limited behavioural and physical changes in a stressor-specific manner. These alterations are normally adaptive and improve the individual's chance for survival (Chrousos, 1998). In the following sections the effector pathways of the SNS and the HPA axis will be explained in more detail.

2.1.1 The sympathetic nervous system (SNS)

The SNS is part of the autonomic nervous system (ANS), which also includes the parasympathetic and the enteric nervous system. The SNS provides thereby the most immediate response to stressful stimuli to control a wide range of functions within the organism. Thus, it regulates cardiovascular, gastrointestinal, renal, respiratory and other somatic systems (Chrousos, 1998). The central control station of the SNS is located in the brain stem and termed LC, a cluster of norepinephrine (NE)-containing neurons in the upper dorso-lateral pontine tegmentum (Benarroch, 2009). Interestingly, the LC is the major noradrenergic nucleus in the brain with widespread projections to the entire neuroaxis and has been shown to be activated by diverse stressors (Valentino and Van

Bockstaele, 2008). For instance, stressors like restraint or noise were shown to increase extracellular NE levels in LC terminal regions such as the hippocampus (Abercrombie et al., 1988; Britton et al., 1992). Moreover, a strong activation was also found after stressors like shock, immune challenge, water avoidance or stressors of social nature, indicated by an increased mRNA and protein expression of the immediate-early gene c-fos and the catecholamine synthesizing key enzyme tyrosine hydroxylase (TH) in the LC itself (Valentino and Van Bockstaele, 2008). Importantly, the LC sends direct projections to the sympathetic preganglionic neurones, which are located in the intermediolateral column (thoracic and lumbar spinal cord) (Pacak et al., 1998a), and increases their activity via releasing NE and binding of the latter to α_1-adrenoceptors (Lewis and Coote, 1990).

Most of the sympathetic preganglionic neurons synapse in paravertebral ganglia (ventral and lateral to the spinal cord) with postganglionic sympathetic neurons, which innervate then the peripheral organs. The remaining preganglionic fibers synapse in prevertebral ganglia (lying in front of the vertebral column) (see Fig. 1). However, an exception is the greater splanchnic nerve, which arises from the thoracic spinal cord (segment 5-9) and projects directly, without synaptic contact in the sympathetic ganglia, to the chromaffin cells of the adrenal gland to provide the control of medullary function by the SNS (Holgert et al., 1998).

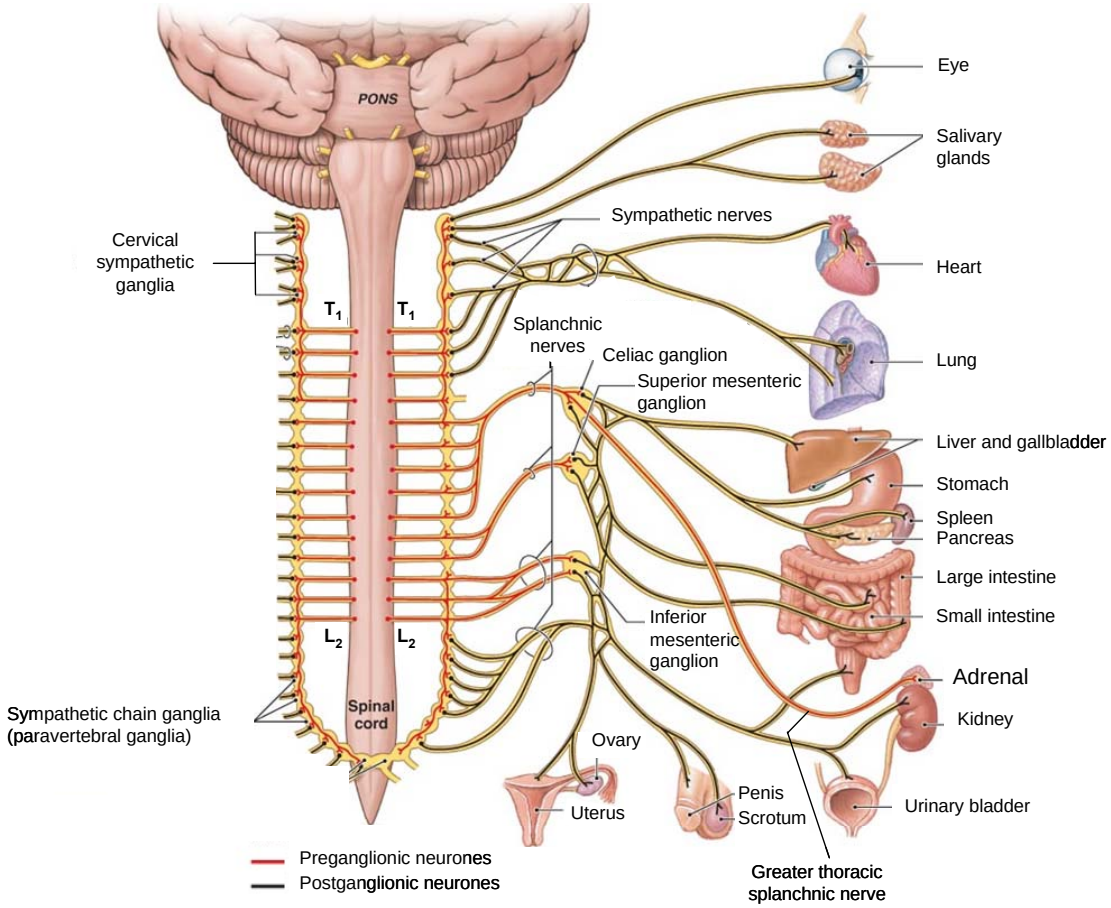

Figure 1: Schematic illustration of the sympathetic division of the autonomic nervous system (ANS). Preganglionic neurons arising from thoracic and upper lumbal regions of the spinal cord synapse with postganglionic neurons either at the paravertebral ganglia next to the spinal cord or at the prevertebral ganglia (celiac ganglion, superior mesenteric ganglion, inferior mesenteric ganglion) lying in front of the vertebrae. The postganglionic fibers innervate then the peripheral organs. The greater splanchnic nerve arises from the thoracic spinal cord and travels directly, without synaptic contact in the sympathetic ganglia, to the chromaffin cells of the adrenal medulla. [adapted from http://www.highlands.edu/academics/divisions/scipe/biology/faculty/harnden/2121/images/sympathetic.jpg]

Preganglionic neurons of the SNS are cholinergic, using acetylcholine (ACh) as neurotransmitter, whereas the noradrenergic postganglionic neurons release NE (Elenkov et al., 2000; Elenkov and Chrousos, 2006). A special feature of the SNS poses the adrenal

medulla, which provides an additional humoral limb. The medullary chromaffin cells are embryologically and anatomically homologous to the sympathetic postsynaptic neurons and also secrete NE (20 %), but to a higher extend epinephrine (80 %), directly into the bloodstream (Elenkov et al., 2000) (see Fig. 2).

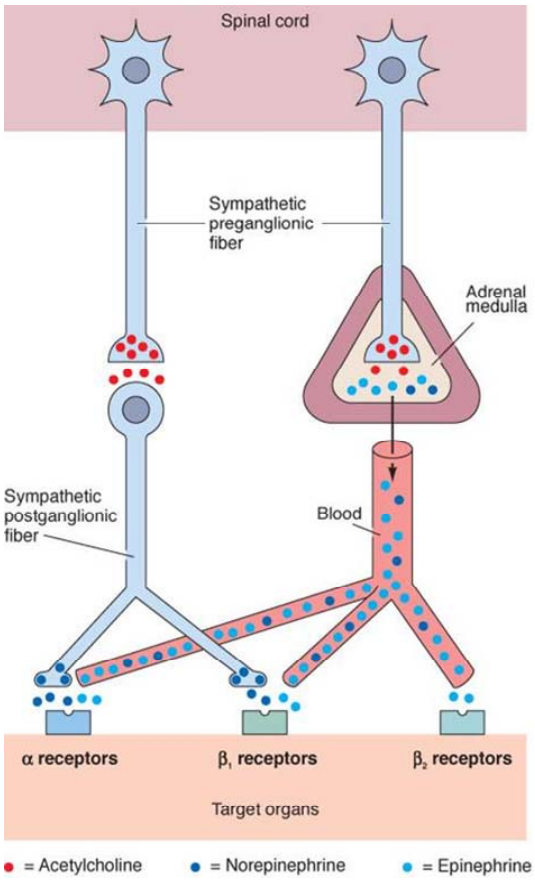

Figure 2: Sympathetic preganglionic fibers arising from the spinal cord are cholinergic, releasing the neurotransmitter acetylcholine (ACh).
Norepinephrine (NE) is the major neurotransmitter released by the postganglionic neurons at the level of the effector cells of the target organs.
Medullary chromaffin cells, representing modified postganglionic neurons, release in addition to NE also epinephrine directly into the bloodstream. [adapted from http://www.austincc.edu/rfofi/NursingRvw/PhysText/PNSefferent.html]

NE and epinephrine, both catecholamines (synthesis pathway see chapter 3.1), exert their physiological effects by binding to adrenoreceptors, which can be divided into two main classes, α- and β-adrenoreceptors, with several subtypes (α_1, α_2, β_1, β_2, β_3). All types belong to the family of seven-transmembrane domain receptors, coupled to guanosine triphosphate-binding proteins (G-proteins), stimulating or inhibiting different downstream signalling pathways (Molinoff, 1984). Given the different expression patterns of these receptors in different tissues, catecholamines exert physiological responses in an organ-specific manner. All in all, activation of the SNS and subsequent release of catecholamines

immediately increases heart and breathing rate, attention, mobilization of energy resources and slows down digestion to enable the pivotal fight-or-flight response.

2.1.2 The hypothalamic-pituitary-adrenal (HPA) axis

The HPA axis poses one of the key components of the stress reaction. Upon stressor exposure, the hypothalamus receives information from the prefrontal cortex and limbic system to produce and release corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) from parvocellular neurons of the paraventricular nucleus (PVN) into the hypophyseal portal blood system (see Fig. 3) (Tsigos and Chrousos, 2002; Baumann and Turpin, 2010). In the anterior pituitary, CRH and AVP stimulate the production and secretion of adrenocorticotropic hormone (ACTH) into the bloodstream through binding to CRH type 1 (CRHR1) and vasopressin type 1b (V1b) receptors, expressed on corticotropic cells (Aguilera, 2011). Interestingly, even though it was believed for a long time that CRH is the principal stimulus of ACTH secretion and AVP exhibiting only additive effects (Kyrou and Tsigos, 2007), nowadays it is supposed that CRH and AVP rather act in a synergistic fashion (Lightman, 2008; Spiga et al., 2009). The adrenal cortex is the principal target for pituitary-derived ACTH, where the latter triggers the synthesis and secretion of GCs through binding to melanocortin-2-receptors (Mc2r) (Gorrigan et al., 2011) (see also chapter 3.3). The GCs (cortisol in humans, corticosterone (CORT) in rats and mice) are the final effector molecules of the HPA axis with a variety of effects on the organism. For instance, GCs play an important role in energy mobilization by rapidly enhancing blood glucose and lipid levels and are known to modulate immune as well as cardiovascular functions. These peripheral effects are mediated via binding of GCs to intracellular GC receptors (GRs), which are widely distributed all over the organism. In addition, increased

GC levels affect also emotional responses and cognitive processes (Sapolsky et al., 2000), because their lipophilic nature allows crossing the blood-brain barrier.

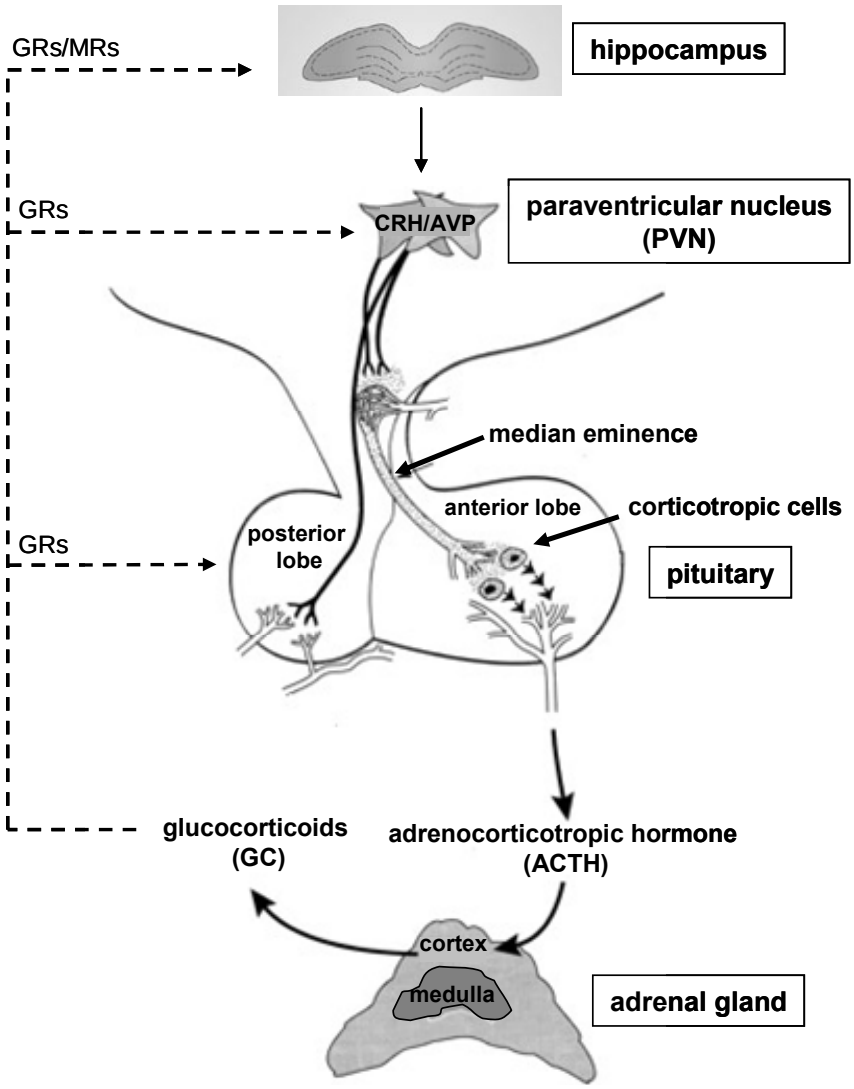

Figure 3: Schematic illustration of HPA axis activation. Corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP) are released from parvocellular neurons of the paraventricular nucleus (PVN) of the hypothalamus into the portal blood system at the levels of the median eminence to reach the corticotropic cells of the anterior pituitary. Here, they trigger the release of adrenocorticotropic hormone (ACTH) into the bloodstream through binding to their respective receptors. ACTH in turn binds to its receptors expressed in the adrenal cortex, stimulating the synthesis and release of glucocorticoids (GC), cortisol in humans and corticosterone (CORT) in

rats and mice. The GCs bind at different levels of the HPA axis to GC receptors (GRs) and at the level of the hippocampus also to mineralocorticoid receptors (MRs) to down-regulate HPA axis activity (negative feedback loop). [adapted from http://www.med.uni-magdeburg.de/~cschulz/lectures/neuroendocrinology/hpa/mat/hpa_2006.pdf]

In the brain, GCs exert their effects via binding to the GR and, in addition, also by binding to the intracellular mineralocorticoid receptor (MR), both differing in their affinity and distribution (Young et al., 1998). These receptors play an important role in mediating the effects of GCs on basal HPA axis activity and on termination of the stress response. GCs initiate several feedback loops by binding to GRs and MRs, expressed at different levels of the HPA axis, i.e. the pituitary, the hypothalamus and the hippocampus. The MR is mainly expressed in limbic brain structures, particularly in the hippocampus, and has an extremely high affinity to GCs (dissociation constant (k_d) ~ 0.5 nM) and is, thus, already occupied at low levels of circulating GCs. Therefore, the MR is thought to mediate tonic feedback, dampening HPA axis activity during the diurnal trough. The GR is most abundant in the hypothalamus and the pituitary. This receptor has a 10-fold lower affinity to GCs (k_d ~ 5.0 nM) than MR and is primarily occupied when circulating GC levels are high as it is the case at the diurnal peak and during stress. Thus, the GR is thought to play a major role in termination of the stress response, recovery from stress, and preparation to following stressors (De Kloet et al., 1998; Ladd et al., 2004). It has to be emphasized that the presence of these two receptor types with varying affinities to GCs and different local distribution enables a high flexibility in regulating basal and stress-induced HPA-axis activity with the right balance in MR- and GR-mediated effects being of critical importance (de Kloet et al., 1993; De Kloet et al., 1998).

Interestingly, is has been shown that GRs are also expressed in the adrenal glands (Loose et al., 1980; Paust et al., 2006; Riester et al., 2012), suggesting the existence of a feedback loop also at this level of the HPA axis. Nevertheless, the exact role of GRs in the adrenals is discussed controversially (Darbeida and Durand, 1987).

2.2 Acute and chronic stress

The maintenance of homeostasis within the body is of importance for the survival of an organism, but is consistently challenged by intrinsic and extrinsic forces, the stressors. The primary function of the brain is thereby to perceive the stressor and enable the organism to deal with its consequences. Thus, after exposure to stressful stimuli, independent of the nature of the stressor, the SNS and the HPA axis are activated resulting in a rapid increase of circulating stress hormones, the catecholamines and GCs. Subsequently, these stress hormones trigger physiological and behavioural changes, including energy mobilization, increased cardiovascular tone and respiratory rate. Moreover, arousal and alertness are increased, and attention is heightened. In contrast, digestive and reproductive functions are inhibited (Charmandari et al., 2005). Therefore, Sterling and Eyer (1988) introduced the term “allostasis” to describe the active processes of the body to adapt to the new situation and to restore homeostasis (Sterling and Eyer, 1988). These changes are generally beneficial in the short run and increase an individual’s chance of survival. Nevertheless, in case that these processes are excessive or prolonged, like during chronic or repeated stressor exposure, the cost of restoring homeostasis becomes too high and may result in disease. For this condition, McEwen and Stellar (1993) introduced the term “allostatic load” to refer to the wear and tear of the body resulting from excessive or prolonged activation of the stress systems and consequent endocrine imbalance (McEwen and Stellar, 1993). Thus, an important distinguishing criteria of stress is its duration and also its

intensity. Acute stress is defined to last for minutes to hours, whereas chronic stress persists for days to month (Dhabhar, 2000). Moreover, stress intensity can be determined by the magnitude of the cardiovascular response, i.e. the heart rate and blood pressure, or by measuring stress hormone levels in blood (GCs, catecholamines). For example, after acute stressor exposure blood GC levels rise and peak after 15-30 min and decline slowly to baseline values in the following hours, depending on the nature and intensity of the stressor (de Kloet et al., 2005).

In contrast to acute stressor exposure, prolonged activation of the stress systems may result in persistently elevated levels of plasma GCs. For instance, rats exposed to chronic or repeated stressors such as chronic subordination for 14 days using the visible burrow system (Albeck et al., 1997) or repeated restraint for 7 days (1.5 h/ day) (Zelena et al., 1999) are characterized by significantly increased basal plasma CORT concentrations compared with unstressed controls. Such long-lasting elevations in plasma GC levels can have deleterious effects on both physical and mental health (see section 5).

However, chronic or repeated stressor exposure not necessarily results in a persistent elevation of basal plasma GCs. There are also studies showing unaffected or initially increased, but over time declining basal plasma GC concentrations during chronic or repeated stressor exposure. For instance, Armario et al. (1986) showed that basal plasma CORT levels were not affected after repeated exposure of rats to noise (4h/ day for 21 days). Moreover, acute exposure of these rats to the same (homotypic) stressor on day 21 resulted in a significantly attenuated CORT response in repeatedly stressed compared with previously unstressed rats. Furthermore, although basal plasma CORT levels were found to be increased in mice exposed to chronic mild stress for 1 or 2 weeks, as early as 3 weeks after the start of stressor exposure basal plasma CORT levels returned to pre-stress levels (Silberman et al., 2003). These findings indicate a mechanism of adaptation, which enables

the organism to habituate to familiar innocuous stressors to prevent the negative outcomes of hypercorticism. Importantly, habituation only occurs if chronic/ repeated stressor exposure is of homotypic and not heterotypic (series of different stressors) nature. Moreover, it is generally accepted that an organism only habituates, if the homotypic stressor is of non-social nature (Bartolomucci, 2007).

Interestingly, despite habituation to a familiar stimulus, it was shown that these animals are still able to adequately respond to a novel challenge. For instance, rats which were habituated to repeated noise (see above) showed a significantly increased CORT response comparable to that of previously unstressed rats when exposed to forced swimming (10 min) (Armario et al., 1986). Such a sensitization of the CORT response to novel challenges was also observed after other stress paradigms such as repeated cold exposure for 21 days (4h/ day). Here, plasma CORT levels in response to heterotypic stressor exposure (20 min restraint) were even higher in previously stressed compared with unstressed rats (Bhatnagar et al., 1995).

Until now the mechanisms underlying adaptation of the CORT response to familiar and sensitization of the same to novel threats are not well understood. Nevertheless, it is known that ACTH secretion from the pituitary can also be down-regulated in response to chronic or repeated homotypic stressor exposure and sensitized in response to subsequent heterotypic stressors. These changes, which in turn may influence secretion of GCs from the adrenal cortex, are hypothesized to be regulated by alterations in the secretion of hypothalamic CRH and AVP or other hypothalamic regulators and of their interactions, by changes in pituitary CRHR1 and/ or V1b receptors, or alterations in the GC feedback mechanism under these conditions (Aguilera, 1994). However, at least to my knowledge, nothing is known so far, if such mechanisms of adaptation/ sensitization also occur directly at the level of the adrenal glands.

Importantly, the outcome of prolonged stressor exposure is not only dependent on the type and duration of the stressor, but also depends on the individual itself. A challenging situation can be perceived as stressful by some individuals, whereas others are highly resilient and cope with the same situation easily. These differences in stress coping are dependent on the genetic background, developmental influences, early stress experiences, and also on gender (McEwen and Stellar, 1993). For example, the influence of the genetic background on stress coping mechanisms is supported by an animal model using mice selected and bred for differences in their attack latency towards an intruder (Bohus et al., 1987). It was shown that short and long attack-latency mice are characterized by different coping styles and HPA axis responsiveness during stressor exposure. In detail, HPA axis responsiveness to chronic social defeat was significantly higher in long attack-latency mice, displaying a passive coping style, than in the active coping short attack latency mice. Therefore, the authors speculated that long attack-latency mice are more susceptible to stress-related diseases (Feldker et al., 2003; Veenema et al., 2003). In terms of early life experiences the group of Levine could already show in 1957 that short maternal separation of rat pups leads to a reduced emotional and neuroendocrine reactivity to common stressors in adulthood. Levine explained this outcome as a result of enhanced maternal care during the time when the pups were returned to their mothers (Levine, 1957). In contrast, another group could demonstrate that rats that were repeatedly separated from their mothers daily for 3 h during postnatal day 2 to 14 were characterized by an enhanced emotional and HPA axis reactivity to stressors in adulthood and, thus, also by a more vulnerable phenotype for stress-related diseases (Ladd et al., 2004).

2.3 Psychosocial stress

In 1936, Hans Selye first mentioned his theory of the "general adaptation syndrome" to describe the typical reactions of an organism (in this case rats) to different stressors to adapt to the new situation (Selye, 1936a). Importantly, the stressors he used at this time were only of physical nature, such as exposure to cold, surgical injury or intoxication. Although today still many physical stressors are in use in the laboratory such as footshock, restraint or forced swim, it became increasingly clear over the years that most of these stress paradigms have only little to do with the natural conditions of the animals and may also elicit different responses from those using psychological or social stressors (Tamashiro et al., 2005). Moreover, using only physical stressors made it also very difficult to transfer the outcomes to the human situation.

Importantly, psychosocial stress comprises the two most important aspects of stress in human life, a psychological as well as a social one. Psychological stress is thereby the result of a cognitive appraisal of environmental demands, which strains or exceeds the adaptive capacity of the individual (Cohen et al., 2007) and is thought to be a very potent stimulus for activation of the HPA axis (Mason, 1968). In contrast, social stress is deriving from interactions between different individuals, i.e. competition for resources or social rank and is thought to be a major risk factor for development of a wide variety of somatic as well as affective disorders in humans (Bennett et al., 1998a; Heim and Nemeroff, 2001; Bitton et al., 2003; Heim et al., 2009). Thus, animal models of chronic psychosocial stress are of special clinical relevance and may provide a promising tool to reveal the mechanisms underlying stress-induced disorders in humans. One appropriate animal model seems to be the chronic subordinate colony housing (CSC) paradigm, which induces chronic psychosocial stress in male mice by housing them in a group of four together with a dominant male mouse. Importantly, the CSC paradigm was shown to induce

physiological, immunological as well as behavioural consequences. To name a few, CSC mice are characterized by a reduced body-weight gain, atrophy of the thymus, and increased adrenal weight (Reber et al., 2007). CSC also induces development of spontaneous colonic inflammation (Reber et al., 2007) and increases anxiety-related behaviour (Reber et al., 2007; Reber and Neumann, 2008; Singewald et al., 2009). However, the most important consequence of CSC for the present thesis is a decrease in GC signalling. More precisely, CSC leads to basal evening hypocorticism as well as GC resistance of target cells (Reber et al., 2007). Such consequences are also observed in humans exposed to chronic psychosocial stress and seem to be involved in the development of a variety of chronic-stressed induced diseases (Heim et al., 2000a). Therefore, it seems that the CSC paradigm provides an appropriate and clinically relevant tool to gain more insight into the mechanisms underlying chronic stressed-induced disorders in humans.

3 The adrenal gland

The adrenals are paired glands located in the retroperitoneum. In humans, they lie superior to the left and right kidney with direct contact (see Fig. 4A), whereas in rodents the adrenals are located bilaterally in front of the kidneys without direct contact. Interestingly, it was shown in humans (Rubin and Phillips, 1991) and also in rats and mice (Coleman et al., 1998; Droste et al., 2003; Droste et al., 2007) that the left adrenal dominates the right one in size, which is speculated to be due to a higher sympathetic drive to the left compared with the right adrenal (Droste et al., 2003). The mammalian adrenal gland consists of two endocrine tissues, which are of different embryological origin. The adrenal cortex, producing primarily steroid hormones, is of mesodermal origin, whereas the

centrally located catecholamine-producing chromaffin cells of the adrenal medulla originate from neural crest precursor cells (Mitani et al., 1999) (see Fig. 4B).

The hormones synthesized in the adrenal glands can reach the systemic circulation and exert their physiological effects because of the rich blood supply to the adrenals. In humans, the adrenals receive blood from three separate groups of arteries: the superior, middle, and inferior arteries arising from the inferior phrenic artery, the aorta, and the renal artery, respectively. Furthermore, venous drainage is provided by the inferior vena cava and the renal vein (Bassett and West, 1997). However, the exact origin of the blood vessels innervating the adrenal glands varies depending on the species (Yamaguchi, 1993).

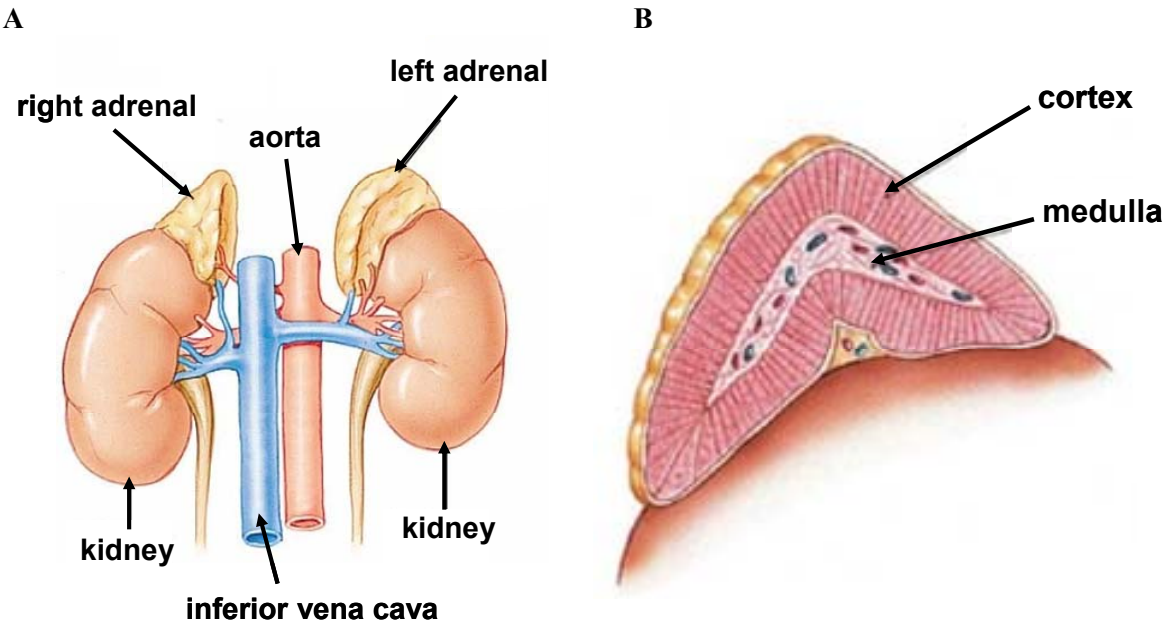

Figure 4: Schematic illustration of location and anatomy of the adrenal glands in humans. The adrenal glands are located bilaterally superior to the kidneys, in direct contact to them (**A**). Blood supply to the glands is assured by the aorta, and also by the inferior phrenic artery and the renal artery (not shown), venous drainage is provided by the inferior vena cava and the renal vein (not shown). Furthermore, the adrenal consists of two embryologically distinct tissues, the cortex and the medulla (**B**). [adapted from http://www.laurelalexander.co.uk/membersarticles/article_adrenal_fatigue.php (**A**) and http://www.endocrinesurgery.net.au/adrenal-anatomy (**B**)]

In the following chapters the innervation, morphology and functioning of the adrenal medulla and the adrenal cortex will be explained in detail to allow a better understanding of the results obtained in the present dissertation.

3.1 The adrenal medulla

Structure and innervation

The adrenal medulla consists mainly of chromaffin cells, which are embryologically and anatomically homologous to the sympathetic ganglia. Therefore, they are also designated as modified postganglionic sympathetic neurons (Diaz-Flores et al., 2008). The preganglionic innervation of the adrenal medulla is thereby mainly mediated by cholinergic fibers of the splanchnic nerve, more precisely of the greater splanchnic nerve, which arises from the thoracic spinal cord (segment 5-9) and travels directly, without synaptic contact in the pre- and paravertebral ganglia, to the chromaffin cells to provide the control of medullary function by the SNS (Holgert et al., 1998).

Types of chromaffin cells

Interestingly, medullary chromaffin cells can be divided into two separate populations, adrenergic and noradrenergic cells, which have the capacity to synthesize, store and release epinephrine or NE, respectively (de Diego et al., 2008) (see Fig. 5A). The proportion of these two types is thereby varying between species. Nevertheless, in most cases 15-20 % of the chromaffin cells are noradrenergic, while 75-80 % represent the adrenergic phenotype (Diaz-Flores et al., 2008). Several lines of evidence suggest that these two populations are regulated by distinct neural pathways to the adrenal medulla. For instance, Edwards et al. (1996) found chemically distinct populations of preganglionic neurons either negative or

positive for the calcium-binding protein calretinin, which are located in different segments of the spinal cord of the cat and innervate either adrenergic or noradrenergic chromaffin cells (Edwards et al., 1996).

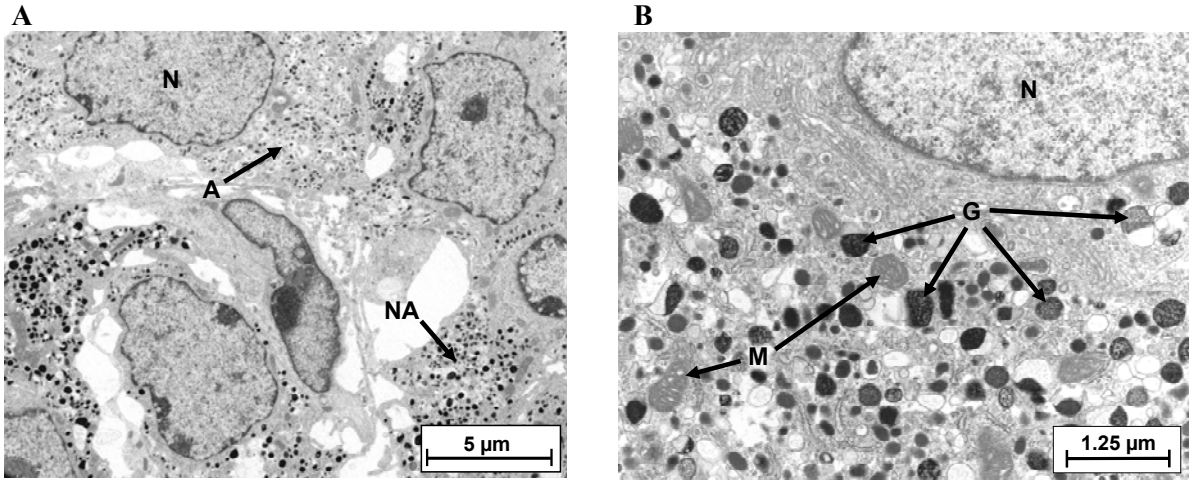

Figure 5: Electron microscopy images from adrenal medullary chromaffin cells. Chromaffin cells can be divided into adrenergic and noradrenergic cells releasing epinephrine or NE, respectively. Adrenergic cells are characterized by moderately electron-dense granules, whereas noradrenergic ones show intensely electron-dense granules (Capaldo et al., 2004) (**A**). After synthesis both catecholamines are stored in granules within the cytoplasm of the chromaffin cells until released (**B**). N, nucleus; A, adrenergic cell; NA, noradrenergic cell; G, granule; M, mitochondrion. [own images in collaboration with Prof. Ernst Tamm, Laboratory for Human Anatomy and Embryology, University of Regensburg]

Enzymatic pathway of catecholamine synthesis

The synthesis of NE and epinephrine, both catecholamines, in adrenal chromaffin cells starts with the amino acid tyrosine, which is converted to dihydroxyphenylalanin (DOPA) by the enzyme tyrosine hydroxylase (TH) (see Fig. 6). This initial step is rate-limiting and controls the synthesis of NE and epinephrine through the entire pathway. Afterwards,

DOPA is decarboxylated into dopamine by the enzyme DOPA decarboxylase and further converted into NE by the enzyme dopamine-ß-hydroxylase. The difference between noradrenergic and adrenergic cells is the existence of the enzyme phenylethanolamine-N-methyltransferase (PNMT), which enables further processing of NE to epinephrine. After synthesis, NE and epinephrine are stored in secretory granules, located in the cytoplasm of the chromaffin cells, until released in response to stimuli (Fernstrom and Fernstrom, 2007) (see also Fig. 5B).

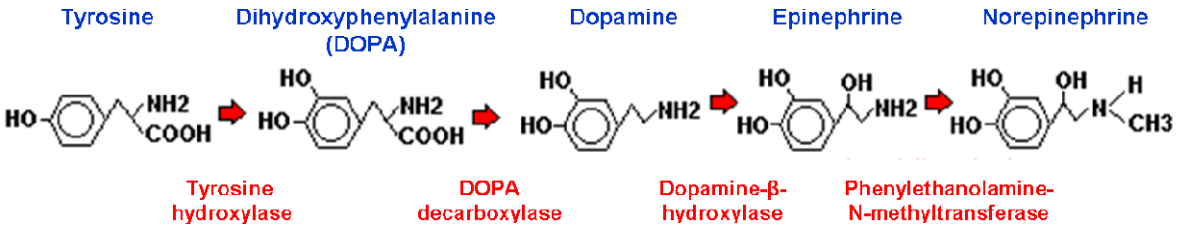

Figure 6: Schematic illustration of the enzymatic pathway of catecholamine synthesis. Synthesis starts with the conversion of tyrosine to dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase, the rate-limiting step in the production of catecholamines. DOPA decarboxylase further decarboxylates DOPA to dopamine, which is then converted to NE by dopamine-ß-hydroxylase. The existence of the enzyme phenylethanolamine-N-methyltransferase (PNMT) in the adrenal medulla is necessary for the final conversion of NE to epinephrine. [adapted from www.vivo.colostate.edu/hbooks/pathphys/endocrine/adrenal/medhormones.html]

Stimulation of catecholamine release from secretory granules

The release of catechoamines from chromaffin cells is triggered by binding of ACh, released from the presynaptic membrane of the preganglionic neurons into the synaptic cleft, to its receptors expressed on the chromaffin cells. The ACh receptors on the chromaffin cells are thereby of the nicotinic and of the muscarinic type (Olivos and

Artalejo, 2008; Sala et al., 2008). Stimulation of nicotinic receptors, which are ligand-gated ion channels, results in membrane depolarization and opening of voltage-dependent calcium (Ca^{2+}) channels. This leads to increased levels of intracellular Ca^{2+}, which trigger translocation of catecholamine-storing secretory granules to and their fusion with the plasma membrane (exocytosis) (Aunis and Langley, 1999). Stimulation of G-protein coupled muscarinic ACh receptors results in upregulation of phospholipase C and, therefore, increased levels of inositol triphosphate. The latter activates proteinkinase C, resulting in increased intracellular Ca^{2+} levels through Ca^{2+} influx and internal Ca^{2+} mobilization, and in the end to exocytosis of the chromaffin granules (Olivos and Artalejo, 2008). Importantly, to ensure termination of synaptic transmission between the preganglionic neurons and the chromaffin cells, the enzyme acetylcholinesterase (AChE) is also present on chromaffin cells. AChE inactivates the neurotransmitter ACh through hydrolyzing the latter into choline and acetate. The liberated choline is then taken up again by the presynaptic neurone were ACh is newly synthesized by combining choline and acetyl-CoA through the actions of choline acetyltransferase (Ferguson et al., 2003).

Chromaffin cells and the release of neuropeptides

It was also shown that in addition to catecholamines, adrenomedullary chromaffin cells are able to produce, store and release a great variety of neuropeptides. For example, the first neuropeptides that were discovered to be present in the adrenal medulla were enkephalins (Schultzberg et al., 1978). Moreover, neuropeptide Y (NPY) (Varndell et al., 1984; Renshaw and Hinson, 2001), vasointestinal peptide (VIP) (Kondo et al., 1986; Heym et al., 1994), atrial natriuretic peptide (ANP) (Morel et al., 1988; Nguyen et al., 1990), pituitary-adenylate-cyclase activating peptide (PACAP) (Ghatei et al., 1993; Frodin et al., 1995), substance P (Saria et al., 1980; Kondo et al., 1986), vasopressin and oxytocin (Hawthorn et

al., 1987), and many more neuropeptides could be detected in the adrenal medulla. They seem to play a major role in intra-adrenal interactions and, thus, the paracrine regulation of adrenocortical function (for details see chapter 3.3). Interestingly, these neuropeptides are co-stored and co-released with epinephrine or NE, thus, multiple populations of chromaffin cells exist within the medulla with varying peptide composition (Ehrhart-Bornstein et al., 1998).

3.2 The adrenal cortex

Structure

The structure of the mammalian adrenal cortex was first described in the year 1866 by Arnold et al., (1866), who divided the cortex in three major zones of cells arranged as concentric shells and termed them zona glomerulosa (ZG), zona fasciculata (ZF) and zona reticularis (ZR) (see Fig. 7).

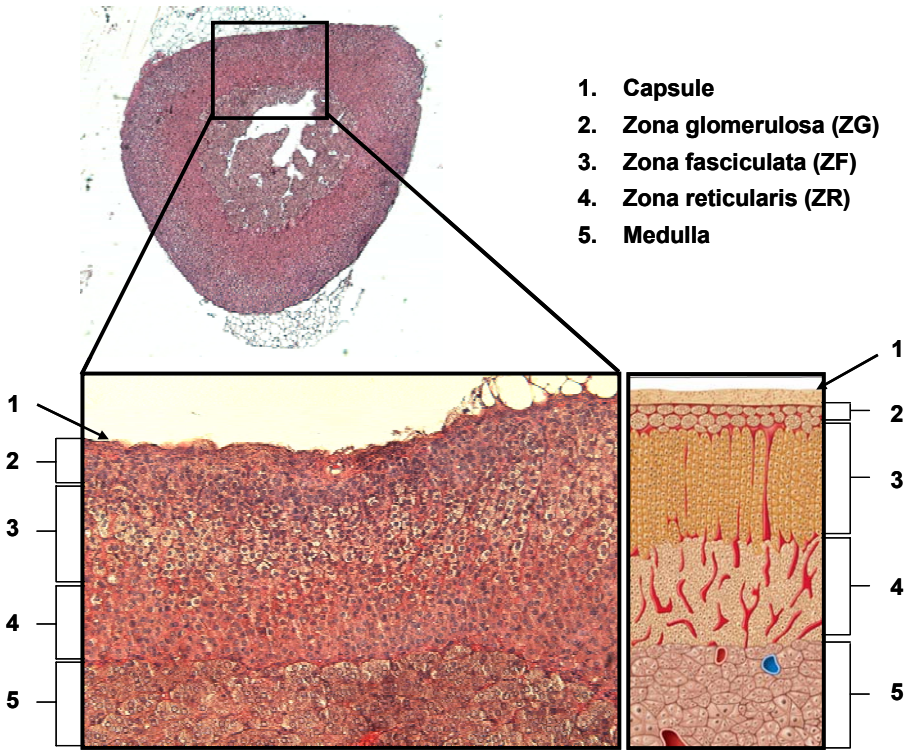

Figure 7: Mouse adrenal cryo-section stained with hematoxylin-eosin (left side). The adrenal gland can be divided into the centrally located medulla and the surrounding cortex. The cortex can be further divided into three zones: the zona glomerulosa (ZG) producing mineralocorticoids, the zona fasciculata (ZF) producing GCs, and the zona reticularis (ZR) producing also GCs [own image]. Additionally, on the right, a schematic illustration of the different zones and their respective cell types is shown. [adapted from http://antranik.org/the-endocrine-system]

The ZG, the outermost layer of the cortex, consists of small angular cells and produces the main mineralocorticoid (MC) aldosterone, which plays an important role in the regulation of salt and water balance in the kidney. The medial and major part of the cortex, the ZF, consists of large lipid-laden cells arranged in columns and is the major source of GCs, cortisol in humans and CORT in rats and mice. The innermost layer, termed ZR, is composed of cells arranged in cords around vascular sinusoids and is also able to produce GCs and in some species, like humans, also androgens, estrogens and progestins (Rosol et al., 2001; McNicol, 2008).

Cholesterol utilization

The common precursor for the synthesis of all steroid hormones in adrenal tissue is cholesterol (see Fig. 10), which can be derived from multiple sources (see Fig. 8). One possibility is the endogenous de novo synthesis from acetyl coenzyme A (acetyl CoA) via the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (Rosol et al., 2001). Furthermore, cholesterol can also be obtained via receptor-mediated endocytic uptake of low-density lipoproteins (LDLs). Here, the intact lipoprotein is internalized from the blood by the LDL receptor (LDL-R) and then degraded in lysosomes to cholesteryl esters. These cholesteryl esters are hydrolyzed by lysosomal acid lipase, which leads to free cholesterol available for steroidogenesis (Kovanen et al., 1979). Large amounts of

lipoprotein-derived cholesteryl esters are also processed through the "selective" uptake via the scavenger receptor class B type I (SR-BI). Cholesterol-rich LDLs or high-density lipoproteins (HDLs) bind here to the SR-BI, located in the plasma membrane, which leads to the release of cholesteryl esters directly into the cell without internalizing the lipoprotein particle itself (Reaven et al., 1996; Williams et al., 1999). It is believed that the SR-BI forms a hydrophobic channel in which the cholesteryl esters move down a concentration gradient, but the exact mechanism is still unknown (Rodrigueza et al., 1999). Once delivered to the cytoplasm via the SR-BI, the cholesteryl esters have to be hydrolyzed to free cholesterol, which is mediated by the hormone-sensitive lipase (HSL) (Krieger, 1999).

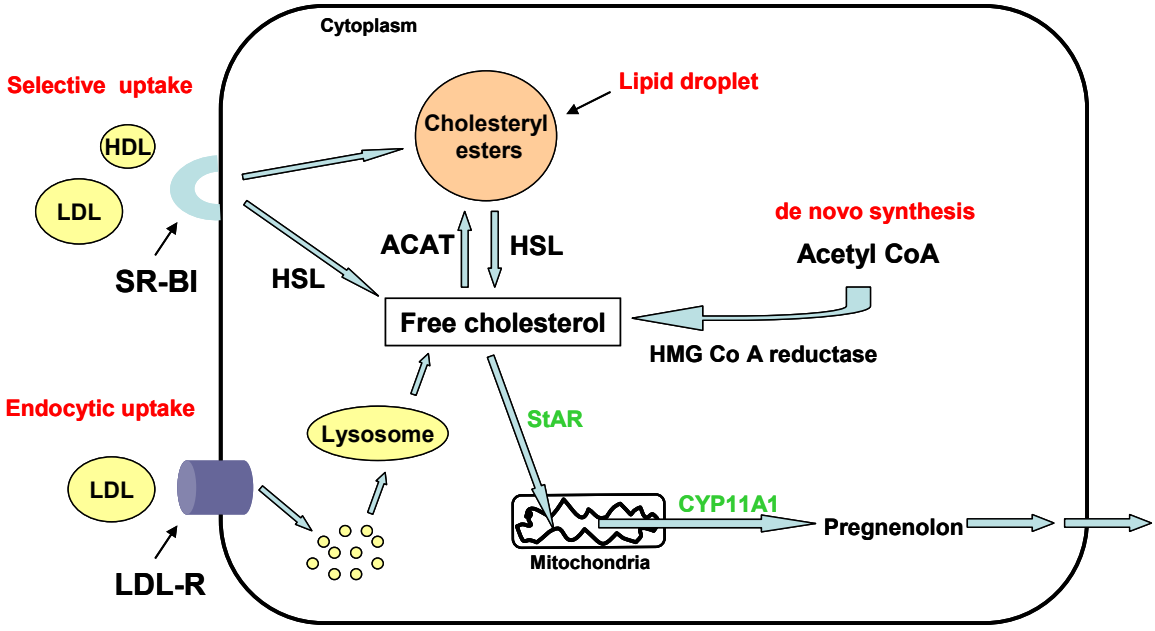

Figure 8: Schematic illustration of cholesterol metabolism for steroidogenesis. Cholesterol can be obtained via de novo synthesis from acetyl coenzyme A (acetyl CoA) catalysed by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, via endocytic uptake of lipoproteins from the blood through the LDL-R or via selective uptake of low-density lipoproteins (LDLs) and high-density lipoproteins (HDLs) from the blood through the scavenger receptor class B type I (SR-BI). Excessive cholesterol can be stored in lipid droplets within the cytoplasm after being esterified by

acyl CoA cholesterol acyltransferase (ACAT) and again hydrolyzed when cholesterol supply is needed by the hormone-sensitive lipase (HSL). Free cholesterol is then transported to the inner mitochondrial membrane via steroidogenic acute regulatory protein (StAR), where the conversion of cholesterol to pregnenolone, catalysed by cholesterol side-chain cleavage enzyme (CYP11A1), takes place. [adapted from (Kraemer, 2007)]

Additionally, free cholesterol can also be obtained through mobilization of cholesteryl esters stored in lipid droplets within the cytoplasm, mostly in cells of the zona fasciculata (see Fig. 9). In these droplets cholesterol can be stored after esterified by acyl CoA cholesterol acyltransferase (ACAT). Later on, when cholesterol supply is needed, these esters can also be hydrolyzed by the HSL.

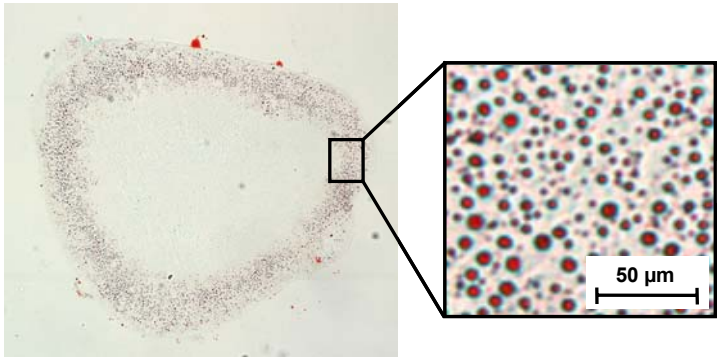

Figure 9: Mouse cryo-section stained with oilred solution to visualize the cholesterol containing lipid droplets stored in cortical cells, mostly in the zona fasciculata [own image]

Interestingly, each of the mechanisms explained above contributes quantitatively different to cholesterol utilization for steroidogenesis. Although de novo synthesis and cholesterol from lipid droplets would provide enough precursors for steroidogenesis, it seems that the adrenal glands preferentially utilize cholesterol derived from uptake of plasma lipoproteins.

Here, the selective uptake of cholesterol via the SR-BI seems to be quantitatively the most important source of cholesterol in rodent adrenal glands under both basal conditions and also in ACTH-stimulated adrenal glands (Hu et al., 2010), whereas in humans the endocytic uptake via the LDL-R seems to be most prominent (Miller, 2008).

The first step in steroidogenesis is the convertion of cholesterol to pregnenolone mediated by the cholesterol side-chain cleavage enzyme (CYP11A1), located in the inner mitochondrial membrane. Thus, the transportation of cholesterol from the outer to the inner membrane of the mitochondria is rate-limiting and it seems that this is mediated by an interplay of two factors. There is a lot of evidence that steroidogenic acute regulatory protein (StAR) is one of those factors, as, for instance, depletion of the murine StAR gene led to impaired steroidogenesis and accumulation of lipid droplets in the adrenal glands of mice (Hasegawa et al., 2000). However, it seems that StAR is not solely responsible for this movement. Several studies also indicated that the translocator protein (TSPO), a high affinity cholesterol binding protein, is also involved in mitochondrial import of cholesterol. The critical role of TSPO in steroidogenesis was indicated by an attenuation of cholesterol transport into mitochondria and, thus, steroidogenesis, after knocking down TSPO expression. This phenomenon was even observed in the presence of StAR (Hauet et al., 2005). Therefore, it is suggested that the transport of cholesterol to the inner mitochondrial membrane is mediated through protein-protein interactions between StAR and TSPO, but there is still a lot of speculation about the exact underlying mechanism (Hauet et al., 2002). One suggested possibility is that StAR binds to cholesterol at the outer membrane forming a complex, which is then transported to the inner membrane via TSPO acting thereby as a channel (Papadopoulos, 2004; Papadopoulos et al., 2007).

Enzymatic pathway of steroidogenesis

In the following section, the enzymatic pathway of steroidogenesis is explained in detail, thereby focusing especially on the synthesis of GCs and MCs. Interestingly, all enzymes involved in this pathway are members of the cytochrome P-450 (CYP) superfamily, mainly with hydroxylase activities and located either in the mitochondria or in the smooth endoplasmic reticulum (McNicol, 2008) (see also Fig. 10).

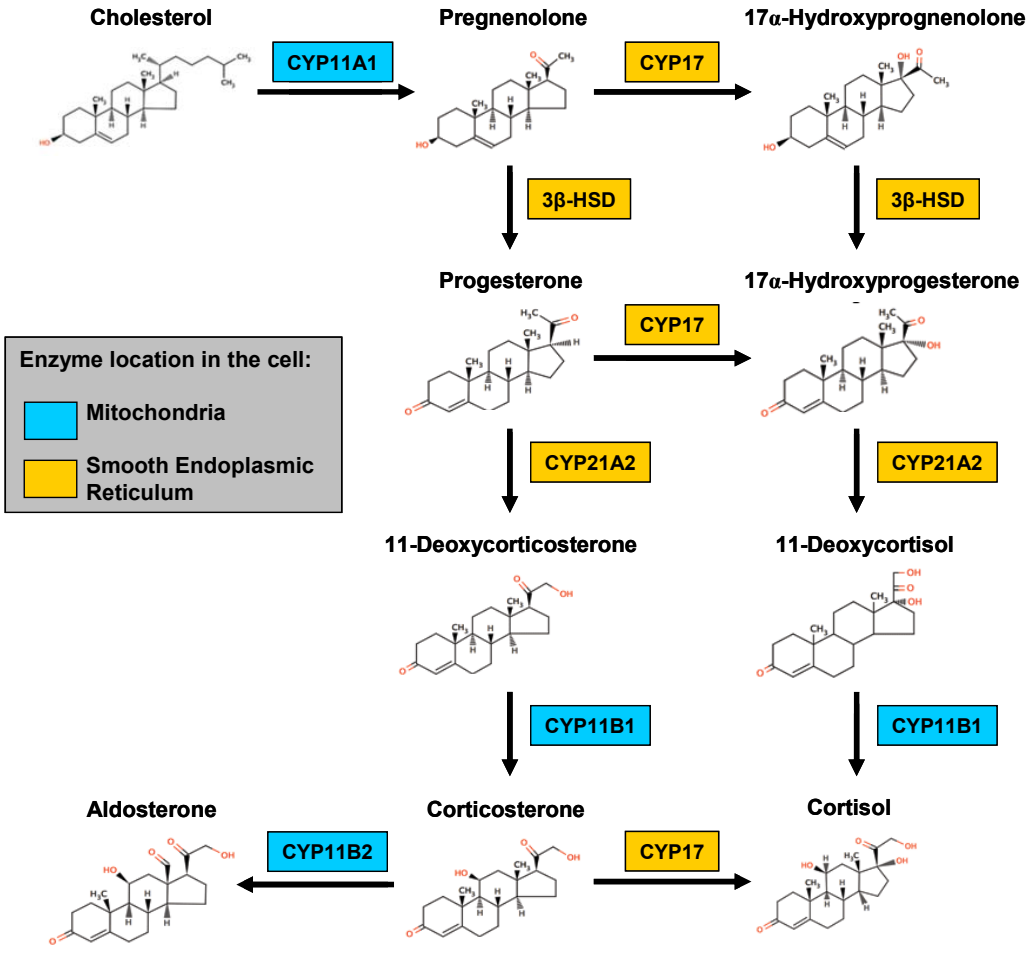

Figure 10: Principal enzymatic steps involved in the synthesis of GCs and MCs in the adrenal gland. The cholesterol side-chain cleavage enzyme (CYP11A1) catalyses the rate-limiting 20α-hydroxylation, 22-hydroxylation and scission of the 20,22 carbon-carbon bond of cholesterol, resulting in pregnenolone. Pregnenolone is then converted into progesterone by the enzyme 3β-

hydroxysteroid dehydrogenase (3β-HSD). Progesterone is then hydroxylated to 11-deoxycorticosterone by the enzyme 21α-hydroxylase (CYP21A2), and further metabolized to CORT by 11β-hydroxylase (CYP11B1). Cortisol can be obtained either via hydroxylation of progesterone to 17α-hydroxypregnenolone via the enzyme 17α-hydroxylase (CYP17), which is then converted by 3β-HSD to 17α-hydroxyprogesterone and further metabolized to 11-deoxycortisol by CYP21A2 and subsequently to cortisol by CYP11B1. Another possibility is the direct hydroxylation of CORT to cortisol mediated by CYP17. The different enzymes are either located in mitochondria or in the smooth endoplasmic reticulum. [adapted from (Hu et al., 2010)]

As mentioned before, the enzyme CYP11A1 is located in the inner mitochondrial membrane and catalyses there the rate-limiting 20α-hydroxylation, 22-hydroxylation and scission of the 20,22 carbon-carbon bond of cholesterol, resulting in pregnenolone (Miller, 2008). Afterwards, pregnenolone is converted to progesterone, mediated by the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD). Progesterone is then hydroxylated to 11-deoxycorticosterone by the enzyme 21α-hydroxylase (CYP21A2), which is subsequently metabolized to CORT by 11β-hydroxylase (CYP11B1). Cortisol can be obtained either via hydroxylation of progesterone to 17α-hydroxypregnenolone via the enzyme 17α-hydroxylase (CYP17), which is then converted by 3β-HSD to 17α-hydroxyprogesterone. Afterwards, 17α-hydroxyprogesterone is metabolized to 11-deoxycortisol by CYP21A2 and further to cortisol by CYP11B1. Another possibility is the direct hydroxylation of CORT to cortisol also mediated by CYP17 (Payne and Hales, 2004; Hu et al., 2010). Importantly, CORT is the principal GC in rats and mice, whereas in humans approximately 80 % of the circulating GCs represent cortisol (Rosol et al., 2001).

The synthesis of the MC aldosterone is mediated by the aldosterone-synthase (CYP11B2), an enzyme with two distinct activities. CYP11B2 possesses an 11β-hydroxylase activity to enable the conversion of 11-deoxycorticosterone to CORT and additionally also an 18-

hydroxylase activity to further metabolize CORT to aldosterone (Curnow et al., 1991; Domalik et al., 1991).

Importantly, the type of steroid hormone synthesized is thereby dependent on the expression patterns of the steroidogenic enzymes, which varies in a species-dependent manner among the different cortical zones, and also on the peptide hormone receptor expressed on adrenal cells. For example, CYP11B1 is mainly expressed in cells of the ZF and to a lesser extent also in cells of the ZR, resulting in the synthesis of GCs after binding of ACTH to its receptor also expressed on cells of both zones. In contrast, in the ZG the main enzyme is CYP11B2, leading to the synthesis of aldosterone, if ACTH, angiotensin II or potassium bind to their respective receptors expressed in this zone (Gallo-Payet and Payet, 2003; Hu et al., 2010).

The adrenal cortex and ACTH action

As mentioned above, adrenal GC synthesis is mainly regulated by ACTH through binding to the Mc2r, a seven-transmembrane receptor belonging to the superfamily of G-protein-coupled receptors (GPCRs) (Buckley and Ramachandran, 1981; Gorrigan et al., 2011) (Fig. 11). It has been shown that Mc2r is highly expressed in the ZF of the adrenal and to a lesser extend also in the ZR (Xia and Wikberg, 1996). The intracellular signal cascade of Mc2r has been extensively studied and it is known that binding of ACTH lead to activation of adenylate cyclase, which in turn results in increased conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). Intracellular cAMP serves as a second messenger and activates further cAMP-dependent protein kinase A (PKA) (Gallo-Payet and Payet, 2003). It is important to know that ACTH initiates two types of responses, an acute response, occurring rapidly within seconds or minutes after binding of ACTH to Mc2r, and a long-term response (Simpson and Waterman, 1988). The

acute response implies rapid activation of the above mentioned enzymes important for cholesterol utilization and steroidogenesis via phosphorylation through PKA, leading in the end to a fast increase in steroid synthesis. For example, it has been shown that the HSL, which triggers the conversion of esterified cholesterol stored in lipid droplets to free cholesterol, is stimulated by ACTH via PKA-mediated phosphorylation (Huttunen et al., 1970; Jefcoate et al., 1992). Moreover, already in 1954 Stone and Hechter showed that CYP11A1 activity is increased after ACTH stimulation in bovine adrenals and claimed that this is also mediated via phosphorylation through PKA (Stone and Hechter, 1954).

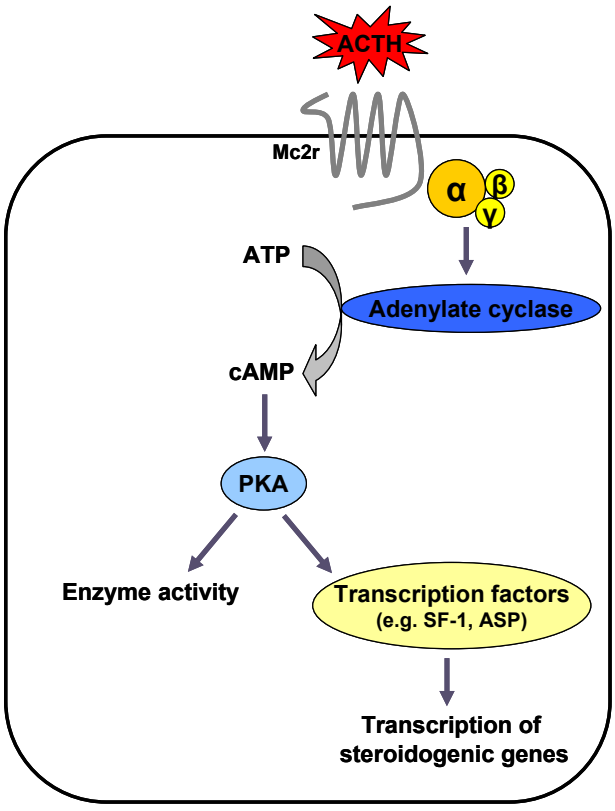

Figure 11: Schematic illustration of ACTH action in adrenal cells. Binding of ACTH to Mc2r leads to G-protein-mediated activation of the adenylate cyclase, which leads, in turn, to increased intracellular levels of cyclic adenosine monophosphate (cAMP). After activation of cAMP-dependent proteinkinase A (PKA), the latter enhances the activity of enzymes important for cholesterol utilization and steroidogenesis and also activates transcription factors via phosphorylation. Both mechanisms contribute to increase steroid synthesis and release in adrenocortical cells. [adapted from (Gallo-Payet and Payet, 2003; Sewer and Waterman, 2003; Sewer et al., 2007)]

Long-term actions of ACTH involves the maintenance of optimal expression levels of steroidogenic enzymes mediated via activation of various transcriptions factors, like adrenal-specific protein (ASP), specificity protein (Sp) family members, cAMP response element-binding protein (CREB), and steroidogenic factor 1 (SF-1), by PKA (Sewer and Waterman, 2003). Thus, it has been shown that activation of the signalling cascade through binding of ACTH to its receptor induces transcription of almost all enzymes needed for steroidogenesis, like StAR, TSPO, CYP11A1, CYP17, CYP11B1, and CYP11B2 (Sewer et al., 2007).

Interestingly, in 2005 Metherell and co-workers elucidated that Mc2r is dependent on the co-expression of an accessory protein, which is termed Mc2r accessory protein (MRAP) (Metherell et al., 2005). Later on, it was shown that MRAP plays an important role in Mc2r trafficking, cell surface expression and function (Webb and Clark, 2010). This was also indicated by the fact that mutations in MRAP cause familial GC deficiency type 2, an autosomal recessive syndrome characterized by GC deficiency despite high plasma ACTH levels (Clark et al., 2005a; Metherell et al., 2005).

In addition to the stimulatory effect of ACTH on GC synthesis, ACTH also seems to play an important role in the control of adrenocortical growth. Thus, it was shown that ACTH induces hypervascularization as well as cellular hypertrophy and hyperplasia in the adrenal cortex (Nussdorfer et al., 1971; Bornstein and Chrousos, 1999). The importance of ACTH for adrenocortical growth was further supported by the finding that a loss of endogenous ACTH as a result of hypophysectomy lead to atrophy of cortical zones, especially of the ZF and ZR (Bornstein et al., 1990b; Ceccatelli et al., 1995).

Neural innervation of the adrenal cortex

It has also to be mentioned that in addition to the direct hormonal control by ACTH, the adrenal cortex receives also an extensive extrinsic and intrinsic neural innervation, both contributing to the regulation of adrenocortical sensitivity to ACTH (Engeland and Arnhold, 2005). Extrinsic innervation is thereby mainly provided by sympathetic nerve fibers, consisting of cholinergic preganglionic fibers of the splanchnic nerve as well as catecholaminergic postganglionic fibers (Kondo, 1985; Holgert et al., 1995; Holgert et al., 1998). Intrinsic innervation is provided by nerve fibers arising from the adrenal medulla, which release catecholamines as well as a great variety of neuropeptides like NPY, VIP, and SP (Bornstein and Chrousos, 1999) (for details see section 3.3).

3.3 Intra-adrenal interactions

For a long time it was believed that the mesodermally derived steroid-producing adrenal cortex and the catecholamine-producing medullary chromaffin cells, originating from neural precursor cells, are completely distinct functional units. However, it turned out that this oversimplified view is not true and that adrenocortical and adrenomedullary cells interact in very close contact (see Fig. 12). Already in 1968 it was shown that adrenal chromaffin cells can also be found in all zones of the adrenal cortex, distributed as islets or as single cells or radiating from the medulla through the cortex (Fortak and Kmiec, 1968; Gallo-Payet et al., 1987; Bornstein et al., 1991). Later on, Bornstein and co-workers (1994) could also demonstrate the presence of cortical cells in the medulla. Because of these findings, it was suggested that the cortical-medullary interaction is a bidirectional phenomenon. This was confirmed by various studies demonstrating the influence of the adrenal cortex on the functionality of the medulla and vice versa (Bornstein and Chrousos,

1999). Such intra-adrenal interactions play a very important role, i.e. under stress conditions, because they enable an organism to synchronize the response of the cortex and the medulla and, therefore, of the HPA axis and the SNS.

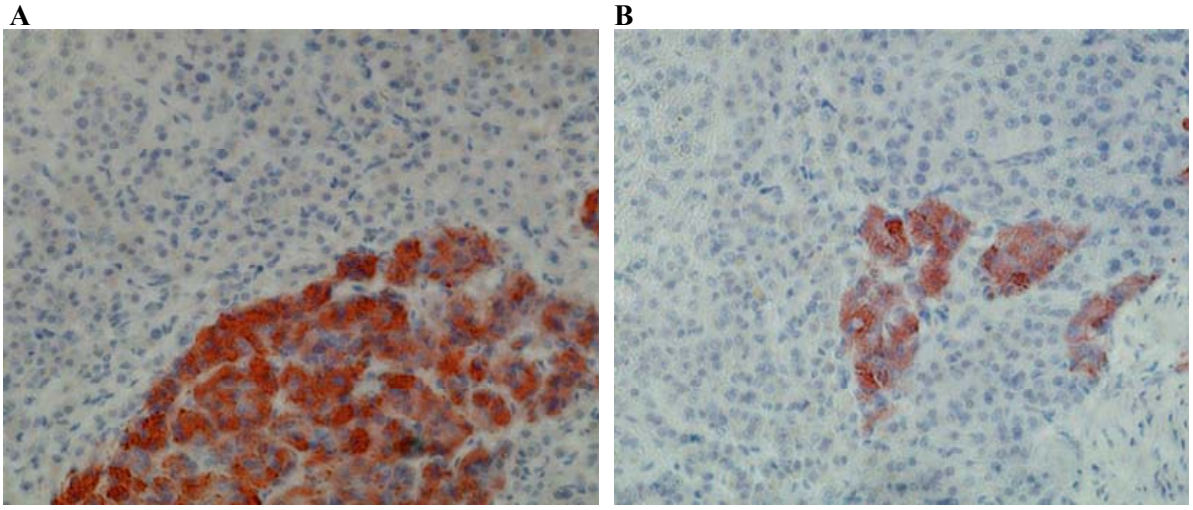

Figure 12: Representative images of chromaffin cells stained with an antibody to chromogranin A (red) using immunohistochemistry. Adrenal medullary and cortical cells are in very close contact (**A**). Moreover, chromaffin cells located in the adrenal cortex are completely surrounded by adrenocortical cells (**B**). [adapted from (Haase et al., 2011)]

Regulation of adrenomedullary function by the adrenal cortex

Regulation of adrenomedullary function by the adrenal cortex was first demonstrated by Wurtman and Axelrod (1965). By using an *in vitro* approach they could show that GCs from the cortex reach the adrenal medulla to exert influence on the enzyme PNMT, which catalyzes the conversion of NE to epinephrine. Thereby, both gene transcription as well as degradation of PNMT is regulated by GCs (Berenbeim et al., 1979; Wong et al., 1992). Especially during stress the influence of the GCs on PNMT expression seems to be very important as stress-induced increases in PNMT mRNA are not dependent on splanchnic

nerve integrity but on extreme high levels of GCs (Wurtman and Axelrod, 1965; Wong, 2006).

Regulation of adrenocortical function by the medulla

The idea of the involvement of other factors than circulating hormones like ACTH in the regulation of adrenocortical function emerged as it became more and more clear that discrepancies exist between the concentrations of these circulating hormones and the secretion of GCs (Vinson et al., 1994). Various studies gave evidence that nerve supply to the cortex is involved, and nowadays it is generally accepted that the mammalian adrenal cortex is highly innervated by nerves, whose vesicle-containing terminals directly contact cortical cells (Kondo, 1985; Parker et al., 1993; Hinson et al., 1994b). Large proportions of these nerves originate from the adrenal medulla, more precisely from medullary ganglion neurons projecting to the cortex (Holgert et al., 1998), and are, thus, dependent on splanchnic nerve activity (Holzwarth et al., 1987). Interestingly, the importance of splanchnic nerve activity in regulating adrenocortical sensitivity to ACTH was suggested by functional studies in different species. For example, sectioning of the splanchnic nerve led to a decreased response of the adrenal glands to ACTH (Edwards et al., 1986; Edwards and Jones, 1987a), whereas stimulating the splanchnic nerve results in an enhanced ACTH responsiveness (Edwards and Jones, 1987b; Engeland and Gann, 1989). Moreover, Ulrich-Lai et al. (2006a) could demonstrate, also by sectioning the splanchnic nerve in rats, that its integrity is of importance for the increase in plasma CORT in response to the diurnal rhythm by modulating adrenal sensitivity to ACTH in the active phase of the animals.

As already mentioned before, the nerve fibers innervating the adrenal cortex are able to release in addition to catecholamines a great variety of neuropeptides. Enkephalins, PACAP, SP, ANP, NPY, and VIP are only a few to name (Ehrhart-Bornstein et al., 1998).

Many of these neuropeptides and also the catecholamines have been shown to influence the release of steroids from the adrenal cortex by regulating adrenocortical function (Bornstein et al., 1990a; Hinson et al., 1994a; Hinson et al., 1994b; Andreis et al., 1995). In accordance, respective receptors could be detected in the adrenal cortex. For instance, already in 1984 Shima et al. (1984) discovered the expression of β-adrenergic receptors in the adrenal cortex.

Thus, it seems that splanchnic nerve activity regulates, at least partly, adrenocortical sensitivity to ACTH by triggering the release of catecholamines as well as of the mentioned neuropeptides from nerve endings innervating the adrenal cortex or directly from medullary cells to act in a paracrine manner.

4 Stress and the adrenal glands

As already mentioned before, exposure to stressful stimuli, real or perceived, lead to activation of the primary systems responsible for maintaining homeostasis, the HPA axis as well as the SNS. The adrenal glands are thereby the end-organs of both systems releasing GCs and catecholamines and are, thus, directly affected by stressor exposure.

Effects of stressor exposure on adrenal morphology

The most visible effects of stressor exposure on the adrenal glands are changes in their morphology. However, it is necessary to distinguish between acute and chronic stressor exposure. Acute stressor exposure, like 60 min heat stress, has been shown to decrease adrenal gland mass and volume as a consequence of adrenal cortex reduction, especially of the ZF. Koko et al. (2004) could further show that this decrease in volume of the ZF was

mainly mediated by a depletion of lipid droplets, where the CORT precursor molecule cholesterol is stored as cholesteryl esters, and accompanied by a pronounced increase in plasma CORT levels.

In contrast to these acute effects, prolonged stressor exposure has often been shown to result in adrenal enlargement (Gamallo et al., 1986; Retana-Marquez et al., 2003; Westenbroek et al., 2005; Reber et al., 2007; Slattery et al., 2011). Interestingly, an enlargement of the adrenal glands in response to long-lasting challenges, like fasting or intoxication, was already observed in 1936 by the "father of stress" Hans Selye (Selye, 1936b). Today the increase in adrenal weight, as a result of cellular hypertrophy and/or hyperplasia, in response to chronic stressor exposure is believed to be mainly mediated by the trophic hormone ACTH, the principal regulator of postnatal adrenal growth and function (see also section 3.2). This assumption was supported by studies, in which treatment with exogenous ACTH (up to 36 days) led to a strong increase in adrenal mass (Nussdorfer et al., 1974), whereas hypophysectomy and subsequent loss of endogenous ACTH resulted in adrenal atrophy, mainly due to apoptosis (Cater and Stack-Dunne, 1953; Tchen et al., 1977). In 1983 Nussdorfer and Mazzocchi could further demonstrate that prolonged ACTH treatment (3 to 15 days) increased the volume of ZF cells (Nussdorfer and Mazzocchi, 1983). Furthermore, Ulrich-Lai et al. (2006) showed in their study that male rats exposed to a 14-day chronic variable stress (CVS) paradigm exhibited significantly enlarged adrenals. Stereological counting of cells labelled for Ki67 (cell proliferation marker) or 4, 6-diamidino-2-phenylindole (DAPI, nuclear marker) combined with specific markers for the different adrenal zones in their study showed that adrenal enlargement was mediated by hypertrophy and hyperplasia in a subregion-specific manner. In detail, CVS induced hypertrophy in the inner ZF and hyperplasia of cells from the outer ZF (Ulrich-Lai et al., 2006b). Cellular hypertrophy of the ZF was also observed after

toluene administration for 7 days, mediated via an increased ACTH input to the adrenal glands (Gotohda et al., 2005) and both hypertrophy and hyperplasia of ZF cells were also found in rats with streptozotocin-induced diabetes (15 days after induction). For the latter, the investigators also speculated that this effect was mediated by a concurrent enhancement of ACTH release (Rebuffat et al., 1988). Last but not least, hypertrophy of the ZF was also shown in mice exposed to combined acoustic and restraint stress on 4 successive days (2h twice a day). Interestingly, although the size of the ZF was increased, Depke and co-workers could show that the size of lipid droplets within this zone was decreased along with elevated plasma CORT concentrations (Depke et al., 2008). In contrast to the effects on the ZF, chronic stressor exposure seems to decrease the size of the ZG and its cells. For instance, a reduced size of ZG cells was found in the above mentioned study where rats were exposed to 14 days of CVS (Ulrich-Lai et al., 2006b). Moreover, chronic hypoxia (up to 136 days) was also shown to decrease the size of the ZG and its cells (Wolman et al., 1993; Lorente et al., 2002). To conclude, it seems that chronic stressor exposure can exert different effects on adrenal morphology, with cells of the ZF undergoing growth, whereas cells of the ZG decreasing in size, which seems in both cases to be mainly mediated by ACTH.

Interestingly, in contrast to the enlargement of the adrenal cortex in response to chronic stressor exposure, it was shown that ACTH seems not to be required for the compensatory growth of the remaining adrenal after unilateral adrenalectomy, as this phenomenon occurs, for instance, also after unilateral adrenalectomy in hypophysectomised rats (Engeland et al., 1975). It is suggested that adrenal compensatory growth is mainly neurally mediated by a reflex consisting of afferent and efferent neural connections between the adrenal and the hypothalamus (Dallman et al., 1976; Ulrich-Lai et al., 2002).

Furthermore, also the cellular mechanisms seem to be different. ACTH has been shown to firstly stimulate increases in adrenal ribonucleic acid (RNA) and protein content followed later on by increases in deoxyribonucleic acid (DNA), which means that ACTH induces primarily adrenal growth by causing cellular hypertrophy followed by cellular hyperplasia (Farese and Reddy, 1963; Imrie et al., 1965). In contrast, compensatory growth induces rapid increases in all three components, DNA, RNA and protein (Dallman et al., 1976; Engeland et al., 2005).

In addition to the adrenal cortex, chronic stress has also been shown to affect the morphology of the adrenal medulla. For example, 14 days of CVS was shown to result in an increased size of the medulla, mediated by cellular hypertrophy, but not hyperplasia, of medullary chromaffin cells (Ulrich-Lai et al., 2006b). Moreover, it seems that an increased medullary size may be a general consequence of chronic stress, as medullary hypertrophy was also observed after other types of chronic stressors like chronic hypoxia (up to 136 days) (Gosney, 1985; Wolman et al., 1993) or chronic cold exposure (Scaria and Premalatha, 1967) and seems to be mediated, at least partly, by the increased sympathetic drive to the adrenal medulla as a consequence of repeated activation of the SNS (Ulrich-Lai et al., 2006b).

Effects of stressor exposure on adrenal function

Beside the effects on adrenal morphology, exposure to stressful stimuli can also affect the functionality of the adrenal glands. During acute stressor exposure adrenal functionality is normally increased to enable an adequate response, important for adaptation of the organism to the adverse situation and to restore homeostasis. Various studies describe thereby changes in the expression levels of steroidogenic enzymes important for GC synthesis in response to acute stressor exposure. For instance, mRNA and protein

expression levels of StAR were found to be strongly increased and mRNA expression of CYP11A1 was also found to be slightly increased in adrenal homogenates after an acute ACTH injection *in vivo* in rats. In accordance to that, plasma CORT levels were significantly increased in these animals compared with basal values (Lehoux et al., 1998). Moreover, StAR mRNA was found to be increased in neonatal rats after acute hypoxia (4 h) on postnatal day 8 and, in addition to that, mRNA expression of LDL-R was increased in these animals. The authors speculated that these effects, resulting in increased levels of plasma CORT, were mainly mediated by the increased levels of plasma ACTH in response to acute hypoxia (Bruder et al., 2008). Furthermore, a single ACTH injection or surgical lapratomy was shown to significantly increase CYP11B1 mRNA expression in adrenal tissue (Engeland et al., 1997). Beside the effects on steroidogenic enzymes, Jaroenporn et al. (2009b) revealed that an acute stressor like restraint for 15 min results in increased mRNA expression levels of Mc2r, the ACTH receptor in the adrenal glands. This was accompanied by increased plasma CORT levels, suggesting an increased ACTH responsiveness in acutely stressed rats. Importantly, acute stressor exposure was also shown to affect expression levels of enzymes important for catecholamine synthesis. For example, cold exposure for 1 h was shown to elicit a rapid induction of adrenomedullary TH mRNA expression (Baruchin et al., 1990). This effect was also present after a single immobilization session (2 h), and in addition to that, TH protein expression and TH activity were also found to be increased (Nankova et al., 1994; Kvetnansky et al., 2003). Moreover, TH mRNA expression and TH activity were also increased after a 5-h cold exposure or a single insulin injection (5 h after injection) (Kvetnansky et al., 2003). These changes in TH expression are in accordance to the increased levels of plasma epinephrine/NE in response to acute stimuli like cold exposure (up to 6 h) or immobilization (1 h) (Avakian et al., 1984; McCarty, 1985; Morimoto et al., 2000). All in

all, the above described changes that occur in the adrenal glands in response to acute stressor exposure enable the organism to mount adequate GC and catecholamine responses to better cope with challenging situations.

During chronic stressor exposure it is often observed that a dissociation exist between plasma levels of the steroid hormones synthesized in the adrenal glands and their direct secretagogues. It has been shown that during chronic stress GC levels can be markedly increased despite normal or decreased plasma ACTH levels (Bornstein et al., 2008). For example, Chatelain and co-workers (2003) showed that a 3-day water deprivation led to increased levels of plasma CORT in female rats, whereas plasma ACTH levels remained unchanged, suggesting an increased sensitivity of the adrenal glands to ACTH. Furthermore, exposure to chronic hypoxia for 7 days in neonatal rats was shown to result in increased plasma CORT but not plasma ACTH levels compared with normoxic controls, indicating an increased steroidogenesis in chronically-stressed rats despite the ACTH-input seems to be unaffected (Raff et al., 2003). This increase in plasma CORT was further accompanied by increased mRNA and/ or protein expression levels of StAR, TSPO and LDL-R (Raff et al., 2003; Bruder et al., 2007). Increased plasma CORT levels were also found in rats subjected to repeated immobilization (2 h/day for 14 days). Consistent with elevated plasma CORT levels, Aguilera and co-workers (1996) also revealed increased mRNA expression levels of CYP11A1 in the ZF from these chronically-stressed rats and isolated adrenal fasciculata cells also showed higher cAMP, higher pregnenolone and higher CORT responses to ACTH *in vitro*, suggesting an increased sensitivity to ACTH. Interestingly, the group of Kapoor (2005; 2008) revealed that even prenatal stress during critical windows of neuroendocrine development can affect HPA axis activity during adulthood in male guinea pigs. Repeated exposure of the mother to high frequency strobe light on gestational days 50-52 or 60-62 thereby resulted in increased basal or ACTH-

induced plasma CORT levels during adulthood, respectively. In both cases, mRNA expression of Mc2r and SF-1 were significantly increased, suggesting also an increased sensitivity of the adrenal cortex to ACTH following prenatal stress. Unfortunately, plasma ACTH levels mere not determined in the last two studies.

Other studies also provide evidence for a dissociation of plasma ACTH and CORT levels during repeated or chronic stressor exposure, but in contrast to the ones mentioned above plasma CORT levels were found to be rather decreased or normal despite normal or increased plasma ACTH levels, respectively. For example, a single social defeat (30 min) followed by 14-18 h of sensory contact was shown to result in normal plasma ACTH levels, whereas plasma CORT concentrations were significantly decreased. Moreover, subsequent exposure to the stressor of forced swim (5 min) resulted in a diminished plasma CORT response, despite increased levels of plasma ACTH. Therefore, the authors speculated that the adrenal cells of socially defeated rats are hyposensitive to ACTH (Berton et al., 1999). Hyposensitivity of the adrenals to ACTH was also suggested in Lewis rats exposed to repeated immobilization (duration not known). Although basal serum ACTH levels were significantly increased, CORT concentrations were not altered in these animals (Gomez et al., 1996). The same phenomenon was observed in rats exposed to uncontrollable intermittent foot shock for 14 days (one trail per day with 0.16, 0.32, 0.65, 1.3, 2.6 mA each for 5 sec). Despite elevated plasma ACTH levels, plasma CORT was found to be unaffected, suggesting a decreased adrenal sensitivity to ACTH in chronically-stressed rats (Anderson et al., 1996). Unfortunately, in these studies no further adrenal parameters were assessed, thus, no information on the mechanism underlying this decreased adrenal ACTH sensitivity is available. Interestingly, decreases in plasma CORT levels and adrenal CORT content despite increased plasma ACTH levels were also found in adult mice exposed to prenatal (repeated restraint of the mother 6 h/ day over 11-12 days

before parturition) as well as postnatal (repeated restraint 6 h/ day for 14 days) stress. However, adrenal mRNA expression of Mc2r and CORT secretion from adrenal fragments to different doses of ACTH *in vitro* were not altered compared with unstressed controls. Therefore, the authors assumed that it is unlikely that the decrease in plasma CORT levels in repeatedly-stressed mice is mediated by a reduced adrenal responsiveness to ACTH itself, but rather mediated by dysfunctions in the CORT synthesis or exhaustion as a result of prolonged CORT secretion in response to repeated stressor exposure. However, further adrenal parameters like expression of steroidogenic enzymes were not measured in this study to enable a more precise conclusion (Chung et al., 2005). Last but not least, Khorram et al. (2011) showed in their rat study that chronic maternal undernutrition (food restricted diet from pregnancy day 10 until delivery) resulted in decreased plasma CORT concentrations in the offspring on day 1 of life. This effect seemed to be mediated by a reduced mRNA expression of Mc2r and key enzymes of steroidogenesis (StAR, CYP11A1, CYP 11B1, and CYP11B2) in adrenal tissue. Unfortunately, plasma ACTH concentrations were not determined.

In summary, stressor exposure can exert direct effects on both morphology as well as functionality of the adrenal glands. However, the direction and magnitude of these effects seem to be dependent on the type of stressor and its duration.

Interestingly, although not addressed in the studies described in this section, it has to be mentioned that body side-specific differences in adrenal morphology have not only been described under basal (see section 3 of the general introduction) but also under stimulated conditions. For example, it was shown in mice and rats that prolonged voluntary wheel running results in a body side-specific weight gain of especially the right adrenal gland (2003; 2006; Droste et al., 2007). Furthermore, body side-specific differences in adrenal weight gain were also described in humans who committed suicide. Here, specifically the

left adrenal was increased compared with humans who died of sudden death (Szigethy et al., 1994; Dumser et al., 1998).

5 Chronic stress and HPA axis dysregulation

HPA axis dysregulation – hyperfunction and hypofunction

Life exists by maintaining a complex dynamic equilibrium, called homeostasis, which is daily challenged by different kinds of stressors, real or perceived. To restore homeostasis, the HPA axis, one of the key components of the stress system, is activated to regulate a complex repertoire of physiological and behavioural responses. HPA axis activation is thereby normally adaptive in the short-run, but chronic or repeated activation can result in its own dysregulation (Chrousos and Gold, 1992). This leads to alterations in hormone secretion and, as a consequence, affects regulation of end-organ function. The predominant opinion of the past decades was thereby that chronic stress generally results in an increased activity of the HPA axis and consequently in basal hypercorticism (Heim et al., 2000a). As almost every organ system is negatively affected by chronically-elevated levels of GCs, hypercorticism is thought to be involved in the development of a great variety of stress-related somatic as well as affective disorders (Chrousos and Kino, 2007). For example, one system, which is highly affected by increased levels of GCs is the immune system. Chronically-elevated levels of GCs are in general immunosuppressive, resulting in an impaired cytokine production, atrophy of tissue important for immune cell production like the thymus or the spleen, and also impaired leukocyte trafficking (Chrousos, 1995). Moreover, prolonged high levels of GCs impair cellular immunity by suppressing type 1 helper T-lymphocyte function and promote humoral immunity by enhancing the function

of type 2 helper T-lymphocytes (Elenkov et al., 1999), together contributing to an increased susceptibility of the organism to infections and neoplastic disease (Chrousos, 2000b). Furthermore, elevated GC levels have deleterious effects on metabolism. Hypercorticism is associated with the entire spectrum of the metabolic syndrome, including visceral obesity, insulin resistance, dislipidemia, dys-coagulation and hypertension (Friedman et al., 1996; Chrousos, 2000a). Other conditions which are associated with hypercorticism and prolonged HPA axis activation include anorexia nervosa (Gold et al., 1986), panic disorder (Gold et al., 1988b), chronic alcoholism (Wand and Dobs, 1991), and melancholic depression (Gold et al., 1988a; Chrousos and Gold, 1992).

Nevertheless, over the past years there is growing evidence from both preclinical and clinical studies for a novel and paradox phenomenon challenging the concept of a hyperactive HPA axis as a general consequence of chronic stress. Thus, besides HPA axis hyperactivity, more and more studies indicate that chronic stress can also result in a state of a decreased HPA axis activity (Heim et al., 2000a). It is even discussed that this state might develop after periods of stress with an initially hyperactive HPA axis state (Hellhammer and Wade, 1993) and might, thus, represent an over-adjustment of the organism to counteract the prolonged increased levels of GCs and their deleterious effects (Fries et al., 2005). Here, one mechanism appears in the focus of chronic stress-induced physiological, immunological and behavioural alterations, namely a decreased GC signalling. In their review, Raison and Miller (2003) describe an insufficient GC signalling as any state in which the signalling capacity of GCs is inadequate to restrain relevant stress-responsive systems. Moreover, they claim that a decrease in GC signalling can be either a result of decreased hormone bioavailability, called hypocorticism, as a result of

GC resistance of target cells, or a combination of both. Thus, the existence of GC insufficiency is also possible in the case of GC hypersecretion.

Chronic stress and a decreased GC signalling

There is a variety of studies giving evidence for chronic stress-induced hypocorticism. For example, already studies from the 1960s and 1970s revealed hypocorticism in healthy individuals living under conditions of ongoing stress. Friedman and co-workers (1963) demonstrated decreased urinary and plasma cortisol levels in parents of children with neoplastic disease, with further decreasing cortisol levels during periods of heightened stress. Other groups revealed that high work load employees have decreased basal morning plasma cortisol levels as well as blunted cortisol responses to increases in their work responsibilities (Caplan et al., 1979) and a blunted cortisol secretion was further observed in soldiers in the mist of combat (Bourne et al., 1967, 1968). Furthermore, Yehuda and colleagues have described the phenomenon of hypocorticism in patients who developed post-traumatic stress disorder (PTSD) as a result of combat exposure, natural disaster, or following childhood abuse (Yehuda, 1997, 2001). In line with the latter, studies by Heim and colleagues also demonstrated a link between early life stress in women, like emotional neglect and sexual or physical abuse, and reduced basal morning cortisol levels in adulthood (Heim et al., 2000b; Heim et al., 2001). Despite an exaggerated ACTH response to an acute stressor exposure, these women also produce less cortisol in response to an ACTH challenge test, suggesting that hypocorticism is here exclusively mediated by alterations at the level of the adrenal glands (Heim et al., 2000b; Heim et al., 2001). In addition to hypocorticism, there are also studies showing chronic stress-induced decreases in GC responsiveness of target cells. Although stress-related disorders like major depression are characterized by basal hypercorticism (Holsboer, 2001), GC responsiveness

assessed *in vitro* and *in vivo* seems to be impaired in these patients (Holsboer, 2000; Pariante and Miller, 2001).

Thus, considering all these studies, there is a large body of evidence for an overall decrease in GC signalling as one of the main outcomes of chronic psychosocial stress, independent if experienced early in life or in adulthood.

Decreased GC signalling and disease

Importantly, a lot of studies provide evidence for the involvement of a reduced GC signalling in the development of several somatic as well as affective disorders (Heim et al., 2000a). Decreased basal salivary cortisol levels were observed, for instance, in patients suffering from burnout (Kudielka et al., 2006a) or chronic fatigue syndrome (Poteliakhoff, 1981). Moreover, hypocorticism has also been linked to the development of fibromyalgia and other chronic pain syndromes, like idiopathic pain or chronic headache (Valdes et al., 1989; Elwan et al., 1991; Crofford et al., 1994; Griep et al., 1998). In the case of fibromyalgia, studies provide evidence for adrenocortical impairment, thus, patients with fibromyalgia showed a reduced adrenocortical responsiveness to a CRH stimulation test, despite normal ACTH responses (Crofford et al., 1994; Griep et al., 1998). Additionally, hypocorticism seems also to be involved in the etiology of inflammatory disorders, like rheumatoid arthritis, asthma, as well as inflammatory bowel disease (Chikanza et al., 1992; Kruger and Spiecker, 1994; Mayer, 2000).

In addition to hypocorticism, GC resistance also contributes to a decreased GC signalling and has been shown to be involved in the etiology of many disorders. For example, GC resistance is associated with the inflammatory states of rheumatoid arthritis and asthma, with Crohn´s disease and ulcerative colitis (Kino and Chrousos, 2002). Importantly, the most persuasive disorder in the context of GC resistance seems to be major depression.

Although a common and reliable finding in patients with major depression is a hypersecretion of GCs, numerous *in vitro* and *in vivo* studies observed a reduced responsiveness to GCs in these patients (Holsboer, 2000; Pariante and Miller, 2001). Thus, they are characterized by non-suppression of cortisol in the dexamethasone-suppression/CRH-challenge test *in vivo* (Heuser et al., 1994; Holsboer, 2000), as well as by an impaired inhibition of natural killer cell activity, lymphocyte proliferation, and cytokine production by peripheral immune cells after GC exposure *in vitro* (Lowy et al., 1984; Miller et al., 1991; Maes et al., 1993).

Given that all these disorders are thought to be linked to chronic psychosocial stress strongly suggests that the latter, by decreasing GC signalling and consequently up-regulating the immune system, increases susceptibility to affective, inflammatory and autoimmune diseases (Chrousos, 2000b).

Nevertheless, the mechanisms underlying chronic stress-induced decrease in GC signalling, and whether or not this is causally involved in the development of these somatic and affective disorders are still unknown. One powerful tool to gain more insight into these mechanisms is the use of appropriate and clinically relevant animal models of chronic psychosocial stress. Importantly, the CSC paradigm, which was established to induce chronic psychosocial stress in male mice (Reber et al., 2007), seems to represent such a model. The detailed effects of the CSC paradigm on behavioural, immunological and physiological parameters supporting this assumption are explained later on (see section 6).

Protective effects of a decreased GC signalling?

Interestingly, beside the above described negative symptoms associated with a decreased GC signalling, a hypoactive state of the HPA axis might also have protective effects for the

organism. For example, beneficial effects of a decreased GC signalling seem to occur in pregnant women with daily high stress load. Thus, in these women the cortisol awakening response is attenuated compared with pregnant women with normal or low daily stress load, suggesting the prevention of pre-term birth due to harmful stimulatory effects of maternal cortisol on placental CRH, which is known to play a major role in the onset of delivery (Fries et al., 2005). Moreover, Hellhammer and colleagues could demonstrate that the development of stress-induced hypocorticism can also protect the organism against a high allostatic load index (index of biomarkers representing neuroendocrine, immune, metabolic, and cardiovascular system functioning), which is associated with a higher risk for mortality. Thus, although they scored higher on perceived stress scales, patients with hypocorticism had a much lower allostatic index than patients with normal hormone status (Hellhammer et al., 2004). Therefore, the authors suggested that a hypoactive state of the HPA axis represents a kind of protective response to chronic stress reducing the damaging effects of high GC levels at the expense of symptoms like inflammation, pain, and fatigue.

Development of HPA axis hyper- or hypofunction – individual differences

Interestingly, if an individual develops a chronic stress-induced hyper- or hypofunction of the HPA axis might be dependent on the intensity and duration of the stressor, the timing of stressor exposure (e.g. during critical periods of development), the genetic background, and also on the social environment (Chrousos and Gold, 1992). In this context, Chrousos and Gold illustrated in their review sigmoidal dose-response curves between the potency of a stressor (dependent on the factors mentioned above) and the activity of the stress system (HPA axis activity) with shifting to both sides would result in an altered vulnerability to stress (see Fig. 13 A). In hyperresponsive individuals the curves would therefore shift to the left, whereas a shift to the right would reflect a hyporesponsiveness.

In addition, corresponding inverse *U*-shaped dose-response curves picture the coherence between sense of well-being/performance and stress system activity with an optimal level of arousal for normally reactive individuals (see Fig. 13 B). However, shifting the activity of the stress system in either direction could produce discomfort or decreased performance. Individuals whose curves are shifted to the left may therefore be more vulnerable to diseases associated with a hypoactive state of the HPA axis like atypical depression or inflammatory diseases, whereas individuals whose curves are more shifted to the right would be more prone to hyperactivity illnesses like melancholic depression or the metabolic syndrome (Chrousos and Gold, 1992; Chrousos, 2009).

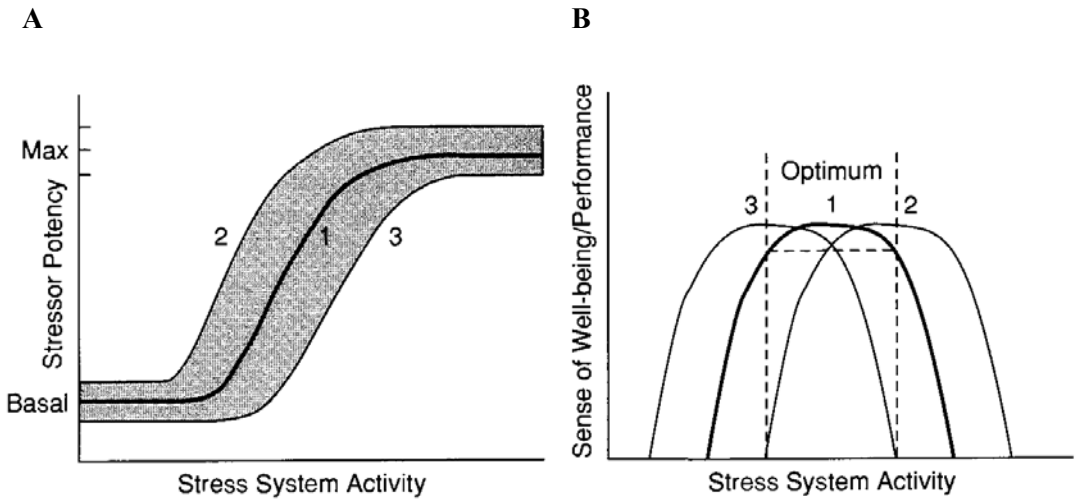

Figure 13: Sigmoidal dose-response curves between stressor potency (dependent on its intensity and duration, timing of stressor exposure, genetic background, and social environment) and activity of the stress system (HPA axis) (**A**) and inverse *U*-shaped dose-response curves between sense of well-being/performance and activity of the stress system (HPA axis) (**B**) in normally reactive (1), hyperreactive (2) and hyporeactive (3) individuals. Dependent on the individual responsiveness the curves shift to the left or right, respectively. [adapted from (Chrousos and Gold, 1992)]

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

CSC paradigm

The CSC paradigm, which was also employed in the experiments of the present thesis, was established by Reber and colleagues (2007) as an appropriate and clinically relevant model to induce chronic psychosocial stress in male C57BL/6 mice. Thereby, four experimental male mice are exposed to chronic psychosocial stress by housing them in a colony together with a larger and more aggressive, and therefore, dominant male mouse (resident) for 19 consecutive days. To avoid habituation, the resident is exchanged once per week (see Fig. 14).

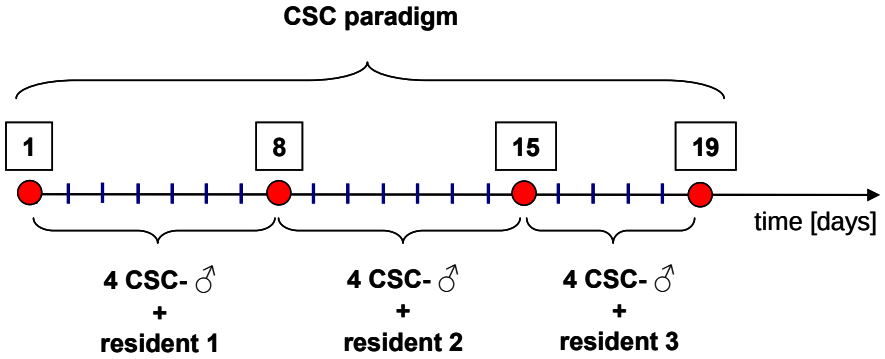

Figure 14: Time-schedule of the CSC paradigm. To induce chronic psychosocial stress, four male C56BL/6 mice are housed together with a larger, dominant male (resident) for 19 consecutive days. To avoid habituation, the resident is replaced by a novel one on days 8 and 15.

A subordinate position of the four experimental mice is thereby the main requisite for the success of the CSC paradigm. This is ensured by behavioural observations during the first 30-60 min after setting up the CSC colonies. The dominant male displays offensive behaviours like chasing, mounting, and attacking its four cage-mates, whereas the four

experimental CSC mice display defensive behavioural strategies like flight and defensive upright (Reber and Neumann, 2008) (see Fig. 15). Thus, the experimental CSC mice live in chronic subordination over 19 consecutive days and are characterized by typical signs of chronic stress, including physiological, immunological, and behavioural alterations, when compared with single-housed control (SHC) mice (Reber et al., 2007; Reber and Neumann, 2008; Veenema et al., 2008; Singewald et al., 2009; Slattery et al., 2011). Importantly, in line with other studies showing that single housing is less stressful than group housing in male mice (Bartolomucci et al., 2003; Gasparotto et al., 2005), SHC mice were considered to be appropriate controls for the CSC paradigm (Singewald et al., 2009).

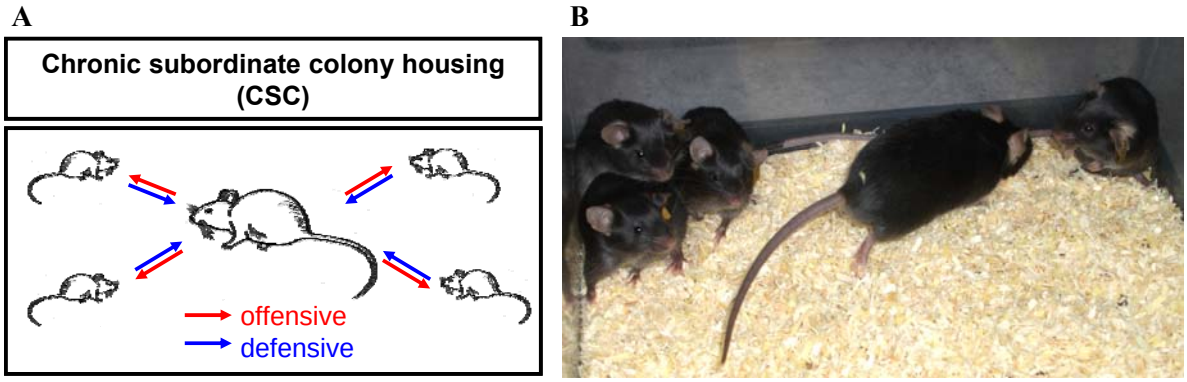

Figure 15: Schematic illustration (**A**) and a representative image (**B**) of the CSC paradigm. The four experimental mice (left and right corners) display almost exclusively defensive behavioural strategies like defensive upright, whereas the resident (middle) shows offensive behaviours like chasing its four cage-mates.

CSC-induced physiological alterations

With respect to physiology, the CSC paradigm has been shown to result in profound alterations of different physiological parameters. For example, CSC mice display a reduced body weight gain (Fig. 16 A) and relative thymus weight (Fig. 16 B). Furthermore, relative

adrenal weight is increased as early as 24 h after the start of CSC (Reber et al., 2007) (Fig. 16 C).

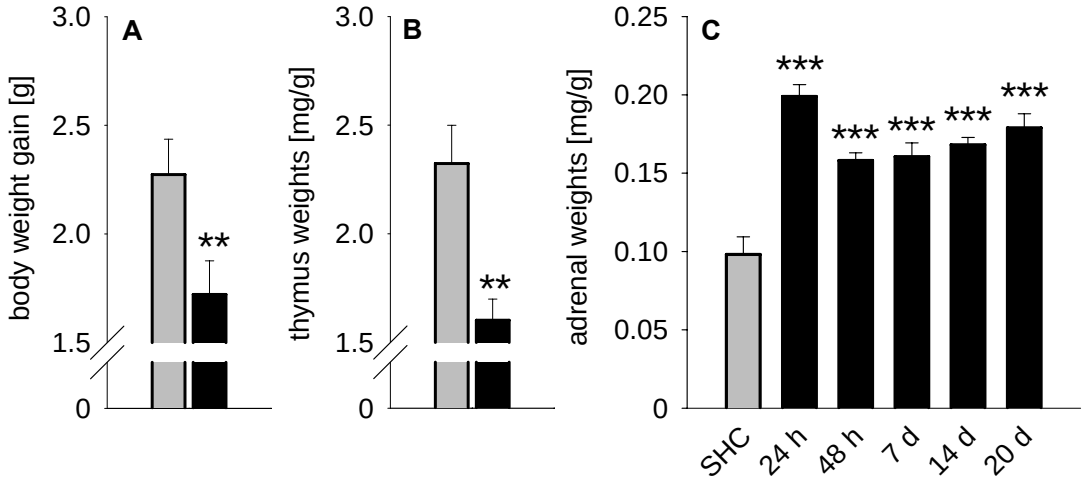

Figure 16: 19 days of CSC result in decreased body weight gain (**A**), decreased relative thymus weight (**B**), and increased relative adrenal weight (**C**) compared with SHC mice. Relative adrenal weight was already increased as soon as 24 h after the start of CSC (**C**).). ■ SHC; ■ CSC. Data represent means + SEM; **, $P < 0.01$; ***, $P < 0.001$ *vs.* respective SHC mice. [adapted from (Reber et al., 2007)]

Importantly, it was also shown that CSC exposure affects the functionality of the HPA axis, which seems to be mediated mainly at the level of the adrenal glands. Thus, despite constantly enlarged adrenals, basal morning plasma CORT levels are only increased during the initial 24 h of CSC exposure (Reber et al., 2007; Reber et al., 2011) (Fig. 17 A). Moreover, following 19 days of CSC exposure these mice are incapable to mount the circadian rise in plasma CORT, indicated by significantly reduced evening plasma CORT levels in CSC compared with SHC mice (Reber et al., 2007) (Fig. 17 B/C), suggesting adrenal dysfunction. In line, *in vitro* stimulation of isolated adrenal cells (pooled from left and right adrenal glands) revealed a strongly attenuated ACTH responsiveness following

19 days of CSC exposure, indicated by lowered CORT concentrations in the supernatants of CSC compared with SHC adrenal cells (Reber et al., 2007) (Fig. 17 D).

In addition, 19 days of CSC exposure have also been shown to result in GC resistance of target cells. Thus, isolated splenocytes stimulated with lipopolysaccharides as well as Th2 cells from peripheral lymph nodes of CSC mice showed a reduced sensitivity to different physiological and pharmacological doses of GCs *in vitro* (Reber et al., 2007; Schmidt et al., 2010a). These results clearly demonstrate that CSC exposure results in an overall impairment of GC signalling, as it is also observable in humans living under conditions of ongoing stress.

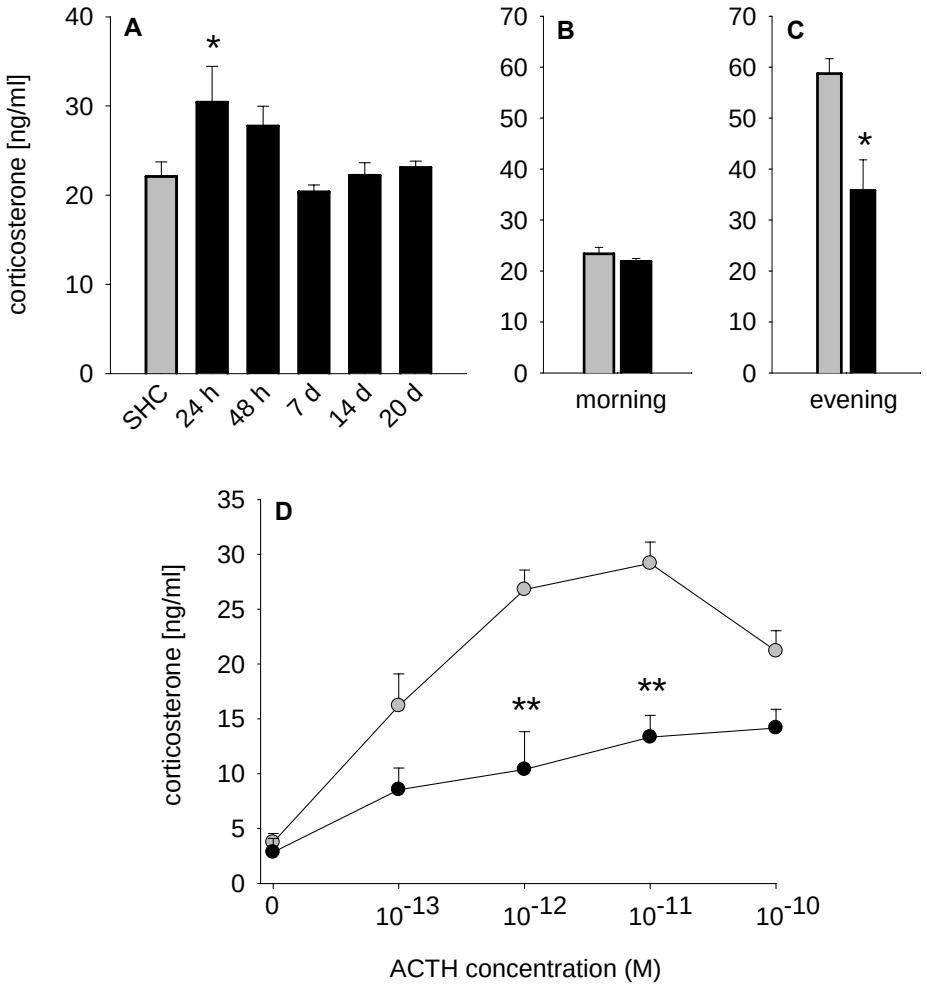

Figure 17: Basal morning plasma CORT levels are significantly increased during the initial 24 h of CSC exposure compared with baseline levels (Reber et al., 2007; Reber et al., 2011). However, this effect is not present anymore as early as 48 h after the start of CSC (**A**). Following 19 days of CSC basal morning plasma CORT levels are unaffected (**A**/**B**), but CSC mice show significantly reduced basal evening plasma CORT levels compared with SHC mice (**C**). Moreover, CORT secretion of isolated adrenal cells in response to different doses of ACTH is significantly reduced following 19 days of CSC (**D**). ■ SHC; ■ CSC. Data represent means + SEM; *, $P < 0.05$; **, $P < 0.01$ *vs.* respective SHC mice. [adapted from (Reber et al., 2007)]

CSC-induced behavioural and immunological alterations

In addition to physiological alterations, it has also been shown that CSC exposure affects behavioural and immunological parameters. The most obvious behavioural consequence of 19 days of CSC exposure is a profound and robust increase in anxiety-related behaviour when compared with SHC mice. This effect was evident in four independent behavioural tests used to determine anxiety-related behaviour in rodents: the light-dark box (Costall et al., 1989; Henniger et al., 2000), the elevated plus-maze (Salome et al., 2006), the open-field test (Jaszberenyi et al., 2007), and the novel object test (van Gaalen and Steckler, 2000). In detail, CSC mice spent less time in the bright zone of the light-dark box (Reber and Neumann, 2008) and on the open arms of the elevated plus-maze (Reber et al., 2007; Reber and Neumann, 2008; Slattery et al., 2011). Furthermore, CSC mice entered the inner zone of the open field less often than SHC mice and the time of novel object exploration was also found to be decreased after CSC exposure (Veenema et al., 2008). Although the detailed mechanisms underlying the CSC-induced increase in anxiety-related behaviour are not known so far, there is evidence for the involvement of CSC-induced alterations in neuronal activity of different brain regions involved in processing of emotionality, like the parvocellular PVN, ventral and intermediate parts of the lateral septum, the hippocampal

dorsal CA3 region, and different subregions of the periaqueductal grey and of the nucleus accumbens (Singewald et al., 2009).

Beside these behavioural alterations, 19 days of CSC exposure also affect different immunological parameters, as for instance atrophy of the thymus gland (Reber et al., 2007) and development of spontaneous colonic inflammation. This was indicated by an increased histological score in the colon (Reber et al., 2007), indicative of epithelial damage and leukocyte infiltration into colonic tissue, and an increased *in vitro* cytokine secretion from isolated mesenteric lymph node (Reber et al., 2007) and lamina propria mononuclear (Reber et al., 2011) cells in CSC compared with SHC mice. Moreover, the number of macrophages, dendritic and Th cells in colonic tissue are increased following CSC (Reber et al., 2011). In addition to spontaneous colitits, CSC mice further developed a more severe chemically-induced colitis when treated with dextran sodium sulphate (Reber et al., 2007; Veenema et al., 2008).

Taken together, 19 days of CSC exposure reliably result in basal hypocorticism, at least in the evening, and GC resistance of target cells and, thus, in an overall impairment of GC signalling. Furthermore, CSC mice are characterized by an increased anxiety-related behaviour and an increased inflammatory state. As the human literature supports the view of an overall impairment of GC signalling as consequence of chronic psychosocial stress to be causally involved in the development of a great variety of diseases (Heim et al., 2000a), the CSC paradigm seems to represent an adequate and clinically relevant tool to gain more insight into the mechanisms underlying chronic stress-induced alterations in GC signalling and its consequences on the development of the affective and somatic disorders already mentioned before.

7 Aim of the present thesis

As already stated before, chronic psychosocial stress represents a high risk factor for the development of a variety of somatic as well as affective disorders. Thereby, a decrease in GC signalling to be causally involved in the development of chronic stress-related disorders like burnout, chronic fatigue syndrome, fibromyalgia, inflammatory bowel disease, rheumatoid arthritis and also major depression, becomes more and more prominent. However, the detailed mechanisms underlying chronic stress-induced decreases in GC signalling, either as a result of hypocorticism or GC resistance of target cells, and its consequences on the development of the above mentioned disorders are largely unknown.

As described in detail in section 6 of the general introduction, male mice exposed to the CSC paradigm, a clinically relevant tool to induce chronic psychosocial stress, are characterized, amongst others, by basal evening hypocorticism. Thus, despite enlarged adrenals, CSC mice have unaffected basal morning and even decreased basal evening plasma CORT concentrations when compared with SHC mice. In line, the *in vitro* CORT response of isolated adrenal cells to ACTH was found to be strongly attenuated in CSC compared with SHC mice. Together, these finding suggest CSC-induced attenuation/ loss of adrenal ACTH responsiveness to mediate basal evening hypocorticism. Therefore, the CSC paradigm serves as an adequate and promising tool to gain more insight into the mechanisms underlying chronic stress-induced hypocorticism with the main focus of the present dissertation lying thereby on CSC-induced alterations at the level of the adrenal glands.

In **chapter 2**, the effects of 19 days of CSC exposure on adrenal function are investigated in more detail and possible mechanisms underlying the CSC-induced attenuation/ loss of adrenal ACTH responsiveness *in vitro* are discussed.

For this, SHC and CSC mice were implanted with a jugular vein catheter for repeated blood sampling and plasma CORT concentrations were assessed under basal conditions and in response to an acute heterotypic stressor (elevated platform (EPF), 5 min). Furthermore, adrenal CORT content was analyzed in SHC and CSC mice under both conditions. In addition, *in vitro* stimulation (6 h) was performed with physiological as well as pharmacological ACTH doses in adrenal explants instead of isolated adrenal cells. Moreover, expression levels of Mc2r, MRAP and key enzymes of steroidogenesis were assessed for revealing the mechanism underlying the attenuation/ loss of adrenal ACTH responsiveness following 19 days of CSC exposure.

Chapter 3 deals with the alterations of basal morning plasma CORT concentrations during the initial 48 h of CSC exposure.

Thus, adrenal weight, adrenal ACTH responsiveness, availability of the CORT precursor molecule cholesterol for steroidogenesis and expression patterns of key steroidogenic enzymes in adrenal tissue were assessed to gain more insight into the mechanism underlying the increase in plasma CORT during the first 24 h and the subsequent return to baseline levels following 48 h of CSC exposure.

In **chapter 4**, it is assessed, whether the discrepancy between adrenal ACTH responsiveness under *in* vitro (6 h) and acute stress *in vivo* conditions (EPF, 5min) following 19 days of CSC, described in chapter 2, is due to the timing schedule employed to assess adrenal CORT secretion under these conditions. Thus, relative and absolute plasma CORT concentrations were investigated in CSC and SHC mice in response to prolonged heterotypic stressor exposure (4 h shaking/ restraint stress). Furthermore,

relative adrenal CORT secretion during acute (30 min) *in vitro* ACTH stimulation is assessed in adrenal explants from SHC and CSC mice.

Moreover, to further investigate general functionality of the adrenal glands in chronically-stressed mice, the availability and mobilization capacity of the CORT precursor molecule cholesterol is assessed in adrenal tissue of SHC and CSC mice following 19 days of CSC.

An important part of the present thesis was also the establishment of a series of techniques to enable the investigation of adrenal parameters mentioned above and of adrenal parameters which will be assessed in future investigations. Thus, in the course of my dissertation I established the following techniques:

- Stimulation of adrenal explants *in vitro* with ACTH and with the cAMP analogue $(Bu)_2$ cAMP
- Determination of adrenal cAMP content in response to ACTH *in vitro*
- Determination of *in vivo* adrenal CORT content
- Oilred staining in adrenal cryo-sections (for quantification of adrenal lipid droplet content)
- 4´6-diamidino-2-phenylindol (DAPI) staining in adrenal cryo-sections (for quantification of cell numbers per given area)
- Acetylcholinesterase (AChE) histochemistry in adrenal cryo-sections (for quantification of AChE activity in the adrenal medulla)

The performance of these techniques and subsequent data analysis are also described and discussed in detail in section 2 of chapter 5.

Chapter 2

Chronic psychosocial stress results in sensitization of the HPA axis to acute heterotypic stressors despite a reduction of adrenal *in vitro* ACTH responsiveness

Uschold-Schmidt N.: Study design, performance of experiments and data analysis (performance of the CSC paradigm, determination of adrenal weight (Fig. 22), cryo-sectioning of adrenal tissue and subsequent quantification of number of adrenal cells per given area in DAPI-stained sections (Fig. 23), *in vitro* adrenal ACTH stimulation and subsequent determination of CORT levels in supernatant via ELISA (Fig. 24/25), determination of adrenal CORT content and plasma CORT and ACTH levels in trunk blood following EPF exposure via ELISA (Fig. 26), mRNA expression analysis via TaqMan-qPCR (Fig. 27A, 28, Tab. 1), determination of plasma CORT in repeated blood samples via ELISA and of anxiety-related behaviour during EPF exposure in catheterized mice (Fig. 21, in cooperation with Nyuyki)), writing the first draft of the manuscript
Nyuyki K.D.: Performance of experiments and data analysis (determination of plasma CORT in repeated blood samples via ELISA and of anxiety-related behaviour during EPF exposure in catheterized mice (Fig. 21, in cooperation with Uschold-Schmidt))
Füchsl A.M.: Performance of experiments and data analysis (Western blotting for adrenal Mc2r protein expression (Fig. 27B/C)
Neumann I.D.: Study design, revision of manuscript
Reber S.O.: Study design, revision of manuscipt
Jugular vein catheterization was performed by the technical assistant Maloumbi R.

[taken and partly adapted from Uschold-Schmidt N., Nyuyki K.D., Füchsl A.M., Neumann I.D., Reber S.O., 2012. Chronic psychosocial stress results in sensitization of the HPA axis to acute heterotypic stressors despite a reduction of adrenal *in vitro* ACTH responsiveness. *Psychoneuroendocrinology*. 37, 1676-1687]

Abstract

Although chronic psychosocial stress is often accompanied by changes in basal HPA axis activity, it is vital for a chronically-stressed organism to mount adequate GC responses when exposed to acute challenges. The main aim of the study described in the following chapter was to test whether this is true or not for the CSC paradigm (19 days), an established and clinically relevant mouse model of chronic psychosocial stress. As shown previously, CSC mice are characterized by unaffected morning, and decreased evening plasma CORT levels despite enlarged adrenals, suggesting a maladaptive breakdown of adrenal functioning.

Plasma CORT levels, determined by repeated blood sampling via jugular vein catheters, as well as relative right adrenal CORT content were increased in CSC compared with SHC mice in response to acute elevated platform (EPF, 5 min) exposure. However, *in vitro* stimulation of adrenal explants with physiological and pharmacological doses of ACTH revealed an attenuated/ lost responsiveness of both the left and right adrenal glands following CSC, despite mRNA and/ or protein expression of Mc2r, MRAP, and key enzymes of steroidogenesis were not down-regulated.

Taken together, chronic psychosocial stressor exposure was shown to impair *in vitro* ACTH responsiveness of both the left and right adrenal glands, whereas it increases adrenal responsiveness to an acute heterotypic stressor *in vivo*. This suggests that an additional factor present during acute stressor exposure *in vivo* rescues left and right adrenal ACTH sensitivity, or itself acts as CORT secretagogue in chronically-stressed CSC mice.

Introduction

Exposure to repeated or chronic stressors has been shown to alter the activity and responsiveness of the HPA axis. For example, ACTH secretion from the pituitary gland is down-regulated in response to repeated homotypic stressor exposure (Aguilera, 1994), whereas subsequent exposure to an acute heterotypic stressor results in ACTH responses that match or even exceed those of control animals exposed to the same acute stressor (Berton et al., 1999; Chen et al., 2008). Moreover, repeated or chronic stressors often result in an increased sensitivity of the adrenals to ACTH (Zelena et al., 2003; Engler et al., 2005; Droste et al., 2006; Vining et al., 2007) and, thus, chronically elevated basal plasma GC levels (Dhabhar and McEwen, 1997; Engler et al., 2005). These observations suggest that the attenuated ACTH response during repeated stress reflects habituation to familiar stimuli to prevent excessive levels of deleterious GC, while the resultant sensitized ACTH response to a heterotypic stressor allows a sufficient acute GC response to novel threats (Berton et al., 1999; Chen et al., 2008). Interestingly, in contrast to the level of the pituitary, to my knowledge, nothing is known so far about the role of the adrenal glands with respect to adaptation/ sensitization of the HPA axis during chronic and subsequent heterotypic stressor exposure.

Unlike repeated homotypic stressor exposure, the CSC paradigm represents a mouse model of chronic psychosocial stress. Importantly, it has already been shown that CSC exposure alters HPA axis functionality, particularly at the adrenal level. In detail, despite enlarged adrenals (Reber et al., 2007) CSC mice show normal basal morning GC levels and are incapable of mounting the circadian rise in plasma GC (Reber et al., 2007). In support, the *in vitro* CORT response to various ACTH doses of isolated adrenal cells (pooled from both left and right glands) was found to be strongly attenuated following CSC (Reber et al., 2007). Although very likely it still has to be proven, whether the latter finding indeed

reflects the mechanism underlying CSC-induced dark phase hypocorticism, as cell-to-cell contacts and adrenal medullary cells are missing in isolated adrenal cortical cell preparations. These are known to substantially influence the responsiveness of the adrenal gland to ACTH (Ehrhart-Bornstein et al., 1998; Ehrhart-Bornstein and Bornstein, 2008).

Taken together, these data strongly suggest that CSC exposure causes adrenal insufficiency, mediated by a reduction/ loss of adrenal ACTH responsiveness. Therefore, it is hypothesized that the CSC-induced physiological changes underlying the attenuated adrenal responsiveness to ACTH represent a maladaptive consequence of, rather than a beneficial adaptation to, chronic psychosocial stress. Consequently, CSC mice should not be able to mount an adequate CORT response to heterotypic stressors. This hypothesis is in line with previous findings showing no attenuation of HPA axis activity and, thus, no adaptation to repeated social stressors (Keeney et al., 2001; Bailey et al., 2004; Engler et al., 2005).

It is important to mention that there are body side-specific differences in adrenal functioning both during basal and stimulated physiological conditions. For example, an increased weight of the left compared with the right adrenal has been found for instance in both rats (Droste et al., 2007) and mice (Droste et al., 2003; 2006) under unstressed conditions. Moreover, there is evidence from the same two rodent species for a body side-specific weight gain of only the right adrenal during prolonged voluntary wheel running (Droste et al., 2003; 2006; 2007) and an increase in specifically the left adrenal weight has been described in humans who committed suicide (Szigethy et al., 1994; Dumser et al., 1998). Therefore, development of body side-specific differences in the reduction/ loss of adrenal ACTH responsiveness between left and right adrenals following CSC is not unlikely. Importantly, this hypothesis is not in contrast to previous data showing an

attenuated, but not totally abolished, ACTH responsiveness of pooled left and right adrenal cells of CSC mice (Reber et al., 2007).

Possible mechanisms underlying the reduction/ loss of adrenal ACTH responsiveness could be a general or a body side-specific reduction in the expression of the Mc2r, the main receptor for ACTH in the adrenal glands (Xia and Wikberg, 1996; Gorrigan et al.), or MRAP. As already mentioned before, MRAP is known to play an important role for Mc2r trafficking, cell surface expression and function, and mutations in MRAP have been shown to cause familial GC deficiency type 2 (Metherell et al., 2004; Clark et al., 2005a; Clark et al., 2005b; Metherell et al., 2005). Furthermore, effects of CSC on expression levels of StAR, CYP11A1, CYP11B1 and CYP11B2 in the left and right adrenal have also to be taken into consideration. These enzymes are essential in the progress of CORT synthesis (Miller, 1988; Biason-Lauber, 1998) and their expression and activity is controlled by ACTH signalling (Sewer and Waterman, 2003; Sewer et al., 2007).

Therefore, one aim of the study described in this chapter is to reveal that (i) there is a lack or at least a reduction of CORT response to an acute heterotypic stressor (EPF, 5 min) after CSC exposure using repeated jugular venous blood sampling in mice. A further aim is to show that (ii) CSC exposure affects adrenal *in vitro* responsiveness to ACTH in a body-side specific manner. To test this adrenal explants are used instead of isolated adrenal cells to keep an intact adrenal architecture. Furthermore, (iii) it is tested if possible body side-specific alterations in adrenal ACTH responsiveness seen *in vitro* following CSC are accompanied by respective alterations in the *in vivo* response to EPF exposure. Therefore, relative adrenal CORT content is quantified in the left and right adrenal before and after EPF exposure. Finally, (iv) CSC effects on left and right adrenal mRNA and/ or protein expression of Mc2r, MRAP, StAR, CYP11A1, CYP11B1, and CYP11B2 are investigated to reveal the body side-specific mechanism underlying CSC-induced adrenal insufficiency.

Material and Methods

Animals

Male C57BL/6 mice (Charles River, Sulzfeld, Germany) weighing 19-22 g (experimental mice) or 30-35 g (dominant mice) were individually housed in standard polycarbonate mouse cages (16 x 22 x 14 cm) for at least one week before the experimental procedure started. 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 performed according to international guidelines on the ethical use of animals. All efforts were made to minimize the number of animals used and their suffering.

Experimental procedures

All experimental mice were either chronically stressed by 19-day exposure to the CSC paradigm or single-housed for control (SHC).

To determine whether CSC affects plasma CORT levels in response to an acute heterotypic stressor, one set of SHC and CSC mice underwent jugular vein catheterization on day 20 (Nyuyki et al., 2012). To recover from surgery all mice were single-housed for one day. In the morning of day 21 mice were exposed to the EPF for 5 min. Blood was drawn before (basal) and 5 min after termination of EPF exposure. Anxiety-related behaviour was assessed during EPF exposure, in order to verify catheter surgery itself did not affect well known CSC effects.

In a second set of animals, body side-specific effects of CSC on adrenal weight, number of adrenal cells per given area, *in vitro* adrenal ACTH responsiveness, and mRNA and/ or protein expression of Mc2r, MRAP, StAR, CYP11A1, CYP11B1, and CYP11B2 were assessed in non-operated SHC and CSC mice, which were, therefore, decapitated on day 20 of CSC between 0800 and 1000 h.

In order to determine the *in vivo* adrenal CORT content per mg tissue (= relative adrenal CORT content) under both basal conditions and following acute stressor exposure, a third set of SHC and CSC mice were single-housed in the evening of day 19 and decapitated on day 20 of CSC between 0800 and 1000 h or 5 min after termination of an EPF exposure (5 min), respectively. Additionally, plasma ACTH and CORT levels were assessed following EPF exposure.

Chronic subordinate colony housing (CSC)

The CSC paradigm was conducted as described recently (Reber et al., 2007; Reber and Neumann, 2008; Reber et al., 2008; Veenema et al., 2008; Singewald et al., 2009). Briefly, one week after arrival, experimental mice were weighed and randomly assigned to the SHC or the CSC group. SHC mice remained undisturbed in their home cage except for change of bedding once a week. CSC mice were housed in groups of four together with a dominant male for 19 consecutive days, in order to induce a chronic stressful situation. To avoid habituation during the chronic stressor exposure, each dominant male was replaced by a novel larger male at days 8 and 15. SHC and CSC mice were again weighed on day 20, either before undergoing surgery or before being killed by decapitation.

Jugular vein catheterization and repeated blood sampling

In the morning of day 20 SHC and CSC mice were implanted with a jugular vein catheter (Nyuyki et al., 2012). Briefly, surgery was performed under isoflurane anaesthesia using semi-aseptic conditions. The jugular vein was exposed by blunt dissection, a catheter consisting of silicone tubing (Dow Corning Corp., Midland MI, USA) and PE-10 polyethylene tubing was inserted approximately 1.5 cm into the vessel through an incision in cardiac direction and exteriorized dorsally at the neck of the mouse (see Fig. 18). The catheter was filled with sterile 0.9 % saline containing heparine (30 IU/ml, Heparin-Natrium, Ratiopharm, Ulm, Germany) and gentamycin (30.000 IU/ml; Centravet, Bad Bentheim, Germany) and sealed afterwards. Following surgery, mice were kept individually in transparent plexiglas observation cages (24 × 40 × 36 cm) until the end of experiment. On day 21, at 0800 h, catheters were attached to an extension tube connected to a 1-ml plastic syringe filled with heparinized 0.9 % saline (30 IU/ml, Heparin-Natrium, Ratiopharm, Ulm, Germany) (see Fig. 18).

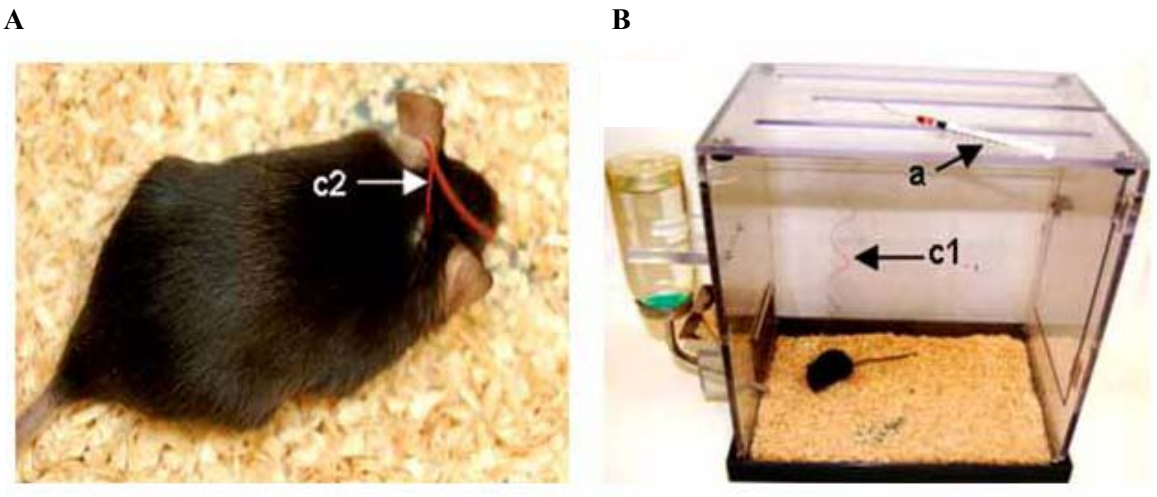

Figure 18: Representative image of a C56BL/6 mouse with implanted catheter exteriorized through the nape of the neck (**A**, c2) and also of a catheterized mouse in a plexiglas observation cage with extended connection tubing (**B**, c1) attached to the syringe (**B**, a). [adapted from (Nyuyki et al., 2012)]

Each mouse was then left undisturbed for 90 min. Blood samples (0.1 ml) were taken immediately before mice were placed on the EPF for 5 min (basal) and 5 min following termination of EPF exposure. All blood samples were collected in EDTA-coated tubes (Sarstedt, Nümbrecht, Germany) on ice, immediately substituted by i.v. infusion of sterile 0.9 % saline, and centrifuged at 4° C (5000 rpm, 10 min). Plasma samples were stored at – 20° C until assayed. Basal CORT concentrations were set to 100 % and the percentage rise in CORT was calculated.

Elevated platform (EPF) exposure

In the morning of day 20 or 21 (catheterized mice) between 0800 and 1000 h, SHC and CSC mice were exposed to an EPF for 5 min (Neumann et al., 2000). Each mouse was individually placed in the centre of the EPF (diameter 18 cm; elevation 75 cm; 160 lux), consisting of an inner (diameter 12 cm) and an outer zone (see Fig.19). The behaviour of the catheterized mice was videotaped and subsequently analysed for the time spent in the outer zone. The EPF was cleaned thoroughly before each trial.

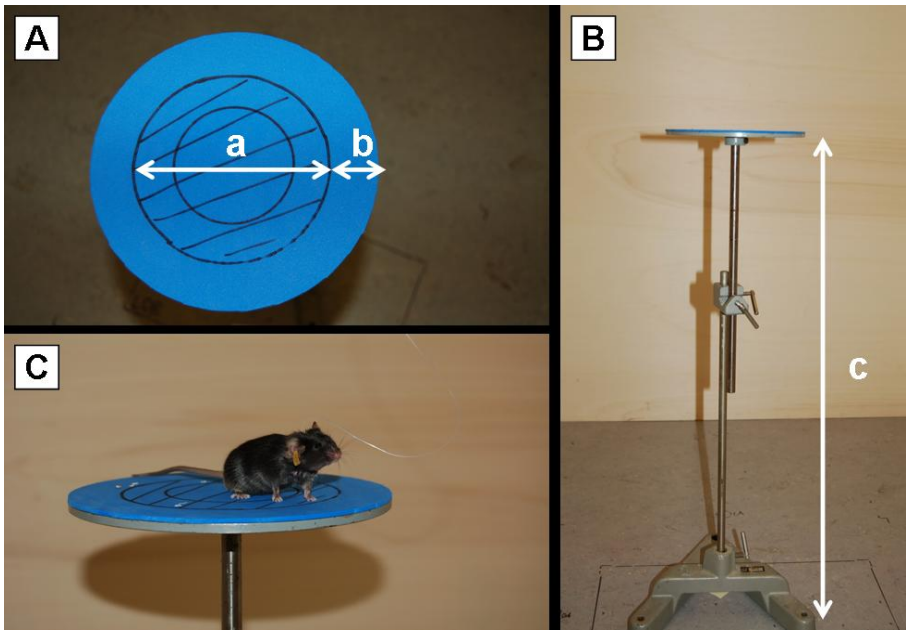

Figure 19. Representative images of the EPF. The platform consists of an inner zone (**A**, a) with a diameter of 12 cm and an outer zone (**A**, b), which is elevated 75 cm (c) from the floor (**B**). The experimental mice were placed in the centre of the platform (**C**).

Trunk blood sampling

On day 20, after all mice were single-housed in the evening of day 19, SHC and CSC mice were rapidly killed by decapitation under CO_2-anaesthesia 5 min following termination of acute stressor exposure (EPF; 5 min) between 0800 and 1000 h. Blood drawing for each mouse was finished within 3 min after entering the animal room. Trunk blood was collected in EDTA-coated tubes (Sarstedt, Nümbrecht, Germany) on ice and centrifuged at 4° C (5000 rpm, 10 min). Plasma samples were stored at – 20° C until assayed.

ELISA for CORT and ACTH

Plasma samples were analyzed using commercially available ELISA for CORT (Analytical sensitivity < 1.631 nmol/L, intra-assay and inter-assay coefficients of variation ≤ 6.35 %, IBL International, Hamburg, Germany) and ACTH (Analytical sensitivity 0.22 pg/ml, intra-assay and inter-assay coefficients of variation ≤ 7.1 %, IBL International, Hamburg, Germany).

Determination of adrenal weight

After decapitation on day 20, the left and right adrenal glands of each animal were removed, pruned of fat, and weighed separately. The adrenals were then used either for *in vitro* ACTH stimulation, determination of adrenal CORT content, number of adrenal cells

per given area, or mRNA/ protein expression analysis. Values represent absolute adrenal weight (milligram). In addition, the sum of left and right absolute adrenal weight was calculated for each mouse.

Cryo-sectioning of adrenal tissue

After removal and pruning of fat, left and right adrenal glands were embedded in protective freezing medium (Tissue-Tek, Sakura Finetek Europe, Zoeterwoude, The Netherlands) and stored at – 80° C. Subsequently, for each left and right adrenal one series of five 5-µm cryo-sections (containing both adrenal cortex and medulla) were cut using a cryostat (at – 20° C) and then thaw-mounted onto pre-coated slides (SuperFrost Plus; Menzel-Gläser, Braunschweig, Germany).

DAPI staining in adrenal cryo-sections

To assess the effects of CSC on adrenal cell number, nuclear staining was performed in adrenal cryo-sections. Briefly, one series of 5-µm adrenal cryo-sections of each adrenal gland were air dried for 10 min at RT and then fixed in cold acetone for 15 min at 8° C. Afterwards, the sections were again air dried before being stained with a solution of the nuclear dye 4´6-diamidino-2-phenylindol (DAPI) for 30 sec. Finally, sections were mounted with fluorescence mounting medium (Fluorescent Mounting Medium, Dako, Glostrup, Denmark) and covered with a glass cover slip. DAPI staining results in nicely stained cell nuclei (blue) (Fig. 20 A), as also shown by others (Battista et al., 2005; Schulte et al., 2007; Huang et al., 2010). Quantification was performed as illustrated in Fig. 20 B. Per section the positive stained area was assessed in the cortex and the medulla per given area [5000 μm^2] for three times in digitized images using Leika QWin V3 Software (Leika

Microsystems, Wetzlar, Germany). In addition, determination of the average nucleus size allows calculation of the number of cells [n] per given area. The results of each zone of two to five adrenal sections per mouse were pooled to provide individual means. In enlarged adrenals a comparable number of cells per given area indicates cell hyperplasia, whereas a decreased number of cells per given area due to their increase in size indicates cell hypertrophy.

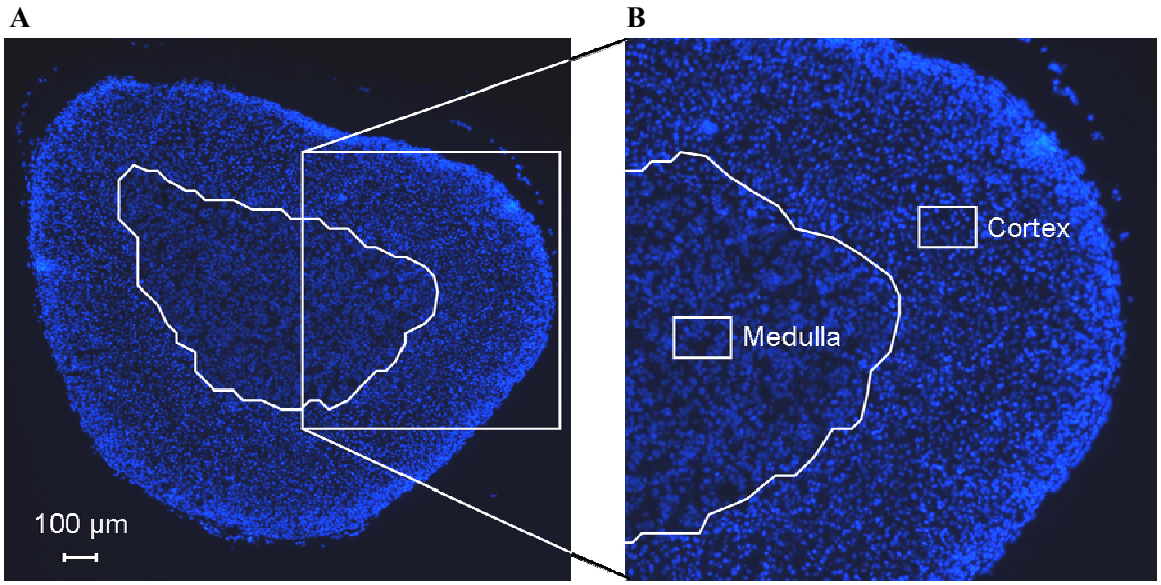

Figure 20: Representative image of an adrenal cryo-section stained with the nuclear dye 4´6-diamidino-2-phenylindol (DAPI) with the border between the cortex and the medulla marked in white (**A**). The positive stained area (blue) can be assessed per given area [5000 µm^2] in the cortex and in the medulla (**B**).

ACTH stimulation of adrenal explants *in vitro*

ACTH stimulation was adapted from a study by Richter et al. (2008). Briefly, left and right adrenals were pruned of fat, separately weighed and stored in ice-cold Dulbecco´s Modified Eagle Medium (DMEM/F-12, Life Technologies, Inc., Grand Island, NY, USA) containing 0.1 % BSA until all mice were killed and adrenals removed. Afterwards, each

left and right adrenal gland was cut into two halves each containing cortical and medullary tissue. The halves were then weighed and pre-incubated in 200 µl DMEM/F-12 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 (0.0022 nM, 0.0100 nM, 0.1500 nM or 100 nM) for 6 h at 37° C (95 % O_2, 5 % CO_2). The ACTH concentrations were chosen to be either equivalent to basal morning (0.0022 nM), basal evening (0.01 nM), or acute stress (forced swim, 10 min, 0.15 nM) plasma ACTH levels (own unpublished data). Additionally, 100 nM ACTH was used, representing a pharmacological dose.

Afterwards, supernatants were carefully removed and stored at – 20° C until analyzed using a commercially available ELISA for CORT (IBL International, Hamburg, Germany). CORT concentrations [ng/ml] were calculated in relation to the weight of the respective adrenal explants [ng/ml/mg tissue] (= relative *in vitro* CORT secretion).

Determination of relative adrenal CORT content

Adrenal CORT content was determined either under basal conditions or 5 min after termination of EPF exposure (5 min). Therefore, left and right adrenal glands were removed, pruned of fat, separately weighed and immediately frozen in liquid nitrogen. Further processing was adapted from Torres-Farfan et al. (2011). Briefly, left and right adrenals were homogenized with 20 % ethanol in 1 x phosphate buffered saline (PBS) on ice, centrifuged at 4° C (4000 rpm, 5 min) and supernatants were collected and stored at – 20° C until analyzed using a commercially available ELISA for CORT (IBL International, Hamburg, Germany). CORT content [ng/ml] was calculated in relation to the weight of the respective adrenal gland [ng/ml/mg tissue] (= relative adrenal CORT content).

Quantitative real-time polymerase chain reaction (qPCR) for Mc2r, MRAP, StAR, CYP11A1, CYP11B1, and CYP11B2 using TaqMan technology

The qPCR was performed as described recently (Reber et al., 2011). Briefly, total RNA was prepared from adrenal tissue using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse transcribed into first-strand cDNA (Reverse Transcription System; Promega, Mannheim, Germany). Expression levels of murine Mc2r, MRAP, StAR, CYP11A1, CYP11B1, and CYP11B2 were quantified by TaqMan-qPCR (ABI PRISM 7900HT Sequence Detection System; Applied Biosystems, Foster City, CA, USA) in single-tube reactions (20 µl) in 384-well plates. Primers and probes used were as follows: Mc2r forward: GCT CCA TGT TCT CTT AAT GAC CTT CT, Mc2r reverse: GAC CTG GAA GAG AGA CAT GTA GC, Mc2r probe: CCC AAA TAA CCC TTA CTG TGT; MRAP forward: CCC GCT CAC CAG CTA TGA GT, MRAP reverse: GCT TCT TCT CGT CCA CAG GAA T, MRAP probe: TTA CCT GGA CTA CAT AGA CCT; StAR forward: GGA GCT CTC TGC TTG GTT CTC A, StAR reverse: CAC CTC TCC CTG CTG GAT GTA G, StAR probe: TCT ATA GTG ACC AGG AGC TGT; CYP11A1 forward: CCG GAG CGG TTC CTT GTG CC, CYP11A1 reverse: CAG GAC CCC AAT GGG CCT CTG A, CYP11A1 probe: CTG GGT GGC CTA TCA CCA GTA; CYP11B1 forward: GCA GAG ATG ATG CTC CTG C, CYP11B1 reverse: CCG CAC ATC CTC TTT CTC TTG, CYP11B1 probe: TGT GCT GAA ATC CTT CCA CGT; CYP11B2 forward: GGC AGC CTG AAG TTT ATC C, CYP11B2 reverse: GGG TAA GAA CAG GAG CTG TG, CYP11B2 probe: CAT TCT ATG TTC AAG TCC AC. The probes were labelled 5´ with 6-carboxy-fluorescein (FAM) and 3´ with 6-carboxytetramethyl-rhodamine (TAMRA). TaqMan-qPCR was performed using 1 µl cDNA, 1 µl forward and reverse primer (each 18 µM), 1 µl probe (5 µM), 10 µl Taqman Mastermix (Applied Biosystems, Foster City, CA,

USA), 1 µl glyceralaldehyd-3-phosphatdehydrogenase (GAPDH)-Mix (served as reference; Applied Biosystems, Foster City, CA, USA) and made up to the final volume of 20 µl with sterile H_2O. Cycling was as follows: 50° C for 2 min, 90° C for 10 min followed by 40 repeats of 95° C for 15 sec and 60° C for 1 min. Average expression value of three individual measures normalized to GAPDH mRNA expression was quantified for each mouse.

Western blotting for adrenal Mc2r protein expression

Left and right adrenal glands were removed, pruned of fat, and immediately shock-frozen in liquid nitrogen and stored at – 80° C until assayed. For protein extraction frozen adrenals were homogenized in ethylendiamintetraacetic acid (EDTA) lysis buffer (50 mM EDTA, 250 mM NaCl, 0.5 mM 2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethansulfonsäure (HEPES), 0.5 % Igepal, 10 % Complete Mini Protease Inhibitor (Roche Diagnostics GmbH, Mannheim Germany)) and total protein concentration was determined using a commercial kit (Bicinchoninic Acid Protein Assay Kit, Thermo Scientific, Rockford, USA). Western blot analysis was carried out using 20 µg of protein per adrenal. Samples were loaded on sodium dodecyl sulphate polyacrylamide gels (10 %) and transferred on nitrocellulose membranes. The membranes were then blocked for 2 h at RT in 5 % milk powder diluted in Tris-buffered saline (TBS) with 0.05 % Tween 20 (TBST, Sigma-Aldrich, Milan, Italy) before being probed with primary anti-Mc2r antibody (1:200; Santa Cruz Biotechnology, Inc., Heidelberg, Germany) overnight at 4° C. Visualization was performed using horseradish peroxidase-conjugated donkey anti-rabbit antibody (1:3000; GE Healthcare, Freiburg, Germany) followed by ECL Western Blotting Detection Reagents (GE Healthcare, Freiburg, Germany). Immunoblots were digitized using Molecular Imager® ChemiDoc™ XRS+ system and analysed using Image Lab™ software

(Bio Rad Laboratories GmbH, München, Germany). Afterwards, each membrane was stripped using Re-Blot Plus Mild Antibody Stripping Solution ((Millipore GmbH, Schwalbach, Germany), blocked twice with 5 % milk powder in TBST for 5 min, and probed with primary rabbit anti-ß-tubulin antibody (1:1000, Cell Signaling Technology, New England Biolabs GmbH, Frankfurt am Main, Germany) for 1 h at RT. Visualization and digitization was performed as explained above (horseradish peroxidase-conjugated donkey anti-rabbit antibody 1:1000). Bands were detected at ~ 42 kDA for Mc2r and at ~ 50 kDA for the loading control ß-tubulin, as specified by the manufacturers and also described in the literature (Cote et al., 1997; Elias and Clark, 2000). Mc2r protein expression of each left and right adrenal gland was normalized to ß-tubulin protein expression and averaged per group.

Statistics

For statistical comparisons, the software package PASW statistics (version 18.0) was used. Data of two experimental groups (SHC versus CSC) were compared by using the Student's *t*-test. Absolute adrenal weights, Mc2r protein expression, mRNA expression of Mc2r, MRAP and selected enzymes of steroidogenesis (factors CSC and body side each), number of adrenal cells per given area (factors CSC and adrenal zone), *in vitro* adrenal CORT secretion (factors CSC and ACTH concentration), and adrenal CORT content (factors CSC and acute stressor exposure) were compared using a two-way analysis of variance (ANOVA) followed by a *post hoc* Bonferroni test when appropriate. Data represent the mean + SEM. Significance was taken at $P < 0.05$.

Results

Effects of CSC on plasma CORT levels in response to EPF exposure

Absolute basal levels of CORT before EPF exposure were not statistically different between SHC and CSC mice (Fig. 21 A). Importantly, the percentage rise relative to basal values (set to 100 %) in plasma CORT 5 min following termination of EPF exposure (5 min) was found to be significantly higher in CSC compared with SHC mice ($P = 0.040$; Fig. 21 B).

Effects of CSC on anxiety-related behaviour

Prior CSC increased anxiety-related behaviour during 5-min EPF exposure indicated by a significantly reduced time spent in the outer zone of the EPF in CSC compared with SHC mice ($P = 0.046$, Fig. 21 C).

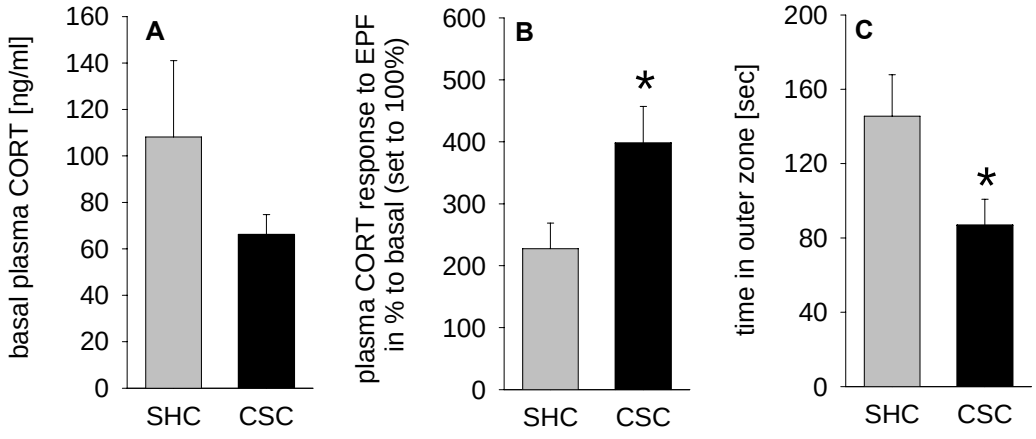

Figure 21: Effects of CSC on plasma CORT levels in response to EPF exposure and on anxiety-related behaviour

Following one day of recovery from catheter surgery (day 20), SHC and CSC mice were exposed to an elevated platform (EPF) for 5 min on day 21. Blood was taken via jugular vein catheter before (basal) and 5 min after termination of EPF exposure. Shown are absolute basal plasma

CORT values before EPF exposure (SHC: n = 8; CSC: n = 7; **A**) and the percentage rise in plasma CORT relative to basal values (set to 100%) 5 min following termination of EPF exposure (SHC: n = 6; CSC: n = 7; **B**). Furthermore, anxiety-related behaviour, indicated by the time the mice spent in the outer zone of the EPF, was assessed during EPF exposure (SHC: n = 8; CSC: n = 9; **C**). ■ SHC; ■ CSC. Data represent the mean + SEM. * represent $P < 0.05$ *vs.* respective SHC mice. Experiments performed in cooperation with Kewir. D. Nyuyki. [adapted from (Uschold-Schmidt et al., 2012)]

Effects of CSC on absolute adrenal weight

Absolute weight of both adrenals (left and right adrenal weights of each mouse were summed up) was significantly increased in CSC compared with SHC mice ($P < 0.001$, Fig. 22 B). Statistical analysis, considering the factors body side as well as CSC, revealed a significant main effect of both factor body side ($F_{1, 542} = 148.96$; $P < 0.001$) and factor CSC exposure ($F_{1, 542} = 116.24$; $P < 0.001$) with increased absolute weights of left ($P < 0.001$) and right ($P < 0.001$) adrenals in CSC compared with SHC mice (Fig. 22 A). Moreover, the absolute weight of the left adrenal was increased compared with the right adrenal gland in both SHC and CSC mice (for both $P < 0.001$; Fig. 22 A).

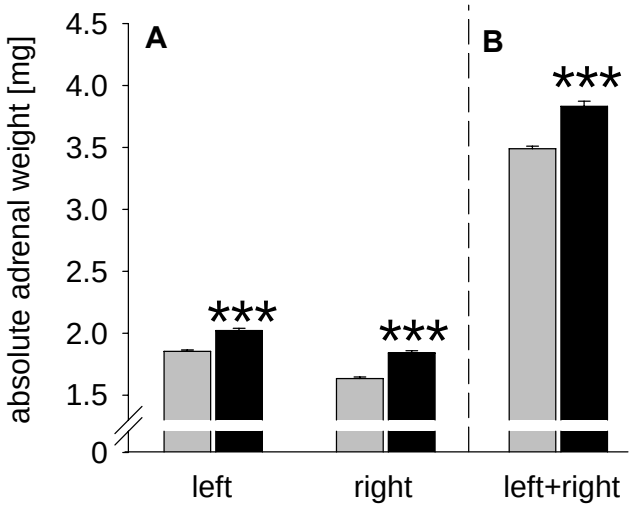

Figure 22: Effects of CSC on absolute weight of the left and right adrenal glands

Following decapitation on day 20 the left and right adrenal glands of SHC and CSC mice were removed, pruned of fat and weighed separately. Shown is the absolute weight [mg] of left and right adrenal glands of SHC (n = 128-134; **A**/**B**) and CSC (n = 135-143; **A**/**B**) mice. Symbols indicating significant differences are only shown for effects between the groups. ■ SHC; ■ CSC. Data represent the mean + SEM. *** represent $P < 0.001$ *vs.* respective SHC mice. [adapted from (Uschold-Schmidt et al., 2012)]

Effects of CSC on the number of cells per given area

Statistical analysis, considering the factors adrenal zone as well as CSC exposure, revealed that the number of adrenal cells per given area in both the left and right adrenal gland was only dependent on factor adrenal zone (left: $F_{1, 66} = 68.09$; $P < 0.001$; right: $F_{1, 66} = 85.03$; by trend $P < 0.001$; Fig. 23 A/B), with increased number of cells per given area in the cortex compared with the medulla in both SHC and CSC mice (for each $P < 0.001$; Fig. 23 A/B).

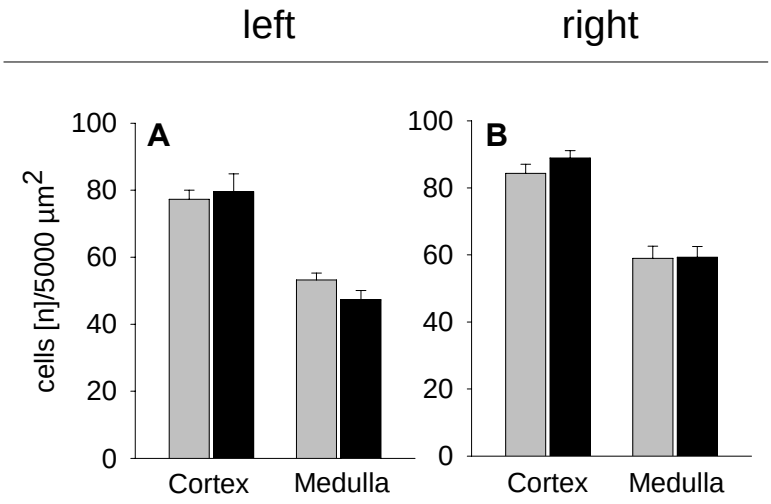

Figure 23: Effects of 19 days of CSC on adrenal cell number

Following decapitation on day 20 the left and right adrenal glands of SHC (n = 18; **A**/**B**) and CSC (n = 17; **A**/**B**) mice were removed and pruned of fat. One series of cryo-sections of each left and

right adrenal gland were stained with DAPI solution and the number of cells [n] per given area [5000 μm^2] was determined in the adrenal cortex and in the medulla. ■ SHC; ■ CSC. Data represent the mean + SEM.

Effects of CSC on *in vitro* adrenal CORT secretion in response to different physiological doses of ACTH

Following stimulation of adrenal explants with different physiological doses of ACTH (basal, 0.0022, 0.0100 or 0.1500 nM) statistical analysis revealed a significant effect of both factor CSC exposure and factor ACTH dose on *in vitro* CORT secretion from left (CSC: $F_{1,50} = 29.53$; $P < 0.001$; ACTH dose: $F_{3,50} = 3.22$; $P = 0.031$) and right (CSC: $F_{1,50} = 11.42$; $P = 0.001$; ACTH dose: $F_{3,50} = 4.53$; $P = 0.007$) adrenal explants (Fig. 24 A/B).

In SHC mice, CORT secretion from both left and right adrenal explants was significantly increased in response to 0.1500 nM ACTH (left: $P = 0.007$; right: $P = 0.042$ Fig. 24 A/B) compared with respective basal values. Moreover, CORT secretion from left and right adrenal explants was significantly reduced in CSC compared with SHC mice in response to 0.0100 nM (left: $P = 0.015$; right: $P = 0.046$) and 0.1500 nM (left: $P < 0.001$; right: $P = 0.004$) ACTH (Fig. 24 A/B).

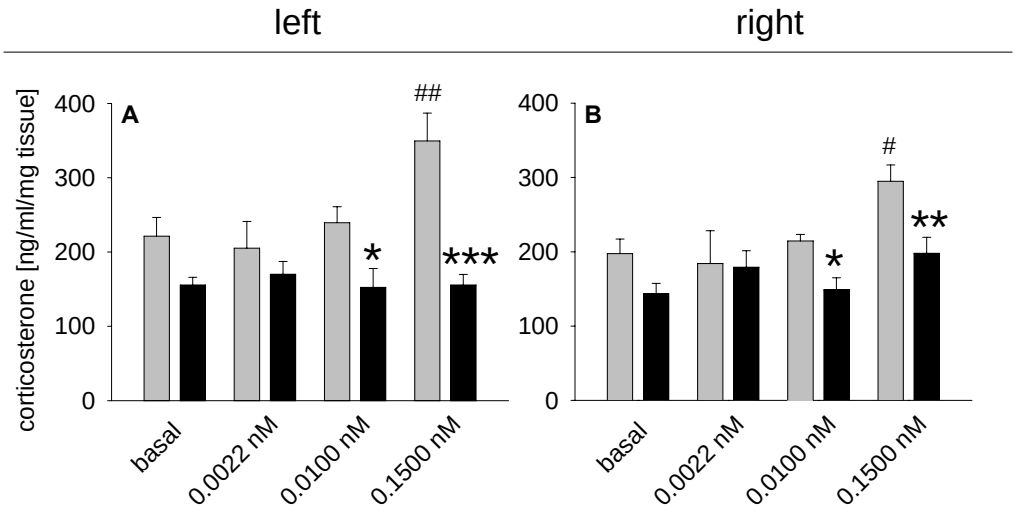

Figure 24: Effects of CSC on *in vitro* adrenal CORT secretion in response to different physiological doses of ACTH

Following decapitation on day 20 the left and right adrenal glands of SHC and CSC mice were removed, pruned of fat, weighed separately, and cut into two halves. Left (**A**) and right (**B**) adrenal halves of the SHC (left and right: n = 6-7) and CSC (left and right: n = 7-8) group were weighed again and incubated for 6 h in the presence of either saline (basal) or 0.0022 nM, 0.0100 nM or 0.1500 nM ACTH. CORT concentrations [ng/ml/mg tissue] were determined in supernatants. ▒ SHC; ■ CSC. Data represent the mean + SEM. * represent $P < 0.05$, ** represent $P < 0.01$, *** represent $P < 0.001$ *vs.* respective SHC mice, # represent $P < 0.05$, ## represent $P < 0.01$ *vs.* respective basal values. [adapted from (Uschold-Schmidt et al., 2012)]

Effects of CSC on *in vitro* adrenal CORT secretion in response to a pharmacological (100nM) ACTH dose

In vitro CORT secretion from both left and right adrenal explants was found to be dependent on factor CSC exposure (left: $F_{1, 74} = 28.66$; $P < 0.001$; right: $F_{1, 67} = 6.49$; $P = 0.013$) and factor ACTH treatment (left: $F_{1, 74} = 67.26$; $P < 0.001$; right: $F_{1, 67} = 101.73$; $P < 0.001$) (Fig. 25 A/B). *Post hoc* analysis revealed that in both SHC and CSC mice ACTH-stimulated CORT secretion from left and right adrenal explants was significantly increased compared with respective basal values (SHC left and right: $P < 0.001$; CSC left: $P = 0.006$; CSC right: $P < 0.001$). However, in both left and right adrenal explants ACTH-stimulated CORT secretion was significantly lower in CSC compared with SHC mice (left: $P < 0.001$; right: $P = 0.003$) (Fig. 25 A/B).

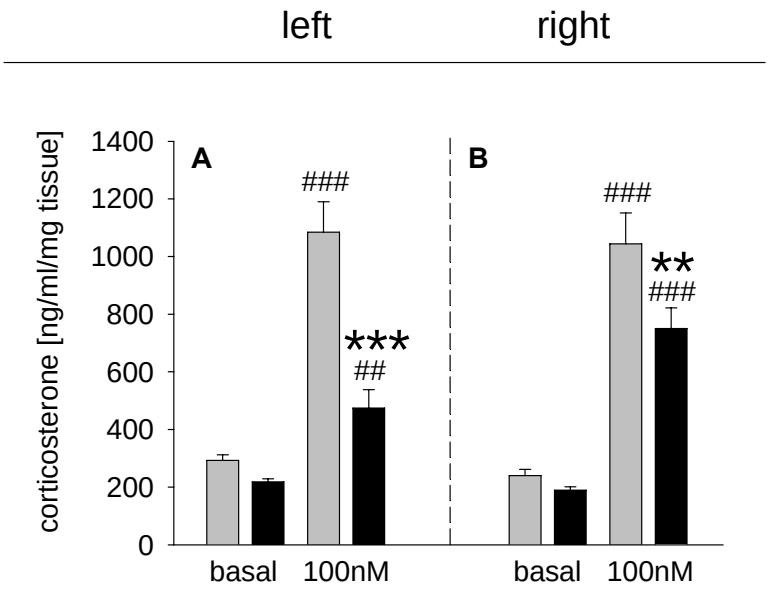

Figure 25: Effects of CSC on *in vitro* adrenal CORT secretion in response to a pharmacological dose (100 nM) of ACTH

Following decapitation on day 20 the left and right adrenal glands of SHC and CSC mice were removed, pruned of fat, weighed separately, and cut into two halves. These left (**A**) and right (**B**) adrenal halves of the SHC (left: n = 19-20; right: n = 19-20) and CSC (left: n = 19-20; right: n = 16) group were weighed again and incubated for 6 h in the presence of either saline (basal) or 100 nM ACTH. CORT concentrations [ng/ml/mg tissue] were determined in supernatants. ■ SHC; ■ CSC. Data represent the mean + SEM. ** represent $P < 0.01$, *** represent $P < 0.001$ *vs.* respective SHC mice, ## represent $P < 0.01$, ### represent $P < 0.001$ *vs.* respective basal values. [adapted from (Uschold-Schmidt et al., 2012)]

Effects of CSC on basal and EPF-induced adrenal CORT content

Whereas left adrenal CORT content was found to be dependent only on the factor EPF exposure ($F_{1, 44} = 82.22$; $P < 0.001$; Fig. 26 A), right adrenal CORT content was dependent on both factor CSC exposure and factor EPF exposure (CSC: $F_{1, 44} = 6.06$; $P = 0.018$; EPF: $F_{1, 44} = 170.84$; $P < 0.001$; Fig. 26 B).

Post hoc analysis revealed that in both SHC and CSC mice CORT content of left and right adrenal glands was significantly increased 5 min after EPF exposure compared with basal levels (for each $P < 0.001$; Fig. 26 A/B). Moreover, right adrenal CORT content was significantly increased after EPF exposure in CSC compared with SHC mice ($P = 0.008$; Fig. 26 B).

Effects of CSC on plasma CORT and ACTH levels measured in trunk blood 5 min after EPF exposure

Compared with SHC mice plasma CORT levels in response to EPF exposure were significantly increased in CSC mice ($P < 0.001$; Fig. 26 C). In contrast, plasma ACTH levels in response to EPF exposure were not affected by CSC exposure (Fig. 26 D).

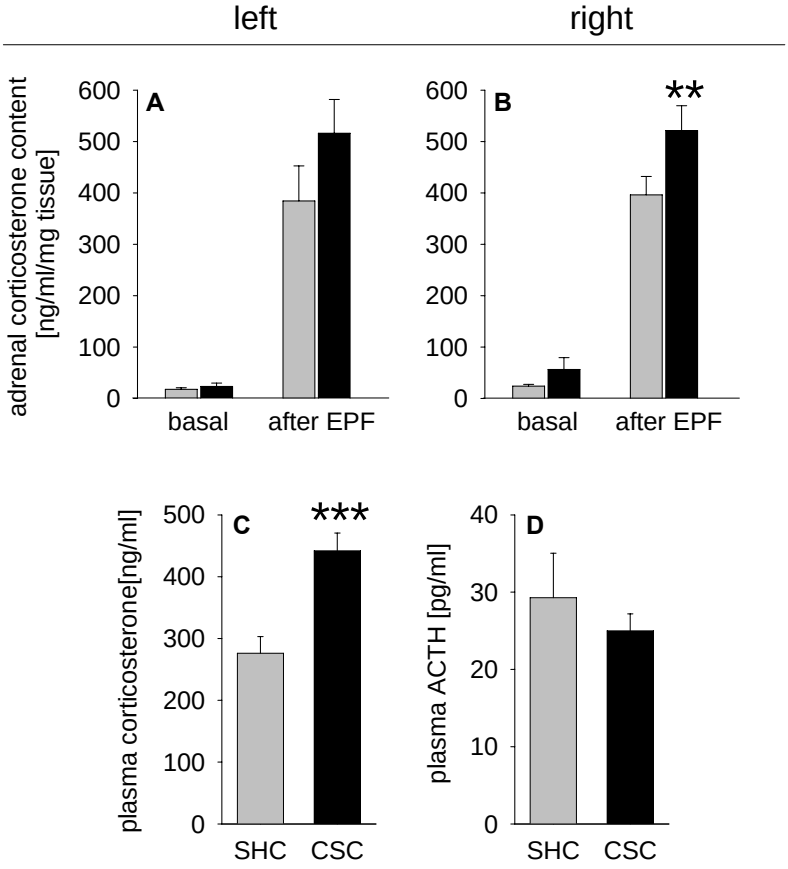

Figure 26: Effects of CSC on basal and EPF-induced adrenal CORT content and EPF-induced plasma CORT and ACTH concentrations

Following decapitation on day 20 between 0800 and 1000 h under basal conditions or 5 min after termination of an EPF exposure (5 min) the left (**A**) and right (**B**) adrenal glands of SHC (n = 12) and CSC (n = 12) mice were removed, pruned of fat, and weighed separately. Afterwards, adrenal glands were homogenized and adrenal CORT content [ng/mg tissue] was determined in supernatants. Furthermore, plasma CORT [ng/ml] (**C**) and ACTH [pg/ml] (**D**) concentrations were determined in trunk blood following EPF exposure (SHC and CSC: n = 12). Symbols indicating significant differences are only shown for effects between the groups. ▒ SHC; ■ CSC. Data represent the mean + SEM. ** represent $P < 0.01$, *** represent $P < 0.001$ *vs.* respective SHC mice. [adapted from (Uschold-Schmidt et al., 2012)]

Effects of CSC on Mc2r mRNA and protein expression

Statistical analysis, considering the factors body side as well as CSC exposure, revealed that Mc2r mRNA expression was dependent only on factor CSC exposure ($F_{1,24} = 6.82$; $P = 0.015$). Compared with SHC mice, Mc2r mRNA expression was significantly decreased in the left adrenal of CSC mice ($P = 0.035$) (Fig. 27 A).

However, Mc2r protein expression was neither affected by factor CSC exposure nor by factor body side (Fig. 27 B/C).

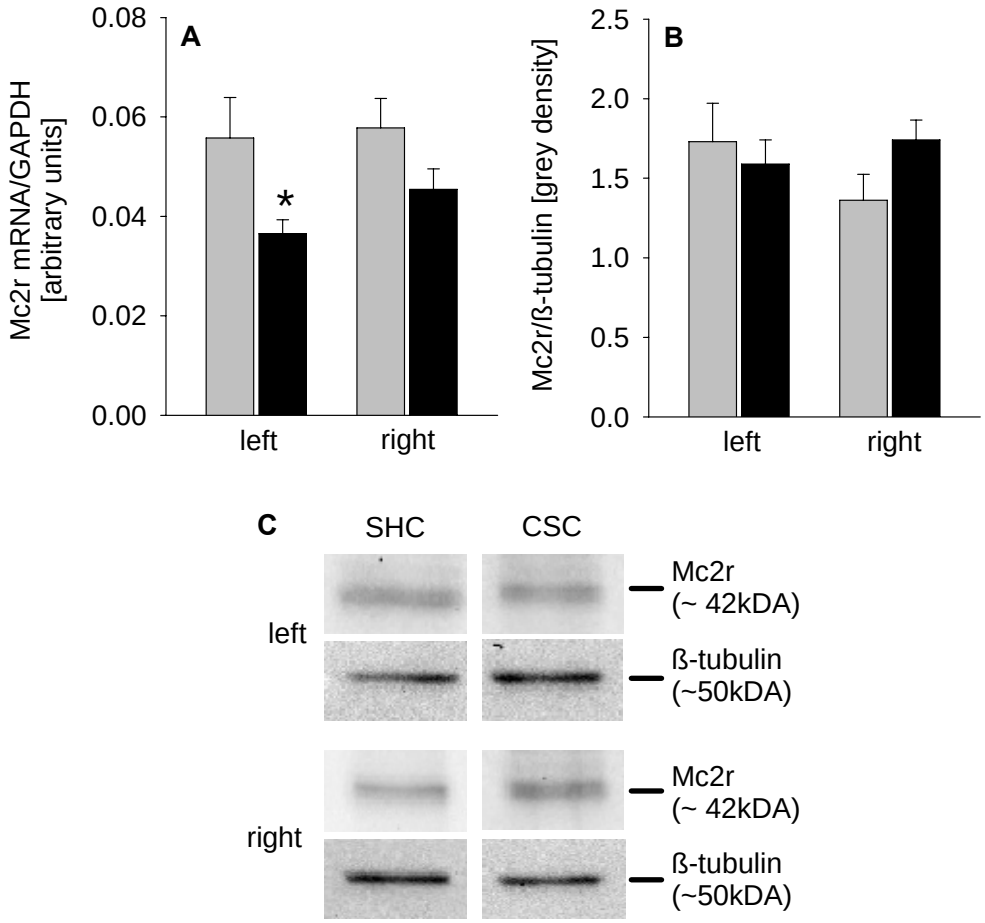

Figure 27: Effects of CSC on Mc2r mRNA and protein expression

Following decapitation on day 20 the left and right adrenal glands of both SHC and CSC mice were removed and pruned of fat. Afterwards, either RNA of each left and right adrenal of SHC (n = 7-8) and CSC (n = 6-7) mice was extracted and reversed transcribed into cDNA for determination of Mc2r mRNA expression [arbitrary units] via qPCR using TaqMan technology normalized to the mRNA expression of the housekeeping gene GAPDH (**A**) or protein was extracted from the left and right adrenal glands of SHC (n = 7) and CSC (n = 8) mice for determination of Mc2r protein expression [grey density] normalized to the loading control ß-tubulin (**B**). ■ SHC; ■ CSC. Data represent the mean + SEM. * represent $P < 0.05$ *vs.* respective SHC mice. Additionally, representative images of the bands detected for Mc2r (~ 42 kDA) and the loading control ß-tubulin (~ 50 kDA) are shown for both the left and right adrenal glands of SHC and CSC mice (**C**). Western blotting of Mc2r (**B**/**C**) was performed by Andrea M. Füchsl. [adapted from (Uschold-Schmidt et al., 2012)]

Effects of CSC on MRAP mRNA expression

A significant main effect of factor CSC exposure was found for MRAP mRNA expression ($F_{1,22}$ = 8.698; P = 0.007; Fig. 28). *Posthoc* analysis revealed a significant increase in MRAP mRNA expression in both the left (P = 0.053) and right (P = 0.045) adrenal glands in CSC compared with SHC mice.

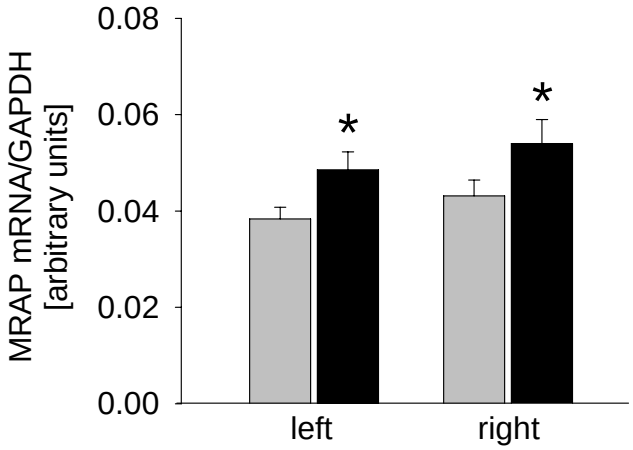

Figure 28: Effects of CSC on MRAP mRNA expression

Following decapitation on day 20 the left and right adrenal glands of SHC (n = 7-8) and CSC (n = 5-6) mice were removed and pruned of fat. Afterwards, RNA was extracted of each adrenal gland and reverse transcribed into cDNA for determination of MRAP mRNA expression [arbitrary units] via qPCR using TaqMan technology normalized to the mRNA expression of the housekeeping gene GAPDH. ■ SHC; ■ CSC. Data represent the mean + SEM. * represent $P < 0.05$ *vs.* respective SHC mice. [adapted from (Uschold-Schmidt et al., 2012)]

Effects of CSC on mRNA expression of selected steroidogenic enzymes

No statistical differences between SHC and CSC mice were found in mRNA expression of StAR, CYP11A1, and CYP11B2 (Tab. 1). However, CYP11B1 mRNA expression was found to be dependent on both factor body side ($F_{1,24}$ = 5.741; P = 0.025) as well as factor

CSC exposure ($F_{1,24}$ = 14.782; P = 0.001), with an increased expression in both left (P = 0.005) and right (P = 0.027) adrenal glands of CSC compared with SHC mice (Tab. 1).

Table 1. Effects of CSC on mRNA expression of selected steroidogenic enzymes

	SHC		CSC	
	left (7-8)	right (7-8)	left (7)	right (7)
StAR	0,260 + 0,012	0,259 + 0,012	0,259 + 0,018	0,279 + 0,012
CYP11A1	0,251 + 0,016	0,265 + 0,016	0,298 + 0,022	0,288 + 0,015
CYP11B1	0,404 + 0,021	0,363 + 0,010	0,500 + 0,026 **	0,436 + 0,026 *
CYP11B2	0,056 + 0,008	0,053 + 0,005	0,076 + 0,010	0,059 + 0,006

Following decapitation on day 20 the left and right adrenal glands of SHC and CSC mice were removed and pruned of fat. RNA of each adrenal was extracted and reversed transcribed into cDNA for determination of mRNA expression [arbitrary units] of StAR, CYP11A1, CYP11B1, and CYP11B2 in adrenal tissue via qPCR using TaqMan technology normalized to mRNA expression of the housekeeping gene GAPDH. Numbers in parentheses indicate group sizes. Data represent the mean + SEM. * represent $P < 0.05$, ** represent $P < 0.01$ *vs.* respective SHC mice. [adapted from (Uschold-Schmidt et al., 2012)]

Discussion

In the study of the present chapter it was revealed, and this is in contrast to the initial hypothesis, that the plasma CORT response to an acute heterotypic stressor, namely EPF exposure, is exaggerated in CSC compared with SHC mice. Furthermore, both left and right adrenal mass is increased following CSC, which seems to be mediated by cell proliferation, and the rise in relative (per mg of tissue) adrenal CORT content in response to EPF exposure was increased in the right adrenal of CSC compared with SHC mice. In

contrast, the *in vitro* CORT secretion of both left and right adrenals in response to physiological as well as pharmacological ACTH doses was reduced following CSC. Although it was not possible to unravel the mechanism underlying the general attenuation of adrenal *in vitro* ACTH responsiveness, the data indicate that changes in Mc2r, MRAP, StAR, CYP11A1, CYP11B1, and CYP11B2 expression are not likely to play a causal role. Taken together, the data show that chronic psychosocial stressor exposure causes a general impairment of *in vitro* adrenal ACTH responsiveness, whereas increasing adrenal responsiveness to an acute heterotypic stressor *in vivo*. This suggests that an additional factor present during heterotypic stressor exposure rescues adrenal ACTH sensitivity, or itself acts as CORT secretagogue in chronically stressed CSC mice.

The responsiveness of the HPA axis to EPF exposure on day 21, one day after termination of CSC, has been assessed by quantification of plasma CORT concentrations in repeated blood samples drawn from the jugular vein. In order to demonstrate that jugular vein catheterization performed on day 20 did not interfere with the known behavioral and physiological consequences of CSC exposure, anxiety levels were assessed and basal plasma CORT samples were measured. In agreement with previous findings (Reber et al., 2007; Reber and Neumann, 2008; Reber et al., 2008; Singewald et al., 2009; Slattery et al.), morning plasma CORT concentrations were comparable between catheterized SHC and CSC mice one day following surgery and before EPF exposure, with CSC mice even showing a tendency towards basal morning hypocorticism. Furthermore, CSC mice spent less time in the outer zone of the EPF one day following catheter surgery compared with SHC mice, indicative of increased levels of anxiety (Ennaceur et al., ; Michalikova et al.). Together these findings demonstrate that the surgical procedure *per se* did not interfere with the main CSC effects. Therefore, the increased plasma CORT response to EPF exposure in CSC compared with SHC mice on day 21 represents a specific effect of CSC.

This is further supported by higher plasma CORT concentrations in trunk blood of another set of non-catheterized CSC versus SHC mice, taken 5 min following termination of EPF exposure on day 20 of CSC. Taken together, the present data indicate that chronic psychosocial stressor exposure compromises basal adrenal function (Reber et al., 2007) with a concurrent increase in adrenal functionality in response to acute heterotypic stressors.

It is becoming apparent that chronic stressor exposure can result in both elevated (Zelena et al., 1999; Schmidt et al., 2010b) and compromised (Berton et al., 1999; Krishnan et al., 2007) basal adrenal functions. Given that adrenals of CSC mice are not insufficient in response to an acute heterotypic stressor, one likely explanation for the specific reduction of basal adrenal function in CSC mice is to protect stressed individuals from chronically elevated basal CORT levels mediated by the increased adrenal mass (Reber et al., 2007). In contrast to our initial hypothesis this might, thus, reflect a beneficial adaptation to, rather than a maladaptive consequence of chronic psychosocial stress. However, in favor of the latter speaks that lowered basal GC levels have been described, for instance, in a mouse model of atypical depression (Touma et al., 2008) and also in a wide range of somatic and affective disorders like burnout (Pruessner et al., 1999), chronic fatigue syndrome (Demitrack et al., 1991), fibromyalgia (Crofford et al., 1994), and posttraumatic stress disorder (PTSD) (Yehuda, 1997). Moreover, besides hypocorticism, patients suffering from PTSD are often also characterized by an over-reactive HPA axis in response to acute stressful stimuli (Yehuda and Seckl, 2011).

Furthermore, CSC mice have been shown to develop a robust increase in state anxiety (Reber et al., 2007; Reber and Neumann, 2008; Slattery et al.), a diagnostic criteria also used for PTSD patients (Golier et al., 2001).

Although future studies are needed to clarify the adaptive or maladaptive character of these physiological changes and to uncover the detailed underlying mechanisms, the available data indicate that CSC-induced changes in adrenal responsiveness to ACTH might be at least partly involved. A previous study showed that *in vitro* CORT secretion from pooled left and right adrenal cells of CSC mice in response to different doses of ACTH (Reber et al., 2007) is strongly attenuated compared with SHC mice. This was confirmed with the present experiments using adrenal explants instead of isolated adrenal cells. Adrenal explants were used for reasons of better mimicking the *in vivo* situation as cell-to-cell contact and the presence of adrenal medullary cells are known to highly influence the responsiveness of the adrenal gland to ACTH (Ehrhart-Bornstein et al., 1998; Ehrhart-Bornstein and Bornstein, 2008). However, in contrast to the initial hypothesis this effect is not body side-specific. Both left and right adrenals of CSC mice showed a decreased adrenal *in vitro* CORT secretion in response to the physiological ACTH concentrations 0.01 nM and 0.15 nM, as well as to the pharmacological ACTH concentration 100nM. Basal and 0.0022 nM ACTH-induced adrenal CORT secretions were not different between CSC and SHC mice.

For optimal mimicking of *in vivo* conditions, physiological ACTH concentrations used in this *in vitro* experiment were chosen to be either equivalent to basal morning (0.0022 nM), basal evening (0.01 nM) or acute stress (forced swim, 10 min) plasma ACTH levels (0.15 nM) (own unpublished data). Together, this data extends the results of the previous study (Reber et al., 2007) by revealing that the attenuated adrenal *in vitro* CORT response is mediated by both adrenals. In addition, these *in vitro* data support previous *in vivo* findings showing that basal evening, but not basal morning, plasma CORT levels are reduced in CSC compared with SHC mice (Reber et al., 2007). This is indicated by the fact that CORT secretion from adrenal explants stimulated with basal morning ACTH levels

(0.0022 nM) is not different between CSC and SHC mice, whereas CORT secretion in response to basal evening ACTH levels (0.01 nM) is significantly reduced following CSC. Surprisingly, despite the attenuated adrenal *in vitro* ACTH responsiveness and decreased Mc2r mRNA expression after CSC, no changes could be detected in adrenal Mc2r protein expression, suggesting no causal involvement of changes in the ACTH receptor itself. Moreover, mRNA expression of MRAP was found to be increased in both the left and right adrenal glands, indicating that a reduction in trafficking or cell surface expression of Mc2r is also not likely to be involved. Furthermore, it is unlikely that the attenuated adrenal *in vitro* ACTH responsiveness is due to a reduction in the steroidogenic enzymes StAR, CYP11A1, CYP11B1 and CYP11B2, as neither in the left nor right adrenal glands a decreased mRNA expression could be detected. CYP11B1 mRNA expression was even found to be increased in both the left and right adrenal glands of CSC mice. Therefore, future studies have to be conducted to reveal the detailed mechanism underlying the attenuated/ lost adrenal ACTH responsiveness of both adrenal glands under *in vitro* conditions.

Importantly, determination of the left and right *in vivo* adrenal CORT content under basal morning conditions and following acute EPF exposure indicated that CSC does neither cause a body side-specific reduction in basal morning CORT production nor in adrenal stress responsiveness. Again, this data support previous findings showing comparable basal morning plasma CORT levels in CSC and SHC mice (Reber et al., 2007). Moreover, and in line with the increased plasma CORT levels found in catheterized CSC compared with SHC mice following EPF exposure, the EPF-induced rise in relative (per mg tissue) adrenal CORT content was more pronounced in CSC compared with SHC mice, at least in the right adrenal. Given the increase in both left and right adrenal mass due to cell proliferation, indicated by a comparable number of adrenal cells in the medulla and the

cortex per given area, and increased plasma CORT response to acute heterotypic stressors following CSC, it is more than likely that absolute adrenal CORT content of both left and right adrenals is increased following EPF exposure in CSC compared with SHC mice.

In contrast to what is generally accepted for repeated homotypic stressors (Aguilera, 1994), a promoting effect on adrenal CORT secretion by a sensitized and, thus, exaggerated ACTH response of the pituitary to heterotypic stressor exposure is not likely in the current study, as plasma ACTH levels were found to be similar in SHC and CSC mice after EPF exposure.

Taken together, chronic psychosocial stressor exposure seems to cause an impairment of *in vitro* ACTH responsiveness in both left and right adrenal glands. However, it seems that the *in vivo* situation implies an additional, yet unknown, factor that is enhanced in CSC mice during acute heterotypic stressor exposure, not in response to the diurnal rhythm, which rescues the attenuated adrenal ACTH responsiveness. For example, sympathetic innervation of the adrenal medulla, for instance, via the splanchnic nerve is known to play a critical role in modulating adrenocortical sensitivity to ACTH (Edwards et al., 1986; Edwards and Jones, 1987b; Ulrich-Lai et al., 2006a). Following activation, adrenal medullary cells secrete neurotransmitters and neuropeptides such as adrenaline/ NE, NPY, VIP or SP, which may, in a paracrine manner (Ehrhart-Bornstein et al., 1998), influence adrenocortical CORT secretion (Bornstein et al., 1990a; Neri et al., 1990; Mokuda et al., 1992; Hinson et al., 1994b). Moreover, neuropeptides such as prolactin and oxytocin, which are released during acute stressor exposure (Jahn and Deis, 1986; Stachowiak et al., 1995; Hashiguchi et al., 1997; Jaroenporn et al., 2009b; Zelena et al., 2009), act as direct CORT secretagogues (Glasow et al., 1998; Lo et al., 1998; Jaroenporn et al., 2009a). Therefore, instead of rescuing ACTH signaling, it is also possible that this unknown factor is a CORT secretagogue itself, thereby simply replacing ACTH in the process of adrenal

activation during heterotypic stressor exposure. However, future studies are required to elucidate the identity of this currently unknown factor.

Chapter 3

Restoration of normal functional adrenal mass during prolonged psychosocial stressor exposure in male mice – a novel strategy to prevent basal hypercorticism?

Uschold-Schmidt N.: Study design, performance of experiments and data analysis (determination of plasma CORT and ACTH in trunk blood via ELISA (Fig. 30), determination of body weight (Fig. 31) and adrenal weight (Fig. 32), *in vitro* adrenal ACTH stimulation and subsequent determination of CORT in supernatant via ELISA (Fig. 33), cryo-sectioning of adrenal tissue and subsequent quantification of adrenocortical cholesteryl esters in oilred-stained sections (Fig. 34), mRNA expression analysis via TaqMan-qPCR (Tab. 2)), writing the first draft of the manuscript

Peterlik D.: experimental assistance (performance of the CSC paradigm)

Reber S.O.: Study design, revision of manuscript

[from Uschold-Schmidt N., Peterlik D., Reber S.O., Restoration of normal functional adrenal mass during prolonged psychosocial stressor exposure in male mice – a novel strategy to prevent basal hypercorticism?, in preparation]

Abstract

19 days of CSC result in unaffected basal morning CORT levels despite a pronounced increase in adrenal mass, mediated by an attenuation/loss of relative adrenal ACTH responsiveness. Given that the pronounced increase in basal morning plasma CORT returns to baseline levels as early as 48 h after the start of CSC, it is likely that the attenuated ACTH responsiveness develops already in this initial phase. This was tested in the experiments described in the present chapter.

In line with previous findings, basal morning plasma CORT concentrations were elevated following 10 h, but returned to baseline following 48 h of CSC exposure, whereas basal morning plasma ACTH concentrations were unaffected following both time points. Furthermore, relative *in vitro* CORT secretion of both left and right adrenals in response to ACTH (100 nM) was unaffected after both time points. Interestingly, absolute left and right adrenal weights were increased following 10 h, but not 48 h of CSC. Together with an unaffected relative amount of cortical cholesteryl esters and unaffected relative mRNA expression of the steroidogenic enzymes StAR, CYP11A1 and CYP11B1 following 10 h, these findings suggest that the increased GC levels following 10 h are due to an increase in functional adrenal tissue.

Taken together, our data suggests that fluctuating basal morning plasma CORT levels during the initial phase of CSC are neither mediated by changes in plasma ACTH nor alterations in adrenal ACTH responsiveness, but mediated by adrenal weight changes and, thus, changes in the quantity of functional adrenal tissue.

Introduction

Exposure to acute stressful stimuli leads to the activation of both the SNS and the HPA axis and, consequently, to the systemic release of catecholamines and GCs. These stress hormones, in turn, trigger physiological alterations enabling an organism to adjust to the new situation and to show an adequate behavioural response (Chrousos, 1998; Charmandari et al., 2005). Together this indicates that the acute stress response is generally beneficial and increases an individual's chance of survival. However, if the stress systems are activated over a prolonged period of time, chronically-elevated levels of plasma GC can promote development of somatic and affective disorders (Sapolsky, 1996; McEwen, 2000; Vanitallie, 2002). Thus, it is adaptive for an individual to habituate possibly fast to prolonged and not life threatening homotypic stressors (Kudielka et al., 2006b; Sasse et al., 2008).

Although it is generally accepted that habituation to a prolonged homotypic stressor does not occur if the stressor is of social nature (Bartolomucci, 2007), we just recently provided first evidence that this holds not true for the chronic subordinate colony housing (CSC) paradigm, an adequate and clinically relevant mouse model of chronic psychosocial stress (Reber et al., 2007; Reber and Neumann, 2008; Slattery et al., 2012). This is indicated by unaffected basal morning plasma CORT levels, despite significantly enlarged adrenals in CSC compared with SHC mice. A general break down of HPA axis functionality in chronically stressed mice, explaining this controversy, can be excluded here, as CSC compared with SHC mice show an even more pronounced CORT response to an acute heterotypic stressor. The latter finding, indicating functionally not compromised adrenals in CSC mice, is further supported by data showing that relative mRNA expressions of the main steroidogenic enzymes StAR, CYP11A1, CYP11B1 (Miller, 1988; Biason-Lauber, 1998) are not affected or even increased following CSC (see chapter 2). In concert with the

lower relative adrenal *in vitro* ACTH responsiveness following 19 days of CSC, these data strongly suggest that CSC mice prevent the health compromising consequence of enlarged and fully functional adrenals, namely increased basal plasma CORT levels, by reducing relative adrenal sensitivity to the main CORT secretagogue ACTH. Unaffected protein expression levels of Mc2r, the main receptor for ACTH in the adrenal gland, as well as increased mRNA expression levels of MRAP, important for Mc2r trafficking and cell surface expression (Clark et al., 2005a; Clark et al., 2005b; Metherell et al., 2005), thereby suggest that this is rather mediated by a reduction of the Mc2r sensitivity to ACTH than by down-regulation of receptor expression itself (see chapter 2).

Although elevated basal morning plasma CORT levels in CSC mice return to baseline as early as 48 h after the start of stressor exposure (Reber et al., 2007; Reber et al., 2011), the reduction in adrenal ACTH responsiveness has been investigated and described only after 19 days of CSC so far (see chapter 2). It is, thus, the aim of the present chapter to reveal the development of CSC-induced reduction in relative adrenal ACTH responsiveness during the initial 48 h of CSC, the time period in which elevated morning basal plasma CORT levels return to baseline.

To test this, the effects of 10 h, as basal morning plasma CORT levels were found to be strongly increased at this time point (Reber et al., 2011), and 48 h of CSC exposure were investigated on i) basal morning plasma CORT and ACTH concentrations ii) body weight, (iii) absolute and relative adrenal weight, and (iv) relative adrenal *in vitro* ACTH responsiveness. In addition, (v) the relative amount of cortical cholesteryl esters in the adrenals and (vi) relative adrenal mRNA expression of the main key enzymes of steroidogenesis (StAR, CYP11A1 and CYP11B1) were investigated following 10 h of CSC to reveal possible changes involved in basal morning hypercorticism at this time point. Given that the attenuated ACTH responsiveness after 19 days of CSC exposure was

much more pronounced in the left compared with the right adrenal (see chapter 2), all adrenal parameters were assessed in a body side-specific manner.

Material and Methods

Animals

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. The male offspring (weighing 30-35 g) of high anxiety-related behavior 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.

Experimental procedures

Experimental mice were either chronically stressed by exposure to the CSC paradigm or single-housed for control (SHC) in a weight matched setup.

Experimental mice were killed in the morning between 0800 and 1000 h either following 10 h or 48 h of CSC exposure and body weight was assessed. To assess the effects of CSC

on absolute and relative adrenal weight, *in vitro* adrenal ACTH responsiveness, amount of cortial lipid droplets (only 10 h), and adrenal mRNA expression of StAR, CYP11A1, and CYP11B1 (only 10 h), left and right adrenal glands were removed, pruned of fat and treated according to the respective readout parameter as described below. Furthermore, trunk blood was collected from SHC and CSC mice for determination of basal morning plasma CORT and ACTH levels following 10 h and 48 h of CSC exposure.

Chronic subordinate colony housing (CSC)

The CSC paradigm was conducted as described recently (Reber et al., 2007; Reber and Neumann, 2008; Singewald et al., 2009; Slattery et al., 2012). Briefly, one week after arrival, experimental mice were weighed and in a weight-matched manner assigned to the SHC or the CSC group. SHC mice remained undisturbed in their home cages. CSC mice were housed in groups of four together with a dominant male for either 10 h or 48 h, in order to induce a prolonged stressful situation. SHC and CSC mice were again weighed in the morning between 0800 and 1000 h following 10 h or 48 h of CSC exposure, immediately before being killed by decapitation.

Trunk blood sampling

In the morning between 0800 and 1000 h following either 10 h or 48 h of CSC exposure, SHC and CSC mice were rapidly killed by decapitation under CO_2-anaesthesia within 3 min after entering the animal room. Trunk blood was collected in EDTA-coated tubes (Sarstedt, Nümbrecht, Germany) on ice and centrifuged at 4° C (5000 rpm, 10 min). Plasma samples were stored at – 20° C until assayed for plasma CORT and ACTH.

Determination of adrenal weight

After decapitation, the left and right adrenal glands of each mouse were removed, pruned of fat, and weighed separately. The adrenals were then used either for *in vitro* ACTH stimulation, determination of adrenal lipid content or mRNA expression analysis. Values represent absolute adrenal weight [mg] or relative adrenal weight [mg/ body weight in g]. In addition, the sum of left and right absolute and relative adrenal weight was calculated for each mouse.

ACTH stimulation of adrenal explants *in vitro*

ACTH stimulation was performed as described before (see chapter 2). Briefly, left and right adrenals were pruned of fat, weighed separately and stored in ice-cold Dulbecco´s Modified Eagle Medium (DMEM/F-12, Life Technologies, Inc., Grand Island, NY, USA) containing 0.1 % BSA until all mice were killed and adrenals removed. Afterwards, each left and right adrenal gland was cut into two halves each containing cortical and medullary tissue. The halves were then weighed and pre-incubated in 200 µl DMEM/F-12 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 (100 nM) for 6 h at 37° C (95 % O_2, 5 % CO_2). Afterwards, the supernatant of each adrenal explant was carefully removed and stored at – 20° C until assayed for CORT.

ELISA for CORT and ACTH

Plasma and supernatant samples were analyzed using commercially available ELISA for CORT (Analytical sensitivity < 1.631 nmol/L, intra-assay and inter-assay coefficients of variation ≤ 6.35 %, IBL International, Hamburg, Germany) and ACTH (plasma samples only) (Analytical sensitivity 0.22 pg/ml, intra-assay and inter-assay coefficients of variation ≤ 7.1 %, IBL International, Hamburg, Germany). Supernatant CORT concentrations [ng/ml] were calculated in relation to the weight of the respective left and right adrenal explants [ng/ml/mg] (= relative *in vitro* CORT secretion).

Cryo-sectioning of adrenal tissue

After removal, left and right adrenal glands were embedded in protective freezing medium (Tissue-Tek, Sakura Finetek Europe, Zoeterwoude, The Netherlands) and stored at – 80° C. Subsequently, for each left and right adrenal one series of five 5-µm cryo-sections (containing both adrenal cortex and medulla) were cut using a cryostat (at – 20° C) and then thaw-mounted onto pre-coated slides (SuperFrost Plus; Menzel-Gläser, Braunschweig, Germany).

Oilred staining in adrenal tissue

To assess the effects of CSC on the availability of cortical cholesteryl esters, adrenal lipid droplets were stained with oilred as previously described (Ramirez-Zacarias et al., 1992). Briefly, one series of 5-µm adrenal cryo-sections of each adrenal gland were fixed in 4 % paraformaldehyd for three days. Afterwards, the sections were washed in distilled water, rinsed in 60 % isopropyl alcohol for 5 min and then stained in a freshly filtered oilred

solution (Certistain Oilred O, Merck, Darmstadt, Germany) for 10 min. The sections were then differentiated in 60 % isopropyl alcohol and washed again in distilled water. Sections were mounted with glycerine jelly and covered with a glass coverslip. As shown in Fig. 29 A, this staining protocol results in red staining of the lipid droplets within the adrenal cortex as also described by others (Bland et al., 2000; Kocher et al., 2003; Lee et al., 2005). Quantification was performed as illustrated in Fig. 29 B. Per section the entire area of all lipid droplets [pixel] as well as the cortex area [pixel] containing these lipid droplets (= relative cortical lipid expression per area) were measured in digitized images using Leika FW4000 Software (Leika Microsystems, Wetzlar, Germany). The results of two to five adrenal sections per mouse were pooled to provide individual means.

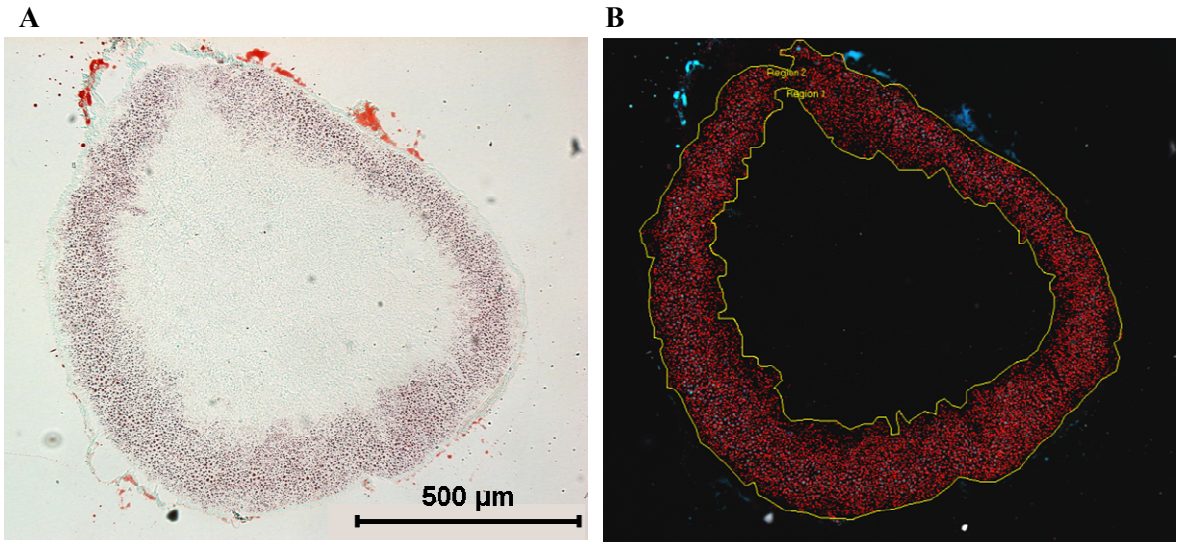

Figure 29: Representative image of an adrenal cryo-section stained with oilred to visualize the lipid droplets (red) within the adrenal cortex (**A**). For quantification the entire area of all lipid droplets [pixel] as well as the area [pixel] of the adrenal cortex containing these droplets (surrounded in yellow) was quantified as illustrated (**B**).

Quantitative real-time polymerase chain reaction (qPCR) for StAR, CYP11A1 and CYP11B1 using TaqMan technology

The qPCR was performed as described recently (Reber et al., 2011). Briefly, total RNA was prepared from adrenal tissue using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse transcribed into first-strand cDNA (25 ng/µl RNA for each mouse, AffinityScript Multiple Temperature cDNA Synthesis Kit, Agilent Technologies, Waldbronn, Germany). Expression levels of murine StAR, CYP11A1, and CYP11B1, were quantified by TaqMan-qPCR (7500 Fast Real-Time PCR System; Applied Biosystems, Foster City, CA, USA) in single-tube reactions (20 µl) in 96-well plates. Primers and probes used were as follows: StAR forward: GGA GCT CTC TGC TTG GTT CTC A, StAR reverse: CAC CTC TCC CTG CTG GAT GTA G, StAR probe: TCT ATA GTG ACC AGG AGC TGT; CYP11A1 forward: CCG GAG CGG TTC CTT GTG CC, CYP11A1 reverse: CAG GAC CCC AAT GGG CCT CTG A, CYP11A1 probe: CTG GGT GGC CTA TCA CCA GTA; CYP11B1 forward: GCA GAG ATG ATG CTC CTG C, CYP11B1 reverse: CCG CAC ATC CTC TTT CTC TTG, CYP11B1 probe: TGT GCT GAA ATC CTT CCA CGT. The probes were labelled 5′ with 6-carboxy-fluorescein (FAM) and 3′ with 6-carboxytetramethyl-rhodamine (TAMRA). TaqMan-qPCR was performed using 1 µl cDNA, 0.15 µl forward and reverse primer (each 18 µM), 1.4 µl probe (5 µM), 10 µl Mastermix + 0.3 µl ROX reference dye (Brilliant III Ultra-Fast QPCR Master Mix, Agilent Technologies, Waldbronn, Germany), 0.6 µl glyceralaldehyd-3-phosphatdehydrogenase (GAPDH)-Mix (served as reference; Applied Biosystems, Foster City, CA, USA) and made up to the final volume of 20 µl with sterile H_2O. Cycling was as follows: 95° C for 3 min followed by 40 repeats of 95° C for 15 sec and 60° C for 30 sec. Expression value normalized to GAPDH mRNA expression was quantified for each mouse.

Statistics

For statistical comparisons, the software package SPSS statistics (version 19.0) was used. Data of two experimental groups (SHC versus CSC) were compared by using the Student's *t*-test. Absolute and relative adrenal weights, amount of cortical cholesteryl esters, mRNA expression of the main enzymes for steroidogenesis (factor CSC and factor body side), and *in vitro* adrenal CORT secretion (factor CSC and factor ACTH) were compared using a two-way analysis of variance (ANOVA) followed by a *post hoc* Bonferroni test when appropriate. Data represent the mean + SEM. Significance was taken at $P < 0.05$.

Results

Plasma CORT levels are increased following 10 h, but not 48 h of CSC exposure, whereas plasma ACTH levels are not affected at all.

Following 10 h ($P = 0.006$; Fig. 30 A), but not 48 h (Fig. 30 B), of CSC exposure basal morning plasma CORT levels were significantly increased in CSC compared with SHC mice. However, basal morning plasma ACTH levels were comparable between SHC and CSC mice following both 10 h (Fig. 30 C) and 48 h (Fig. 30 D) of CSC exposure.

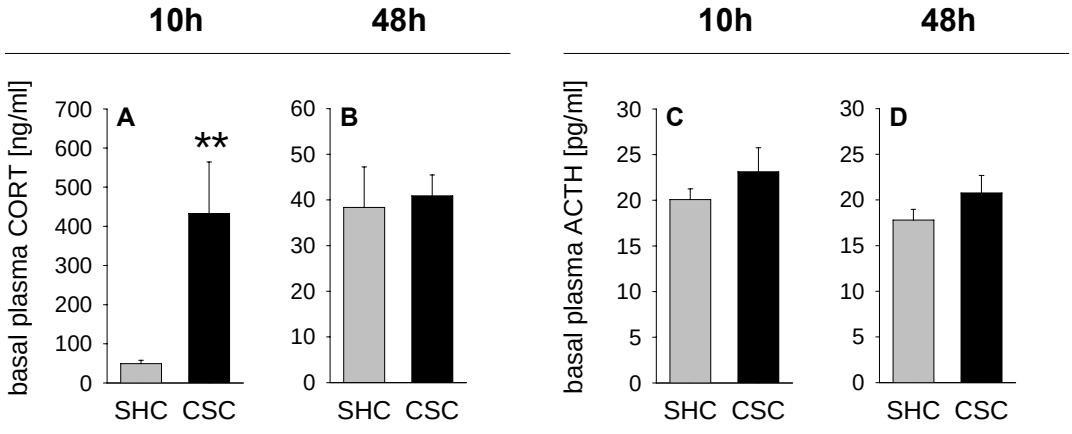

Figure 30: Effects of 10 h and 48 h of CSC exposure on basal morning plasma CORT and ACTH concentrations

SHC and CSC mice were decapitated in the morning between 0800 h and 1000 h either following 10 h (**A**, **C**) or 48 h (**B**, **D**) of CSC exposure and blood was collected. Afterwards, plasma CORT [ng/ml] (SHC: n = 12-14; CSC: n = 16-19; **A, B**) and plasma ACTH [pg/ml] (SHC: n = 13; CSC: n = 18-20; **C, D**) concentrations were determined. ■ SHC; ■ CSC. Data represent the mean + SEM. ** represent $P < 0.01$ *vs.* respective SHC mice.

Body weight is decreased following both 10 h and 48 h of CSC exposure

Following both 10 h ($P < 0.001$; Fig. 31 B) and 48 h ($P = 0.001$; Fig. 31 D) of CSC exposure body weight was significantly decreased in CSC compared with SHC mice. Importantly, body weight between SHC and CSC mice was neither different at the beginning of the 10-h nor the 48-h CSC exposure (Fig. 31 A/C).

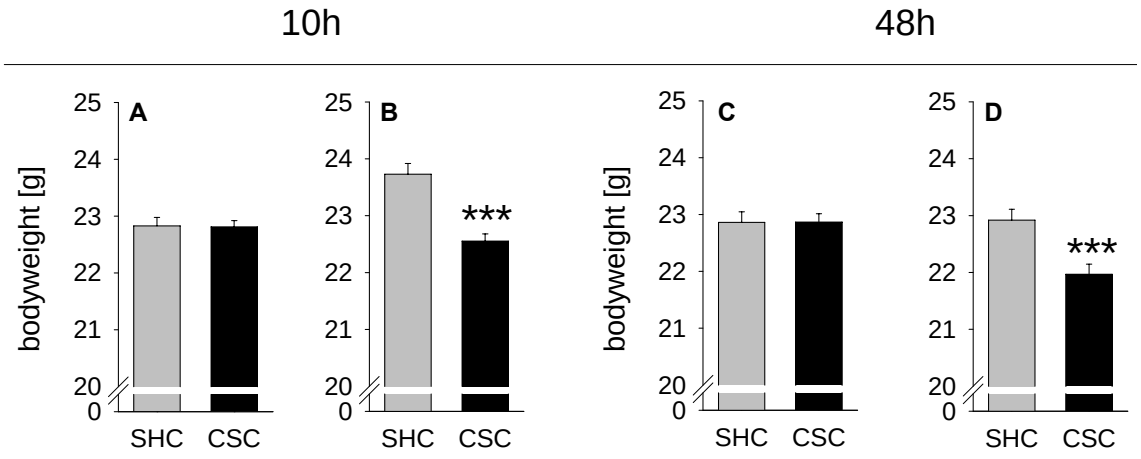

Figure 31: Effects of 10 h and 48 h of CSC exposure on body weight

Body weight [g] was assessed in SHC (10 h: n = 81; 48 h: n = 36) and CSC (10 h: n = 81; 48 h: n = 36) mice before the start of CSC (**A**/**C**) and following 10 h (**B**) and 48 h (**D**) of CSC exposure before being killed by decapitation. ■ SHC; ■ CSC. Data represent the mean + SEM. *** represent $P < 0.001$ *vs.* respective SHC mice.

Relative left and right adrenal weight is increased following 10 h and 48 h of CSC exposure

Following 10 h ($P < 0.001$) and 48 h ($P = 0.003$) of CSC exposure relative adrenal weight of both adrenals (left and right adrenal weights of each mouse were summed up) was significantly increased in CSC compared with SHC mice (Fig. 32 B/D).

Statistical analysis, considering the factors body side as well as CSC exposure, further revealed a significant main effect of both factors (Fig. 32 A/C) following 10 h (body side: $F_{1, 320} = 166.59$; $P < 0.001$; CSC: $F_{1, 320} = 104.72$; $P < 0.001$) and 48 h (body side: $F_{1, 140} = 58.12$; $P < 0.001$; CSC: $F_{1, 140} = 15.53$; $P < 0.001$). Relative weight of both left (10 h: $P < 0.001$; 48 h: $P = 0.013$) and right (10 h: $P < 0.001$; 48 h: $P = 0.003$) adrenals was increased in CSC compared with SHC mice. Moreover, following both time points the relative weight of the left adrenal was increased compared with the right adrenal in both SHC and CSC mice (for each $P < 0.001$; Fig. 32 A/C).

Absolute left and right adrenal weight is increased following 10 h, but not 48 h of CSC exposure

Following 10 h ($P < 0.001$) of CSC exposure absolute weight of both adrenals (left and right adrenal weights of each mouse were summed up) was significantly increased in CSC compared with SHC mice (Fig. 32 F).

Statistical analysis, considering the factors body side as well as CSC exposure, further revealed a significant main effect of both factors following 10 h of CSC (body side: $F_{1, 320} = 229.25$; $P < 0.001$; CSC exposure: $F_{1, 320} = 50.53$; $P < 0.001$). Absolute weight of both left ($P < 0.001$) and right ($P = 0.024$) adrenals were increased in CSC compared with SHC

mice. Moreover, the absolute weight of the left adrenal was increased compared with the right adrenal gland in both SHC and CSC mice (for both $P < 0.001$; Fig. 32 E).

Interestingly, following 48 h of CSC exposure absolute weight of both adrenals (left and right adrenal weights of each were summed up) was found to be comparable between SHC and CSC mice (Fig. 32 H). Moreover, statistical analysis considering the factors body side as well as CSC exposure revealed only a significant main effect of factor body side ($F_{1, 140} = 67.30$; $P < 0.001$) with increased absolute weight of the left compared with the right adrenal gland in both SHC and CSC mice (for both $P < 0.001$; Fig. 32 G).

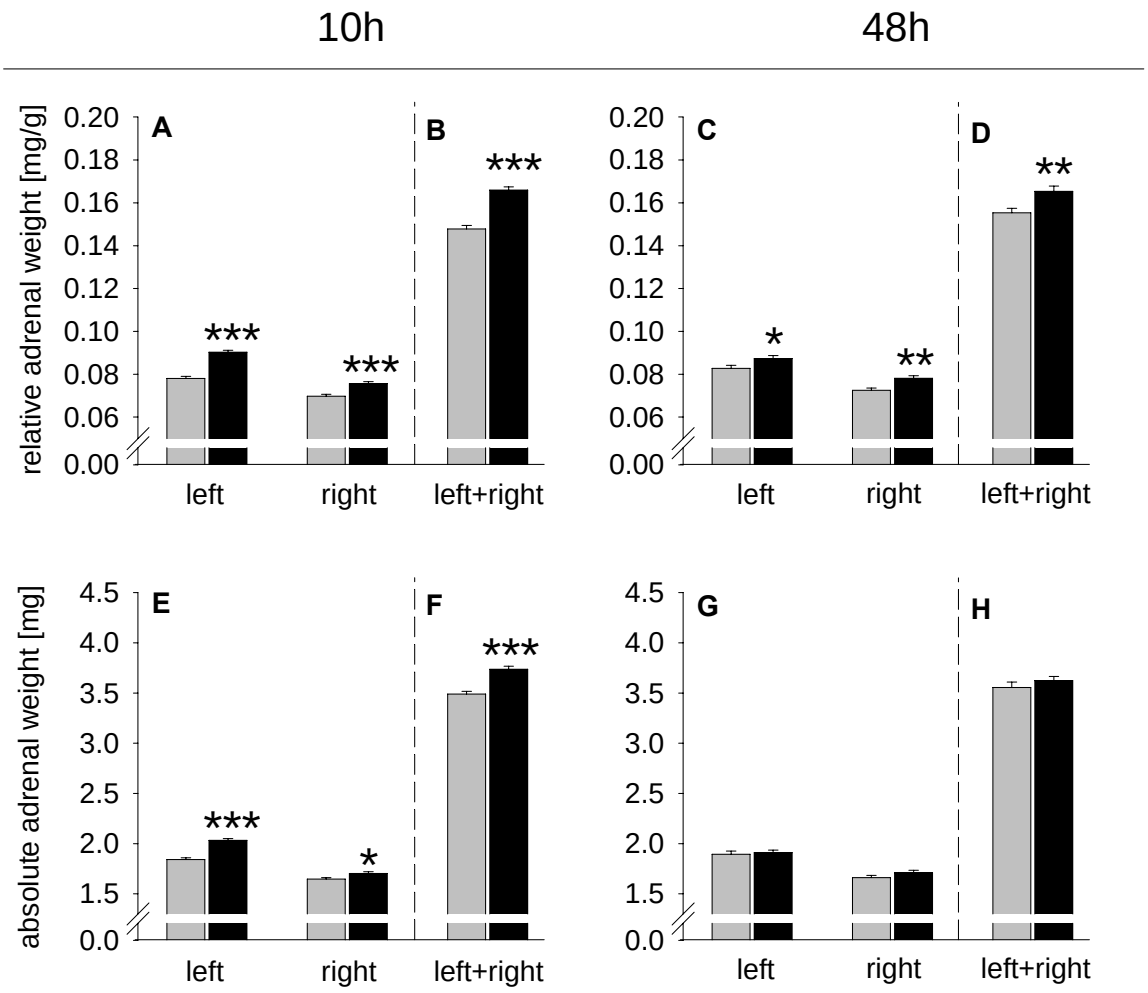

Figure 32: Effects of 10 h and 48 h of CSC exposure on relative and absolute adrenal weight

SHC and CSC mice were killed either following 10 h or 48 h of CSC exposure. Adrenals were

removed, pruned of fat and weighed separately. Shown is the relative [mg/g] and absolute weight [mg] of left and right adrenal glands of SHC (10 h: n = 81; 48 h: n = 36; **A**/**C**/**E**/**G**) and CSC (10 h: n = 81; 48 h: n = 36; **A**/**C**/**E**/**G**) mice. In addition, the sum of relative (**B**/**D**) and absolute (**F**/**H**) left and right adrenal weight of SHC and CSC mice is also shown for both time points. Symbols indicating significant differences are only shown for effects between the groups. ■ SHC; ■ CSC. Data represent the mean + SEM. * represent $P < 0.05$, *** represent $P < 0.001$ *vs.* respective SHC mice.

Relative *in vitro* adrenal CORT secretion in response to ACTH is not affected following both 10 h and 48 h of CSC exposure

Following both 10 h and 48 h of CSC exposure relative (per mg tissue) *in vitro* CORT secretion from both left and right adrenal explants was found to be only dependent on factor ACTH treatment (10 h left: $F_{1, 58} = 19.51$; $P < 0.001$; 10 h right: $F_{1, 62} = 22.51$; $P < 0.001$; 48 h left: $F_{1, 64} = 19.74$; $P < 0.001$; 48 h right: $F_{1, 62} = 36.50$; $P < 0.001$; Fig. 33 A-D). *Post hoc* analysis revealed that in both SHC and CSC mice relative *in vitro* CORT secretion from both left and right adrenal explants was significantly increased following ACTH treatment compared with respective basal values (for each $P \leq 0.004$; Fig. 33 A-D).

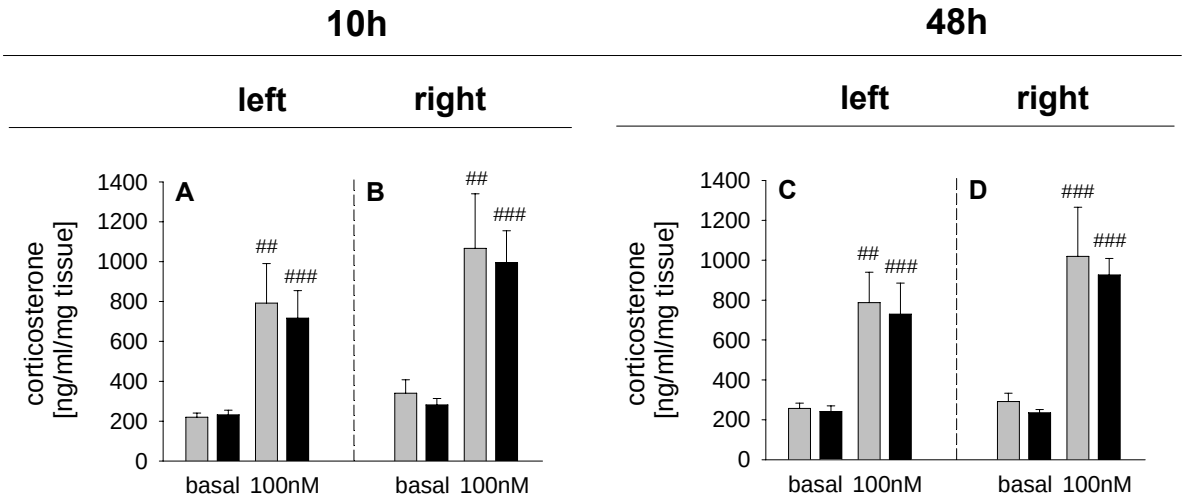

Figure 33: Effects of 10 h and 48 h of CSC exposure on *in vitro* adrenal CORT secretion in response to ACTH

SHC and CSC mice were killed either following 10 h (**A**, **B**) or 48 h (**C**, **D**) of CSC exposure. Left and right adrenal glands were removed, pruned of fat, weighed separately, and cut into two halves, respectively. These left (**A, C**) and right (**B, D**) adrenal halves of the SHC (10 h left: n = 11; 10 h right: n = 13; 48 h left and right: n = 14) and CSC (10 h left and right: n = 20; 48 h left: n = 20; 48 h right: n = 19) group 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. ▒ SHC; ■ CSC. Data represent the mean + SEM. ## represent $P < 0.01$, ### represent $P < 0.001$ *vs.* respective basal values.

Relative amount of cortical cholesteryl esters is not affected following 10 h of CSC exposure

Statistical analysis, considering the factors body side as well as CSC exposure, indicated a significant main effect of factor body side on the relative amount of cortical cholesteryl esters after 10 h of CSC exposure ($F_{1, 26} = 5.56$; $P = 0.026$; Fig. 34), with a trend towards an increased amount in the left compared with the right adrenals. However, *post hoc* analysis revealed no significant differences in the left and right adrenal between both groups.

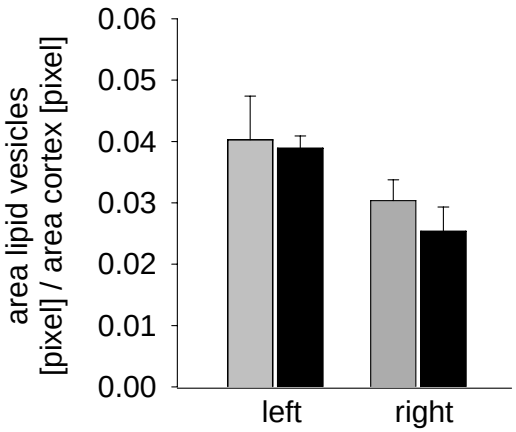

Figure 34: Effects of 10 h of CSC exposure on the amount of cortical cholesteryl esters

SHC and CSC mice were killed following 10 h of CSC exposure. Left and right adrenal glands were removed and pruned of fat. One series of cryo-sections of each left (n = 8) and right (n = 7) adrenal gland were stained with oilred solution and the area of lipid droplets [pixel] per cortex area [pixel] was determined. ■ SHC; ■ CSC. Data represent the mean + SEM.

StAR, CYP11A1 and CYP11B1 mRNA expression are not affected following 10 h of CSC exposure

Statistical analysis indicated that StAR, CYP11A1 and CYP11B1 mRNA expression were neither dependent on factor body side nor CSC exposure after 10 h of CSC (Table 2).

Table 2. Effects of 10 h of CSC exposure on mRNA expression of selected steroidogenic enzymes.

	SHC		CSC	
	left (6)	right (6)	left (5)	right (5)
StAR	0.535 + 0.055	0.560 + 0.053	0.573 + 0.028	0.667 + 0.070
CYP11A1	0.660 + 0.047	0.652 + 0.048	0.679 + 0.047	0.666 + 0.027
CYP11B1	1.405 + 0.051	1.429 + 0.074	1.467 + 0.029	1.518 + 0.069

SHC and CSC mice were killed following 10 h of CSC exposure. Afterwards, left and right adrenal glands were removed and pruned of fat. Total RNA of each adrenal of the SHC and CSC group was extracted and reverse transcribed into cDNA for determination of mRNA expression [arbitrary units] of StAR, CYP11A1, and CYP11B1 in adrenal tissue via qPCR using TaqMan technology normalized to mRNA expression of the housekeeping gene GAPDH. Numbers in parentheses indicate group sizes. Data represent the mean + SEM.

Discussion

The experiments described in the present chapter reveal, and this is in line with previous findings, that basal morning plasma CORT levels are significantly increased following 10 h, but not anymore following 48 h of CSC exposure. Moreover, absolute left and right adrenal mass is significantly increased following 10 h, but also not any more following 48 h of continuous psychosocial stressor exposure. Interestingly, basal plasma ACTH levels as well as relative *in vitro* CORT secretions of both left and right adrenal glands in response to ACTH (100 nM) are comparable between CSC and SHC mice following both 10 h and 48 h of CSC.

Taken together, the present data suggest that increased basal morning plasma CORT levels during the initial 10 h (present study) to 24 h (Reber et al., 2007) of CSC exposure are simply the consequence of an increase in functional adrenal mass. Furthermore, they provide evidence that the restoration of normal basal morning CORT levels as early as after 48 h of CSC is due to normalization of this increased adrenal mass and not to a reduction in the *in vitro* ACTH responsiveness of adrenal cells, as it was previously shown following 19 days of CSC.

For investigation of basal morning plasma CORT concentrations during the initial phase of CSC exposure, mice were killed in the morning either following 10 h or 48 h of CSC exposure and plasma CORT concentrations were determined. As shown before (Reber et al., 2007; Reber et al., 2011), following 10 h of CSC basal morning plasma CORT concentrations were significantly (8 to 9-fold) increased in CSC compared with SHC mice, whereas this effect was absent following 48 h of continuous CSC exposure. In contrast, basal morning plasma ACTH levels were comparable between SHC and CSC mice following both 10 h and 48 h of CSC exposure. This indicates that the increase in basal morning plasma CORT following 10 h of CSC, and the subsequent decline to baseline

levels until the 48 h time point, are not mediated by changes in the release patterns of ACTH from the pituitary.

In a previous study Reber and co-workers (2007) showed that relative adrenal mass was significantly increased during CSC at all time points assessed, beginning as early as 24 h after the start of the chronic psychosocial stressor. In the present chapter the increase in relative adrenal weight following 48 h could be confirmed. Moreover, the data showed that this effect is even present following 10 h of CSC. However, in contrast to relative adrenal weight, absolute weight of both the left and right adrenal was increased only after 10 h, but not 48 h, of CSC exposure. Considering the CSC-induced reduction in body weight following both 10 h and 48 h, which is in line with previous findings (Slattery et al., 2012), this suggests that the increase in relative left and right adrenal weight observed following 48 h of CSC is due to changes in body weight and not to changes in absolute adrenal weight. Interestingly, these findings seem to be in line also with the study by Reber and co-workers assessing the time course of relative adrenal weight during CSC exposure. Although relative adrenal weight was increased at all time points assessed, having a closer look at this parameter during the initial CSC phase clearly indicates a pronounced drop of relative adrenal weight at the 48 h time point (Reber et al., 2007).

Taken together, the changes in absolute adrenal weight during the initial phase of CSC run in parallel with the changes in basal morning plasma CORT levels. Following 10 h both parameters are significantly increased, whereas following 48 h both parameters return back to baseline values, suggesting that CSC mice reverse the early increase in adrenal mass (10 h) to prevent the organism from prolonged exposure to elevated plasma CORT levels. Future studies are needed to clarify if the increase in absolute adrenal weight after 10 h is mediated by hypertrophy or hyperplasia of adrenal cells and if the subsequent restoration

of normal adrenal weight after 48 h is mediated by a decrease in cell size or apoptosis of adrenal cells.

Given that following 19 days of CSC hypercorticism is prevented rather by a reduction of adrenal responsiveness to the main CORT secretagogue ACTH (see chapter 2), than by a reduction of adrenal mass, it was assessed in a next step whether this mechanism, at least partly, contributes to this initial return to baseline concentrations of plasma CORT. However, relative *in vitro* CORT secretion of both left and right adrenal explants in response to ACTH (100 nM) was comparable between SHC and CSC mice following both 10 h and 48 h of CSC exposure. This suggests that the plasma CORT profile during the initial phase of CSC, with increased concentrations following 10 h and unaffected ones following 48 h, is mediated by an increase in functional adrenal mass at the 10 h time point and its reversal at the 48 h time point, but not by alterations in adrenal ACTH responsiveness.

Support for this hypothesis is provided by a comparable relative amount of cortical cholesteryl esters as well as mRNA expression of StAR, CYP11B1, and CYP11B2 following 10 h of CSC, suggesting an overall elevated rate of steroidogenesis when considering the increased adrenal mass in CSC mice at this time point. As already mentioned, cortical lipid droplets store cholesterol, the precursor molecule for steroidogenesis, in form of cholesteryl esters, which can be hydrolyzed if increased amounts of cholesterol are needed, for example under stress conditions (Kraemer, 2007; Hu et al., 2010). Moreover, StAR, CYP11A1, and CYP11B1 are key enzymes in the process of steroidogenesis.

Taken together, the data of the present chapter suggest that elevated basal plasma CORT levels following 10 h of CSC are mediated by an increase in functional adrenal tissue in both the left and right adrenal gland and not by an increase in basal plasma ACTH

concentrations or adrenal ACTH responsiveness. Furthermore, in contrast to the 19-day CSC time point, at which hypercorticism is prevented by a reduction in adrenal ACTH responsiveness, basal hypercorticism during the initial phase of CSC seems to be prevented by restoration of normal functional adrenal mass. Thus, the data suggest that short- and long-term adaptations in response to prolonged homotypic psychosocial stressor exposure are mediated by different mechanisms.

Chapter 4

Male mice exposed to chronic psychosocial stress show a faster HPA axis habituation during prolonged heterotypic stressor exposure *in vivo*

Uschold-Schmidt N.: Study design, performance of experiments and data analysis (performance of the CSC paradigm, cryo-sectioning of adrenal tissue and subsequent quantification of adrenocortical cholesteryl esters in oilred-stained sections (Fig. 36), determination of adrenal weight and plasma CORT and ACTH in trunk blood via ELISA following shaking/ restraint stressor exposure (Fig. 39), *in vitro* adrenal ACTH stimulation and subsequent determination of CORT in supernatant via ELISA (Fig. 40)), writing the first draft of the manuscript

Füchsl A.M.: Performance of experiments and data analysis (Western blotting for adrenal HSL, LDL-R, SR-BI, and HMG-CoA protein expression (Fig. 37/38))

Reber S.O.: Study design, revision of manuscript

[taken and partly adapted from Uschold-Schmidt N., Füchsl A.M., Reber S.O., Male mice exposed to chronic psychosocial stress show a faster HPA axis habituation during prolonged heterotypic stressor exposure *in vivo*. *Journal of Physiology*, submitted]

Abstract

Exposure of male mice to the CSC paradigm for 19 days results in an exaggerated adrenal CORT response to an acute heterotypic stressor (elevated platform, 5 min), despite a loss of adrenal responsiveness to stress-equivalent ACTH doses (0.15 nM) during prolonged (6 h) *in vitro* stimulation. Therefore, the main aim of the study of the present chapter was to test, if this *in vivo*/ *in vitro* discrepancy in CSC mice is due to differences in the time schedule employed to assess adrenal responsiveness under these conditions. However, first adrenal mobilization capacity of the CORT precursor molecule cholesterol in CSC mice was assessed, as this represents a prerequisite for our hypothesis to prove true.

In terms of the latter unchanged or even increased cortical lipid droplets and protein expression of HSL, HMG-CoA reductase, LDL-R, and SR-BI were revealed in CSC compared with SHC mice, indicating, if at all, an enhanced availability and mobilization capacity of cholesterol in chronically-stressed mice.

With respect to the main aim, I could show that plasma CORT was lower in CSC compared with SHC mice following prolonged heterotypic stressor exposure (4 h shaking/ restraint stress), despite plasma ACTH levels were increased following CSC. Moreover, relative adrenal CORT secretion during a 30-min *in vitro* challenge was significantly decreased in CSC compared with SHC mice in response to 0.15 nM, but not 100 nM (pharmacological dose), ACTH.

These data clearly indicate that adrenal *in vitro* responsiveness to stress-equivalent ACTH doses in CSC mice is totally lost, and not gradually declining over time, arguing against my initial hypothesis. In contrast, they support the idea of an additional *in vivo* factor, released/ activated during the acute phase of a heterotypic stressor and metabolized/ deactivated when stressor exposure is prolonged, restoring adrenal ACTH responsiveness in a time-limited fashion and, thus, enabling a chronically-stressed organism to show an

adequate plasma CORT response to acute challenges and a faster habituation to prolonged ones.

Introduction

Repeated exposures to a homotypic stressor are known to result in adaptation of the HPA axis (Aguilera, 1994; Wood et al., 2010). Moreover, considering the deleterious effects of a prolonged elevation of plasma GCs on physical and mental health (McEwen, 1998), it seems to be the better for an organism the faster such HPA axis habituations to repeated innocuous homotypic stressors occur (Kudielka et al., 2006b; Sasse et al., 2008). However, a phenomenon called sensitization enables the adapted organism to even show an increased HPA axis response to a subsequent heterotypic and possibly life-threatening challenge. To date the mechanisms underlying these adaptive/ sensitizing processes are at least partly understood at higher HPA axis levels, i.e. the PVN and the pituitary gland (Aguilera, 1994; Wood et al., 2010).

Interestingly, I recently provided first evidence for such processes to occur also at the level of the adrenal glands, at least during acute heterotypic stressor exposure subsequent to chronic psychosocial stress (see chapter 2). Male mice exposed to the CSC paradigm (19 days), an adequate and clinically relevant mouse model of chronic psychosocial stress (Reber et al., 2007; Reber and Neumann, 2008; Reber et al., 2008; Slattery et al., 2011), are characterized by a loss of relative (per mg tissue) adrenal *in vitro* responsiveness to basal and stress-equivalent ACTH doses (see chapter 2). Although these findings at the first glance suggest a general breakdown of adrenal functionality or at least adrenal ACTH responsiveness following CSC, an exaggerated plasma CORT response and an increased

adrenal CORT content in CSC compared with single housed control (SHC) mice following acute heterotypic stressor exposure (elevated platform exposure (EPF), 5 min) clearly argue against this hypothesis (see chapter 2). Comparable plasma ACTH concentrations in EPF-exposed SHC and CSC mice even suggest an increased adrenal ACTH sensitivity in CSC mice, at least during acute heterotypic stressor exposure. Thus, there seems to be a discrepancy between adrenal ACTH responsiveness under *in vitro* and acute stress *in vivo* conditions following 19 days of CSC exposure.

As already discussed in chapter 2 of the present thesis, a possible mechanism underlying this phenomenon might be that CSC mice release/ activate a factor during acute heterotypic *in vivo* challenges, which is absent under *in vitro* conditions, either rescuing relative adrenal ACTH responsiveness or itself acting as CORT secretagogue.

However, another possible explanation for the above described discrepancy in CSC mice might be that *in vitro* data result from adrenals being challenged with an acute stress-dose of ACTH (0.15 nM) for 6 h, whereas *in vivo,* adrenals were exposed to stress-doses of ACTH for only 10 min (mice were killed 5 min after a 5-min EPF exposure). Consequently, the lack of an adrenal CORT response during 6 h of continuous *in vitro* stimulation with stress-equivalent ACTH doses (0.15 nM) in CSC mice might be due to an initially unaffected or even increased but over time gradually declining ACTH responsiveness. Given this is true, one would expect lower plasma CORT concentrations in CSC compared with SHC mice if the heterotypic stressor becomes prolonged and, in turn, a comparable or even increased relative CORT secretion between SHC and CSC adrenal explants during the initial phase of a 6-h *in vitro* ACTH (0.15 nM) stimulation.

Importantly, both explanations presume intact and functional adrenal tissue in mice exposed to 19 days of CSC. In line, I could reveal comparable expression patterns of the adrenal ACTH receptor (Mc2r), (Xia and Wikberg, 1996; Gorrigan et al.) and an even

increased expression of the Mc2r accessory protein MRAP (Clark et al., 2005a; Clark et al., 2005b; Metherell et al., 2005) in CSC compared with SHC mice. Moreover, expression of key enzymes essential in the progress of steroidogenesis (Miller, 1988; Biason-Lauber, 1998) was also not affected or even enhanced following 19 days of CSC (see chapter 2). However, it is currently not known whether the availability and/ or mobilization capacity of the CORT precursor molecule cholesterol is affected by CSC exposure (Gwynne and Strauss, 1982; Brown and Goldstein, 1986; Azhar and Reaven, 2002; Kraemer, 2007).

To address this question in the present chapter I (i) analyzed if the amount of cortical cholesteryl esters and protein expression levels HSL, HMG CoA reductase, LDL-R, and SR-BI in left and right adrenal tissue are affected by 19 days of CSC. Moreover, to reveal if the *in vitro/ in vivo* discrepancy in CSC mice is due to differences in the time schedule employed to assess adrenal CORT secretion during *in vitro* ACTH challenge and *in vivo* acute stressor exposure, I assessed (ii) if CSC mice show lower plasma CORT levels compared with SHC mice after prolonged heterotypic stressor exposure (4 h of shaking/ restraint), and (iii) if relative adrenal CORT secretion during an acute (30 min) *in vitro* challenge with stress-equivalent (0.15 nM) or pharmacological (100 nM) doses of ACTH is comparable between SHC and CSC mice.

Material and Methods

Animals

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. The male offspring (weighing 30-35 g) of

high anxiety-related behavior 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 resident/ 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.

Experimental procedures

Experimental mice were either chronically stressed by 19-day exposure to the CSC paradigm or single-housed for control (SHC).

To assess the effects of CSC on acute *in vitro* ACTH responsiveness, the amount of adrenal lipid droplets, and protein expression of HSL, HMG-CoA reductase, LDL-R, and SR-BI, one set of SHC and CSC mice were killed in the morning of day 20 between 0800 and 1000 h. Afterwards, left and right adrenal glands were removed, pruned of fat and treated according to the respective readout parameter as described below.

A second set of SHC and CSC mice was exposed to 4 h of continuous shaking/ restraint stress on day 20, starting at 1500 h, and decapitated immediately afterwards for quantification of plasma CORT and ACTH levels. Moreover, adrenals were removed, pruned of fat and weighed.

Chronic subordinate colony housing (CSC)

The CSC paradigm was conducted as described previously (Reber et al., 2007; Reber and Neumann, 2008; Reber et al., 2008; Veenema et al., 2008; Singewald et al., 2009; Schmidt et al., 2010a). Briefly, one week after arrival, experimental mice were weighed and in a weight-matched manner assigned to the SHC or the CSC group. SHC mice remained undisturbed in their home cage except for change of bedding once a week. CSC mice were housed in groups of four together with a dominant male for 19 consecutive days, in order to induce a chronic stressful situation. To avoid habituation during the chronic stressor exposure, each dominant male was replaced by a novel one at days 8 and 15. SHC and CSC mice were again weighed on day 20, immediately before being killed by decapitation.

Cryo-sectioning of adrenal tissue

After removal on day 20 of CSC, left and right adrenal glands were pruned of fat and embedded in protective freezing medium (Tissue-Tek, Sakura Finetek Europe, Zoeterwoude, The Netherlands) and stored at – 80° C. Subsequently, for each left and right adrenal one series of five 5-µm cryo-sections (containing both adrenal cortex and medulla) were cut using a cryostat (at – 20° C) and then thaw-mounted onto pre-coated slides (SuperFrost Plus; Menzel-Gläser, Braunschweig, Germany).

Oilred staining in adrenal tissue

To assess the effects of CSC on the availability of cortical cholesteryl esters, adrenal lipid droplets were stained with oilred as described in chapter 3. Briefly, one series of 5-µm adrenal cryo-sections of each left and right adrenal gland were fixed in 4 %

paraformaldehyd for three days. Afterwards, the sections were washed in distilled water, rinsed in 60 % isopropyl alcohol for 5 min, and then stained in a freshly filtered oilred solution (Certistain Oilred O, Merck, Darmstadt, Germany) for 10 min. The sections were then differentiated in 60 % isopropyl alcohol and washed again in distilled water. Sections were mounted with glycerine jelly and covered with a glass cover slip. Per section the entire area of all lipid droplets [pixel] as well as the cortex area [pixel] containing these lipid droplets (= relative cortical lipid expression per area) were measured in digitized images using Leika FW4000 Software (Leika Microsystems, Wetzlar, Germany). The results of two to five adrenal sections per mouse were pooled (individually for left and right adrenals) to provide individual means.

Western blotting for adrenal protein expression of HSL, LDL-R, SR-BI, and HMG-CoA reductase

Left and right adrenal glands were removed on day 20 of CSC, pruned of fat, immediately shock-frozen in liquid nitrogen, and stored at – 80° C until assayed. For protein extraction frozen left and right adrenals were homogenized separately in ethylendiamintetraacetic acid (EDTA) lysis buffer (50 mM EDTA, 250 mM NaCl, 0.5 mM 2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethansulfonsäure (HEPES), 0.5 % Igepal, 10 % Complete Mini Protease Inhibitor (Roche Diagnostics GmbH, Mannheim Germany)) and total protein concentration was determined using a commercially available detection kit (Bicinchoninic Acid Protein Assay Kit, Thermo Scientific, Rockford, USA). Western blot analysis was carried out using 20 µg of protein per adrenal. Samples were loaded on sodium dodecyl sulphate polyacrylamide gels (10 %) and subsequently transferred on nitrocellulose membranes. The membranes were then blocked for 1 h at RT in 5 % BSA (for HSL protein expression)

or 5 % milk powder (for LDL-R, SR-BI, and HMG-CoA reductase), both diluted in Tris-buffered saline (TBS) with 0.05 % Tween 20 (TBST, Sigma-Aldrich, Steinheim, Germany), before being probed with primary rabbit anti-HSL (1:1000; Cell Signaling Technology, New England Biolabs GmbH, Frankfurt am Main, Germany), anti-LDL-R (1:500; Abcam, Cambridge, UK), anti-SR-BI (1:1600; Abcam, Cambridge, UK), or anti-HMG-CoA reductase (1: 500; Santa Cruz Biotechnology, Inc., Heidelberg, Germany) antibodies overnight at 4° C. Visualization was performed using horseradish peroxidase-conjugated donkey anti-rabbit antibody (1:3000; GE Healthcare, Freiburg, Germany) followed by ECL Western Blotting Detection Reagents (GE Healthcare, Freiburg, Germany). Immunoblots were digitized using Molecular Imager® ChemiDoc™ XRS+ system and analysed using Image Lab™ software (Bio Rad Laboratories GmbH, München, Germany). Afterwards, each membrane was stripped using Re-Blot Plus Mild Antibody Stripping Solution (Millipore GmbH, Schwalbach, Germany), blocked twice with 5 % milk powder in TBST for 5 min, and probed with primary rabbit anti-ß-tubulin antibody (1:1000, Cell Signaling Technology, New England Biolabs GmbH, Frankfurt am Main, Germany) for 1 h at RT. Visualization and digitization was performed as described above (horseradish peroxidase-conjugated donkey anti-rabbit antibody 1:1000). Bands were detected at ~ 82 kDA for HSL, at ~ 150 kDA for LDL-R, at ~ 76 kDA for SR-BI, at ~ 97 kDA for HMG-CoA reductase, and ~ 50 kDA for the loading control ß-tubulin, as specified by the manufacturers. Expression of all proteins was normalized to the respective ß-tubulin protein expression and averaged separately for both left and right adrenal per group.

Prolonged shaking/ restraint stressor exposure

This stress protocol was chosen based on a previous study, showing strong activation of the HPA axis in response to a combination of shaking and restraint stressor exposure in rats (Dhabhar and McEwen, 1997). Briefly, SHC and CSC mice, the latter were directly taken out of the CSC colony, were restraint in a well ventilated 50ml-Falcon tube (3 cm diameter) (see Fig. 35) and placed on an oscillatory shaker (~ 150 U/min) for 4 h on day 20 of CSC, starting at 1500 h.

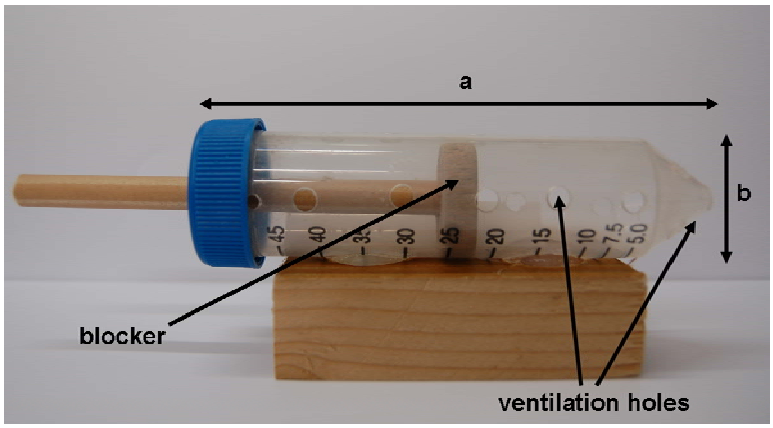

Figure 35: Representative image of a well-ventilated (see ventilation holes) 50ml-Falcon restraint tube with a length of 12 cm (a) and diameter of 3 cm (b). The tube length can be varied individually (blocker).

Trunk blood sampling

Following 4 h of shaking/ restraint stressor exposure on day 20 of CSC, both SHC and CSC mice were rapidly killed by decapitation under inhalation-anaesthesia. Trunk blood was collected in EDTA-coated tubes (Sarstedt, Nümbrecht, Germany) on ice and centrifuged at 4° C (5000 rpm, 10 min). Plasma samples were stored at – 20° C until assayed for plasma CORT and ACTH (see below).

ACTH stimulation of adrenal explants *in vitro*

ACTH stimulation was performed as already described (see chapter 2/3). Briefly, left and right adrenals were pruned of fat, weighed separately, and stored in ice-cold Dulbecco´s Modified Eagle Medium (DMEM/F-12, Life Technologies, Inc., Grand Island, NY, USA) containing 0.1 % BSA until all mice were killed and adrenals removed. Afterwards, each left and right adrenal gland was 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 medium containing saline (basal) or medium containing ACTH (0.15 nM or 100 nM) for 30 min (37 °C, 95 % O_2, 5 % CO_2). The ACTH concentrations were chosen to be either equivalent to acute stress plasma ACTH concentrations (forced swim, 10 min, 0.15 nM) or to represent a pharmacological dose of ACTH (100 nM). Afterwards, supernatants were carefully removed and immediately stored at – 20° C until analyzed using a commercially available ELISA for CORT (see below).

ELISA for CORT and ACTH

Plasma and supernatant samples were analyzed using commercially available ELISA kits for CORT (plasma & supernatants; analytical sensitivity < 1.631 nmol/L, intra-assay and inter-assay coefficients of variation ≤ 6.35 %, IBL International, Hamburg, Germany) and ACTH (plasma; analytical sensitivity 0.22 pg/ml, intra-assay and inter-assay coefficients of variation ≤ 7.1 %, IBL International, Hamburg, Germany). Plasma CORT concentrations [ng/ml] were additionally calculated in relation to the respective sum of left and right adrenal weight of each mouse (= relative plasma CORT; [ng/ml/mg]). As basal as well as

ACTH-induced adrenal CORT secretion *in vitro* was not different between left and right explants, we decided to sum the CORT concentrations in the supernatants [ng/ml] of left and right adrenal explants per mouse up and to express them in relation to the respective adrenal explant weight (sum of respective left and right explants) (= relative *in vitro* CORT secretion; [ng/ml/mg]).

Statistics

For statistical comparisons, the software package SPSS statistics (version 19.0) was used. Data of two experimental groups (SHC versus CSC) were compared using the Student's *t*-test. Data of four experimental groups such as i*n vitro* adrenal CORT secretion (factors CSC and ACTH concentration), amount of cortical cholesteryl esters, protein expression of HSL, HMG-CoA reductase, LDL-R, and SR-BI, absolute adrenal weight after 4 h restraint/ shaking stressor exposure (factors CSC exposure and body-side) were compared using a two-way analysis of variance (ANOVA) followed by a *post hoc* Bonferroni test when appropriate. Data represent the mean + SEM. Significance was taken at $P < 0.05$.

Results

CSC does not affect relative amount of cholesteryl esters in the adrenal cortex

Statistical analysis, considering the factors body side as well as CSC exposure, revealed no effects on the relative (per area) amount of cholesteryl esters in the left and right adrenal cortex (Fig. 36 A/B).

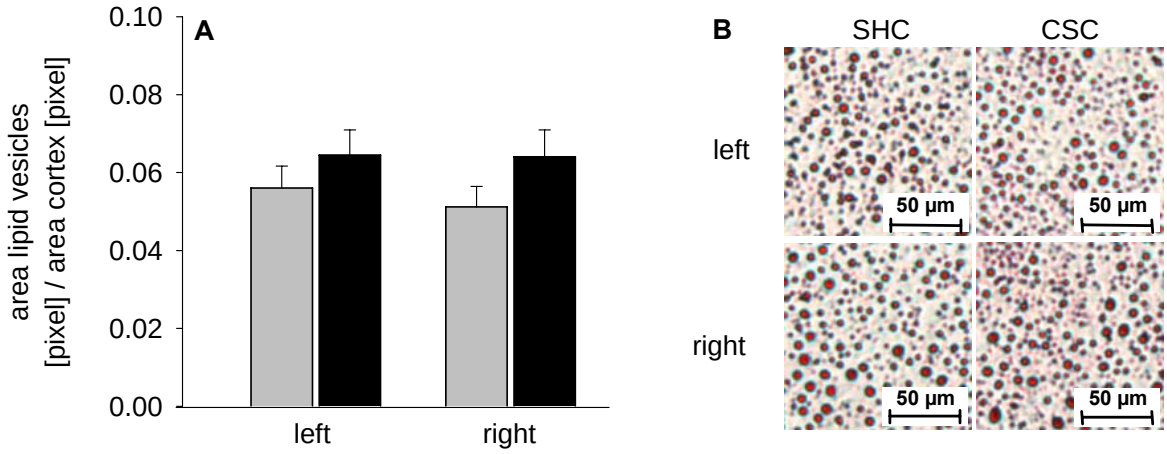

Figure 36: Effects of CSC on the amount of cortical lipid droplets

Following decapitation on day 20 the left and right adrenal glands of SHC (n = 18; **A**) and CSC (n = 20; **A**) mice were removed and pruned of fat. One series of cryo-sections of each left and right adrenal gland were stained with oilred solution and the area of lipid droplets [pixel] per cortex area [pixel] was determined. ■ SHC; ■ CSC. Data represent the mean + SEM. In addition, representative images of cryo-sections stained with oilred are shown for both the left and right adrenal glands of SHC and CSC mice (**B**). [adapted from (Uschold-Schmidt et al., submitted)]

CSC does not affect relative adrenal protein expression of HSL and HMG-CoA reductase

No effects of factor CSC exposure or factor body side were found on relative (per 20 µg of total protein) protein expression of HSL (Fig. 37 A/B) or HMG-CoA reductase (Fig. 37 C/D).

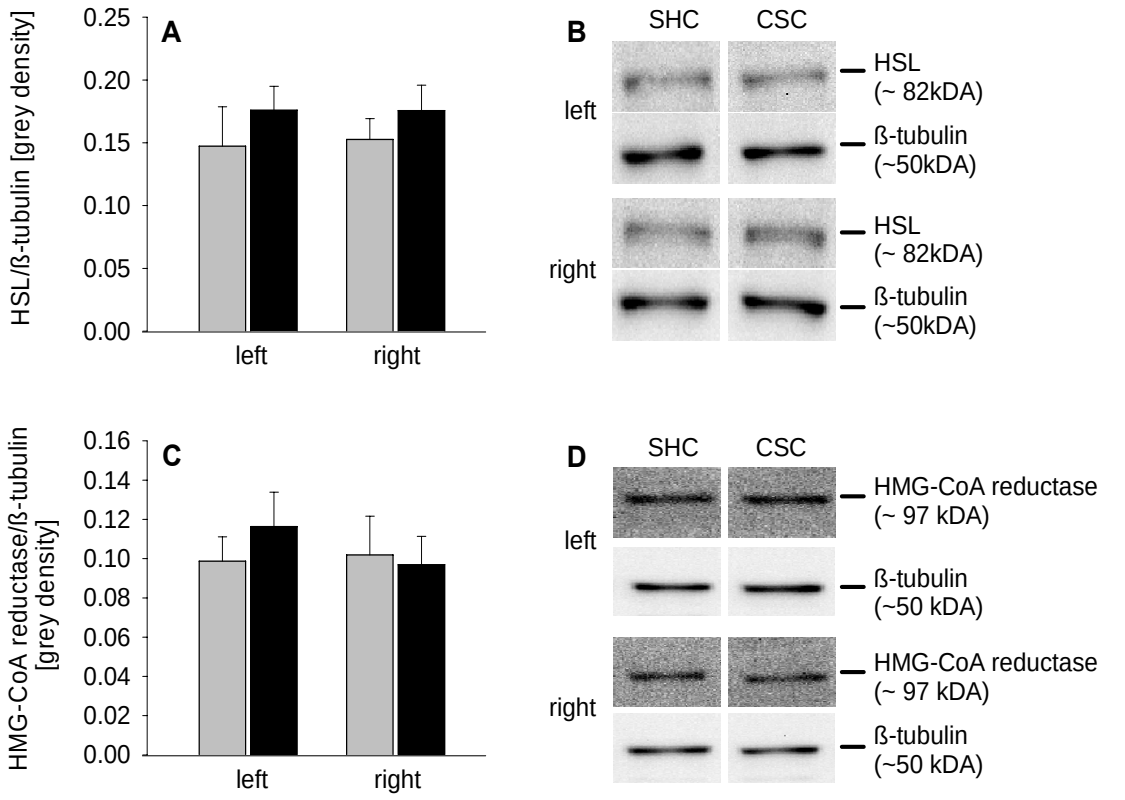

Figure 37: Effects of CSC on adrenal HSL and HMG-CoA reductase protein expression

Following decapitation on day 20 the left and right adrenal glands of both SHC and CSC mice were removed and pruned of fat. Afterwards, protein was extracted from the left and right adrenal glands of SHC (n = 8) and CSC (n = 8) mice for determination of HSL (**A**) and HMG-CoA reductase (**C**) protein expression [grey density] normalized to the loading control ß-tubulin. ■ SHC; ■ CSC. Data represent the mean + SEM. In addition, representative images of the bands detected for HSL (~ 82 kDA; **B**), HMG-CoA reductase (~ 97 kDa; **D**) and the loading control ß-tubulin (~ 50 kDA; **B**/**D**) are shown for both the left and right adrenal glands of SHC and CSC mice. Experiments performed by Andrea M. Füchsl. [adapted from (Uschold-Schmidt et al., submitted)]

CSC does not affect relative adrenal LDL-R protein expression, but increases relative adrenal SR-BI protein expression

Relative (per 20µg of total protein) LDL-R protein expression in both the left and right adrenal glands was neither affected by factor CSC exposure nor by factor body side (Fig. 38 A/B).

In contrast, relative SR-BI protein expression was found to be dependent on factor CSC exposure ($F_{1,28}$ = 22.27; P < 0.001; Fig. 38 C/D). *Post hoc* analysis revealed a significant increase in relative SR-BI protein expression in both the left (P = 0.008) and right (P = 0.001) adrenal glands in CSC compared with SHC mice.

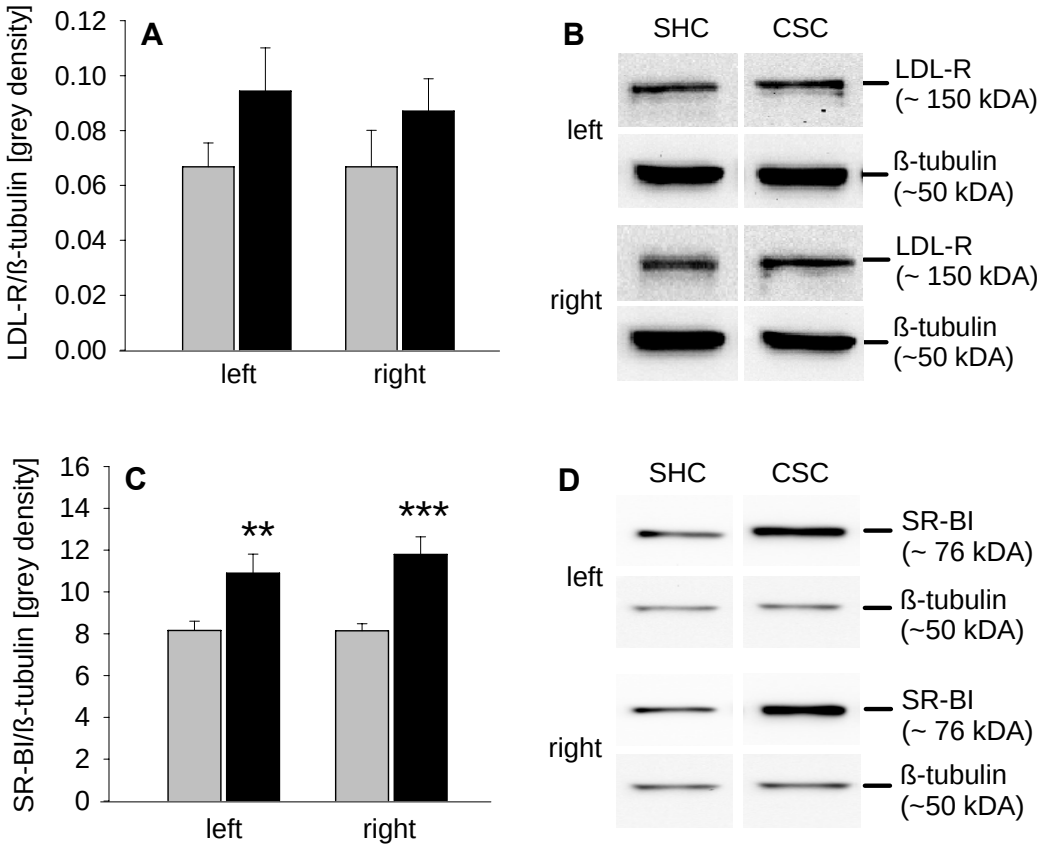

Figure 38: Effects of CSC on adrenal LDL-R and SR-BI protein expression

Following decapitation on day 20 the left and right adrenal glands of both SHC and CSC mice were removed and pruned of fat. Afterwards, protein was extracted from the left and right adrenal glands of SHC (n = 8) and CSC (n = 8) mice for determination of LDL-R (**A**) and SR-BI (**C**)

protein expression [grey density] normalized to the loading control ß-tubulin. ■ SHC; ■ CSC. Data represent the mean + SEM. ** represent $P < 0.01$, *** represent $P < 0.001$ *vs.* respective SHC mice. In addition, representative images of the bands detected for LDL-R (~ 150 kDA; **B**), SR-BI (~ 76 kDA; **D**), and the loading control ß-tubulin (~ 50 kDA; **B**/**D**) are shown for both the left and right adrenal glands of SHC and CSC mice. Experiments performed by Andrea M. Füchsl. [adapted from (Uschold-Schmidt et al., submitted)]

CSC increases absolute adrenal weight following 4 h of shaking/ restraint stressor exposure

Absolute adrenal weight was found to be significantly increased in CSC (4.11 + 0.12 mg) compared with SHC (3.55 + 0.06 mg) mice following 4 h of shaking/ restraint stressor exposure ($P = 0.002$; not shown).

CSC increases plasma ACTH but decreases plasma CORT concentrations following prolonged shaking/ restraint stressor exposure

Statistical analysis revealed a significant decrease in plasma CORT concentrations [ng/ml] ($P = 0.025$; Fig. 39 A), despite plasma ACTH concentrations [pg/ml] were significantly increased ($P = 0.002$; Fig. 39 B), in CSC compared with SHC mice following 4 h of shaking/ restraint stressor exposure.

Moreover, relative plasma CORT concentrations (calculated in relation to adrenal weight to enable a better comparability to relative adrenal *in vitro* CORT secretion) were also found to be significantly decreased in CSC (175.75 + 11.99 ng/ml/mg tissue) compared with SHC (270.06 + 21.07 ng/ml/mg tissue) mice ($P = 0.003$; not shown).

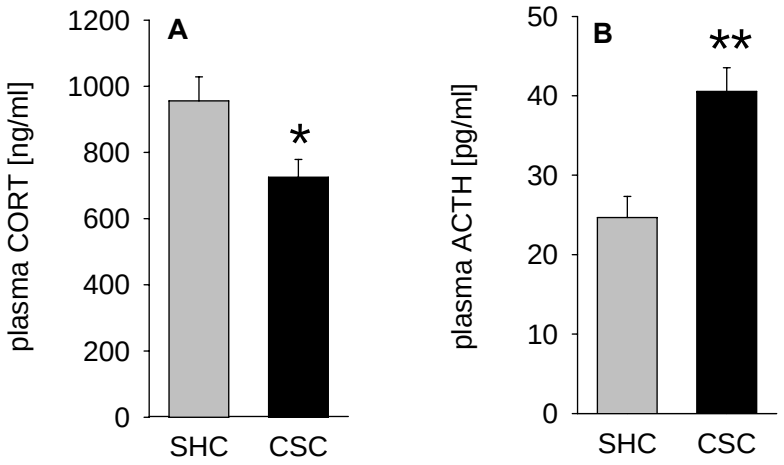

Figure 39: Effects of CSC on plasma CORT and ACTH levels following prolonged shaking/ restraint stressor exposure

On day 20 of CSC, both SHC (n = 7-8) and CSC (n = 7-8) mice were decapitated following continuous shaking/ restraint stressor exposure for 4 h, starting at 1500 h, and plasma CORT [ng/ml] (**A**) and plasma ACTH [pg/ml] (**B**) were determined in trunk blood. ■ SHC; ■ CSC. Data represent the mean + SEM. * represent $P < 0.05$, ** represent $P < 0.01$ *vs.* respective SHC mice. [adapted from (Uschold-Schmidt et al., submitted)]

CSC reduces acute *in vitro* adrenal CORT secretion in response to 0.15 nM but not 100 nM ACTH

Relative (per mg tissue) *in vitro* adrenal CORT secretion in response to physiological stress doses of ACTH (0.15 nM) was significantly increased (compared with basal values) in SHC ($P = 0.011$; interaction of factor CSC and ACTH treatment: $F_{1, 24} = 4.49$; $P = 0.045$), but not CSC mice ($P = 0.002$ vs. respective ACTH-treated SHC group) (Fig. 40 A). However, relative *in vitro* adrenal CORT secretion in response to pharmacological ACTH concentrations (100 nM) was significantly increased (compared with respective basal

values) in both SHC and CSC mice (both: $P < 0.001$; factor ACTH treatment: $F_{1, 28} = 45.81$; $P < 0.001$; Fig. 40 B).

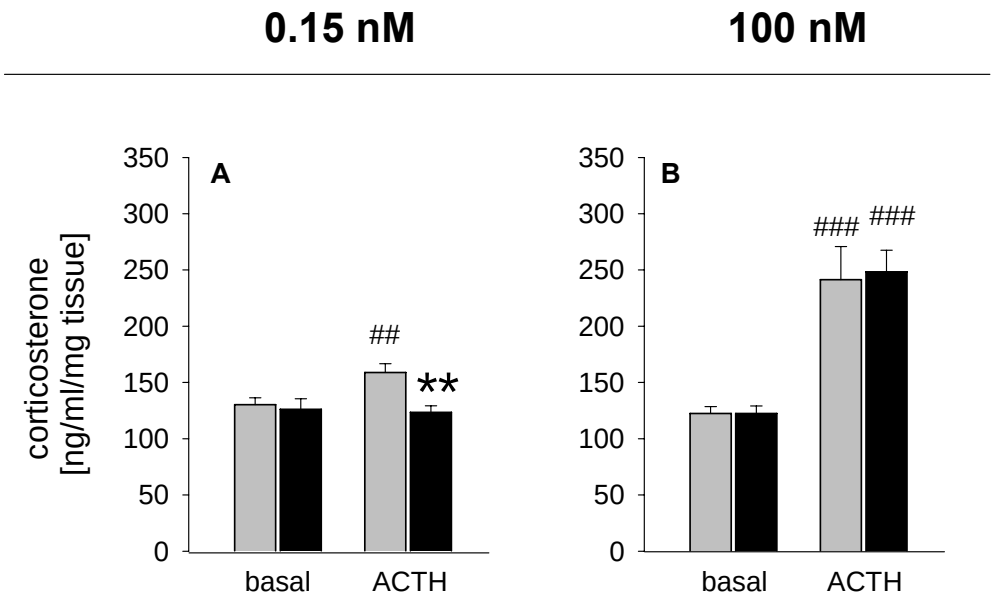

Figure 40: Effects of CSC on acute *in vitro* adrenal CORT secretion in response to 0.15 nM and 100 nM ACTH

Following decapitation on day 20 adrenal glands of SHC and CSC mice were removed, pruned of fat, weighed separately, and cut into two halves. These adrenal halves of the SHC (n = 7-8) and CSC (n = 7-8) group were weighed again and pre-incubated for 4 h. Afterwards, each half of one adrenal was incubated for 30 min in the presence of either saline (basal) or 0.15 nM (**A**) or 100 nM (**B**) ACTH. CORT concentrations [ng/ml/mg tissue] were determined in supernatants. ▒ SHC; ■ CSC. Data represent the mean + SEM. ** represent $P < 0.01$ *vs.* respective SHC mice; ## represent $P < 0.01$, ### represent $P < 0.001$ *vs.* respective basal values. [adapted from (Uschold-Schmidt et al., submitted)]

Discussion

In the present chapter, I revealed that the relative amount of cortical cholesteryl esters, as well as relative HSL, HMG-CoA reductase, LDL-R, and SR-BI protein levels are

unaffected or even enhanced after CSC exposure, in line with previous data supporting the idea of CSC exposure if at all increasing overall adrenal functionality. Moreover, relative and absolute plasma CORT concentrations following prolonged heterotypic stressor exposure (4 h of shaking/ restraint stress) are significantly lower in CSC compared with SHC mice. Together with previous data indicating an increased HPA axis response to acute heterotypic stressors following CSC, this suggests a faster habituation to prolonged innocuous heterotypic challenges in chronically-stressed mice, despite an initially increased HPA axis response to the same. Finally, relative adrenal CORT secretion during 30 min of *in vitro* stimulation with stress-equivalent ACTH doses (0.15 nM) is lower in adrenal explants of CSC compared with SHC mice. This clearly rules out the possibility that the *in vitro*/ *in vivo* discrepancy described in the introduction is due to differences in the time schedule employed to assess adrenal CORT secretion *in vitro* during ACTH stimulation (6 h) and *in vivo* during acute heterotypic stressor exposure (5min EPF). Together, and given that adrenal *in vitro* CORT secretion in response to a pharmacological ACTH dose (100 nM; 30 min) was not different between SHC and CSC mice, the data of the present chapter suggest that an additional *in vivo* factor is activated/ released in CSC mice during acute heterotypic stressor exposure, itself not acting as CORT secretagogue but restoring adrenal ACTH responsiveness.

In chapter 2 it was revealed that 19 days of CSC result in an exaggerated CORT response to acute heterotypic stressor exposure (EPF, 5 min), despite a complete loss of *in vitro* adrenal responsiveness to physiological ACTH doses. I hypothesized this discrepancy to be either due to an additional *in vivo* factor, driving adrenal CORT secretion during acute stressor exposure, or due to differences in the time schedule employed to assess adrenal CORT secretion during *in vitro* ACTH challenge and *in vivo* acute stressor exposure.

Given that both explanations presume intact and fully functional adrenal tissue in CSC mice, in a first step it was assessed if CSC exposure affects adrenal cholesterol availability and mobilization capacity. Oilred staining in adrenal cryo-sections thereby revealed a comparable relative amount (in relation to the cortex area) of cortical lipid droplets in SHC and CSC mice and, thus, an undisturbed availability of cholesteryl esters following CSC exposure. Moreover, relative (in relation to the loading control) protein expression of HSL was also not affected by CSC exposure, suggesting that metabolism of cholesterol esters to the CORT precursor cholesterol is also not impaired in adrenal tissue of CSC mice. In addition, relative protein expression of LDL-R and SR-BI was unaffected or even increased, respectively, following 19 days of CSC, suggesting that cholesterol deriving from endocytic and selective cellular uptake of blood LDLs and/ or HDLs, respectively, is not impaired or even enhanced in CSC mice. Finally, relative protein expression of HMG-CoA reductase was comparable between SHC and CSC mice, suggesting that endogenous cellular *de novo* synthesis of cholesterol from acetyl CoA was also not affected by CSC exposure. Thus, given the increased adrenal mass in CSC mice it seems that the overall availability and mobilization capacity of cholesterol is rather enhanced than compromised after 19 days of CSC exposure. Together with the results of unaffected or even enhanced expressions of adrenal Mc2r, MRAP and key steroidogenic enzymes following CSC (see chapter 2), these data clearly suggests that the adrenal glands of CSC mice overall have even more capacity to produce and secrete CORT. This is in line with the adrenal CORT response to an acute heterotypic challenge *in vivo*.

To gain more insight into the mechanisms underlying the discrepancy between adrenal hyper-responsiveness during acute heterotypic stressor exposure on the one, and a lack of *in vitro* adrenal ACTH responsiveness on the other hand, the major aim of the present chapter was to reveal if differences in the time schedule, employed to assess adrenal CORT

secretion under these conditions, are involved. *In vitro* adrenals were challenged with acute stress-doses of ACTH (0.15 nM) for 6 h, whereas *in vivo* for only 10 min (mice were killed 5 min after a 5-min EPF exposure) (see chapter 2). Thus, if the timing issue plays a crucial role, one would expect lower plasma CORT levels in CSC compared with SHC mice after prolonged heterotypic stressor exposure and, in turn, a comparable or even higher CORT secretion in response to a 30 min *in vitro* ACTH (0.15 nM) challenge in CSC compared with SHC mice.

In support of this hypothesis, 4 h of shaking/ restraint stressor exposure indeed resulted in significantly lower relative and absolute plasma CORT concentrations in CSC compared with SHC mice. A combination of shaking and restraint was chosen, as this type of stressor was shown to highly activate the HPA axis over a prolonged period of time (Dhabhar and McEwen, 1997) and, thus, to adequately mimic continuous *in vitro* adrenal ACTH stimulation. Moreover, an involvement of higher HPA axis levels in mediating the decrease in plasma CORT concentrations following 4 h of shaking/ restraint can be excluded, as plasma ACTH levels were found to be even increased in this set of CSC compared with SHC mice. Importantly, and independent of how the *in vitro*/ *in vivo* discrepancy is finally mediated, an increased HPA axis response to acute (chapter 2) and a decreased one to prolonged heterotypic stressors (present chapter), indicating faster habituation, strongly suggest that CSC-induced changes at the adrenal level represent a beneficial adaptation to rather than a maladaptive consequence of chronic psychosocial stress.

In contrast to the current *in vivo* data, *in vitro* data obtained in the present chapter strongly argue against the idea of the *in vitro*/ *in vivo* discrepancy being due to differences in the time schedule employed to assess adrenal CORT secretion under these conditions. In line with 6 h of adrenal *in vitro* ACTH (0.15 nM) stimulation, relative *in vitro* adrenal CORT

secretion during a 30-min ACTH (0.15 nM) challenge was significantly lower in CSC compared with SHC mice. These findings clearly show that the loss of adrenal *in vitro* CORT secretion following 6 h of continuous ACTH (0.15 nM) stimulation is not due to an initially unaffected and over time gradually decreasing ACTH responsiveness, but due to a general loss of adrenal *in vitro* ACTH responsiveness following CSC. Although this has to be investigated in detail in future studies, the present data further strengthen the idea of an additional factor, absent under *in vitro* conditions and activated or released during acute heterotypic stressor exposure in chronically-stressed mice, acting itself as a CORT secretagogue or restoring adrenal ACTH sensitivity. For the latter speaks that even though adrenal explants of CSC mice do not respond to an acute stress-dose of ACTH (0.15 nM; 30 min) they are still able to respond to high pharmacological ACTH doses. This was indicated by a comparable increase in CORT secretion from adrenal explants in SHC and CSC mice during a 30-min stimulation with 100 nM ACTH compared with respective basal values.

A possible *in vivo* factor restoring adrenal ACTH sensitivity during acute heterotypic challenges in CSC mice constitutes the sympathetic nervous system (SNS). Sympathetic innervation of the adrenal medulla via the splanchnic nerve is known to play an important role in modulating adrenocortical sensitivity to ACTH (Edwards et al., 1986; Edwards and Jones, 1987a; Ulrich-Lai et al., 2006a). Thus, an increased sympathetic drive to the enlarged adrenal glands during acute stressor exposure in CSC compared with SHC mice and the accompanied restoration of adrenal ACTH sensitivity would enable an adequate/ enhanced CORT response under these conditions despite the loss of adrenal ACTH responsiveness under *in vitro* conditions.

In summary and together with the findings of chapter 2, the current data indicate that i) adrenal functionality following CSC is rather enhanced than decreased, ii) adrenal *in vitro*

sensitivity to acute stress-doses of ACTH in CSC mice is generally lost and does not gradually decrease over time during prolonged stimulation, iii) CSC adrenals are able to, at least acutely, respond to pharmacological doses of ACTH *in vitro*, iv) CSC mice show a faster HPA axis habituation during prolonged innocuous heterotypic challenges, despite an initially increased HPA axis response. Therefore, the present data strongly support the idea that an additional factor, released/ activated during the acute phase of a heterotypic stressor and metabolized/ deactivated during prolonged heterotypic stressor exposure, restores adrenal ACTH sensitivity in a time-limited fashion and, therefore, enables a chronically-stressed organism to show an adequate plasma CORT response to acute challenges and a faster habituation to prolonged ones.

Chapter 5

General Discussion

Content

1 Discussion of the present results

1. 1 Short- and long-term adaptations to CSC exposure at the adrenal level – different mechanisms

Exposure of an organism to acute stressful stimuli leads to the activation of the SNS and the HPA axis. The subsequent release of catecholamines and GCs into the systemic circulation enables the organism to adjust to the new situation by triggering physiological and behavioural responses. This acute stress response is generally accepted to be beneficial, as it increases an individual's chance to survive. However, problems emerge, if the stress systems are activated over a prolonged period of time, because chronically-elevated levels of plasma GCs are known to have deleterious effects on both physical and mental health (McEwen, 2000; Vanitallie, 2002). Thus, if an individual is exposed to an innocuous homotypic stressor over a prolonged period of time it is adaptive to show fast habituation (Kudielka et al., 2006b; Sasse et al., 2008). Such processes of habituation of the stress response have been described in response to various homotypic stressors of non-social nature, such as repeated exposure to restraint (Bhatnagar et al., 2002; Fernandes et al., 2002), cold (Bhatnagar et al., 1995), noise (Armario et al., 1986) or water immersion (De Boer et al., 1990). In contrast, less is known about habituation to homotypic stressors of social nature, which was until recently believed not to occur (Bartolomucci, 2007). Nevertheless, Wood et al. (2010) could show using the social defeat paradigm (30 min/ day for 7 days) that a subgroup of rats in their study habituated to this repeated social stressor. In line, the present thesis provides evidence for such mechanisms of adaptation to occur also to continuous exposure to chronic psychosocial stress induced by the CSC paradigm. Beyond that, these mechanisms of adaptation to CSC exposure seem to happen at the level of the adrenal glands.

In detail, despite significantly enlarged left and right adrenal glands in CSC compared with SHC mice, a phenomenon generally observed following prolonged stressor exposure (Zelena et al., 2003), basal morning plasma CORT concentrations were only increased following 10 h and 24 h (Reber et al., 2007), but returned to baseline levels as early as 48 h after the start of CSC and remained unaffected until day 19. Interestingly, 19 days of CSC also resulted in an attenuated relative responsiveness of adrenal explants to pharmacological (100 nM) and an even lost relative responsiveness to physiological (0.01 and 0.15 nM) ACTH doses during prolonged (6 h) *in vitro* stimulation compared with SHC mice. These findings indeed suggest a mechanism of adaptation in response to chronic stressor exposure to protect CSC mice from chronically-elevated plasma CORT concentrations due to their increased left and right adrenal mass, which seems to be mediated by proliferation of generally functional adrenal cells.

Considering that basal morning plasma CORT levels returned to baseline as early as 48 h after the start of CSC, one might assume that the attenuation/ loss of relative adrenal ACTH responsiveness already develops in this initial phase of CSC exposure. However, this is rather unlikely as relative *in vitro* CORT secretion in response to 6-h stimulation with ACTH (100 nM) was neither affected in the left nor the right adrenal gland following 48 h of CSC exposure. Moreover, as the same results were obtained following 10 h of CSC, it is also unlikely that the increase in basal morning plasma CORT following 10 h of CSC was mediated by an increased relative ACTH responsiveness of the adrenal glands. Furthermore, the influence of different release patterns of ACTH from the pituitary following 10 h and 48 h seem also not likely to be involved, because basal morning plasma ACTH concentrations were comparable between SHC and CSC mice following both time points.

However, more clarity was obtained by investigating relative and absolute adrenal weights at this time points. Although relative adrenal weight of both left and right adrenal glands was found to be increased following 10 h as well as 48 h, absolute adrenal weight of both adrenal glands was only increased following 10 h but not 48 h of CSC exposure. This suggests that the increase in relative adrenal weight following 48 h of CSC was just mediated by the loss of body weight in CSC compared with SHC mice at that time point and not by changes in adrenal weight itself. Moreover, this is in line with the study of Reber et al. (2007) assessing the relative adrenal weight at different time points during CSC exposure. Although relative adrenal weight was found to be increased at all time points assessed, beginning as early as 24 h after the start of CSC, a pronounced drop was observed in this study at the 48 h compared with the 24 h time point.

Together, it seems that the changes in basal morning plasma CORT concentrations in the initial phase of CSC exposure run in parallel to the changes in absolute adrenal weight. This suggests that the increase in basal plasma CORT levels following 10 h of CSC is mediated by an increase in adrenal weight and, thus, by an increase in functional adrenal tissue in both the left and right adrenal glands. This is further supported by a comparable relative amount of cortical cholesteryl esters as well as relative mRNA expression of StAR, CYP11B1, and CYP11B2 following 10 h of CSC, indicating an overall elevated rate of steroidogenesis, when considering the increased adrenal mass in CSC mice at this time point. Furthermore, the decline in basal plasma CORT to baseline values following 48 h of CSC seems to be mediated by restoration of normal functional adrenal mass. As already mentioned, support for this hypothesis comes also from rat studies showing a positive correlation between plasma CORT concentrations and adrenal weight under stress conditions (Schwartz et al., 1997; Baranyi et al., 2005).

Importantly, all these findings indicate different mechanisms in mediating short- and long-term adaptations to prolonged stressor exposure to prevent basal hypercorticism. In the initial phase of CSC exposure basal hypercorticism seems to be prevented by restoration of normal functional adrenal mass, a strategy that has to my knowledge not been described before. In contrast, at the 19-day time point basal hypercorticism seems to be prevented by a reduction in adrenal ACTH responsiveness.

Moreover, I am indeed able to support the finding of Wood et al. (2010) that habituation occurs if the prolonged homotypic stressor is of social nature.

Unfortunately, so far I am not able to give evidence if the increase in absolute adrenal weight following 10 h of CSC is also mediated by hyperplasia, like following 19 days of CSC, or rather by hypertrophy of adrenal cells or a mixture of both and if the subsequent decline to baseline levels following 48 h is mediated by cell apoptosis and/ or by a decrease in cell size. To gain more insight into that matter one possibility might be to perform the DAPI staining in adrenal cryo-sections to assess the number of cells per given area following 10 h and 48 h of CSC exposure. Furthermore, cell apoptosis could be determined performing a terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL assay) in adrenal cryo-sections. Using this method, DNA fragmentation due to cell apoptosis can be detected by colorimetric or fluorescent labelling of the terminal end of nucleic acids (Peters et al., 2005; Loo, 2011).

1. 2 Attenuation/ loss of adrenal ACTH responsiveness in CSC mice – when does it develop?

The attenuation/ loss of ACTH responsiveness of left and right adrenal explants observed following 19 days of CSC exposure does not seem to develop in the initial phase of CSC,

i.e. not until 48 h of prolonged stressor exposure. Nevertheless, the time frame can already be narrowed more precisely. Thus, it seems that adrenal ACTH responsiveness is also not altered until day 14 of CSC exposure. This was indicated by an unaffected relative *in vitro* CORT secretion in response to a 6-h stimulation with ACTH (100 nM) from both left and right CSC compared with SHC adrenal explants following 7 days (Fig. 41 A/B) as well as 14 days (Fig. 41 C/D) of CSC exposure. These results indicate that the attenuation/ loss of adrenal responsiveness to prolonged (6 h) ACTH stimulation *in vitro* develops between day 14 and 19.

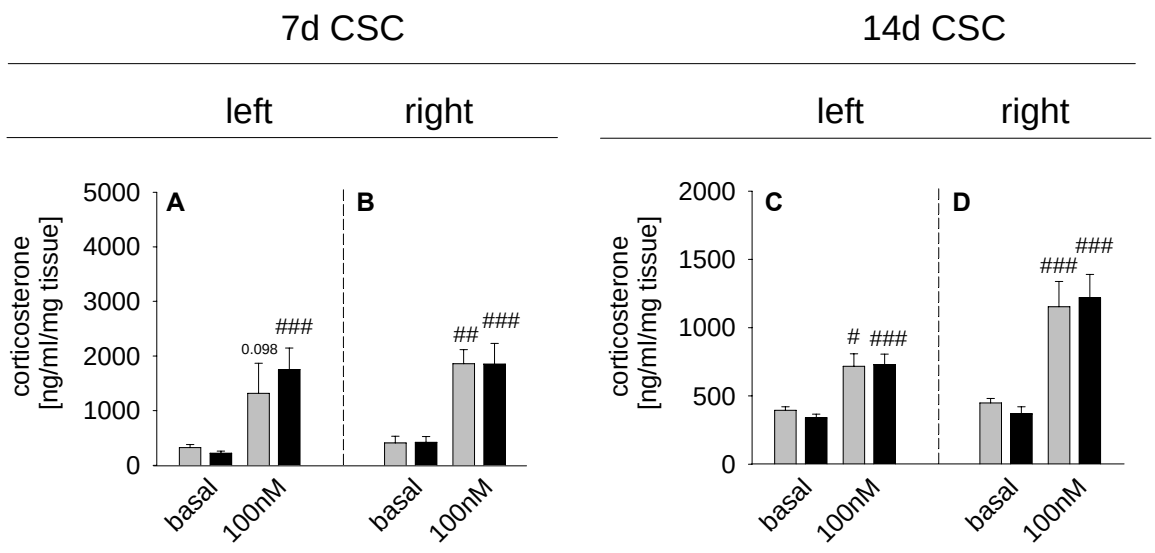

Figure 41: Effects of 7 days and 14 days of CSC on *in vitro* adrenal CORT secretion in response to a pharmacological dose (100 nM) of ACTH

Following decapitation on day 8 or 15 the left and right adrenal glands of SHC and CSC mice were pruned of fat and cut into two halves. These left (**A**/**C**) and right (**B**/**D**) adrenal halves of the SHC (left: n = 5-8; right: n = 5-7) and CSC (left and right: n = 8-11) group were weighed and incubated for 6 h in the presence of either saline (basal) or 100 nM ACTH. CORT concentrations [ng/ml/mg tissue] were determined in supernatants. ■ SHC; ■ CSC. Statistics: Two-way ANOVA followed by a *post hoc* Bonferroni test when appropriate. A significant effect was found only for factor ACTH treatment (7 days CSC left: $F_{1,28}$: 12.98; p = 0.001; 7 days CSC right: $F_{1,28}$: 21.65; p < 0.001;

14 days CSC left: $F_{1,28}$: 21.39; $p < 0.001$; 14 days CSC right: $F_{1,26}$: 35.79; $p < 0.001$). Data represent the mean + SEM. # represent $P < 0.05$, ## represent $P < 0.01$, ### represent $P < 0.001$ *vs.* respective basal values.

Support for this late development of adrenal adaptation is also provided by other studies. For instance, Ottenweller et al. (1992) showed in their study using a stress paradigm of repeated exposures to immobilisation and tail shock (2h/ day) that habituation to this stressor, indicated by a decreased rate of weight loss with increasing numbers of sessions applied, does not occur until the fourth session of stressor exposure. Furthermore, Silberman et al. (2003) showed that at least 3 weeks of chronic mild stress were necessary to induce a decline of basal plasma CORT concentrations to baseline values and, thus, habituation in mice to this stressor. Together, these studies demonstrate the need of a certain amount of stress received, strongly depending on the type and intensity of the stressor, sufficient to induce habituation to the respective stressor. Therefore, in case of the CSC paradigm the amount of stress received to induce habituation by reducing adrenal *in vitro* ACTH responsiveness seems to be reached somewhere between day 14 and 19.

1. 3 Mechanism underlying the attenuation/ loss of adrenal ACTH responsiveness in chronically-stressed mice

One intention of the present thesis was to reveal the mechanism underlying the attenuation/ loss of ACTH responsiveness of both left and right adrenal glands in CSC compared with SHC mice observed on day 19 of chronic psychosocial stressor exposure.

As explained in detail in the general introduction, the main receptor responsible for the actions of ACTH in the adrenal glands is the Mc2r. Thus, one possible cause for the altered

adrenal ACTH responsiveness in CSC mice might be a reduced expression of this receptor. Support for this hypothesis comes from a study showing a decreased mRNA expression of Mc2r in adrenal tissue accompanied by a reduced CORT response of isolated adrenal cells to ACTH *in vitro* in rats repeatedly injected with insulin to induce hypoglycaemia (Lehmann et al., 2011). However, although relative Mc2r mRNA expression was found to be decreased in both left and right adrenal glands of CSC compared with SHC mice, no changes in relative Mc2r protein expression could be detected. This suggests that changes in the number of adrenal Mc2r itself can be excluded as underlying mechanism. Another important factor regulating the responsiveness of the adrenal glands to ACTH is the Mc2r associated protein MRAP, which was shown to play an important role for trafficking and cell surface expression of Mc2r (Metherell et al., 2004; Metherell et al., 2005). Moreover, mutations in MRAP have been shown to cause familial GC deficiency type 2, an autosomal-recessive inherited disease also called syndrome of ACTH insensitivity (Clark et al., 2005b; Clark and Metherell, 2006). However, despite protein expression could not be assessed as there is currently no functional antibody against MRAP available at the market, an increased relative mRNA expression of MRAP in both left and right adrenal glands of CSC mice suggests that a reduction in trafficking or cell surface expression of the Mc2r is also not likely to play a crucial role. Another approach was to investigate the expression levels of key enzymes of steroidogenesis, as these enzymes were already shown to be decreased in response to chronic stressor exposure such as chronic undernutrition (Khorram et al., 2011). Nevertheless, following 19 days of CSC relative mRNA expression of StAR, CYP11A1, CYP11B1 and CYP11B2 was either unaffected or even enhanced in CSC compared with SHC mice (CYP11B1) in left and right adrenal tissue, suggesting that a decreased expression of these enzymes is also not likely to be involved.

In addition to the parameters mentioned above, an adequate availability and mobilization capacity of adrenal cholesterol is critical in the process of steroidogenesis (Kraemer, 2007). Here, an important aspect is the quantity of cortical lipid droplets storing the CORT precursor molecule cholesterol in form of esters. Interestingly, Kiank and co-workers (2006) revealed that repeated exposure of mice to a combination of acoustic and restraint stress on 4 successive days (2 h/ day) resulted in a decreased size of these lipid droplets within the ZF of the adrenal gland, accompanied by a less pronounced plasma CORT response to ACTH injection in these mice compared with unstressed controls. These findings suggest a reduced adrenal ACTH responsiveness due to the lack of the CORT precursor cholesterol. However, in contrast to the repeated stressor applied in the study above, 19 days of chronic psychosocial stress did not result in an impaired availability of cholesterol. This was indicated by a comparable relative amount (in relation to the cortex area) of cholesteryl esters in left and right adrenal cryo-sections of SHC and CSC stained with oilred.

Another approach was to investigate, if the attenuated/ lost adrenal ACTH responsiveness is mediated by an altered metabolism of the cholesteryl esters to cholesterol. Evidence for this hypothesis comes from studies using HSL-deficient mice. Kraemer et al. (2004) showed that HSL-deficiency and, thus, an impaired mobilization capacity of cholesterol from adrenal lipid droplets results in a reduced basal as well as ACTH-stimulated CORT secretion from isolated adrenal cells. Moreover, another group revealed an impaired adrenal CORT response of HSL-deficient mice in response to ACTH injection *in vivo* (Li et al., 2002). However, although HSL phosphorylation (= activation) has not been assessed so far, an impaired mobilization capacity of cholesterol in mediating the attenuated ACTH responsiveness in CSC mice is not likely in the present thesis, as relative HSL protein expression was not altered in left and right adrenal tissue following CSC exposure. With

respect to the availability of cholesterol it was also assessed if other sources of cholesterol, namely the endocytic and selective cellular uptake of blood LDLs and/ or HDLs mediated by the LDL-R and the SR-BI, respectively, and the endogenous cellular *de novo* synthesis of cholesterol from acetyl CoA via the enzyme HMG-CoA reductase are affected following 19 days of CSC. Importantly, it was already shown that deficiency for LDL-R or SR-BI in mice leads to blunted plasma CORT concentrations in response to ACTH due to deficient cholesterol delivery (Dichek et al., 2006; Cai et al., 2008). A similar pattern was obtained by inhibition of HMG-CoA reductase in cultured bovine adrenocortical cells. Here, ACTH-stimulated steroidogenesis was also blunted, indicated by a significantly reduced CORT response to ACTH in cells with inhibition of HMG-CoA reductase (Rainey et al., 1992). But again, relative left and right adrenal protein expression of LDL-R, SR-BI and HMG-CoA reductase were also found to be unaffected or even increased (SR-BI) in CSC compared with SHC mice.

It has to be mentioned that it is not possible to discuss all of the findings of the present thesis above in the context of continuous stressor exposure, because partially no studies are available assessing these parameters in the adrenal glands with regard to the latter.

Nevertheless, with respect to all parameters assessed in the present experiments, it seems that the left and right adrenal glands of CSC mice are generally functional and that the reduction/ lack of adrenal ACTH responsiveness is not due to adrenal insufficiency. Moreover, given the increased adrenal mass, which seems to be due to proliferation of functional cells, these data suggest an overall enhanced adrenal capacity to produce and secrete CORT when challenged after CSC exposure. This is also in line with the exaggerated CORT response to an acute heterotypic stressor *in vivo*, namely EPF exposure, in CSC compared with SHC mice (discussed below).

As the initial question of the mechanism underlying the attenuated/ lost adrenal ACTH responsiveness could not be fully answered in the present thesis, future studies are needed to clarify this. Although a down-regulation of the Mc2r protein itself seems not to be involved, a possible mechanism might be a decreased sensitivity of Mc2r to ACTH and/ or a disrupted Mc2r signalling. As explained in detail in the general introduction, binding of ACTH to Mc2r leads to activation of the adenylyl cyclase and hence to increased levels of the second messenger cAMP, resulting in activation of cAMP-dependent PKA. Chronic stressed-induced alterations in this signalling pathway might, therefore, result in altered cAMP levels in response to ACTH. Such alterations were, for instance, shown after repeated immobilization in rats (2 h/day for 14 days). Here, the cAMP response of adrenal cells from these animals to ACTH *in vitro* was significantly increased and accompanied by an increased CORT secretion compared with unstressed controls (Aguilera et al., 1996). In contrast to the repeated stressor above, a decreased cAMP response to ACTH following chronic psychosocial stressor exposure might be possible. Thus, it needs to be assessed in future *in vitro* studies, whether the CSC-induced attenuation/ loss of adrenal CORT response to ACTH is mediated by a decreased cAMP response to ACTH. This method is already established (see section 2 of the general discussion).

Moreover, in case a decreased sensitivity of the Mc2r to ACTH and/ or a disrupted signalling in the pre-cAMP pathway and, thus, a decreased cAMP signal are involved in mediating the attenuated/ lost adrenal ACTH responsiveness in CSC mice after 19 days, one would expect a comparable CORT response of adrenal explants from SHC and CSC mice stimulated with a cAMP analogue such as dibutyryl cAMP ($(Bu)_2$ cAMP) *in vitro*. This procedure is also already established as detailed in section 2 of the general discussion.

1. 4 Sensitization of the adrenal CORT response to acute heterotypic stressor exposure in chronically-stressed mice

Habituation to prolonged innocuous homotypic stressor exposure occurs to protect the organism from the negative outcomes of chronically-elevated plasma CORT levels. However, it is indispensable for an individual's survival to be able to generate an adequate CORT response when challenged by a novel stimulus. Such a sensitized CORT response to acute heterotypic challenges has been described after various types of prolonged homotypic stressors. For example, although rats repeatedly exposed to restraint stress over 15 days (2 h/ day) displayed marked habituation of the CORT response to this homotypic stressor, they showed an increased CORT response to a subsequent heterotypic stressor, in this case lipopolysaccharide-injection (Fernandes et al., 2002). Similarly, rats exposed to repeated noise for 21 days (4 h/ day) also showed habituation to this homotypic stressor, indicated by a decreased CORT response to noise on day 21 compared with previously unstressed controls, but they were still able to respond to the subsequent heterotypic challenge of forced swimming (Armario et al., 1986).

To date, the mechanisms underlying such processes of habituation to familiar and sensitization to novel stimuli are only partly understood at higher HPA axis levels, for example the pituitary gland (Aguilera, 1994; Wood et al., 2010).

Interestingly, in the experiments performed during my dissertation I am able to provide first evidence that such processes also occur at the level of the adrenal glands. Male mice exposed to the CSC paradigm for 19 days are characterized by a loss of relative adrenal responsiveness to basal and stress-equivalent ACTH doses, suggesting at first glance a general breakdown of adrenal function. However, in contrast to this assumption plasma CORT concentrations as well as the *in vivo* adrenal CORT content in response to acute

heterotypic stressor exposure (EPF, 5 min) were found to be even higher in CSC compared with SHC mice. Moreover, comparable plasma ACTH concentrations in EPF-exposed SHC and CSC mice suggest an even increased adrenal ACTH sensitivity, at least during acute heterotypic stressor exposure.

It has to be mentioned that the lost adrenal ACTH responsiveness in CSC compared with SHC mice was observed following prolonged (6 h) *in vitro* stimulation with an acute stress-dose of ACTH (0.15 nM), whereas *in vivo* adrenals were challenged acutely with stress-doses of ACTH (mice were killed 5 min after a 5-min EPF exposure). Thus, one might assume that this *in vitro*/ *in vivo* discrepancy in CSC mice is mediated by the differences in the time schedule employed to assess adrenal ACTH responsiveness under these conditions. The loss of relative adrenal CORT response during 6 h of continuous *in vitro* stimulation with stress-equivalent ACTH doses (0.15 nM) in CSC mice might, thus, just be a consequence of an initially unaffected or even increased but over time gradually declining ACTH responsiveness. However, this could be excluded in the present thesis as relative *in vitro* adrenal CORT secretion was found to be decreased in CSC compared with SHC adrenal explants already during acute *in vitro* stimulation (30 min) with the acute stress-dose of 0.15 nM ACTH, suggesting a general loss of adrenal responsiveness to stress-equivalent ACTH doses *in vitro* in chronically-stressed mice.

Interestingly, despite an exaggerated plasma CORT response in CSC compared with SHC to acute heterotypic stressor exposure (5 min EPF), plasma CORT levels in response to prolonged heterotypic stressor exposure (4 h shaking/ restraint) were even found to be decreased in CSC compared with SHC mice.

Together, all these findings support the idea of an additional *in vivo* factor activated or released during acute, but deactivated or metabolized during prolonged heterotypic stressor exposure, either acting itself as a CORT secretagogue or restoring adrenal ACTH

responsiveness. This would enable a chronically-stressed mouse, on the one hand, to adequately respond to acute challenges, but, on the other hand, also to show faster habituation if the challenge is prolonged. Moreover, the present data suggests that this still unknown *in vivo* factor rather restores adrenal ACTH responsiveness in CSC mice than acts as a direct CORT secretagogue. This was indicated by the fact that, although adrenal ACTH responsiveness was generally lost in CSC mice in response to the acute stress-dose of 0.15 nM ACTH, CSC adrenal explants were still able to respond to the pharmacological dose of 100 nM ACTH with an increased CORT secretion compared with basal values.

Even though I am not able to exactly define this *in vivo* factor to date, one possibility constitutes the SNS. It is known since decades that sympathetic innervation of the adrenal glands via the splanchnic nerve plays an important role in modulating adrenocortical sensitivity to ACTH. Indications for this assumption came from studies showing that sectioning of the splanchnic nerve or chemical sympathectomy led to a decreased responsiveness of the adrenal cortex to ACTH (Edwards et al., 1986; Edwards and Jones, 1987a; Walker, 1995), whereas electrical stimulation of the splanchnic nerve resulted in an increased adrenocortical ACTH responsiveness (Edwards and Jones, 1987b; Engeland and Gann, 1989). It is suggested that the SNS modulates adrenocortical sensitivity to ACTH through the release of neurotransmitters and neuropeptides, either from sympathetic nerve endings directly innervating the adrenal cortex or from adrenomedullary cells to act in a paracrine fashion. These neurotransmitters and neuropeptides, such as epinephrine/ NE, VIP, SP or NPY, in turn seem to influence Mc2r activity and/ or the affinity of Mc2r to ACTH (Ehrhart-Bornstein et al., 1998). In support, it was shown that splanchnic nerve transection leads to a decrease in adrenal cAMP levels, suggesting that the splanchnic nerve acts proximal to the generation of cAMP (Ulrich-Lai et al., 2006a).

Interestingly, it was shown in rats that splanchnic nerve transection reduces the plasma CORT response to exogenous ACTH *in vivo* (injected subcutaneously) in dexamethasone-blocked rats, indicating a reduced adrenal ACTH sensitivity (Ulrich-Lai et al., 2006a). However, in these animals plasma CORT response was not affected by splanchnic nerve transection in vehicle-injected rats, suggesting that splanchnic nerve-mediated effects require the presence of ACTH (Ulrich-Lai and Engeland, 2002). Furthermore, basal plasma CORT levels were found to be higher in sham-operated compared with splanchnic nerve-transected rats, despite basal plasma ACTH levels were not affected. However, this effect was prevented by adrenal demedullation, suggesting that in this case splanchnic nerve-mediated facilitation of plasma CORT in sham-operated rats requires the presence of the adrenal medulla (Ulrich-Lai and Engeland, 2002).

With respect to these findings, it might be that a higher sympathetic input to the adrenal glands via the splanchnic nerve in CSC mice mediates the exaggerated adrenal CORT response to acute heterotypic stressor exposure through the release of neurotransmitters/ neuropeptides from the adrenal medulla. The latter consequently act in a paracrine manner to enhance Mc2r activity and/ or affinity to ACTH and, thus, to restore adrenocortical ACTH responsiveness.

However, future studies are required to reveal if this hypothesis is indeed appropriate following 19 days of CSC exposure. To gain more knowledge about sympathetic innervation to the adrenal glands in SHC and CSC mice, one possibility is the performance of an AChE staining in adrenal cryo-sections, as described in detail in section 2 of the general discussion, for assessing AChE activity in the adrenal medulla.

Moreover, in case the activation of the SNS and, thus, an increased sympathetic drive to the adrenal glands during acute heterotypic stressor exposure restores adrenal ACTH

responsiveness in CSC mice, one would expect that the observed decrease of plasma CORT in CSC compared with SHC mice during prolonged heterotypic stressor exposure *in vivo* is prevented by an additional acute heterotypic stressor in between. Therefore, experiments are planned for the determination of plasma CORT levels in SHC and CSC mice exposed to shaking/ restraint for 4 h, but additionally stressed, for instance, by tail vein blood sampling 2 h after the start of stressor exposure, to see if the fast habituation of plasma CORT levels in CSC mice is prevented. Tail vein blood sampling as acute stressor would further enable to compare plasma CORT levels before and after additional acute stressor exposure.

Another option might also be the transection of the splanchnic nerve or chemical sympathoectomy (Edwards et al., 1986; Edwards and Jones, 1987a; Walker, 1995) following CSC exposure. If the SNS is indeed the yet unknown factor restoring adrenal ACTH sensitivity, the exaggerated adrenal CORT response to acute heterotypic stressor should be prevented in sympathoectomized CSC mice.

Furthermore, if the restoration of adrenal ACTH responsiveness during acute stressor exposure is mediated through neurotransmitters/ neuropeptides arising from the adrenal medulla, one would also expect that the lost *in vitro* responsiveness of CSC adrenal explants to the stress-equivalent dose of ACTH (0.15 nM) can be rescued by adding the crucial neurotransmitters and/ or neuropeptides to the medium. Therefore, *in vitro* stimulations will be performed with varying combinations of 0.15 nM ACTH and the neurotransmitters/ neuropeptides mentioned above.

1. 5 CSC-induced adaptations at the adrenal level – two sides of one coin

As indicated before, the loss of adrenal responsiveness to physiological doses of ACTH in CSC mice seems to be adaptive, as this enables habituation to the chronic homotypic

stressor, indicated by unaffected basal morning plasma CORT concentrations following 19 days of CSC (Reber et al., 2007). Thus, it protects CSC mice from chronically-elevated plasma CORT concentrations, which are likely to occur as consequence of enlarged functional adrenal tissue in chronically-stressed mice, and its deleterious effects on the organism. Therefore, CSC mice should be protected to develop somatic and affective diseases associated with prolonged elevations of plasma GC levels, such as depressive-like behaviour (Keeney et al., 2001; Dadomo et al., 2011) and typical characteristics of the metabolic syndrome-like adiposity, hyperinsulinemia, and hyperglycemia (Karatsoreos et al., 2010; Lee et al., 2010). Indeed, it was shown that CSC mice do not develop depressive-like behaviour, indicated by comparable times spent immobile in the tail suspension and the forced swim test, and a comparable anhedonic-like behaviour, assessed in the saccharin preference test, between SHC and CSC mice (Slattery et al., 2012). Moreover, CSC mice are also not characterized by metabolic syndrome-like adiposity, indicated by a comparable body weight gain during 19 days of CSC (Slattery et al., 2012). Unfortunately, nothing is known so far about blood insulin and glucose levels in CSC mice. Furthermore, they should be also less susceptible to infections, which are promoted by chronically-elevated plasma CORT levels due to their immunosuppressive effects (Ben-Eliyahu et al., 1991; Malisch et al., 2009). The CSC-induced chances at the level of the adrenal glands, therefore, seem to represent at first glance a beneficial adaptation to rather than a maladaptive consequence of chronic psychosocial stress.

However, as explained in detail in the general introduction, 19 days of CSC do very well result in behavioural and immunological alterations, despite these adrenal adaptations. With respect to behavioural changes the most obvious one is an increased anxiety-related behaviour (Reber et al., 2007; Reber and Neumann, 2008; Veenema et al., 2008; Slattery et al., 2011). Furthermore, CSC mice develop spontaneous colonic inflammation and, in

addition to that, an even more severe chemically-induced colitis when treated with dextran sodium sulphate (Reber et al., 2007; Veenema et al., 2008).

The question arises, how these behavioural and immunological alterations are mediated. It has to be emphasized that even though basal morning plasma CORT levels are unaffected, CSC mice are not able anymore to mount the circadian rise in plasma CORT, resulting in basal evening hypocorticism (Reber et al., 2007). Based on these results, it seems that the adaptive changes occurring at the adrenal level in CSC mice in the end result in an over-adjustment to chronic psychosocial stress. Thus, the loss of adrenal responsiveness to physiological ACTH doses on the one hand reduces the deleterious effects of high basal morning GC levels in CSC mice, but on the other hand makes the latter more susceptible to diseases associated with a hypoactive state of the HPA axis, such as an increased inflammatory state.

Considering the sigmoidal dose-response curves describing the link between the potency of a stressor and the activity of the stress system (HPA axis activity) of Chrousos and Gold (1992) (see Figure 13 A), this would mean that 19 days of CSC exposure rather shift the curve to the left, reflecting hyporesponsiveness of the HPA axis. Moreover, the corresponding inverse *U*-shaped dose-response curves, picturing the coherence between sense of well-being/performance and stress system activity (see Figure 13 B), is also more shifted to the left in CSC mice and therefore reflects a greater vulnerability to diseases associated with a hypoactive state of the HPA axis.

1. 6 Conclusion

In the present thesis I am able to show that mechanisms of adaptation to homotypic and sensitization to acute heterotypic stressor exposure occur, in addition to higher HPA axis levels such as the pituitary, also at the level of the adrenal glands; a phenomenon, at least

to my knowledge, never described before. Exposure to 19 days of CSC result in a loss of adrenal responsiveness to stress-equivalent ACTH doses (0.15 nM), thereby protecting the chronically-stressed mice from being exposed to prolonged high levels of GCs, a likely consequence of adrenal enlargement. However, an additional factor, present during acute heterotypic stressors *in vivo*, restoring adrenal ACTH responsiveness enables a chronically-stressed mouse to generate an adequate plasma CORT response to a novel, possibly life-threatening, challenge. One likely candidate constitutes here the SNS, but this has to be revealed in future studies.

Nevertheless, even though the lost adrenal responsiveness to physiological ACTH doses in CSC mice seems to be in the first place adaptive, it is likely that these changes at the same time result in an over-adjustment to the homotypic stressor and consequently in basal evening hypocorticism. Although I am not able to reveal the exact mechanism underlying the loss of adrenal ACTH responsiveness in CSC mice, the findings of the present thesis give a deeper insight into chronic stress-induced alterations at the level of the adrenal glands contributing to basal hypocorticism. Therefore, I am convinced that the present results obtained in mice are pioneering to improve the understanding of the underlying mechanisms of chronic stress-induced hypocorticism in humans, at least at the level of the adrenal glands.

Finally, I am able to present a novel strategy for short-term adaptations during the initial phase of prolonged homotypic stressor exposure. Thus, in contrast to 19 days of CSC were basal hypercorticism seems to be prevented by the attenuation/ loss of adrenal ACTH responsiveness, basal hypercorticism during the initial phase of CSC exposure is likely to be prevented by restoration of normal functional adrenal mass.

2 Established techniques – performance and analysis

In the course of my dissertation I established a series of techniques, enabling me to assess various adrenal parameters, which I will discuss in detail in the following. These newly established techniques were not only employed in the experiments of the present thesis, but also in many studies of colleagues in our laboratory (e.g. Bartlang et al., *Journal of Endocrinology*, in press; Perani et al., unpublished data; Peters et al., unpublished data, Reber & Lowry, unpublished data) and international collaborators (e.g. Donner et al., unpublished data).

Oilred and DAPI staining in adrenal cryo-sections are explained in detail in the respective method sections in chapter 2, 3 and 4. These techniques were also performed in our laboratory for the first time in the experiments of the present thesis, but worked immediately without the need of further modifications.

2. 1 ACTH stimulation of adrenal explants *in vitro*

Although *in vitro* stimulation with ACTH was already established in our laboratory for isolated adrenal cells (Reber et al., 2007), I established this technique in adrenal explants because of two reasons. First, it is claimed in the literature that cell-to-cell contacts play an important role for the functionality of adrenocortical cells, which are not existent anymore in cell suspension. Second, intra-adrenal interactions are also of great importance for the sensitivity of the adrenal cortex to ACTH (Ehrhart-Bornstein et al., 1998; Ehrhart-Bornstein and Bornstein, 2008). Therefore, the presence of medullary cells seems to be indispensable for the responsiveness of the adrenal glands to ACTH *in vitro*. However, in cell suspensions medullary cells are also not present anymore, because they do not survive the isolation procedure (Oitzl et al., 1995).

The protocol I used for ACTH stimulation of mice adrenal explants *in vitro* was adapted from a previous study of Richter et al. (2008), in which adrenal stimulation was performed in rat adrenal explants. Importantly, with the protocol used in their study they were able to show a 3-fold increased CORT secretion in response to 100 nM ACTH compared with basal values.

Although rat adrenals were quartered in the study of Richter et al., due to the smaller size of mouse adrenals I decided to halve them. It was thereby ensured that each half contained cortical and medullary tissue to enable the important intra-adrenal interactions and cell-to-cell contacts explained above. These adrenal 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). Afterwards, culture medium was replaced and each half of one adrenal was incubated with medium (100 µl) containing saline (basal) or ACTH for 6 h (37 °C, 95 % O_2, 5 % CO_2). As I used saline as vehicle for ACTH, I decided to use saline for basal instead of medium alone to improve comparability between basal and stimulated conditions. Moreover, in addition to the pharmacological dose of 100 nM used in the rat study, I used also 0.1 nM, 1 nM and 10 nM ACTH in my pre-experiment to test if adrenal CORT secretion is also increased in response to lower and physiological ACTH concentrations. Furthermore, the incubation periods of the rat study, which were 6 h for pre-incubation and 12 h for the stimulation itself, were shortened to prevent degradation of secreted CORT. After stimulation, supernatants were collected and stored at – 20 °C until assayed using a commercial available ELISA for CORT. CORT concentrations were calculated, as described in the rat study, in relation to the weight of the respective adrenal explant [ng/ml/mg tissue] (= relative *in vitro* CORT secretion) to compensate for differences in the size of the explants.

As shown in Figure 42, relative CORT secretion was increased at all ACTH doses tested compared with respective basal values. Moreover, CORT concentrations increased steadily with increasing doses of ACTH. Although statistical comparisons were not performed because of the small group sizes (each n = 2), these findings clearly confirm the reliability of the established mouse protocol for assessing the *in vitro* responsiveness of adrenal explants to physiological as well as pharmacological ACTH doses.

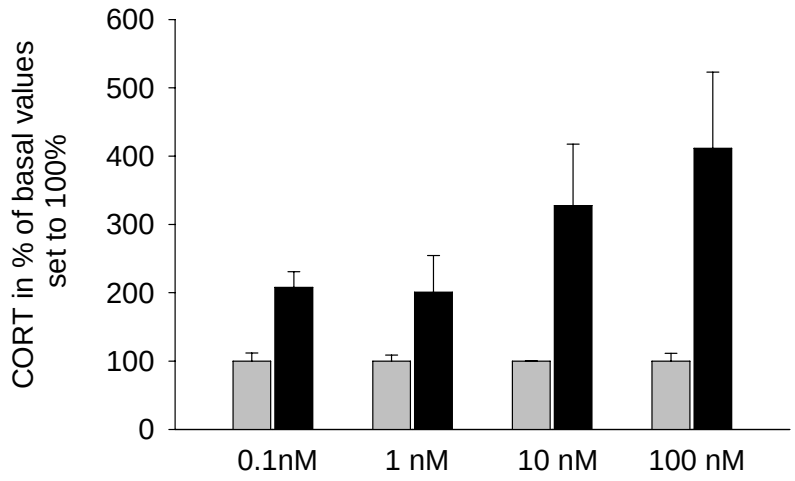

Figure 42: Establishment of ACTH stimulation of mouse adrenal explants *in vitro*

Adrenals from naive mice were pruned of fat and cut into two halves, each half containing cortical and medullary tissue. These halves were weighed and pre-incubated for 4 h in 200 µl DMEM/F-12. Afterwards, culture medium was replaced and each half of one adrenal (n =2 for each) was incubated with medium (100 µl) containing either saline (basal) or different doses of ACTH (0.1 nM, 1 nM, 10 nM or 100 nM) for 6 h. CORT concentrations were calculated in relation to the weight of the respective explant [ng/ml/mg tissue] and expressed as CORT in % of basal set to 100 %. ■ basal; ■ ACTH; Data represent the mean + SEM.

2. 2 $(Bu)_2$ cAMP stimulation of adrenal explants *in vitro*

As explained in the general introduction, binding of ACTH to the G-protein-coupled Mc2r in adrenal tissue leads to activation of the adenylate cyclase and, hence, to increased levels of the second messenger cAMP, resulting in activation of cAMP-dependent PKA. This finally leads to an increased rate of GC synthesis (Gallo-Payet and Payet, 2003). For investigating the responsiveness of adrenal tissue to cAMP one possibility is the *in vitro* stimulation with membrane permeable cAMP analogues such as dibutyryl cAMP ($(Bu)_2$ cAMP) or 8-bromo-adenosine 3:5 cyclic monophosphate (8-Br-cAMP), which normally results in an increased GC response in case the cAMP pathway is not impaired at any point (Arai and Widmaier, 1993; Kan et al., 2003). For example, Arai et al. (1993) showed that stimulation of isolated rat adrenal cells with different concentrations of $(Bu)_2$ cAMP lead to a dose-dependent increase in CORT secretion compared with basal values.

In the present thesis, *in vitro* stimulation with $(Bu)_2$ cAMP was established to reveal possible differences in the CORT response of adrenal explants to cAMP between SHC and CSC mice and, therefore, to reveal possible CSC-induced changes in Mc2r signalling. This will be done in future studies as outlined in section 1 of the general discussion. For $(Bu)_2$ cAMP stimulation adrenal halves were treated exactly as described before. After pre-incubation for 4 h, culture medium was replaced and each half was incubated with medium (100 µl) either containing saline (basal) or different doses of $(Bu)_2$ cAMP (0.01 mM, 0.1 mM, 1 mM or 10 mM) for 2 h (37 °C, 95 % O_2, 5 % CO_2) (Fig. 43 A). Incubation time was adapted from Arai et al. (1993). In line with the Arai study, relative *in vitro* CORT secretion was significantly increased in response to 1 nM and 10 nM $(Bu)_2$ cAMP compared with basal values (Fig. 43 A). Moreover, I could show in a follow up experiment that a 30-min stimulation with 10 nM $(Bu)_2$ cAMP was already sufficient to induce a significantly increased CORT response compared with basal values (Fig. 43 B). In future

studies CORT response to 30-min $(Bu)_2$ cAMP will allow a better comparison with results obtained from the determination of adrenal cAMP content in response to 30 min ACTH *in vitro* (explained in detail below) in SHC and CSC mice. Together, this will provide more insight into possible CSC-induced changes in Mc2r sensitivity to ACTH (discussed in section 1 of the general discussion).

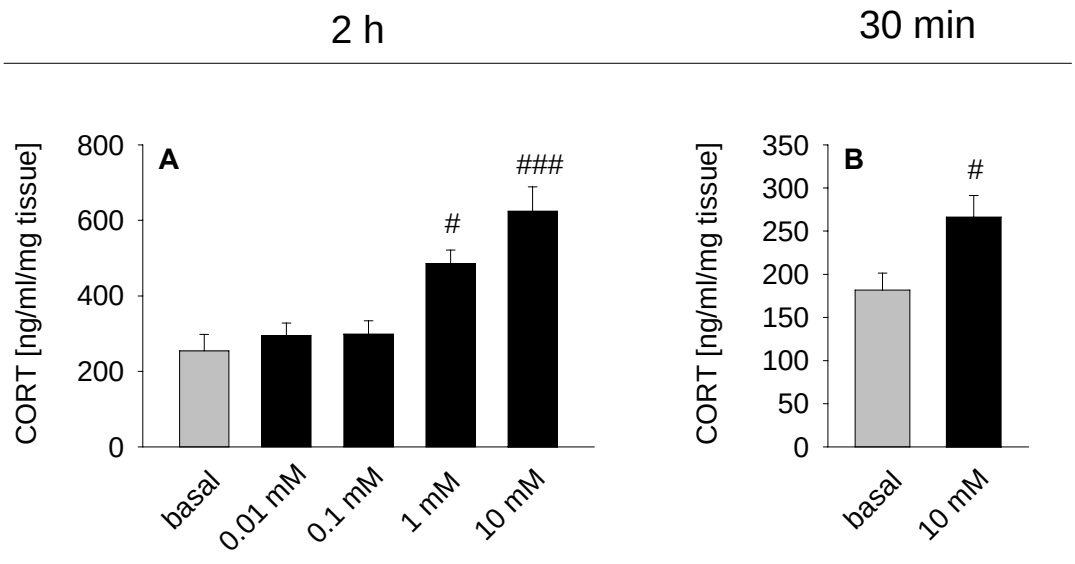

Figure 43: Establishment of $(Bu)_2$ cAMP stimulation of adrenal explants *in vitro*

Adrenal from naive mice were pruned of fat and cut into two halves. These adrenal halves were weighed, pre-incubated for 4 h in 200 µl DMEM/F-12. Afterwards, medium was replaced and each half of one adrenal was incubated for either 2 h (**A**; n = 6-7) or 30 min (**B**; n = 10-11) in the presence of either saline (basal) or different doses of $(Bu)_2$ cAMP. CORT concentrations [ng/ml/mg tissue] were determined in supernatants. ■ basal; ■ $(Bu)_2$ cAMP. One-way ANOVA followed by a Bonferroni *post hoc* test showed a significant effect of factor $(Bu)_2$ cAMP dose ($F_{4,31}$: 12.44; $p < 0.001$) with an increased CORT secretion in response to 1 mM and 10 nM $(Bu)_2$ cAMP compared with basal values following 2 h of stimulation (**A**). Moreover, 30 min of stimulation with 10 mM $(Bu)_2$ cAMP were also sufficient to induce an increased CORT response compared with basal values (**B**; Student's *t*-test). Data represent the mean + SEM. # represent $P < 0.05$, ### represent $P < 0.001$ *vs.* respective basal values.

2. 3 Determination of adrenal cAMP content in response to ACTH *in vitro*

As mentioned above, binding of ACTH to Mc2r in the adrenal glands results in increased levels of the second messenger cAMP trough activation of the adenylate cyclase. Thus, determination of endogenous cAMP in response to ACTH in adrenal tissue is often assessed to investigate the sensitivity of Mc2r to ACTH and the functionality of the pre-cAMP pathway (Dallman et al., 1978; Aguilera et al., 1996). In the present thesis, this method was established to reveal possible CSC-induced changes in Mc2r sensitivity to ACTH in future studies, as outlined in section 1 of the general discussion and, thus, to gain more insight into the mechanisms underlying the CSC-induced attenuation/ loss of ACTH responsiveness. In detail, adrenal explants of naive mice were stimulated with 100 nM ACTH for 30 min as described before. Afterwards, adrenal explants were immediately frozen in liquid nitrogen to inhibit cAMP degradation. For cAMP-extraction the frozen explants were homogenized in 5 % trichloroacetic acid (TCA) on ice. After centrifugation (4000 rpm, 10 min, 4 °C) the supernatant of each sample was collected and TCA was removed using water-saturated ether (five volumes ether and one volume sample were mixed and the top ether layer was discarded for three times). After removing the residual ether by heating (70 °C, 5 min), samples were analyzed for their cAMP content using a commercial available ELISA according to the manufacturer´s manual (Cyclic AMP EIA Kit, Cayman Chemical Company, USA). Adrenal cAMP content was calculated in relation to the weight of the respective explant [pmol/ml/mg tissue] (= relative cAMP content) to compensate for differences in the size of the explants. As shown in Figure 44, stimulation with 100 nM ACTH for 30 min resulted in a significantly increased cAMP content in both left and right adrenal explants compared with basal levels, which fits nicely to the literature (Dallman et al., 1978; Aguilera et al., 1996; Matthys et al., 1998).

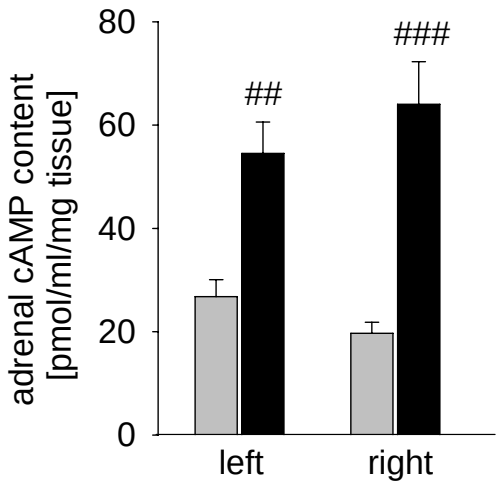

Figure 44: Establishment of cAMP-determination in adrenal explants following ACTH stimulation *in vitro*

Left and right adrenal glands from naive mice were pruned of fat and cut into two halves. These left (n = 4) and right (n = 5) adrenal halves were weighed and incubated for 30 min in the presence of either saline (basal) or 100 nM ACTH. After cAMP-extraction samples were analyzed for their cAMP content [pmol/ml/mg tissue] using commercial available ELISA. ■ basal; ■ ACTH. Statistics: Two-way ANOVA followed by a *post hoc* Bonferroni test when appropriate. A significant effect was found for factor ACTH ($F_{1,14}$: 40.26; $p < 0.001$) with increased cAMP levels in response to ACTH compared with basal values in both left and right adrenal explants. Data represent the mean + SEM. ## represent $P < 0.01$, ### represent $P < 0.001$ *vs.* respective basal values.

2. 4 Determination of *in vivo* adrenal CORT content

For investigating *in vivo* adrenal function under basal conditions or also in response to stressor exposure the determination of plasma CORT concentrations is a common method (Levine et al., 1991; Dhabhar et al., 1993; Levine, 2001). However, plasma CORT levels allow no conclusion about body side-specific differences in adrenal CORT synthesis and

secretion. As this was an important issue for the present dissertation, I established the determination of *in vivo* adrenal CORT content. In pre-experiments I tested first the optimal time interval between acute stressor exposure and adrenal CORT content assessment. As it was previously shown that plasma CORT levels are significantly increased at 5 and 30 min following termination of acute stressor exposure (5 min EPF exposure) (Nyuyki et al., 2012), adrenal CORT content was determined at these time points and compared with basal values. The extraction protocol was adapted from a previous study of Torres-Farfan and colleagues (2011). Briefly, adrenals were removed, pruned of fat, weighed separately, and immediately frozen in liquid nitrogen to avoid degradation of CORT in the tissue. Afterwards, adrenals were homogenized with 20 % ethanol in 1 x PBS on ice, centrifuged at 4 °C and the supernatants, containing the extracted CORT, were collected. CORT content was measured with ELISA and calculated in relation to the weight of the respective adrenal gland (= relative adrenal CORT content). As shown in Figure 45, the extraction procedure worked properly and I was able to demonstrate a strong increase in relative adrenal CORT content 5 min, but not 30 min, after termination of EPF compared with basal values. This suggests that adrenal CORT synthesis was already shut down 30 min following termination of EPF exposure and the synthesized CORT completely released into the blood (Sibley et al., 1980). Based on these results, I decided to determine adrenal CORT content 5 min following termination of EPF exposure to reveal possible differences in the CORT content of the left and right adrenal gland under basal conditions and in response to acute stressor exposure in SHC and CSC mice (see chapter 2).

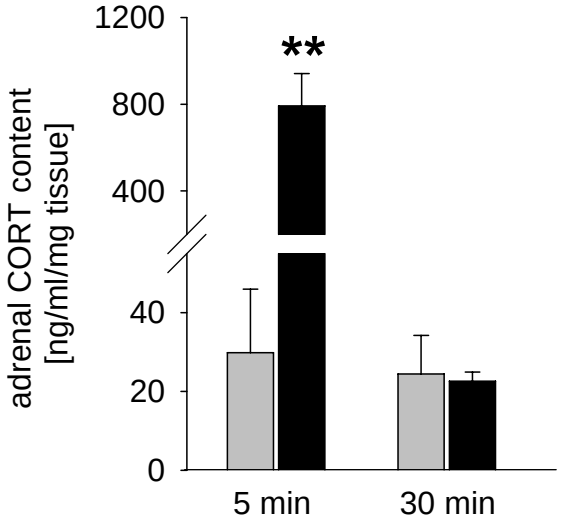

Figure 45: Establishment of adrenal CORT content determination in naive mice

Adrenal CORT content [ng/ml/mg tissue] was determined under basal conditions and 5 or 30 min after termination of EPF exposure (5 min) (n = 3 for each). Basal and EPF-induced values were compared using the Student's *t*-test. ■ basal; ■ EPF exposure; Data represent the mean + SEM. ** represent $P < 0.01$ *vs.* respective basal values.

2. 5 AChE histochemistry in adrenal cryo-sections

As already explained in the general introduction, synaptic transmission from preganglionic neurons to adrenal medullary chromaffin cells is mediated by the neurotransmitter ACh. ACh is thereby released from the pre-synaptic membrane into the synaptic cleft and subsequently binds to its respective receptors located on the chromaffin cells inducing the release of catecholamines into the bloodstream (de Diego et al., 2008). The serine protease AChE, present in the synaptic cleft, plays thereby an important role in the termination of signal transmission as it hydrolyzes the neurotransmitter ACh into acetate and choline, which are taken up again by the pre-synaptic neurone (Mizobe and Livett, 1980; Ferguson et al., 2003). Interestingly, the activity of AChE is often used to assess sympathetic innervation to the adrenal glands (Iwasa et al., 1999; Murabayashi et al., 2009) and can be

determined using the direct coloring thiocholine method established by Karnovsky and Roots (Karnovsky and Roots, 1964). AChE activity is thereby detected by providing an artificial substrate, namely acetylthiocholine iodide. In the present thesis AChE staining was established to reveal possible differences in the sympathetic innervation to the adrenal glands between SHC and CSC mice in future studies as outlined in section 1 of the general discussion. To obtain an optimal AChE staining in adrenal cryo-sections different fixatives (4 % paraformaldehyde, ethanol-glacial acetic acid, Zamboni's fixative) and different durations of fixation (5 min – 24 h) and staining (30 min – 240 min) were tested first. The best results were obtained using the following protocol. Briefly, adrenal cryo-sections were fixed in Zamboni's fixative (5 min, RT) and washed in 0.1 M Tris-maleate buffer (pH 5.0, containing 0.1 % Triton X-100). Afterwards, sections were stained in a solution containing 0.1 M Tris-maleate buffer (pH 5.0, containing 0.1 % Triton X-100), 0.4 M sodium citrate, 0.12 M copper sulphate, 0.16 M potassium ferricyanid and acetylthiocholine iodide (adapted from Schober et al. (1997)) for 1 hr at 37° C. The sections were then washed again in Tris-maleate buffer (pH 5.0, containing 0.1 % Triton X-100), counterstained with eosin (to enable differentiation between cortex and medulla), mounted with glycerine jelly and covered with a glass coverslip. During staining the following reaction occurs: the substrate acetylthiocholine iodide is cleaved by the AChE present in the medulla of the adrenal sections into thiocholine and acetate. Thiocholine in turn reduces then ferricyanide to ferrocyanide. The latter combines with copper ions to form the insoluble precipitate copper ferrocyanide, also termed Hatchett's Brown. It is important to mention that especially the fixation duration is of critical importance for a successful staining. More than 5 min of fixation dramatically decreased the amount of precipitate in the adrenal medulla probably due to inactivation of AChE. Moreover, staining for more than 1 h resulted in such an intense precipitate that possible differences between groups will be

hardly detectable. However, performance of the above described protocol resulted in a nice AChE staining in the adrenal medulla (Fig. 46 A/B) as also shown in other studies (Schober et al., 1997; Iwasa et al., 1999; Murabayashi et al., 2009), underpinning its adaptability.

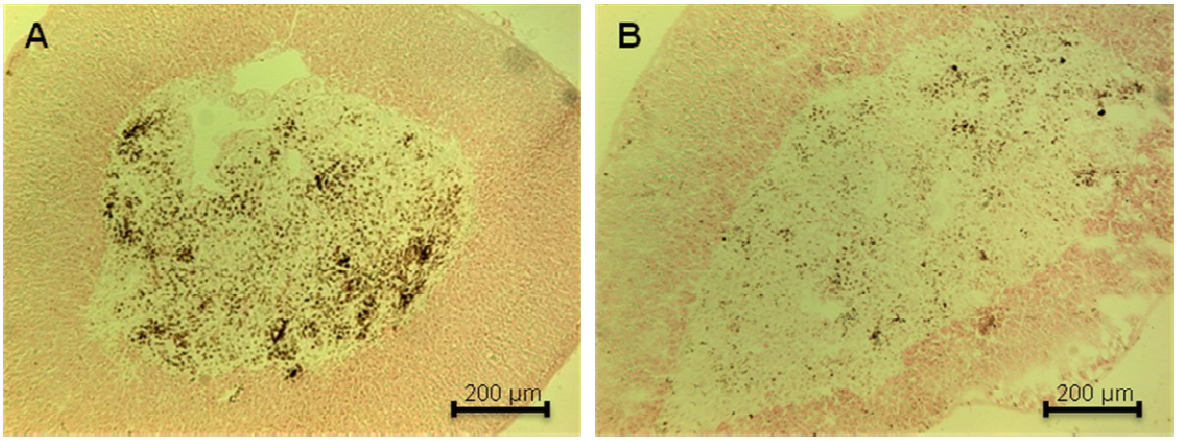

Figure 46: Representative images of AChE staining in adrenal cryo-sections. The precipitate Hatchett's Brown is visible in the adrenal medulla (**A**/**B**), indicative for AChE activity.

Summary in German

Zusammenfassung auf Deutsch

Chronischer psychosozialer Stress stellt in unserer heutigen Zeit einen hohen Risikofaktor dar, affektive sowie auch körperliche Erkrankungen zu entwickeln. So werden zum Beispiel chronisch entzündliche Darmerkrankungen, Erkrankungen des Herz-Kreislaufssystems, aber auch Angst- und Depressionserkrankungen mit chronischer psychosozialer Stressbelastung in Zusammenhang gebracht. Diese Erkrankungen wurden dabei über Jahrzehnte mit einer Hyperaktivität der Hypothalamus-Hypophysen-Nebennierenrinden (HPA)- Achse und somit chronisch erhöhten Konzentrationen an Glucocorticoiden (GC) im Organismus assoziiert. In den letzten Jahren wurde es jedoch zunehmend deutlicher, dass sich bestimmte durch chronischen Stress ausgelöste Erkrankungen mit diesem Phänomen nicht erklären lassen. Ein Mechanismus der dabei immer mehr in den Vordergrund rückt ist eine verminderte Wirkung der GC. Diese kann entweder durch eine verminderte Verfügbarkeit der GC, dem sogenannten Hypocortizismus, oder durch eine verminderte Sensitivität der Zielzellen gegenüber den GC (GC-Resistenz) vermittelt sein. So konnte zum Beispiel in klinischen Studien eine verminderte GC-Wirkung in Patienten mit chronischer Stressbelastung gezeigt werden. Darüber hinaus wurde eine verminderte GC-Wirkung auch in Patienten mit Erkrankungen, welche mit einer chronischen Stressbelastung assoziiert sind, nachgewiesen. Beispiele hierfür wären das Burnout-Syndrom, das chronische Erschöpfungssyndrom oder auch Fibromyalgie. Der genaue Mechanismus dieser stress-induzierten Verminderung der GC-Wirkung und deren Effekte auf die Pathogenese dieser Krankheiten sind jedoch bis heute weitgehend ungeklärt. Eine Möglichkeit um mehr Einsicht in diese Mechanismen zu erhalten ist der Einsatz geeigneter Tiermodelle. Ein Beispiel hierfür wäre die in der vorliegenden Arbeit verwendete chronisch-subordinierte Koloniehaltung (chronic subordinate colony housing (CSC)), ein validiertes und klinisch-relevantes Modell um

chronischen psychosozialen Stress bei männlichen Mäusen zu induzieren. Im CSC-Modell werden dazu 4 männliche C57BL/6-Mäuse gleichen Alters zusammen mit einem älteren, schwereren und aggressiveren Männchen, dem sogenannten Resident, für 19 Tage in einer Kolonie gehalten. Der Resident nimmt dabei die dominante und die 4 Versuchsmäuse eine subordinierte Position ein. Es konnte bereits gezeigt werden, dass die CSC-Exposition zu einer veränderten Funktionalität der HPA-Achse führt, was dem Anschein nach durch Veränderungen auf Ebene der Nebenniere vermittelt wird. So führte die CSC-Exposition zu einer Zunahme des Nebennierengewichts, ein Effekt welcher bereits nach 24 Stunden sichtbar war. Die basale Corticosteron (CORT)-Konzentration im Plasma in der inaktiven Phase der Tiere war jedoch nur in den ersten 24 Stunden der CSC-Exposition erhöht und ging dann auf Werte der ungestressten Kontrollen (single-housed control (SHC)) zurück. Weiterhin waren die CSC-Mäuse nach 19-tägiger Exposition nicht mehr in der Lage den circadianen Anstieg im Plasma CORT zu bewältigen, was zu einer erniedrigten basalen CORT-Konzentration im Vergleich zu SHC-Mäusen und somit zu einem basalen Hypocortizismus in der aktiven Phase führte. Interessanterweise waren die basalen ACTH-Werte im Plasma gleichzeitig unverändert. Diese Ergebnisse deuten auf eine CSC-induzierte Dysfunktion der Nebennieren hin, eine Annahme welche durch eine verminderte CORT-Sekretion isolierter Nebennierenzellen auf ACTH *in vitro* verstärkt werden konnte.

Zusätzlich zu dem stress-induzierten basalen Hypocortizismus konnte in den CSC-Mäusen auch eine verminderte Sensitivität der Zielzellen auf GC festgestellt werden. So zeigten isolierte und mit Lipopolysacchariden stimulierte Milzzellen, sowie auch isolierte Th2-Zellen aus peripherem Lymphknotengewebe eine verminderte Sensitivität auf verschiedene physiologische und pharmakologische GC-Konzentrationen. Diese Ergebnisse deuten auf eine allgemein verminderte GC-Wirkung in CSC-Mäusen hin, eine Auswirkung welche auch bei Menschen mit anhaltender Stressbelastung beobachtbar ist.

Neben den oben genannten Effekten konnte ebenfalls gezeigt werden, dass eine 19-tägige CSC-Exposition in Verhaltensänderungen und in Veränderungen von immunologischen Parametern resultiert. So zeigten CSC-Mäuse eine erhöhte Ängstlichkeit, welche in verschiedensten Verhaltenstests bestimmt werden konnte. Immunologische Veränderungen betreffend zeigten CSC-Mäuse neben einer Größenzunahme der Thymusdrüse, eine spontane Entzündung des Colons. Darüber hinaus entwickelten diese im Vergleich zu SHC-Mäusen auch eine viel stärkere Dextran-Sodium-Sulfat-induzierte Colitis.

Zusammenfassend ist also zu sagen, dass die 19-tägige CSC-Exposition in einem basalen Hypocortizismus in der aktiven Phase der Mäuse, sowie in einer verminderten GC-Sensitivität der Zielzellen und somit in einer allgemein verminderten GC-Wirkung resultiert. Zusätzlich führt die CSC-Exposition zu einer erhöhten Ängstlichkeit und einem erhöhtem Entzündungsstatus.

Wie bereits erwähnt, deuten immer mehr humane Studien darauf hin, dass eine verminderte GC-Wirkung als Resultat chronischer Stressbelastung an der Pathogenese verschiedenster Erkrankungen beteiligt ist. Somit stellt das CSC-Paradigma ein geeignetes und klinisch-relevantes Tiermodell dar, um mehr Einblick in die zugrundeliegenden Mechanismen der stress-induzierten Verminderung der GC-Wirkung und deren Auswirkungen auf den Organismus zu erhalten.

Der Fokus der vorliegenden Arbeit lag dabei vor allem auf möglichen CSC-induzierten Veränderungen auf Ebene der Nebennieren mit dem Ziel mehr über die Mechanismen zu erfahren, welche dem stress-induzierten Hypocortizismus zugrundeliegen.

Um zunächst die CSC-induzierte Verminderung der ACTH-Reaktivität der Nebennieren *in vitro* bestätigen zu können, wurde die *in vitro*-Stimulation mit verschiedenen physiologischen (0.0022, 0.01, 0.15 nM) Konzentrationen, sowie auch einer

pharmakologischen (100 nM) Konzentration an ACTH wiederholt. Die physiologischen Konzentrationen entsprachen dabei den ACTH-Werten von männlichen C57BL/6-Mäusen im Plasma unter basalen Bedingungen in der inaktiven Phase (0.0022 nM), der aktiven Phase (0.01 nM), oder nach akuter Stressexposition (0.15 nM). Darüber hinaus wurden diese Stimulationen in Nebennierenexplants (Nebennierenhälften mit kortikalem und medullärem Anteil) und nicht wie in vorhergehenden Experimenten in isolierten Nebennierenzellen durchgeführt. Zell-Zell-Kontakte sowie auch die Kommunikation zwischen kortikalen und medullären Zellen spielen eine sehr wichtige Rolle für die Sensitivität der Nebennierenrindenzellen gegenüber ACTH. Zwei Vorraussetzungen, welche in einer Zellsuspension nicht mehr gegeben sind. Im Vergleich zu Kontrolltieren zeigte sich, dass die CORT-Sekretion der Nebennierenexplants von CSC-Mäusen auf eine pharmakologische ACTH-Konzentration (100 nM) signifikant vermindert war. Darüber hinaus zeigten die CSC-Explants überhaupt keine Reaktion mehr auf physiologische ACTH-Konzentrationen, die CORT-Sekretion auf ACTH war also im Vergleich zu basalen Werten (Stimulation mit Saline) nicht erhöht. Diese Ergebnisse deuten in der Tat auf eine Insuffizienz der Nebennieren nach 19-tägiger CSC-Exposition hin. Interessanterweise konnte in der vorliegenden Arbeit jedoch auch gezeigt werden, dass die Nebennieren der chronisch gestressten Mäuse auf einen akuten heterotypischen Stressor, also einen neuen unbekannten Stressor, in diesem Falle eine 5-minütige Exposition auf einer erhöhten Plattform, reagieren konnten. So zeigten SHC- als auch CSC-Mäuse im Vergleich zu basalen Werten signifikant erhöhte Konzentrationen an CORT im Plasma sowie einen signifikant erhöhten Gehalt an CORT in den Nebennieren. Diese Effekte waren darüber hinaus in den CSC-Mäusen deutlich stärker ausgeprägt und sprechen gegen eine CSC-induzierte Nebenniereninsuffizienz. Zusammen mit der CSC-induzierten Erhöhung des Nebennierengewichtes, welche der Wahrscheinlichkeit nach durch Proliferation der

Nebennierenzellen vermittelt ist, deuten diese Ergebnisse vielmehr an, dass der Verlust der ACTH-Reaktivität der Nebennieren eine Adaptation auf den homotypischen Stressor der 19-tägigen CSC-Exposition, zum Schutz vor den negativen Konsequenzen einer lang anhaltenden Erhöhung von basalen CORT-Konzentrationen im Organismus, darstellt.

Wie bereits erwähnt, zeigte die Untersuchung der basalen CORT-Konzentration im Plasma in der inaktiven Phase der Mäuse, dass diese im Vergleich zu Kontrolltieren am Anfang der CSC-Exposition zwar erhöht war, jedoch bereits nach 48 Stunden auf SHC-Werte zurückging. Diese Veränderungen der CORT-Konzentration in der Anfangsphase der CSC-Exposition konnten in der vorliegenden Arbeit bestätigt werden. So war die basale CORT-Konzentration im Plasma im Vergleich zu Kontrolltieren zwar nach 10-stündiger, jedoch nicht nach 48-stündiger, CSC-Exposition erhöht. Es wurde deshalb in der vorliegenden Arbeit untersucht, wie diese Veränderungen im Plasma-CORT in der Anfangsphase der CSC-Exposition vermittelt werden. Dazu wurde zunächst nach 10- und 48-stündiger Exposition eine *in vitro*-Stimulation von Nebennierenexplants durchgeführt. Es zeigte sich, dass bei beiden Zeitpunkten die CORT-Sekretion der Explants von SHC- und CSC-Mäusen auf ACTH im Vergleich zu basalen Werten gleichermaßen anstieg. Dies deutet darauf hin, dass sowohl der Anstieg im Plasma-CORT in den ersten 10 Stunden, wie auch der Abfall auf SHC-Werte nach 48 Stunden nicht durch eine veränderte Sensitivität der Nebennierenzellen auf ACTH vermittelt sein kann. Weiterhin konnte auch ausgeschlossen werden, dass eine veränderte ACTH-Sekretion der Adenohypophyse involviert ist, da die ACTH-Konzentrationen im Plasma sowohl nach 10 als auch nach 48 Stunden in den SHC- und CSC-Tieren vergleichbar waren. Die Analyse des relativen (in Relation zum Körpergewicht) Nebennierengewichtes brachte zunächst auch keinen Hinweis auf die zugrundeliegenden Mechanismen, da dieses sowohl nach 10 als auch nach

48 Stunden CSC im Vergleich zu den Kontrolltieren signifikant erhöht war. Interessanterweise zeigte sich jedoch, dass der Anstieg im relativen Nebennierengewicht nach 48 Stunden lediglich durch einen Rückgang im Körpergewicht vermittelt wurde. So war das absolute Gewicht der Nebennieren zwar nach 10-stündiger CSC-Exposition erhöht, nach 48 Stunden konnten aber keine Unterschiede mehr zu Kontrolltieren festgestellt werden. Die beschriebenen Veränderungen im Plasma-CORT in der Anfangsphase der CSC-Exposition laufen also parallel zu den Veränderungen im Nebennierengewicht. Es scheint also, als würde der CORT-Anstieg im Plasma in den ersten 10 Stunden durch eine Zunahme des Nebennierengewichts und somit einer Zunahme an funktionellem Gewebe vermittelt werden. Eine unveränderte Menge an Cholesterinestern pro Fläche in der Nebennierenrinde und eine unveränderte relative mRNA Expression der Schlüsselenzyme der GC-Synthese in den Nebennieren (StAR, CYP11A1 und CYB11B1) nach 10 Stunden CSC verstärkt diese Annahme zusätzlich. Der Abfall der CORT-Konzentration im Plasma auf Kontrollwerte nach 48 Stunden scheint dagegen durch eine Abnahme des Nebennierengewichtes, und somit durch Abnahme an funktionellem Gewebe, vermittelt zu sein. Wie genau die Zunahme (Hyperplasie oder Hypertrophie der Zellen) und die darauffolgende Abnahme des Nebennierengewichtes (Atrophie oder Apoptose) in der Anfangsphase der CSC-Exposition zustande kommt, muss leider erst noch untersucht werden. Dennoch zeigen diese Ergebnisse zwei unterschiedliche Strategien der Adaptation in chronisch gestressten Tieren auf. Es scheint, als würde in der frühen Phase der CSC-Exposition einer lang anhaltenden Erhöhung an CORT im Plasma durch die Abnahme an funktionellem Nebennierengewebe entgegengewirkt. Nach 19 Tagen hingegen verhindert der Verlust der Reaktivität der Nebennierenzellen auf physiologische ACTH-Konzentrationen die Entwicklung eines basalen Hypercortizismus. Ab welchem Zeitpunkt sich dieser Verlust entwickelt konnte in

der vorliegenden Arbeit leider nicht genau bestimmt werden. Es deutet sich jedoch an, dass dies erst in der späten Phase der CSC-Exposition geschieht, genauer gesagt zwischen Tag 14 und Tag 19.

Es stellte sich natürlich auch die Frage, wie dieser Verlust der Nebennierenreaktivität auf ACTH in den chronisch gestressten Mäusen vermittelt ist. Um dies zu klären wurden in der vorliegenden Arbeit verschiedenste Parameter in der Nebenniere analysiert. So wurde die relative mRNA Expression und/ oder Proteinmenge des ACTH-Rezeptors, des ACTH-Rezeptor assoziierten Proteins MRAP und der Schlüsselenzyme der GC-Synthese (StAR, CYP11A1, CYP11B1, CYP11B2) nach 19-tägiger CSC-Exposition untersucht. Darüber hinaus wurde untersucht, ob Veränderungen in der Verfügbarkeit des Cholesterins, dem Vorläufermolekül der Steroidhormonsynthese, auftreten. So wurde die Menge an Lipidvesikeln pro Fläche in der Nebennierenrinde und die relative Proteinexpression des LDL-Rezeptors, des Scavenger-Rezeptors B1, der Hormon-sensitiven Lipase, und der HMG-CoA-Reduktase bestimmt. Die Untersuchung all dieser Parameter brachte jedoch keinen Hinweis auf den zugrundeliegenden Mechanismus, da diese im Vergleich zu den SHC-Mäusen nach 19-tägiger CSC-Exposition entweder unverändert oder sogar signifikant erhöht waren. Bedenkt man die Zunahme im Nebennierengewicht nach 19 Tagen CSC, so scheint es als wäre die Kapazität der Nebennieren CORT zu synthetisieren in den chronisch gestressten Tieren deutlich erhöht, was wiederum im Einklang mit der überhöhten adrenalen CORT-Sekretion bei akuter heterotypischer Stressbelastung in CSC-Mäusen steht. Weitere mögliche Ursachen für den Verlust der ACTH-Reaktivität der Nebennieren stellen eine verminderte Aktivität des ACTH-Rezeptors oder eine geringere Affinität desselben auf ACTH dar. Dies muss allerdings erst noch untersucht werden.

Wie bereits erwähnt, zeigen die Nebennieren der CSC-Mäuse am Tag 19 eine übermäßige CORT-Antwort auf einen akuten heterotypischen Stressor, obwohl die Reaktivität auf physiologische ACTH-Konzentrationen *in vitro* praktisch nicht mehr vorhanden ist. Es stellte sich die Frage, wie diese Unterschiede in der Reaktivität der Nebennieren auf ACTH *in vitro* und *in vivo* vermittelt sind. Ein wichtiger Punkt der hier unbedingt erwähnt werden muss, sind die zeitlichen Differenzen zwischen der *in vitro*-Stimulation und dem *in vivo*-Versuch. Bei der *in vitro*-Stimulation wurden die Nebennierenexplants für 6 Stunden mit 0.15 nM ACTH stimuliert, eine Konzentration vergleichbar mit den ACTH-Werten im Plasma nach akuter Stressbelastung. *In vivo* hingegen wurde die CORT-Konzentration im Plasma sowie der CORT-Content in den Nebennieren der Mäuse 5 Minuten nach Ende einer 5-minütigen Exposition auf einer erhöhten Plattform bestimmt. Es könnte also der Fall sein, dass die Nebennieren der CSC-Mäuse zu Beginn der *in vitro*-Stimulation noch auf ACTH reagieren, die Reaktivität jedoch mit der Zeit abnimmt. Diese Annahme konnte in der vorliegenden Arbeit jedoch nicht bestätigt werden. Eine 30-minütige *in vitro*-Stimulation der Nebennierenexplants von SHC- und CSC-Tieren mit 0.15 nM ACTH zeigte zwar eine erhöhte CORT-Sekretion der Explants von SHC-Tieren im Vergleich zu basalen Werten (mit Saline stimuliert), Explants der CSC-Mäuse zeigten allerdings keine Reaktion. Es scheint also, als wäre die ACTH-Reaktivität der Nebennieren *in vitro* nach 19 Tagen CSC generell beeinträchtigt.

Interessanterweise konnte auch gezeigt werden, dass die CORT-Konzentrationen im Plasma der CSC-Mäuse im Vergleich zu den SHC-Mäusen nach einem länger andauernden heterotypischen Stressor, in diesem Falle eine Kombination aus Immobilisierungs- und Schüttelstress für 4 Stunden, signifikant niedriger waren. Zusammengenommen deuten alle Ergebnisse auf einen zusätzlichen Faktor hin, der *in vivo* bei akuter heterotypischer Stressbelastung aktiviert oder sekretiert wird und die Reaktivität der Nebennieren auf

ACTH wieder herstellt oder selbst die CORT-Synthese in den Nebennieren anregt. Hält die heterotypische Stressbelastung jedoch länger an, so scheint es als wird dieser Faktor wieder inaktiviert bzw. abgebaut. Dies ermöglicht chronisch gestressten Tieren auf der einen Seite adäquat auf eine neue Situation reagieren zu können, auf der anderen Seite sind die CSC-Mäuse dadurch aber auch in der Lage bei einer länger andauernden Stressbelastung die CORT-Konzentration im Plasma schneller zu senken und somit den Organismus vor dauerhaft erhöhtem Plasma-CORT zu bewahren. Dabei ist es wahrscheinlicher, dass der noch unbekannte Faktor eher die ACTH-Reaktivität der Nebennieren wieder herstellt. So zeigten die Nebennierenexplants der CSC-Mäuse in den ersten 30 Minuten der *in vitro*-Stimulation zwar keine Reaktion auf physiologische ACTH-Konzentrationen, die CORT-Sekretion auf die pharmakologische ACTH-Konzentration von 100 nM war jedoch im Vergleich zu basalen Werten erhöht. Leider konnte dieser Faktor in der vorliegenden Arbeit nicht bestimmt werden. Eine Vermutung ist, dass die sympathische Innervierung der Nebennieren eine wichtige Rolle spielt. Dies muss allerdings erst noch genauer untersucht werden.

Auf den ersten Blick scheint der Verlust der Reaktivität der Nebennieren auf physiologische ACTH-Konzentrationen nach 19 Tagen CSC also adaptiv zu sein, da die chronisch gestressten Mäuse dadurch vor den negativen Konsequenzen dauerhaft erhöhter CORT-Konzentrationen im Plasma bewahrt werden, welche vermutlich auf Grund des Nebennierenwachstums auftreten würden. So scheinen die CSC-Mäuse vor affektiven und somatischen Krankheiten geschützt, welche mit einem basalen Hypercortizismus assoziiert sind, wie depressions-ähnliches Verhalten und das metabolische Syndrom. Dennoch ist festzustellen, dass die CSC-Mäuse, trotz der adaptiven Veränderungen auf Ebene der

Nebennieren, alles andere als gesund sind. So zeigen CSC-Mäuse sowohl eine erhöhte Ängstlichkeit als auch einen erhöhten Entzündungsstatus im Colon.

Wie bereits angedeutet, sind die basalen CORT-Konzentrationen im Plasma in der inaktiven Phase der CSC-Mäuse zwar vergleichbar mit den Werten der SHC-Mäuse, die CSC-Mäuse sind jedoch durch einen basalen Hypocortizismus in der aktiven Phase charakterisiert. Es scheint also, als würden die adaptiven Veränderungen am Ende in einer Überadaptation als Antwort auf die chronische Stressbelastung resultieren. So schützt der Verlust der adrenalen ACTH-Reaktivität die chronisch gestressten Tiere zwar vor den Auswirkungen dauerhaft erhöhter CORT-Konzentrationen, macht diese auf der anderen Seite aber anfälliger für Krankheiten, welche mit einer hypoactiven HPA-Achse assoziiert sind, wie zum Beispiel einem erhöhten Entzündungsstatus.

Abschließend ist zu bemerken, dass die Ergebnisse der vorliegenden Arbeit einen tieferen Einblick in die Veränderungen auf Ebene der Nebenniere bei chronischer psychosozialer Stressbelastung gewähren, welche an der Entwicklung eines basalen Hypocortizismus mitwirken. So tragen die vorliegenden Ergebnisse in C57BL/6-Mäusen dazu bei, das Verständnis über die zugrundeliegenden Mechanismen des stress-induzierten Hypocortizismus beim Menschen zu erweitern.

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Abbreviations

Abbreviations

A	Adrenergic
ACAT	Acyl CoA cholesterol acyltransferase
ACh	Acetylcholine
AChE	Acetylcholinesterase
ACTH	Adrenocorticotropic Hormone
ANOVA	Analysis Of Variance
ANP	Atrial Natriuretic Peptide
ANS	Autonomic Nervous System
ASP	Adrenal-Specific Protein
AVP	Arginin Vasopressin
$(Bu)_2$	Dibutyryl
Ca	Calcium
cAMP	cyclic Adenosine Monophosphate
cDNA	complemetary Deoxyribonucleic Acid
CO_2	Carbon Dioxide
CoA	Coenzyme A
CORT	Corticosterone
CREB	cAMP Response Element Binding Protein
CRH	Corticotropin Releasing Hormone
CRHR1	CRH Receptor type 1
CSC	Chronic Subordinate Colony housing
CVS	Chronic Variable Stress
CYP	Cytochrome P-450
CYP11A1	Cholesterol side-chain cleavage enzyme
CYP11B1	11β-Hydroxylase
CYP11B2	Aldosterone synthase
CYP17	17α-hydroxylase
CYP21A2	21α-hydroxylase
DAPI	4, 6-diamidino-2-phenylindole
DMEM	Dulbecco´s Modified Eagle Medium

DNA	Deoxyribonucleic Acid
DOPA	Dehydroxyphenylalanine
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
EPF	Elevated Platform
FAM	6-Carboxy-Fluorescein
G	Granule
GAPDH	Glyceralaldehyd-3-Phosphatdehydrogenase
GC	Glucocorticoid
GPCR	G-Protein-Coupled Receptors
GR	Glucocorticoid Receptor
h	hour
HDL	High-Density Lipoprotein
HEPES	2-(4-(2-Hydroxyethyl)-1-Piperazinyl)-Ethansulfonsäure
HMG-CoA	3-Hydroxy-3-Methylglutaryl Coenzyme A
HPA	Hypothalamo-Pituitary-Adrenal
HSL	Hormone-Sensitive Lipase
IBD	Inflammatory Bowel Disease
k_d	Dissociation constant
kDA	Kilo Dalton
LC	Locus Coeruleus
LDL	Low-Density Lipoprotein
LDL-R	Low-Density Lipoprotein-Receptor
M	Mitochondrion
Mc2r	Melanocortin-2-Receptor
min	minute
mM	millimolar
MR	Mineralocorticoid Receptor
MRAP	Melanocortin-2-Receptor Accessory Protein
mRNA	messenger Ribonucleic Acid
N	Nucleus
NA	Noradrenergic

NE	Noradrenaline
nM	nanomolar
NPY	Neuropeptide Y
PACAP	Pituitary-Adenylate-Cyclase Activating Peptide
PBS	Phosphate Buffered Saline
PKA	Proteinkinase A
PNMT	Phenylethanolamine-N-Methyltransferase
PTSD	Post-Traumatic Stress Disorder
PVN	Paraventricular Nucleus
qPCR	quantitative Polymerase Chain Reaction
RNA	Ribonuleic Acid
RT	Room Temperature
sec	second
SF-1	Steroidogenic Faktor 1
SHC	Single-Housed Control
SP	Substance P
SR-BI	Scavenger Receptor class B type I
StAR	Steroidogenic Acute Regulatory Protein
TAMRA	6-Carboxytetramethyl-Rhodamine
TBS	Tris Buffered Saline
TH	Tyrosine Hydroxylase
TSPO	Translocator Protein
V1b	Vasopressin 1b
VIP	Vasointestinal Peptide
ZF	Zona Fasciculata
ZG	Zona Glomerulosa
ZR	Zona Reticularis
3β -HSD	3β-Hydroxysteroid Dehydrogenase

CV, publications and awards

Curriculum vitae

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Publications

Schmidt D, Reber SO, Botteron C, Barth T, Peterlik D, Uschold N, Neumann ID, Männel DN, Lechner A. 2010. Chronic psychosocial stress drives systemic immune activation and the development of inflammatory Th cell responses. *Brain, Behavior, and Immunity*. 24:1097-1104

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Author's declaration – Eidesstattliche Erklärung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als 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.

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(Schmidt Nicole)