# Consequences of Chronic Psychosocial Stress in Male Wistar rats

## DISSERTATION

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# Dissertation

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Unter Anleitung von

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# Dedication

To our son: bundle of joy & treasure

*A'len*

*In adversity keep motivated, for often the best comes from difficulty*

*Norman Vincent Peale ‘The power of positive thinking’.*

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# Abstract

In humans, chronic stress is regarded as a major risk factor for the development of a variety of affective and somatic disorders, among them anxiety, depression, post traumatic stress disorder, inflammatory bowel disorders, chronic fatigue syndrome, chronic pelvic pain and fibromyalgia. In order to determine the exact underlying mechanisms and pharmacological treatment, a large number of models have been used to induce chronic stress in rodents. However, conventional rodent models of chronic stress including restraint, forced swim and inescapable footshock for example; are unnaturalistic, of time-limited exposure and do not consider the etiology of human stress-associated disorders, where the main source of stress stimuli is of social origin. Consequently, a greater amount of attention has been focused upon developing animal models that utilize more naturalistic experimental paradigms to model stress that is ethologically relevant to the model organism.

Accordingly, a clinically relevant animal model of chronic psychosocial stress (CPS); chronic subordinate colony housing (CSC) has recently been established and extensively characterized in our group. It consists of housing 4 experimental adult male mice together with a slightly larger and more dominant conspecific over 19 consecutive days, exchanging the dominant male weekly in order to prevent habituation. This consequently results in a reduced body weight gain, thymus atrophy and increased adrenal weight, long-lasting anxiety-like behaviour, and spontaneous colitis. Of particular importance to this thesis is the unchanged morning basal plasma

corticosterone, but a reduced adrenal response to corticotrophin (ACTH) *in vitro*. With respect to the growing use of genetic mouse models in general, and need the study *in vivo* responsiveness of the HPA axis especially with regards to the CSC model, one of the objectives of this thesis was to devise a technique by which blood samples can be repeatedly drawn from conscious mice under stress-free conditions. Therefore, a simplified jugular vein catheterization technique was established and validated. This thesis showed that repeated blood sampling is possible in mice 24 h after surgery, and that corticosterone concentrations in repeated 100-µl venous blood samples were similar to trunk blood samples both under basal conditions and after stressor exposure collected at identical time points (at 5, 15 and 60 min). Further, anxiety-related behavior, as assessed on the elevated plus-maze 3 to 4 days after surgery did not differ between catheterized and non-catheterized mice.

In parallel, as the main objective of this thesis, the CSC model was extended to male Wistar rats in order to i), investigate if the reported behavioural, physiological, immunological and neuroendocrine changes accompanying CSC in mice are species specific and ii), characterize in substantial detail, *in vivo* neuroendocrine changes following CSC which till moment have not been possible in mice e.g, plasma ACTH. Consistent with the results in mice, 19 days of CSC exposure in rats resulted in a decreased body weight gain and absolute thymus mass, mild colonic barrier defects and intestinal immune activation. Moreover, no changes in depressive-like behaviour or social preference were observed. Most importantly, CSC rats showed an increased plasma corticosterone response to an acute heterotypic stressor, despite

displaying similar basal levels and similar basal and stressor-induced plasma corticotrophin (ACTH) levels in samples repeatedly collected via a chronically implanted jugular vein catheter. In contrast to CSC mice, anxiety-related behaviour and absolute adrenal weights remained unchanged in CSC rats. In this regard, risk factors that could influence the susceptibility to CSC were further investigated.

There is a plethora of literature addressing the involvement of adverse early life events and genetic predisposition as risk factors in the vulnerability to chronic stress in adulthood. Therefore, this thesis made use the high (HAB) and low (LAB) anxiety-related behaviour animal models of anxiety, as well as prenatally stressed male Wistar offspring. Specifically, HAB and LAB rats also show robust differences in depressive-like behaviour, neurobiochemical responses and neurogenetic constitution. It was therefore revealed that after 19 days of CSC, prenatally stressed rats gained less body weight and had a higher histological damage score. No changes in adrenal weight, basal plasma corticosterone levels, as well as anxiety-related behaviour were observed. The present thesis could not reveal any differences between HAB and LAB rats in their response to CSC in adulthood, but both lines were independently affected by CSC. Anxiety-related behaviour tested on the light-dark box after CSC was not altered, thus confirming the results in non-selected rats. CSC induced a decrease in body weight gain and thymus mass, but no changes in adrenal mass, basal plasma corticosterone and colonic morphology in both HAB and LAB rats.

In a nutshell, this thesis demonstrates that chronic jugular vein catheterization and stress-free repeated blood sampling is possible in conscious mice using our relatively simple catheter and sampling technique. It also establishes the CSC paradigm as an adequate model of CPS in male rats. Consistent with mice data, our data from repeated blood sampling supports the initial hypothesis that adrenal hyper-responsiveness to ACTH during acute heterotypic stressors represents despite unchanged basal plasma corticosterone levels after CSC represents a general adaptation, which enables a chronically-stressed animal to adequately respond to novel challenges. We further reveal that early life stress increases the vulnerability to chronic psychosocial stressors during adulthood, with respect to inflammatory disorders like colitis and that genetic predisposition, in terms of genetically determined differences in innate anxiety do not shape susceptibility to chronic psychosocial stressors during adulthood.

# Chapter 1

# General introduction

1 Introduction
1.1 Stress: a recapitulation of its concepts
1.1.1 The hypothalamo-pituitary-adrenal axis
1.1.2 The sympathetic nervous system
1.2 Acute versus chronic stress
1.3 Monitoring stress hormones
1.4 Chronic psychosocial stress
1.4.1 Importance of psychosocial stress models
1.5 Risk factors shaping adult stress vulnerability
1.5.1 Early life experiences
1.5.2 Genetic predisposition
1.6 Aims and outline of the present thesis

# 1. Introduction

In humans, psychosocial stress arises due to, but is not limited to, exposure to adverse or tragic life events in the social surrounding. It can also be a result of the inability to appropriately manage day-to-day demands when they go beyond coping resources. In view of the huge socio-economic and cultural differences which exist between given populations for example; the developing and developed countries, the perception of psychosocial stress may widely differ between these populations. Independent of these differences and irrespective of the population, chronic exposure to psychosocial stressors leads to mal-adaptations in various physiological and immunological systems. Chronic psychosocial stress (CPS) has been shown to raise blood pressure, suppress immune functions, increase the risk of: sleep disturbances, obesity, heart attack, stroke, infertility, and the development of inflammatory bowel disorders, just to name a few possible consequences. Long-term stress can even rewire the brain, resulting in an increased vulnerability to psychopathologies.

Due to the complex pathological consequences of CPS, its aetiology has been at the center of chronic stress research in the last 2 decades. In this context, genetic predisposition and environmental events are considered as risk factors modulating an individual's vulnerability to CPS.

The most common and acceptable approach in the study of CPS-induced and disease-related consequences and their underlying mechanisms has been the use of relevant animal models of CPS. Therefore, a variety of animal models for CPS have been established in the last decade including;

the social defeat paradigm, visible burrow system and the chronic subordinate colony housing paradigm. The reliability of an animal model is based on: i), face validity (display certain symptoms characteristic of a particular human clinical condition) ii), predictive validity (show reduced symptoms when treated with a clinically efficacious drug) and iii), construct validity (identical neurobiological mechanisms underlying symptomatology and psychological causes). In this thesis, the recently established model for CPS in mice has been extended to male Wistar rats, as this allows various additional and detailed physiological measures which are not possible in mice.

In this chapter, the reader will be introduced to the basic concept of stress, the main pathways activated in response to acute stressors as well as to the techniques applied to study neuroendocrine stress responses. Moreover, I will differentiate between acute versus chronic stress and outline the importance of social stressors in particular psychosocial stress as well as models used to study CPS-induced consequences. Furthermore, factors influencing the vulnerability to chronic stress such as adverse early life stress and genetic predisposition are also presented as a prerequisite to understanding my work.

## 1.1 Stress: a recapitulation of its concepts

Over the years, various concepts have been put forward in an attempt to understand an organism's response to environmental changes and challenges. As early as the 19$^{th}$ century, Claude Bernard (1813 - 1878), a French physiologist, developed the concept of '*milieu intérieur*" (translated

as: internal environment). It is constituted mainly of circulating body fluids, whose constancy is essential for the functional integrity of an organism independent of its external environment. Walter Cannon (1871 – 1945) then later coined the term 'homeostasis' to describe the maintenance of this internal environment within an acceptable physiological range. He was the first to discuss that the disruption of homeostasis due to perceived threats leads to the rapid 'flight or fight' response, associated with activation of the sympathetic nervous system (SNS). Then in the early half of the 20$^{th}$ century, the term ''stress'' was properly addressed by Hans Selye (1907 - 1982). He defined stress as the ''non specific response of the body to any demand'' [1] and further elaborated that any agent that produces stress at anytime is known as a stressor [2]. The stress response is therefore the set of physiological and emotional changes the body makes in response to a threat or stressor. This therefore throws the body out of its normal balance and as a consequence, the SNS and the hypothalamo-pituitary-adrenal (HPA) axis is activated [for review see 3].

Recent comprehensive theoretical outlines approach the concept of stress with a strong commonality among them. They share an interest in the process in which environmental demands tax or exceed the adaptive capacity of an organism, resulting in psychological and physiological changes that may place persons at risk for disease. Therefore, when an organism has appraised a situation as threatening or otherwise demanding and is uncertain of its capability of coping with this particular situation, stress is experienced [4]. In one of the latest premises, stress is viewed as a consciously or

unconsciously sensed threat to homeostasis [5, 6], which can either be internal or external in nature. The body, therefore, responds to these threats, adapting in an attempt to maintain the constancy of its internal environment [7]. This response involves activation of the central and autonomic nervous systems as well as endocrine systems, resulting in complex physiological and behavioural outcomes. The magnitude of activation depends on the specific challenge to homeostasis, the animal's perception of the stressor and the perceived ability to cope with it [8].

Measuring parameters that describe the activity of the HPA axis [9, 10] and of the SNS [11, 12] are commonly employed to assess stressor response levels of an animal, combined with the observation of behavioural alterations especially in the context of emotionality

### *1.1.1 The hypothalamo-pituitary-adrenal (HPA) axis*

The HPA axis as the central stress regulatory unit of animals links the central nervous system (CNS) and endocrine system. A tonic activation of the HPA axis occurs in a diurnal pattern, resulting in diurnal changes in plasma glucocorticoids (GC). The fine-tuned interaction between the hypothalamus, pituitary and adrenal gland, modulated by neuronal inputs from mainly limbic brain regions, determines the basal and stress-induced activity of the HPA axis. Under stress conditions, corticotrophin-releasing hormone (CRH) is secreted from the hypothalamus, which in turn provokes adrenocorticotrophic hormone (ACTH) release from the anterior pituitary gland. ACTH then triggers GC (cortisol in humans and corticosterone in rodents) release from the adrenal cortex into systemic circulation (figure 1).

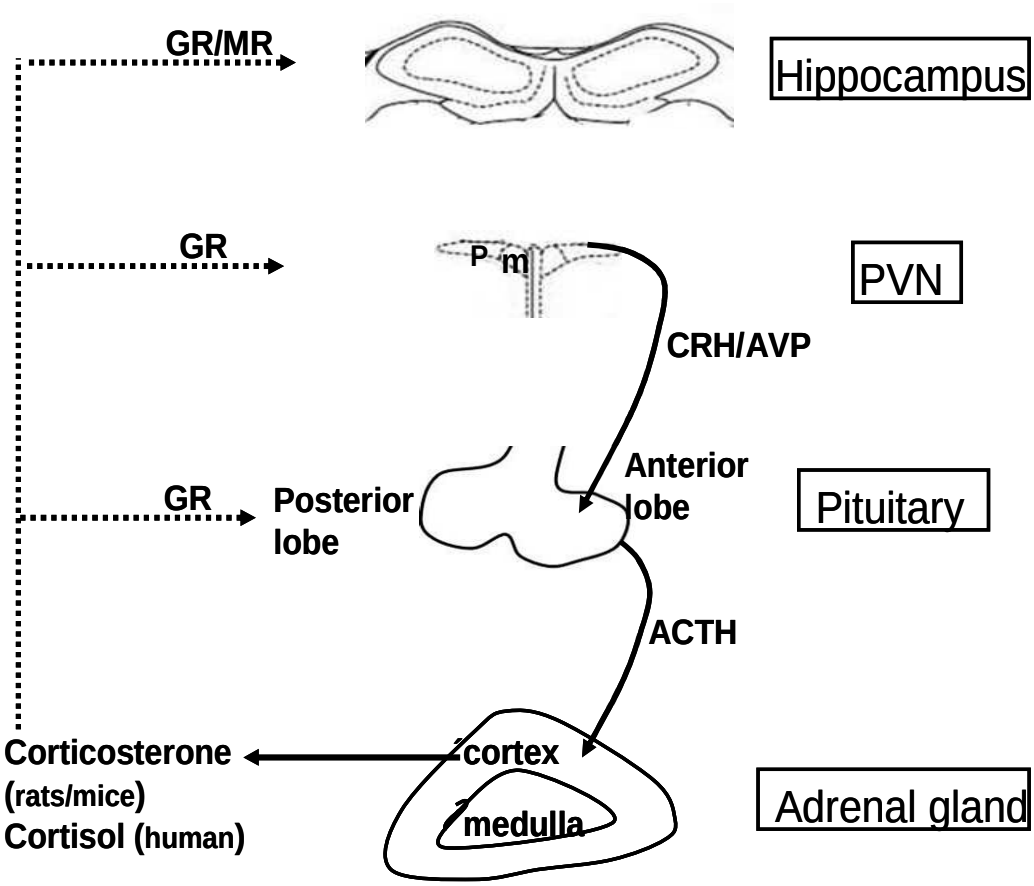


Figure 1: A schematic representation of the HPA axis. Corticotrophin releasing hormone (CRH) is synthesized in the parvocellular nuclei (p) of the hypothalamic paraventricular nucleus (PVN). Released into the portal circulation, it binds to receptors in the corticotroph cells of the anterior pituitary and initiates the synthesis and release of corticotrophin (ACTH). ACTH is released into systemic circulation and binds to its receptors in the adrenal cortex, leading to the synthesis and release of glucocorticoids (GC). Arginine vasopressin (AVP) contributes to the stimulation of the HPA axis in synergy with CRH especially under conditions of chronic stress. At various levels in the brain, GC bind to their receptors to shut down the activity of the HPA axis via a negative feedback reaction (dashed lines). m; magnocellular PVN

In more detail, the neurosecretory cells within the parvocellular subdivision of the hypothalamic paraventricluar nucleus (PVN) in response to stress

synthesize and release the 41-amino acid peptide CRH and the nonapeptide arginine vasopressin (AVP; figure 1). These peptides are then transported via axonal projections into the external zone of the median eminence where CRH and AVP are released into the hypophyseal portal circulation which lies in close proximity to the anterior pituitary. Within the anterior pituitary, both peptides bind to their respective receptors [13-15] on the plasma membrane of the pituitary corticotroph cells and provoke synthesis and release of ACTH through activation of the adenylate cyclase-cAMP-dependent protein kinase system [15, 16]. Important to mention is the fact that AVP acts synergistically with CRH to amplify the effects of CRH [17], especially under chronic stress situations [7, 18, 19].

Primarily, the corticotroph cells synthesize the large precursor protein proopiomelanocortin (POMC). POMC is enzymatically cleaved in a tissue- and species-specific manner into 2 peptides; beta-lipotrophic pituitary hormone (ß-LPH: an endogenous opioid) and ACTH, both released into systemic circulation. Whilst the bioactive products of the former (gamma-LPH and, especially ß-endorphin) exert widespread analgesic and euphoric effects in the brain, the later binds to its receptors in the adrenal cortex (melanocortin 2 receptors). Once bound, it stimulates the synthesis and release of GCs into systemic circulation via an intracellular chain of reactions. The steroids are synthesized from cholesterol stores (cholesterol esters), which are made locally from acetate or transported into the cell by lipoproteins [20]. About 95 % of cortisol released into systemic circulation predominantly binds to proteins called cortisol binding proteins, rendering it inactive, whereas 5 % circulates as unbound, biologically active ‘free’ cortisol [21]. A number of

functions are attributed to GC: they play a critical role in metabolism by mobilizing glucose and lipid resources to provide energy [22, 23], regulate the immune system, the cardiovascular system, as well as the SNS by stimulating catecholamine synthesis and modulate affective and cognitive functions [21, 24].

In addition to stress-induced alteration of the HPA axis, its activity shows a clear circadian pattern principally controlled by the hypothalamic suprachiasmatic nucleus, which is the central circadian clock in mammals. The HPA axis, in turn, coordinates circadian events such as sleep/wake cycle and food intake. This is possible through binding of GC to glucocorticoid receptors (GR) distributed throughout peripheral clock organs (spleen, liver, heart, adrenal glands). Moreover, the lipophylic nature of GC permits them to cross the blood brain barrier to bind to their central receptors in different brain regions and to exert profound effects on the HPA axis and other brain systems. Two types of GR exist in the brain: the mineralocorticoid (MR) and the glucocorticoid (GR) receptors. MR, with an approximately 10-fold higher affinity than GR [25] are found in the dentate gyrus, lateral septal nuclei, pyramidal neurons and the amygdala, as well as limbic brain regions especially the hippocampus [26, 27]. GR are distributed in brain regions involved in the central regulation of the stress response such as the hippocampus, hypothalamus, pituitary as well as the amygdala and various brain stem nuclei [28]. At low concentrations, GC bind primarily to MR which are therefore believed to be stimulated under basal conditions [29]. On the contrary, high levels of corticosterone around the circadian peak and during stress [25] bind to GR and regulate the feedback functions of the HPA axis as

well as appropriate behavioural responses [29, 30]. Corticosterone therefore mediates recovery from stress response and prepares the body for the next encounter by regulating homeostatic disturbance.

Especially at the level of the pituitary, hypothalamus and hippocampus, corticosterone binds to GR, as well as MR, shutting down the activity of the HPA axis (see figure 1) in a mechanism known as the negative feedback [31, 32]. Moreover, several feedback reactions occur in the various organs of the HPA axis to adequately control its overall functioning as illustrated in figure 1 [33].

In laboratory animals, plasma ACTH and corticosterone can be monitored in plasma using various invasive experimental approaches (see 1.3).

### *1.1.2 The Sympathetic Nervous System (SNS)*

The second major stress system of the body is the SNS. It is a branch of the autonomic nervous system, composed of nerves originating in the thoracic and lumbar regions of the spinal cord (figure 2). Presynaptic neurons originating from the spinal cord synapse with postsynaptic neurons at the level of the synaptic trunk or abdominal cavity ganglia, which in turn extends to the target organs including adrenal glands, eyes, kidney etc. Its primary function is to mobilize energy resources in the body, preparing it for the flight or fight response in stress situations. Moreover, it accelerates heart rate, constricts blood vessels and increases blood flow, enabling the body to rise to emergency demands.

In acute stress situations, catecholamines are the main executors of the flight or fight response. The major neurotransmitter secreted by the peripheral

postganglionic sympathetic fibres, at the level of the effector cell synapse is norepinephrine (NE). On the other hand, the adrenal medulla which consists of a mass of sympathetic postganglionic neurons provides most of the systemic circulating epinephrine and NE to a lesser extent [34]. Synthesis of both catecholamines begins with the amino acid L-tyrosine, which undergoes a 4 to 5-step enzymatic reaction to produce NE and epinephrine respectively. When bound to specific receptors on the surface of the target cells, they exert various physiological reactions like increased heart rate, blood glucose and pressure.

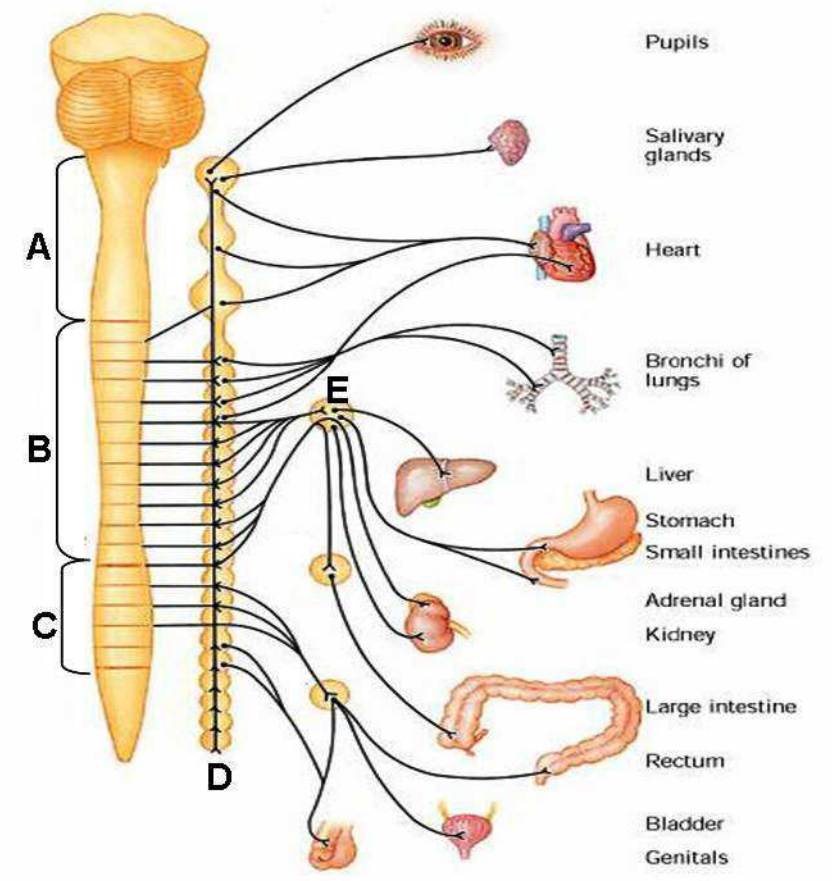


Figure 2: The sympathetic nervous system (SNS). Sympathetic innervation of various effector organs in the body. Preganlgionic neurons originating in the thoracic (B) and lumbar (C) divisions of the spinal cord synapse with postsynaptic neurons at the level of the synaptic trunk (D) or abdominal cavity ganglia (E), and postsynaptic neurons innervate various

effector organs. A: cervical division of the spinal cord. Adapted from http://www.ehs.net/2231/pdf/autonomic.pdf.

Another important system of the autonomic arm, which mediates stress responses, is the locus coeroleus/norepinephrine (LC/NE) system. It is composed of the LC and cell groups of the medulla and pons (synthesizing NE). It is the central arousal system of the sympathetic division in the brain stem and spinal cord. Reciprocal neural projections exist between the hypothalamic CRH system and the LC/NE system (figure 3). As CRH receptors extend to limbic brain areas and the LC [35], both systems act as effectors of the stress response. To this effect, they participate in a positive feedback loop, whereby activation of one tends to activate the other (figure 3). Efferent preganglionic fibres of the LC whose cell bodies lie in the intermediolateral column of the spinal cord innervate peripheral organs like the liver, kidney, heart, adrenal glands etc. Basically, activation of the LC/NE system enhances arousal and increases vigilance [36], memory and adaptive responses to stress [37].

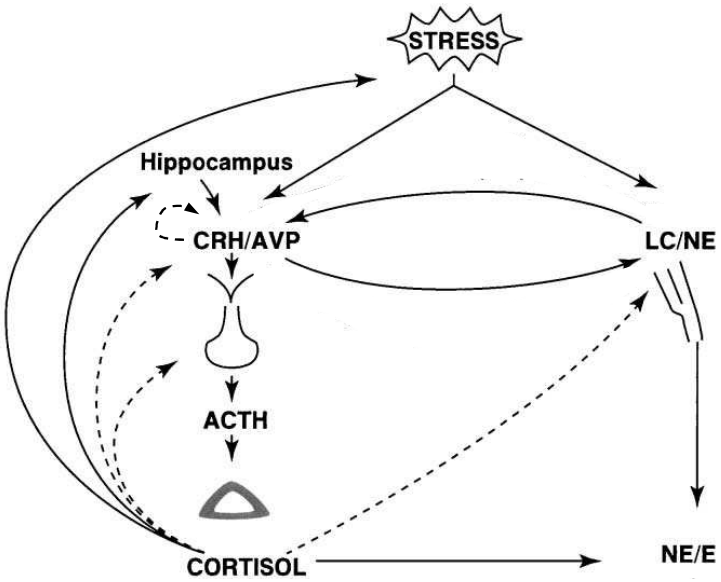


Figure 3: Interaction between the central and peripheral components of the stress system. Reciprocal innervation and activation of the CRH/AVP (Corticotrophin releasing hormone/Arginine vasopressin) neurons and the central catecholaminergic neurons of the LC/NE (Locus coeruleus/Norepinephrine) system. Solid lines represent activation, whereas broken lines represent inhibition. [Adapted from 34]

## 1.2 Acute versus chronic stress

Stress, according to Dhabhar and McEwen [38] is a constellation of events, which begins with a stimulus (stressor) that precipitates a reaction in the brain (stress perception), which subsequently results in the activation of certain physiologic systems in the body (stress response) i.e., the SNS and HPA axis. In acute stress situations, these physiological responses lead to protection and promote adaptation of the organism to stressors in order to maintain homeostasis (allostasis). However, overactivity of these systems in chronic stress situations leads to an accumulation of allostatic load, thereby

overexposing the body to increased concentrations of mediators of neural, endocrine, and immune stress which can have adverse effects on various organ systems, leading to disease [6, 39, 40]. Therefore, the duration of stressor exposure is an important characteristic in distinguishing stressors. In this line, stressors applied from sec to hours are termed acute, whereas chronic stress situations are defined by; i) frequent or repeated exposure to different acute stressors (heterotypic) and ii), continuous exposure to a given stressful condition (homotoypic) i.e., over days to months [38]. Moreover, stressors can also differ in intensity. Quantification of peak concentrations of stress hormones, neurotransmitters, and other physiological changes (increased heart rate, blood pressure and stroke volume), and the length of time these changes persist further gives a clue on the type of stressor. In this respect, increased activity of the physiologic systems is maintained during, and for several hours (~ 6) after the stressor exposure is terminated in response to acute stimuli [41, 42]. On the other hand, the stress response in chronic stressful situations may persist for longer periods after the stressor has subsided, or may be repeatedly activated. As a consequence, the organism is exposed to increased levels of stress hormones [43] and stands a higher risk for disease susceptibility.

In humans, continuous exposure to stressful stimuli is an acknowledged risk factor for a variety of affective and somatic disorders (Kessler, 1997) including anxiety- and depression-related disorders [44, 45], cardiovascular diseases [46], chronic fatigue syndrome [47], bronchial asthma [48], fibromyalgia [49], inflammatory bowel diseases [50], as well as frequent diarrhoea or constipation, chronic pelvic and abdominal pain [51, 52].

Moreover, a suppression of the immune system has also been reported [53], and dys-regulation of the HPA axis [54].

There are multiple animal models mimicking acute and chronic stress conditions, respectively. Acute stressors for laboratory rats and mice include for example; brief exposure to emotional/psychological (novel environment, noise, light, smell, predators) or physical (restraint, forced swimming, shock, cold, swimming, and social conflict) stressors, whereas continuous or repeated exposure to thesame stressors, for example: forced swim, restraint, social conflict are among some examples of chronic stressors. Depending on the intensity and type of stressor exposure, chronic stressful stimuli lead to several consequences. These include characteristic physiological changes in rodents like decreased body weight gain, adrenal hypertrophy [55, 56], atrophy of the thymus and spleen [57], as well as increased anxiety-related [58] and depression-like behaviour [59]. Additionally, chronic homotypic stressor exposure has been shown to suppress the immune system [38, 57], decrease growth and reproductive system activity [60] and is a major risk factor for colitis [61]. Lastly, prolonged increases in GC and excitatory amino acids in the brain as a result of chronic stress may lead to remodeling of neurons in the hippocampus and impairment of cognitive function [62].

In a nutshell, the consequences of acute stress are generally adaptive [63], whereas chronic stress precipitates enormous disease situations [64]

There are different views as to how the HPA axis is regulated in response to prolonged stressor exposure. In response to chronic stress stimuli, several studies have controversially reported either an increased [65-67] or unchanged [68, 69] basal plasma GC levels, whereas ACTH response

remains thesame, desensitizes or transiently increases [70]. These inconsistent effects seem to depend on the nature of the stressor: under repeated homotypic stress situations, ACTH response is down regulated, but it increases in response to subsequent heterotypic stressors [7]. In response to an acute stimulus, CRH neuron activation is usually followed by increased CRH synthesis, indicated by increases in CRH mRNA [71, 72]. On the other hand, during homotypic stressor exposure, decreased CRH mRNA and increased AVP mRNA expression levels have been reported. This shift was previously thought to mediate adaptation to homotypic and sensitization to heterotypic stressors [70]. Together with CRH, AVP has been shown to coactivate ACTH synthesis and release during heterotypic stressor exposure, following homotypic stressors. [17]. Furthermore, increased AVP mRNA has been directly linked to hypersensitivity of the HPA axis [73], suggesting that AVP may act as a determining factor for the responsiveness of corticotroph cells to CRH during heterotypic stressor exposure [7]. However, this claim has been recently contradicted by Chen and colleagues [74]. They showed that administration of a non-peptidergic AVP antagonist via minipumps throughout 14 days of restraint did not alter ACTH responses to hypertonic saline, compared to handled controls. Control handled rats however showed reduced plasma ACTH responses. Therefore, CRH and not AVP is the driving force mediating hypersensitivity of the HPA axis to a novel stressor.

To sum it up, chronic exposure to stressful events is primarily regarded as risk factor for a wide scope of diseases. Thus, adapting to stressful events can result in positive outcomes such as personal growth, reprioritization of life

goals, increased feelings of self-esteem and –efficiency, as well as strengthening social networks.

## 1.3 Monitoring stress hormones

An important tool in behavioural neuroendocrinology entails the monitoring of stress hormones such as GC (cortisol in humans, corticosterone in rodents), as well as epinephrine or NE. GC can be quantified in blood, feces, urine, hair, feathers or saliva, depending on the requested dynamics of time schedule, the choice of the appropriate sampling protocol and most especially, on the species studied, while epinephrine and NE can be quantified in blood, urine and feces.

Approaches employed to collect samples for the estimation of these hormones can either be invasive (for blood samples) or non-invasive (for feces, urine, hair, feathers or saliva). Generally, non-invasive approaches are performed in humans and non-human primates [75-78] and have an advantage over the invasive in that sample collection is done easily and repeatedly under stress-free conditions. However, they have a number of drawbacks. Primarily, only unbound free corticosterone levels can be quantified in saliva [21], thus limiting the use of this non-invasive technique. In addition, collection of saliva in non-human primates requires training and in rodents, it is not yet applicable because of the distress this protocol produces to the animal. Furthermore, measuring GC in feces as well as catecholamines in urine still poses a problem because the ability to excrete feces/urine cannot be systematically controlled and to this effect, the time-

course cannot be effectively investigated due to the inconviniences associated with individual variability and lack of precision.

With regards to the fact that most preclinical and biomedical research is performed in rodents- especially the progressive use of transgenic mice models as of the last decade, the need for a reliable and stress free technique to obtain blood samples for the estimation of hormones becames imperative, an issue that has been addressed by the present thesis (chapter 2). Moreover, in order to detailly study the HPA axis response to stressors, determination of plasma corticosteorne levels has 3 main advantages: i) plasma corticosterone concentrations reflect the degree of functionality of the adrenal glands, ii) mirror the fast dynamics in hormonal fluctuations and iii), can be used to estimate the ratio of unbound and bound GC. Therefore, invasive stress-free techniques in conscious animals are of paramount importance in stress research as they, in addition to providing an overview of the functioning of the HPA axis, can also be employed to compare in parallel, *in vivo* and *in vitro* adrenal gland activity.

There are several invasive techniques available for obtaining blood samples from rodents including, for example: collection of trunk blood or chronic catheterization of the jugular vein or the femoral artery (table 1). Depending on the species in question and the purpose of study, the choice of method of blood collection is at the epicenter for successful results.

| Technique | Reference Rats | Reference mice |
|---|---|---|
| Orbital sinus | [79, 80] | [81, 82] |
| Tail snip/clip | [83, 84] | [85, 86] |
| Saphenous vein | [87, 88] | [89, 90] |
| Cardiac puncture | [87, 91] | [92, 93] |
| Carotid artery | [83, 94] | [95, 96] |
| Trunk blood | [97, 98] | [99, 100] |
| Jugular vein | [101, 102] | [103, 104] |

Table 1: Methods for blood collection used in rats and mice.

Employing techniques which are stressful to the animal either through the use of anesthesia or restraint prior to sample collection may introduce a dependent variable that can be overlooked or difficult to be corrected for. This therefore produces erroneous results which do not actually depict physiological functions. This claim is supported by previous studies in rats, which showed that in trunk blood collection, decapitation with prior administration of anesthesia significantly increased plasma ACTH and corticosterone levels compared with non-anesthetized rats [97]. Further, they revelaed similar basal plasma ACTH and corticosterone levels in samples collected via indwelling vena cava catheters and tail nick as well as profile response after a-30 min retraint stress, only if the tail nick procedure was successfully performed in less than 3 min (time required to initiate the pituitary stress response). Thus, blood sampling in rats from either venous catheters or tail vein can be used in stress studies without confounding results. However, this is not the case in mice as recent studies from Pryce

and colleagues [86] showed elevated plasma corticosterone and ACTh levels in samples repeatedly collected via tail vein. Therefore, the optimal method for collecting blood with minimal stress to the mouse is particulary important quantification of stress hormones as well as other hormones.

Jugular vein catheterization, one of the most important invasive techniques for collecting blood uses fewer animals as compared with trunk blood, provides the possibility for repeated collection of samples, reduces the range of inter-indivdual variability in results and enables the study of time course of the HPA axis response to a given stressor. Additionally, blood is collected from an undisturbed freely moving animal, thus ruling out the effects of anesthesia or restraint. Again, drawing blood via the implanted jugular vein catheter is quick (~60 s), stress-free and additionally provides an opportunity for stress-free intravenous infusions which can be adequately employed in pharmacokinetic studies.

With regards to the emerging expansion of research on genetic mouse models in the last decades, establishment of a simple and stress-free method of blood collection is necessary in mice. Additionally, in order to properly study *in vivo* alterations in the recently established clinically relevant mice model of CPS in our lab [68], one of the aims of this thesis was therefore to establish a simplified method for jugular vein catheterization in mice (chapter 2). Although catheterization of the jugular vein has been performed in mice (see table 1), the techniques described involve either tethering the exteriorized catheter to a freely moving wheel system [104], or additionally performing head surgery to anchor the catheter [105] and inserting it in polyvinyl chloride tubing for extra protection [106]. These rather complicated

constructions are probably the reason why the above mentioned techniques have not been routinely used in neuroendocrinological research. Our catheter is simply built from commercially available material and our technique for catheterization elaborately described (chapter 2) and has been recently used [107].

## 1.4 Chronic psychosocial stress

The term ‘psychosocial’ is a mélange of the words ‘psychological’ and ‘social’. The study of psychosocial stress focuses on environmental events of social origin which an individual subjectively judges as taxing its ability to cope, subsequently challenging its homeostatic state [4]. Basically, psychosocial stressors are characterized by the lack of i), controllability, ii) predictability of the stress onset and iii), outlet for frustration and or social relationships.

In humans, psychosocial stressors include, but are not limited to, exposure to adverse environments and life experiences such as crowding or isolation, relative position in a social hierarchy, stigma and discrimination, family violence and child abuse. Empirical evidence reveals that chronic stress, specifically CPS can lead to multiple mal-adaptations of physiological, immunological and brain systems, resulting in psychopathologies and chronic somatic diseases [108-110]. In rodents, mal-adaptations have been described in response to exposure to CPS including increased anxiety- and depression-like behaviour [111, 112], immunological [113] and

neuroendocrine disorders [58] as well as colonic inflammatory symptoms including acute colitis [68, 114].

In order to better reveal the behavioral, neuroendocrine or immunological consequences of chronic stress seen in humans, attempts have been made to establish animal models, which to some extent mirror human psychosocial stress-related diseases [115, 116]. The most frequently employed models of CPS in rodents include the visible burrow system [65, 117], chronic social instability [58, 118] and the social defeat system [119-121].

In our lab, a clinically relevant animal model of CPS based on the principle of defeat/subordination was recently established in male C57BL6 mice [68]. The consequences of CSC in mice have been extensively studied and summarized in table 2 below.

| System | CSC adaptations | Reference |
|---|---|---|
| Behaviour/ Physiology | - consistent subordinate behaviour ,<br>- increased anxiety-related behaviour (plus-maze, separate open arm exposure, open field/novel object, light-dark box)<br>- no depression-related symptoms and social avoidance behaviour<br>- reduced body weight gain | [68, 100, 122-125] |
| HPA axis | - adrenal hypertrophy, but adrenal insufficiency *in vivo* and *in vitro*<br>- unchanged basal plasma ACTH and GC, but<br>-Increased adrenal responsiveness to an acute heterotypic stressor *in vivo*<br>-increased pituitary mass<br>- decreased levels of AVP mRNA in the PVN | [68, 100, 107, 124, 125] |
| Neuronal functions | -altered neuronal responsiveness (Fos) to acute emotional stimuli in relevant brain regions<br>- unchanged CRH, reduced AVP mRNA expression in PVN | [122, 123] |
| Immune System | - thymus atrophy<br>- increased cytokine secretion from mesenteric lymph node cells *in vitro*<br>- reduced GC sensitivity of splenocytes $CD8^+$ T-cells<br>- increased number of granulocytes in spleen<br>- increased number of $CD11b^+/Ly6c^+$ (myeloid suppressor cells) and decreased number of $CD11c^+/CD11b^+$ splenocytes | [68, 100, 126] |
| Colon Functions | - higher histological damage score in colonic tissue<br>- reduced colonic length<br>- increased vulnerability to pharmacologically induced colitis<br>-decreased colonic mucus production<br>-increased bacterial translocation<br>-increased risk for inflammation – related colon carcinogenesis | [68, 100, 127, 128] |
| Retina | - reduced illumination-induced apoptosis after 10 h of CSC, but no effects after 20 days | Tembei et al, in preparation |

**Table 2:** Consequences of CSC in mice

Given the increasing evidence purporting particularly chronic psychosocial stress to be a risk factor for the development of stress-related pathologies in humans, recent attempts have focused on the development of particularly such paradigms [55, 58, 68, 117, 129-136]. Therefore, I want to emphasize the necessity to employ psychosocial stress models.

### *1.4.1 Importance of psychosocial stress models*

First of all, the use of animal stress models employing stressors of physical origin like restraint, electric shock and water deprivation for instance, does not really reflect human stressors. It has been suggested that research aimed at uncovering the bio-behavioural consequences of stress should be focused on the type of events which are likely to serve as stressors across mammalian species: humans inclusive [137]. Additionally, most physical stressors differ in the duration of application in the case of restraint, ranging from 5 to 30 days [138-140]. In fact, social stress in the lives of many higher species is a chronic and recurrent factor, and is thought to represent an ethologically valid stress stimulus [141]. Secondly, stimuli of social origin are thought to be the most widespread in humans [142] as well as primates [143]. Moreover, stressful stimuli of social nature have been shown to increase the risk for psychiatric disorders [144], and associated to increased activity of the HPA axis [65].

It is therefore crucial that in an animal model, the neurobiological mechanisms underlying the symptomatology, as well as the psychological causes of a particular disease are identical: an aspect of construct validity which is very much portrayed in psychosocial stress models. The growing

increase in the quest to understand the etiology of CPS-induced effects has stepped up the establishment of animal models. Therefore, as the main objective of this thesis, I extended the CSC model in mice to male Wistar rats (chapter 3) in order to substantiate our mouse data and characterize in greater detail, the neuroendocrine changes through repeated blood sampling in higher volumes as this is not possible in mice. Successful establishment of CSC in rats strengthens the clinical validity of this animal model for future use in the study of neurobiological mechanisms involved in the study of physiological, behavioural and immunological alterations associated with CPS.

## 1.5 Risk factors shaping adult stress vulnerability

The individual perception of a given stressor may depend on previous stress experiences as well as his genetic background. These main factors therefore determine the degree of stress vulnerability, making some individuals resistant, and others more or less susceptible to affective as well as somatic disorders.

### *1.5.1 Early life experiences*

Both positive and adverse early life experiences shape an individual's emotional and cognitive development. Human and preclinical studies consistently report that negative early life events increase the emotional and neuroendocrine stress vulnerability in adulthood and, consequently, the

susceptibility to psychopathology [145, 146]. A correlation exists between adverse early life events and illness later in life, suggesting that life events may account for most of the variations in disease conditions. In humans, for example, exposure to childhood adversities like abnormal parental attachment behaviour [147], childhood neglect, maltreatment, sexual and or emotional abuse [148, 149] may underlie an increased risk for the development of adult psychiatric disorders, inflammatory diseases [150] and cancer [151]. Recent studies from the group of King suggest that prenatal stress i.e., chronic stress exposure of the mother during pregnancy (the Quebec ice storm natural disaster) increases the risk for obesity [152] and negatively correlates with general intellectual and language abilities in toddlers [153].

In non-human primates, pregnancy stress as well as early adverse life effects (maternal separation) decreased motoric functions and also reduced exploration, locomotion and play behaviour in offspring [154]. Additionally, several rodent studies revealed compelling evidence that prenatal stress leads to behavioural alterations and dys-regulation of the HPA axis in adult offspring [155]. Similarly, postnatal stress in rodents performed by periodic maternal separation of pups from their mothers causes behavioural and neuroendocrine alterations in both adult rats [156] and mice [157], as well as a reduction in hippocampal neurogenesis in rats [158]. Recently, maternal separation has been shown to increase the susceptibility to CPS in mice in our lab [125]. Whether a similar additive effect of early life stress and CPS in adulthood can be found after prenatal stress in male rats hasn’t been studied and has been addressed in this thesis (chapter 4).

### *1.5.2 Genetic predisposition*

Genetic predisposition strongly contributes to individual variations in stress susceptibility and the stress-induced development of psychiatric illness in humans [108, 159], as well as disorders associated with dys-regulation of the stress system [54]. As the mechanisms underlying these disorders are difficult to diagnose in humans for ethical reasons, appropriate rodent models are currently used to provide a better understanding of gene x environmental interactions affecting emotional behaviour and stress sensitivity.

A broad range of mouse [160-162] as well as rat [163-166] models with remarkable genetically-driven differences in behavioural and endocrine responses to stressors have been established by different laboratories. As an example, in this thesis, I have used male Wistar rats selectively and bi-directionally bred on the basis for high (HAB) or low (LAB) anxiety-related behaviour on the elevated plus-maze (EPM) [163, 167-169]. In this regard, HAB rats with an anxiety level of less than 10 % and LAB rats with more than 35 % time on the open arms during testing at the age of 9 weeks were used in experiments. The extremes in anxiety-related behaviour in HAB and LAB rats have been confirmed in a number of relevant behavioural tests including the open field, light-dark box and modified holeboard [170, 171]. In addition, HAB animals show higher depression-like behaviour in the forced swim test [171-173], impaired fear extinction [174] and increased maternal care [175-177]. Furthermore, HAB rats, to a lesser extent show abnormal aggressive behaviour compared with LABs [178]. These robust behavioural profiles have been persistent over the past 13 years [167, 169, 173], and are independent of age, gender and laboratory [179]. In addition, the behavioural differences

are accompanied by distinct neuroendocrine, neuronal, and genetic characteristics. The HPA axis response to acute emotional stressors is heightened in HAB compared with LAB rats [180], but the reverse is true when exposed to an acute social stimuli [181]. HAB rats also show an abnormal hormonal secretion during the dexamethasone-suprression/CRH challenge test [172, 182] and an elevated CRH mRNA expression in the PVN [183] compared with LAB rats. Also, brain regions belonging to the anxiety/ fear circuitry (paraventricular nucleus of the hypothalamus, the lateral and anterior hypothalamic area, and the medial preoptic area) show increased neuronal activation in response to emotional stimuli in HAB compared with LAB rats [184]. These differences are thought to be brought about by the genetically determined increased AVP mRNA expression and release within the hypothalamic PVN of HAB compared with LAB rats [172, 185]. Thus, a single nucleotide polymorphism in the promoter region of the HAB rats was identified to contribute to the high AVP activity and elevated HPA axis responses [186, 187] for review, see [169, 188].

In consideration of the above mentioned characteristics, this thesis investigates for the first time, if this genetically-determined differences influence the susceptibility to CPS in adulthood (chapter 4).

## 1.6 Aims and Outline of the present thesis

The first objective of the studies described in this thesis was to establish a technique for repeated blood sampling from conscious mice under stress free conditions. This was done in order to monitor *in vivo,* CSC-induced alterations in HPA axis responses in the established CSC mice model. **Chapter 2** describes a simplified jugular vein catheterization procedure in mice, as a technique for repeatedly collecting blood samples. In order to validate this technique, plasma corticosterone has been compared in venous and trunk blood samples under basal and acute stress conditions i.e., 5 min exposure to an elevated platform (psychological stressor) and 60 sec forced swim (physical stressor). Finally, the effect of long term catheterization on state anxiety is investigated 4 days after surgery.

As there is increasing interest to reveal in great detail the consequences of CPS and to uncover underlying cellular and neuronal mechanisms, I extended the recently established clinically relevant animal model of CPS in male mice to male Wistar rats. My main goal was to establish a rat model of CSC using the standardized 3-week stress procedure in order to study the dynamics of the HPA axis, and also to monitor various neuroendocrine parameters, as repeated blood sampling in moderate quantities for the estimation of ACTH, corticosterone, or oxytocin is only possible in rats. In **chapter 3**, the CSC procedure in male Wistar rats is described, and the consequences of CSC exposure on established physiological, neuroendocrine and immunological biomarkers of chronic stress investigated. Furthermore, the dynamics of HPA axis responsiveness to an acute

heterotypic stressor (open arm; 5 min) after CSC exposure is studied by analyzing blood samples collected via chronically implanted jugular vein catheters. Lastly, CSC effects on variable behavioural outcomes including anxiety, depression, social preference and inter-male aggression are investigated.

Another objective of the research presented in this thesis was to investigate what factors influence vulnerability to CPS. For this purpose, the use of prenatally stressed Wistar, and availability of HAB and LAB rats enabled me to study the contribution of adverse early life treatment and genetically determined differences respectively, in vulnerability to CSC-induced symptoms. **Chapter 4** investigates relevant physiological and immunological parameters after CSC exposure in genetically characterized HAB and LAB rats, and in prenatally stressed adult male Wistar rats. Furthermore, the effects of CSC on anxiety-related behaviour have also been studied in these animals.

# Chapter 2

# Comparison of Corticosterone Responses to Acute Stressors: Chronic Jugular Vein versus Trunk Blood Samples.

**Author's contribution:**

**Nyuyki:** study design (figure 4), establishment of venous catheter set up (figure 4), and jugular vein surgery (5), performing all blood sampling experiments, stress procedures, behavioural tests, plasma corticosterone quantifications (figures 6, 7, 8), analyzing all data and writing first draft of manuscript

**Maloumby:** assisted in jugular vein surgery and blood sampling (figures 5, 6, 7)

**Reber**: assisted in blood sampling (figure 6), revision of manuscript

**Neumann**: study design (figure 4), revision of manuscript

[Adapted from Nyuyki KD, Maloumby R, Reber SO, and Neumann ID; 2012; Stress DOI: 10.3109/10253890.2012.655348]

## Abstract

A commonly used method for obtaining blood samples from mice is decapitation. However, there is an obvious need for repeated blood sampling in mice under stress-free conditions. Here, we describe a simple technique to repeatedly collect blood samples from conscious, freely-moving mice *via* a chronically implanted jugular vein catheter. Further, we compare plasma corticosterone concentrations in samples obtained via the catheter 1 day after surgery with samples taken from trunk blood obtained under basal or acute stress conditions. Corticosterone concentrations in repeated 100-µl venous blood samples were found to be similar to trunk blood samples both under basal conditions and after stressor exposure collected at identical time points (at 5, 15 and 60 min). Using both techniques, we demonstrate a progressive increase in corticosterone levels until 15 min after termination of stressor exposure and a decrease towards baseline values 60 min later. Anxiety-related behaviour, as assessed on the elevated plus-maze 3 to 4 days after surgery did not differ between catheterized and non-catheterized mice. Our results provide evidence for application of jugular vein catheterization as a technique for repeated blood sampling in conscious laboratory mice. Use of this technique will greatly reduce the number of animals required for such endocrine endpoints.

## Introduction

The collection of blood from laboratory rats or mice for the estimation of immunological or neuroendocrine parameters, including hormones of the HPA axis, the hypothalamo-neurohypophysial system (HNS), or the SNS is an essential experimental tool in preclinical neurobiology, endocrinology or immunology. In rodents, this can be achieved by collection of trunk blood [68] and of blood from the tail vein [85, 189] or orbital sinus [82], resulting in either termination of the experiment or a severe stressor exposure. More elegant methods for monitoring neuroendocrine parameters in rats are blood microdialysis [190], or withdrawal of blood samples from the femoral [191] or jugular vein via acutely or chronically implanted vein catheters [180, 192]. Only the latter technique allows chronic sampling of sufficient amounts of blood under stress-free conditions from freely moving animals. However, this powerful method, which provides the opportunity to reveal the dynamics of circulating concentrations of a given substance, has only been effectively applied in rats so far [192, 193]. Despite a similar need in mice, especially given the progressive use of valuable transgenic animals, and despite repeated approaches to develop a venous blood sampling method in mice [189, 194], a simplified and reliable method for chronic blood sampling in mice has not been successfully employed until now.

Here, we report a simplified technique for repeated or chronic blood sampling in conscious freely moving mice via a previously implanted jugular vein catheter which can be applied in every neuroendocrine laboratory. The sampling method has been validated by comparing concentrations of the stress hormone corticosterone in blood samples obtained from the jugular

vein and the trunk under basal conditions and in response to exposure to two different acute stressors (forced swim, FS; elevated platform, EPF). Both stressors have been described to trigger HPA axis activation in rats [195] or mice [99]. In order to investigate the effects of catheterization on anxiety-related behaviour, we used the well established elevated plus-maze (EPM) test. The EPM is an established unconditioned anxiety test for rodents, which is based on inducing a conflict situation to remain in the protected closed arms or to explore the open arms of the maze [196].

## Materials and methods

*Subjects*

Adult male C57BL6/N mice (21-23 g body weight) were obtained from a commercial supplier (Charles River, Sulzfeld, Germany), and were single-housed in polycarbonate cages (21 x 15 x 14 cm) for 2 weeks prior to surgery. Single-housing was previously found to be less stressful compared with group housing in male mice with respect to body weight development and anxiety-related behaviour during open arm exposure [123]. Mice were maintained under standard laboratory conditions (12:12 light/dark cycle, lights off at 6 pm, temperature of 22°C, 40 % humidity, V1535-000 commercial diet for rats/mice maintenance from ssniff®, Soest, Germany, and tap water *ad libitum*). All experiments were approved by the local Bavarian government and performed in accordance with the *Guide for the Care and Use of Laboratory Animals* by the National Institutes of Health.

*Reagents and other materials*

Reagents employed during surgery included isoflurane (PZN: 6497131, Baxter Deutschland GmbH, Unterschleißheim, Germany) for anesthesia, softasept® N (article number: 3887138, B.Braun Melsungen AG, Melsungen, Germany) for disinfection of tissue and instruments, betaisodona® (article number: 1970433, Mundipharma GmbH, Limburg-Lahn, Germany) as an antiseptic to reduce the possibility of tissue infection, gentamicin (30 000 IU/ml, PZN: 0536516, 40 HEXAL® SF, Holzkirchen, Germany) for antibiotic treatment, physiological saline (article number: 3200950, 0.9 % NaCl, B. Braun Melsungen AG, Melsungen, Germany) to replace withdrawn blood volume, heparinized saline (30 IU/ml, PZN: 3441331, Liquemin® N 25000, Hoffman-La Roche AG, Grenzach-Wyhlen, Germany) to prevent blood clotting in the tube. Applied volumes are given below when animal treatment procedures are described. Polyethylene (PE-10) tubing (30 m Portex® Non Sterile 0.28 mm I.D x 0.61 mm O.D, Smiths Medical International Ltd, Hythe, Kent, UK, ref: 800/110/100), Dow Corning SILASTIC Silicone laboratory tubing (article number: 2415496 single 50 coil, 0.30 mm I.D x 0.64 mm O.D), and chloroform (article number: 108749, MERCK, Darmstadt, Germany) were used for construction of the catheter, while silk sutures (ref: H4F, 3-0 USP Resorba, Nürnberg, Germany) were used during surgery. Plexiglas cages (38 x 22 x 36 cm) with perforated lids were used for housing the animals after surgery (figure 4).

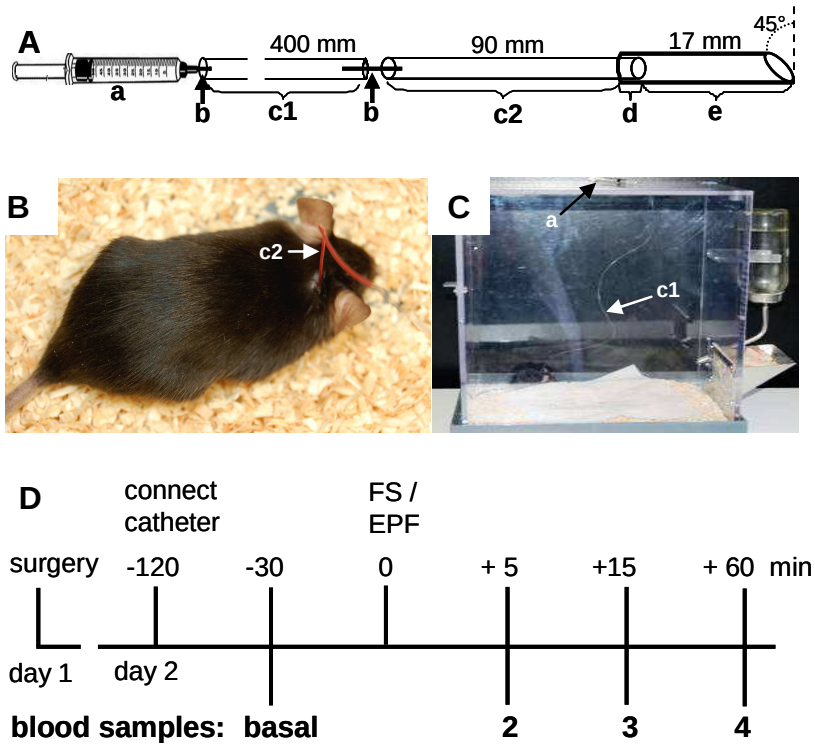


Figure 4: A: Schematic representation of the mouse catheter system adapted for repeated blood sampling in conscious mice; **a**: 1-ml syringe, **b**: a 15-mm piece of a 27-G cannula, **c1**: a 400-mm PE-10 extension tubing (I.D. 0.28 mm; O.D 0.61 mm) which is linked to the catheter via another 15-mm piece of 27-G cannula. The catheter is composed of a PE-10 tubing (**c2**: 90 mm long) connected through a 5-mm overlap (**d**) to a silicone tubing (**e**: I.D. 0.30 mm; O.D. 0.64 mm, 17 mm long), beveled at its tip to an angle of about 45. B: Mouse with already implanted catheter, exteriorized through the nape of the neck. C: Representation of a catheterized mouse in the Plexiglas cage with the extended PE-10 connection tubing (arrow, **c1**) attached to the syringe (arrow, **a**) lying on top of the cage. D: Schematic representation of the blood sampling protocol: About 24 h after surgery, the indwelling jugular vein catheter is connected to the PE-10 extension tubing and the 1-ml syringe 90 min before the start of the experiment. Thirty mins after collection of the basal sample, the mouse is exposed for 5 min to the elevated platform (EPF) or, another group of mice to 60 s of forced swim (FS). At 5, 15 and 60 min after termination of stressor exposure, blood samples 2, 3 and 4 are collected.

*Catheter assembly*

A 90-mm piece of PE-10 tubing and a 17-mm piece of the silicone tubing were used in the preparation of the catheter. One end of the silicone tubing was expanded by dipping in chloroform for about 15 s to facilitate insertion. The PE-10 tubing was then immediately fitted into the expanded end of the silicone tubing to an overlap of 5 mm, and the free end of the silicone tubing beveled to an angle of about 45° to allow easy insertion into the blood vessel (figure 4A), as compared to the flat-tipped catheter. The catheter was stored under aseptic conditions in 70 % alcohol overnight prior to surgery.

*Surgical procedure - jugular vein catheterization*

*Pre-surgical preparations:* Heparinized saline (30 IU/ml), gentamicin (30 000 IU/ml) and sterile saline were prepared before surgery. Prior to surgery, catheters were repeatedly flushed with distilled water, followed by sterile saline in order to remove remaining alcohol. They were filled with heparinized saline by attaching the PE-10 end of the catheter via a sterile blunted 27-gauge (27 G) needle (0.45 x 23 mm BL/LB) to a 1-ml syringe (figure 4A). The filled catheter was placed in sterile saline for at least 10 min before use. All surgical instruments were fumigated with softasept® N, and later placed in 70 % alcohol for about 10 min before surgery. The instruments were rinsed briefly with sterile saline before use.

Mice were briefly anesthetized using isoflurane. The area around the right clavicle and nape of the neck was shaved and the animal placed on a heating pad (figure 5A). Continuous supply of inhalant isoflurane was maintained throughout surgery with careful observation of the breathing frequency of the

animal. The absence of a tail and foot withdrawal reflex when pinched was used an index of sufficient anesthesia. The mouse was laid on its back and the hind paws taped on the surgical board to maintain positioning. Softasept® N was applied on the shaved surface, and the surgical procedure was carried out under stereomicroscopic control (Leica MZ6).

*Jugular vein catheterization:* On average, the entire procedure of catheterization, starting with anesthesia and ending with the return to the home cage lasted about 25 min. A longitudinal skin incision of about 8 - 10 mm was made above the right clavicle and the jugular vein exposed by blunt dissection (figure 5B). After careful isolation from the underlying tissue and exposition of approximately 5 mm of the vein, 2 silk sutures were placed underneath the vein (figure 5C).

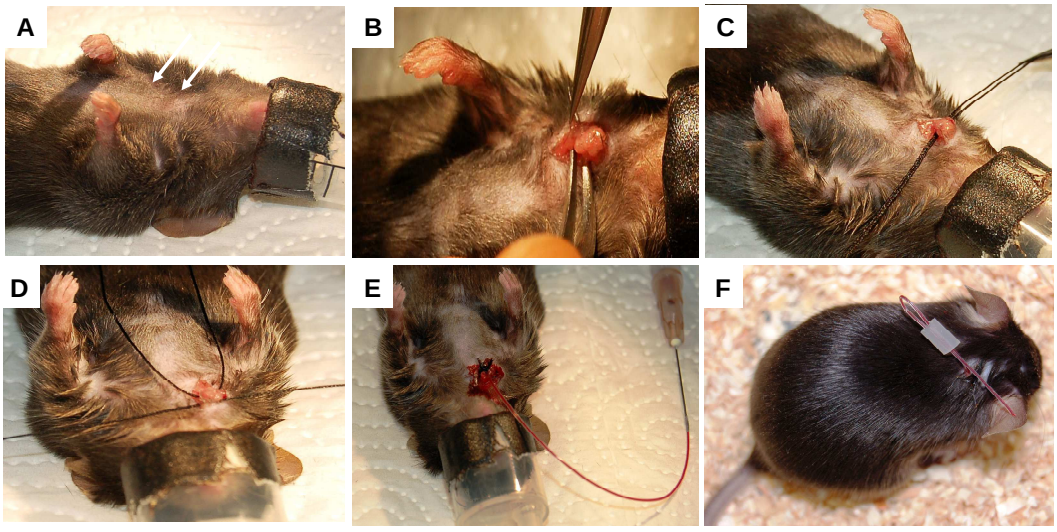


Figure 5: Surgical representation of jugular vein catheterization in mice. A: mouse placed on a surgical pad under the stereoscopic microscope after shaving the area around the neck (arrow). B: isolation of the right jugular vein. C: silk sutures placed beneath identified vein to hold it in pace. D: cranial suture knotted to interrupt blood flow. E: catheter connected to the

syringe successfully inserted into the right jugular vein. F: inserted catheter exteriorized between the ears and crimped to prevent the inflow of air into the right atrium.

One suture was tightly knotted cranially to occlude the vein and interrupt blood flow, while the caudal suture remained untied (figure 5D). Using a micro-scissor, a small v-like incision into the wall of the vein was made in between both ligatures. The beveled end of the catheter was carefully inserted into the jugular vein and advanced with the aid of fine forceps till the tip reached the right atrium. About 13 - 15 mm of the catheter was inserted into the vein, just including part of the silicone-PE tubing overlap (figure 5E). The caudal suture was then knotted once on both the vein and the catheter at the level of the silicone-PE tubing overlap, holding it in place (figure 5E). About 0.05 ml of heparinized saline was injected into the catheter. An attempt to draw blood via the catheter confirmed the correct positioning of the catheter within the jugular vein and its tip within the right atrium. The small amount of blood withdrawn into the tubing was gently flushed back with heparinized saline. If no blood could be drawn, the catheter was repositioned. The flushing with heparinized saline also prevented blood coagulation within the catheter. Next, the caudal suture was repeatedly knotted on top of the silicone-PE tubing overlap and the free ends of the sutures cut off.

To exteriorize the catheter, the mouse was laid on its right side and a guide cannula (18 G, 1.2 x 40 mm, BD Microlance$^{TM}$, Spain, ref: 304622) guided subcutaneously to the nape of the neck and pushed through the skin behind the ears. The catheter was rapidly detached from the syringe, fitted through the guide cannula and exteriorized at the other end. The mouse was turned on its back, the catheter reconnected to the syringe, and trapped air was

drawn into it. Afterwards, 0.03 ml of sterile gentamicin was injected intravenously to prevent infection. Heparinized saline (0.05 ml) was next injected to flush down the gentamicin. The syringe was detached, the exteriorized PE-10 tubing bent at an angle of 180° 20 - 30 mm away from the skin forming a crimp, and a piece of silicone rubber tubing slipped over this crimp to prevent air entering the tubing (figure 5F). The ventral wound was closed with wound clips. Finally, all surgical incisions were swabbed with betaisodona® and the mouse placed into a clean observation Plexiglas cage (figure 4C) for recovery. Mice remained single-housed in these cages after surgery and during blood sampling, and were observed carefully during the immediate postoperative period.

*Blood sampling*

In order to collect blood samples from the undisturbed freely moving mouse, the jugular vein catheter was connected to a 1-ml plastic disposable syringe filled with heparinized saline via a 400-mm piece of PE-10 tubing that extended outside the cage at 8 am of the following day, i.e. 20 - 24 h after venous catheter implantation. In more details, the exteriorized free end of the catheter was attached via a 15-mm piece of 27 G cannula to the 400-mm PE-10 extension tubing, which was attached to the 1-ml plastic syringe via a 27-G cannula (figure 4A). This allowed blood sampling without disturbance of the conscious mouse. The first baseline sample (100 µl) was collected 90 min after connection of the catheter to the syringe. The same volume of blood was replaced with sterile saline, before the extension tubing was filled with heparinized saline again. Thirty min after collection of the basal sample, the

animals were exposed to either the EPF (5 min) or to FS (60 s). Subsequent samples were collected 5, 15 and 60 min after termination of stressor exposure (figure 4D, experimental setup).

Blood samples were collected into EDTA-coated Eppendorf tubes, centrifuged at 5000 rpm for 10 min at 4°C, and plasma aliquots (10 µl) were stored at -20°C until corticosterone concentrations were assayed by ELISA (DRG Instruments GmbH, Marburg).

*Stress procedures and behavioural tests*

*Elevated Platform (EPF):* Exposure of rodents to the EPF has been used as a mild psychological stressor in order to assess the stress response of the HPA axis as described in detail before [195]. Briefly, the EPF consisted of a circular platform, 25 cm in diameter and elevated 75 cm above the ground. Mice were exposed to the EPF for 5 min and blood samples were drawn 5, 15 and 60 min following termination of EPF exposure. The EPF was cleaned thoroughly before each test.

*Forced Swimming (FS):* represents an ethologically relevant combined physical and emotional stressor for rats [101, 197] and mice [198]. The FS tank consisted of an open top cylinder (25 cm height × 13 cm diameter) filled with tap water (21 ± 1°C) to about 12 cm to the brim. Mice were immersed into the water tank for 60 s and blood samples collected 5, 15 and 60 min following termination of FS exposure. The water was changed after every 3$^{rd}$ FS trial and fecal boli removed after every each swim.

*The elevated plus maze (EPM):* has been used in order to assess the anxiety-related behaviour of rodents [196]. As described before [68], our

mouse EPM consisted of two open (6 x 30 cm) and two closed (6 x 30 x 17 cm) arms radiating from a central platform (6 x 6 cm) to form a plus-shaped figure elevated 130 cm above the floor. Each mouse with or without the catheter was placed on the central platform facing the closed arm. All animals were tested between 0800 and 1100 h. During the 5-min test period, the latency to the first open arm entry, the number of entries into the open and closed arms, and the time spent on the respective arms were recorded by means of a video/computer setup to allow calculation of the percentage of time spent on, and the percentage of entries performed into open arms of the maze as parameters of anxiety-related behaviour. Closed arm entries were taken as a parameter of locomotion. The maze was cleaned thoroughly before each test.

*Experimental protocols*

*Plasma corticosterone concentrations immediately and 24 h after surgery and stressor exposure*

In a pilot experiment, we obtained blood samples immediately after surgery (post surgery) and 24 h later (basal), in order to determine the time point of decreased plasma corticosterone concentrations after surgery. Further, to determine whether the sampling technique was stressful to the animals, we measured corticosterone concentrations in blood samples repeatedly collected under basal conditions in 15-min intervals and each replaced by sterile saline (100 µl) in another set of animals 24 h after surgery.

A new set of animals was used to measure plasma corticosterone responses to an acute stressor as early as 24 h after surgery. During this experiment 30

min after collection of the basal blood sample, mice were exposed to the EPF for 5 min and subsequent blood samples were collected 5, 15 and 60 min following termination of EPF.

*Plasma corticosterone concentrations in venous and trunk blood in response to EPF or FS exposure*

Next, we compared corticosterone concentrations in plasma samples obtained either repeatedly from the jugular vein catheter or, in separate sets of mice, from trunk blood. Venous or trunk blood samples were taken under basal conditions (sample 1) or 5, 15 and 60 min following termination of either EPF or FS exposure. These established stressors differ in the quality (psychological versus physical) and intensity (mild versus severe). As the pilot experiment revealed that plasma corticosterone levels 24 h after surgery were comparable to baseline levels in mice without prior surgery, blood sampling was already performed on the day after surgery. Trunk blood was collected from separate groups of single-housed mice killed at the respective time points of blood sampling from the vein. Mice were briefly (5 - 10 s) narcotized by $CO_2$ inhalation and decapitated within 50 s from the onset of transportation from the experimental room.

*Effects of catheterization on anxiety-related behaviour*

Three to four days after surgery and blood sampling, mice were tested on the EPM for anxiety-related behaviour as described above. Catheterized and non-catheterized control mice received the same stress treatment (5 min EPF

3 days prior to testing). The mice were transported to the EPM room for habituation in the evening before testing.

*Statistical analysis*

Data are represented as group means ± S.E.M. For statistical analysis the software package SPSS 18.0 (SPSS Inc., Chicago, USA) was used. Comparisons were done using either a two tailed t-test, one-way analysis of variance (ANOVA: factor time), ANOVA for repeated measures, or a two-way ANOVA (factors sampling technique and factor time) followed by *Bonferroni's post hoc* test if appropriate. $P \leq 0.05$ was considered statistically significant.

## Results

*Experimental success*

Of all operated mice, 91 % survived this procedure, and repeated blood sampling could be successfully performed in 87 % of the survived mice 24 h later. Eighty % of catheterized mice were kept for EPM testing performed 3 days after blood sampling.

*Plasma corticosterone levels immediately and 24 h after stress*

Statistical analysis revealed significantly lower plasma corticosterone concentrations in catheter blood withdrawn 24 h after surgery compared with blood withdrawn immediately after surgery ($P = 0.01$; figure 6A). Twenty-four hours after surgery, repeated blood sampling and replacement with sterile saline did not affect corticosterone concentrations in venous blood samples

($F_{2,4}$ = 0.25, P = 0.79; figure 6B). A 5-min exposure to the EPF significantly altered plasma corticosterone concentrations ($F_{3,15}$ = 20.74, P < 0.01; figure 6C) with increased levels found at 5 and 15 min (P = 0.01) following termination of stressor exposure. Importantly, 24 h after surgery, basal plasma corticosterone levels in catheter blood were comparable to those in trunk blood samples collected under basal conditions in another set of non-catheterized animals (P = 0.06, figure 7A/B). Based on these findings and the high success rate of blood sampling after 24 h, we decided to perform all repeated blood sampling experiments on the day following surgery.

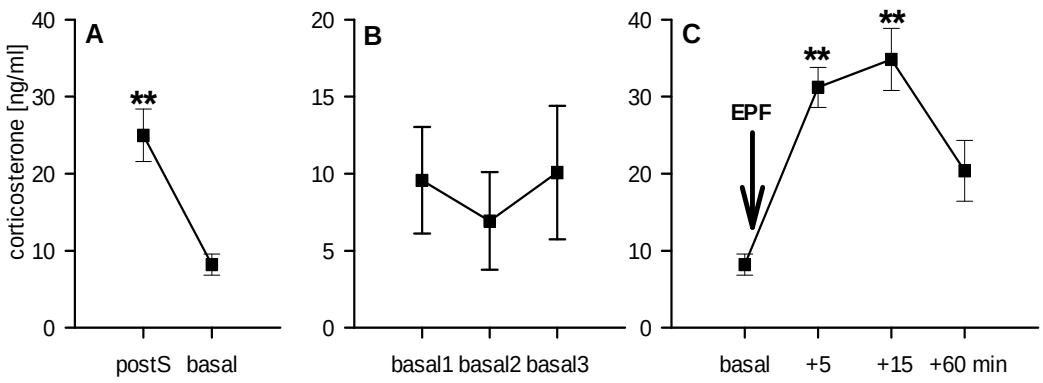


Figure 6: Corticosterone concentrations in mouse venous blood repeatedly collected via a chronically implanted indwelling jugular vein catheter immediately (post surgery; postS) and 24 h after surgery (A: n = 6), 24 h after surgery under basal conditions at 15-min intervals with replacement of 100 µl sterile saline after each blood sampling (B, n = 3); and 24 h after surgery under basal conditions as well as 5, 15 and 60 min after exposure to an elevated platform (C; EPF, arrow, 5 min,; n=6. Data represent means ± S.E.M.; ** P<0.01 versus basal.

*Plasma corticosterone concentrations in venous versus trunk blood samples in response to EPF or FS exposure*

Plasma corticosterone concentrations did not differ between venous and trunk blood samples collected under basal conditions (P = 0.06). Exposure to EPF significantly increased plasma corticosterone in venous (factor time: $F_{3,27}$ = 21.62, P < 0.01) and trunk blood ($F_{3,25}$ = 3.81, P = 0.02) samples. Post hoc comparison revealed increased corticosterone levels in venous blood at 5, 15 and 60 min (P = 0.01 versus basal), whereas, in contrast, in trunk blood samples, elevated corticosterone concentrations were only evident 5 min after EPF exposure (P = 0.05 versus basal). However, venous and trunk blood plasma corticosterone concentrations differed in response to acute EPF exposure (factor sampling technique: $F_{3,61}$ = 16.71, P < 0.01; factor sampling technique x time: $F_{3,61}$ = 0.93, P = 0.43; figure 7A/B).

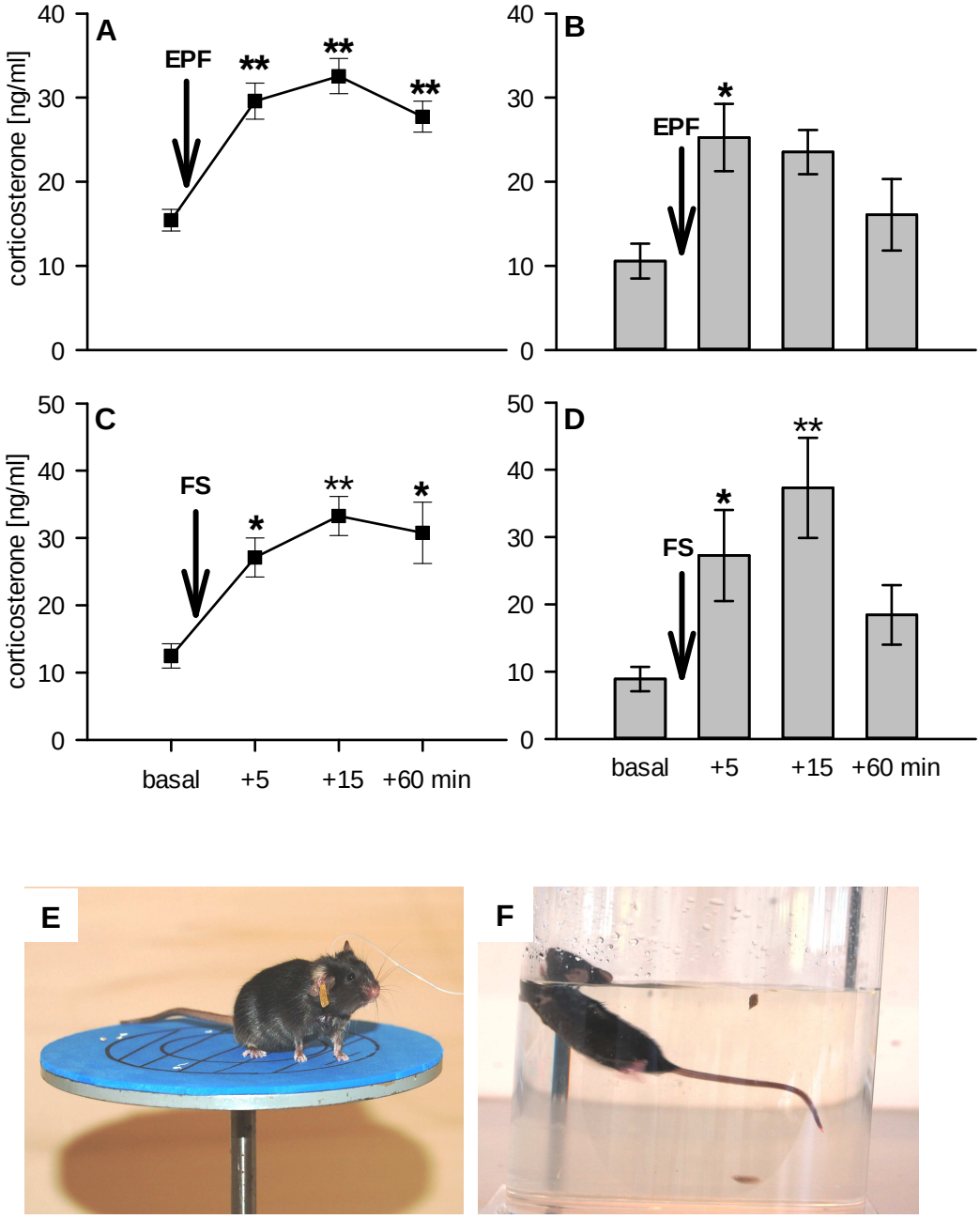


Figure 7: Corticosterone concentrations in mouse blood collected via a chronically implanted indwelling jugular vein catheter (A/C, n=10) or from trunk blood (B/D, n = 7 to 10) under basal conditions as well as 5, 15 and 60 min after stressor exposure (arrow): A and B show plasma corticosterone concentrations after exposure to the elevated platform (EPF, 5 min), C and D after exposure to forced swim (FS, 60 s). E and F show mouse exposed to the EPF

and to FS in the FS tank respectively. Data represent means ± S.E.M.; ** P < 0.01, * P<0.05 versus basal.

In order to investigate in detail corticosterone responses to an acute stressor in venous versus trunk blood samples, we exposed another group of mice to a more severe physical/emotional stressor (FS). Again, basal plasma corticosterone did not differ between venous and trunk blood samples (P = 0.29, figure 7C/D). FS increased plasma corticosterone concentrations in both venous ($F_{3,27}$ = 9.62, P < 0.01; figure 7C) as well as trunk blood ($F_{3,32}$ = 5.28; P = 0.01, figure 7D), with increased corticosterone concentrations at 5 min (P = 0.04 for venous, P = 0.05 for trunk blood vs. basal). FS-induced changes in plasma corticosterone concentration were independent of the sampling technique (factor sampling technique: $F_{3,68}$ = 0.81, P = 0.37; factor sampling technique x time: $F_{3,68}$ = 1.12, P = 0.32; figure 7C/D), and comparable peak concentrations found at 15 min after FS exposure (P = 0.01, vs. basal).

In contrast to trunk blood samples (figure 7B/D), plasma corticosterone levels in venous blood samples were still elevated 60 min following EPF or FS exposure (P = 0.01; figure 7A, P = 0.03; figure 7C). Statistical analysis revealed no significant difference in the area under the curve (AUC) of plasma corticosterone concentrations in venous blood samples obtained in response to EPF or FS exposure (P = 0.72, figure 7A/C).

*Effects of catheterization on anxiety-related behaviour*

The anxiety-related behaviour on the EPM tested 3 days after repeated blood sampling was not different from that found in non-catheterized mice

(percentage of time on open arms: P = 0.85, percentage of entries into open arm: P = 0.29). However, a significantly reduced number of closed arm entries performed by catheterized compared with non-catheterized mice (P = 0.02, figure 8) indicated a decreased locomotion in the former.

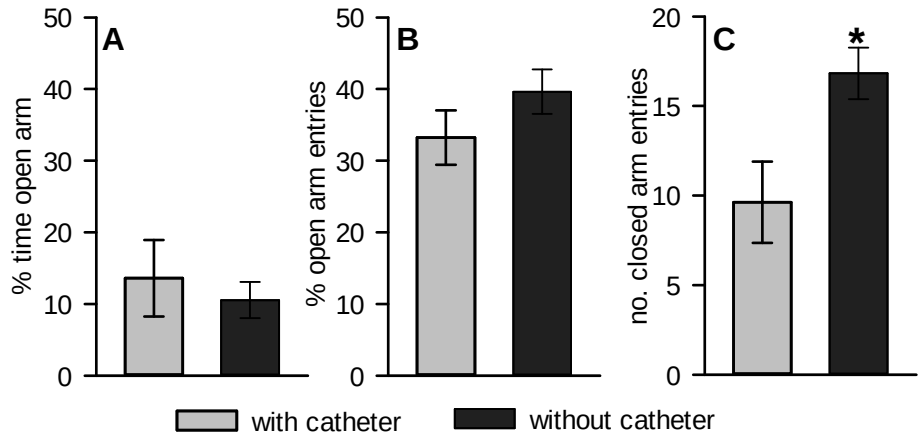



Figure 8: Anxiety-related behaviour of mice with (n = 8) and without (n = 11) a chronic jugular vein catheter 4 days after surgery represented by the percentage of: (A); time spent on and (B); entries performed into the open arms of the elevated plus-maze. Closed arm entries (C) reflect locomotor activity. D shows a less-anxious mouse exploring the open arm of the maze. Data represent means ± S.E.M.; *P<0.05 versus catheter group.

## Discussion

In this study, we demonstrate that chronic jugular vein catheterization and repeated blood sampling is possible in conscious, freely-moving mice. Using our relatively simple catheter and sampling technique, we could repeatedly withdraw as much as 100-µl blood samples up to 4 times within approximately 120 min; this is sufficient blood volume for the reliable quantification of plasma corticosterone as well as other plasma hormones which are far less concentrated in plasma than corticosterone (e.g. corticotrophin (ACTH), oxytocin, and vasopressin). Additionally, under these conditions, fluctuations in any given plasma factor can also be monitored in response to pharmacological treatment after completely stress-free intravenous infusion via the implanted jugular vein catheter. Importantly, in 3 mice (40% of catheterized mice tested at this time point), blood sampling could even be performed up to 14 days after catheterization (data not shown). The jugular vein catheter could also be useful in experiments employing chronic intravenous drug infusion using osmotic minipumps connected to the catheter.

Due to their small size, blood sampling in mice has been frequently limited to nonsurgical methods so far. Thus, repeated blood sampling has been performed from the mouse tail vein, or via cardiac puncture among others (http://www.theodora.com/rodent_laboratory/blood_collection.html), – all being stressful procedures not allowing basal, stress-free sampling [189]. Small amounts of blood can also be collected from the orbital sinus of an anesthetized or firmly restrained mouse [82], or from the carotid artery [95, 96]. Although studies have been published more than two decades ago

showing that serial blood samples can be collected from the right jugular vein of a freely moving mouse [103], the techniques described in these studies are rather complicated and have consequently, not been routinely applied since then. For example, MacLeod and Shapiro [104] showed that serial blood sampling could only be performed in the mouse when the inserted catheter is tethered to a freely moving wheel system built above the cage. Also, Mokhatarian and colleagues [105] reported the possibility of collecting blood samples via a right jugular vein catheter fixed to the skull by a special dental cement, and Bardelmeijer and colleagues [106] designed a special holding cage system, which enabled the collection of blood samples via a previously implanted jugular vein catheter. These rather complicated experimental constructions with anchoring of the venous catheter with dental cement and additional surgery, tethering which requires a special build up system and hot water coiled polyethylene tubing as extension tubing probably prevented the aforementioned protocols from routine use in life science research. They were however, important steps towards the development of the relatively simple blood sampling technique introduced in this study.

In comparison to arterial catheterization commonly employed in metabolic studies, jugular vein catheterization may present some advantages including simpler catheterization procedure. Moreover, the tip of the catheter rests in the right atrium. This allows ‘first pass’ venous blood collection that should have higher concentrations of plasma hormones originating in the pituitary or the adrenal glands. Moreover, the risk to induce stroke seen after arterial catheterization [199] is low after jugular vein catheterization.

Our catheter set up, originally designed for rats is easily and rapidly prepared, and most importantly, made of inexpensive commercially available materials. We also use special experimental cages (Plexiglas 38 x 22 x 36 cm, figure 4C) that are routinely employed for blood sampling in rats [180, 192], and microdialysis in rats [190, 200] and mice [201, 202]. These cages are higher (36 cm) than regular mouse cages (21 x 15 x 14 cm) and equipped with a perforated lid allowing unrestricted movement of the mouse with the extension tubing of the catheter and the sampling syringe attached (figure 4C). However, the sampling system should be transferable to any cage which prevents the freely moving animal from escaping while having the possibility to collect blood samples from the indwelling catheter via extension PE-10 tubing terminating just above cage. Though relatively simple, it is worth mentioning that our blood sampling technique requires a greater level of expertise for successful experimental performance than obtaining trunk blood.

In order to further validate the method of repeated blood sampling from the jugular vein, we compared corticosterone concentrations in venous and trunk blood samples collected under basal conditions and at identical time points following acute stressor exposure. Corticosterone is the main stress hormone of the HPA axis, which is an excellent indicator of any external disturbance. Importantly, we could demonstrate that the plasma corticosterone concentrations obtained from both the jugular and trunk blood samples were almost identical under basal conditions and following exposure to a physical/emotional stressor (FS), and at least comparable in response to exposure to the (EPF). Therefore, the method of blood collection should also

be considered in dependence on the stressor applied. Moreover, the observable elevation in plasma corticosterone at 60 min after stressor exposure in response to EPF and FS in venous blood samples (figure 7A/C) indicates the possibility of altered HPA axis feedback, for example as a result of repeated sampling or prior surgery. Although further testing is needed at later time points, the technique might be more feasible for assessing acute HPA axis responses, rather than for studying the recovery of the HPA axis. It is also important to mention that mice could require a longer recovery period (maybe 2 h or more), but we did not investigate this in more detail.

Compared with trunk blood collection, serial collection of blood samples from a chronically implanted jugular vein has a lot of advantages. It allows intra-individual comparisons and monitoring of the temporal dynamics of a given blood-born factor. Moreover, a substantial reduction in the number of experimental mice, which has clear ethical and financial benefits, is a major factor.

High corticosterone concentrations in plasma sampled immediately after surgery (figure 6A) indicated that anesthesia and/or surgery are stressful. This underlines that blood sampling under conditions of long-term anesthesia (more than 60 s), or of restraint, as necessarily performed for blood collection from the tail vein, orbital sinus or via cardiac puncture are likely to provide false basal values. Therefore, anesthesia or restraint may result in conflicting basal results to those obtained in freely moving non-stressed mice. To our surprise, catheterized mice show low basal plasma corticosterone levels already 24 h after surgery. Therefore, one day of recovery seems enough, and blood sampling experiments can already be performed the day following

surgery. This poses an advantage over rats in which repeated blood sampling is routinely performed 4 to 5 days after jugular vein surgery [192].

According to the literature [203], the average total volume of blood of an organism is 6-8% of its body weight i.e., 1.5. – 2 ml in a 25 g mouse. To avoid serious alterations in cardiovascular functions and haemorrhage of the experimental mouse, not more than 10-15 % of total blood volume or 1 % of body weight should be collected at one time. Therefore, we chose to withdraw 100-µl blood samples from the catheterized mouse at a time, which was immediately replaced by sterile saline, as suggested before [104, 203]. Alternate options for replacing removed blood and to avoid blood dilution are donor blood from mice of the same strain [106] or serum from the same mouse [105], but this is labor and cost intensive, and interference with the donor's plasma hormones cannot be avoided. This may provide additional source of (donor's) hormones as discussed above.

It is important to mention that anxiety-related behaviour on the EPM did not differ between catheterized mice 4 days after surgery and 3 days after repeated blood sampling and mice without surgery and blood sampling (figure 8A). However, long-term catheterization and prior blood sampling reduced locomotion (figure 8B); therefore, we would not recommend the use of catheterized mice for detailed behavioural observation and the assessment of subtle behavioural parameters 4 days after surgery and blood sampling.

In conclusion, repeated or chronic blood sampling is also possible in mice using a simplified technique of indwelling catheterization of the jugular vein. We are convinced that the routine application of catheterization is an

advantageous and important technique in the field of endocrinological research.

## Acknowledgements

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# Chapter 3

# Chronic subordinate colony housing (CSC) represents a model of chronic psychosocial stress in male rats

**Author's contribution:**

**Nyuyki:** study design (figure 9), establishing and performing the CSC paradigm (figures 10, 11, 12, 13), organ removal and preparation (figures 11, 13), jugular vein surgery and blood sampling (figure 12), assisted in plasma corticosterone and ACTH quantifications (figures 11, 12), analyzing aggressive behaviour during CSC (figure 10) as well as performing the elevated plus-maze, light-dark box, forced swim, social preference and resident intruder tests after CSC (table 3), analyzing data, writing first draft of the manuscript

**Beiderbeck:** assisted in performing and analyzed the resident intruder test (table 3)

**Lukas**: assisted in performing and analyzing the social preference test (table 3)

**Neumann**: study design (figure 9), revision of manuscript

**Reber**: study design (figure 9), assisted in performing the CSC paradigm, quantification of histological damage score (figure 13), revision of manuscript

Adapted from: Kewir D. Nyuyki, Daniela I. Beiderbeck, Michael Lukas, Inga D. Neumann and Stefan O. Reber, PLoS ONE: *under review*

## Abstract

Chronic subordinate colony housing (CSC) is an adequate and reliable mouse model of CPS, resulting in reduced body weight gain, reduced thymus and increased adrenal weight, long-lasting anxiety-like behaviour, and spontaneous colitis. Furthermore, CSC mice show increased adrenal ACTH responsiveness to acute heterotypic stressors, suggesting a general mechanism which allows a chronically-stressed organism to adequately respond to a novel threat. Therefore, the aim of the present study was to extend the CSC model to another rodent species, namely male Wistar rats, and to characterize relevant physiological, immunological, and behavioural consequences; placing particular emphasis on changes in HPA axis responsiveness to an acute heterotypic stressor. In line with previous mouse data, exposure of Wistar rats to 19 days of CSC resulted in a decrease in body weight gain and absolute thymus mass, mild colonic barrier defects and intestinal immune activation. Moreover, no changes in depressive-like behaviour or social preference were seen; again in agreement with the mouse paradigm. Most importantly, CSC rats showed an increased plasma corticosterone response to an acute heterotypic stressor (open arm, 5 min) despite displaying similar basal levels and similar basal and stressor-induced plasma ACTH levels. In contrast to CSC mice, anxiety-related behaviour and absolute adrenal weights remained unchanged in CSC rats.

In summary, the CSC paradigm could be established as an adequate model of chronic psychosocial stress in male rats. Our data further supports the initial hypothesis that adrenal hyper-responsiveness to ACTH during acute

heterotypic stressors represents a general adaptation, which enables a chronically-stressed organism to adequately respond to novel challenges.

## Introduction

In humans, chronic stress has been repeatedly shown to be a risk factor for the development of several affective and somatic disorders [for review see 109, 204]. There is also a large body of evidence from rodent studies indicating a link between chronic or repeated stress and emotional, social and physiological, in particular immunological, dysfunctions [44, 58, 135, 136, 205]. However, despite this knowledge and substantial research efforts in the last decades, the aetiology of stress-based disorders remains poorly understood. This has led to a resurgence of interest in developing more clinically relevant animal models of chronic stress. Given the increasing evidence for chronic psychosocial stress being a risk factor for the development of stress-related pathologies in humans [for review see 45, & 206], recent attempts have focused on the development of novel psychosocial stress paradigms believed to better mimic the human situation [58, 117, 133, 135].

We have recently established chronic subordinate colony housing (CSC) as an adequate and clinically relevant mouse paradigm for chronic psychosocial stress [68, 100]. During CSC exposure, 4 experimental male mice are housed together with a dominant, and slightly larger resident for 19 consecutive days, whereby, the larger male is replaced by a novel one on days 8 and 15 to avoid habituation [68]. Reliable indicators of chronic stress in CSC mice are a decrease in body weight gain and thymus weight, an increase in adrenal mass, increased anxiety-, but no depression-related symptoms, development of spontaneous colitis and aggravation of a chemically-induced colitis [68, 100, 123, 124]. Interestingly, one key factor

involved in the development of CSC-induced spontaneous colitis has been shown to be bacterial translocation, endorsed by a leaky colonic barrier caused at least partly by a decrease in colonic mucus production [128]. CSC mice further show an increased risk for inflammation-related colon carcinogenesis [207]. Finally, CSC affects adrenal functionality resulting in unaffected basal morning, but decreased basal evening, plasma corticosterone levels [68], and a reduced adrenal *in vitro* corticotrophin (ACTH) responsiveness [68, 107]. These findings, at least at the first glance, suggest the development of adrenal insufficiency and, thus, seem to be in line with the negative immunological and behavioural consequences of CSC in mice.

However, in contrast to the reduced adrenal *in vitro* ACTH responsiveness, we recently showed an increased *in vivo* plasma corticosterone response to an acute heterotypic stressor, namely exposure to an elevated platform, in CSC compared with single-housed control (SHC) mice [107]. As a comparable rise in plasma ACTH was found, CSC-induced changes at the level of the adrenal gland are likely and may include decreased *in vitro* ACTH responsiveness, but increased *in vivo* ACTH sensitivity during acute heterotypic stressors. Such changes might represent beneficial adaptations to, rather than maladaptive consequences of, chronic psychosocial stress, allowing an adequate response to a novel challenge while preventing prolonged exposure to high basal levels of deleterious corticosterone. Attenuated responses of the HPA axis to repeated homotypic [for review see 7], but sensitization to acute heterotypic stressors has been repeatedly described in mice and rats [74, 208-210]. However, in contrast to our findings

in CSC mice, until today, there is the general assumption that these adaptations are not relevant for stressors which are of social nature [211]. Therefore, the biological relevance of our mouse data needs to be further substantiated and confirmed in a different rodent species.

In the present study, we therefore established the CSC model in male Wistar rats grouped into CSC, single-housed (SHC), and group-housed (GHC) controls. In order to confirm that the male Long-Evans rats used as residents obtained the dominant, and the 4 Wistar rats the subordinate positions during CSC exposure, we monitored their offensive and defensive behaviours. Next, we investigated the effects of CSC on well established stress parameters such as body, thymus and adrenal weight, and basal plasma corticosterone levels. Using repeated jugular vein sampling via chronically implanted catheters we further monitored plasma ACTH and corticosterone responses to an acute emotional stressor (open arm exposure). We also assessed CSC effects on socio-emotional behaviours (anxiety- and depression-related behaviours, social preference, inter-male aggression), and on somatic symptoms (signs of colonic barrier defects and of colonic inflammation).

## Materials and methods

*Ethics statement*

All experimental protocols were approved by the Committee on Animal Health and Care of the local government of Bavaria (54–2531.2–16/08), and conformed to international guidelines on the ethical use of animals. Surgery

was performed under isoflurane anesthesia, and all efforts were made to minimize the number of animals used and their suffering.

*Animals*

Male Wistar (300-350 g; experimental rats), and male (400-450 g; dominant rats) and female (300-350 g) Long-Evans rats were purchased from a commercial supplier (Charles River, Sulzfeld, Germany) and kept under standard laboratory conditions (12/12-h light–dark cycle, lights on at 0600 h, 21 ± 1°C, 60 ± 5% humidity, free access to tap water and standard rat chow). Each Long-Evans resident male was permanently housed with a Long-Evans female in order to stimulate territorial behaviour and improve offensive approach [212], and single-housed 1 h before the start of CSC colony formation (see below). All experimental Wistar rats were randomly housed in groups of 4 upon arrival for 1 week before the start of CSC colony formation.

*Chronic subordinate colony housing (CSC)*

The chronic subordinate colony housing (CSC) paradigm in rats was adapted from the CSC paradigm previously described in mice [68]. Briefly, 4 male Wistar rats were housed together with a larger male Long-Evans rat in its home cage (56 x 39 x 20 cm) for 19 consecutive days. Prior to CSC, the male Long-Evans rats were tested for their aggressive behaviour. During 2 training sessions, the female Long-Evans rats were separated from their male counterparts 1 h before lights off. In the dark phase, 4 unfamiliar male Wistar rat intruders were then introduced to each resident Long-Evans rats for about an hour. The offensive behaviours of residents were observed. Non dominant (non offensive) as well as males that started to injure and bite their

opponents were excluded. To avoid habituation, each larger Long-Evans male was replaced by a novel one on days 8 and 15 by introducing the 4 CSC rats into the homecage of the novel dominant Long-Evans male. During the first 2 h of colony formation which was started immediately after lights off on days 1, 8, and 15, the colonies were videotaped for behavioural analysis. As the appropriate and stress free housing condition for male control rats is still under debate, both SHC and GHC rats were used for comparison. SHC rats were housed singly (40 × 25 × 15 cm) for 19 consecutive days, whereas GHC rats were housed in groups of four per cage (55 × 35 × 20 cm) for 19 consecutive days. Both control groups were kept in separate animal rooms from the CSC colonies and remained undisturbed in their home cages except for change of bedding once a week. For assessment of body weight, all rats were weighed in the morning of days 1, 3, 8, 10, 15, 17 and 20, and delta body weight gain between day 20 and day 1 was calculated.

*Experimental procedures*

**Experiment 1**. In order to confirm the consequences of CSC exposure on established physiological and HPA axis parameters in male rats, SHC, GHC and CSC rats were rapidly killed by decapitation (after brief inhalation anesthesia) in the morning of day 20 of CSC exposure between 0800 h and 1000 h (figure 9). Trunk blood was collected for the quantification of plasma corticosterone levels. Moreover, changes in adrenal and thymus weight were determined (see below). The behaviour during the first 2 h after CSC colony

formation was recorded for later analysis of offensive and defensive behavioural patterns.

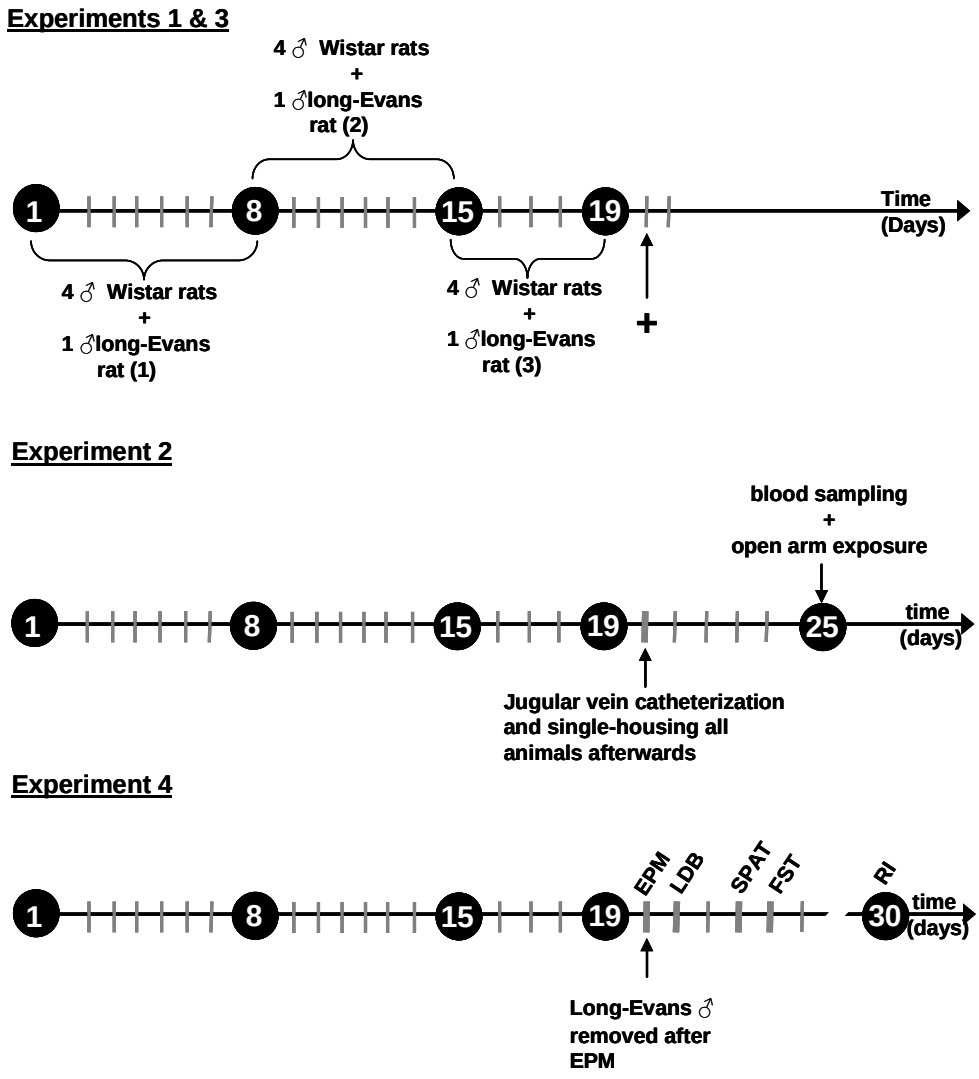


Figure 9: Experimental design. Male Wistar rats were exposed to 19 days of chronic psychosocial stress, i.e. CSC, in which 4 males were housed with a dominant Long-Evans male, which was replaced by a novel dominant male on days 8 and 15. Single-housed and group-housed (4 rats without the dominant Long-Evans) served as controls, i.e. SHC and GHC respectively. In experiments 1 and 3, all rats were killed (+) in the morning of day 20, in order to investigate the effects of CSC exposure on relevant physiological and immunological parameters. In experiment 2, catheters were implanted in the right jugular vein of all rats in the morning of day 20, after which they were single-housed in Plexiglas cages. Five days

after catheterization (day 25), rats were exposed to an acute emotional stressor (open arm; 5 min) in order to investigate plasma ACTH and corticosterone responsiveness. After assessing anxiety-related behaviour on the EPM on day 20 (experiment 4), dominant Long-Evans rats were removed from CSC colonies and this assessment again done the next day (day 21) on the LDB. On days 23, 24 and 30, social preference /avoidance, depressive-like behaviour, and inter-male aggression were analysed using the SPAT, FST and RI test respectively.

**Experiment 2**. In order to investigate plasma ACTH and corticosterone responses to an acute emotional stressor, another set of SHC, GHC and CSC rats, fitted with a chronic jugular vein catheter on day 20 were immediately single-housed thereafter. Five days later (day 25), they were exposed to the open arm of an elevated plus-maze (EPM) for 5 min (figure 9). Blood was repeatedly drawn under basal conditions and 5, 15 and 60 min after termination of open arm exposure.

**Experiment 3.** In order to confirm that CSC exposure also results in colonic inflammation in rats as shown in mice [68, 128], another set of SHC, GHC and CSC rats were killed in the morning of day 20 (figure 9). Colon and mesenteric lymph nodes were removed to determine the histological damage score, colonic mucus production (Alcian Blue staining) and anti-CD3-stimulated interferon-gamma (IFN-γ) secretion from mesenteric lymph node cells (see below).

**Experiment 4.** To study the behavioural consequences of CSC in rats, another set of SHC, GHC and CSC rats was subjected to a series of behavioural tests at the last day of CSC (EPM) and following termination of CSC, respectively. Whereas the housing conditions for SHC and GHC

remained unchanged until termination of all behavioural tests (until day 30), the dominant residents were removed from the CSC colonies on day 20 after EPM testing, and CSC rats remained in groups of 4 (figure 9). The effects of CSC on anxiety-related behaviour were investigated on the EPM and in the light-dark box (LDB) on days 20 and 21, respectively. In addition, CSC effects on social anxiety were assessed employing the social preference/ social avoidance test (SPAT) on day 23, i.e. 3 days after termination of CSC. Furthermore, depression-related behaviour and inter-male aggression were investigated in the forced swim test (FST) and the resident-intruder (RI) test on days 24 and 30, respectively.

*Behavioural observations during CSC*

To confirm the intended dominant/ subordinate hierarchy within each colony, rats of Experiment 1 were videotaped during the first 2 h of colony formation immediately after lights off on days 1, 8, and 15. Their agonistic behaviour was analyzed in terms of number of offensive behavioural patterns like threat, attack, offensive upright and keep down, and number of defensive behavioural patterns like freezing, defensive upright and lying on the back (figure 10A/B). Importantly, in each colony, resident Long-Evans rats exclusively showed offensive behavioural elements, and Wistar CSC rats exclusively showed defensive behavioural elements. The mean number of each behaviour during the 3 x 2-h (6-h) observation period was calculated and depicted in the graphs.

*Blood sampling and Enzyme-Linked Immunosorbent Assay (ELISA) for ACTH and corticosterone*

To determine the effect of CSC on plasma ACTH and corticosterone concentrations, SHC, GHC and CSC rats were either rapidly killed by decapitation under carbon dioxide anaesthesia within 3 min after entering the animal room (experiment 1; basal plasma corticosterone), or chronically implanted with a jugular vein catheter for repeated blood sampling (experiment 2; ACTH and corticosterone response to acute stressor). About 100 µl of trunk blood (experiment 1) or 200 µl of venous blood obtained via the implanted jugular vein catheter (experiment 2) was collected in EDTA-coated tubes on ice (Sarstedt Nümbrecht, Germany) and centrifuged at 4 °C (5000 rpm, 10 min). Plasma samples were stored at –20 °C until assayed using commercially available ELISA kits for ACTH (analytical sensitivity 0.22 pg/ml, intra-assay and inter-assay coefficients of variation ≤ 7.1%, IBL International, Hamburg, Germany) and corticosterone (analytical sensitivity < 1.631 nmol/l, intra-assay and inter-assay coefficients of variation ≤6.35%, IBL International, Hamburg, Germany). ELISA kits with different LOT numbers were used for experiments 1 and 2.

*Determination of thymus and adrenal weights*

To assess CSC effects on thymus and adrenal weight, thymus and both adrenal glands were removed after decapitation, carefully pruned from fat tissue and weighed (experiment 1). The left and right adrenals were weighed together. All values represent absolute (mg) or relative (mg organ weight/g body weight) weights.

*Jugular vein catheterization and repeated blood sampling*

Implantation of the jugular vein catheter in Experiment 2 was performed as described previously [101, 213] on day 20. Briefly, the rat was placed in a perspex box to allow inhalation of isoflurane anaesthesia, and then transferred to the surgery table, where anaesthesia was maintained via a face mask. The right jugular vein was exposed by blunt dissection, and a catheter consisting of a silicone tubing (Dow Corning Corp., Midland MI, USA) and a polyethylene-50 tubing was inserted approximately 3 cm into the vessel through an incision in a cardiac direction and exteriorized at the neck of the animal behind the ears. The catheter was filled with sterile saline containing gentamicin (30,000 IU/ml; Centravet, Bad Bentheim, Germany). Catheterized animals were then single-housed in standard polycarbonate observation cages (38 x 22 x 36 cm) for 5 days (experiment 2).

In the morning of day 25 at 0700 h, i.e. 5 days after termination of CSC exposure, the catheter of each rat was attached to an extension tube connected to a 1-ml plastic syringe filled with sterile heparinized 0.9% saline (30 IU/ml, Heparin-Natrium, Ratiopharm, Ulm, Germany). Each rat was then left undisturbed for 90 min. Two basal blood samples were taken 30 min apart, before rats were placed on the open arm for 5 min. Subsequent samples were collected 5, 15 and 60 min after end of open arm exposure. Collected blood was immediately replaced with sterile 0.9 % saline. All blood samples were collected in EDTA-coated tubes on ice and treated as explained above.

The two basal ACTH and corticosterone concentrations (basal 1 and 2) were averaged to calculate the mean basal concentrations for both ACTH and

corticosterone which was set to 100 %. Data are presented as percentage increase with respect to basal values.

*Determination of the histological damage score of the colon*

Assessment of the histological damage score was performed as previously described [68, 100]. In order to assess the effect of CSC on histological markers of the distal part of the intestinal tract, the colon was removed and mechanically cleaned (experiment 3). Afterwards, 1 cm of the distal third of the colon was cut longitudinally, laid on a filter paper and fixed in 10 % formalin overnight. The next day, the fixed tissue was embedded in paraffin and cut longitudinally. Two 3 µm haematoxylin-eosin stained sections taken at 100 µm distance were evaluated by histological scoring performed by an investigator blind to treatment. For statistics, each individual score represented the mean of the 2 sections. Histological damage score ranges from 0 to 8 and represents the sum of the epithelium score *(*0: normal morphology; 1: loss of goblet cells; 2: loss of goblet cells in large areas; 3: loss of crypts; 4: loss of crypts in large areas) and infiltration score (0: no infiltration; 1: infiltrate around crypt bases; 2: infiltrate reaching to lamina muscularis mucosae; 3: extensive infiltration reaching the lamina muscularis mucosae and thickening of the mucosa with abundant oedema; 4: infiltration of the lamina submucosa).

*Alcian Blue staining*

One aspect of epithelial barrier function, namely the number of mucus producing epithelial cells, was investigated as previously described [128]. Briefly, formalin-fixed colon tissue was embedded in paraffin and cut

longitudinally (experiment 3). Two 3-µm sections of each rat were taken at 100-µm distance and used for acidic Alcian Blue staining. Afterwards Alcian Blue positive cells were counted in the colonic crypt layer of ten non overlapping adjacent fields of view (Leika FW4000 software; magnification 1:20) per section (total of 20 fields of view per rat) and averaged per rat.

*Isolation and incubation of mesenteric lymph node cells*

Mesenteric lymph nodes from 4 rats per treatment group (randomly selected) were harvested under sterile conditions and collected on ice in cell culture medium [RPMI-1640 supplemented with 10 % fetal calf serum (Biochrom, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin (GIBCO-BRL, Eggenstein, Germany) and 3 x $10^{-5}$ M ß-mercaptoethanol (Sigma, Deisenhofen, Germany)] for each individual rat (Experiment 3). Lymph nodes were mechanically disrupted and filtered through a cell strainer (70-µm Nylon, Falcon™, Becton Dickinson, Germany). Afterwards, cells were washed three times in cell culture medium and adjusted to a concentration of $10^6$ cells/ml. 2 x $10^5$ (200 µl) lymph node cells were transferred to wells of a 96-well plate and stimulated by pre-coating wells with 100 µl of 2.5 µg/ml anti-CD3 antibody (final concentration 100 U/ml). Four wells were transferred with the respective number of cells of each rat. After incubation for 24 h (37 °C, 5 % $CO_2$), IFN-γ concentrations were measured in the supernatants of each of the four wells per rat using the MILLIPLEX® MAP Kit for Rat cytokine/chemokine assay (Millipore GmbH, Schwalbach, Germany) and averaged per rat.

*Elevated plus-maze (EPM)*

To assess the effects of CSC on anxiety-related behaviour, SHC, GHC and CSC rats of experiment 4 were transported to the EPM room the evening before testing, i.e. on day 19. The next day, they were tested on the EPM between 0800 and 1200 h for 5 min as described before [196, 214]. The EPM consisted of two opposing open (50 × 10 cm; 140 lux) and closed (50 × 10 × 40 cm; 20 lux) arms connected by a central platform (10 x 10 cm) and elevated 80 cm above the floor. Each rat was placed on the central platform facing a closed arm and allowed to explore the maze for 5 min. Before each test, the maze was thoroughly cleaned. The time spent in the respective arms was recorded by means of a video/ computer setup to allow calculation of the percentage of time spent on the open arms of the maze. Further, the number of entries into the closed arms was recorded as measure for locomotor activity.

*Light-Dark Box (LDB)*

To further assess the effects of CSC on anxiety-related behaviour in another established test [215], SHC, GHC and CSC rats were tested in the LDB (experiment 4) in the morning of day 21 between 0800 and 1200 h as described before [214, 216]. Briefly, the LDB consisted of a brightly lit (40 × 50 cm; 350 lux) and a dark (40 × 30 cm; 70 lux) compartment separated by a partition wall that had an opening (7.5 cm) at floor level. Rats were individually placed in the dark box facing the opening and allowed free exploration for 5 min. The time spent in the respective compartments was recorded by means of a video/ computer setup to allow calculation of the

percentage of time spent in the lit box. The number of line crossings/ minute in the dark compartment was taken as an indicator of locomotor activity. The light–dark box was cleaned thoroughly before each test.

*Social preference/ avoidance test (SPAT)*

The effects of CSC on social anxiety were studied using the SPAT in experiment 4 as described before in rats and mice [124, 217]. Briefly, 1 h after lights off (1900 h) on day 23, SHC, GHC and CSC rats were habituated in a novel arena (40 x 80 x 40 cm, red light) for 30 s, then an empty wire-mesh cage (non-social stimulus) was placed at one side wall of the arena for 4 min. The empty cage was then exchanged by an identical cage containing an unknown male con-specific (social stimulus) for an additional 4 min. Each test procedure was videotaped and scored afterwards by an observer blind to treatment using JWatcher behavioural observation software (V 1.0, Macquarie University and UCLA). Non-social and social stimulus investigation times were scored by measuring the time the rat spent in active olfactory investigation (sniffing). Social preference/ avoidance was calculated based on the following formula: (time investigating the social stimulus [s]/ time investigating the empty cage [s]) x 100 %. A value less than 100 % was taken to represent social avoidance, while a value above 100 % was taken to indicate social preference. The arena was cleaned thoroughly before each test.

*Forced swim test (FST)*

In order to assess the effects of CSC on depression-like behaviour, SHC, GHC and CSC rats were tested in the modified FST as described before

[171, 218]. Briefly, between 0800 and 1100 h of day 24 (experiment 4), rats were individually placed into a Plexiglas tank (50 cm high x 29 cm in diameter) filled with 25 °C water to a depth of 30 cm for 10 min. Each session was recorded using a video camera placed in front of the cylinder for subsequent analysis. Afterwards, the behaviour, floating, struggling or swimming was manually quantified in a 5-s interval in the 600-s test period (total of 120 scores/ events) by an observer blind to treatment. Water was changed before each trial. The animal was removed, towel-dried and placed in a clean observation Plexiglas cage (40 x 24 x 36 cm) for a 2-h recovery period.

*Resident Intruder (RI) test*

In order to assess aggressive-like behaviour in SHC, GHC and CSC rats (experiment 4), we employed the RI test as described before [219, 220] on day 30. Therefore, after the 2-h recovery period on day 24, SHC, GHC and CSC rats in the Plexiglas cages (known as residents in this test) were transported to the neighboring experimental room and each housed with a sexually receptive female for 6 days in order to stimulate territorial behaviour [212]. The 12/12-h light-dark cycle switched to lights off at 1200 h. The RI test was performed on day 30 in the early dark phase (1 h after lights off), which is the most active phase in rats. At about 1300 h, the female was removed 30 min before the onset of the RI test. An unfamiliar, lighter (~10%) male con-specific was placed in the resident's home cage for 10 min. The test was videotaped and afterwards the aggressive behavioural patterns of the residents were scored by an experienced observer blind to treatment as explained in detail before [220]. These patterns consisted of attack, lateral

threat, offensive upright, keep down, threat, aggressive grooming and were scored in real-time using preset keys on a computer (Eventlog; Version 1.0, 1986, R. Hendersen) and calculated as percentage of overall test time. Additionally, the attack latency and number of attacks were counted.

*Statistical analysis*

For statistical analyses, the software package SPSS 18.0 (SPSS Inc., Chicago, IL, USA) was used. The following tests were used: the Mann-Whitney U test for behavioural analysis during CSC, the two-way analysis of variance (ANOVA) for repeated measures for ACTH and corticosterone levels in blood samples obtained from catheterized rats (factors treatment x time), and the one-way ANOVA (factor treatment) for all other readouts. Both ANOVA were followed by *Bonferroni post hoc* test if appropriate. Data are presented as mean ± SEM; $P \leq 0.05$ was considered statistically significant.

## Results

**Experiment 1:**

*Behavioural analysis during CSC.* During the first 2 h of colony formation on days 1, 8 and 15 of CSC exposure, only the larger male Long-Evans rats displayed offensive behaviours (Figure 10C), thus establishing the dominant position in each CSC colony. In contrast, CSC male Wistar rats only displayed defensive behaviours resulting in subordinate positions in each colony. Consequently, the numbers of threats, attacks, offensive uprights and keep downs shown by the dominant Long-Evans rats were significantly

higher compared with CSC rats (P < 0.001). *Vice versa*, CSC rats displayed significantly more freezing, defensive upright, and lying on the back postures compared with Long-Evans residents (P < 0.001; Figure 10C).

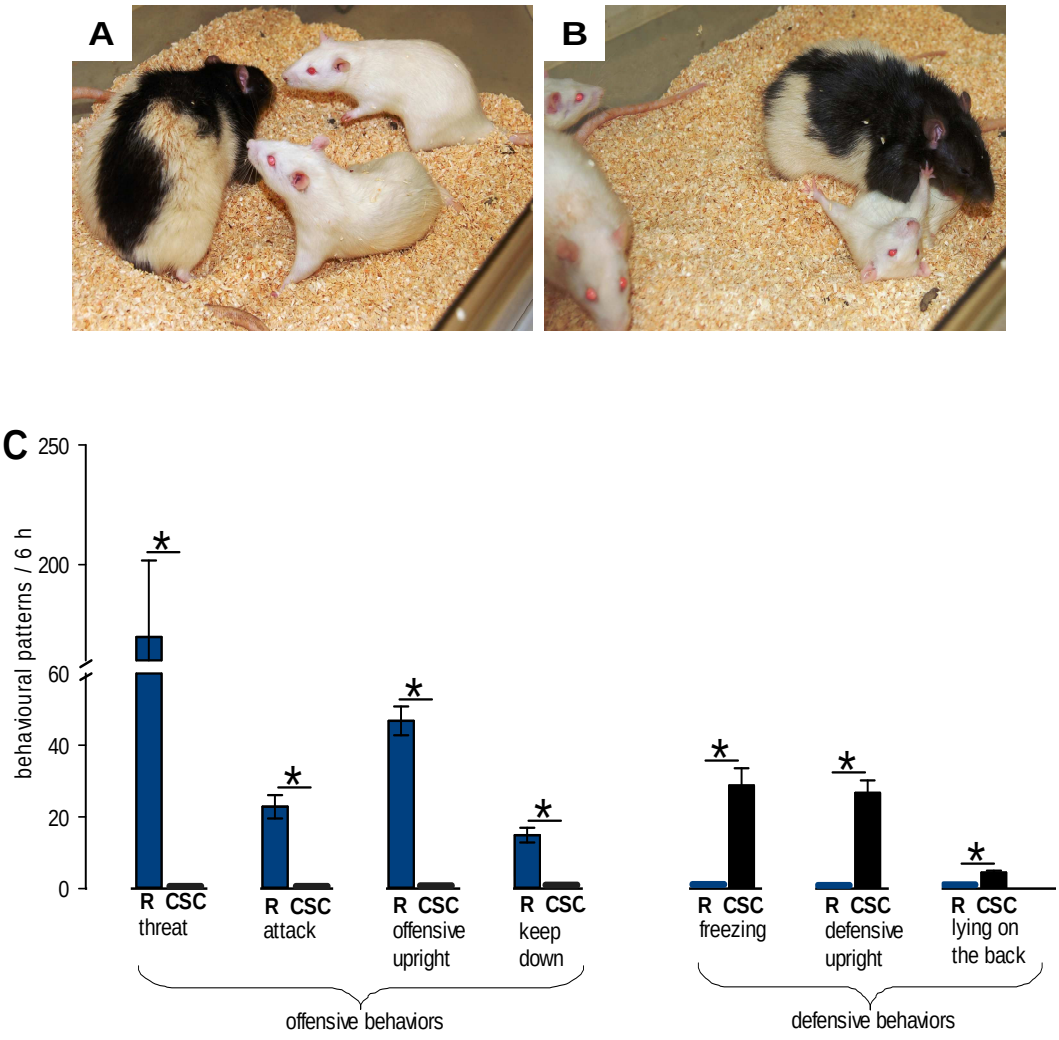


Figure 10: Offensive and defensive behavioural patterns displayed during chronic subordinate colony housing (CSC). Dominant Long-Evans rat threatens (A) and subsequently defeats intruder, maintaining it in a 'keep-down' position (B). Mean offensive and defensive behavioural patterns displayed by the Long-Evans residents (R; blue columns) and the CSC (black columns) rats during the first 2 h of CSC colony formation on days 1, 8 and 15 (3 x 2 h = 6 h). Long-Evans residents (n = 27) exclusively showed offensive behaviours including threat, attack, offensive and keep down. CSC rats (n = 35) exclusively

displayed defensive and submissive behaviours such as freezing, defensive upright and lying on the back. Data represent means ± S.E.M. * P < 0.05 vs. respective behaviour shown

*CSC effects on body weight gain, absolute thymus and adrenal weight and basal plasma corticosterone.* Statistical analysis revealed that CSC compared with both SHC and GHC rats showed a decrease in body weight gain ($F_{2,97}$ = 20.09; p < 0.001; p < 0.001 vs. both SHC and GHC; figure 11A) and absolute thymus weight ($F_{2,97}$ = 3.44; p = 0.036; p = 0.050 vs. SHC; p = 0.016 vs. GHC; figure 11B), whereas absolute adrenal weight ($F_{2,97}$ = 2.09; p = 0.129; figure 11C) and basal plasma corticosterone ($F_{2,97}$ = 0.28; p = 0.756; figure 11D) in trunk blood were not different between the groups. Relative thymus (SHC: 1.11 ± 0.06; GHC: 1.12 ± 0.04; CSC: 1.03 ± 0.04; $F_{2,97}$ = 1.43; p = 0.24) and adrenal (SHC: 0.15 ± 0.01; GHC: 0.14 ± 0.00; CSC: 0.15 ± 0.00; $F_{2,97}$ = 0.53; p = 0.590) weights remained unchanged after CSC.

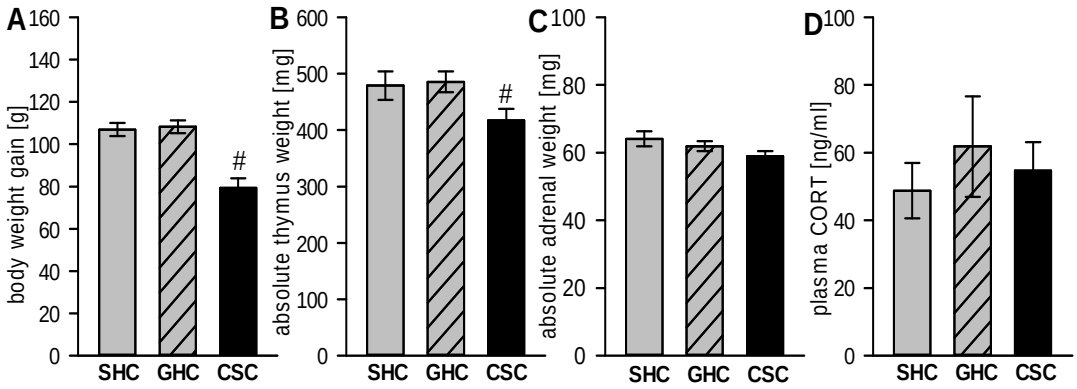


Figure 11: Effects of CSC on body weight gain, absolute thymus and adrenal weight, and basal plasma corticosterone. Nineteen days of CSC exposure (n = 35) resulted in a decreased body weight gain (A) and decreased absolute thymus weight (B) compared with single-housed controls (SHC, n = 25) and group-housed controls (GHC, n = 40), but caused

no changes in absolute adrenal weight (C) and basal plasma corticosterone (D). Data represent means ± S.E.M.; # $p < 0.05$ versus SHC and GHC.

**Experiment 2:**

*CSC effects on ACTH and corticosterone responses to an acute heterotypic stressor (open arm).* Open arm exposure (5 min) altered plasma ACTH (factor time: $F_{3,87} = 17.11$; $p < 0.001$; figure 12A) and corticosterone ($F_{3,93} = 33.90$; $p < 0.001$; figure 12B) levels and increased ACTH levels at 5 min (SHC: $p = 0.001$; GHC, CSC: $p < 0.001$ versus basal). Similarly, corticosterone levels were significantly increased at 5 (SHC, GHC, CSC: $p < 0.001$) and 15 min (SHC: $p = 0.003$; GHC: $p = 0.002$, CSC: $p < 0.001$ versus basal).

CSC prior to open arm exposure did not affect ACTH (factor treatment: $F_{2,29} = 0.48$; $p = 0.622$; figure 12A), but increased corticosterone responses (factor treatment: $F_{2,31} = 3.37$; $p = 0.047$; figure 12B) to open arm exposure. *Post hoc* comparisons revealed that CSC rats had higher plasma corticosterone levels compared with SHC (5 min: $p = 0.050$; 15 min: $p = 0.012$) and GHC (5 min: $p = 0.009$; 15 min: $p = 0.010$) rats.

In confirmation of results from experiment 1, basal plasma ACTH ($F_{2,29} = 1.88$; $p = 0.171$) and corticosterone ($F_{2,31} = 0.80$; $p = 0.457$) levels did not differ between groups (see insert, figures 12A/ B, respectively).

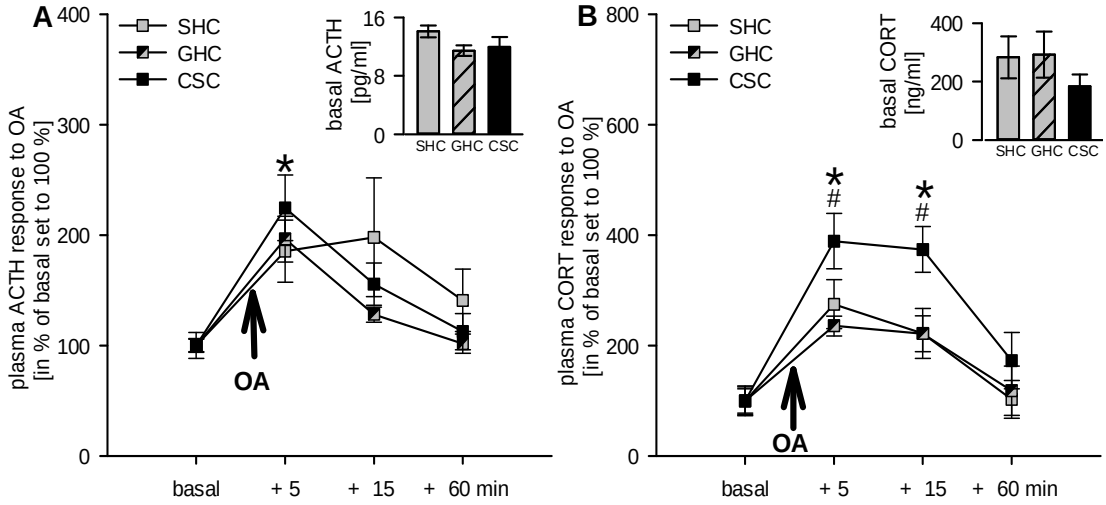


Figure 12. Effect of CSC on plasma ACTH and corticosterone responses to an acute heterotypic stressor. On day 20 after 19 days of CSC exposure, CSC (n = 11), together with single-housed (SHC; n = 11) and group-housed (GHC; n = 10 - 12) control rats were chronically catheterized. On day 25, i.e. 5 days after recovery from jugular vein catheter surgery, blood was sampled under basal conditions (2 basal 200-µl samples were taken 30 min apart and averaged), and 5, 15 and 60 min after open arm exposure (5 min). Whereas basal plasma ACTH (insert A) and corticosterone (CORT) (insert B) were not different between the groups, CSC rats showed a more pronounced plasma corticosterone (B) response 5 and 15 min following termination of open arm exposure compared with both SHC and GHC rats, despite comparable ACTH (A) responses between the different groups at all time points measured. Data represent means ± S.E.M.; # $p < 0.05$ versus SHC and GHC, * $p < 0.05$ versus respective basal values.

**Experiment 3:**

*Effects of CSC on colonic damage score, colonic mucus production and IFN-γ secretion from mesenteric lymph node cells***.** The histological damage score of the colon was not different between SHC, GHC and CSC rats ($F_{2,22} = 0.28$; $p = 0.759$; figure 13A). However, a decrease in the number of Alcian Blue positive epithelial cells in the colon ($F_{2,22} = 7.04$; $p = 0.004$; figure 13B) of

CSC compared with both SHC (p = 0.048) and GHC (p = 0.004) rats provides evidence of mild barrier deficits following CSC exposure. Increased *in vitro* IFN-γ secretion ($F_{2,9}$ = 5.19; p = 0.032; figure 13C) from anti-CD3-stimulated mesenteric lymph node cells of CSC compared with both SHC (p = 0.014) and GHC (p = 0.04) further indicates intestinal immune activation following CSC exposure.

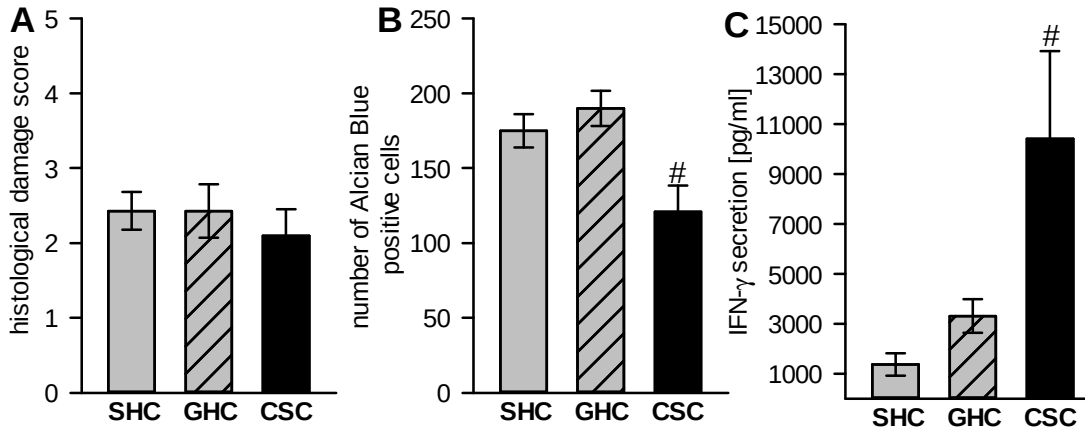


Figure 13. CSC effects on the histological damage score, colonic mucus production, and IFN-γ secretion. Nineteen days of CSC exposure (n = 4 - 7) did not alter the histological damage score in the colon (A), but decreased the number of Alcian Blue positive epithelial cells in the colon per 20 fields of view (B) and increased IFN-γ secretion from anti-CD3-stimulated mesenteric lymph node cells (C) compared with SHC (n = 4 – 7) and GHC (n = 4 – 11) rats. Data represent means ± S.E.M.; # p < 0.05 versus SHC and GHC.

**Experiment 4:**

*Effects of CSC on anxiety- and depression-related behaviours, social preference, and inter-male aggression (Table 3).* The percentage of time spent on the open arms of the EPM ($F_{2,20}$ = 2.34; p = 0.122) and in the light compartment of the LDB ($F_{2,21}$ = 1.64; p = 0.218), respectively, was not

different between SHC, GHC and CSC rats indicating that CSC exposure did not affect general non-social anxiety in rats. With respect to locomotor activity, GHC rats showed an increased number of entries into the closed arms during EPM testing ($F_{2,20}$ = 4.91; p = 0.018; p = 0.016 vs. SHC), but the number of line crossings / min in the dark compartment of the LDB of did not differ among groups ($F_{2,21}$ = 0.44; p = 0.648).

Furthermore, the number of floating events during the FST, indicative of depression-like behaviour ($F_{2,21}$ = 0.27; p = 0.767), as well as the number of struggling ($F_{2,21}$ = 0.35; p = 0.708) and swimming events ($F_{2,21}$ = 1.09; p = 0.35), indicative of active coping behaviour were not different between SHC, GHC and CSC rats.

In addition, CSC exposure did not affect social preference behaviour ($F_{2,21}$ = 2:79; p = 0.085), and did not induce signs of social avoidance.

Finally, no differences were found in the percentage time CSC rats (here used as residents during the RI test on day 10 after CSC) spent with aggressive behaviour ($F_{2,20}$ = 0.02; p = 0.977), in the attack latency ($F_{2,20}$ = 0.06; p = 0.946), and in the number of attacks ($F_{2,20}$ = 1.82; p = 0.189) during the RI test between SHC, GHC and CSC rats, indicating no CSC effect on inter-male aggression.

| Test | day | Readout | SHC | GHC | CSC |
|---|---|---|---|---|---|
| EPM | 20 | Time open arm (%)<br>closed arm entries (n) | 14.40 ± 5.89<br>5.38 ± 1.31<br>(n = 8) | 25.44 ± 4.88<br>11.29 ± 1.27*<br>(n = 7) | 29.92 ± 4.99<br>8.50 ± 1.36<br>(n = 8) |
| LDB | 21 | Time light box (%)<br>Entries dark box/min (n) | 13.54 ± 3.22<br>13.73 ± 1.30<br>(n = 8) | 16.50 ± 4.87<br>14.63 ± 1.71<br>(n = 8) | 22.84 ± 2.54<br>12.57 ± 1.62<br>(n = 8) |
| SPAT | 23 | Social preference (%) | 165.41 ± 14.07<br>(n = 8) | 154.85 ± 22.93<br>(n = 8) | 182.41±26.25<br>(n = 8) |
| FST | 24 | Floating events (n)<br>Struggling events (n)<br>Swimming events (n) | 69.25 ± 3.15<br>39.50 ± 3.28<br>11.25 ± 2.01<br>(n = 8) | 71.00 ± 1.18<br>36.25 ± 3.12<br>12.75 ± 3.06<br>(n = 8) | 72.75 ± 4.78<br>39.63 ± 3.29<br>7.63 ± 2.38<br>(n = 8) |
| RI test | 30 | Time aggression (%)<br>Attack latency (s)<br>Number of attacks (n) | 22.19 ± 7.06<br>424.58 ± 64.01<br>2.28 ± 0.92<br>(n = 8) | 18.93 ± 5.72<br>461.17 ± 69.16<br>5.0 ± 2.38<br>(n = 8) | 20.76 ± 5.48<br>473.09± 47.66<br>1.0 ± 0.44<br>(n = 7) |

Table 3: Effects of 19 days of CSC on non-social anxiety-related behaviour, social preference, depressive-like and aggressive behaviours. CSC did not alter non-social anxiety on the elevated plus-maze (EPM; day 20) and light-dark box (LDB; day 21), social preference behaviour as evaluated in the social preference/ avoidance test (SPAT; day 23), depressive-like behaviour as assessed in the forced swim test (FST; day 24), and inter-male aggression as measured during the resident-intruder test (RI test; day 30). Group size is presented in parentheses. Data represent mean ± S.E.M. * $p < 0.05$ versus SHC.

## Discussion

Our study shows that chronic subordinate colony housing (CSC) is an efficient chronic psychosocial stress paradigm also in male Wistar rats. In addition to a decrease in body and absolute thymus weight observed after 19 days of CSC, there are several other physiological, immunological and behavioural parameters which were found to be comparable between

chronically stressed rats and mice [68, 124]. Thus, CSC rats showed increased IFN-γ secretion from isolated and anti-CD3-stimulated mesenteric lymph node cells, a decreased number of mucus producing epithelial cells in the colon, and unaffected basal plasma corticosterone levels, whereas, most importantly, plasma corticosterone response to subsequent acute open arm exposure was found to be exaggerated. Further in line with our mice data, CSC rats neither showed a reduction in social preference nor developed depressive-like behaviour, but, and this is in contrast to CSC mice, stressed rats did not show an increase in general anxiety-related behaviour.

Extending the finding of a more pronounced corticosterone response to a heterotypic stressor to another species, namely rats, in the present study substantially strengthens our hypothesis drawn from recent mouse data [107]. Thus, CSC-induced changes at the level of the adrenal gland, i.e. increased *in vivo* ACTH responsiveness during acute heterotypic stressor exposure, but no significant changes in basal plasma corticosterone levels may represent a beneficial adaptation to, rather than a maladaptive consequence of chronic psychosocial stressor exposure. This allows a chronically stressed organism to adequately respond to a novel threat, while preventing prolonged exposure to high basal levels of deleterious glucocorticoids. Thus, the present study provides support for the general value of the CSC paradigm to reveal physiological or behavioural adaptations as a consequence of chronic psychosocial stress not only in mice, but also in rats.

*Choice of adequate control group*

For meaningful data interpretation, it is essential to choose an adequate control group. As this issue is still controversially discussed for rats, we decided to consistently employ both SHC and GHC rats as adequate controls. This is in contrast to studies performed in male mice, where we [123] and others [221, 222] convincingly showed that single housing is an adequate and stress-free housing condition lacking any stress-relevant behavioural, physiological or immunological symptoms. However, there are several studies demonstrating that single housing of rats *per se* is stressful and may result in anxiety- and depressive-like phenotypes [223, 224], as well as in major deficits in sexual behaviour [225]. One possible explanation might be that the consequences of housing conditions are strain- [226] or sex-specific [227, 228]. Of considerable importance seems to further be the duration of social isolation, which varies in the above mentioned studies from 8 to 15 days [224, 227] up to 10 or even 13 weeks [223, 225, 228]. Given that SHC and GHC rats in the current study did not differ in any physiological, neuroendocrine or immunological parameters investigated, we are convinced that both control groups can still be employed, with consideration of the above mentioned factors.

*Confirmation of chronic stress consequences*

For validation of a chronic stress model, for example in a novel species, several key parameters need to be affected. For example, a decrease in body weight gain, as observed in the subordinate male CSC compared with

both SHC and GHC rats, is a relevant physiological marker of chronic psychosocial stress [55, 68, 229, 230].

Another important indicator for CSC depicting a relevant chronic psychosocial stress model for male rats [231, 232] is the development of a pronounced thymus atrophy which we could find in CSC compared with both SHC and GHC rats. Previous studies employing 24-h social defeat stress suggested that thymus atrophy is mediated by a decrease in the number of thymocytes, specifically the $CD4^+$ and $CD8^+$ subpopulation [133, 231] and, thus, reduces the functional capacity of T cells. Given that in this respect glucocorticoids have been shown to play an important role [233], we hypothesize that, like in CSC mice [68], increased plasma corticosterone levels during the initial phase of CSC exposure may initiate thymus atrophy. Moreover, the medullary part of the thymus exhibits a high density of ß-adrenergic receptors [234] involved in cAMP-mediated thymocyte apoptosis [235] and loss of thymus mass [231]. Although further studies need to investigate this in detail, it is further likely that the sympathetic-adrenomedullary system is chronically activated [236, 237] in CSC rats. Therefore, glucocorticoid-initiated thymus involution could be maintained during CSC exposure by an increased drive of the sympathetic nervous system.

Although adrenal hypertrophy is generally referred to as an accepted indicator of chronic stress in rats and mice [56, 58], and has recently been shown in CSC mice [107], there are studies showing that especially in rats, an increase in adrenal mass is not a reliable biomarker for chronic stress. For instance, 19 to 28 days of subordination during colony housing in male Wistar rats did not result in adrenal hypertrophy, despite a decreased body weight

gain and thymic involution [232]. Furthermore, exposing rats to the visible burrow system as an accepted animal model of chronic psychosocial stress does not reliably result in an increase of adrenal mass [117]. Support comes also from recent own data, showing that repeated restraint and overcrowding stress increases adrenal mass in lactating female rats, but not in virgins [238]. Thus, the lack of a CSC effect on absolute rat adrenal mass as seen in the present study is in contrast to what is repeatedly described for CSC mice, but is in line with other rat studies showing no effect of chronic stress on adrenal weight. Further studies need to be carried out; perhaps even in different rat strains, to properly dissect the underlying adrenal mechanisms during CSC exposure in the rat.

In contrast to the species-dependent effects of CSC exposure on absolute adrenal mass in mice and rats, CSC did not affect basal, but acute stress-induced HPA axis function in both species. In detail, comparable to CSC mice [68, 107], CSC rats showed unaffected basal morning plasma corticosterone levels, but an exaggerated plasma corticosterone response to an acute heterotypic emotional stressor, relevant for rats [180], i.e. 5-min exposure to the open arm of an EPM (compared with both SHC and GHC rats). Given that plasma ACTH response to open arm exposure was comparable between SHC, GHC, and CSC rats, this suggests either an increased *in vivo* ACTH responsiveness of the CSC rat adrenal cortex during acute heterotypic stressor exposure or glucocorticoid secretion to be driven by an additional corticosterone secretagogue different from ACTH. Again, this is strongly in line with our mouse CSC data, showing an increased corticosterone, but unchanged ACTH secretion in response to acute elevated

platform exposure following CSC [107]. However, in contrast to CSC mice, absolute adrenal mass is not increased in CSC rats. This suggests development of morphological/ histological/ cellular adrenal changes different from just an increase in absolute adrenal weight, enabling rat CSC adrenals to show an exaggerated response to acute heterotypic stressors. Given that basal plasma corticosterone levels are not increased in CSC rats, adrenal ACTH responsiveness and/ or release of the above mentioned corticosterone secretagogue seems to be enhanced specifically during acute stress. This allows a chronically stressed organism to adequately respond to a novel threat, but protects it from being exposed to deleterious consequences of basally elevated plasma corticosterone levels. However, detailed investigation is required to confirm the *in vitro* reactivity of the rat adrenals to ACTH.

Increased secretion of the pro-inflammatory cytokine IFN-γ from isolated mesenteric lymph node cells and decreased colonic sulfomucins have turned out to be reliable and sensitive indicators of mild colonic inflammation and breakdown of epithelial barrier function, induced by CSC exposure in mice [55, 68, 128]. Similarly, the finding of increased IFN-γ secretion from isolated mesenteric lymph node cells together with the decreased number of colonic sulfomucin positive epithelial crypt cells in CSC compared with SHC and GHC rats observed in the present study suggest that CSC, in a species-independent manner, causes colonic barrier defects coupled with intestinal immune activation. Additional studies are needed to characterize CSC-induced cellular alterations in the colon in more detail, by investigating, for instance, mast cell activation/ infiltration and mechanisms of intestinal barrier

dys-functions as performed in comparable studies following chronic crowding stress in rats [114, 239]. However, the lack of detectable histological damage in CSC rats, which is in contrast to our findings in CSC mice [68], may either represent a species-specific protection of epithelial barrier function, or higher stress resilience in male Wistar rats compared with male C57Bl6 mice.

The latter is further supported by the fact that CSC rats - in contrast to CSC mice [68, 100, 107, 122-125] - do not develop increased anxiety-related behaviour, which has been monitored right at the end of (EPM, day 20 of CSC) or one day after termination of CSC exposure (LDB, day 21). We have recently shown that elevated levels of anxiety-related behaviour can appear as late as 9 days following termination of chronic psychosocial stress in mice [124]. Therefore, the possibility exists that alterations in anxiety-related behaviour develop at a later time point in CSC rats. Support for this option comes from McCormick and colleagues [240] showing increased anxiety-related behaviour on the EPM in male rats 25 days after termination of a 2-week social stress procedure. Although the lack of behavioural alterations following chronic stress exposure is rather untypical in rodent studies, it offers the unique possibility to dissect the mechanisms underlying stress-induced somatic pathologies without conflicting affective alterations. However, future studies are needed to assess CSC effects on anxiety-related behaviour also at later time points.

Our finding of unchanged social preference behaviour in the SPAT and lack of a depressive-like phenotype in the FST following CSC in rats are well in line with our recent mice studies [124]. Moreover, CSC rats displayed adequate aggressive behaviour, reflected by unchanged attack latencies and

number of attacks during the RI test comparable to those seen in both control groups. Whether the unknown con-specific is confined in a wire mesh cage (as in the SPAT) or interacts as intruder actively with the experimental animal (as in the RI test), CSC exposure does not seem to influence both the passive and active social interaction behaviour of these animals.

In summary, our results further confirm and extend the validity of the CSC paradigm as a relevant chronic psychosocial stressor also in male Wistar rats. The present study reveals that CSC rats (i) show adaptive, rather than mal-adaptive HPA axis changes at the level of the adrenal gland, (ii) develop mild colonic barrier defects and intestinal immune activation and (iii), show no anxiety- or depressive-like behaviours, no increase in aggression, and do not lose their social preference.

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# Chapter 4

# Risk factors increasing the vulnerability to chronic psychosocial stress in male Wistar rats.

**Author's contribution:**

**Nyuyki:** study design (figure 14), performing CSC paradigm and assisted in prenatal stress procedure (figure 15), organ preparation (figures 15, 16), assisted in plasma corticosterone quantification (figures 15, 16), performing all behavioural experiments during CSC (table 4), analyzing data, writing first draft of manuscript

**Hillerer:** performing prenatal stress procedure (figure 15)

**Slattery**: performing prenatal stress procedure (figure 15)

**Neumann**: study design (figure 14), revision of manuscript

**Reber:** study design (figure 14), assisted in performing CSC paradigm, organ preparation and quantification of histological damage score (figures 15, 16), revision of manuscript

[Adapted from: Kewir D. Nyuyki, Katharina M. Hillerer, David A. Slattery, Inga D. Neumann and Stefan O. Reber; Stress, in preparation]

## Abstract

Exposure to adverse early life events, as well as genetic predisposition is a documented risk factor for the development of psychiatric and somatic disorders in adulthood. Additionally, stress exposure during pregnancy can program adult behaviour and HPA axis responsiveness later in life. Maternal separation, a model of adverse early life treatment, together with chronic subordinate colony housing (CSC) in adult mice leads to increased CRH mRNA expression in the paraventricular nucleus, and exacerbated colonic inflammation and immune dys-regulation following administration of dextran sodium sulphate in mice. In the present study, therefore, we exposed prenatally stressed [prenatal stress: alternating overcrowding (24 h) and restraint from postnatal day 4 to 14] male Wistars to the CSC paradigm. Further, we used selectively bred Wistar rats for high (HAB) and low (LAB) anxiety-related behaviour to study genetic influences on CSC vulnerability, and analyse changes in relevant behavioural, physiological and immunological parameters. Nineteen days of CSC exposure resulted in a decreased body weight gain and pronounced histological damage score in Prentally stressed rats, but no alterations in adrenal weight and plasma corticosterone compared with non-prenatally stressed rats. On the other hand, no gene x environment interactions were observed in HAB nor LAB animals, but CSC exposure resulted in a decreased body weight gain, thymus atrophy, unchanged adrenal and plasma corticosterone in both lines. In conclusion, prenatal stress and subsequent exposure to chronic psychosocial stressor in adulthood worsens the outcome of CSC in rats,

induce colonic damage, but genetically determined differences in innate-anxiety do not influence the susceptibility to CSC in adulthood.

## Introduction

The vulnerability to stress in adulthood can be affected by several factors. In humans for example, exposure to early life stress is known to increase the risk for developing stress-related psychopathologies including anxiety and depression disorders [149, 241, 242], and schizophrenia [243, 244]. Moreover, adverse early life experiences in the form of social isolation, maltreatment and socioeconomic disadvantage have been shown to greatly increase the risk for colonic inflammation and clustering of metabolic risk markers [245].

In line, numerous rodent studies have reported behavioural, endocrine and neurobiological mal-adaptations in adulthood following prenatal stress (PS) [155, 246] and postnatal adverse experiences [157, 247]. In detail, PS in rats, has been shown to alter reproductive behaviour [248, 249], increase anxiety- and depressive-related behaviour [250-253], reduce social behaviours [254], and impair cognitive functions [255]. Moreover, increased reactivity of the HPA axis to acute emotional stressors in rats [165, 183, 256] has also been reported after PS.

In addition to early life events, inborn differences in personality and emotional reactivity strongly shape individual response to stress. In humans, the risk for developing depression after stressful life events has been proved to be greatly magnified, if an individual carries a genetic predisposition for this disorder i.e., polymorphism in the promoter region of the 5-HTT gene [108, 257]. Other human research implicate the role of BDNF Val66Met polymorphism in depression [258, 259] as well as anxiety [260]. In concordance with human reports, inter-strain differences have been shown to

exist in the HPA axis response to acute stressors in rats [164] and mice [261]. Furthermore, besides inter-strain differences, compelling evidence shows that rats and mice within a given strain may exhibit individual differences in stress reactivity, making them more or less sensitive to stress [180, 262, 263]. For example, in high anxious rats and low anxious mice, single nucleotide polymorphisms in the promoter region of the vasopressin gene in the hypothalamic paraventricular nucleus have been associated with increased emotionality and stress responsiveness [168]. Therefore, both environmental and genetic factors pose important risk factors for some individuals to develop affective and/ or somatic pathologies, including anxiety, depression, inflammation-related disorders and a dys-regulated HPA axis response to acute stressors

So far, long lasting effects of PS as well as of genetic factors have been studied mainly on basal etiology, physiology and immunology, or on the response to acute emotional stressors [183, 253, 256, 264, 265]. However, it is chronic stress, especially CPS, during adulthood that has been mainly implicated as a risk factor for developing stress-related pathologies in humans [45, 109, and 204]. Interestingly, own mouse data provided first evidence that early life stress, induced by maternal separation, strongly increases the vulnerability for CPS, induced by the chronic subordinate colony housing (CSC) paradigm, during adulthood in male C57BL6 mice [125]. This was indicated by additive effects of early life and adulthood stressor exposure on increased CRH mRNA expression in the paraventricular nucleus and exacerbated colonic inflammation and immune dysregulation following administration of dextran sodium sulphate in drinking

water. However, the influence of genetic factors on the vulnerability to CSC in mice has not been assessed so far.

The main aims of the present study were, therefore, i) to extend our knowledge on early life stress increasing the vulnerability for CPS during adulthood in the prenatal period and to another species, namely male rats, and ii) to investigate, if genetically determined differences in innate anxiety affect vulnerability to CPS. Early life stress, which in the current study is PS, was induced according to the protocol recently established and validated in our group [238]. Chronic psychosocial stress during adulthood in male offspring of prenatally stressed dams was induced by the CSC paradigm, which we recently validated as an adequate animal model of CPS for male Wistar rats (chapter 3). The influence of genetic factors on the vulnerability to CSC in adulthood was assessed employing male Wistar rats selectively bred for extremes in emotionality i.e., high (HAB) and low (LAB)-anxiety related behaviour [163]. Given that HAB compared with LAB rats show increased depressive-like behaviour [167], and HPA axis responsiveness to acute emotional stressors [177, 180], we tested whether HAB and LAB rats would differ in their vulnerability to CPS.

## Materials and Methods

*Animals*

Experiments were performed using male (400-450 g; dominant) and female (300-350 g) Long Evans; male (300-350 g) and female (200-250 g) Wistar rats (Charles River, Sulzfeld, Germany), and male Wistar rats selectively

bred for high (HAB: 300-350 g) or low (LAB: 300-350 g) anxiety-related behaviour in the animal facilities of the University of Regensburg [266].

Each Long-Evans resident male was permanently housed with a Long Evans female and single-housed 1 h before the start of CSC colony formation (see below). Male and female Wistar rats were mated, and pregnant rats housed together with nulliparous females (otherwise known as virgins) in groups of 4 i.e., 2 pregnant and 2 virgin rats until pregnancy day (PD) 4, the onset of chronic prenatal stress (see below).

After weaning HAB and LAB pups on post natal day (PND) 21, they were housed in groups of 3 to 4 up till the age of 9 weeks, when they were tested on the elevated plus-maze (EPM) by an observer blind to breeding line. All animals were kept under standard laboratory conditions (12:12 light–dark cycle, lights on at 0600 h, 22°C, 60 ± 5 % humidity) and had free access to tap water and standard rat chow. Unless otherwise mentioned, animals were mostly housed in standard rat cages (cage size 55 × 35 × 20 cm). All experimental protocols were approved by the Committee on Animal Health Care of the local government of Oberpfalz and conformed to international guidelines on the ethical use of animals

*Prenatal stress procedure*

This was done as previously described by Hillerer KM et al [238]. Briefly, from PD 4–PD 16, stressed rats were exposed alternatively to daily restraint stress (RS: 2 × 1 h at least 3 h apart during the light phase; Plexiglas column with ventilation holes; 12 cm diameter) and overcrowding (OC: 4 unfamiliar rats housed together for 24 h; cage size: 40 × 25 × 15 cm). On the days RS was

done, rats were single- housed (cage size: 40 × 25 × 15 cm) in their respective cages and taken from individual housing into overcrowding conditions the following day, resulting in social instability. From PD16, stressed rats were single-housed, the same way unstressed controls housed in groups of 4 were treated.

After birth, litters from either mums exposed to PS and control (non-prenatal stress: NS) were culled to 8 pups to ensure comparable conditions across all dams. On PND 21, pups were weaned and housed in groups of 4 as previously mentioned until the start of experiments in adulthood. Not more than 2 pups per litter were used for each experimental subgroup.

*Chronic Subordinate colony housing (CSC)*

The CSC paradigm was performed as described in chapter 3. Briefly, 4 male Wistar rats were housed together with a larger male Long Evans rat in its home cage (cage size: 56 x 39 x 20 cm) for 19 consecutive days. Prior to CSC, the male Long Evans rats were tested for their aggressive behaviour. Males that started to injure and bite their opponents were excluded. To avoid habituation, each larger Long Evans male was replaced by a novel one on days 8 and 15. Both single housed (SHC: cage size 40 × 25 × 15 cm) and group housed (GHC) control rats were used for comparison. Both control groups were kept in separate animal rooms from the CSC colonies and remained undisturbed in their home cages except for change of bedding once a week. Body weight development was assessed.

*Experimental procedures*

**Experiment 1**. PS effects on anxiety-related behaviour, physiological, and neuroendocrine parameters after CSC. At adulthood (week 12), NS rats, and those exposed to PS were assigned to 3 experimental groups i.e., SHC, GHC and CSC for 19 consecutive days. In the morning (between 0800 h and 1200 h) of the 19$^{th}$ day, all experimental groups were exposed to the EPM, to test anxiety-related behaviour and the next day (day 20), they were rapidly decapitated between 0800 and 1000 h (figure 14). Trunk blood was collected on pre-chilled EDTA-coated tubes for subsequent quantification of plasma corticosterone levels. Changes in adrenal and thymus weights were determined (see below). Moreover, the colon was removed to determine the histological damage score as an already established indicator of colonic inflammation in rodents [68, 267].

**Experiment 2.** Effects of gene x environment interactions on anxiety-related behvaviour, physiological, and immunological parameters after CSC

HAB and LAB rats were assigned into control (SHC and GHC) or CSC groups for 19 consecutive days. In the morning of day 19, a randomly selected subset of the animals was exposed to the light dark box (LDB), in order to determine CSC effects on anxiety-related behaviour. The following day (day 20), all experimental animals were rapidly decapitated between 0800 h and 1000 h and collected trunk blood handled as previously described in experiment 1 (see figure 1). Similarly, adrenal and thymus weights were determined, and the histological damage score of the colon in a randomly selected subset of animals was also quantified.

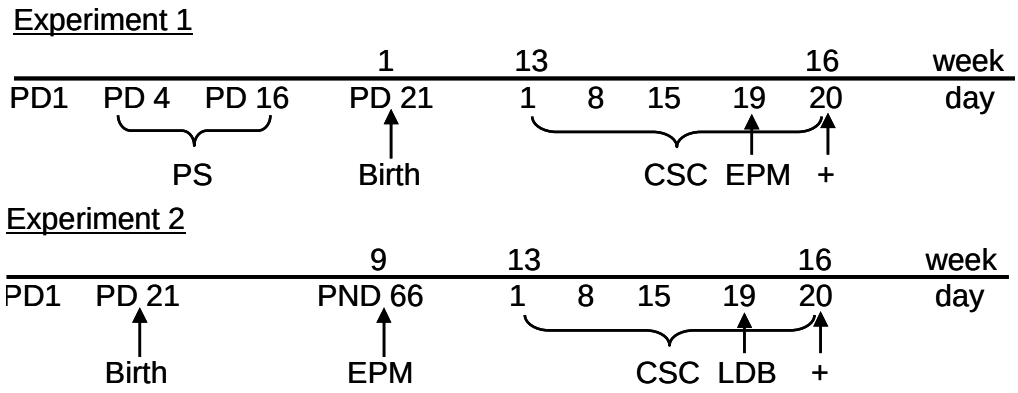


Figure 14: Experimental design. PS [alternating overcrowding (24 h) and restraint (2 x 1 h)] was carried out from PD 4 to 16 (experiment 1). In experiment 2, offspring from the HAB and LAB rats were selected on the EPM based on their anxiety-related behaviour 66 days (PND 66) after birth (which occurred on PD 21). On week 12, male offspring, from both PS and NS dams (experiment 1), and those selected from experiment 2 (HAB/LAB), were exposed to 19 days of CPS (CSC, in which 4 males were housed with a dominant Long Evans male, which was replaced by a novel dominant male on days 8 and 15). Single housed and grouped housed (4 rats without the dominant Long Evans) served as controls i.e., SHC and GHC respectively. In the morning of day 19, anxiety-related behaviour of PS/ NS (Experiment 1), and HAB/ LAB (experiment 2) rats, subjected to CSC as well as their controls was assessed on the EPM and LDB respectively. In the morning of day 20, all rats were killed (+) in order to investigate the effects of CSC exposure on relevant physiological and immunological parameters. PS; prenatal stress, NS; non-prenatally stress, PD; pregnancy day, PND; post natal day.

*Elevated plus-maze (EPM)*

In the evening of day 18, SHC, GHC and CSC rats of experiment 1 were transported to the EPM room. The next day, they were exposed to the EPM between 0800 and 1200 h for 5 min as described before [268], and anxiety-related behaviour was assessed. The EPM consisted of two opposing open

(50 × 10 cm; 140 lux) and closed (50 × 10 × 40 cm; 20 lux) arms connected by a central platform (10 x 10 cm) and elevated 80 cm above the floor. In the beginning of the 5-min test period, each rat was placed in the central platform facing a closed arm and allowed to explore the maze. Before each test, the maze was thoroughly cleaned. The time spent in the respective arms was recorded by means of a video/ computer setup to allow calculation of the percentage of time spent on the open arms of the maze. Further, the number of entries into the closed arms was recorded as a measure of locomotor activity.

*Light-Dark Box (LDB)*

The LDB is also a well established and validated test for anxiety in rodents [215]. In a similar way to experiment 1, SHC, GHC and CSC rats were moved to the LDB room 24 h before they were tested on day 19. In the morning of day 19 between 0800 and 1200 h as described before [268], the animals were exposed to the LDB. Briefly, the LDB consisted of a brightly lit (40 × 50 cm; 350 lux) and a dark (40 × 30 cm; 70 lux) compartment separated by a partition wall that had an opening (7.5 cm) at floor level. Rats were individually placed in the dark box facing the opening and allowed a 5-min free exploration time. The time spent in the respective compartments was recorded by means of a video/ computer setup to allow calculation of the percentage of time spent in the lit box. The number of line crossings/ minute in the dark compartment was taken as an indicator of locomotor activity. The LDB was cleaned thoroughly before each test.

*Blood sampling and Enzyme-Linked Immunosorbent Assay (ELISA) for corticosterone*

To determine the effect of CSC on plasma corticosterone concentration, SHC, GHC and CSC rats were rapidly killed by decapitation. About 100 µl of trunk blood was collected in EDTA-coated tubes on ice (Sarstedt Nümbrecht, Germany) and centrifuged at 4 °C (5000 rpm, 10 min). Plasma samples were stored at –20 °C until assayed using a commercially available ELISA kit for corticosterone (analytical sensitivity < 1.631 nmol/l, intra-assay and inter-assay coefficients of variation ≤6.35%, IBL International, Hamburg, Germany).

*Determination of thymus and adrenal weights*

After decapitation, the thymus and both adrenal glands were removed, carefully pruned from fat tissue and weighed in order to assess CSC effects on thymus and adrenal weight. The left and right adrenals were weighed together. All values represent absolute (g) weights.

*Determination of the histological damage score of the colon*

Assessment of the histological damage score was performed as previously described [68, 100]. In order to assess the effect of CSC on histological markers of the distal part of the intestinal tract, the colon was removed and mechanically cleaned. Afterwards, 1 cm of the distal third of the colon was cut longitudinally, laid on a filter paper and fixed in 10 % formalin overnight. The next day, the fixed tissue was embedded in paraffin and cut longitudinally. Two 3 µm haematoxylin-eosin stained sections taken at 100

µm distance were evaluated by histological scoring performed by an investigator blind to treatment. For statistics, each individual score represented the mean of the 2 sections. Histological damage score ranges from 0 to 8 and represent the sum of the epithelium score *(*0: normal morphology; 1: loss of goblet cells; 2: loss of goblet cells in large areas; 3: loss of crypts; 4: loss of crypts in large areas) and infiltration score (0: no infiltration; 1: infiltrate around crypt basis; 2: infiltrate reaching to lamina muscularis mucosae; 3: extensive infiltration reaching the lamina muscularis mucosae and thickening of the mucosa with abundant oedema; 4: infiltration of the lamina submucosa).

*Statistics*

For statistical analysis, the software package SPSS 20.0 (SPSS Inc., Chicago, IL, USA) was used. A two-way analysis of variance (ANOVA; factor PS/strain and CSC) were used unless otherwise stated, followed by *Bonferroni post hoc* test if appropriate. Data are presented as mean + SEM; $P \leq 0.05$ was considered statistically significant.

## Results

### Experiment 1.

*PS effects on behavioural, physiological and immunological parameters*.

*EPM.* Anxiety-related behaviour, reflected by the percent time spent on the open arms of the maze, and locomotion, as seen in the number of closed arm entries was neither affected by prenatal stress nor CSC exposure, as well as

an interaction of both (percent open arm: factor PS x CSC $F_{2,64}$ = 0.172; P = 0.842, closed arm entries: factor PS x CSC $F_{2,64}$ = 0.909; P = 0.408). See table 4

*Body weight gain*. Regardless of the experimental group, PS rats significantly gained less body weight compared with NS rats (factor PS $F_{1,69}$ = 12.017; P = 0.001, figure 15A). Furthermore, CSC exposure significantly reduced the body weight gained (factor CSC $F_{2,69}$ = 3.195; P = 0.047), although *post hoc* testing revealed no further significance. However, separate statistics revealed that CSC-stressed PS rats gained less body weight compared with their respective controls (SHC: P = 0.026, GHC: P = 0.024; one way ANOVA) and CSC-stressed NS rats (P = 0.004; student's t test).

*Thymus, Adrenal weights and plasma corticosterone.* No interactive effect of PS and CSC were found on absolute thymus (factor PS x CSC $F_{2,69}$ = 2.081; P = 0.133; figure 15B) and adrenal weights (factor PS x CSC $F_{2,69}$ = 0.344; P = 0.710; figure 15C). However, separate statistics on the absolute thymus weight revealed that CSC-stressed PS rats had lower thymus weights compared with CSC-stressed NS rats (P = 0.051 [tendency]; student's t test. Moreover, neither prior stress exposure (factor PS $F_{1,67}$ = 0.121; P = 0.729) nor CSC (factor CSC $F_{2,67}$ = 0.186; P = 0.831; figure 15D) significantly affected plasma corticosterone concentrations.

*Histological damage score*. An effect of CSC was found after scoring the degree of damage of the colon (factor CSC $F_{2,69}$ = 5.449; P = 0.006; figure

15E). This effect was more pronounced only in CSC-stressed PS rats whereby, *post hoc* comparisons revealed a significantly higher histological damage score compared with their respective controls (P = 0.017 vs. SHC; and P = 0.003 vs. GHC). Additionally, separate statistics revealed that CSC-stressed PS rats had significantly higher histological scores (P = 0.021; student's t test) compared with CSC-stressed NS rats.

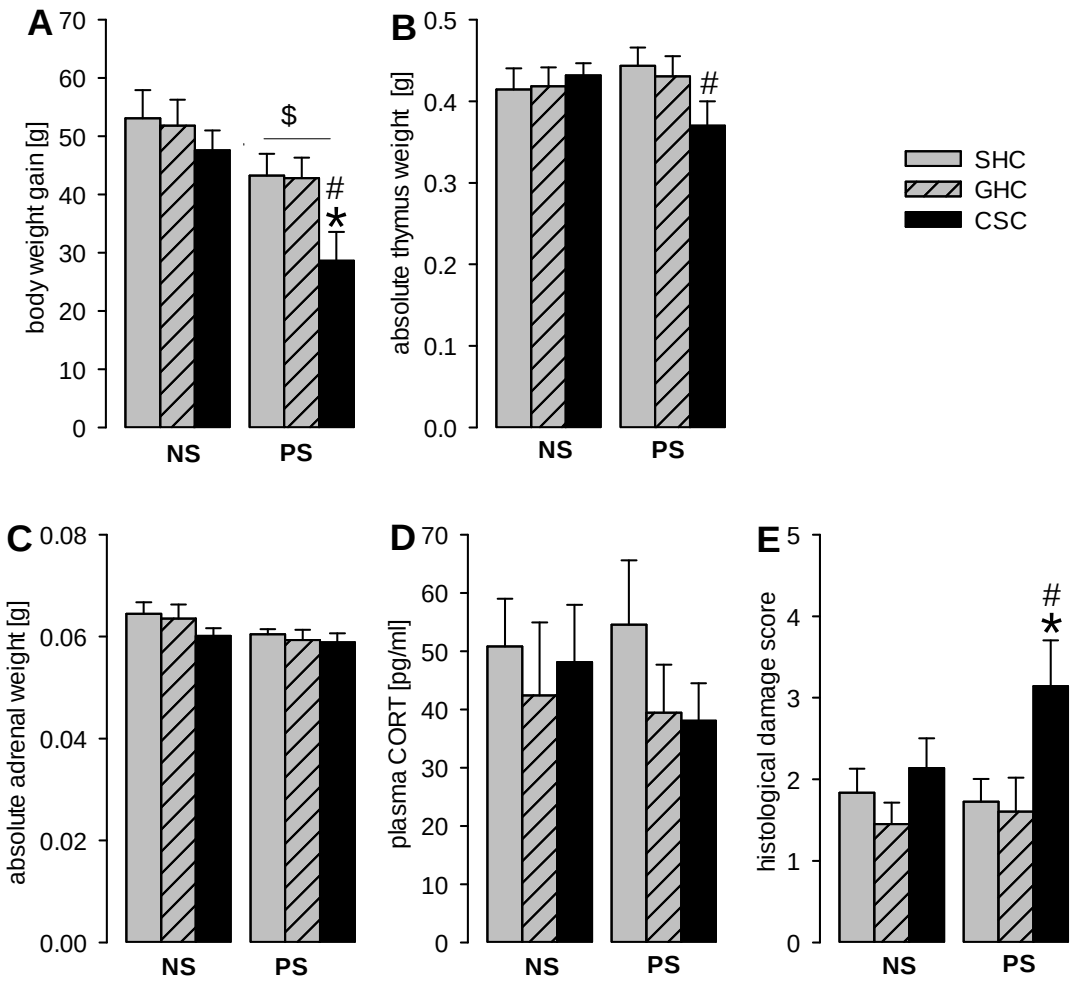


Figure 15: Effects of PS on body weight gain (A), absolute thymus (B) and adrenal weights (C), basal plasma corticosterone (D) and histological damage score (E) after exposure to 19 days of CSC. Number of rats in all groups ranges from 10 and 16. Data represent mean +

SEM. * P < 0.05 vs. respective single-housed controls (SHC) and group-housed controls (GHC), # P < 0.05 vs. NS CSC and $ P < 0.05 vs. all NS groups

**Experiment 2**.

*Gene x environment interaction on state anxiety, physiological, and immunological parameters after CSC*

*LDB:* Strain differences in anxiety-related behaviour between HAB and LAB rats were confirmed on the LDB, as HAB rats spent significantly less time in the lit compartment compared with the LAB rats (factor strain $F_{1,41}$ = 96.423; P < 0.001). However, CSC exposure did not further alter the anxiety-related behaviour of these animals, regardless of the strain (factor strain x CSC $F_{2,41}$ = 1.541; P = 0.226), but separate statistics revealed that CSC-stressed LAB animals spent significantly more time in the lit compartment of the LDB (P < 0.001; student's t test). Moreover, locomotion, assessed by the number of closed arm entries did not differ in both strains, irrespective of CSC treatment (factor strain $F_{2,41}$ = 1.748; P = 0.187). See table 4.

| Test | Day | Readout | Group | SHC | GHC | CSC |
|---|---|---|---|---|---|---|
| EPM | 19 | time OA [%] | NS | 18.7 + 8.6 (n = 7) | 25.6 + 5.3 (n = 16) | 33.8 + 10.6 (n = 15) |
| | | | PS | 12.4 + 5.0 (n = 10) | 27.0 + 5.0 (n = 12) | 33.4 + 7.9 (n = 10) |
| | | CA entries [n] | NS | 9.7 + 0.7 (n = 7) | 7.8 + 0.6 (n = 16) | 9.1 + 0.7 (n = 15) |
| | | | PS | 14.5 + 4.0 (n = 10) | 8.1 + 1.0 (n = 12) | 9.7 + 1.3 (n = 10) |
| LDB | 19 | time LB [%] | HAB | 1.9 + 1 (n = 7) | 2.9 + 2 (n = 8) | 4.5 + 2 (n = 8) |
| | | | LAB | 33.0 + 5.0$^{\$}$ (n = 8) | 22.8 + 4.0$^{\$}$ (n = 8) | 32.1 + 4.0$^{\$}$ (n = 8) |
| | | line crossings DB/min [n] | HAB | 8.4 + 2.0 (n = 7) | 10.7 + 1.0 (n = 8) | 8.7 + 1.0 (n = 8) |
| | | | LAB | 10.5 +3.0 (n = 8) | 9.7 + 2.0 (n = 8) | 17.0 + 5.0 (n = 8) |

Table 4. Anxiety-related behaviour analysed in the EPM and LDB on day 19 of CSC. Neither prior PS exposure nor genetic differences influenced state anxiety on the EPM (percent time open arm) and LDB (percent time spent in light compartment) respectively. Locomotion indicated by the number of closed arm (CA) entries and line crossings in the dark box (DB)/min also remain unaltered. Group sizes are represented in parenthesis. $ P < 0.05 HAB animals.

*Body weight gain*. Irrespective of the strain differences, exposure to CSC significantly decreased the body weight gained (factor CSC $F_{2,90}$ = 35.312; P < 0.001). *Post hoc* comparisons further revealed that CSC stressed animals gained significantly less body weight compared with both SHC (HAB/LAB: P < 0.001) and GHC (HAB P = 0.002, LAB: P < 0.001; figure 16A).

*Thymus, Adrenal weights and plasma corticosterone*. The absolute weight of the thymus of HAB and LAB animals significantly differed (factor strain $F_{1,90}$ = 51.157, P < 0.001) from each other, and exposure to CSC further led to thymus atrophy (factor CSC $F_{2,90}$ = 11.255, P < 0.001; figure 16B). Pair wise *post hoc* comparisons revealed that CSC animals had significantly reduced thymus weights compared with both SHC (HAB: P = 0.003, LAB: P = 0. 076 [tendency]) and GHC (HAB: P = 0.004, LAB: P = 0. 030). Again, separate statistics (student's t test) revealed that CSC-stressed LAB animals had significantly lower thymus weights (P = 0.002) compared with CSC-stressed HAB rats. There were no significant changes in the absolute adrenal weights with respect to the strain differences, nor CSC exposure (factor strain x CSC $F_{2,90}$ = 0.165, P = 0.846, figure 16C). However, plasma corticosterone levels

differed between HAB and LAB rats (factor strain $F_{2,90}$ = 4.808, P = 0.031; figure 16D), but CSC treatment did not further alter its concentration.

*Histological damage score*. The morphology of the colon was not affected by CSC independent of the rat strain (factor strain x CSC $F_{2,41}$ = 1.876, P = 0:166; figure 16E)

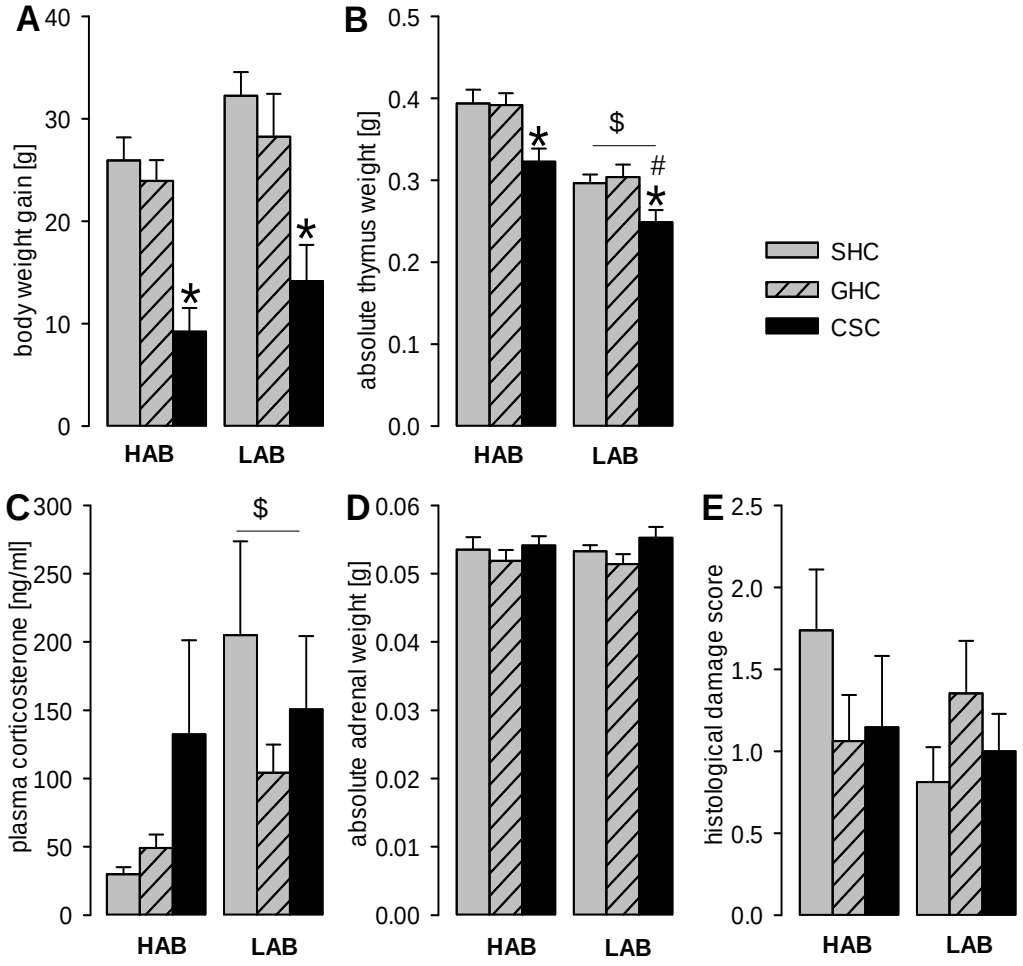


Figure 16: Effects of genetic predisposition on body weight gain (A), absolute thymus (B) and adrenal weights (C), basal plasma corticosterone (D) and histological damage score (E) after exposure to 19 days of CSC. Number of HAB and LAB rats in all groups; n = 16, except for for E: n = 7-8. Data represent mean + SEM. * P < 0.05 vs. respective single-housed controls

(SHC) and group-housed controls (GHC), # P < 0.05 vs. NS CSC and $ P < 0.05 vs. all HAB animals.

## Discussion

The present study investigated 2 important risk factors likely to shape the vulnerability to CPS, i.e., early life stress and genetic factors. We could reveal that PS exposure enhanced the vulnerability to CSC in adult male Wistar rats. Accordingly, only PS, but not NS rats, exposed to CSC in adulthood gained less body weight and had a higher histological damage score of the colon. However, we could not reveal any gene x environment interactions with regards to differences in chronic stress susceptibility in HAB and LAB rats.

*CSC effects on anxiety, physiological and immunological parameters in NS rats*

In accordance with our recent study (chapter 3), CSC exposure did not alter anxiety-related behaviour, absolute adrenal weight, basal plasma corticosterone concentrations, and did not increase the histological damage score of the colon in NS rats. However, in contrast to our previous data, body weight gain and absolute thymus weight were not affected in this study. Although decreased body weight gain [68, 230] and thymus atrophy [231] are generally considered as adequate indicators of chronic stress in rodents, there are studies showing no alterations in these parameters following repeated/ chronic stressor exposure. For instance, 10 min of daily social

defeat over 10 consecutive days in male C57BL6 mice did not alter the body weight gain [121]. Similarly, simultaneous application of chronic variable and restraint stress (1 h daily) for 10 days in male Wistar rats did not affect thymus and adrenal weights [269]. Support comes also from recent own data [238] showing that repeated exposure to a restraint/ overcrowding does not alter thymus weight in virgin female rats. It is important to mention that the present studies use in-house bred rats, and not commercially supplied rats used in our first study (chapter 3). We suggest that there may be minor differences in early-life handling of these rats which may at least partly explain the disparity in some readout parameters such as body weight gain. Support for this also comes from recent reports from Olfe and colleagues [270]. Comparing commercially supplied and in-house bred female BALB/C mice, they revealed that the former, same age-matched with later, gained less body weight in adolescence, and showed less corticosterone response to an acute psychological stressor (a 2-h combined restraint and acoustic exposure) in adulthood.

*PS effects on anxiety, physiological and immunological parameters in SHC and GHC rats*

The PS-induced decrease in body weight gain is in agreement with previous findings of PS on male and female rats showing a decreased weight gain throughout adolescence till adulthood [271, 272]. Although this decrease in body weight could be attributed to restrictions in maternal nutrient during PS [273], it is more likely that, increased corticosterone reaching the foetus as a result of decreased 11-beta-hydroxysteroid-type 2 expression during

gestational stress [274, 275] may produce harmful developmental effects *in utero* [276], consequently resulting in a decreased body weight [277]. The absence of higher anxiety-related behaviour in PS rats is likely explained by the differences in housing conditions i.e., single versus group housing, which may mask known PS-induced increased anxiety-related behaviour [253]. No PS-induced changes were found in other physiological parameters studied like thymus, adrenal weights and basal plasma corticosterone levels, which is in line with some previous reports [264, 278]. PS alone did not affect inflammatory parameters of the colon.

*CSC effects on anxiety, physiological, and immunological parameters in PS rats*

In contrast to NS rats, CSC in PS rats resulted in a decreased body weight gain and signs of colonic inflammation, indicated by a pronounced increase in the histological damage score. However, no additive effects of PS and subsequent CSC exposure during adulthood were seen in terms of adrenal and thymus weight, plasma corticosterone concentrations and anxiety-related behaviour during EPM testing.

Our findings are in line with recent data [279], revealing unchanged adrenal weights and plasma corticosterone concentrations in PS Wistar rats following 10 days of immobilization (3 times per day; 45 min each) in adulthood. However, a number of studies have reported that in male rats, PS enhances the HPA axis responsivity to stressors [253, 256, 280, 281], observations which are at the first glance contrary to our present study. However, one plausible explanation for these discrepancies in HPA axis responsiveness

might be the different types of stressors employed during adulthood. An increased HPA axis reactivity in PS rats has been described mainly in response to acute stressors (5 min elevated platform [281], 10 min open field [256] and 30 min restraint [253, 280]), whereas plasma corticosterone concentrations revealed in the present study represent basal morning values following a chronic psychosocial stressor (CSC). Support for this hypothesis is provided by Ladd and colleagues [282] showing an increased HPA axis response of early life-stressed (maternal separation) rats to acute air-puff startle (3 x 1 s), but an attenuated responsiveness, if the acute air-puff was applied to early life-stressed rats, additionally exposed to chronic variable stress during adulthood (a rotating schedule of stressors presented twice per day i.e., between 0700-1100 h and 1400-1700 h). Moreover, a range of paradigms including suspension [251], immobilization [283], restraint [279], social defeat [284], as well as flashing lights/noise [285] have been extensively used to study foetal programming. Due to the fact that some stressors inflict pain, others comprise a physical and/or social component, their consequences on offspring are, therefore, not readily comparable. Furthermore, the timing, duration, and frequency of stress application during pregnancy [286] is also an important consideration. These variable methodological aspects could probably lead to different physiological stress responses in pregnant females, and as a consequence, differentially programme offspring response to stressors [287].

The most important finding of this study is the increased histological damage score of the colon in rats exposed to both PS and CSC, indicating additive effects of several adverse life events on immune functions. An increase risk

for developing colonic inflammation has been reported in adults maltreated at childhood [150, 245]. In line, recent own data have shown that in C57BL6 mice, maternal separation during days 1-14, and CSC during adulthood have also additive effects in terms of the severity of a subsequent chemically-induced colitis. This was reflected by a more pronounced body weight loss, inflammatory reduction of colon length, and increase in IFN-gamma secretion from draining mesenteric lymph node cells in maternally separated compared with non-maternally separated CSC mice [125]. Therefore, additive effects of early life stress and CPS during adulthood in rats and mice represent a common feature of the CSC paradigm in both rats and mice, and, thus, further support the validity and relevance of this animal model for studying the neurobiological mechanisms underlying stress-induced pathology.

*Gene x environment interactions in terms of behavioural, physiological and immunological parameters after CSC exposure*

The risk for developing psychopathologies can be enhanced by the presence of certain gene polymorphisms as shown in humans as well as rodents [288]. Therefore, assessing the interaction of gene and environment factors employing relevant animal models could help to diagnose the neurobiological mechanisms underlying both affective and somatic disorders. HAB and LAB rats used in the present study have been extensively characterized on the basis of their behavioural, neuroendocrine, neuronal and genetic differences. Selected on the EPM for differences in extremes in anxiety-related behaviour [167], hyper-anxiety of HAB rats has been confirmed in relevant behavioural tests [170, 171], and shown to be accompanied by a depressive-like

phenotype [171, 172]. Furthermore, HAB and LAB males show also high and abnormal aggressive behaviour [178]. The HPA axis response to an acute emotional stressor is heightened in HAB compared with LAB males [180], but the reverse is true when exposed to an acute social stimuli [181]. Also, brain regions belonging to the anxiety/ fear circuitry (paraventricular nucleus of the hypothalamus, the lateral and anterior hypothalamic area, and the medial preoptic area) show increased neuronal activation in response to emotional stimuli in HAB compared with LAB rats [184].

In the present study, we extend our knowledge on these mostly acute stressor-related changes to chronic psychosocial stress by investigating whether HAB and LAB rats differ in their vulnerability to CSC. In line with our recent study, CSC exposure, independent of genetic background, resulted in a decreased body weight gain and thymus weight, but unchanged adrenal weight, histological score of the colon, and anxiety level. However, we could not reveal any line specific differences, which is in contrast to what has been reported following acute stressor exposure [180, 181]. HAB and LAB rats did not differ in their vulnerability to a chronic psychosocial stressor, at least with respect to the parameters assessed in the present study. A plausible explanation for this may be that continuous exposure of HAB and LAB rats to a severe stressor like CSC over 19 days may mask consequences, which could be evident after an acute stressor exposure. However, this hypothesis still needs to be investigated.

In conclusion, the present study reveals that i) early life stress, i.e. prenatal stress, increases the vulnerability to chronic psychosocial stressors during adulthood also in rats, at least with respect to inflammatory colonic

parameters, and ii) genetic predisposition, at least in terms of genetically determined differences in innate anxiety-related behaviour, does not shape susceptibility to chronic psychosocial stressors during adulthood.

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# Chapter 5

# General Discussion

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5.5 Summary and future perspectives

## 5.1 Summary of Results

The last decade has seen resurgence in the establishment of animal models for CPS, on the one hand, to study the exact neurobiological mechanisms involved in CPS-induced mal-adaptations and on the other hand, develop therapeutic strategies for already existing diseases. The results presented in this thesis therefore describe my efforts to contribute to this growing research interest in the field of neurobiology. This, I have approached from a technical (chapter 2), and experimental (chapter 3 and 4) perspective.

Generally, estimation of hormones and catecholamines as well as cytokines in venous blood samples is an essential experimental approach in preclinical neurobiology, endocrinology and immunology. Given the progressive use of valuable transgenic mice models, need to study pharmacodynamics and most importantly, *in-vivo* responsiveness of the HPA axis to stressors, the necessity to develop a technique for repeatedly sampling venous blood in mice increased in the last years. I, therefore, demonstrated in chapter 2 that it is possible to repeatedly withdraw blood samples from conscious and freely-moving mice using a simply-constructed catheter, chronically implanted in the right jugular vein. I further provided a detailed description of the construction of the catheter, and of the implementation and sampling procedure. I could provide evidence that it is possible to estimate relatively reliable basal corticosterone concentrations in plasma samples as early as 24 h post surgery. Moreover, although 100 µl of blood was withdrawn for up to 4 times (total 400 µl) within 120 mins, the technique in itself was proven to be non-stressful to the mice. Data showing that saline injection alone did not

increase venous plasma corticosterone concentrations supported this claim. Notably, corticosterone concentrations in repeated 100-µl venous blood samples were found to be similar to trunk blood samples both under basal conditions and after acute stressor exposure (5 min elevated platform or 60 s open arm) collected at identical time points (at 5, 15 and 60 min). Additionally, I could show a progressive increase in corticosterone levels until 15 min after termination of stressor exposure, and a decrease towards baseline values 60 min later in both venous and trunk blood samples, thus validating my technique. Importantly, anxiety-related behaviour, as assessed on the EPM 4 days after surgery remained unchanged when compared with non-catheterized animals, but locomotion was decreased. In conclusion, repeated or chronic blood sampling is also possible in mice using a simplified technique of indwelling catheterization of the jugular vein.

In chapter 3, I describe my efforts to adapt the already established CSC paradigm in mice (Reber et al.; 2007) to male Wistar rats, a project performed in parallel to the one described in chapter 2. This was done in order to further substantiate the biological relevance of our mouse data. Moreover, for detailed neuroendocrine characterization of hormones like ACTH and oxytocin for example, repeated blood sampling in higher volumes and for a longer duration is necessary, which is only possible in rats. Thus, we could analyse important physiological, immunological and behavioural parameters after CSC exposure. I was able to prove that this model is effective in male Wistar rats, as analysis of aggressive behaviour during the onset of colony formation (3 x 2 h) showed that experimental rats indeed

assumed a subordinate position, whereas the Long-Evans residents assumed the dominant position. Nineteen days of CSC exposure resulted in decreased body weight gain and absolute thymus mass, mild colonic barrier defects and intestinal immune activation. However, the absolute adrenal weight and basal plasma corticosterone remained unchanged. Furthermore, detailed neuroendocrine changes in the HPA axis responsiveness, especially to an acute heterotypic stressor (open arm, 5 min) were investigated in venous blood samples repeatedly drawn via a catheter implanted into the right jugular vein. Notably, CSC rats showed an increased plasma corticosterone response to open arm *in vivo*, despite displaying similar basal levels and similar basal and stressor-induced plasma ACTH levels. I observed no changes in anxiety-related behaviour as assessed on the EPM and LDB on days 20 and 21 respectively after CSC exposure. Moreover, no changes in social preference or depressive-like behaviour were seen, as well as inter-male aggression as investigated on the SPAT, FST and RI test, on days 23, 24 and 30 respectively, after CSC. Therefore, our results further confirm and extend the validity of the CSC paradigm as a relevant chronic psychosocial stressor also in male Wistar rats

In chapter 4, we studied whether prenatal stress increases the susceptibility to CPS in adulthood. I revealed that male offspring from chronically stressed mothers during pregnancy [chronic stress paradigm: overcrowding (24 h) and restraint stress (2 x 1 h) employed on alternate days, between pregnancy days 4-16] i.e., prenatally stressed rats subjected to CSC in adulthood showed no changes in anxiety-related behaviour as evaluated on the EPM on

day 19. Again, as seen in rats without prenatal stress during this particular study, CSC exposure neither affected the thymus or adrenal weight, as well as basal plasma corticosterone levels. Notably, prenatally stressed CSC rats gained less body weight and showed an increased histological damage score in the colon, indicative of an additive effect of prenatal stress on CSC susceptibility. Therefore, prenatal stress increases the vulnerability to chronic psychosocial stressors in adulthood, at least with respect to colonic parameters investigated.

In the other line of investigation, I aimed to reveal the genetic factors possibly contributing to differential stress susceptibility. I could show that HAB and LAB animals did not differ in their response to CSC, at least in the parameters investigated. But both rat lines were independently affected by CSC. In both breeding lines, CSC exposure did not alter anxiety-related behaviour tested on the LDB on day 19, thus confirming the results in non-selected rats. We found a CSC-induced decrease in body weight gain and thymus mass, but no changes in adrenal mass, basal plasma corticosterone and colonic morphology in both HAB and LAB rats after CSC. Therefore, genetic predisposition, at least in terms of genetically determined differences in innate anxiety, does not shape susceptibility to chronic psychosocial stressor during adulthood.

## 5.2 Chronic jugular vein catheterization as a reliable method for blood sampling in mice

The jugular vein catheterization technique presents many advantages over other methods of obtaining blood from mice (summarized in table 5). One of the most important advantages is that few animals are used, making the procedure cost effective. Moreover, anesthesia is not employed, thus producing physiologically relevant values (influence of anesthesia on plasma corticosterone levels are shown in chapter 2, figure 6A).

| Technique | Collected repeatedly? | Anesthesia employed? | Stressful? |
|---|---|---|---|
| Jugular vein catheterization | **yes** | **no** | **no** |
| Saphenous Vein | Yes | No | Yes |
| Dorsal pedal vein | Yes | No | Yes |
| Orbital sinus | No | Yes | No |
| Cardiac puncture | Yes | Yes | No |
| Decapitation | No | Yes | No |
| Tail clip | Yes | No | Yes |

Table 5: Pros and Cons of some techniques used to collect blood samples in mice.

It is important to mention that, as early as 1979 [103], attempts have been made to establish chronic jugular vein catheterization as a technique for repeatedly collecting blood samples from freely moving and conscious mice. About 10 years later, MacLeod and Shapiro [104], showed that serial blood sampling could only be performed in the mouse when the inserted catheter is

tethered to a freely moving wheel system built above the cage. Also, Mokhatarian and colleagues [105] reported the possibility of collecting blood samples via a right jugular vein catheter fixed to the skull by a special dental cement, and Bardelmeijer and colleagues [106] designed a special holding cage system, which enabled the collection of blood samples via a previously implanted jugular vein catheter. However, the aforementioned techniques have not been routinely applied in neuroendocrinological research probably due to their complicated and time consuming build-ups, but have actually contributed to the establishment of our present technique.

Conclusions about the validation of this technique came from the comparison made in plasma corticosterone concentrations between venous and trunk blood samples obtained via the implanted jugular vein catheter and trunk blood respectively, under basal as well as after acute stress exposure (5 min elevated platform, 60 s FS, chapter 2 figures 6 and 7). Statistical computation revealed the reliability of this technique, as basal and acute stress-induced plasma corticosterone levels in venous as well as trunk blood samples did not differ from each other and showed similar profiles. Support for the validity and reliability of this technique also comes from our recent studies investigating *in vivo* adrenal responsiveness to an acute heterotypic stressor (elevated platform, 5 min) *(*Uschold-Schmidt N, Nyuyki KD et al.,2012*)* [107]. In this study, we implanted catheters in the right jugular vein of the mice at the end of CSC (day 20). Twenty four hours later, blood samples were collected before (basal), and 5 min following termination of elevated platform exposure for the estimation of plasma corticosterone levels. We were able to

show that absolute basal levels of corticosterone in venous samples were not different between SHC and CSC mice (figure 17A). However, the percentage rise, relative to basal values (set to 100 %) in plasma corticosterone 5 min following termination of elevated platform was significantly higher in CSC compared with SHC mice (figure 17B). Similarly, plasma corticosterone concentrations in trunk blood collected 5 min following termination of a 5-min elevated platform exposure were significantly increased in CSC mice compared with SHC (figure 17C). Thus, the similar response trends in CSC mice with and without catheters during basal as well as after termination of acute stress exposure extends the reliability of our technique.

Notably, increased anxiety-related behaviour, repeatedly reported after CSC in mice as one of the main effects [68, 100, 122-124] was reconfirmed 24 h post surgery [107]. In this study, we assessed anxiety-related behaviour on the elevated platform [289], and it was observed that CSC mice spent less time on the outer zone of the elevated platform, indicative of increased levels of anxiety. This therefore proves that surgery *per se* does not interfere with known CSC effects and confirms previous data, where we observed a stable anxiety state even up to 4 days post surgery on the EPM [290]. However, locomotion was affected. Therefore, our simplified technique for jugular vein catheterization can be used for neuroendocrinological research, but care must be taken when designing experiments requiring detailed behavioural observations.

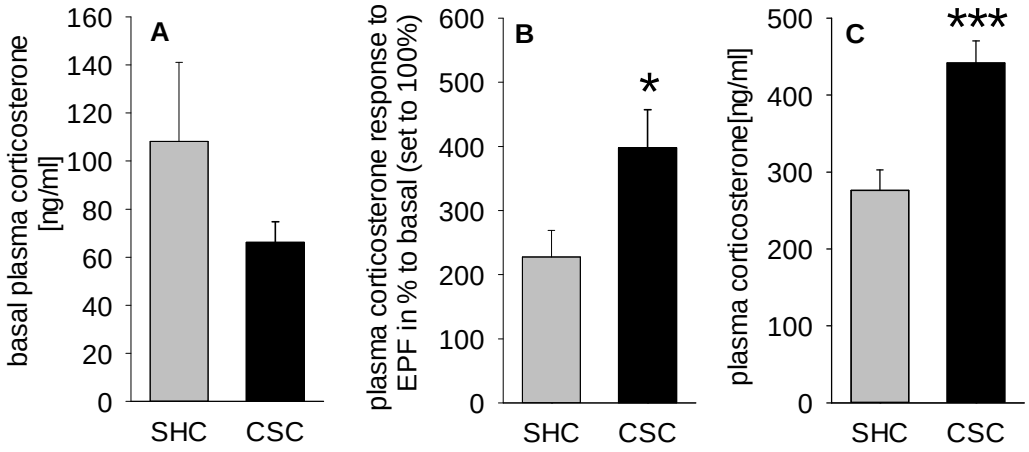


Figure 17. Effects of CSC on plasma corticosterone levels in response to elevated platform. SHC (n = 6-12) and CSC (n = 7-12) were exposed to an elevated platform for 5 min (on day 21), 24 h after catheter surgery (day 20; A; B) or without surgery (C). Venous blood was collected via jugular vein catheter before (basal, A) and 5 min after termination of elevated platform exposure (B), as well as trunk blood after stressor exposure (C) for quantification of plasma corticosterone. Data represents mean + S.E.M, * $P < 0.05$ and *** $P < 0.005$ vs. SHC. Adapted from Uschold-Schmidt N, Nyuyki KD et al, 2012 [107].

## 5.3 CSC as model of CPS in rats

### *5.3.1 Choice of control group*

Our choice for the use of SHC and GHC in this thesis was guided by growing literature, since the best control-housing condition for rats is controversially discussed till date. Some rat studies have reported increased anxiety- and depressive-like behaviour [223, 224], as well as deficits in sexual behaviour [225] after single-housing. Alterations of the HPA axis functioning as reflected by higher basal levels of ACTH, and enhanced release of ACTH and

corticosterone following stress in socially isolated male Sprague-Dawley rats has also been reported [228]. With regards to data presented in this thesis in favour of both single and group housing, one possible explanation may be that the consequences of housing conditions may be strain- or sex-specific [226-228, 291]. Moreover, the duration of social isolation seems to be of considerable importance, which varies in the above mentioned studies from 8 to 15 days [224, 227] up to 10 or even 13 weeks [223, 225, 228]. In our preliminary studies, we employed pair-housed controls i.e., housing male Wistar rats with ovariectomised females for 19 consecutive days during the course of the chronic stress procedure. Compared with SHC and GHC, pair-housed controls showed a high tendency of decreased body weight gain similar to CSC stressed rats. Based on these findings, pair-housed group was eliminated from further studies. In the subsequent sections, therefore, reference is made to SHC and GHC as appropriate control groups, at least for the CSC model in rats, especially when comparing its validity to the mice model.

### *5.3.2 Comparison of CSC consequences in rats and mice (table 6)*

***Physiological parameters:*** In mice, it has been repeatedly shown that 19 days of CSC exposure results in typical signs of chronic stress including decreased body weight gain, thymus atrophy and adrenal hypertrophy [68, 100, 122, 124, 125]. Similarly, the present thesis reveals that CSC exposure is also stressful for male Wistar rats as it results in a decreased body weight gain and thymus atrophy, but no changes in adrenal weight. Although some chronic stress models, for instance, 20 days of chronic predatory stress [292],

repeated swimming (22°C, 45 min per session x 7 days) [293], and forced treadmill running (8 weeks) [294] have reported that adrenal hypertrophy, besides thymus atrophy and decreased body weight gain is a reliable readout for chronic stress, these findings cannot be generalized. Support for this claim comes from studies performed by De Goeij and colleagues [232], who revealed that 19 to 28 days of subordinate housing did not change adrenal weight, despite decreased body weight gain and thymus atrophy. It might therefore be feasible that 19 days of subordination, as in our CSC model may differentially regulate the morphology and physiology of the adrenal glands in a species dependent manner.

***Neuroendocrine parameters:*** With regards to the adaptations of the HPA axis as a result of CSC exposure, both mice and rats showed similar patterns. Compared with their respective controls, the basal plasma ACTH and corticosterone levels were not different. Interestingly, an increased corticosterone, but not ACTH response to an acute heterotypic stressor was observed in CSC rats and mice. Our findings are in line with previous reports from Spiga and colleagues [208], who also describe both habituation of the HPA axis in response to a homotypic stressor (restraint: 1h daily x 11 days), and sensitization to a heterotypic stressor. In more detail, we report a sensitization of the adrenal response to an acute open arm exposure (5 min), and Spiga et al, report a sensitization of the pituitary response to white noise, i.e., increased ACTH release and no changes in plasma corticosterone. Therefore, particular note has to be taken when making conclusions about sensitization/adaptation of the HPA axis, as similar discrepancies between

pituitary and adrenal responses to stress have also been previously described [295]. It can, therefore, be postulated that stressor type and intensity differentially activate the HPA axis, especially under conditions of chronic stress. Moreover, mechanisms responsible for the regulation of ACTH response during CSC are still under investigation. Whether changes in hypothalamic CRH/AVP secretion, its pituitary receptor density and alteration of the sensitivity of the GR feedback mechanism, accompanying CSC-induced effects and the HPA axis remains to be shown. Also, the involvement of the LC/NE system cannot be completely ignored. The interaction between the CRH/AVP system and LC/NE system has been elaborately described before (chapter 1 section 1.1.2). It can, therefore, be speculated that CSC sensitization of the adrenals following acute OA exposure is possible through interaction of these systems. Support for this also comes from mounting evidence showing the modulation of adrenal responses to ACTH via adrenal sympathetic innervations [296, 297].

Arguing for the importance of the CSC model, unchanged basal plasma corticosterone, but increased adrenal sensitivity to a heterotypic stressor, may curtail the activity of endogenous cytokines and other stress reactive systems [298], eventually mounting immune protective effects in the body. Therefore, low basal plasma corticosterone may be an adaptive mechanism of the HPA axis to protect the body from increasingly deleterious amounts of GC, while effectively responding to novel threats.

| CSC readout parameter | Mice | Rats |
|---|---|---|
| Body weight gain | ⇩ | ⇩ |
| Absolute adrenal weight | ⇧ | ⇔ |
| Relative adrenal weight | ⇧ | ⇔ |
| Basal plasma corticosterone | ⇔ | ⇔ |
| Basal plasma ACTH | ⇔ | ⇔ |
| Corticosterone response to acute heterotypic stressor (open arm) | ⇧ | ⇧ |
| ACTH response to acute heterotypic stressor | ⇔ | ⇔ |
| Absolute thymus weight | ⇩ | ⇩ |
| Relative thymus weight | ⇩ | ⇔ |
| Proinflammatory cytokine secretion from mesenteric lymph node cells | ⇧ | ⇧ |
| Histological damage score (colon) | ⇧ | ⇔ |
| Mucus production (colon) | ⇩ | ⇩ |
| Anxiety-related behaviour | ⇧ | ⇔ |
| Depressive-like behaviour | ⇔ | ⇔ |
| Social preference/avoidance behaviour | ⇔ | ⇔ |

Table 6. Comparison of CSC consequences in rats and mice.

***Immunological parameters:*** Reliable and sensitive indicators of colonic inflammation and breakdown of epithelial barrier function in mice after CSC are reportedly, increased pro-inflammatory cytokine secretion from isolated and stimulated mesenteric lymph node cells, elevated histological damage scores and decreased colonic sulfomucin positive epithelial crypt cells (alcian blue positive cells) [68, 128]. Alcian blue positive cells are glandular epithelial

cells secreting mucus which forms a protective layer and acts as a medium for lubrication on the colonic epithelium [299]. In rats, CSC exposure did not change the histological damage score of the colon, but decreased the number of alcian blue positive cells and increased IFN-γ secretion from isolated mesenteric lymph node cells. This, therefore, indicates an activation of the enteric immune system, coupled to mild colonic barrier defects. Our present findings substantiate the general importance and relation of psychosocial stress to colonic inflammation, as a recent report revealed that 15 days of crowding decreases the Alcian blue positive cell population in the colon [239]. The direct role of immune cells in epithelial functioning had been established more than a decade ago [300]. In this study, *in vitro* analysis performed with the human T84 colonic epithelial cell line revealed that the application of neutralizing Abs to IFN-γ increased the secretory response of T84 cells to carbachol and forskolin, resulting in a decreased permeability. This indicates that activated IFN-γ cells partially promote epithelial membrane transport abnormalities. Therefore, higher levels of IFN-γ, in association with decreased number of alcian blue positive cells after CSC in rats suggests that our CSC model can at least provide the possibility to study mechanisms underlying CPS-induced colonic barrier dysfunction.

***Behavioural parameters:*** Mice studies have revealed that 19 days of CSC exposure increases anxiety-related behaviour [68, 100, 122], but does not affect depressive-like behaviour or social preference [124]. Except for anxiety-related behaviour, which remains unchanged after CSC in rats, most consequences are similar to the mice model (table 6). Several rodent studies have reported an increased state anxiety [301, 302] as one reliable indicator

for the efficiency of stress response. As it is not the case after CSC exposure in rats despite the presence of other reliable biomarkers of stress like thymus atrophy and decrease body weight gain, I therefore speculate that testing animals immediately following termination of CSC exposure might not be relevant in revealing CSC-induced behavioural alterations in rats. This claim is supported by recent findings [58, 126, 210], which speak in favour of the fact that measurement of long term alterations of behavioural parameters due to chronic stress is crucial. In these studies, the authors revealed increased anxiety-and depressive-like behaviour 7 days after termination of a 7-week chronic stressor exposure. This is particularly important as human pathologies often develop with latency to the experienced stressor exposure for instance, childhood trauma.

In the context of emotional regulation, activation of the brain areas involved in emotionality and stress processing should be mentioned. Increased neuronal activation of the amygdala, prefrontal cortex and hippocampus has been reported after acute and chronic stressor exposure in rodents [303-305]. Our lab has reported that acute open arm exposure significantly increased activation in brain regions like hippocampus and PVN to a higher extent in CSC compared with SHC or GHC in mice [123]. It would be interesting to study whether such differences in activation are found also in CSC rats despite unchanged anxiety- and depression-related behaviour.

In summary, the CSC model in rats enables us to reveal species-dependent effects especially with regards to anxiety-related behaviour. In order to further

investigate possible risk factors that could affect the vulnerability to CSC, I employed various models, i.e., genetic predisposition and adverse early environment. Since exposure to adverse early life (maternal separation) enhances susceptibility to CSC in mice [125], our primary motivation was to employ prenatal stress as a model of adverse early life experience. Further, I used HAB and LAB rats, with genetically determines differences in vasopressin expression in order to reveal the influence of the genetic predisposition on CSC susceptibility (chapter 4).

## 5.4 Adverse early life events and genetic factors affecting vulnerability to chronic stress

**Adverse early life:** Generally, the use of the term ‘early life’ denotes a period before (pre) and immediately after (post) birth. In humans, psychopathologies including anxiety, depression, drug abuse, post traumatic stress disorder and schizophrenia are among some of the documented mal-adaptive consequences in children exposed to some form of postnatal stress for instance, childhood trauma, maltreatment, neglect, sexual abuse or abnormal parenting [149, 241, 306]. In addition, childhood maltreatment has also been associated with an increased risk for developing colonic inflammation and metabolic diseases in adulthood [150, 245]. Moreover, it has recently been revealed that prenatal stress (PS) increases the risk for developing diabetes [152]. In accordance, numerous rodent studies have reported behavioural, endocrine and neuronal mal-adaptations following PS and postnatal stressor

exposure including increased anxiety-related and depressive-like behaviour, impaired social behaviour [155, 307-309] and increased responsiveness of the HPA axis to acute stressors [253, 256, 280-282, 310]

Although prenatal stress as well as postnatal stress has long-lasting adverse consequences in offspring, this thesis investigates prenatal stress as a risk factor for increased susceptibility to CPS exposure in adulthood for 2 main reasons. First of all, pregnancy is a very sensitive period of development, and stressor exposure during this period affects the neuroendocrine development, as this principally occurs *in utero* [311]. Secondly, chronic stress during pregnancy has been attributed to increased foetal exposure to corticosterone through reduced placental expression of 11beta-hydroxysteroid dehydrogenase type 2 [274, 275], which normally inactivates more than 90 % of maternal corticosterone delivered to the placenta [312]. Consequently, increased levels of corticosterone lead to long-term programming of foetal HPA axis development [313], increasing stress responsiveness in adulthood.

The present thesis reports a decreased body weight gain and increased histological damage, which is a sign of colonic inflammation, following CPS exposure in adulthood (19 days of CSC) in prentally stressed male rats compared with non-prenatally stressed rats. These observations are in line with recent own data which reports that, postnatal stress induced by maternal separation (3 h/ day, days 1-14) and CSC during adulthood have also additive effects in mice. This was reflected by a more pronounced body weight loss, inflammatory reduction of colon length, and increase in IFN-gamma secretion from draining mesenteric lymph node cells in maternally

separated compared with non-maternally separated CSC mice [125]. Moreover, there was an increased severity of a subsequent chemically-induced colitis after CSC in postnatally stressed mice. Therefore, additive effects of early life stress and chronic psychosocial stress during adulthood in rats and mice represent a common feature of the CSC paradigm and, thus, further support the validity and relevance of this animal model for studying the neurobiological mechanisms underlying stress-induced colonic dys-function.

As discussed above, our CSC model in rats failed to induce anxiety-related behaviour, nor alterations in basal plasma corticosterone. Importantly, prior prenatal stress exposure did not alter these behavioural and neuroendocrine readouts. At present, we can, therefore, only speculate that the degree of maternal care may compensate stress vulnerability in rats. In adulthood, the offspring of high licking/grooming/arched back nursing mothers showed a reduced plasma corticosterone and ACTH responsiveness to acute stressors, increased hippocampal GR mRNA expression, enhanced GC feedback sensitivity, and decreased hypothalamic CRH mRNA levels [314]. Recent data shows that prenatal stress increases arched back nursing, at least in the early light phase of lactation [238]. Though the exact mechanisms as to how this happens is still elusive, it can be speculated that increased arched-back nursing may directly affect GR mRNA expression and GC feedback, consequently compensating behavioural or HPA axis alterations after CSC exposure.

**Genetic predisposition:** There is compelling evidence that the vulnerability to stress and disease is also influenced by genetic factors. In humans, interactions between early life stress episodes and genetic variations in the serotonin transporter and brain-derived neurotrophic factor genes have been associated with the risk of developing mood disorders in adulthood [259, 260, 288]. In our well characterized rat model for genetic differences in anxiety- and depression- like behaviour [163, 186], I could not reveal any influence of gene x environment interactions, at least, with respect to the parameters investigated (body weight gain, thymus and adrenal gland weight changes, plasma corticosterone and colonic damage. Thus, there was no difference in vulnerability to CSC between HAB and LAB rats. One plausible reason for this observation may be the fact that inborn, genetically driven differences in anxiety and stress coping are so robust and resistant to CSC. Secondly, a 19-day exposure to CSC may mask the effects, at least in terms of differences in HPA axis response which have been previously shown in response to acute stressors [180, 181] .

Comparing these observations to human psychopathologies associated with genetic predisposition, care has to be taken before making any conclusive statements. In this line, it is important to note that most human studies that report gene x environment interactions in psychopathologies make reference to long-term and, or repeated stressor exposure (childhood maltreatment, abuse, poor parenting etc) throughout childhood and adolescence [149]. Moreover, such psychopathologies develop later in life, implying that stressor exposure may induce some epigenetic remodeling of the gene, with long-term consequences later evident in life. Therefore, repeated episodes of 19-

day exposure of HAB and LAB rats to CSC may be required to evoke differential physiological, behavioural and immunological alterations.

Not withstanding, recent years have received a burgeoning amount of literature on rodent models, with different genetic modifications and characteristics [160, 166, 188, 315-317]. Due to these differences, disparities in response to different types of stressors are bound to exist, but nonetheless, progress in understanding their pathophysiology are being made.

## 5.5 Summary and future perspectives

In summary, using various experimental techniques and establishing novel techniques and paradigms, I could show that CPS affects in particular neuroendocrine functions in both rats and mice. Repeated *in vivo* blood sampling allowed the demonstration of beneficial adrenal adaptations to CSC in both species, allowing an acute rise in plasma glucocorticoids in response to heterotypic stressors, while basal levels remain low.

Further, exposure to stressful events early in life, both prenatally as well as postnatally, are a major risk factor for elevated susceptibility to chronic stress later in life. In contrast, in the genetic model used, I could not reveal any genetic factors shaping chronic stress vulnerability.

However, future studies need to reveal the distinct epigenetic regulations shaping CSC susceptibility as well as the involvement of neuropeptides oxytocin and vasopressin in HPA axis regulation during and after CSC. Moreover, long-term characterization of neuroendocrine parameters will

reveal the profile of such neurobiological adaptations after CSC, which are important to determine the persistence of CSC effects. Lastly, the CSC paradigm in rats is a promising model to study CPS-induced colonic dysfunctions without comoborbidity of psychopathologies like anxiety or depression. Therefore, detailed analysis including epithelial ion transport, colonic permeability and CRF receptor type 1 expression could reveal the underlying pathogenic mechanisms associated with functional gastrointestinal disorders.

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# Abbreviations

## Abbreviations

| | |
|---|---|
| ACTH | adrenocorticotrophic hormone |
| ANS | autonomic nervous system |
| CNS | central nervous system |
| CPS | chronic psychosocial stress |
| CRH R1 | CRH 1 receptors |
| CRH | corticotrophin-releasing hormone |
| CRH | corticotropin releasing hormone |
| CSC | chronic subordinate colony housing |
| EPF | elevated platform |
| EPM | elevated plus-maze |
| FS | forced swimming |
| FST | forced swim test |
| GC | glucocorticoids |
| GHC | group housed controls |
| GR | glucocorticoid receptor |
| HAB | high anxiety-related behaviour |
| HPA | Hypothalamo-pituitary-adrenal |
| Lab | laboratory |
| LAB | low anxiety-related behaviour |
| LC | locus coeruleus |
| LC-NE | locus coeruleus-norepinephrine |
| LDB | light dark box |
| MR | mineralocorticoid receptor |
| mRNA | messenger ribonucleic acid |

| | |
|---|---|
| NE | norepinehrine |
| NS | non-prenatal stress |
| PD | pregnancy day |
| PND | post natal day |
| PS | prenatal stress |
| PVN | paraventricular nucleus |
| RI | resident intruder |
| SHC | single housed controls |
| SNS | sympathetic nervous system |
| SPAT | social preference /avoidance test |
| vs | versus |

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# Curriculum Vitae

# List of publications

# Awards

# Curriculum Vitae

Personal details

| | |
|---|---|
| Date/place of birth: | march 2nd 1984 in Sop/Cameroon |
| Nationlaity: | Cameroonian |
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**Scientific education**

**2008 – present**

PhD student at the department of Behavioural and Molecular Neurobiology in the laboratory of Prof. Dr. I.D. Neumann, University of Regensburg, Germany (topic: Consequences of chronic psychosocial stress in male Wistar rats)

**2006-2007**

M Sc. in Experimental and Clinical Neurosciences (ECN) at the university of Regensburg, Germany (Master thesis: Effects of mating on emotionality, stress-coping behaviour, neuroendocrine and stress-induced neuronal activity in male Wistar rats).

**2005-2006**

Tutor of Biology and Chemistry at the Saint Sylvester's Catholic College Sop, Cameroon

**2004-2005**

Laboratory Technician at the Good Samaritan Polyclinic Bamenda, Cameroon

**2001-2004**

Undergraduate studies in Biochemistry and Medical Laboratory Technology at the University of Buea, Cameroon

# Publications

**Nyuyki K.D.,** Maloumby R., Reber S.O., Neumann, I.D **(2012**). Comparison of corticosterone responses to acute stressors: Chronic jugular vein versus trunk blood samples in mice. Stress, DOI: 10.3109/10253890.2012.655348

**Kewir D. Nyuyki,** Daniela I. Beiderbeck, Michael Lukas, Inga D. Neumann and Stefan O. Reber. Chronic subordinate colony housing (CSC) represents a model of chronic psychosocial stress in male rats. *PLoS ONE, under review*

**Kewir D. Nyuyki**, Katharina M. Hillerer, David A. Slattery, Inga D. Neumann and Stefan O. Reber. Risk factors increasing the vulnerability to chronic psychosocial stress in male Wistar rats. *In preparation*

**Nyuyki KD,** Waldherr M, Baeuml S, Neumann ID (**2011**). Yes, i am ready now: differential effects of paced versus unpaced mating on anxiety and central oxytocin release in female rats. *PLoS One 6(8):e23599.*

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 **(2012).** *Psychoneuroendocrinology 37, 1676-1687.*

Waldherr M, **Nyuyki KD,** Maloumby R, Bosch OJ, Neumann ID. Attenuation of the neuronal stress responsiveness and corticotrophin releasing hormone synthesis after sexual activity in male rats **(2010).** *Horm Behav. 57(2):222-9.*

Landgrebe M, **Nyuyki KD,** Frank E, Steffens T, Hauser S, Eichhammer P, Hajak G, Langguth B. Effects of colour exposure on auditory and somatosensory perception--hints for cross-modal plasticity. *Neuro Endocrinol Lett.* ***2008****; 29(4):518-21.*

## Awards/Schoolarships

**2002**

Academic excellence; Ministry of Education-top 30% female science students Ministry of education awards to the top 30% female students in the department of life sciences, University of Buea, Cameroon

**2008-2011**

Fellow of the Bayerische Forchungsstiftung (Bavarian Research Foundation) from the Bavarian State Ministry of Sciences, Research and the Arts

**2011-2012**

Promotion abschluss stipendium, Frauenförderung University of Regensburg, Germany

# Declaration/Erklärung

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

*While some stress is inevitable, being "stressed out" is not*

By McEwen BS and Elizabeth N.Lasley.

‘2002. The end of stress as we know it.