Chronic psycho-social stress & colitis:

Physiological, neuroendocrine, and immunological studies with male C57BL/6 mice

DISSERTATION

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Gewidmet meiner Familie

Contents

Chapter 1

General Introduction

Contents

1. Introduction

Inflammatory Bowel Disease (IBD) develops in approximately 0.1 percent of the Western population either as ulcerative colitis (UC) or Crohn's Disease (CD). While IBD limits quality of life due to diarrhea, blood stools, ulceration, fever, tiredness, abdominal cramps, enormous pain, and other socially unacceptable symptoms, it additionally can be linked to complications such as toxic megacolon, bowel perforation and/or surgical complications. IBD may also cause a delay in puberty or growth problems, because it can interfere with appropriate uptake of nutrients.

The pathogenesis of IBD is still not completely understood, but it is generally accepted that IBD has a complex and multi-factorial aetiology, involving genetic and environmental factors, which are associated with dysregulation of the mucosal immune system. Interestingly, one of these environmental factors that has often been linked to the pathogenesis of human IBD during its long history of research, is the level of perceived stress. Although there has been a rapid expansion of animal studies in the recent years, providing evidence for a link between stress and experimentally induced colitis, the detailed mechanisms are also still unknown. Additionally, the use of unnatural and time-limited stressors in these animal studies complicates the transferability to the human situation, where mainly chronic psycho-social stressors are hypothesized to play a role in the pathogenesis of IBD. Therefore, in the present thesis the mechanisms by which chronic psycho-social stress affects either the severity of a chemically-induced or the development of spontaneous colonic inflammation in male mice were investigated.

In this chapter, the history of what is nowadays referred to as "stress" and the role of the hypothalamo-pituitary-adrenal (HPA) axis as well as of the autonomic nervous system (ANS) in the adaptation process during acute and chronic stress will be described. Furthermore, the reader will be introduced into the architecture and function of the intestinal tract, providing the basic background knowledge for the understanding of the complex changes and dysregulations occuring during IBD. Finally, the question of a link between stress and IBD is addressed by reviewing the existing human and non-human literature.

2. Stress is "the non-specific response of the body to any demand" (Hans Selye, 1975)

Already in the 19th century the French physiologist Claude Bernard (1813 – 1878) noticed that the relative constancy of the internal environment is critical for the functional integrity of an organism and its independent existence. This constancy of the internal milieu was defined by Bernard as "homeostasis" (1878). In his "emergency" concept Walter Cannon (1915) described that fear- or rage-induced fight and flight reactions pose a threat to this steady state within the body. This was found to be paralleled by an activation of primarily the adrenomedullary system and serves to mobilize metabolic resources for preparing the body rapidly for action in response to acute threat. The first definition of stress was given by Hans Selye (1907 – 1982). According to him, stress is a typical defensive reaction that occurs whenever an organism is placed in a critical situation. He referred to this, in his eyes stereotypic response to multiple stressors, as the general adaptation syndrome [1]. Selye emphasized that in this respect it does not matter whether this response is caused by pleasant or unpleasant things [2]. He later defined the general adaptive

syndrome as stress, further defining the term stress as "the non-specific response of the body to any demand" and the term stressor to describe the challenges that cause stress in the body [3].

A more recent definition comes from Sapolsky (1994), another eminent stress scientist: A stressor can be defined as anything that throws your body out of balance – e.g. an injury, an illness or a great psychological demand. The stress response, in turn, is the bodies attempt to restore this balance [4] or in other words: The preservation of the constancy of the internal environment requires continues adaptation to external or internal stimuli or stressor [5]. These adaptations are characterized by an activation of the central and autonomic nervous and endocrine systems, subsequently resulting in a complex pattern of species-specific behavioural changes. Therefore, the stress level of an organism could be assessed by either measuring parameters that describe the activity state of the HPA axis [6, 7] and of the ANS [8, 9], or by behavioural observations. The delay or latency, for instance, of resuming normal behaviour following an environmental challenge has been shown to pose a useful measure of stress [10]. However, for proper interpretations of behavioural changes due to stress, knowledge of species-specific behaviour is required.

2.1 The hypothalamo- pituitary-adrenal axis

If an individual is exposed to a stressor, meaning it experiences a challenging situation that leads to the disruption of homeostasis, the HPA axis (Fig. 1) is being activated [6, 7]. The magnitude of physiological activation in this situation represents the adversity of the threat. When the HPA axis is activated during stress, importantly without reference to the nature of the stressor, parvocellular neurons within the paraventricular nucleus (PVN) of the hypothalamus are stimulated.

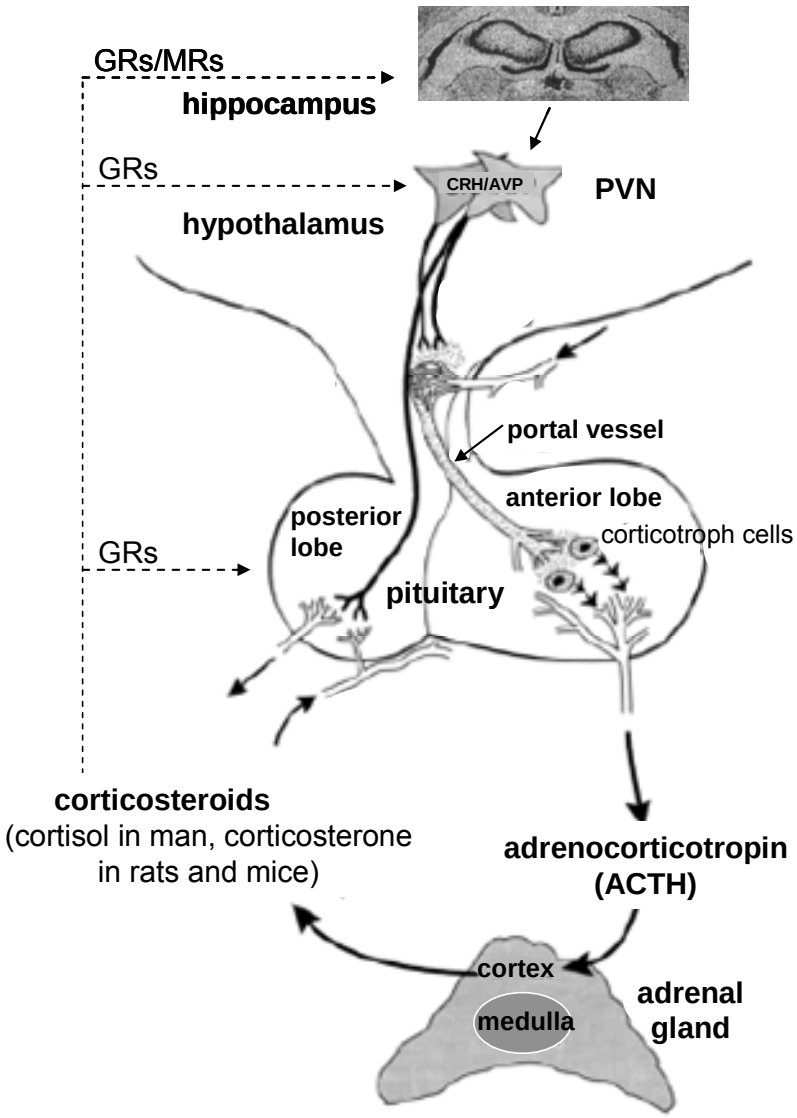


Fig. 1: Schematic representation of activation of the HPA axis. In the hypothalamus corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) are released by the parvocellular neurons of the paraventricular nucleus (PVN). These are secreted into vessels of the hypophyseal portal circulation. After binding to their respective receptors on the corticotroph cells within the anterior lobe of the pituitary adrenocorticotropic hormone (ACTH) is secreted in the blood stream. At the level of the adrenal glands ACTH binds to its receptors in the cortex and induces the secretion of glucocorticoids (GCs), cortisol in man, corticosterone in rats and mice. These act on different levels of the HPA axis via negative feed back (dashed arrows). [adapted from http://www.med.uni-magdeburg.de/~cschulz/lectures/ neuroendocrinology/hpa/mat/hpa_ 2006.pdf]

The main peptides produced in these cells are corticotropin releasing hormone (CRH) and arginine-vasopressin (AVP). After production both peptides are transported along the axons of the parvocellular neurons to the median eminence, a

region at the bottom of the brain. After stimulation, CRH and AVP are released into the hypophyseal portal circulation, which links the median eminence directly with the anterior part of the pituitary gland. In the anterior pituitary both peptides bind to their respective receptors located in the plasma membrane of pituitary corticotroph cells [11], thereby activating the production and secretion of adrenocorticotropic hormone (ACTH) into the bloodstream. CRH is thought to be the main activator of ACTH release, but the effects of CRH are amplified by AVP, which after prolonged stressor exposure is co-expressed and co-secreted from hypothalamic parvocellular neurons [5] (for detailed explanation see Chapter 1, Sec. 2.3). In the adrenal cortex, ACTH binds to its receptors, thereby stimulating the synthesis of glucocorticoids (GCs; cortisol in humans, corticosterone in rats and mice; Fig. 2C, D) from cholesterol (Fig. 2B) and increasing the subsequent secretion of these steroids into the systemic circulation. The substrate cholesterol can enter the cell via lipoprotein or can be made locally from acetate [12]. In any case, stores of cholesterol as ester constitute the immediate starting point for the synthesis of steroid hormones. Corticosterone synthesis (Fig. 2A) can be seen as a branch of the pathway to cortisol in which 17α-hydroxylation does not occur. However, corticosterone appears to act qualitatively in exactly the same way as cortisol [13]. Corticosterone is also an intermediate in the synthesis of the mineralocorticoid aldosterone [13] (Fig. 2E), which serves as the principal regulator of the salt and water balance of the body. Increased GC concentrations play an important role in maintaining energy supply by enhanced catabolism, mobilizing lipid and glucose reserves [14-16]. Furthermore, GCs modulate immune and cardiovascular responses [14], and contribute to the visceral responses to stress by stimulating catecholamine synthesis and modulating the metabolic and cardiovascular effects of catecholamines [5, 17].

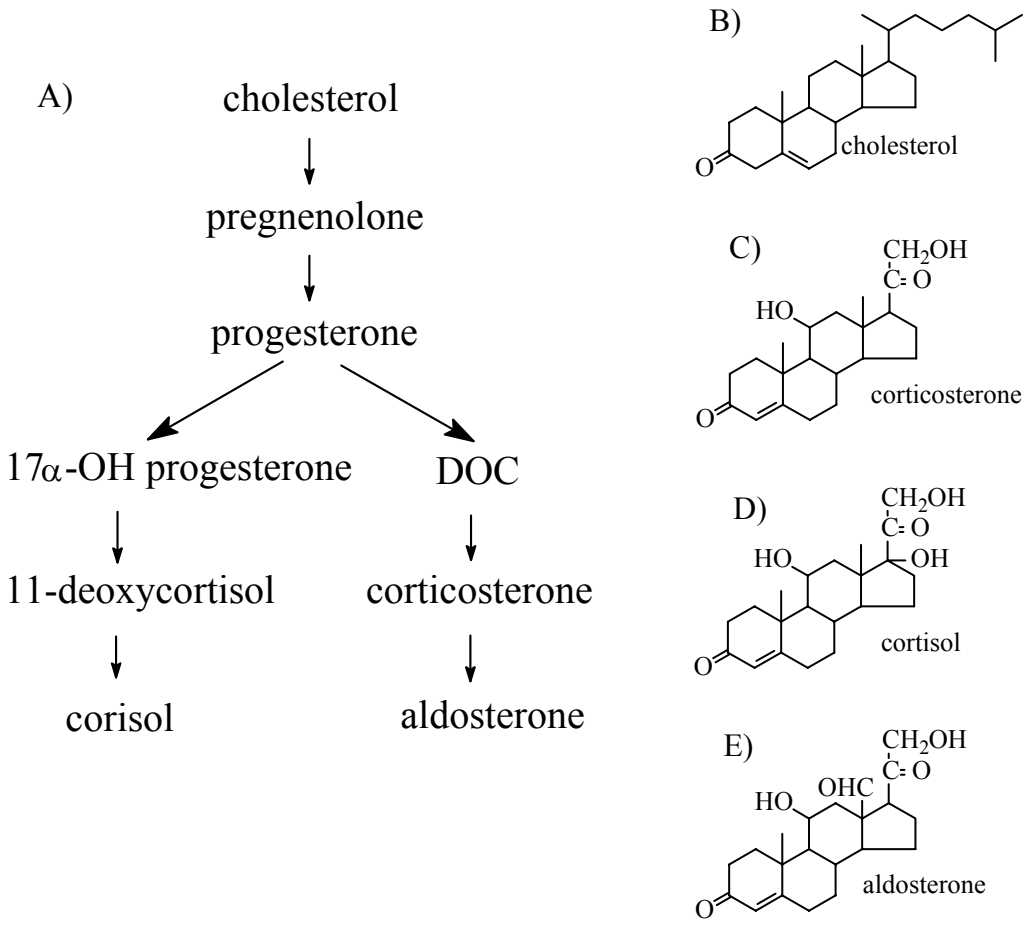


Fig. 2: A) Schematic illustration of the pathway to corticosterone, cortisol, and aldosterone. The pathway starts with the conversion of cholesterol to pregnenolone. Pregnenolone is then converted to progesterone by type 1 3β-hydroxysteroid dehydrogenase. Afterwards progesterone is converted either into 17α-hydroxyprogesterone (17 α-hydoxylase), followed by 11-deoxycortisol (21-hydroxylase) and cortisol (11β-hydroxylase) or into 11-deoxycorticosterone (DOC; 21-hydroxylase), followed by corticosterone (11β-hydroxylase) and aldosterone (18-Hydroxylase; 18-OH-Dehydrogenase, 18-Aldehyd-Synthetase). Additionally, the chemical structures of the GCs corticosterone (**C**; in rodents) and cortisol (**D**; in humans), the mineralocorticoid aldosterone (**E**), and cholesterol (**B**), as the precursor of all the steroid hormones, are shown. [adapted from http://www.megru.unizh.ch/j3/module/endokrinologie/endo.php?uniId=E42100&di=10]

Additionally, the HPA axis also coordinates circadian events, such as food intake and the sleep/wake cycle. During this diurnal rhythm the HPA axis activity is highest at the onset of the activity period. These peripheral effects of GCs are mainly mediated via binding to intracellular GC receptors (GRs) which are found in most peripheral organs. In addition, stress-induced increase in GCs has a profound effect on

emotional responses and cognitive processes [14], because due to their lipophilic nature, they can easily enter the brain. The intracellular corticosteroid receptor system in the brain consists of two different types of receptors: the mineralocorticoid receptor (MR) and the GR [18-20]. Both receptor types differ in primary structure, localization, and affinity. Paradoxically, MRs *in vitro* bind corticosterone with an approximately 10-fold higher affinity (0.5 – 1 nM) than GRs [19]. In the brain MRs are predominantly present in the limbic system, particularly in hippocampus, in the pyramidal neurons and the dentate gyrus, but also in lateral septal nuclei and the amygdala [21, 22]. In contrast, GRs have a widespread distribution and are abundantly expressed in brain regions involved in the stress response such as the hypothalamus, hippocampus, amygdala, various brain stem nuclei, and in the pituitary [23, 24]. Due to the differences in affinity and distribution of the two receptors, corticosterone has distinct functions after binding to MRs or GRs. MRs are predominantly occupied at low corticosterone levels and therefore are thought to be involved in evaluation of environmental stimuli under basal conditions [25], whereas additional GR activation by high levels of corticosterone around the circadian peak and during stress [19] is involved in mediating the termination of HPA axis activation, recovery from stress, facilitation of behavioural adaptations, and preparation to following stressor [25, 26]. Termination of HPA axis activity is mediated via negative feedback actions of activated MRs and GRs at various levels within the HPA axis (Fig. 1). In this respect, MR activation within the hippocampus exerts a tonic inhibitory control on the HPA during basal conditions [27, 28]. In response to high levels of corticosterone GR activation at the level of the hippocampus, hypothalamus, and pituitary prevents the HPA axis from overshooting [20, 26].

2.2 The autonomic nervous system

The ANS is divided anatomically into the sympathetic nervous system (SNS), the parasympathetic nervous system (PNS), and the enteric nervous system (ENS).

The ANS is activated by many of the same stimuli which activate the HPA axis. This is probably due to the fact that a variety of neural structures and circuits are involved in the activation of both HPA and autonomic functions, in general referred to as stress response, during various challenges. The involved brain structures are characterized by a stress-induced expression of immediate-early genes such as c-fos. In addition to the PVN, in this respect the particular reactivity of the locus coeruleus (LC) system to stressful situations has to be mentioned, which underlines its important role in mediating the stress response [29]. A high stress-induced neuronal activity in the LC has further been demonstrated by electrophysiology as well as biochemistry, revealing increased expression of the catecholamine synthesizing key enzyme tyrosine hydroxylase [30-32]. Anatomically, the LC is located in the dorsal pons, on the floor of the fourth ventricle. It is the major noradrenergic nucleus in the brain [33, 34] with widespread projections to both the brain [34] and spinal cord [35]. These spinal projections are responsible for influencing the peripheral preganglionic neurons of the SNS and PNS [35] (Chapter 1, Sec. 2.2.1).

Immediate-early gene expression in the PVN and brain stem LC is in fact correlated with the stimulation of HPA and ANS activity [36, 37] during exposure to novel and potentially stressful situations. The activity of these brain regions during stressor exposure is controlled by limbic structures, which are comprised of the olfactory areas, the hippocampus and amygdaloid complex, the cingulated cortex, and the septal region. Furthermore, regions within the cortex and spinal cord were shown to be responsible for HPA axis and ANS activity control.

Interestingly, it has been shown that LC neurons project into the PVN [38] and that afferents of the PVN *vice versa* monosynaptically innervate catecholamine-containing dendrites in the LC [39]. In addition, some CRH neurons in the PVN were found to project to the LC [39]. This circuitry underlines the importance of the dual activation of the ANS together with the HPA axis in terms of stress for allostasis, the process by which the organism adapts to challenges within the internal milieu. Thus, in response to stress PVN neurons, known to be critical in the integration of autonomic, physiologic, and endocrine regulations [40] as well as in generating a stress response [41], are activated and in turn lead to an activation of LC noradrenergic neurons [42] and vice versa. Through widely divergent projections, LC neurons innervate the entire neuroaxis [34] and are involved in global brain functions such as emotion, vigilance [43], memory and adaptive responses to stress [44].

2.2.1 The sympathetic and parasympathetic nervous system

The ANS functionally overlaps with both afferent (sensory) and efferent (motor) systems. It includes regions within a central regulatory brain stem that determine motor output via parasympathetic and sympathetic nerves to visceral organs, after interpreting information from sensory nerves regarding the status of an organ. The sympathetic and parasympathetic components (Fig. 3) of the ANS act as antagonists and mainly control the involuntary body functions via the distribution of nerve fibers to the various organs and glands. The SNS originates in the thoracic and lumbar regions of the spinal cord and tends to reduce digestive secretions, speeding up the heart, contracting blood vessels and therefore enables the body to rise to emergency demands encountered in flight, combat, pursuit, and pain.

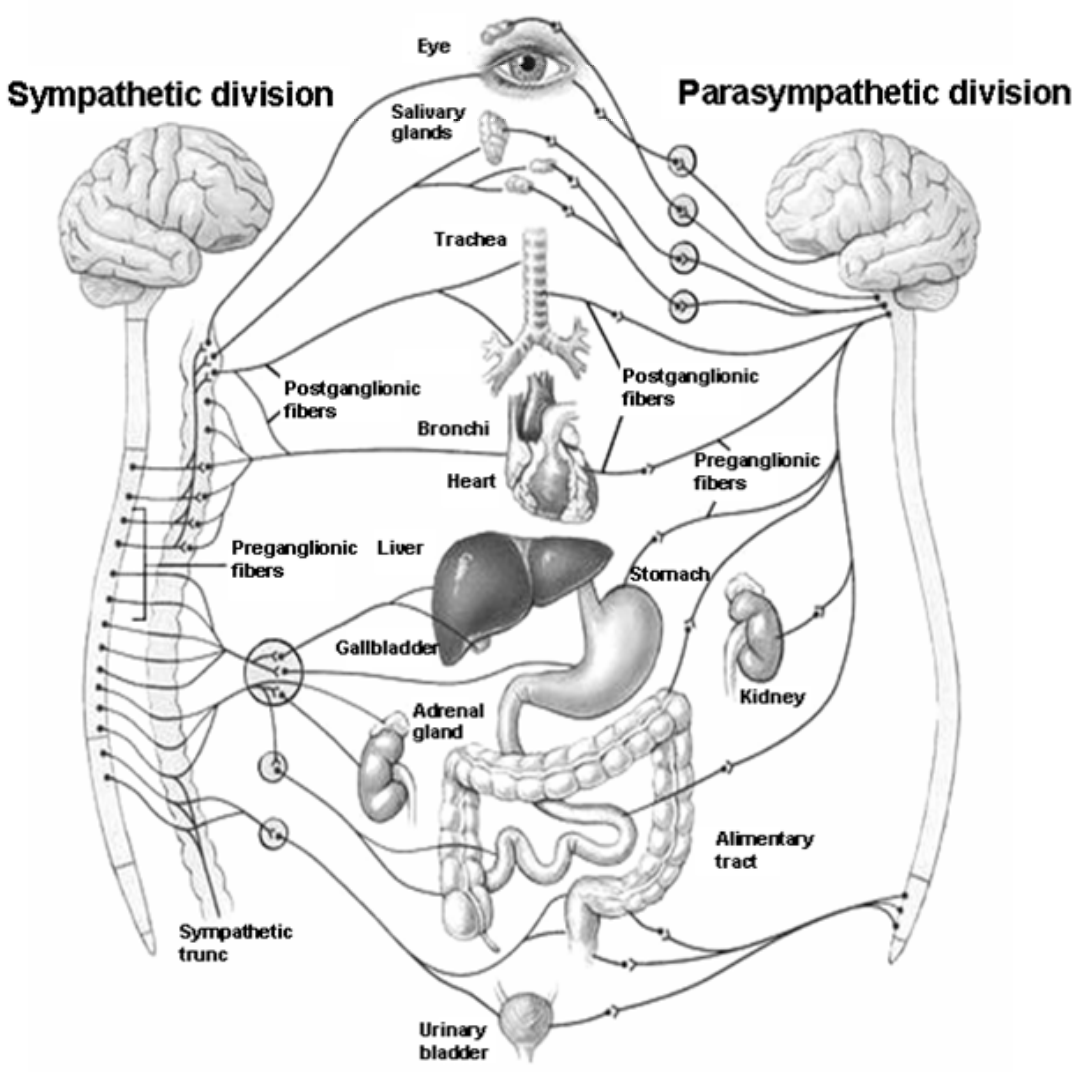


Fig. 3: Schematic representation of the SNS and PNS as parts of the ANS. The ANS is customarily divided into two parts: the sympathetic and the parasympathetic systems. The pathways of both usually have two efferent neurons; a first (presynaptic) neuron exits from the CNS and synapses with a second (postsynaptic) neuron that innervates the target organ.The presynaptic neurons of the sympathetic system exit from the thoracic and upper lumbar regions of the spinal cord, and synapse with the postsynaptic neurons in a series of small ganglia lying near the cord and forming the sympathetic trunk or in larger ganglia in the abdominal cavity; the postsynaptic neurons then run from the ganglia to the target organs. The presynaptic neurons of the parasympathetic system exit from the medulla of the brain and from the sacral region of the spinal cord. These are very long neurons that run all the way to the target organ, where they synapse with short postsynaptic neurons. Most internal organs are innervated by both the sympathetic and the parasympathetic system. [adapted from ardb.bjmu.edu. cn/main/ARDisease.htm]

In contrast, the PNS appears to be in control during such pleasant periods as digestion and rest. Its nerve fibers originate in the brain stem and the lower part of

the spinal cord and tend to stimulate digestive secretions, slow the heart, constrict the pupils, induce secretion, increase the tone and contraction of smooth muscles, and dilate blood vessels, in general, inhibit or oppose the physiological effects of the SNS. Therefore, it cannot be said that one system, the sympathetic, always has an excitatory role and the other, the parasympathetic, an inhibitory role; the situation depends on the organ in question (Fig. 3).

Within the sympathetic nervous system end organs during “fight or flight” situations are mainly stimulated by catecholamines. Norepinephrine (NE) is the major neurotransmitter secreted by peripheral postganglionic sympathetic fibers at the level of the effector cells, whereas epinephrine is the primary hormone secreted by the adrenal medulla in mammals after sympathetic stimulation. The adrenal medulla consists of masses of postganglionic neurons that are part of the sympathetic branch of the autonomic nervous system. Instead of releasing their neurotransmitters at an effector cell synapse, these neurons release them into the blood stream. Thus, although part of the nervous system, the adrenal medulla functions as an endocrine gland.

Synthesis of both catecholamines, epinephrine and NE, begins with the amino acid L-tyrosine (Fig. 4). Tyrosine hydroxylase is the enzyme responsible for catalysing the conversion of L-tyrosine to dihydroxyphenylalanine (DOPA). This initial reaction is the rate limiting step in the production of catecholamines. Therefore, the enzyme is found in the cytosol of all cells containing catecholamines. DOPA afterwards is decarboxylated into dopamin and dopamine-ß-hydoxylase further converts dopamine into NE. Whether or not epinephrine and/or NE are secreted by neurons of the SNS depends on the existence of the enzym phenylethanolamin-N-methyltransferase, which catalyzes the final step from NE to epinephrine.

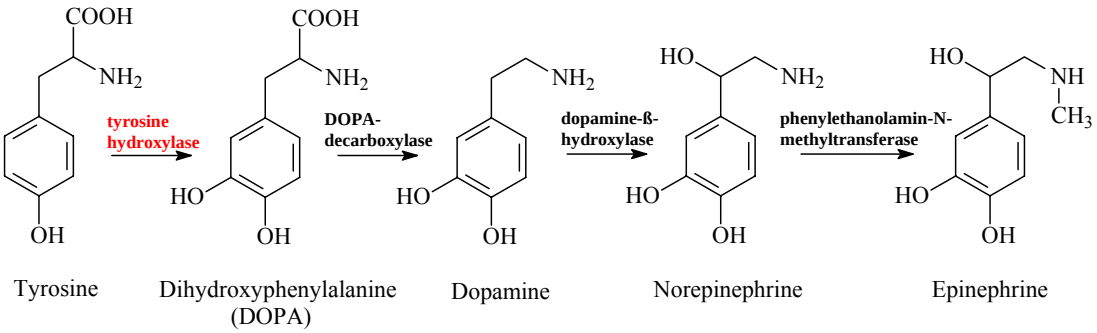


Fig. 4: Schematic illustration of catecholamine synthesis. Synthesis of catecholamines begins with the amino acid tyrosine, which is converted by tyrosine hydroxylase to dihydroxyphenylalanine (DOPA). This initial reaction is the rate limiting step in the production of catecholamines. Afterwards DOPA is decarboxylated to dopamine by the enzyme DOPA-decarboxylase. Dopamine is converted into norepinephrine (NE), the main catecholamine secreted by parasympathetic nerve fibers, by dopamine-ß-hydroxylase. The existence of the enzyme phenylethanolamin-N-methyltransferase in cells of the adrenal medulla is responsible for converting NE into epinephrine. Therefore, after sympathetic stimulation the adrenal medulla is able to secret both, NE and epinephrine into the blood stream. [adapted from [45]]

The physiologic effects of epinephrine and NE are initiated by their binding to adrenergic receptors on the surface of target cells. These receptors are prototypical examples of seven-pass transmembrane proteins that are coupled to guanosine triphosphate-binding proteins (G-proteins), which stimulate or inhibit intracellular signalling pathways. Different physiological responses of distinct organs of the body to catecholamines are due to multiple receptor types (α_1, α_2, β_1, β_2, β_3) which are differentially expressed in different tissues and cells. The alpha- and beta-adrenergic receptors and their subtypes were originally defined by differential binding of various agonists and antagnonists and, more recently, by the analysis of molecular clones.

End-organ stimulation by acetylcholine in the parasympathetic nervous system is more "vegetative", e.g. assisting digestion. Acetylcholine receptors are of two types: a fast-acting ion-channel controlling receptor and a slow-acting receptor that acts through a G-protein, stimulating second-messengers (often cyclic AMP) to indirectly

open ion-channels. Direct ion-channel controlling receptors can respond in microseconds, whereas indirect second-messenger controlling receptors take milliseconds to produce a response. The two acetylcholine receptor classes are named for artificial toxins that selectively activate them (Fig. 5). The fast-acting receptor is named "nicotinic", because it is in addition to acetylcholine specifically activated by the toxin found in tobacco. The slow-acting receptor is named "muscarinic", because the toxin muscarine (found in poisonous mushrooms) will activate it, but nicotine will not.

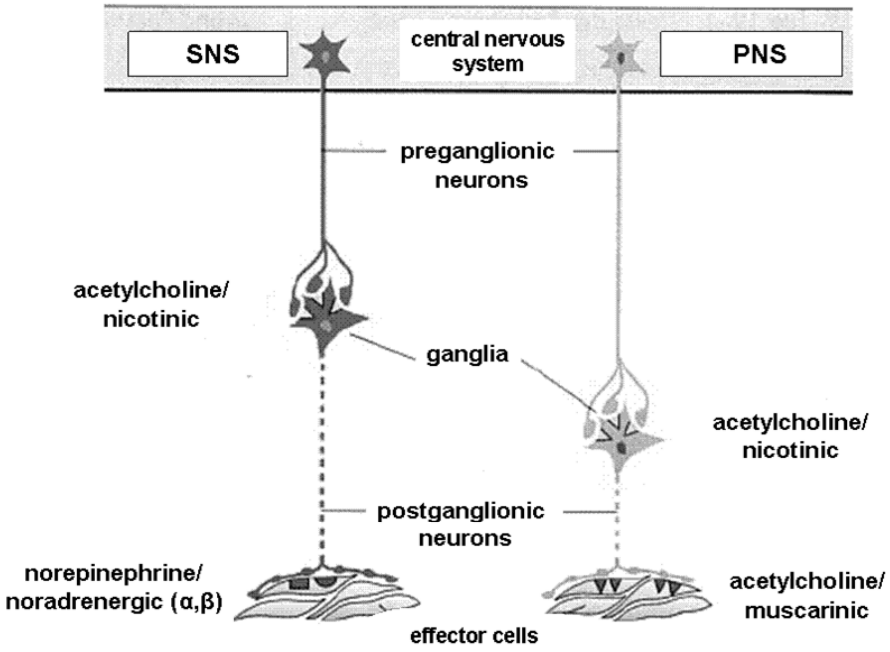


Fig. 5: Effector cells are stimulated by norepinephrine via noradrenergic receptors in the SNS and by acetylcholine via muscarinic receptors in the PNS. The cell bodies of the preganglionic neurons of both the parasympathetic and sympathetic systems are located within the central nervous system. The cell bodies of the postganglionic neurons of both systems are located outside the CNS in autonomic ganglia. In the SNS these ganglia are located in bilateral trunks of paravertebral ganglia that lie beside or on the vertebral column. Within the PNS these ganglia are located in the vicinity of end organs. The postganglionic neurons of both the SNS and PNS are stimulated by acetylcholin via nicotinic receptors. [adapted from [46]]

The long parasympathetic preganglionic fibers travel to end-organs containing ganglia but it is the short postganglionic nerves from the ganglia to the smooth

muscles in the end-organs which are muscarinic. The preganglionic fibers are nicotinic. Similarly, the preganglionic fibers of the sympathetic nervous system are nicotinic, although the sympathetic ganglia exist as distinct nodules within the sympathetic trunk (Fig. 3) closer to the spinal cord or as ganglia near large blood vessel in the abdominal cavity (celiac, superior mesenteric, inferior mesenteric).

2.2.2 The enteric nervous system

The ENS is a collection of neurons in the gastrointestinal tract [47] that constitutes the "brain of the gut" and can function independently of the CNS [48]. This system controls the motility [49], exocrine and endocrine secretions [50], and microcirculation [51] of the gastrointestinal tract; interestingly it is also involved in the regulation of immune and inflammatory processes [52].

The ENS was originally thought to be part of the autonomic component of the peripheral nervous system with the neurons in the gut wall being postganglionic parasympathetic neurons [53]. Interestingly, at the beginning of the 20th century, intestinal peristaltic contractions were found to be coordinated reflexes involving only the intramural nerves [53], and the majority of enteric neurons were not found to contact the parasympathetic axons of the CNS [53]. This strongly suggests that the morphology of the ENS is more complex than just consisting of postganglionic parasympathetic neurons. Subsequent examination of the functional and chemical diversity of the some 100 million enteric neurons, which is approximately the number of neurons found in the spinal cord [47], revealed that the ENS closely resembles the CNS [48]. In contrast, the British physiologist J.N. Langley (1852 – 1925) thought that the ENS is separated from the rest of the ANS on the basis of its unique morphological and functional characteristics, including an ability

to function independently of the autonomic influence; normal function does, however, require an intact autonomic innervation [54].

Therefore, the ENS may perhaps best be regarded as a displaced part of the CNS that retains communication with it through sympathetic and parasympathetic afferent and efferent neurons [53]. Together with these connections, the ENS provides neural control of all functions of the gastrointestinal tract [53], which can be described in terms of preprogrammed motor patterns [54]. These patterns are generated by the intrinsic circuitry of the ENS and are modified by signals impinging on them from extrinsic (autonomic) nerves, hormones, immune mediators, and other factors.

In the ENS, the nerve cell bodies are grouped into small ganglia that are connected by bundles of nerve processes, forming two major plexuses, called the myenteric (or Auerbach's) plexus and the submucous (or Meissner's) plexus [53] (Fig. 6). The myenteric plexus lies between the longitudinal and circular muscle layers and extends the entire length of the gut. It contains the majority of enteric neurons and principally controls for motor functions [54] by primarily providing motor innervation to the two muscle layers and secretomotor innervation of the mucosa [47]. Additionally, myenteric neurons project to submucosal ganglia, enteric ganglia of the gallbladder and pancreas [55], and to sympathetic ganglia [56]. The submucous plexus, located in the submucosa between the circular muscle layer and the muscularis mucosa, is best developed in the small intestine, where it plays an important role in secretory control [53]. In various compartments of the intestinal tract neurons of the submucosal plexus also coordinate absorption [54]. Besides innervating the glandular epithelium, neurons in the submucous plexus innervate the muscularis mucosa, intestinal endocrine cells, and submucosal blood vessels [53].

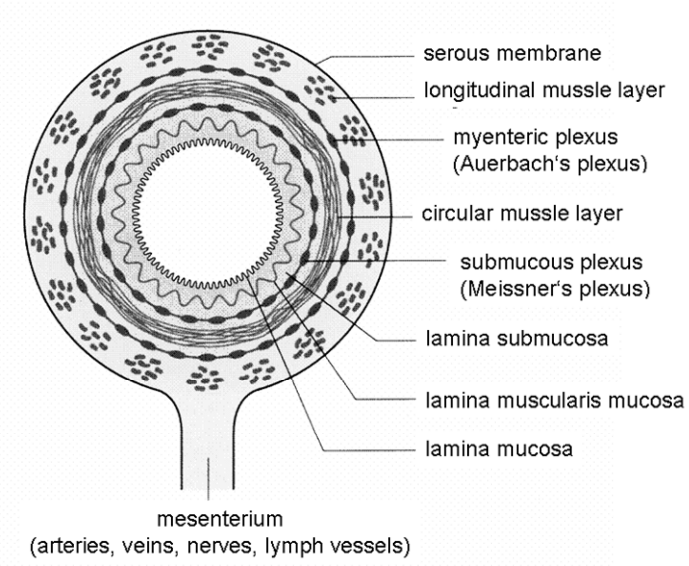


Fig. 6: Schematic representation of the different layers of the intestinal tract. Additionally, the myenteric (Auerbach's) plexus, which is located between the circular and longitudinal mussle layer, and the submucous (Meissner's) plexus, which is located in the submucosa between the circular mussle layer and the muscularis mucosa, are shown. [adapted from [57]]

As described before, the other two branches of the autonomic nervous system, the SNS and the PNS, regulate the ENS in a hierarchical manner through an interaction at the level of the enteric ganglia [58, 59]. For a more detailed understanding of these regulatory processes, a major task of neurohistologists, who study the ENS, has been to identify the different morphological types of neurons in the enteric ganglia and to correlate these with the functional classes of the neurons. In this respect many scientists attempted to establish an adequate classification of the different types of enteric neurons over the past years.

The original morphological classification of plexus neurons by Dogiel (1899), who identified at least three main classes of nerve cell bodies (type I, II, III) [60], seems still to be the most useful one [61]. Type I neurons have numerous short dendritic processes and a single long axon, type II neurons have a smoother surface with 3-10 long smooth processes, and type III neurons have 2-10 branching fine dendrites and a smooth axon [61].

Goyal and Hirano (1996) classified in their review the neurons that make up the enteric nervous system as intrinsic afferent neurons, interneurons, and motorneurons [53]. The intrinsic afferent neurons that form the sensory limb of all intrinsic motor and secretomotor reflexes are located in both the myenteric and submucous plexuses. They project to interneurons in the surrounding myenteric and submucous plexuses. They are all cholinergic and may or may not contain substance P [53]. Additionally, several subgroups of interneurons have been defined on the basis of their neurotransmitter content, but their various physiologic roles are unknown [53]. Interneurons are interposed between the primary afferent neurons and the motor or secretomotor neurons. The motor neurons are either excitatory or inhibitory. The excitatory motor neurons project locally or orally to the circular muscle, and their main neurotransmitters are acetylcholine and substance P. The inhibitory motor neurons always project caudally to the circular muscle and contain vasoactive intestinal peptide (VIP) and nitric oxide [53].

In a recently published textbook the authors distinguished between three major classes of nerve fibers present in the intestinal tract [62]: cholinergic, adrenergic, and "non-cholinergic-non-adrenergic fibers (NCNA-fibers)". The long cholinergic fibers found outside the two enteric plexuses were referred to as preganglionic axons of parasympathetic neurons, projecting to further cholinergic neurons within the myenteric ganglia. These cholinergic plexus neurons mainly control for gut motor function and are suggested to represent the postganglionic parasympathetic neurons [53]. The adrenergic fibers are suggested to be mainly postganglionic fibers of the SNS with cell bodies in the prevertebral ganglia [53] (Fig. 3). They innervate blood vessels, cells of smooth musculature, and other plexus neurons [62]. The neurons belonging to the NCNA-fibers were all referred to as plexus neurons, whereby excitatory fibers on smooth muscle postsynaptic potentials were found to secret

substance P, dynorphin, and enkephalin and inhibitory fibers to secret vasoactive intestinal peptide and adenosine tri-phosphate [62].

However, concerning the regulatory role of the PNS on these enteric plexus neurons Goyal and Hirano (1996) mentioned that the parasympathetic preganglionic neurons are all cholinergic and exert excitatory effects on enteric neurons through nicotinic and, in some regions, muscarinic receptors [53]. Interestingly, the authors of the above mentioned textbook described that, although the preganglionic fibers of the PNS are all excitatory, they can either activate intestinal function by further projecting to the excitatory cholinergic plexus neurons (postganglionic parasympathetic neurons) or damping it by projecting to inhibitory NCNA neurons [62].

Sympathetic nerve fibers are shown to enter the intestinal wall along the arteries and terminate in the myenteric and submucosal plexuses, and in the mucosa [63-65]. As mentioned above, they have different targets in the gut: motorneurons in the myenteric plexus (acetycholin or substance P containing), secretomotor neurons (conaining VIP), presynaptic cholinergic nerve endings, submucosal blood vessels, and gastrointestinal sphincters [53]. Through α_2-adrenergic inputs the SNS inhibits postsynaptic potentials of motorneurons in the myenteric plexus (acetycholin or substance P containing) and of secretoneurons in the submucosal plexus (VIP containing neurons) [65]. Furthermore, via α_1-adrenergic pathways the SNS counteracts vasodilatation induced by substance P [65, 66]. Interestingly, adrenergic nerve-cell bodies are not found in the enteric plexuses [53]. Sympathetic noradrenergic nerve fibres are also involved in the regulation of the gut associated lympoid tissue by plunging directly through the T cell zones and ramifying profusely among lymphocytes, enterochromaffin cells, and plasma cells in the interdomal regions [65]. In this respect it has to be mentioned that the influence of the SNS on the immune response largely depends on the time point of SNS activation in relation to the

immune response [65]. In a very early phase of an inflammation, for instance, activation of the SNS supports the directed migration into the inflamed tissue (via ß adrenoceptors and NPY Y1 receptors), whereas in later phases of the inflammation SNS activation inhibits the tissue destruction by cells of the innate immune system (via ß adrenoceptors) [65].

In addition to the regulation of intestinal function by the SNS and PNS, primary afferent neurons carry sensory information to the CNS within the sympathetic (splanchnic nerves) and parasympathetic (vagal nerves) nerve fibers [53]. It has been estimated that 80 percent of the fibers in the vagal trunk are afferent fibers with their cell bodies being located to the nodose ganlia [53]. These primary afferent fibers are for example sensitive to mechanical distension of the gut, when they innervate the smooth-muscle layer [67], or to luminal concentrations of glucose, amino acids, or long-chain fatty acids, when they innervate the mucosa [68].

Finally, glia cells, outnumbering the enteric neurons [69], are an integral component of the ENS and resemble the astrocytes of the central nervous system [53]. They produce interleukins and express major histocompatibility complex (MHC) class II antigens in response to stimulation by cytokines [70], suggesting a modulatory role in inflammatory responses in the intestine.

2.3 Acute vs. chronic stress

As outlined before stress is defined as any situation in which the metabolic integrity of an organism is threatened. The body's responses to this disruption of homeostasis include a chain of events that always occurs regardless of the nature of the stressful stimulus [71]. This chain involves the central nervous system to which the experience of stress is conveyed, and that responds by promoting the activation of the HPA axis and the ANS, especially its sympathetic tree [72]. This results in an increased

production/secretion of corticosterone by the adrenal cortex and catecholamines by the adrenal medulla and sympathetic nerve endings [73].

According to Dhabhar *et al.* (1999) stress is a constellation of events, which begins with a stimulus (stressor) that precipitates a reaction in the brain (stress perception), and subsequently activates physiologic systems in the body (stress response) [74]. The consequences of this physiologic stress response are generally adaptive in the short run [75], but can be deleterious, when stress is chronic and longlasting [76, 77]. Therefore, important distinguishing characteristics of stress include its duration and intensity, referring to acute stress when stressor exposure lasts for minutes to hours and to chronic stress when stressor exposure persists for days to months [77]. In this respect, the magnitude of a stressor can easily be estimated by the peak levels of stress hormones, neurotransmitters, and other physiological changes, such as an increased heart rate and blood pressure, and by the amount of time which these changes persist during and following stressor exposure [78].

Acute stressor exposure induces an increase in plasma concentrations of ACTH and corticosterone with peak levels after 5 to 30 min, which decline to baseline again within the following 6 hours, also depending on the nature and intensity of the stimulus [79]. This kind of stress is also referred to as *eustress* and was shown to result even in immunopreparatory, or immunoenhancing physiological conditions [78]. In contrast chronic, repeated, or physiologically exhausting stress is also referred to as *distress* and may cause immunosuppression [78]. An important characteristic of *distress* is that the physiological stress response either persists long after the stress has subsided, or is activated repeatedly, with the consequence of an increased exposure of the organism to stress hormones [78].

Animals subjected to prolonged stress conditions were found to show characteristic changes in physiological and emotional parameters including enlargement of the

cortex of the adrenal glands, atrophy of the thymus and other lymphoid structures, development of bleeding ulcers of the stomach and duodenal lining [80], as well as increased levels of state anxiety [81, 82] and depression [83, 84]. Additionally, it is known that stressful life events are able to suppress several components of the immune response, and that these effects are large enough to have biological and/or health consequences. This is on the one hand based on studies in mice with influenza virus, herpes simplex virus type 1 (HSV-1), and Myobacterium truberculosis [85-87]. On the other hand there are also studies done in rats demonstrating that chronic stress increases the metastatic spread of mammary tumor cells [88, 89]. Depending on the individual situation or predisposition, stress can further be a major contributor to the development of cardiovascular disorders, such as ischemic heart disease, arrhythmia, and sudden death [90]. Furthermore, Sabban & Kvetnansky (2001) showed that stress increases the susceptibility of the body to infection, autoimmune diseases, and cancer, as well as influences the progression of chronic diseases [91]. Interestingly, it was also shown that chronic stress suppresses testosterone secretion in both rodents and primates, including man [92-95], and that prolonged increases in GCs elicited by chronic stress or through exogenous administration affect brain physiology [96], neuronal morphology and viability [97-99], and behaviour [100-102]. Finally, many studies done in human and non-human primates as well as in rodents provide a link between stress and IBD [103-111], which is of potential importance concerning the present thesis. (see Chapter 1, Sec. 4).

Therefore, most modern theories about stress recognize that although stress is not a disease itself, it may be the trigger for a majority of diseases, when stressor exposure becomes chronic [112]. Nevertheless, under normal circumstances, the stress response protects the body and guarantees survival [112].

However, whether or not a prolonged exposure to a stressor always results in a prolonged exposure of the organism to increased stress hormone levels is discussed controversially and may depend on the type of stressor, the duration of stressor exposure, and the genetical predisposition of the individual. Generally, it is thought that plasma GC levels are above basal values whereas plasma ACTH responses may vary after prolonged stress, being preserved or desensitized according to the nature of the chronic stimulus [5, 113]. In this respect, the studies done by Albeck *et al.* (1997) and Zelena *et al.* (1999) have to be mentioned, which show increased levels of basal plasma corticosterone in male rats after 14 days of chronic social stress (subordination) in the visible burrow system [114] and after 7 days of chronic restraint stress (1h/day) [115], respectively.

In contrast, there is also evidence that basal plasma corticosterone is not affected by chronic stressor exposure or is only increased during the first weeks of chronic stress, but returns to baseline afterwards, despite continued stressor exposure [116-118]. As outlined before the exposure to prolonged high levels of corticosterone may be deleterious for an organism and therefore, the downregulation of plasma GCs to baseline levels during chronic stress may represent beneficial adaptation processes. In detail these adaptations are not well understood [5] but thought to permit an organism to remain responsive to novel or severe threats on the one hand, while being able to habituate to familiar or milder threats on the other hand.

Aguilera (1994) hypothesized in her review that relative changes in the secretion of the hypothalamic CRH and AVP, other hypothalamic regulators, changes in pituitary CRH or AVP receptors, and alterations of the sensitivity of the GC feedback mechanism might be mediating these adaptations during chronic stress [5]. During the last three decades evidence has accumulated, indicating that AVP plays an important role in the regulation of pituitary ACTH secretion. Interestingly, in man and rodents,

AVP itself is only a weak stimulus of ACTH secretion under basal conditions, but it participates in the adaptation of the HPA axis during chronic stress. AVP mediates thereby the hyperresponsiveness of the pituitary corticotroph cells to a novel heterotypic stimulus through its ability to potentiate the stimulatory effect of CRH [5, 119]. Therefore, it is not surprising that the expression of AVP in parvocellular CRH secreting neurons of the PVN was found to increase during chronic stress paradigms associated with corticotroph hyperresponsiveness [119]. In contrast, CRH mRNA levels after repeated stressor exposure are controversial, depending on the type and duration of the stressor [5]. In general CRH mRNA is found to be elevated only in chronic stress paradigms which show preserved high plasma hormone responses to the repeated stimulus [120]. Interestingly, even a transient activation of hypothalamic CRH neurons by a single stressor can sometimes cause long lasting increases in AVP co-expression, irrespective of the nature of the stressor, which in most cases is not accompanied by changes in CRH [121].

As mentioned above, ACTH responses to a novel heterotypic stressor are described to be invariably enhanced, irrespective of the responses to a persistent homotypic stimulus [5, 119]. This further supports the hypothesis that adaptation to a chronic stimulus permits an organism to remain responsive to novel or severe threats after prolonged stressor exposure. Thereby, the only exception is prolonged osmotic stimulation, after which an organism shows a marked corticotroph hyporesponsiveness to a novel stimuli [5].

Further evidence for a prominent modulatory role of AVP during chronic stress is the fact that in contrast to pituitary CRH receptors, which undergo downregulation during chronic stress, a correlation exists between changes in pituitary vasopressin receptor levels and pituitary ACTH responsiveness during chronic stress [5]. Thus, stress paradigms (chronic osmotic stimulation) in which ACTH responses are

reduced, show vasopressin 1A (V1A) receptor downregulation in the pituitary, whereas stress paradigms (repeated immobilization) with enhanced ACTH responses to a novel stress are associated with V1A receptor upregulation [5].

In addition, Caggiula *et al.* (1989) were able to show that chronically stressed animals also secreted higher levels of GCs when exposed to a novel herterotypic stressor compared with those, previously not exposed to chronic stress [122]. This could on the one hand be a consequence of the enhanced ACTH secretion in response to a novel heterotypic stressor, but on the other hand this effect could be also due to or at least be amplified by chronic stress-induced hyper-activity of the adrenal cortex [123, 124]. Interestingly, in the above mentioned study done by Albeck *et al.* (1997) only a subgroup of rats exposed to 14 days of chronic social stress (subordinates within mixed-sex groups in the visible burrow system) showed this hyperresponsiveness of the HPA axis to a novel stressor [114], whereas no increase in corticosterone was found in the other animals. This indicates that the regulation of the HPA axis during chronic stress is much more complex than tried to simplify above. This finding also points out that, in addition to the already discussed beneficial adaptation processes during chronic stress dysregulations of the HPA axis are likely to occur at various levels. In case of the subordinate nonresponder rats a deficit of CRH mRNA, but not of AVP mRNA, in the PVN was detected [114].

2.4 Psycho-social stress

The term psycho-social involves a psychological as well as a social aspect. Already Selye (1936) described the adaptations of an organism during stress, calling this phenomenon the general adaptation syndrome, but concerning only stressors of a physical nature [1]. As the years passed, this concept underwent a progressive evolution that made its application also transferable to the human situation,

distinguishing physical stress from psychological stress [80]. As a consequence, the fundamental role of the CNS and of psychological factors in response to stressors was highlighted in a series of further studies [125-127]. In fact, the somatic and behavioural manifestations that characterize stress reactions are not triggered directly by the stressor, but indirectly through its action on the CNS [80]. The stressor induces an activation of the CNS to which the psychological processes of cognitive evaluation and emotional reaction correspond. According to this model any stimulus, able to induce a stress reaction through the mediation of the CNS, is a stressor [80]. When the activation of the CNS occurs purely through cognitive means, without any contact with the organism, the stressor is defined psychological.

Concerning the term social stress it has to be stated that this is probably the most potent and naturalistic type of stress used in experimental sciences [128] (Fig. 7). A single social defeat was shown to have long-lasting behavioural consequences in male rats, including an increased immobility response to a mild change in background noise, an increased immobility in the forced swim test [129], and a reduced activity in an open field for at least one week [130]. In addition, a longer lasting stress-induced increase in the social avoidance behaviour was found in rats exposed to social stress compared with electroshock stress [131].

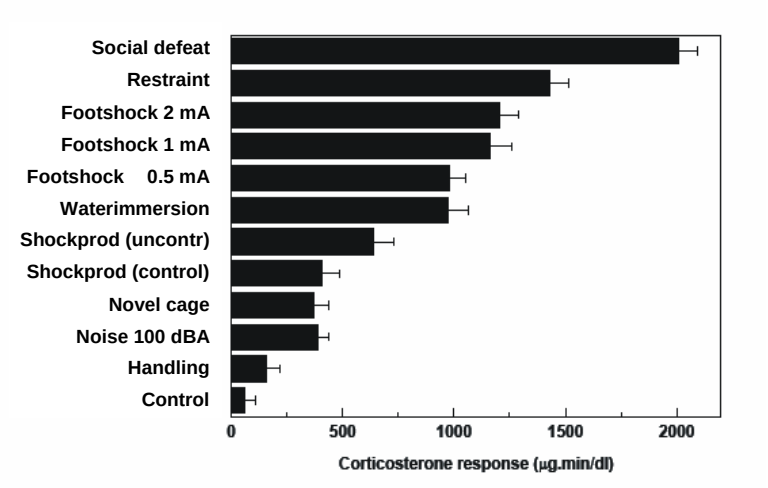


Fig. 7: Plasma corticosterone responses (quantified as area under the response time curve) to a variety of different laboratory stressors. [adapted from [128]]

Among mammals that live in social groups, conspecific threat comprises a common and enduring feature of daily existence for many individuals [132]. Dominance-subordination relationships reflecting social threat may be defined in terms of agonistic behaviour for the interacting dyads, with offensive or aggressive attack characterizing the dominant, and defensive behaviour, the subordinate [133]. Dominance relationships among males within adult, mixed-sex rat groups typically develop within the first several days of grouping, and are often stable over life-span of the group [134]. Although actual fighting may be relatively rare after the hierarchy is set up in colonial groups, the effects of subordination are profound. Subordinates show weight loss, a general increase in defensive behaviours, and major alterations in parameter of sleep, eating, sexual, aggressive, and active behaviours [133]. The magnitude of the subordination effect is also sensitive to the degree of threat: Subordinates often show early mortality, and the duration of their life-span within the groups is related to features that influence group aggression levels such as the presence of females, or the provision of burrowing habitat [133].

Furthermore, grouping, social defeat or subordination appear to produce changes in a variety of stress-linked physiological systems [135-139]. These findings indicate that dominance-subordination relationships may engender a very high level of stress and stress-related behaviours in subordinates. Importantly, subordination has the advantage of face validity in comparison to other experimental or biochemical means of “stress”, because most human stressors appear to be much more closely related to social factors than to unusual and physically painful experiences, or to exogenous biochemical challenges [133].

These considerations suggest that social stressors, such as social defeat and subordination, may provide a very comprehensive and appropriate model for the

analysis of behavioural, endocrine, immunological, and brain system changes associated with chronic stress [133]

3. Inflammatory bowel disease

IBD refers to two chronic diseases that cause inflammation of the gut tissue: UC, which is restricted to the large intestine and CD, which can occur in the small and/or large intestine. As an experimental model for IBD is used in the present dissertation which is restricted to the colon [140], the following section (Chapter 1, Sec 3.1) focuses on the histology and ultrastructural architecture of the large intestine.

3.1 Histology and ultrastructural architecture of the colonic wall

The different sections of the large intestine of mammals consist all of four concentrically arranged layers, as do the other organs of the digestive tract: a) lamina mucosa, b) lamina submucosa, c) lamina muscularis externa, and d) lamina adventitia [141] (Fig. 8).

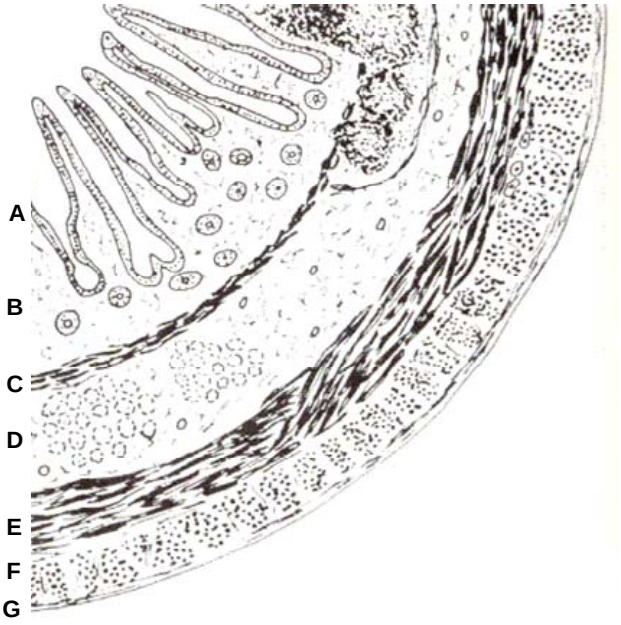


Fig. 8: Schematic representation of the general structure and layers of the large intestine: **lamina mucosa:** A) epithelial layer; B) lamina propria; C) lamina muscularis mucosa; D) **lamina submucosa**; E) **lamina muscularis externa** (circular mussle layer); F) **lamina muscularis externa** (longitudinal mussle layer); G) **lamina adventitia**. [adapted from [141]]

3.1.1 The lamina mucosa

The mucosa lines the large intestinal lumen and lies at the critical interface between the external environment and that of the body's internal milieu. This is reflected in the mucosa being the most dynamic layer of the large intestine. The mucosa is comprised of an epithelium and a basement membrane, supporting connective tissue called the lamina propria, underlain by a sheet of smooth musculature, the lamina muscularis mucosa [141] (Fig. 8). The luminal surface architecture of the large intestine includes rich microflora, particularly in the cecum and colon, but also in the rectum and anal canal [142], important for digesting complex carbohydrates and synthesizing certain products, such as vitamins. Therefore, on the one hand, a barrier is formed so that the deleterious constituents of the digesta, including bacteria, do not invade the parenchyma. On the other hand, water and some ions have to be absorbed while others have to be simultaneously secreted [141]. The simple epithelium lines the lumen and includes absorptive and/or secretory cells (principal cells) as well as varying numbers of goblet cells. The mucin produced by these cells coats the epithelium and is an integral and important part of the innate barrier against the penetration of foreign substances [143]. Scattered lymphoid cells termed intraepithelial leukocytes (IELs; Fig. 9A, B) are also present in the epithelium in variable numbers, containing CD8 positive cells, which are classically associated with cytotoxic or suppressor T lymphocytes, and precursors for mast cells and again cytotoxic T cells [144, 145]. Interestingly, it has been postulated that IELs might be involved in the downregulation of the local immune response, preventing the disastrous effects that could occur as a result of an exuberant reaction to luminal antigens crossing the epithelium [145, 146]. The pool of undifferentiated cells lies within the proliferative zone

at the base of the cryts and by mitosis produces new cells that migrate to their final position within the epithelial layer [141].

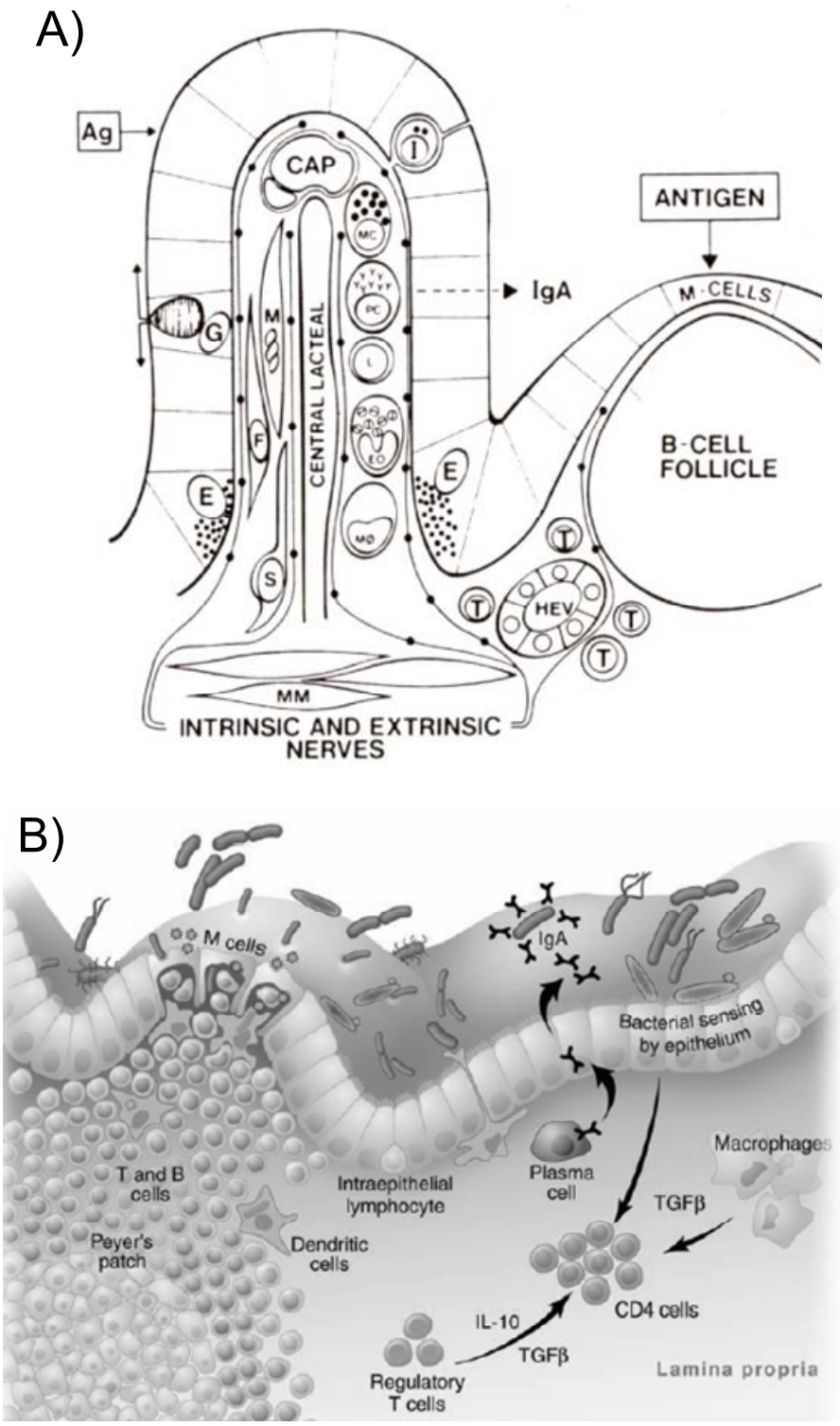


Fig. 9: A) Highly schematic representation of the immune cells and nerves in the gastrointestinal mucosa. Ag, antigen; cap, capillary; E, enteroendocrine cell; EO, eosinophil; F, fibroblast; G, goblet cell; HEV, high endothelial venule; I, intraepithelial leukocyte; L, lymphocyte; M, muscle cell; MC, mast cell; Mø, monocytes; MM, muscularis mucosa; PC, plasma cell; S, Schwann cell; T, T

lymphocyte. [from [145]]; **B**) Highly schematic representation of the mucosa associated lymphoide tissue (MALT). The epithelium overlying organized MALT contains specialized M cells that constantly transport gut bacteria and antigens from the gut lumen into the lymphoid tissue. DC in the lamina propria (LP) extends dendrites through epithelial cells into the lumen and also sample gut bacteria. The epithelium is filled with $CD8^+$ T cells, and the LP contains many $CD4^+$ T cells, macrophages, and immunoglobulin A (IgA)–producing plasma cells. Potentially tissue-damaging T cell responses may be inhibited by immunosuppressive cytokines and regulatory T cells. [from [147, 148]]

The epithelium rests on a basement membrane which is supported by connective tissue elements, collectively termed the lamina propria. This layer includes fibroblasts, endothelial cells, some smooth muscle cells, and a large number of immune cells, including lymphocytes, plasma cells, macrophages, mast cells, eosinophils [145], and dendritic cells [149]. The numbers of the immune competent cells vary, presumably reflecting the relative state of activation of challenged mucosal surfaces. For example, the number of mast cells and eosinophils changes dramatically during numerous inflammatory intestinal disorders such as UC and CD [145, 150]. The predominant lymphoid population in the mucosal lamina propria consists of immunoglobulin A (IgA)-producing plasma cells [145, 146] (Fig. 9B). Secreted dimeric IgA is subsequently transported across the epithelium and is thought to be the important immunological barrier at mucosal surfaces, complexing with luminal antigens and, thus, impeding their penetration [145, 151]. In addition, there are clearly defined lymphoid aggregates, termed Peyer's patches (PP; Fig. 9B) in the gut mucosa, and other solitary lymphoid nodules which are collectively given the term mucosa-associated lymphoid tissues (MALT) [145, 152]. These consist of clearly defined T- and B-cell zones and directly abut the epithelium, which consists of specialized cells, termed M cells (Fig. 9A, B), at such sites. These follicle associated M cells play unlikely a role in antigen presentation, but are more likely a portal for antigen entry into the dome area of the PP [153-155]. Antigen-activated T and B cells

leave the PP and travel via the lymph to the mesenteric lymph nodes, which drain the intestinal tissue [156] and start to induce inflammation by the secretion of proinflammatory cytokines. The outer boundary of mucosa is formed by a sheet of smooth muscle cells, the lamina muscularis mucosa, more commonly called the muscularis mucosa. This layer is very thin, ranging from two to six cells thick [141]. It is more prominent in the large intestine than in the small intestine and becomes progressively thicker in the rectum [157]. Remarkably little is known about the ultrastructure or function of these smooth muscle cells, although several small nerve bundles can be observed piercing the muscularis mucosa and penetrating into the mucosa [141].

3.1.2 The lamina submucosa

The submucosa consists of connective tissue bordering the lamina muscularis mucosa and the outer two layers of smooth musculature, the lamina muscularis externa. The cellular constituents are mainly fibroblasts, some macrophages, mast cells, an occasional eosinophil and basophil, and rarely any plasma cells [141]. All of these cells are embedded in a matrix of loosely packed collagen bundles and some elastin fibers [158]. The submucosa also contains large arteries and veins that run longitudinally, giving perpendicular branches to both the lamina mucosa and lamina muscularis externa [158]. Furthermore, the submucous plexus (Meissner`s plexus) is located in the submucosa abutting the external circular muscle layer [53]. This plexus, together with the myenteric plexus (see Chapter 1, Sec. 2.2.2), is innervated by parasympathetic and sympathetic fibers, whereby the postganglionic nerve cells of the parasympathetic branch are located within the nodes of these plexuses [141]. In addition to these motor neurons, also sensory fibers are coursing in this connective tissue space [141].

3.1.3 The lamina muscularis externa

The lamina muscularis externa consists of an outer longitudinal layer and an inner circular layer of smooth musculature. In between lies the myenteric (Auerbach`s) plexus (see Chapter 1, Sec. 2.2.2). In nearly all mammals, the outer longitudinal muscle layer of the large intestine is segregated into three grossly distinct bands, termed taenae coli, beginning at the ileocaecal junction [141, 159, 160]. Although there is some controversy concerning the disposition of longitudinal muscle layer between the taenae coli, it appears that there is a continuous outer layer around the large intestine [141, 161]. The inner circular muscle layer is also divided into bundles separated by septa. The individual bundles continually merge and divide along the length of the organ [141]. Interestingly, there is a gradual thickening of the muscularis externa from cecum to sigmoid colon [162] and through life span [141], and a specialization of its two layers in the anal canal.

3.1.4 The lamina adventitia

The longitudinal muscle layer is covered by a mat of loose connective tissue containing the usual complement of cells for this type of tissue: macrophages, mast cells, eosinophils, basophiles, monocytes, lymphocytes, fibroblasts, and, in some regions, adipocytes [141]. Aggregations of lymphocytes occasionally form macroscopic “milky spots”. Collectively, this outer layer is termed adventitia and forms one of the four major layers of the large intestine. However, on most of the large intestine the peritoneum covers the adventitia with an additional layer of cells called the mesothelium. In that case, the outermost layer of the large intestine is referred to as serosa (serous membrane) [141].

3.2 Ulcerative colitis and Crohn's disease

In humans IBD classically includes two distinct entities, UC and CD [163, 164]. IBD can be classified as a chronic relapsing inflammatory condition of the intestinal tract and is characterized by mucosal ulceration [165, 166]. Patients suffer from chronic diarrhea, weight loss, abdominal pain, fever, and fatigue[147]. Extra-intestinal manifestations can also occur, including skin ulcers, arthritis, and bile-duct inflammation, the last especially in UC [147]. Epidemiologic studies showed that approximately 0.1 percent of the western population suffer from IBD [167] and that world wide each year 10 – 20 out of 100.000 individuals additionally develop UC, compared to 10 – 200 individuals in case of CD [147]. This incidence rate is stable for UC but increased 8 to 10 fold for CD since the 1960s [147]. The age of onset lies between 15 and 30 years, but both younger and older individuals may also be affected [168].

As described above, one of the core symptoms of IBD is diarrhea. In UC patients these loose stools are slimy and bloody, whereas no blood could be detected in the excrements of individuals suffering from CD [169, 170]. Furthermore, both UC and CD are characterized by mucosal ulceration, which is continuous and restricted to the colon in UC but patchy and possibly spread through the whole intestinal tract in CD [147, 170] (Fig. 10). The downstream effector pathways that drive inflammation and tissue injury are similar to those in immune-mediated diseases in other organs [147]. Thus, immune activation causes an upregulation of different types of cell adhesion molecules on leukocytes and endothelial cells [171], leading to an influx of inflammatory cells from the blood and increasing detectable concentrations of cytokines, free radicals, and lipid mediators [147, 172, 173]. There is also massive overexpression of matrix-degrading enzymes, the matrix metalloproteinases, by fibroblasts, which are ultimately responsible for ulceration and fistulas [147].

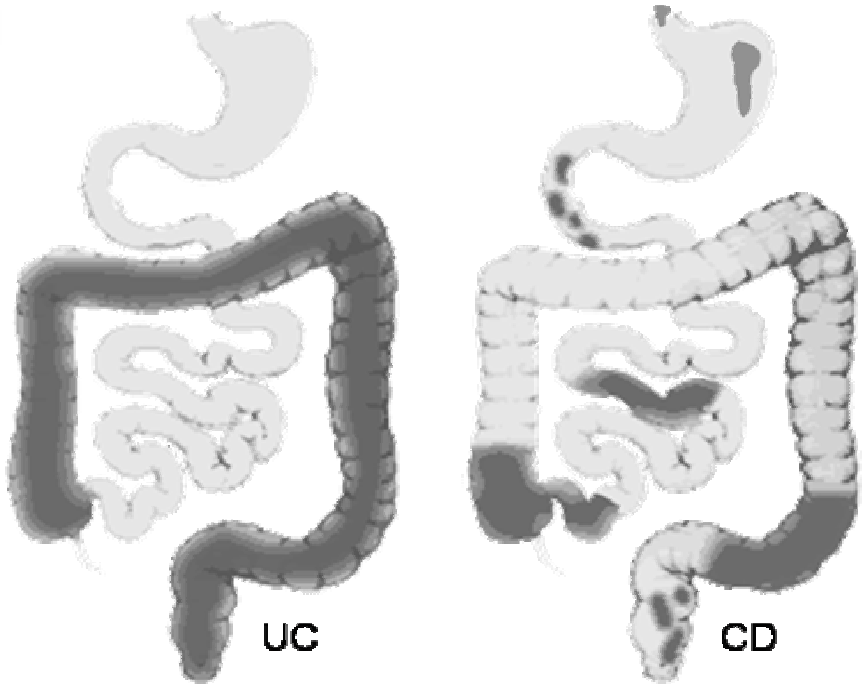


Fig. 10: Schematic representation of the affected areas within the intestinal tract of patients suffering from IBD. Both UC and CD are characterized by mucosal ulceration, which is continuous and restricted to the colon in UC but patchy and possibly spread through the whole intestinal tract in CD. [adapted from www.ced.uni-mainz.de/ced.htm]

Concerning the cytokine secretion by activated lymphocytes, a typical and strong Th1 cytokine profile (increased secretion of tumor necrosis factor (TNF)-α, interleukin (IL)-2, interferon (IFN)-γ, IL-12, IL-18) was described in individuals suffering from CD, in contrast to much weaker support for a T helper type 2 (Th2) pathogenesis (increased secretion of IL-4, IL-5, IL-10) in UC patients [167, 169, 174]. While T helper type 1 (Th1) cytokines do not appear to be raised in UC, there is just little evidence for the presence of increased Th2 cytokines. However, the presence of autoantibodies, such as the anti-neutrophil cytoplasmatic antibody, is suggestive of a Th2 phathogenesis in UC [167]. These conflicting immunological findings are the reason for the paucity of animal models that are able to reproduce the pathological features of human UC [167] (see Chapter 1, Sec. 3.3). However, in murin models of colitis a concomitant decrease in subgroups of suppressor T cells, variously

designated Th3 ($CD4^+$ $CD25^-$) or Tr1 regulatory T cells ($CD4^+$ $CD25^+$), which produce the antiinflammatory cytokines IL-10 and transforming growth factor (TGF)-ß [175], was found.

Macroscopically, UC can be divided in an acute and chronic advanced phase. During the acute phase a reddened and edematous mucosa was described, which starts bleeding after external contact. Mucosal ulcerations were not found during this phase. In contrast, during the chronic advanced phase relapsing ulcerations lead to destructions of the mucosa accompanied by crypt abscesses [170]. The inflammatory process during UC invariably involves the rectum and extends proximally in a continuous fashion, but remains restricted to the colon [148]. Inflammation affects only the superficial (mucosal) layers with infiltration of mainly lymphocytes and granulocytes, paralleled by a loss of goblet cells [148, 176]. In severe case, it leads to "toxic megacolon" and perforations [148].

In CD, ulcers penetrate into the gut wall and fistulous tracts may also develop between loops of bowel or towards the skin [147]. Transmural (affecting all layers of the bowel), dense submucosal infiltration of lymphocytes and macrophages, paralleled by the presence of granulomas in up to 60% of the patients, fissuring ulceration, and fibrosis are typical signs of CD [148]. Any part of the intestinal tract (from mouth to anus) can be affected, whereas the localization within the terminal ileum and colon prevails [170]. CD is further characterized by the presence of segments of normal bowel between affected regions, known as skip lesions [176].

IBD has a complex and multifactorial etiology, comprising genetic [177] and environmental factors [178, 179]. Both UC and CD are shown to represent complex genetic diseases but also tend to run in families [147]. About 20 percent of the individuals with CD have a relative with some form of IBD [148]. Additionally,

concordance in monozygotic twins and ethnic differences strongly suggest the involvement of genetic factors in the development of IBD [180]. Genetic predisposition was further substantiated by the discovery of susceptible loci on chromosome 12 for UC, on chromosome 5, 14, and 16 for CD, and on chromosome 1, 6, and 19 for both UC and CD [181].

In 2001, two groups showed that three major polymorphisms in, or around the external leucine-rich repeat within the recognition region of the nucleotide-binding oligomerization domain (Nod) molecule 2 (Nod2) on chromosome 16 are specifically associated with CD [182, 183]. Nod molecules (Nod1 and Nod2) together with Toll-like receptors (TLR1 to 9) belong to the pattern recognition receptor family, which are expressed by gut epithelial cells, monocytes, and particular dendritic cells (DCs) in the intraepithelial and subepithelial layers. These cells are specialized for sampling microbial and other luminal antigens [184-186] already within the gut by sending long dendrites into the lumen, and they recognize bacteria imported by the M cells into the dome region of the PP (Chapter 1, Sec. 3.1.1). Therefore, a genetically determined disturbance in recognizing the conserved pathogen associated molecular patterns (PAMPs) of these microbial antigens and/or subsequent signaling may result in decreased activation of nuclear factor κB (NF- κB), which is a transcriptional factor for proinflammatory cytokines. A consequence of mutations in Nod2 may therefore be a decreased ability to kill pathogenic gut bacteria [187, 188]. In addition, a possible role for TLR4 polymorphisms in IBD is suggested by the observation that mice with a missense mutation of the *Tlr4* gen have a high incidence of spontaneous colitis [189, 190]. Hence, a picture is emerging that mutations in PAMP signaling in general are associated with IBD and diminished PAMP signaling itself may be a contributing factor to the development of

disease [180]. Interestingly, Nod2 has not been found to be associated with CD disease in Japan [191], highlighting the complex nature of this disease.

Substantial data from clinical observations and animal models additionally incriminate commensal luminal bacteria or their products in the initiation and perpetuation of IBD [192] and associated systemic inflammation [193], although the critical bacterial components or antigens are not yet known. This is quite surprising, because healthy individuals possess an abundant and highly active gut immune system (Fig. 9) that is tightly regulated to prevent excessive immune responses to foods (oral tolerance) and commensal gut bacteria [156, 194-196]. Furthermore, in healthy individuals the commensal flora was shown to even maintain epithelial integrity [147]. This is indicated by an increased epithelial barrier function [197, 198], induction of the cytoprotective heat shock proteins (HSP) 25 and hsp 27 in epithelial cells [199], and other inflammation inhibiting processes due to the presence of the normal gut flora [200, 201].

However, the relationship between the immune system and the commensal flora is a precarious one, and perturbations in immune or epithelial homeaostasis can lead to the onset of gut inflammation. In this situation, the commensal flora appears to act as a surrogate bacterial pathogen, and it is thought that lifelong inflammation ensues because the host response is incapable of eliminating the flora. Therefore, accumulating evidence suggests that the luminal flora is a requisite and perhaps central factor in the development of IBD [164]. This is further supported by the finding that development of spontaneous colitis in rats and mice appears to require the presence of the commensal flora; colitis does not occur in any of several mutant strains when they are maintained in a germ-free environment, but it develops rapidly when these rodents are colonized by commensal bacteria [147, 202].

These findings support the generally accepted hypothesis that IBD results from abnormal immune responses to enteric bacteria in individuals with susceptibility due to polygenic defects, contributing to the onset and perpetuation of IBD [164, 180, 193, 203]. Thereby, an increased intestinal barrier function [204-206], and an activation of T cells instead of T cell tolerance towards bacterial antigens presented by gut epithelial cells [207], both described during IBD, are thought to mediate this overshooting immune response against harmless gut bacteria.

Concerning environmental factors, also blamed to influence the pathogenesis of IBD, it has to be stated that both UC and CD are predominantly associated with industrialized temperate regions and are rare in tropical countries with poor sanitation and a low level of overcrowding [208]. Interestingly, migration to developed countries leads to an increased risk of coming down with IBD [209, 210], further supporting the hypothesis that genetic factors are not solely responsible for the disease. Various environmental factors have been proposed to contribute to the enhanced risk of IBD in industrialized countries, including nutrition, infections, smoking status [211-216], and the level of stress experienced [103-105, 217-220]. The latter finding is of potential interest for the present dissertation and will be outlined in more detail in section 4 of Chapter 1.

3.3 Animal models for inflammatory bowel disease

The study of initiating factors in human IBD is difficult as the clinical materials are in general from the advanced stage of the disease [148]. Therefore, many of the currently known details concerning the early events or the pathogenesis of human IBD have been developed in different types of animal models [167]. These models explained key roles of microflora, epithelial barrier, over reactive lymphocytes, and

the lack of regulatory lymphocytes in colonic mucosal inflammation [148]. Depending on the mechanisms of induction of the inflammatory processes, animal models of IBD can be divided in different categories:

Spontaneous models of IBD are uncommon, with the most studied being the tamarin model [106, 107, 167, 221-223]. Interestingly, in captivity these primates develop a spontaneous colonic inflammation similar to human UC, suggesting an important role of stress (captivity stress) in its pathogenesis [107, 167]. Strains of mice that develop a self-limiting form of right-sided colitis are the C3H/HeJBir [148, 224, 225] and the senescence-accelerated mouse P1/Yit (SAMP) [226].

In adoptive transfer models a colonic inflammation is induced in immunodeficient animals by the transfer of $CD4^+$ T cell subpopulations isolated from healthy animals. The most widely used model in this respect induces colitis within 6-8 weeks by the transfer of $CD4^+$ $CD45RB^{high}$ T cells from an immunocompetent mouse to an immunodeficient SCID or $RAG^{-/-}$ mouse, both deficient for B cells and T cells [227-229]. Recipient mice show a wasting syndrome with transmural intestinal inflammation and a proinflammatory IL-12 driven Th1 response by the transferred $CD4^+$ $CD45RB^{high}$ cells [225]. Interestingly, transfer of the reciprocal population, $CD45RB^{low}$ cells and/or not further purified $CD4^+$ T cells together with the pathogenic $CD45RB^{high}$ cells prevents colitis [167]. This model has helped to define the role of regulatory T cell populations in IBD [230]. However, these transfer models of colitis are very artificial due to the use of immunodeficient mice which are transferred with certain $CD4^+$ T cell subpopulations and therefore do not represent the normal clinical situation of immunocompetent IBD patients.

Another group of animal models for IBD generates animals with a single deletion of a target gene (knockout animals) or increased expression of a particular gene (transgenic animals) [231]. Animal models in which there is a defect in N-cadherin, an epithelial barrier protein, develop IBD [232], suggesting that enteric bacteria then can easily cross the colonic barrier and trigger an immune response [233]. Additionally, deletion of either the cytokine IL-10 (IL-$10^{-/-}$) [234] or IL-2 (IL-$2^{-/-}$) [234] or parts of their receptor complex, $CRFB4^{-/-}$ [235] or IL-$2R\alpha^{-/-}$ [167] respectively, also causes colitis [167]. The IL-$2^{-/-}$ mice, for instance, develop severe colonic inflammation after 8 – 10 weeks of age, characterized by a Th2 driven immune response and therefore similar to human UC [236]. In contrast, IL-$10^{-/-}$ mice develop a severe form of IBD which is mediated by a Th1 immune response and therefore comparable to human CD [236]. Furthermore, a murine model in which a stable form of the TNF-α mRNA is expressed develops arthritis and granuloma formation in the terminal ileum [237]. These findings support a major role for IL-10, IL-2, and TNF- α in the pathogenesis of the different types of IBD. Finally, there are also models using knockout animals with defects in the T-cell receptors ($TCR\alpha^{-/-}$) or the epithelial cell function ($mdr1a^{-/-}$) [165]. Importantly, manipulations in these genetically engineered or transgenic models of IBD (mostly carried out in mice) have also contributed to the study of candidate genes in IBD [167].

In a further animal model of IBD colonic inflammation is induced by oral or rectal administration of different chemicals (inducible models). The latter way of administration is used for dinitrobenzene sulphonic acid (DNBS) [111], trinitrobenzene sulphonic acid (TNBS) [238, 239], acetic acid or oxazolone [240], and is thought to pose an additional and uncontrollable stressor for the animals. Therefore, colitis induction by the rectal instillation of different chemicals is not suitable for investigating the

effects of a defined stress procedure on the pathogenesis or severity of colonic inflammation. This is different for colitis induction by the administration of the sodium salt of a sulphated polysaccharide, dextran sulphate sodium (DSS; Fig. 11), via the drinking water, which has been described for mice [140], rats [241, 242], and hamsters [243, 244]. DSS colitis is restricted mainly to the large intestine and therefore was considered to be a useful animal model for human UC [140, 245]. Interestingly, the finding of a Th1 shifted immune response in animals treated with DSS also suggests a similarity to human CD [246]. However, animals treated with DSS show a marked loss of body weight, rectal bleeding, reduction of colonic length, and destruction of the epithelial layer and glandular architecture of the large intestine [140, 245, 247].

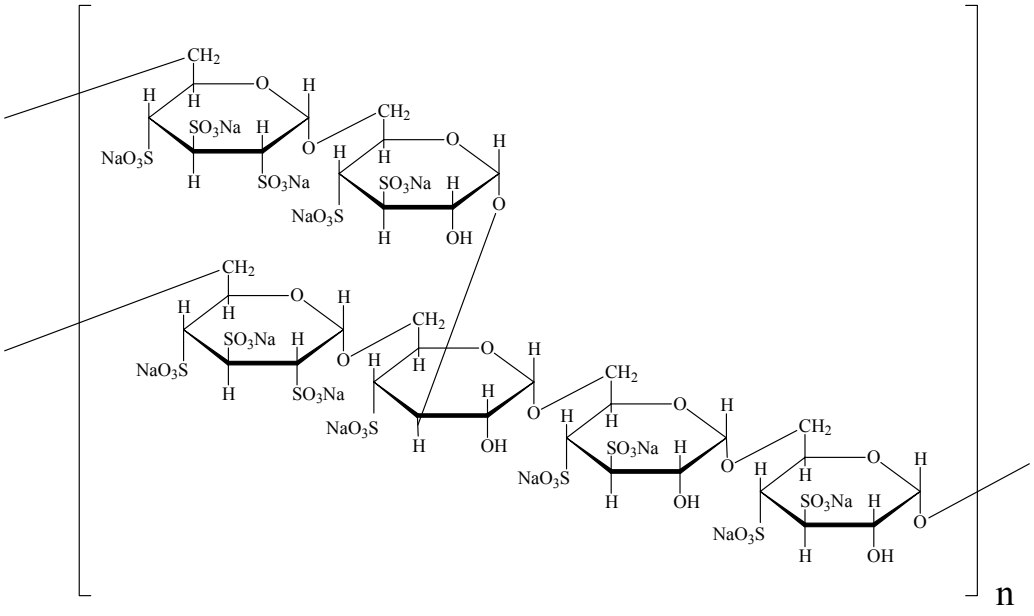


Fig. 11: Chemical structure of dextran sulphate sodium (DSS). [adapted from http://www.sigmaaldrich.com/fluka/product%20information%20sheet/31403_31395_128kb.pdf]

One of the first hypothesized key mechanisms of DSS-induced colitis was a direct toxic effect on mucosal epithelial cells [248-250]. Later on Kitajima *et al*. (1999) showed that colonic mucosal permeability was found to be increased after three days of DSS treatment (BALB/c mice, 5%), although at that time point the superficial mucosal

epithelial cells were still morphologically intact [245]. Therefore, a new hypothesis on the mechanism by which DSS induces colitis emerged [245]: DSS may cause mild injury to the colonic epithelial cells resulting in an increase in colonic mucosal permeability and in toxic luminal bacterial products, such as endotoxin and peptideglycan-polysaccheride polymers, permeating into the colonic mucosa [251]. These permeated substances may have toxic effects and cause destruction of the epithelial cells of basal crypts and induce an inflammation in the colonic mucosa. Due to the uncomplicated and stress-free induction of colitis by DSS, I decided to use this model for the experiments described in the present thesis, investigating the influences of prior chronic psycho-social stress on various parameters of an intestinal inflammation. Colitis was induced by the application of 1% DSS in the drinking water from day 20 to 27, thus, starting one day after termination of the chronic psycho-social stress paradigms.

4. Stress & colitis

The ability of an organism to respond to environmental threats, such as predation or natural disaster, enhanced its chance of survival and consequently its reproductive capacity. As a consequence, the various forms of adaptations that supported such responses were preserved and selected for during evolunitonary processes [252]. As fighting and fleeing carry a high risk of injury, a stressful situation is often accompanied by subsequent entry of infectious agents into the bloodstream or skin. Therefore, it may be that alterations in the immune system, additionally to the physiological support for fight or flight reaction, have been part of these adaptations in terms of stress and were consequently preserved over time [252].

Modern humans rarely encounter many of the stimuli that evoked fight or flight responses for their ancestors. However, human physiological responses continue to reflect the demands of earlier environments, and situations that do not require a fight-or-flight response may therefore have physical consequences, including changes in the immune system [252].

Indeed, over 300 studies in the past 30 year showed that psychological challenges are capable of modifying various features of the immune response in humans [252]. The first theory of stress being broadly immunosuppressive [253] enjoyed long popularity, but would not have been evolutionarily adaptive in life-threatening circumstances [252]. Dhabar & McEwen (1997, 2001) [77, 254] proposed a biphasic model after a series of experiments with mice in which acute stress enhances, and chronic stress suppresses the immune response. However, this model also insufficiently explains findings that link chronic stress with both disease outcomes associated with inadequate immunity (infectious diseases) and disease outcomes associated with excessive immune activity (allergic, autoimmune, inflammatory diseases) [252]. To resolve this paradox, it is hypothesized that chronic stress elicits simultaneously enhancement and suppression of the immune response by altering the patterns of cytokine secretion [255]. Th1 cytokines, which activate cellular immunity to provide defense against many kinds of infection, are suppressed during chronic stress, resulting in permissive effects on production of Th2 cytokines, which activate humoral immunity and exacerbate allergy and many kinds of autoimmune diseases [255]. This shift is mediated via stress hormones such as cortisol [256] and changes the balance between cell mediated and humoral immune response without necessarily influencing the overall level of activation or function within the system [252]. Interestingly, human UC is also described to be mediated by a Th2 shifted immune response [167, 169, 174], maybe suggesting a possible link to chronic stress exposure.

Evidence for a role of stress in the modulation of disease onset and severity in IBD comes from numerous studies done in human and non-human primates as well as in different rodent species. In 1958, Porter *et al.* [257] reported the development of gastrointestinal lesions in 11/19 rhesus monkeys who were either restrained in chairs or placed in a conditioned anxiety situation [106]. Most of these were gastroduodenal erosions, but two of the monkeys, which were conditioned for anxiety, developed a wasting syndrome and chronic colitis [106]. The first hint for a link between stress and human IBD was provided by Salem and Shubair in 1967 who showed that Arab Bedouins frequently developed UC after they were forced to leave their familiar environment in the desert and live in houses provided by the government [105]. Two years later, Stout and Snyder (1969) described extensive colonic ulceration in Siamong gibbons that died within a few weeks after the death of their mating pair [258]. Furthermore, the development of spontaneous colonic inflammation (in humans shown to be a precancerous lesion) and carcinomas in different tamarin species (New World primates; *Saguinus oedipus* = cotton-top tamarin; *Saguinus mystax* = moustached tamarin) during captivity [106, 107, 259] points out a possible link between stress and the development of colonic inflammation. Importantly, these colonic inflammations are absent in the wild populations [260]. These studies also showed that stress during captivity (mainly due to the low temperatures [261] in temperate climates) is the trigger for the onset of colitis, but further implicated the fecal stream as an important factor in the life-long persistence of colitis in the cotton-top tamarin model [259]. This is further supported by the finding that most of the experimentally induced animal models of intestinal inflammation are not working in germ-free animals [262].

In 1991, Duffy *et al.* clearly demonstrated in a large prospective study with 124 patients (UC and CD) performed over 6 month an increased risk of IBD symptom

exacerbation following severe, sustained life stress [217]. Additionally, a longitudinal human study done by Bennett *et al.* in 1998 provided evidence that the presence of a highly threatening chronic stressor inhibits improvement from irritable bowel syndrome, while its reduction or absence may be a prerequisite for a significant improvement of the disease [263]. Furthermore, in 1998 Levenstein *et al.* showed that among a group of patients with previously diagnosed UC who were currently in complete clinical remission, those with endoscopically visible rectal mucosal abnormalities reported higher levels of perceived life stress [219]. A comparable study done by Bitton *et al.* (2003) also described that among 60 patients with UC in remission phase, those who relapsed showed significantly more severe life events during the time span prior to relapsing compared with patients that did not relapse [220]. Further support for an impact of psychological factors and, thus, brain activity on intestinal disease state comes from the extremely high success rate of treating human colitis with placebo drugs [264, 265].

However, the role of psycho-social factors in the development and modulation of common gastrointestinal disorders, such as irritable bowel syndrome and IBD, remains controversial [266]. For example Campbell *et al.* (1986) investigated in a prospective study whether there is a link between stressful life events and symptoms of pain or diarrhea and failed to find any significant correlations [267]. Additionally, in a review by North *et al.* (1990) they reported that only 8 out of 15 studies, done in patients with UC, provided a link between stressful life events and the disease, whereas the other 7 studies failed [268]. In their own study they also failed to provide evidence for a link between stressful life events and disease activity in 24 patients with CD and 8 patients with UC [269]. Furthermore, Riley *et al.* (1990) and Garret *et al.* (1991) were not able to determine a clear correlation between stress and the pathogenesis of IBD [270, 271]. The existence of these contrary findings is particularly

surprising in view of the unique and well established bidirectional interactions between brain and gut, the prominent role of the gut-associated lymphoid tissue in this brain-gut interaction, and in view of the common clinical impression that certain stressful life events frequently precede exacerbation of symptoms in all of these disorders [266].

In addition to the already mentioned studies done in human and non-human primates, a growing number of rodent studies provides evidence for a link between exposure to different types of defined stress procedures and the pathogenesis of an experimentally induced colitis (see Chapter 1, Sec. 3). Interestingly, the direction of the stress-induced effects on colitis severity is varying, on the one hand resulting in increased colitis severity and on the other hand in a decreased one.

Collins *et al.* (1996) showed in rats that a previous colonic inflammation, which was induced by TNBS administration 6 weeks prior to stressor exposure, was reactivated by restraint stress (3h/day for 3 consecutive days), as indicated by a significant increase in myeloperoxidase (MPO) activity. Interestingly, these stress-induced inflammatory processes were not accompanied by evidence of further tissue damage, suggesting that changes in colonic histology may take longer to establish [272]. Additionally, Gue *et al.* (1997) showed that 4 days of partial restraint stress (PRS; 2h/day) applied either prior to or after TNBS instillation were shown to increase the severity of experimental colitis in rats, with the effect being not mediated by increased brain levels of either CRH or AVP [108]. In contrast, they showed that brain CRH and AVP seem to have colitis ameliorating effects, because central injection of either CRH antagonist or AVP antagonist before PRS (applied prior to TNBS instillation) resulted in further enhancement of colitis severity [108]. The authors therefore hypothesized that 4 days of PRS have proinflammatory effects on a subsequent TNBS-induced colitis, but their observations reinforce that during stress,

brain CRH and AVP act to minimize the stress-induced enhancement of colonic inflammation by activation of the HPA axis and secretion of immunosuppressive corticosterone [108]. These data are further supported by the findings of Million *et al.* (1999) who showed that daily intracerebroventricular injections of CRH during the 6 consecutive days, following colitis induction by TNBS, resulted in a decreased colitis severity in inbred rat strains with hypo (Lewis/N) and hyper (Fischer344/N) CRH responses to stress [273]. Additionally, they underlined the protective role of an increased HPA axis activity during stressor exposure on colitis severity by the finding that daily intermittent stress (3h/day water avoidance stress or wrap restraint alternatively for 6 days) following colitis induction by TNBS exaggerated colitis severity, but the effect being more pronounced in the CRH hypo-responsive Lewis/N rats [273]. Similar to the already mentioned findings of Collins *et al.* (1996) [272], Qui *et al.* (1999) demonstrated that colitis induced in mice by dinitrobenzenesulfonic acid (DNBS) resolves by 6 weeks, but can subsequently be reactivated by stress (2 x 2h/day of combined restraint and sonic stress for 5 consecutive days) plus a sub-threshold dose of DNBS, but not by a sub-threshold dose of DNBS alone [111]. Thereby, the reactivation of colitis was also paralleled by mucosal inflammation and ulceration [111]. Furthermore, they provided evidence for an essential role of $CD4^+$ lymphocytes in the reactivation process, because reactivation by stress and a sub-threshold dose of the sensitizing hapten was absent in CD4-deficient mice. Interestingly, stress-induced reactivation of colitis in naive SCID mice after the transfer of $CD4^+$ cells from mice with previous colitis was possible, in case of a combined treatment with a subclinical dose of hapten [111]. In addition, they have shown that stress reduces the colonic barrier to luminal contents by decreasing mucus production and increasing colonic permeability [111]. In 2002, Milde and Murison [109] extended the above described results from Gue et at. (1997) [108]. They

showed that restraint stress in rats (2h/day for 4 consecutive days) also enhanced the development of a subsequent colitis induced by DSS treatment via the drinking water. However, the results of that study were not consistent with those of Gue *et al.* (1997) [108] and Collins *et al.* (1996) [272] showing that restraint stress after colitis induction exacerbated or sustained the inflammatory condition [109]. This was probably due to the way of inflammation assessment in the Milde and Murison study, because colonic inflammation may have been still present regardless of visible, bloody stools [109].

In addition to the already mentioned studies providing evidence that stress fastens the onset, exacerbates the severity and/or causes reactivation of an experimentally induced colitis, there are also studies showing contrasting effects. For example, Cakir *et al.* (2004) showed that 30 min of water avoidance stress, applied during the 6th hour after colitis induction by acetic acid infusion, ameliorated the severity of the colonic inflammation [274]. Interestingly, by the injection of the GR antagonist RU486 one hour before stressor exposure, they could demonstrate that this effect was mediated by GCs, the most potent endogenous inhibitors of inflammation [274]. In addition, Gülpinar *et al.* (2004) provided evidence that acute stress prior to colitis induction by TNBS reduced its severity, possibly via the stimulation of the HPA axis and/or the SNS, as suggested by an increased colitis severity in rats which were injected with a CRH antagonist (intracerebroventricular) or GC receptor antagonist (RU486) 10 min prior to stressor exposure [275]. They also claimed to have shown that only 30 min of controllable electric shock stress (ES; 20 random foot shocks), but not 2h of uncontrollable PRS was able to diminish the severity of a TNBS-induced colitis. This is surprising because a comparable decrease in MPO activity and in direction also in the macroscopic damage score in PRS and ES rats compared with unstressed rats, all treated with TNBS after stressor exposure, rather suggests a

general down-regulation of colitis severity after short term stress in this model. They further hypothesized that the contrasting effects between the colitis exaggerating effects of prior repeated stress (restraint; 2h/day for 4 days) described in the study done by Gue *et al.* (1997) [108] and the colitis ameliorating effects of repeated stress (PRS after chronic ES; day 1: exposure to electric shocks; days 2, 3, 4: exposure to the same boxes but receiving no shock; day 4: 2 hous of PRS) in their study could be due to the different levels of controllability of these stressors [275]. A more evident explanation might be that during the daily exposure to the homotypic stressors, either restraint stress [108] or ES [275] an adaptation of the HPA axis might have occurred, which resulted in equal or just slightly increased GCs levels compared with unstressed animals. The additional exposure to the heterotypic PRS after 4 days of ES and, thus, immediately before colitis induction, probably may have dramatically increased the levels of antiinflammatory corticosterone. These differences in the levels of GCs at the time point of colitis induction may have caused the contrary effects of repeated stress on colitis severity found in these two studies.

From the above mentioned rodent studies one can conclude that although the effects of stress on the pathogenesis of an experimentally induced colitis are contrasting, a stress-induced increase in HPA axis activity, paralleled by an increase in antiinflammatory GCs, seems to be beneficial rather than detrimental. In this respect it is important to note that endogenous corticosterone was also shown to be a mediator of stress-induced decrease in colonic barrier function [276], which is a main feature in patients suffering from IBD [277]. However, concerning the contrasting effects of endogenous GCs in the above mentioned rodent studies, there is speculation that the onset of IBD might be associated with hypocortisolism [266] rather than hypercortisolism. Interestingly, there is indeed evidence of disturbed HPA-axis activity in patients suffering from functional bowel disease [109, 278].

5. Aim of the present thesis

A rapid expansion of animal studies in the recent years provided evidence that various stressors influence the onset, the severity, and the reactivation of an experimentally induced colitis, but the detailed mechanisms are still poorly understood. These studies substantiated that an increase in plasma levels of GCs following stressor exposure may on the one hand exert proinflammatory effects (for example mediated by an increase in colonic permeability), but on the other hand may be rather beneficial than detrimental due to the antiinflammatory potential of these hormones. However, the stressors described in these studies were very time-limitted (with a maximum of 4 days) and had just little heuristic value because they were not really related to the environmental challenges an animal may meet in its everyday life in a natural environment. Furthermore, the stressors used in these studies had just little or even no face validity, because in the human situation, stressors that have been shown to be linked with IBD were chronic and much more closely related to social factors than to unnatural and physically painful challenges.

Therefore, the present thesis aimed to study the effects of two different models of chronic psycho-social stress on a chemically-induced colitis in male mice. In more detail, I have addressed the question whether prior exposure to chronic psycho-social stress over 3 weeks either influenced the severity of or the regeneration after a subsequent DSS-induced colitis or even caused spontaneous colonic inflammation by itself. Furthermore, I aimed to reveal involved mechanisms, thereby mainly focusing on the different levels of the HPA axis. The two models of chronic psycho-social stress were designed to be as heuristic as possible and to further fulfil the above mentioned criteria of chronicity and face validity in an adequate way.

In **chapter 2**, the effects of prior chronic intermittent psycho-social stress on a subsequent chemically-induced colitis are described, and stress-induced adrenal insufficiency as a possible underlying mechanism is discussed.

Therefore, the social defeat/overcrowding (SD/OC) stress paradigm was established and characterized for its physiological and neuroendocrine effects on generally accepted indicators of chronic stress. Furthermore, SD/OC-induced effects on the severity of and the recovery from a subsequent colitis induced by DSS were investigated. To reveal possible underlying mechanisms, the question was addressed whether 19-day exposure to SD/OC affects the *in vivo* or *in vitro* responsiveness of adrenal cells and, as a consequence, the availability of antiinflammatory GCs.

In **chapter 3**, the effects of another chronic psycho-social stressor, namely chronic subordinate colony housing (CSC), on the development of spontaneous colonic inflammation are described. Furthermore, the interplay of the initial rise in GCs during CSC exposure and the later on developed adrenal insufficiency are discussed as possible mediators of the stress effects.

Therefore, in a first experiment the effects of 19-day exposure to CSC on relevant stress-parameters and colonic inflammation were investigated. Furthermore, the time course of various physiological, neuroendocrine, and immunological parameters was assessed during CSC exposure to reveal possible key events, triggering the onset of spontaneous colonic inflammation. Again, the question was addressed whether 19-day exposure to CSC affects the *in vivo* or *in vitro* responsiveness of adrenal cells and, as a consequence, the availability of antiinflammatory GCs. In addition, adrenalectomy (ADX) was performed prior to CSC exposure to reveal the involvement of the initial rise in GCs in the stress-induced development of spontaneous colonic inflammation. Finally, the sensitivity of immune cells to

immunosuppressive GCs was assessed, as stress-induced GC insensitivity might also be a possible mediator of CSC effects on spontaneous colonic inflammation.

In **chapter 4**, the effects of CSC exposure on the time course of a subsequent chemically-induced colitis are described. Furthermore, the initial rise in GCs during CSC and the later on developed adrenal insufficiency are discussed as possible mediaters of CSC effects on colitis severity.

Therefore, the effects of 19-day exposure to CSC on the severity of a subsequent colitis induced by DSS were assessed after 2, 4, or 8 days of DSS treatment. In parallel, plasma corticosterone concentrations were determined at the same time points, to reveal the involvement of adrenal insufficiency and the resulting lack of antiinflammatory GCs, in the CSC-induced increase in colitis severity. In addition, ADX was performed prior to CSC exposure to investigate whether the initial rise in GCs during CSC exposure is involved in the stress-induced exacerbation of a subsequent DSS colitis.

Chapter 2

Chronic intermittent psycho-social stress (social defeat/overcrowding) in mice increases the severity of an acute DSS-induced colitis and impairs regeneration

[adapted from [279]: **Reber SO, Obermeier F, Straub RH, Falk W., Neumann ID; 2006; Endocrinology 147:4968-4976**]

Abstract

UC is a multi-factorial disease, with immunological, genetic and environmental factors playing an important role in its pathogenesis. In this study the consequences of exposure to a chronic intermittent psycho-social stressor on the severity of a DSS-induced colitis in male C57BL/6 mice were investigated. Chronic stress was induced by repeated exposure to social defeat (SD, 2 h) and overcrowding (OC, 24 h) during 19 consecutive days.

SD/OC mice showed a diminished body weight gain, thymus atrophy, and adrenal hypertrophy but similar light phase plasma corticosterone concentrations compared with unstressed mice. In contrast, the rise in dark phase corticosterone was significantly attenuated in SD/OC mice while plasma ACTH concentrations and hypothalamic CRH mRNA expression did not differ between SD/OC and control mice. Additionally, adrenal cells from SD/OC mice showed a decreased *in vitro* response to ACTH stimulation. Subsequent treatment with 1% DSS from day 20 to 27 resulted in a more severe intestinal inflammation in SD/OC mice, as reflected by an increased body weight loss, histological damage score, secretion of IL-6, TNF-α and IFN-γ from mesenteric lymph node cells, and inflammatory reduction of colon length on day 8 of DSS treatment. The impaired health status of stressed mice was also reflected by a significantly lower survival rate after termination of the DSS treatment. In conclusion, the present findings demonstrate that chronic intermittent exposure to a psycho-social stressor before the induction of an acute DSS colitis results in adrenal insufficiency, increases severity of the acute inflammation, and impairs the healing phase.

Introduction

IBD can be classified as a chronic relapsing inflammatory condition of the small and large intestine that appears either as UC or CD [165, 166]. The pathogenesis of IBD is still unknown but it is generally accepted that it has a complex, multi-factorial aetiology, comprising of genetic [177] and environmental factors [178, 179], which are associated with dysregulations of the mucosal immune system. For example, IBD is predominantly associated with industrialized temperate regions and is rare in tropical countries with poor sanitation and a low level of overcrowding [208]. Migration to developed countries leads to an increased risk of coming down with IBD [209, 210], indicating that genetic factors are not solely responsible for the disease. Various environmental factors have been proposed to contribute to the enhanced risk of IBD in industrialized countries, including the infection rate with nematodes [212, 213, 280] and the level of stress experienced [103-105, 217-220]. However, the role of stress in the pathogenesis of IBD remains controversial. Exposure to a variety of life stressors have been shown to exacerbate colitis [103-105, 217-220]. For example, Salem *et al.* [105] showed that Arab Bedouins frequently developed a spontaneous colitis after they were forced to leave their familiar environment. However, in retrospective human literature, which must be interpreted with caution, there are studies showing contrary findings [269, 270, 281].

Both captivity stress and readjustment to a novel social environment were shown to cause spontaneous colitis in cotton-topped tamarins (*Sanguinus oedipus*) [106, 107]. This is supported by other studies demonstrating that repeated exposure to various time-limited stressors (4 to 5 days), including restraint [108, 109] or a combination of cold and restraint stress [110], applied either before or immediately after induction of an experimentally-induced colitis, exacerbated the colonic inflammation in rats. In

addition, reactivation of a completely resolved acute colitis has been described after a combination of restraint and sonic stress, and a sub-threshold dose of DNBS but not by a sub-threshold dose of DNBS alone [111]. Further support for the above mentioned link between stress and IBD is provided by numerous animal studies showing that stress also influences the pathogensis of multiple other diseases [253, 282-284].

However, the influence of a chronic psycho-social, and therefore clinically relevant, stressor on the development and severity of an experimentally-induced colitis has not previously been examined. Therefore, the aim of the studies described in this chapter is (1) to establish, and to extensively characterize, an ethologically relevant model of chronic intermittent psycho-social stress in male mice, and (2) to investigate whether exposure to this stress procedure prior to the induction of an acute DSS-induced colitis influences the severity of, and the subsequent regeneration process after the colonic inflammation. In particular, the aim is to reveal stress-induced alterations of HPA axis functions, which could be at least partially responsible for mediating the effects of chronic stress on the chemically-induced colitis.

Material and methods

Animals

Male C57BL/6 mice (Charles River, Sulzfeld, Germany) weighing 19-22 g (experimental mice) or 30-35 g (used as residents during the stress procedure) were individually housed in standard polycarbonate mouse cages (16 x 22 x 14 cm; experimental mice) or in polycarbonate observation cages (38 x 22 x 35 cm; residents) for at least one week before the start of the experiment. All mice were kept under standard laboratory conditions (12-h light/dark cycle, lights on at 0600 h, 22 ± 2 °C, 60 ± 5 % humidity) and had free access to tap water and standard mouse

diet. All experimental protocols were approved by the Committee on Animal Health and Care of the local government, and conformed to international guidelines on the ethical use of animals. All efforts were made to minimize the number of animals used and their suffering.

Experimental procedure

Mice were exposed to 19 days of a social defeat/overcrowding (SD/OC) stress paradigm (Fig. 12; control: n = 49; SD/OC: n = 48). Some of the stressed and unstressed control mice (control: n = 5; SD/OC: n = 5) were sacrificed on day 20, at the beginning of the light phase (0700 h), to study the effect of the chronic stress on body weight, relative thymus weight, relative adrenal weight, and basal plasma corticosterone concentration during light phase. In order to investigate basal plasma corticosterone and ACTH concentrations at the beginning of the dark phase, i.e. under stimulated conditions, further animals (control: n = 8; SD/OC: n = 8) were sacrificed on day 20 at the beginning of the dark phase (2000 h). Adrenals of these mice were also taken and examined for their ability to respond to an acute ACTH challenge *in vitro*. Additionally, brains were removed to analyse the effect of the SD/OC stress on hypothalamic CRH mRNA expression.

The effect of prior exposure to SD/OC stress on the severity of an acute DSS-induced colitis (1% from day 20 to 27; Figure 1) was assessed in stressed (n = 9) and control mice (n = 10). Respective controls continued to drink tap water (SD/OC; n = 8; control; n = 8). On day 27, i.e. on day 8 of DSS treatment, all mice were sacrificed for quantification of colon length and histological assessment of the colon (histological score, see below), and plasma corticosterone and ACTH concentrations. Additionally, cytokine secretion by mesenteric lymph node cells of all DSS-treated mice was assessed as described before [285].

To determine whether prior chronic stress exposure affects the regeneration process after a subsequent DSS-induced colitis, the survival rate of control (n = 18) and SD/OC (n = 18) mice was monitored 4 days after the termination of subsequent DSS treatment (1% DSS from day 20 to 27; see Fig. 1).

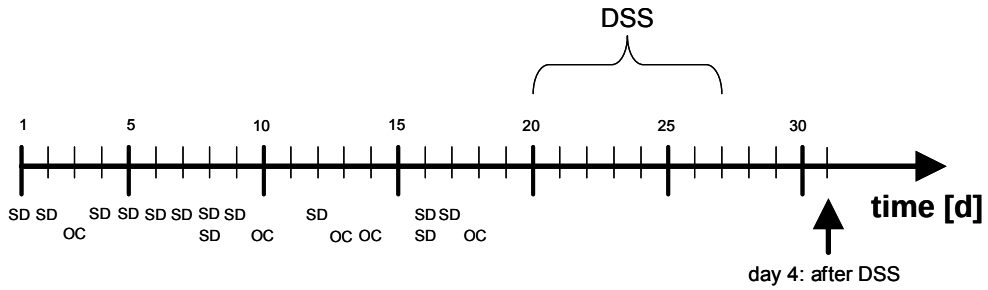


Fig. 12: Schematic illustration of the experimental design of the social defeat (SD)/overcrowding (OC) stress paradigm. Single-housed male C57BL/6 mice were exposed to SD (2 h) and OC (24 h), as indicated, during a 19-day period. Starting on day 20, mice were treated either with 1 % DSS or tap water until they were sacrificed on day 27. In another set of control and SD/OC mice the survival rate on day 4 after termination of subsequent DSS treatment was monitored.

Social defeat/overcrowding (SD/OC) stress paradigm

Mice were randomly assigned to the control or SD/OC group. Control mice were singly housed and remained undisturbed except change of bedding once a week. The SD/OC group was exposed to unpredictable SD and OC during the 19-day stress period (Fig. 12). SD comprised of repeatedly placing mice into a male resident's home cage for 2 h, either once or twice a day, according to the protocol (see Figure 12 for details). In order to avoid physical injuries, the two opponents were separated by a perforated partition wall (Fig. 13B, C) immediately after the first attack of the resident (Fig. 13A), allowing visual, olfactory, and auditory contact. The intruder was confronted with different residents to avoid habituation to the resident. During OC, performed on days 3, 10, 13, 14 and 18 (Fig. 12), large groups (n = 16) of experimental mice were housed together in one observation cage (38 x 22 x 35 cm) for 24 h with free access to water and food (Fig. 13D).

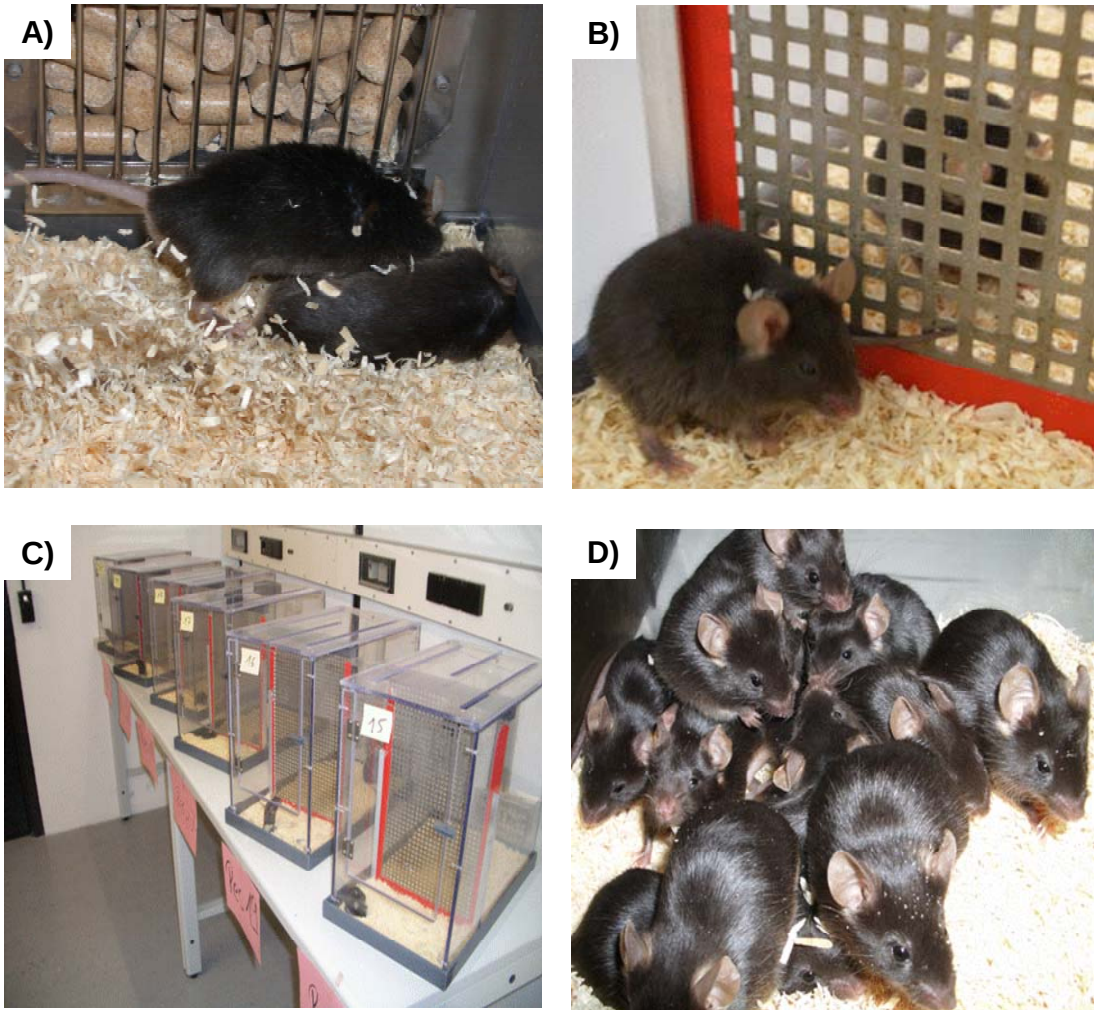


Fig. 13: Chronic intermittent psycho-social stress is induced by 19-day exposure to SD/OC. A) the experimental mouse is attacked and socially defeated by the resident mouse; B) after the first attack the two mice are separated by a partition wall to avoid servere injuries (2h); C) standardized SD/OC procedure in our laboratories; D) 16 experimental mice are housed together for 24 h

Induction of acute colitis

The induction of an acute colitis was achieved by administering 1% DSS (36-50 kDa; ICN, Eschwege, Germany) in the drinking water *ad libitum* from day 20 to 27, as previously described [285].

Blood sampling and radioimmunoassay for corticosterone and ACTH

Mice were rapidly killed by decapitation under CO_2 anaesthesia and approximately 200 µl trunk blood was collected on ice in EDTA-coated tubes (Sarstedt, Nümbrecht, Germany) containing 10 µl aprotinin (Trasylol, Bayer Corp. AG, Leverkusen, Germany). The tubes were then centrifuged at 4 °C (5000 rpm, 10 min) and finally stored at -20 °C until assayed using a commercially available radioimmunoassay for corticosterone and ACTH (ICN Biomedicals, Inc., Costa Mesa, CA) with a detection limit of 10 ng/ml and 4 pg/ml, respectively.

In situ hybridisation of hypothalamic CRH mRNA

After decapitation, brains were removed, snap frozen in iso-pentane cooled on dry-ice and stored at -80°C for subsequent *in situ* hybridisation. A series, with six 16-µm cryocut hypothalamic sections per mouse, was thaw-mounted onto slides in a cryostat at -20°C and afterwards was used to assess hypothalamic CRH mRNA. The hybridisation protocol was adapted from Bosch *et al.* [286]. Briefly, hybridization to CRH mRNA was performed by using a 48-base ^{35}S-labeled oligonucleotide probe complementary to bases 64 - 111 (probe sequence: 5' ggc ccg cgg cgc tcc aga gac gga tcc cct gct cag cag ggc cct gca). Using the National Center for Biotechnology Information basic local alignment and search tool (NCBI BLAST), the sequence was demonstrated to be specific for the mouse CRH transcript. Hybridised slices were exposed to BioMax MR film under 'safe' red light conditions (Eastman Kodak, Rochester, New York, USA) for 21 days. Expression of CRH mRNA was measured as optical density on a Macintosh computer with a computerized image program (ImageJ 1.31, National Institutes of Health, http://rsb.info.nih.gov/ij/). The region of the hypothalamic paraventricular nucleus of four out of six slices per slide was measured bilaterally for each subject to provide individual means. For tissue

background, the optical density of a nonhybridized region outside the PVN was measured.

Determination of colonic length and histological score

As described before [287, 288], the inflammatory reduction of colonic length was used as a parameter to assess colonic inflammation. The colon was removed, mechanically cleaned, and measured to 0.1 cm precision. 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. Three 3-µm haematoxylin-eosin stained sections taken 100-µm apart were evaluated by histological scoring performed by an investigator blind to treatment. For statistics, each individual score represented the mean of the three sections. Histology was scored as follows based on [289, 290]:

Epithelium. 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

Infiltration. 0: no infiltration; 1: infiltrate around crypt basis; 2: infiltrate reaching 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.

The total histological score represents the sum of the epithelium and infiltration score and ranges from 0 to 8.

Isolation and incubation of mesenteric lymph node cells

Mesenteric lymph nodes (pooled from each experimental group) were harvested under sterile conditions and collected on ice in cell culture medium [RPMI-1640 supplemented with 10% fetal calf serum (Biochrom, Berlin, 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)]. Lymph nodes were mechanically disrupted and filtered through a cell strainer (70-µm Nylon, Falcon™, Becton Dickinson, Heidelberg, 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 200 µl of 2.5 µg/ml anti-CD3 antibody in the presence of IL-2 (final concentration 100 U/ml). Eight wells were transferred with the respective number of cells of each experimental group. After incubation for 24 h (37 °C, 5% CO_2), cytokine concentrations were measured in the supernatants by ELISA (all from Endogen, Woburn, MA) using four wells per experimental group.

ACTH stimulation of isolated adrenal cells in vitro

In order to reveal the consequences of chronic stress on the capability of adrenal cells to respond to an ACTH challenge *in vitro*, the stimulation protocol was adapted from Bazhan *et al.* [291]. After decapitation, adrenal glands of four mice of the same experimental group (control vs. SD/OC) were pooled and stored in ice cold Krebs-Ringer bicarbonate-glucose buffer (KRBB, pH 7.4, 0.5% BSA) until they were cleared, cut into small pieces and incubated in 1.5 ml KRBB (4% BSA, 4% collagenase) for 60 min at 37°C in an atmosphere of 95% O_2 and 5% CO_2. Adrenal cells were then dispersed by gentle homogenization (repeated pipetting) and filtered through 5 layers of gauze bandage. The suspensions were then centrifuged (4°C, 10 min at 300 r.p.m.; 10 min at 800 r.p.m.) and washed three times in 2.5 ml cold KRBB (0.5% BSA). Finally, cells were counted (tryphan-blue), cell viability was assessed (tryphan-blue exclusion method), and adjusted to a cell concentration of 4 x 10^5 cells/ml in cold KRBB (0.5% BSA). Two aliquots (a 200 µl) of each group

were incubated for 2 h at 37°C in an atmosphere of 95% O_2 and 5% CO_2 in the presence or absence of different doses of ACTH (10^{-13}, 10^{-12}, 10^{-11} and 10^{-10} M). Afterwards, cells were sedimented by brief centrifugation (5 min at 2000 r.p.m.) and supernatants were stored at -20°C until radioimmunological quantification of corticosterone (triplicates of each sample were analyzed).

Statistics

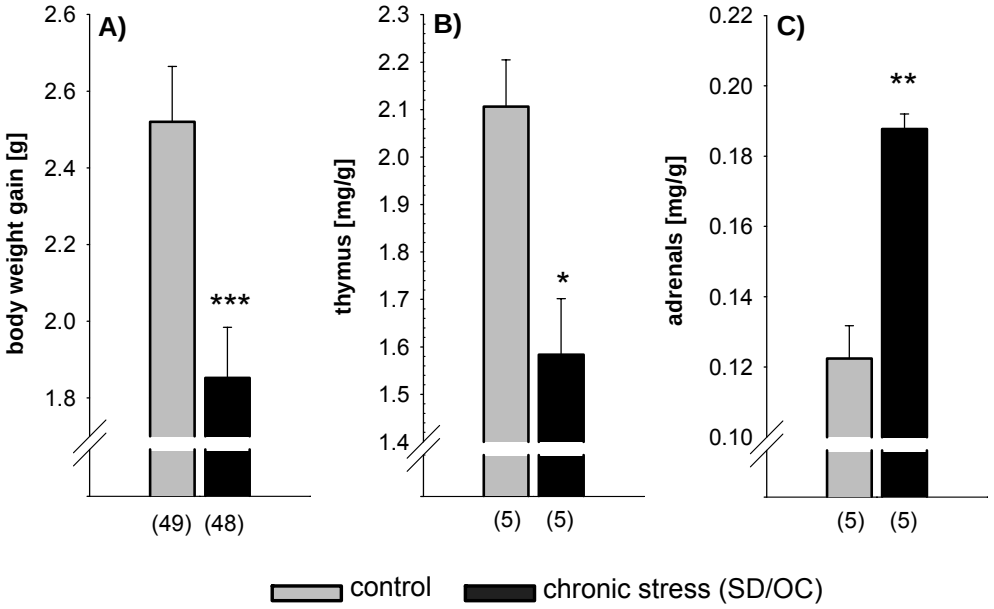
A Mann-Whitney *U*-test was used to compare SD/OC and non-stressed mice (body weight gain during SD/OC, relative thymus and adrenal weights, plasma corticosterone and ACTH concentrations, hypothalamic CRH mRNA expression, number of adrenal cells isolated per animal, cytokine secretion). Two-way ANOVA (factor stress, factor ACTH dose) followed by Tukey honestly significant different (HSD) *post-hoc* testing were appropriate to compare the effect of chronic stress on *in vitro* stimulation of adrenal cells. For comparison of the effects of chronic stress on DSS-induced colitis two-way ANOVA (factor stress, factor DSS) and *post hoc* Tukey-HSD tests have been applied. The survival rates were analysed by Fisher's Exact Test. Data represent mean ± S.E.M. Significance was set at $p < 0.05$. All data were analysed using the software package SPSS (SPSS Inc. Headquarters, 233 S. Wacher Drive, Chicago, Illinois 60606, version 12).

Results

Effects of exposure to SD/OC on body, relative thymus, and adrenal weights, plasma corticosterone and ACTH concentrations, and hypothalamic CRH mRNA expression.

SD/OC mice gained significantly less body weight during the 19-day chronic stress paradigm compared with controls (Fig. 14A). In addition, SD/OC mice, sacrificed

one day after termination of the stress procedure, showed a significantly decreased relative thymus weight (Fig. 14B, D) and a significantly increased relative adrenal weight (Fig. 14C, E) compared with unstressed controls.



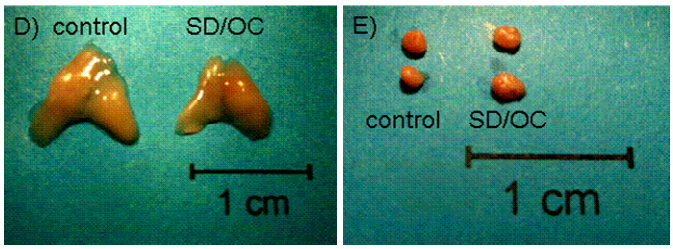


Fig. 14: Effects of exposure to the social defeat/overcrowding (SD/OC) stress paradigm on body weight gain (comparing day 19 with day 1), relative thymus weights (thymus weight [mg]/body weight [g]), and relative adrenal weights (adrenal weight [mg]/body weight [g]) compared with single-housed control mice as measured the day after termination of the stress procedure (day 20). SD/OC mice showed diminished body weight gain, reduced relative thymus weights and increased relative adrenal weights. Furthermore, two photos are shown which are representative for SD/OC-induced thymus atrophy (D) and adrenal hypertrophy (E). Numbers in parentheses indicate group sizes. Data represent mean + S.E.M.; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. unstressed controls.

Basal plasma corticosterone concentrations did not differ between control and SD/OC mice during the light phase at 0700 h (Tab. 1). Consequently, the light phase corticosterone: adrenal weight ratio was significantly lower in SD/OC (Tab. 1) mice

compared with unstressed controls. In the dark phase at 2000h, a 3-fold rise in plasma corticosterone concentrations was observed in unstressed controls, which was attenuated in SD/OC mice (1.5-fold; Tab. 1). In contrast, plasma ACTH concentrations, as well as hypothalamic CRH mRNA expression, did not differ between control and SD/OC mice during the dark phase at 2000 h (Tab. 1).

Table 1. Effects of chronic exposure to social defeat/overcrowding (SD/OC) on plasma light and dark phase corticosterone concentrations, the light phase corticosterone [ng/ml]: adrenal weight [mg] - ratio (lpC : A), dark phase plasma ACTH concentrations, and hypothalamic CRH mRNA expression in male mice on the day after termination of the stressor.

	controls	**SD/OC**
light phase corticosterone [ng/ml]	24.2 ± 3.0 (n=5)	24.9 ± 3.9 (n=5)
dark phase corticosterone [ng/ml]	76.0 ± 15.4 (n=7)	37.5 ± 6.7* (n=5)
lpC : A [1/1000ml]	7.41 ± 0.81 (n=5)	4.84 ± 0.76* (n=5)
dark phase ACTH [pg/ml]	41.9 ± 5.1 (n=7)	52.4 ± 7.1 (n=8)
hypothalamic CRH mRNA expression [grey density]	22.2 ± 1.75 (n=6)	21.9 ± 1.84 (n=8)

Mice were exposed to the stress procedure for 19 days as described in the text, or were kept individually (control). Data represent mean ± S.E.M.; * $p < 0.05$ vs. controls.

Effects of exposure to SD/OC on adrenal corticosterone secretory responses in vitro

The total number of isolated adrenal cells/animal of SD/OC ($5.7 \times 10^5 \pm 9.9 \times 10^4$) compared with control mice ($6.8 \times 10^5 \pm 6.9 \times 10^4$) was not statistically different ($p = 0.667$). No effect of chronic stress was found for baseline corticosterone secretion from adrenal cells *in vitro* (Fig. 15). However, exposure of adrenal cells to ACTH, which has a stimulating effect on the release of corticosterone, was

dependent on prior SD/OC exposure (factor dose x stress: $F_{4, 85} = 5.33$; $p < 0.01$; Fig. 15). *Post hoc* Tukey-HSD analysis showed a significantly diminished corticosterone response of adrenal cells from SD/OC mice at all ACTH doses tested (Fig. 15).

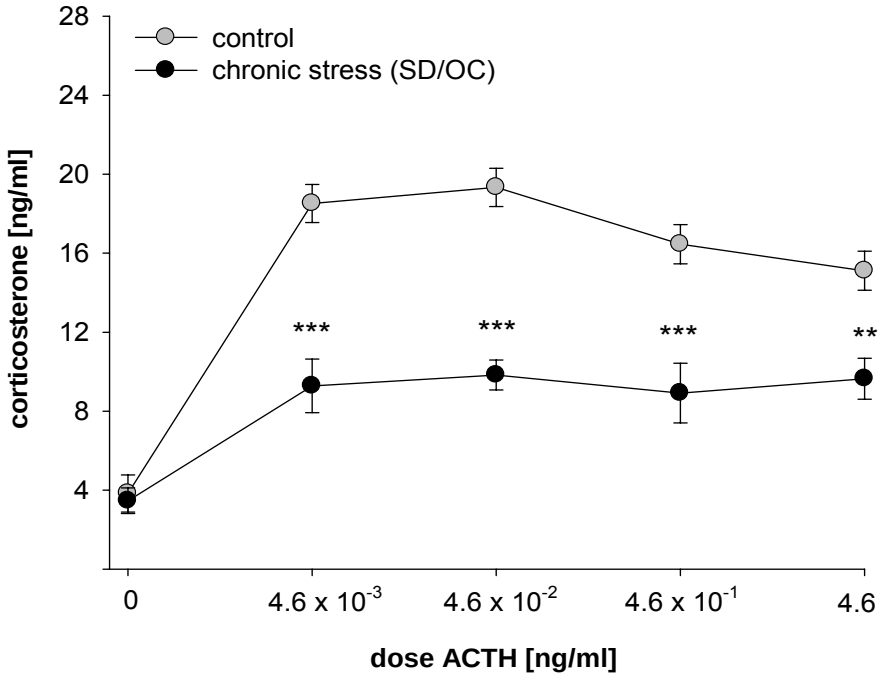


Fig. 15: Effects of exposure to the social defeat/overcrowding (SD/OC) stress paradigm on the *in vitro* release of corticosterone from isolated adrenal cells after stimulation with ACTH. Adrenals of 4 control and 4 SD/OC mice, respectively, were pooled (n = 8; pooled samples), and two aliquots (a 200 µl) of each group were stimulated with ACTH over a concentration range of 4.6×10^{-3} – 4.6 ng/ml (10^{-13} – 10^{-10} M). Supernatants were analyzed by a corticosterone RIA in triplicates. Adrenal cells of SD/OC mice showed a reduced response to ACTH at all doses tested. Data represent mean ± S.E.M.; ** $p < 0.01$; *** $p < 0.001$ vs. respective unstressed controls.

Effects of exposure to SD/OC on the severity of a DSS-induced colitis and on plasma corticosterone and ACTH concentrations assessed on day 8 of DSS treatment

Prior exposure to SD/OC significantly increased the severity of an acute DSS-induced colitis, as indicated by an increased body weight loss, inflammatory reduction of colon length, histological damage score of the colon, and secretion of proinflammatory cytokines by draining mesenteric lymph node cells.

Body weight (Fig. 16A). The body weight gain of mice between experimental days 20 and 27 was found to be dependent on prior stressor exposure and DSS treatment (factor stress x DSS: $F_{1, 67} = 24.03$; $p < 0.01$). DSS treatment resulted in a decrease in body weight in both SD/OC and control mice, which was more pronounced in SD/OC mice compared with unstressed controls. When performing a Mann-Whitney *U*-comparison between the body weight gain of SD/OC and control mice, both receiving no DSS, SD/OC mice gained significantly more body weight from day 20 to 27, the times pan following SD/OC exposure ($p = 0.003$).

Histological score (Fig. 16B). The histological score was depend on prior stressor exposure and DSS treatment (factor stress x DSS: $F_{1, 30} = 53.5$; $p < 0.01$). The histological score of stressed and unstressed mice receiving no DSS indicated no colonic inflammation (Fig. 16D). In contrast, DSS treatment increased the histological score in both SD/OC and control mice. Importantly, SD/OC (Fig. 16F) mice receiving DSS showed a significantly higher histological score than respective non-stressed controls (Fig. 16E) reflecting more severe inflammatory infiltration and increased epithelial damage, i.e. focal disappearance of mucosal crypts.

Colon length (Fig. 16C). Statistical analysis revealed a main interaction of SD/OC exposure and DSS treatment (factor stress x DSS: $F_{1, 31} = 23.32$; $p < 0.01$) on the length of the colon. More specifically, colon length was significantly reduced by DSS application, in both SD/OC and control mice, compared with respective mice treated with tap water. However, the effect of DSS was more severe in SD/OC compared with unstressed control mice.

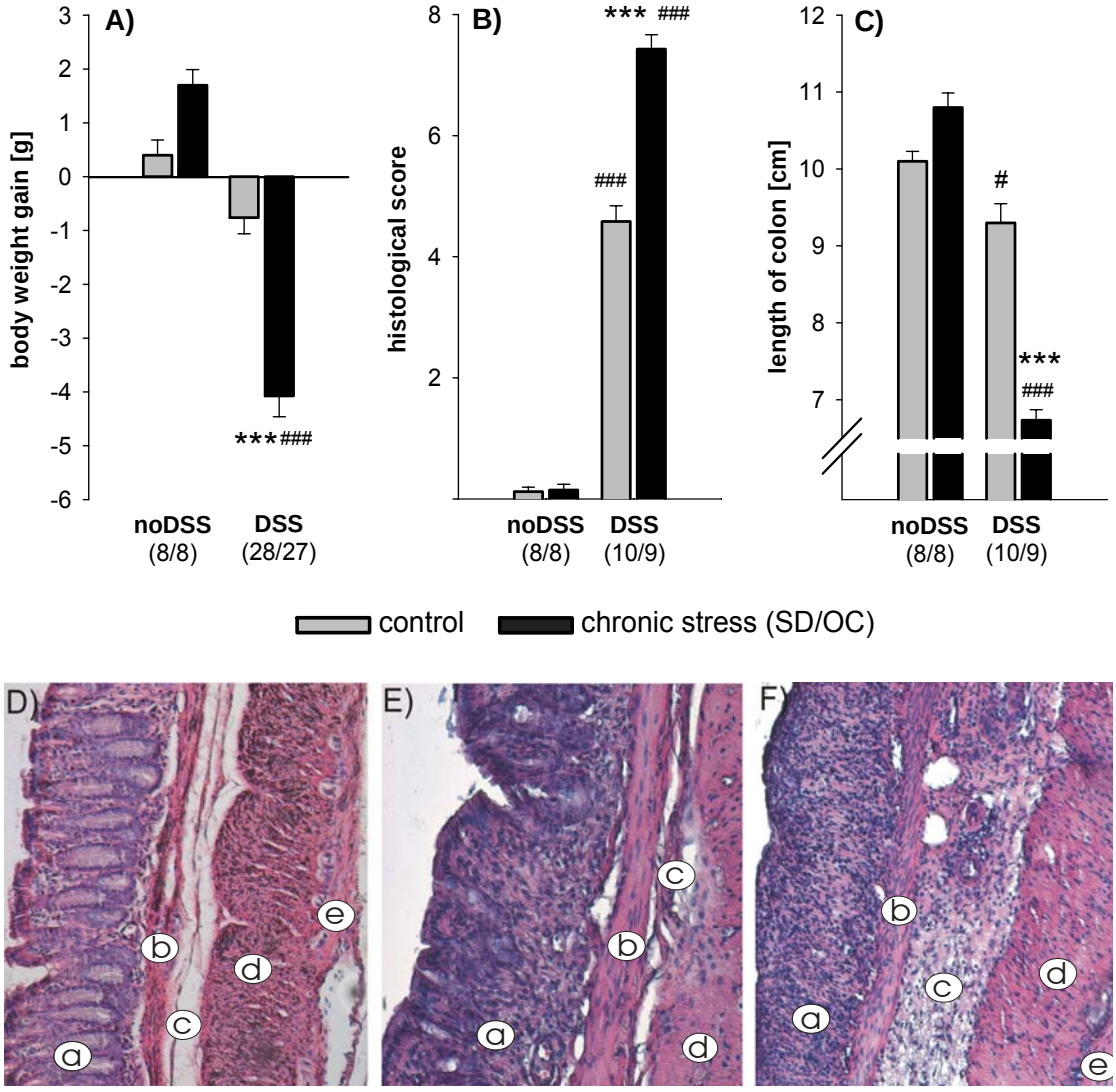


Fig. 16: Effects of exposure to the social defeat/overcrowding (SD/OC) stress paradigm on the severity of an acute DSS-induced colitis. Stress exposure prior to DSS treatment exaggerated the severity of the induced acute colitis, indicated by an enhanced body weight loss (Fig. 4A), a higher histological score (Fig. 4B) and a more severe reduction of colonic length (Fig. 4C). Induction of an acute colitis was achieved by administration of 1% DSS in drinking water from day 20 to 27. Numbers in parentheses indicate group sizes. Data represent mean ± S.E.M.; *** $p < 0.001$ vs. respective unstressed controls; ### $p < 0.001$ vs. respective group without DSS. Furthermore, three representative colonic hematoxylin-eosin sections [a: Lamina mucosa; b: Lamina muscularis mucosa; c: Lamina submucosa; d: Lamina muscularis externa (circular mussle); e: Lamina muscularis externa (longitudinal mussle)] from mice receiving no DSS (Fig. 4D; normal colon histology), DSS-treated control mice (Fig. 4E; goblet cell loss and crypt loss in locally restricted areas; infiltration reaching the Lamina muscularis mucosa) and DSS-treated SD/OC mice (Fig. 4F; crypt loss in large areas; thickening of the mucosa with abundant oedema; infiltration reaching the Lamina submucosa).

Secretion of proinflammatory cytokines from draining mesenteric lymph node cells (Fig. 17). In DSS-treated mice, the secretion of IFN-γ, IL-6 as well as TNF-α from mesenteric lymph node cells was found to be significantly increased in SD/OC compared with control mice.

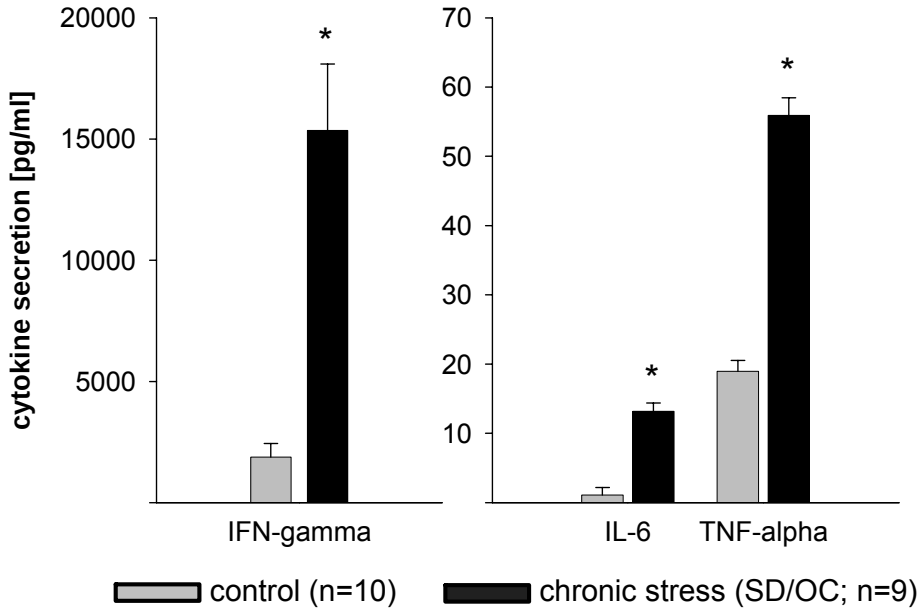


Fig. 17: Effects of exposure to the social defeat/overcrowding (SD/OC) stress paradigm prior to DSS treatment on proinflammatory cytokine secretion by mesenteric lymph node cells. Both, stressed an unstressed mice were given 1% DSS in drinking water from day 20 to 27. SD/OC exposure resulted in increased IFN-gamma, IL-6 as well as TNF-alpha secretion compared with control mice. Data represent mean + S.E.M.; * $p < 0.05$ vs. respective controls.

Plasma corticosterone and ACTH concentrations (Tab. 2). Both, plasma corticosterone and ACTH concentrations were dependent on prior stressor exposure and DSS treatment (corticosterone: factor stress x DSS: $F_{1,31} = 30.6$; $p < 0.001$; ACTH: factor stress x DSS: $F_{1,30} = 11.0$; $p = 0.002$). Hormone concentrations of SD/OC or control mice, which did not receive DSS were not statistically different. However, DSS treatment significantly increased plasma corticosterone and ACTH concentrations only in SD/OC mice.

Table 2. Effects of chronic exposure to social defeat/overcrowding (SD/OC) prior to DSS treatment on plasma light phase corticosterone and ACTH concentrations, in male mice on day 8 of DSS treatment.

	controls no DSS	**SD/OC no DSS**	**controls DSS**	**SD/OC DSS**
corticosterone [ng/ml]	32.4 ± 5.6 (n=8)	27.9 ± 2.7 (n=8)	21.6 ± 12.0 (n=10)	414.8 ± 47.6 (n=9) *** / ###
ACTH [pg/ml]	41.4 ± 2.1 (n=8)	37.4 ± 3.7 (n=8)	65.3 ± 12.4 (n=10)	187.4 ± 37.0 (n=9) *** / ###

Mice were exposed to SD/OC for 19 days as described in Fig. 1, or were singly housed (control). Following the termination of the stress procedure a subgroup of mice from each group (chosen randomly) was treated with 1 % DSS (in drinking water) or tap water from day 20 to 27. Data represent mean ± S.E.M.; *** $p < 0.001$ vs. respective unstressed controls; ### $p < 0.001$ vs. respective group without DSS.

Effects of chronic exposure to SD/OC on the regeneration process after DSS-induced colitis (Fig. 18)

The regeneration process during a 4-day recovery period after termination of DSS treatment was found to be impaired in mice exposed to SD/OC prior to DSS

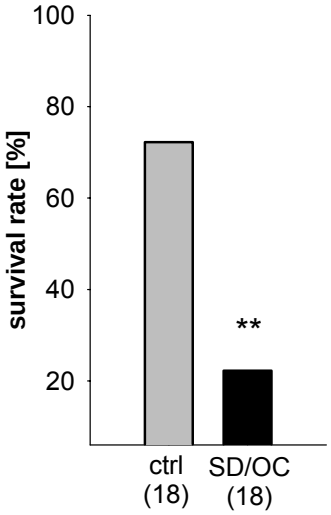


Fig. 18: Effects of exposure to social defeat/overcrowding (SD/OC) on the regeneration process after a subsequent DSS-induced colitis (1% from day 20 to 27). SD/OC mice (black columns) showed a reduced survival rate compared with control mice (grey columns) 4 days after termination of DSS treatment. Data represent survival rate [%] of the respective groups; ** $p \leq 0.01$ vs. respective unstressed controls.

treatment. This was reflected by a significantly reduced survival rate in SD/OC compared with control mice (Fig. 18).

Discussion

The experiments described in chapter 2 were designed to evaluate the impact of prior chronic intermittent psycho-social stress on the severity of a DSS-induced colitis and on the subsequent regeneration period. The findings of this study demonstrate that (i) SD/OC is a relevant chronic psycho-social stressor for male mice, (ii) prior exposure to SD/OC increases the severity of the DSS-induced colitis, and (iii) prior SD/OC impairs the regeneration phase after colitis as indicated by a reduced survival rate.

The present data show that the SD/OC paradigm is a valid and relevant mouse model for chronic psycho-social stress. Various physiological markers are well known indicators of chronic stress, including reduction in body weight gain, thymus involution, and adrenal hypertrophy [66, 81, 292, 293]. Indeed, the mice that underwent 19-day exposure to SD/OC gained less body weight than single-housed control mice. Additionally, SD/OC mice showed severe thymus atrophy and adrenal hypertrophy, as further indicators of chronic stress and activation of the HPA axis [81, 115, 294, 295]. Despite the elevated relative adrenal weight of SD/OC mice, plasma corticosterone concentrations were not different from the respective control mice during the light phase. Therefore, the corticosterone : adrenal weight ratio was found to be significantly lower in SD/OC mice. These results are in agreement with the findings of a comparable study, using chronic subordinant colony housing (CSC; 19 days) as a chronic psycho-social stressor (see Chapter 3). Apart from an initial rise within the first days of CSC exposure, plasma light phase corticosterone concentrations were found to be unaffected by CSC-induced chronic stress (see Chapter 3). Interestingly,

these subordinate mice showed thymus atrophy and adrenal hypertrophy within 24 h after colony formation.

In contrast, Zelena *et al.* [295] demonstrated that repeated restraint stress resulted in increased relative adrenal weights, paralleled also by higher levels of plasma corticosterone. This discrepancy may be due to differences in the stress procedures used, as in their study, animals were stressed for a shorter time-frame (7-8 consecutive days compared with 19 consecutive days in the present study) and a shorter daily duration (1h, restraint compared with 2h SD or 24h OC) [295]. It has also been described that basal plasma corticosterone levels were increased during the first weeks of chronic stress but returned to baseline afterwards, despite continued stressor exposure [117, 118, 296]. Therefore, the finding of similar basal corticosterone levels in SD/OC and control mice might be due to adaptive processes of the HPA axis, protecting the organism from a prolonged exposure to immunosuppressive, and therefore, deleterious, high levels of GCs [74, 77]. Another possible explanation for comparable light-phase plasma corticosterone levels between SD/OC and control mice might be that the adrenal cells of SD/OC mice were simply insufficient to produce appropriate amounts of GCs. Indeed, this is supported by the present finding that plasma corticosterone concentrations during the dark phase were significantly lower in SD/OC mice compared with non-stressed controls. Thus, it is possible that their adrenal glands became insensitive and lost their synthetic and/or secretory capability to appropriately respond to the diurnal rhythm [297]. In support, hypothalamic expression of CRH mRNA, as well as plasma ACTH concentrations, during the dark phase were not different between SD/OC and control mice, indicating an unaffected reactivity of hypothalamic and adenohypophysial corticotroph cells. Thus, the observed corticosterone deficiency at the end of the stress procedure may rather reflect an adrenal than a hypothalamic/adenohypophysial

dysfunction. Moreover, the finding of an attenuated corticosterone secretion by isolated adrenal cells of SD/OC mice in response to an acute *in vitro* ACTH challenge supports the hypothesis that 19-day exposure to SD/OC causes to loss of functional responsiveness of adrenal cells.

Based on the effects of SD/OC on body, relative thymus, and adrenal weights, as well as on adrenal functions, the SD/OC paradigm represents an adequate model of chronic psycho-social stress for male mice. Therefore, the SD/OC paradigm is highly suitable for studying the link between chronic stress and various aspects of the immune system, with the study described in chapter 2 of the present thesis investigating chronic psycho-social stress effects on the outcome of, the severity of, and the regeneration after IBD [103, 210]. Furthermore, the establishment of such a paradigm might be of potential relevance for studying numerous diseases, besides colitis, which are believed to be exacerbated by chronic stress, such as major depression [83, 84].

Although the effects of stress on the severity of an experimentally-induced colitis are controversial [108, 273-275], there is evidence, that a blunted responsiveness of the HPA axis makes the animals more prone to a chemically induced inflammation [108, 273-275]. However, this study is the first which describes the effects of a chronic psycho-social stressor on the severity of a subsequent DSS-induced colitis. Thereby, SD/OC mice showed a significant increase in colonic inflammation on day 8 of DSS treatment with respect to all major relevant physiological, histological, and immunological parameters investigated. For example, the body weight loss was more pronounced and the histological score of the colonic tissue was higher in SD/OC compared with control mice after DSS treatment from day 20 to 27. Moreover, the secretion of the proinflammatory cytokines IFN-γ, IL-6, and TNF-α from stimulated mesenteric lymph node cells was found to be increased in SD/OC compared with control mice

after DSS treatment, indicating an increased activation of the intestinal immune system. Concerning the increased body weight gain observed in SD/OC compared with control mice, both not treated with DSS after termination of SD/OC (from day 20 and 27), it is likely that stressed animals compensated for their reduced body weight gain during the 19 days of SD/OC.

As mentioned above, SD/OC-induced adrenal insufficiency and, thus, attenuation of corticosterone secretion in response to an inflammatory stimulus (DSS), may underlie the increased severity of the DSS-induced colitis. Interestingly, in CSC mice adrenal insufficiency was at least present until day 4 of subsequent DSS treatment (see Chapter 4). However, on day 8 of DSS treatment SD/OC mice showed increased plasma corticosterone and ACTH concentrations, indicating that the adrenals may have recovered from SD/OC-induced adrenal insufficiency. This delayed rise in immunosuppressive GCs did not efficiently counteract the over compensatory production of proinflammatory cytokines in SD/OC mice. It is important to mention that the rise in plasma corticosterone was absent in non-stressed DSS-treated animals, because probably the degree of inflammation was too low to cause HPA axis activation.

The regeneration phase after DSS treatment (from day 20 to 27) was found to be negatively affected by prior exposure to SD/OC, as indicated by a decreased survival rate in SD/OC compared to control mice. On day 4 after termination of subsequent DSS treatment, 72 % of the control mice and only 20% of SD/OC mice survived the experimental procedure. Therefore, the finding of an increased severity of a DSS-induced colitis on day 8 of DSS treatment and an impaired regeneration afterwards in SD/OC compared to control mice provides further evidence for a link between stress and IBD [103-106, 117-120]. Although the detailed mechanisms underlying these chronic stress effects still have to be elucidated, the data shown in this chapter suggest that in

chronically stressed mice, maladaptations of the HPA axis might occur, e.g. at the level of the adrenals, resulting in an insufficient production and/or secretion of immunosuppressive GCs. This is substantiated by the finding of a reduced corticosterone : adrenal weight ratio, lower dark-phase plasma corticosterone concentrations, i.e. during the diurnal rise in corticosterone levels, and diminished reactivity to ACTH-stimulation *in vitro*. Additionally, unchanged plasma ACTH concentrations and hypothalamic CRH mRNA expression provide evidence that this phenomenon reflects an adrenal rather than a hypothalamic disregulation. Consequently, adrenal cortical cells are, at least one day after 19-day exposure to SD/OC, incapable of producing and/or secreting GCs in appropriate concentrations necessary for controlling the DSS-induced inflammatory reaction at the level of the colon. In support, during an inflammatory episode in humans, a rise in plasma cortisol, epinephrine and NE was observed [298, 299], and these endocrine mechanisms were shown to prevent the immune system from overshooting via distinct feedback mechanisms [300]. Therefore, a possibly delayed increase of GC levels might contribute to the development of a more pronounced inflammation in SD/OC mice.

Future experiments are required to reveal the involvement of the sympathetic nervous system in the chronic stress-induced increase in severity of a subsequent DSS colitis. An important role of the sympathetic nervous system in the onset as well as in the control of an already developed inflammation, was recently reported in a model of arthritis in mice [301].

In summary, the data of this study provide evidence that an acute intestinal inflammation induced by DSS is more severe in chronically stressed mice as indicated by physiological, histological and immunological parameters, as well as the subsequent survival rate of the animals. The HPA axis reactivity was blunted in chronically stressed mice, whereby the effect was found to be due to insufficiently

working adrenal cells in SD/OC mice. This implicates that a reduced and/or delayed GC response might be a possible mediator of the SD/OC-induced increase in colitis severity, as this response is essential for the downregulation of inflammatory processes. These results suggest that impaired GC activity following sustained exposure to stressors in humans may be a contributory factor for the development of IBD.

Chapter 3

Adrenal insufficiency and colonic inflammation following a novel chronic psycho-social stress paradigm in mice: implications and mechanisms

[adapted from [302]: **Reber SO, Birkeneder L, Veenema AH, Obermeier F, Falk W, Straub RH, Neumann ID; 2006 ; Endocrinology doi:10.1210/en.2006-0983]**

Abstract

In this chapter chronic psycho-social stress effects on stress-related parameters and on pathohistological changes in the murine colon were investigated. Moreover, it was the aim to reveal the involvement of adrenal GCs in chronic stress effects. Exposure to CSC for 19 days resulted in reduced body weight gain, thymus atrophy, adrenal hypertrophy, increased plasma NE, and increased anxiety. With respect to the time course of CSC effects, CRH mRNA in the hypothalamic paraventricular nucleus, light phase corticosterone and TH expression in colonic tissue were found to be increased, whereas TH expression in the locus coeruleus was found to be decreased on day 2 of CSC; these parameters returned to control levels thereafter. Nevertheless, after 19 days of CSC exposure, the adrenal corticosterone responses *in vivo* and *in vitro*, and GC sensitivity of isolated splenic cells were found to be decreased. Importantly, in CSC mice a significant histological damage of the colon was found beginning on day 14 of CSC exposure. Additionally, pro- and antiinflammatory cytokine secretion by mesenteric lymph node cells was increased after CSC exposure. Adrenalectomy (ADX) prior to CSC at least partially prevented these chronic stress effects as reflected by less increase in proinflammatory cytokine secretion and an equal histological damage score in ADX compared with SHAM operated CSC mice.

In conclusion, chronic exposure to CSC alters relevant neuronal, neuroendocrine, and immune functions which could be directly or indirectly involved in the damage of the histological integrity of the colon comparable with that seen during the development of colitis.

Introduction

Chronic stress is a potential risk factor for the development of gastrointestinal disorders, such as irritable bowel syndrome or IBD [103-105]. Both animal and human studies demonstrate that exposure to various stressors affects the functional integrity of the gastrointestinal tract leading to altered production and release of mucin and impaired colonic mucosal barrier functions, which may result in increased antigen infiltration [110, 221, 303-308]. Thus, acute exposure to 30 min immobilization stress immediately increased mucin release from colonic mucosal tissue [303], which may provide a short-term protection against luminal antigens. In contrast, after an initial increase, colonic mucin production was significantly reduced during repeated cold-restraint stress exposure performed over five consecutive days (1h/day) in Sprague-Dawley rats [110]. Similarly, reduced colonic mucin production has also been reported in both human patients, suffering from ulcerative colitis [304] , and in cotton-top tamarins that have developed a spontaneous, active or quiescent colitis, following capture and readjustment to a novel social environment [221]. In addition, reduced mean net water absorption, and changed mean net sodium and chloride absorption [305] were reported under conditions of experimental stress in human jejunum [306, 307] and rat ileum and colon [308] possibly contributing to stress-induced diarrhea [309].

Moreover, exposure to relatively acute stressors impaired the mucosal barrier function in different intestinal parts of Swiss 3T3 mice [310] and Wistar Kyoto rats [311, 312]. Furthermore, acute forced swimm stress (20 min) increased luminal permeability in all segments of the gastrointestinal tract within 24 hours after stress exposure in mice [276], which was thought to be caused by the stress-induced rise in plasma corticosteroids.

Although these data provide evidence for a link between stressor exposure and impaired intestinal barrier functions pathological alterations of the colonic architectural structure have not been described. This could be due to the fact that the duration of the stressors applied was too short and/or their severity was not efficient to induce histological alterations comparable to those seen, for example, in individuals with IBD.

Therefore, in the studies described in Chapter 3, it was the aim to establish CSC in male mice as a clinically relevant chronic psycho-social stressor measuring relevant behavioural and physiological markers [66, 81, 82, 139, 292, 293, 313-317], and to investigate the influence of exposure to 19 days of CSC on established histological parameters of the colon [289, 290] (experiment 1).

Chronic stress effects on gastrointestinal functions are likely to be mediated by the two main stress systems of the organism, namely the sympathetic nervous system and the HPA axis [108, 273-275, 318-320]. Therefore, also TH immunoreactivity in the brain LC and in the colon as parameters of the sympathetic nervous system activity/function, and hypothalamic CRH mRNA expression, relative adrenal weight and plasma corticosterone as relevant parameters of the HPA axis on days 2, 3, 7, 14, and 20 of CSC exposure were monitored. In parallel, the time course of relative thymus weight and the histological damage score of the colon were assessed throughout the 19 days of stressor exposure (experiment 2)

In order to further investigate the potential adrenal insufficiency in chronically stressed mice [279], adrenal corticosterone secretion was estimated under stimulated conditions *in vivo* and *in vitro*, i.e. during the diurnal rise in plasma corticosterone at the beginning of the dark phase [297, 321] and in response to ACTH, respectively (experiment 3). Additionally, GC sensitivity of isolated splenic cells was determined from the same chronically stressed and control mice, because prolonged exposure to

high concentrations of corticosterone was shown to reduce GC sensitivity of splenocytes [138, 322, 323] (experiment 3).

Finally, cytokine secretion by mesenteric lymph node cells in chronically stressed and control mice was assessed after termination of the CSC procedure as an additional parameter describing the ongoing inflammatory processes in colonic tissue. To investigate whether the stress-induced inflammatory processes in colonic tissue were mediated via adrenal mechanisms adrenalectomy (ADX) was performed one week before the CSC procedure started (experiment 4).

Overall, the experiments of this chapter were designed to determine whether chronic psycho-social stress leads to colonic inflammation in parallel to alterations of the two main stress systems, and whether adrenal GCs are involved.

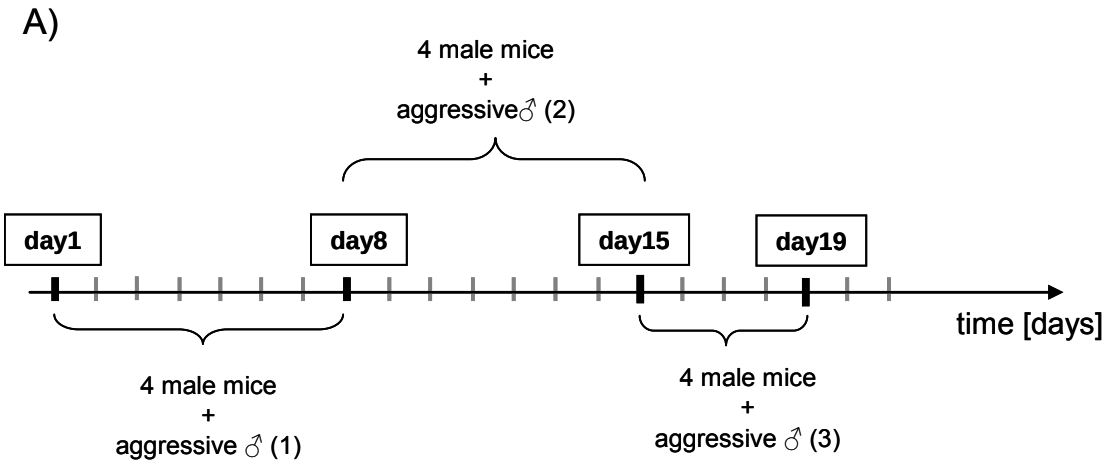
Materials and methods

Animals

Male C57BL/6 mice (Charles River, Sulzfeld, Germany) weighing 19-22 g (experimental mice) or 30-35 g (dominant mice) were individually housed in standard polycarbonate mouse cages (16 x 22 x 14 cm) at least for one week before the experimental procedure started. All mice were kept under standard laboratory conditions (12-h light/dark cycle, lights on at 0600 h, 22 °C, 60% humidity) and had free access to tap water and standard mouse diet. All experimental protocols were approved by the Committee on Animal Health and Care of the local government, and conformed to international guidelines on the ethical use of animals. All efforts were made to minimize the number of animals used and their suffering.

Chronic subordinate colony housing (CSC; Fig 19)

In order to induce a chronic stressful situation, four CSC mice were housed together with a larger dominant male in a polycarbonate observation cage (38 x 22 x 35 cm) for 19 consecutive days. Before starting the chronic stress procedure all male mice selected for becoming the dominants during the CSC paradigm were tested for their aggressive behaviour. Males that started to injure their opponents by harmful bites were not used. To avoid habituation, each dominant male was replaced by a novel dominant male on days 8 and 15 (Fig. 19A). During the first 30 min after colony formation on day 1, 8, and 15, the mice were videotaped for behavioural analyses (Fig. 19B). In all colonies, the larger male mouse established a "dominant" status while it was chasing and attacking all four experimental mice (Fig. 19C). All four experimental mice were considered as 'subordinates' based on their defensive behaviour, including flight, retreat and submissive upright (Fig. 19D, E), according to a recent report in rats [137].



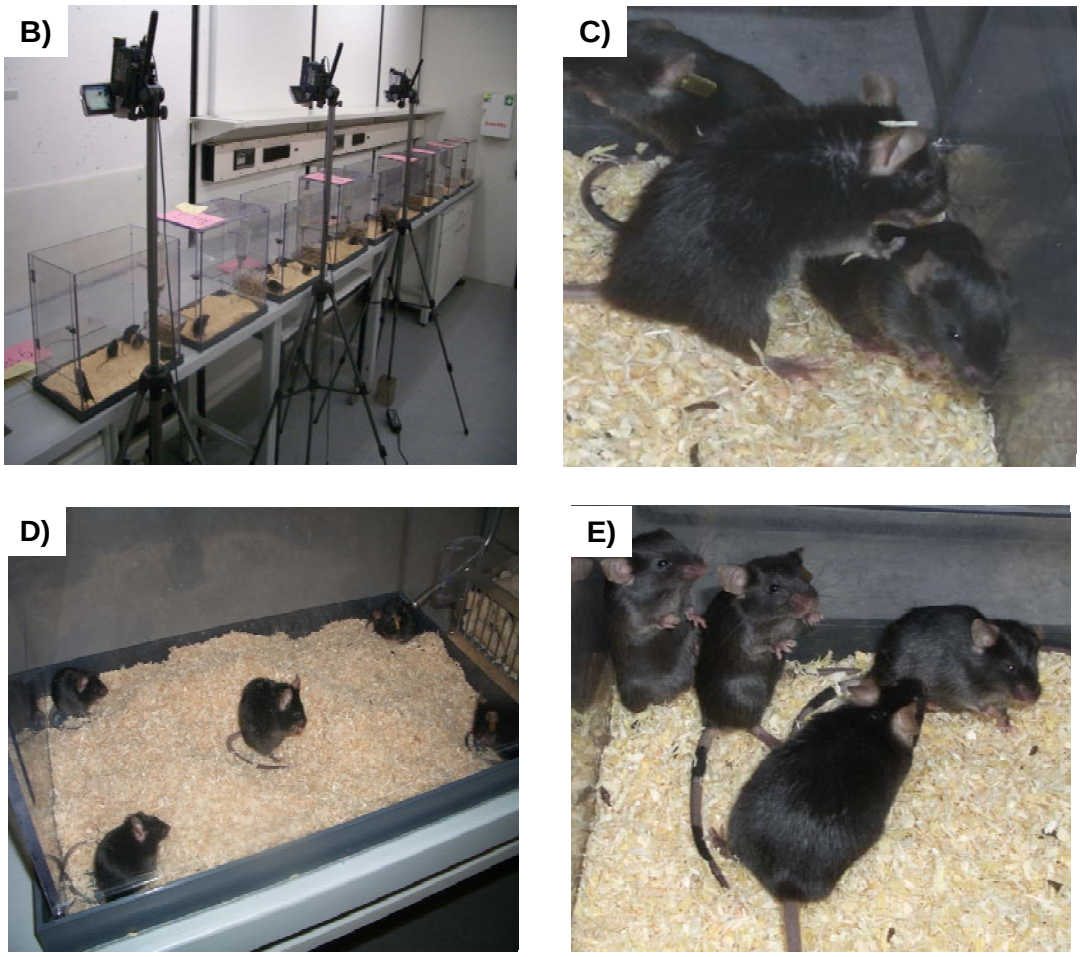


Fig. 19: Chronic psycho-social stress is induced by 19-day exposure to CSC. A) schematic illustration of the experimental design of the CSC paradigm; B) standardized CSC procedure in our laboratories; C) the dominant mouse stands with its forelimbs on the back of a subordinate experimental mouse; D) after establishment of the hierarchy the dominant mouse often shows grooming behaviour in the center of the cage while the subordinate mice are sitting in the corners; E) the subordinate mice often raise their forlimbs and show their ventral side to indicate subordination

Experimental procedure

Experiment 1: Mice were weighed and afterwards randomly assigned to the control or the CSC group. Control mice were single-housed and remained undisturbed in their home cages except for change of bedding once a week. Groups of four CSC mice were housed together with one dominant male over a period of 19 days (see above). On day 19, all mice were weighed again between 1700 h and 1800 h and CSC mice were singly housed afterwards. On the next day (day 20), mice were

sacrificed between 0800 h and 1000 h, and trunk blood was collected for quantification of plasma NE and corticosterone concentrations (light phase). Moreover, relative thymus and adrenal weights, and the histological damage score of the colon were estimated (see below). For assessment of CSC effects on anxiety-related behaviour, an additional set of control and CSC mice was tested on the elevated plus-maze (EPM; see below) between 0800 h and 1100 h on day 20 (EPM data of experiment 3 are included in that additional set of animals).

Experiment 2: In order to study the time-course of CSC effects on TH expression in the LC and in colonic tissue, on CRH mRNA expression in the PVN, on relative adrenal weight, light phase corticosterone concentrations, relative thymus weight, and the histological damage score of the colon, groups of control and CSC mice were sacrificed before (basal) and on days 2, 3, 7, 14, and 20 of CSC exposure between 0800 h and 1000 h.

Experiment 3: In order to study the effects of CSC on plasma corticosterone concentrations during the dark phase, on adrenal corticosterone secretory responses *in vitro*, and on GC sensitivity of splenic cells, mice were weighed and randomly assigned to the control and CSC group. Control mice were singly housed and remained undisturbed in their home cages except for change of bedding once a week. Groups of four CSC mice were housed together with one dominant male over 19 days (see above). On day 19 CSC mice were singly housed. On the next day (day 20) between 0800 h and 1100 h mice were tested on the EPM to confirm the CSC-induced increase in anxiety-related behaviour (data included in Fig. 21 of experiment 1). For assessment of dark phase corticosterone concentrations, i.e. during the diurnal rise in HPA axis activity [297, 321], of adrenal corticosterone secretory responses *in vitro*, and of GC sensitivity of splenic cells, the mice were sacrificed between 0800 h and 1000 h on day 20.

Experiment 4: In order to study an additional marker of inflammation in the colonic tissue at the end of the chronic stress procedure and to investigate adrenal mechanisms mediating CSC-induced effects on colonic inflammation, a new set of animals was either adrenalectomized (ADX) or sham operated (SHAM) one week before CSC exposure. One day after 19-day exposure to either CSC or single housing (controls), the histological score and the pro- and antiinflammatory cytokine secretion by mesenteric lymph node cells was measured in ADX and SHAM mice.

Blood sampling and radioimmunoassay for NE and corticosterone

To determine the effect of CSC exposure on NE and corticosterone concentrations in plasma, all mice of one cage were rapidly killed by decapitation under CO_2 anaesthesia within 3 min after entering the animal room. Approximately 200 µl trunk blood was collected in EDTA-coated tubes on ice (Sarstedt, Nümbrecht, Germany) containing 10 µl aprotinin (Trasylol, Bayer Corp. AG, Leverkusen, Germany) and centrifuged at 4 °C (5000 rpm, 10 min). Plasma samples were stored at -80 °C until assayed using a commercially available radioimmunoassay for NE (IBL Immuno Biological Laboratories, Hamburg, Germany, detection limit: 9 pg/ml) and corticosterone (MP Biomedicals GmbH, Eschwege, Germany; detection limit: 10 ng/ml).

Determination of relative thymus and adrenal weights

To assess chronic stress effects on relative thymus and adrenal weights, thymus and both adrenal glands were removed after decapitation, pruned of fat tissue, and weighed. Both the left and right adrenal glands were weighed together. Values were expressed in relation to body weight (mg/g).

Determination of the histological damage score of the colon

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. Three 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 three sections. Histology was scored as follows [289, 290]:

Epithelium. 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

Infiltration. 0: no infiltration; 1: infiltrate around crypt basis; 2: infiltrate reaching to lamina muscularis mucosae; 3: extensive infiltration reaching the L. muscularis mucosae and thickening of the mucosa with abundant oedema; 4: infiltration of the L. submucosa.

The total histological score of each mouse represents the sum of the epithelium and infiltration score and ranges from 0 to 8.

Elevated plus-maze test

In order to assess the effect of 19 days of CSC on anxiety-related behaviour, CSC mice were singly housed and both controls and CSC mice were transported to the plus-maze test room on day 19 (1800 h). The next day, they were tested on the EPM between 0800 h and 1100 h for 5 min [324, 325]. The EPM consisted of two open (6 x 30 cm) and two closed (6 x 30 x 17 cm) arms radiating from a central platform (6 x 6 cm) to form a plus-shaped figure elevated 130 cm above the floor. The open arm edges were 0.3 cm in height to avoid falling. Each mouse was placed on the

central platform facing a closed arm. The maze was cleaned thoroughly before each test. 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 onto open arms of the maze.

Immunohistochemistry of TH in the LC and colonic tissue

The staining protocol was adopted from Miller *et al.* (2000) [326]. Briefly, the separated brainstem and three colon pieces of each mouse were immediately fixed in 3.75% paraformaldehyde in PBS for 24 h. Tissue was then incubated in 20% sucrose in PBS for another 24 h. Thereafter, brainstem and colonic tissue were embedded in protective freezing medium (Tissue-Tek, Sakura Finetek Europe, Zoeterwoude, the Netherlands) and quick-frozen floating on liquid nitrogen for TH staining.

For assessment of LC TH expression, 5-µm cryosections of the LC were placed on precoated slides, air dryed (1h, room temperature), rehydrated in 0.05 M TBS, and unspecific binding was blocked for 1h with a solution of 10% whole serum (goat and donkey serum, Dako, Glostrup, Dänemark) from the host species of the respective secondary antibody in 0.05 M TBS containing 1%BSA and 0.01% NaN_3 (Sigma, Deisenhofen, Germany). The slides were then washed (10 min, 0.05 M TBS) and immunostained in a humid chamber (24 h, RT) with primary antibodies against TH (Chemicon, Temecula, CA), the key enzyme for neuronal NE production. The primary antibody solution was diluted 1:500 with 0.05 M TBS containing 1% BSA and 0.01% NaN_3. After 3 x 5 min wash with 0.05 M TBS, an Alexa 546 conjugated secondary goat anti-rabbit antibody (Molecular Probes, Leiden, The Netherlands) was used to achieve immunofluorescent staining (conc. 1:600, incubation for 90 min, followed by 3 x 5 min wash in the dark). Under control conditions, the respective

isotype was used in the above mentioned protocol. TH expression was analysed as specific fluorescence (pixels with higher fluorescence than a chosen background-threshold were counted as positive pixels; results are presented as "% positive pixels" of all pixels of each slice) in 10 slices before and 10 slices after the slice showing the highest fluorescence using a computerized image program (MetaVue™, Version 5.0r1, Universial Imaging Corp., Downingtown, PA; Copyright 1999-2002). For determination of TH expression in colonic tissue, 3 cryosections (5 µm, 100 µm distance between the slices, each slice contains all 3 colonic tissues of one mouse) per animal were placed on a precoated slide (SuperFrost Plus, Menzel-Gläser, Braunschweig, Germany). For assessment of colonic TH expression, 17 randomly selected fields of view from each animal were analysed as specific fluorescence (pixels with higher fluorescence than a chosen background-threshold were counted as positive pixels; results are presented as "% positive pixels" of all pixels of each field of view).

In situ *hybridization of hypothalamic CRH mRNA*

After decapitation, brains were rapidly removed, snap frozen in iso-pentane cooled on dry-ice, and stored at -80°C for subsequent *in situ* hybridization. A series of six 16-µm cryocut PVN sections per mouse was thaw-mounted onto slides in a cryostat at -20°C and afterwards was used to assess hypothalamic CRH mRNA expression. The hybridization protocol was adapted from Bosch *et al.* [286]. Briefly, hybridization to CRH mRNA was performed by using a 48-base ^{35}S-labeled oligonucleotide probe complementary to bases 64 - 111 (probe sequence: 5' ggc ccg cgg cgc tcc aga gac gga tcc cct gct cag cag ggc cct gca). Using the NCBI BLAST search the sequence was demonstrated to be specific for the mouse CRH transcript. Hybridized slices were exposed to BioMax MR film under 'safe' red light conditions for 21 days

(Eastman Kodak, Rochester, New York, USA). Expression of CRH mRNA was measured as optical density on a Macintosh computer with a computerized image program (ImageJ 1.31, National Institutes of Health, http://rsb.info.nih.gov/ij/). Bilateral measures were taken from two to four PVN sections for each mouse, which were pooled to provide individual means per mouse. For tissue background, the optical density of a nonhybridized region outside the PVN was measured.

ACTH stimulation of isolated adrenal cells in vitro

In order to reveal the consequences of chronic stress on the capability of adrenal cells to respond to an ACTH challenge *in vitro*, the stimulation protocol was adopted from Bazhan *et al.* (2004) [291]. After decapitation, adrenal glands of four mice of the same experimental group (control vs. CSC; experiment 3) were pooled and stored in ice cold Krebs-Ringer bicarbonate-glucose buffer (KRBB, pH 7.4, 0.5% BSA) until they were cleared, cut into small pieces, and incubated in 1.5 ml KRBB (4% BSA, 4% collagenase) for 60 min at 37°C in an atmosphere of 95% O_2 and 5% CO_2. Adrenal cells were then dispersed by gentle homogenization (repeated pipetting) and filtered through 5 layers of gauze bandage. Afterwards, suspensions were centrifuged (4°C, 10 min at 300 r.p.m.; 10 min at 800 r.p.m.) and washed three times with 2.5 ml cold KRBB (0.5% BSA). Finally, cells were counted (tryphan-blue), cell viability was assessed (tryphan-blue exclusion method), and adjusted to a cell concentration of 4 x 10^5 cells/ml in cold KRBB (0.5% BSA). Two aliquots (a 200 µl) of each group were incubated for 2 h at 37°C (95% O_2, 5% CO_2) in the presence or absence of different doses of ACTH (10^{-13}, 10^{-12}, 10^{-11} and 10^{-10} M). Afterwards, cells were sedimented by brief centrifugation (5 min at 2000 r.p.m.) and supernatants were stored at -20°C until radioimmunological quantification of corticosterone. In order to exclude differences in the adrenal cell preparation between CSC and control groups,

experiments were performed by the same experimentator according to the standardized isolation protocol.

Characterization of GC sensitivity of isolated splenocytes

In order to determine the effects of chronic stress on the GC dependent cell viability of splenic cells, a protocol was adopted from Engler *et al.* (2005) [138]. Briefly, after decapitation, spleens of two mice of the same group (control vs. CSC) were pooled, splenocytes were isolated and adjusted to a final cell concentration of 5 x 10^6 cells/ml.

Cell suspensions were then stimulated with lipopolysaccharide (LPS; final concentration was 1 µg/ml) from Escherichia coli (serotype O111:B4, Sigma-Aldrich, St. Louis, MO) or remained untreated to assess background activity. To determine the GC sensitivity of unstimulated and LPS-stimulated cells, aliquots of each sample were treated with various concentrations of corticosterone (Sigma-Aldrich; final concentrations were 0.005, 0.05, 0.1, 0.5, and 5 µM, respectively) including both physiological and pharmacological doses of the hormone. Triplicates of each treatment were transferred to 96-well flat-bottom microtiter plates and incubated for 48 h in a humidified incubator (37 °C, 5% CO_2) followed by assessment of cell viability.

The cell viability in the splenocyte cultures was determined with a commercially available colorimetric assay (CellTiter 96 AQueous One Solution Assay, Promega G3580). To account for differences in background activity, the mean absorbance of the triplicate set of wells with unstimulated cells for a given treatment was subtracted from the mean absorbance of the three corresponding LPS-stimulated wells.

Surgical procedure/ADX

Surgery was performed under isoflurane anaesthesia. After a 2-cm skin incision was performed on the back of the mice at the level of the kidneys (midline), adrenals were removed bilaterally through two peritoneal incisions, performed on the left and right side of the abdomen of the mouse. SHAM mice underwent the same procedure as ADX mice, but without removal of the adrenals. Following surgery, both ADX and SHAM mice received 0.9% saline in drinking water (until they were sacrificed) and were housed single for one week until the CSC procedure started. Saline helped ADX mice to compensate for loss of mineralocorticoids.

Isolation and incubation of mesenteric lymph node cells

Mesenteric lymph nodes (pooled from each experimental group) 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)]. 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 200 µl of 2.5 µg/ml anti-CD3 antibody in the presence of IL-2 (final concentration 100 U/ml). Eight wells were transferred with the respective number of cells of each experimental group. After incubation for 24 h (37 °C, 5% CO_2), cytokine concentrations were measured in the supernatants by ELISA (all from Endogen, Woburn, MA) using four wells per experimental group.

Statistics

For statistical comparisons, the software package SPSS (version 12) was used. Data of the two groups in experiment 1 were compared using the Mann-Whitney *U*-test. All comparisons of experiment 2 were done using a one-way ANOVA (factor time) followed by a *post hoc* Tukey HSD test when appropriate. In experiment 3, plasma dark phase corticosterone concentrations as well as "delta cell viability" of splenocytes of CSC and control mice were compared using a Mann-Whitney *U*-test. The *in vitro* adrenal corticosterone secretory responses as well as the GC sensitivity data were analyzed by repeated measures ANOVA with "CSC" as between-subject factor and "dose" as repeated measures within-subject factor followed by a *post hoc* Tukey-HSD test when appropriate. All comparisons of experiment 4 were done using a two-way ANOVA (factor ADX; factor CSC) and followed by a *post hoc* Tukey-HSD test when appropriate. Data are presented as means + S.E.M. Significance was taken at $p < 0.05$.

Results

Experiment 1:

Effects of CSC on body, relative thymus and adrenal weights: Compared with single-housed controls, CSC mice gained significantly less body weight during the 19 days of CSC (Fig. 20A). CSC mice also showed a significant decrease in relative thymus weight (Fig. 20B) and a significant increase in relative adrenal weight (Fig. 20C) compared with unstressed control mice as calculated on day 20.

Effects of CSC on plasma NE and corticosterone concentrations: NE concentrations estimated during the light phase between 0800 h and 1000 h were found to be significantly increased in CSC compared with control mice after 19 days of CSC

exposure (Fig. 20D). In contrast, exposure to CSC did not alter plasma corticosterone concentrations in the light phase, i.e. under basal, unstimulated conditions (Fig. 20E).

Effects of CSC on the histological damage score of the colon: 19-day exposure to the CSC procedure resulted in histological abnormalities of the colonic tissue as reflected by a significantly elevated histological score in CSC compared with control mice (Fig. 20F).

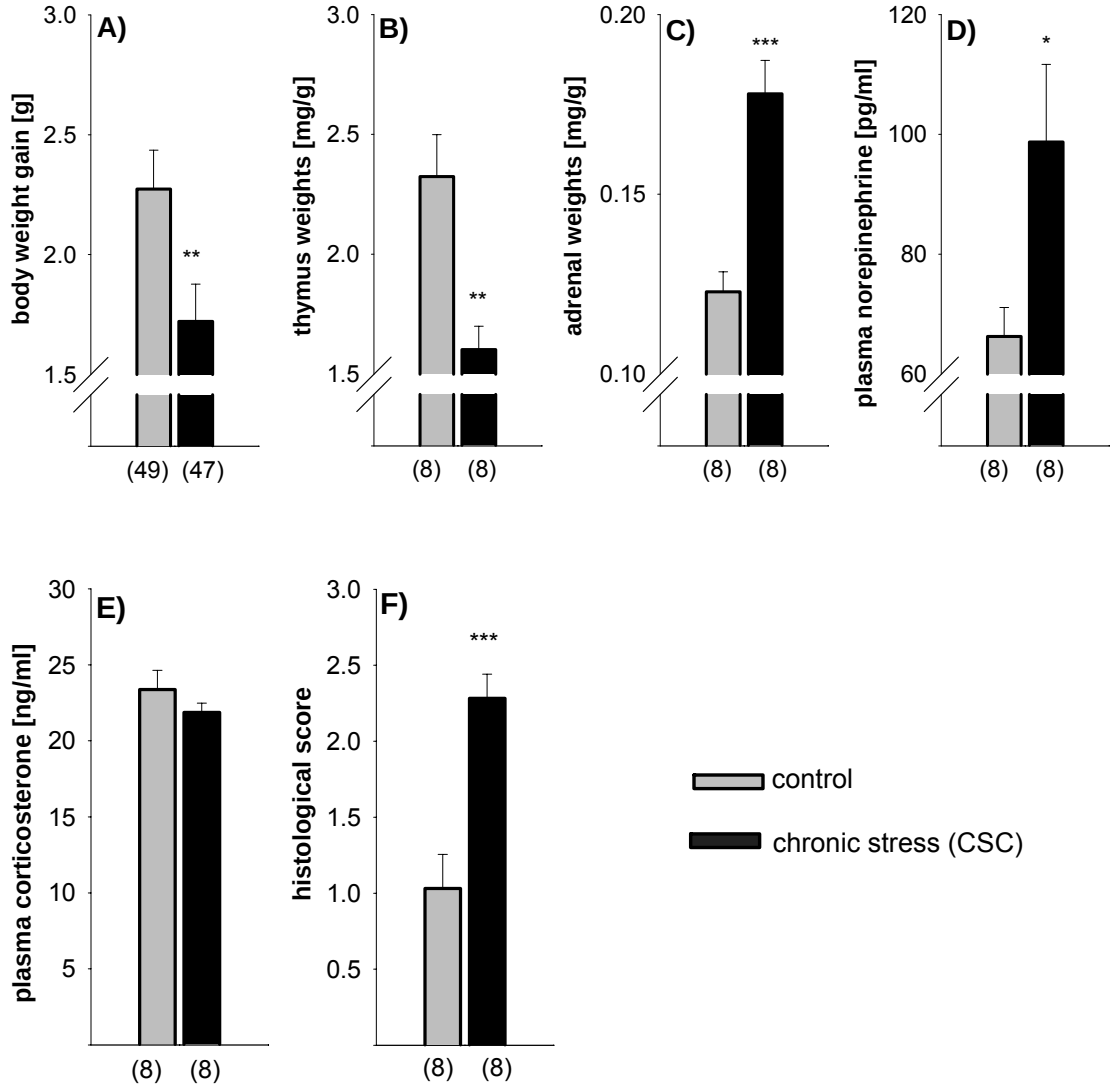


Fig. 20: Exposure to 19 days of chronic subordinate colony housing (CSC) leads to a decreased body weight gain (A), decreased relative thymus weight (B), increased relative adrenal weight (C), increased plasma NE concentrations (D), and an increased histological damage score of the colon (F) in CSC compared with unstressed control mice. Plasma light corticosterone concentrations were found

to be unchanged (E) by CSC exposure. Numbers in parentheses indicate group sizes. Data represent means ± S.E.M.; * p < 0.05; ** < 0.01; *** p < 0.001 vs. single-housed controls.

Effects of CSC on anxiety-related behaviour: 19-day exposure to CSC significantly increased anxiety-related behaviour as reflected by a decrease in the percentage of time spent on the open arms, and in the percentage of open arm entries compared with control mice (Fig. 21). The number of entries into the closed arms (ctrl: 9.3 ± 0.8 vs. CSC: 9.4 ± 0.6; p = 0.873), indicative of locomotor activity, was not altered by CSC exposure.

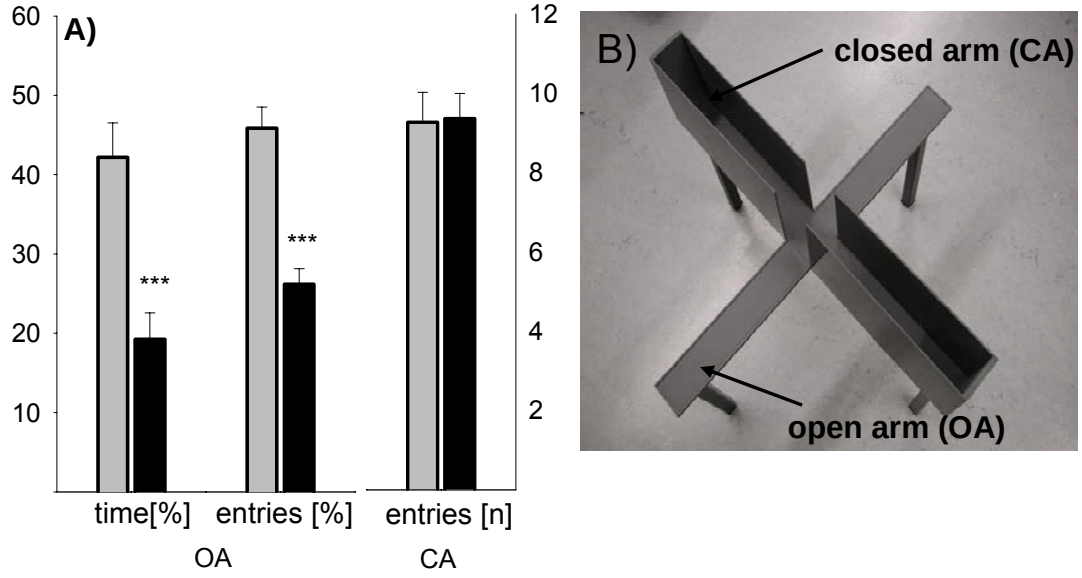


Fig. 21: Exposure to 19 days of chronic subordinate colony housing (CSC; n = 39) leads to an increased anxiety-related behaviour on the elevated EPM compared with control mice (n = 41). This is reflected by a reduced percentage of time (time [%]) spent on and percentage of entries (entries [%]) performed onto the open arms (OA) of the plus-maze. The number of entries onto closed arms (CA) was equal in both groups, indicating that locomotor activity was not affected by CSC exposure. Data represent means ± S.E.M.; * p < 0.05; ** < 0.01; *** p < 0.001 vs. single-housed controls (A). In addition a picture of the mouse EPM is shown (B).

Experiment 2:

Time course of CSC effects on TH expression in the LC and in the colonic tissue: TH immunoreactivity in the LC was found to be significantly affected by CSC exposure ($F_{5, 116} = 4.55$; $p = 0.001$; Fig. 22A). *Post hoc* analysis revealed a significant decrease

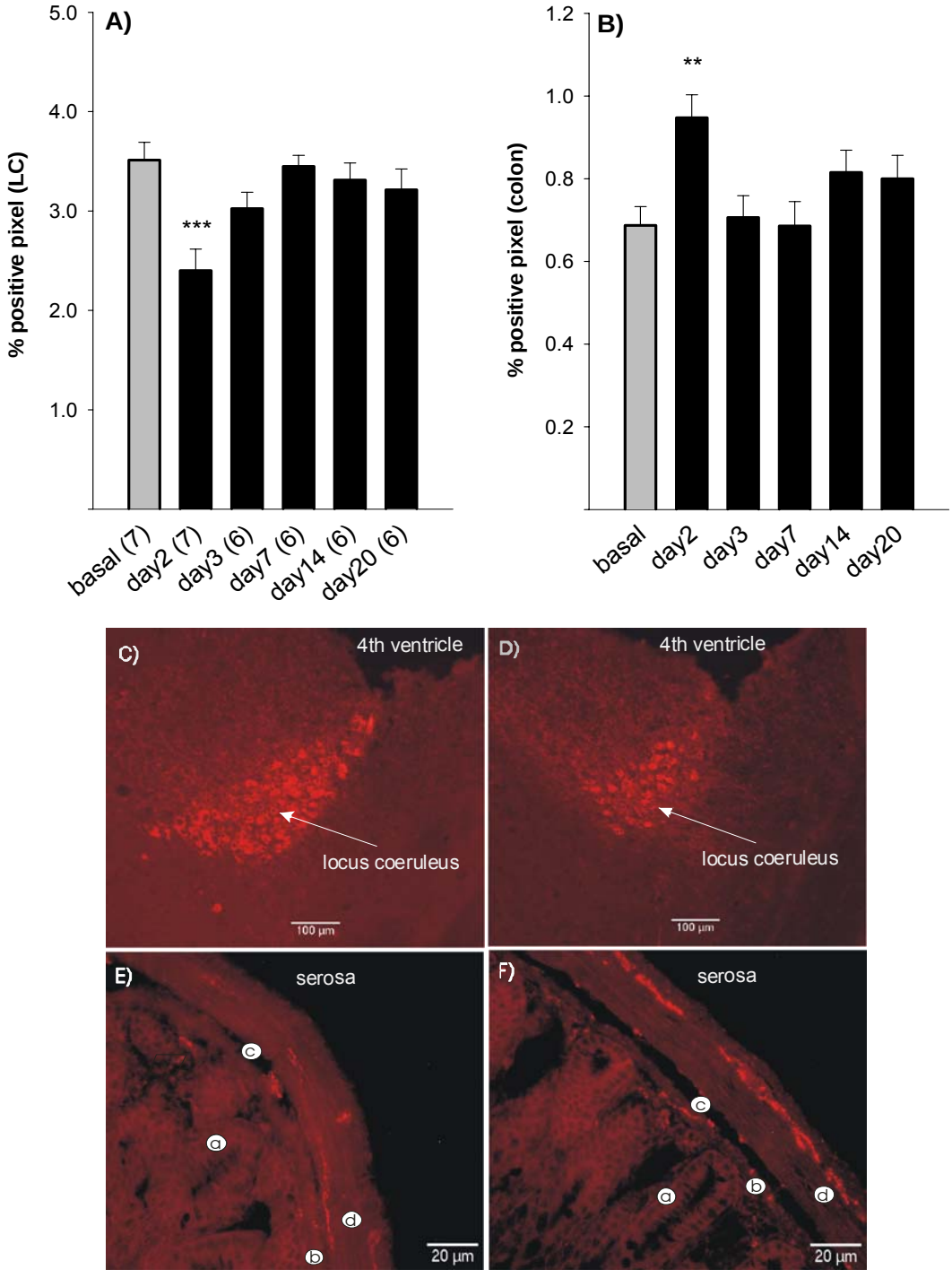


Fig. 22: Effects of 19-day exposure to chronic subordinate colony housing (CSC) on TH expression in the LC (A) and in colonic tissue (B) at various time points (day 2, 3, 7, 14 and 20 of CSC). In A, group sizes are indicated by the numbers in parenthesis, in B, group size was 6 animals each.

Representative images of immunofluorescent TH-staining in the LC and colon [a: Lamina mucosa; b: Lamina muscularis mucosa; c: Lamina submucosa; d: Lamina muscularis (circular and longitudinal mussle)], respectively, are shown in C/E (basal values) and D/F (day 2 values). Data represent means + S.E.M.; ** $p < 0.01$; *** $p < 0.001$ vs. basal values.

in TH expression on day 2 of CSC exposure (Fig. 22A; see also Fig. 22D), but on subsequent days of CSC exposure, the level of TH immunoreactivity returned to baseline (Fig. 22A; see also Fig. 22C). Furthermore, a significant main effect of CSC exposure on TH expression in colonic tissue ($F_{5, 611} = 3.59$; $p = 0.003$; Fig. 22B), with a significant increase on day 2 of CSC exposure (Fig. 22B; see also Fig. 22F) compared with basal values (Fig. 22B; see also Fig. 22E) was determined.

Time course of CSC effects on CRH mRNA in the PVN: Exposure to CSC significantly altered CRH mRNA expression in the PVN ($F_{5, 45} = 3.57$; $p = 0.009$; Fig. 23A). *Post hoc* analysis showed a tendency ($p = 0.055$) to elevated CRH mRNA

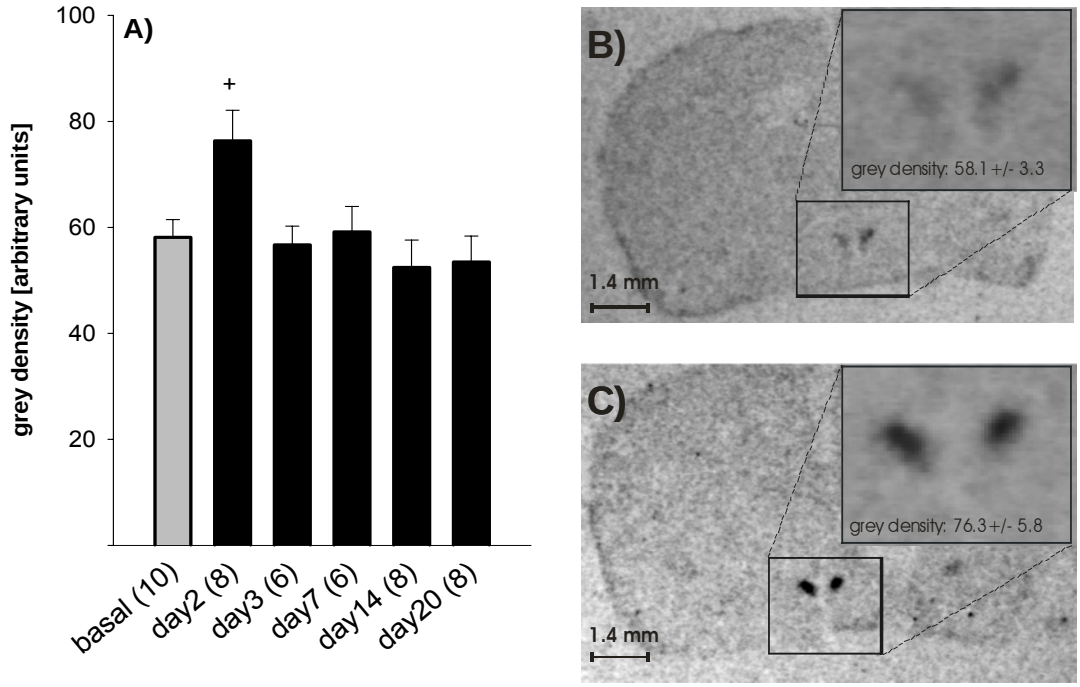


Fig. 23: Effects of 19-day exposure to chronic subordinate colony housing (CSC) on CRH mRNA expression (grey density) in the PVN at various time-points (Fig. 23A; day 2, 3, 7, 14 and 20). Furthermore, two representative images of CRH mRNA expression in the PVN of a basal (B) and a CSC mouse sacrificed on day 2 of CSC (C) are shown. Numbers in parentheses indicate group sizes. Data represent means + S.E.M.; + $p = 0.055$ vs. basal values.

expression on day 2 of CSC-exposure (Fig. 23A). This effect reached statistical significance when performing a Mann-Whitney *U*-comparison between basal (Fig. 23B) and day 2 values ($p = 0.021$; Fig. 23C). At all other days measured no differences in CRH mRNA between stressed and basal mice were found.

Time course of CSC effects on relative adrenal weight and plasma corticosterone concentrations: Relative adrenal weight was found to be significantly increased by CSC exposure ($F_{5, 44} = 18.8$; $p < 0.001$; Fig. 24A). Compared with basal values, the relative adrenal weight was significant increased at all days measured. Plasma corticosterone concentrations during the light phase were also found to be altered by CSC exposure ($F_{5, 45} = 3.41$; $p = 0.012$; Fig. 24B), but significantly elevated hormone levels were only found on day 2, i.e. during the initial phase of CSC.

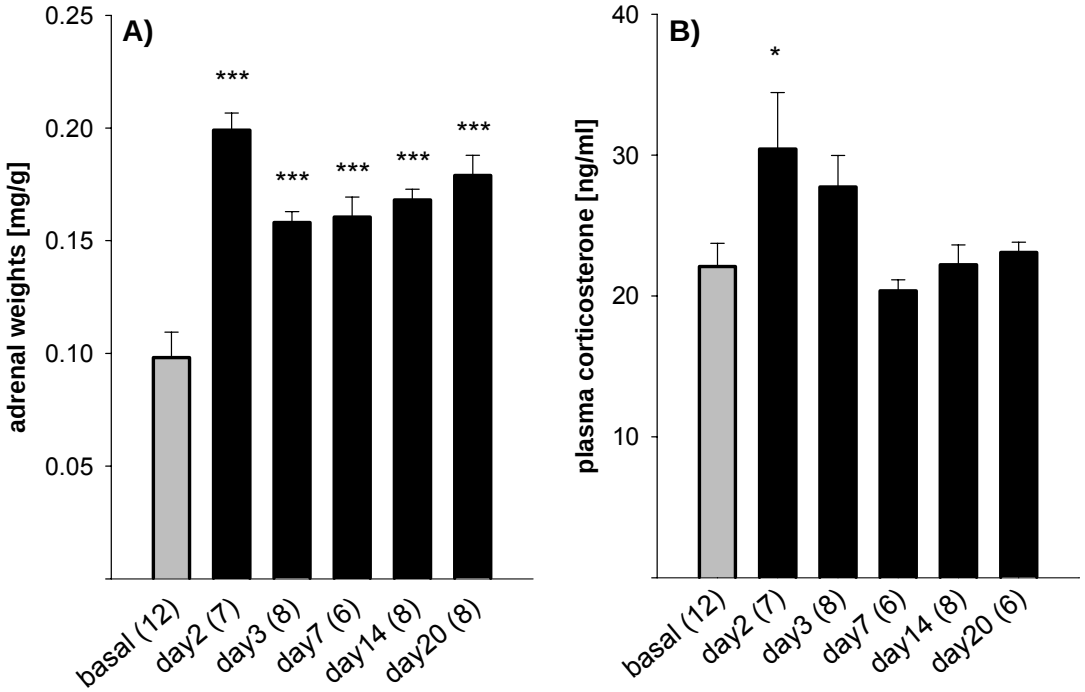
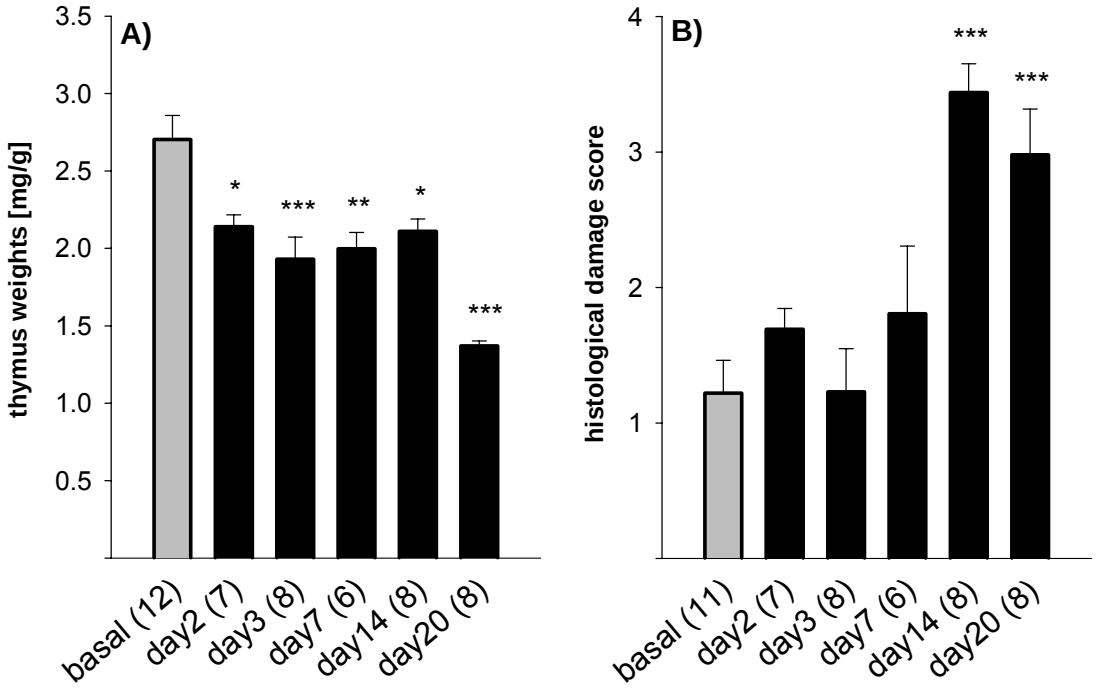


Fig. 24: Effects of 19-day exposure to chronic subordinate colony housing (CSC) on relative adrenal weight (A; adrenal weight [mg]/body weight [g]) and light phase plasma corticosterone (B) concentrations [ng/ml] at various time-points (day 2, 3, 7, 14 and 20). Numbers in parentheses indicate group sizes. Data represent means + S.E.M.; * $p < 0.05$; *** $p < 0.001$ vs. basal values.

Time course of CSC effects on relative thymus weight: Relative thymus weight was significantly reduced during 19 days of CSC exposure ($F_{5, 44}$ = 15.6; $p < 0.001$; Fig. 25A). In comparison to basal values this reduction in relative thymus weight was significant at all days measured.

Time course of CSC effects on the histological damage score: The histological damage score of the colon was also found to be dependent on CSC exposure ($F_{5, 47}$ = 14.6; $p < 0.001$; Fig. 25B). Post hoc analysis revealed that the histological damage score was significantly higher both on day 14 and 20 (Fig. 25D) of CSC exposure compared with values from basal mice (Fig. 25C), reflecting more severe inflammatory infiltration and increased epithelial damage, i.e. focal disappearance of mucosal crypts.



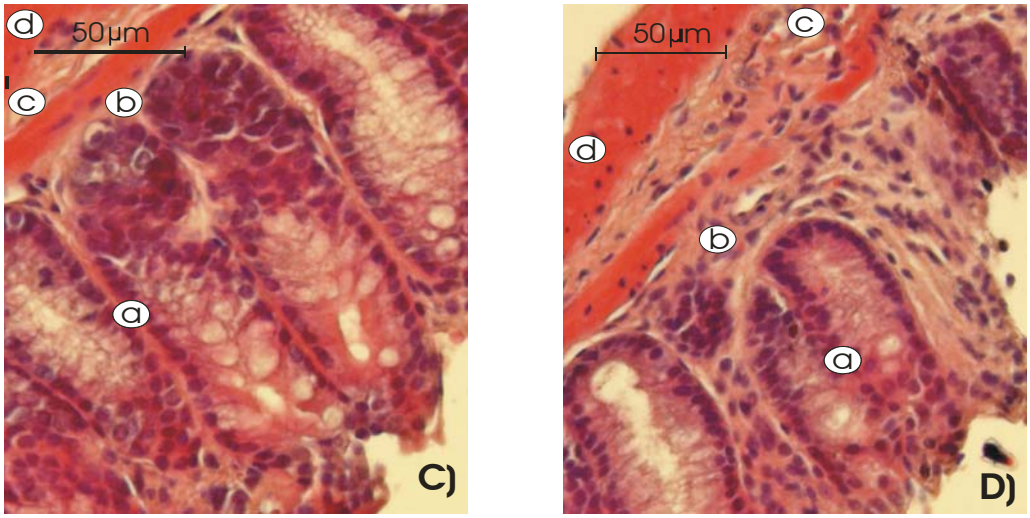


Fig. 25: Effects of 19-day exposure to chronic subordinate colony housing (CSC) on relative thymus weight (A; thymus weight [mg]/body weight [g]) and on the histological damage score (B) at various time-points (day 2, 3, 7, 14 and 20). Numbers in parentheses indicate group sizes. Data represent means + S.E.M.; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. basal values. Furthermore, two representative colonic H&E sections are shown [a: Lamina mucosa; b: Lamina muscularis mucosae; c: Lamina submucosa; d: Lamina muscularis (circular and longitudinal mussle)] from basal mice (Fig. 4C; normal colon histology) and from CSC mice (day 20; Fig. 4D; goblet cell loss and crypt loss in locally restricted areas; infiltration reaching the Lamina muscularis mucosae; thickening of submucosal areas).

Experiment 3:

CSC effects on plasma dark phase corticosterone concentrations and adrenal corticosterone secretory responses in vitro *(day 20).*

Plasma corticosterone concentrations determined at the beginning of the dark phase were found to be significantly lower in CSC compared with control mice (Fig. 26A). No effects of CSC were found on baseline corticosterone secretion from adrenal cells *in vitro* (ACTH dose 0 M; Fig. 26B). Exposure of adrenal cells to ACTH had a stimulating effect on the release of corticosterone in both groups, which was, however, dependent on prior CSC exposure (factor CSC x dose: $F_{4,\ 8} = 6.0$; $p = 0.016$; Fig. 26B). *Post hoc* Tukey-HSD tests showed a significantly attenuated corticosterone response of adrenal cells from CSC mice at 10^{-12} M and 10^{-11} M ACTH compared with unstressed controls (Fig. 26B).

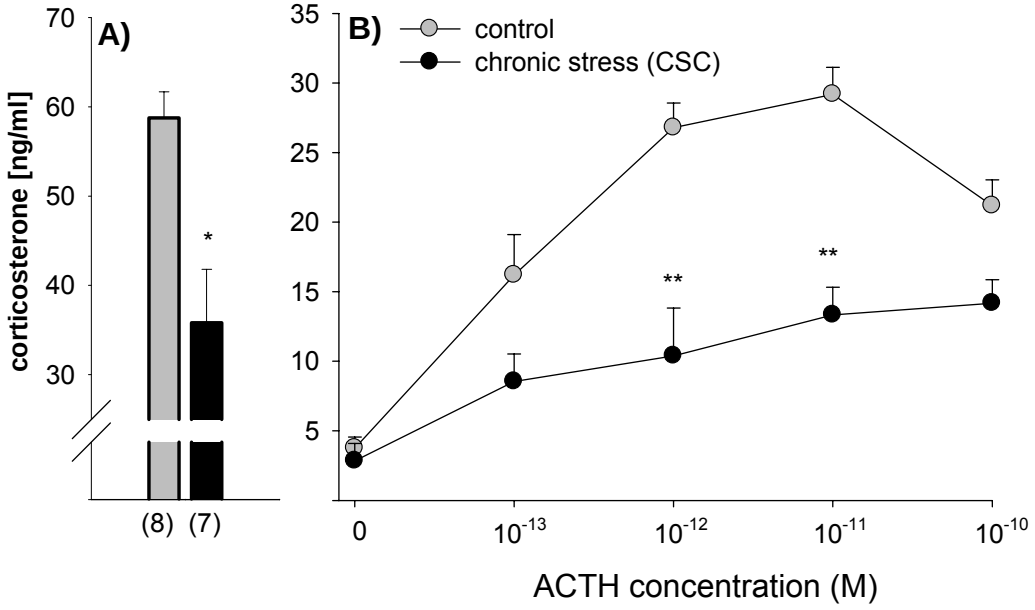


Fig. 26: Effects of 19-day exposure to chronic subdordinate colony housing (CSC) on plasma dark phase corticosterone concentrations (A; numbers in parentheses indicate group sizes.) and on the *in vitro* release of corticosterone from isolated adrenal cells after stimulation with ACTH at varying doses (B). Adrenals of 4 control and 4 CSC mice, respectively, were pooled (n = 8; pooled samples), and two aliquots (a 200 µl) of each group were stimulated with ACTH at concentrations between 4.6 x 10^{-3} – 4.6 ng/ml (10^{-13} – 10^{-10} M). Supernatants were analyzed by a corticosterone RIA in triplicates. Adrenal cells of CSC mice showed a reduced response to ACTH at all doses tested. Data represent means ± S.E.M.; ** $p < 0.01$ vs. respective unstressed controls.

CSC effects on GC sensitivity of LPS-stimulated splenocytes in vitro *(day 20):*

Corticosterone treatment exerted a significant dose-dependent effect on the viability of LPS-stimulated splenocytes ($F_{5, 30} = 83.0$; $p < 0.001$; Fig. 27A), which was, however, dependent on prior CSC exposure (CSC x dose: $F_{5, 30} = 9.85$; $p = 0.001$; Fig. 27A). In unstressed control mice, a significant increase in splenocyte viability was found in cultures treated with 0.005 µM corticosterone compared with untreated cells. This stimulatory effect of corticosterone at the lowest dose tested was absent in splenocytes from CSC mice indicating reduced corticosterone sensitivity after chronic stress (Fig. 27A). At the highest corticosterone doses tested (0.5 and 5.0 µM) cell viability of splenocyte cultures of both CSC and control mice

was significantly diminished compared with respective untreated splenic cells. This effect tended to be less pronounced in CSC mice at 5 µM (Mann-Whitney *U*-comparison, CSC versus unstressed control; p = 0.053). The reduced GC sensitivity of splenic cells of CSC mice is further described by their significantly decreased "delta cell viability" (cell viability at 0.005 µM corticosterone - cell viability at 5 µM corticosterone; Fig. 27B)" compared with splenic cells of control mice.

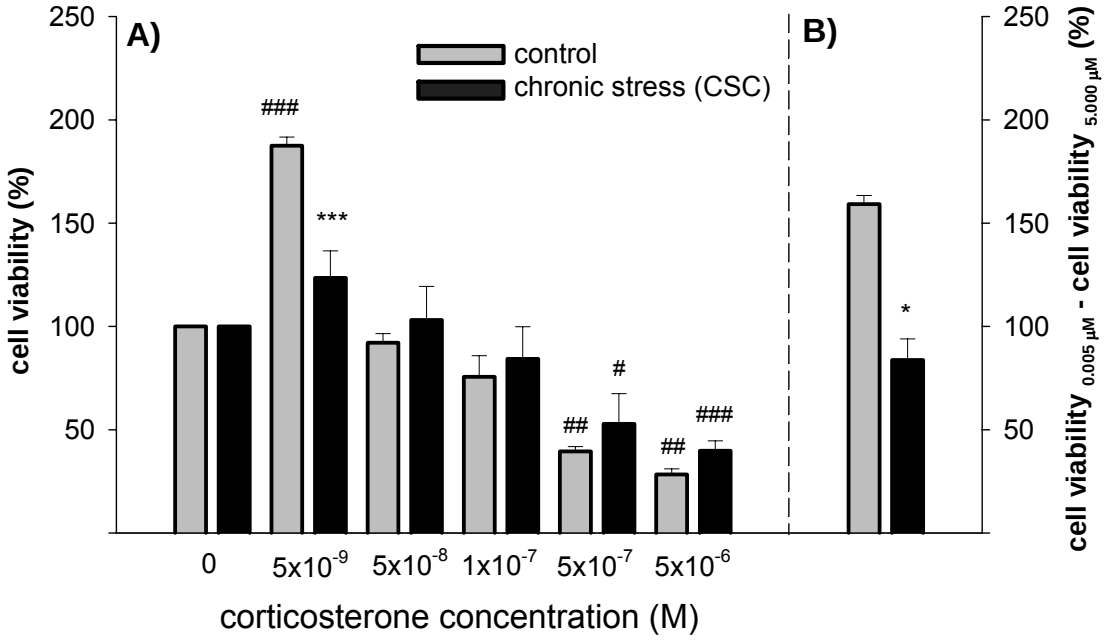


Fig. 27: Effects of 19-day exposure to chronic subordinate colony housing (CSC) on the *in vitro* GC sensitivity of splenocytes. Experimental mice were subjected to 19 days of CSC (n = 8; black bars). One day before (day 19) being tested on the EPM (day 20; from 0800 h to1000 h) to confirm the chronic stress procedure, they were singly housed. 12 hours after the EPM testing was performed (from 0800 h to 1000 h), cells from CSC and control mice (grey bars, n = 10) were cultured with LPS (1 µg/ml) in the presence of various concentrations of corticosterone. After 48 h of incubation, the cell viability was determined using a colorimetric assay. Data are presented as the percentage of cell viability in the absence of corticosterone (A) and "delta cell viability" (cell viability at 0.005 µM corticosterone - cell viability at 5.000 µM corticosterone; B)". Data represent means ± S.E.M.; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$vs. single-housed controls.

Experiment 4:

Effects of prior ADX on CSC-induced changes in relative thymus weight, histological damage score, and cytokine secretion by mesenteric lymph node cells (day 20).

Relative thymus weight was dependent on prior ADX ($F_{1,41}$ = 9.17; $p < 0.004$; Tab. 3) and on CSC exposure ($F_{1,41}$ = 26.2; $p < 0.001$; Tab. 3). CSC mice of both SHAM and ADX group showed a significantly decreased relative thymus weight compared to respective control mice (Tab. 3). Within the non-stressed controls ADX caused a significant thymus hypertrophy compared with SHAM mice (Tab. 3).

Statistical analysis revealed a main interaction of ADX and CSC exposure (factor ADX x CSC: $F_{1,41}$ = 4.78; $p = 0.035$; Tab. 3) on the histological damage score. More specifically, histological damage score was not affected by ADX in non-stressed control mice but the CSC-induced increase, determined within the SHAM group, could be not determined in ADX mice. The histological damage score of CSC-exposed ADX mice was therefore comparable to non-stressed mice of both ADX and SHAM group (Tab. 3).

Both, IFN-γ and TNF-α secretion were dependent on prior ADX and CSC exposure (IFN-γ: factor ADX x CSC: $F_{1,12}$ = 9.15; $p = 0.011$; TNF-α: factor ADX x CSC: $F_{1,12}$ = 33.3; $p < 0.001$; Tab. 1). Proinflammatory cytokine concentrations of SHAM or ADX mice, which were not exposed to CSC, were not statistically different (Tab. 1). However, CSC exposure significantly increased the secretion of IFN-γ and TNF-α in SHAM and ADX mice, with the effect being more severe in the SHAM group (Tab. 1). Secretion of the antiinflammatory cytokine IL-10 was dependent on CSC exposure ($F_{1,41}$ = 26.2; $p < 0.001$; Tab. 1) with CSC mice of both, SHAM and ADX group showing increased secretion compared with controls.

Tab. 3. Effects of adrenalectomy (ADX) prior to 19-day exposure to chronic subordinate colony housing (CSC) on relative thymus weight, histological damage score, and pro- and antiinflammatory cytokine secretion by mesenteric lymph node cells in male mice.

	SHAM/ control	**ADX/ control**	**SHAM/ CSC**	**ADX/ CSC**
thymus weight [mg/g]	2.2 ± 0.1 (n=14)	2.8 ± 0.2 * (n=7)	1.7 ± 0.1 # (n=14)	1.9 ± 0.1 ### (n=10)
histological score	1.0 ± 0.1 (n=14)	1.0 ± 0.2 (n=7)	1.6 ± 0.2 # (n=14)	0.9 ± 0.2 * (n=10)
IFN-gamma [pg/ml]	526.2 ± 69.6 (n=14)	352.7 ± 45.7 (n=7)	8649.0 ± 962.3 ### (n=14)	4350.0 ± 962.9 ##, ** (n=10)
TNF-alpha [pg/ml]	8.9 ± 0.5 (n=14)	8.4 ± 0.6 (n=7)	44.7 ± 4.3 ### (n=14)	18.8 ± 0.5 #, *** (n=10)
IL-10 [pg/ml]	1.0 ± 0.0 (n=14)	1.6 ± 0.6 (n=7)	19.2 ± 2.5 ### (n=14)	15.2 ± 1.8 ### (n=10)

ADX and SHAM mice were housed single for one week before they were exposed to either chronic subordinate colony housing (CSC) or further housed single (control) for 19 consecutive days. Numbers in parentheses indicate group sizes. Data represent mean ± S.E.M.; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. respective SHAM mice; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. respective non-stressed control group.

Discussion

The studies described in this chapter reveal that exposure to CSC housing leads to macroscopic damages of the mucosal layers of the colon and to increased secretion of pro- and antiinflammatory cytokines by mesenteric lymph node cells in male mice. In confirmation of studies performed in human [105] and non-human primates [106, 107], this is the first report of chronic stress effects on the development of a spontaneous colonic inflammation in a relevant chronic psycho-social stress model

for rodents. Furthermore, temporal alterations during the duration of the chronic stress manipulation were shown in the two main stress response systems, the HPA axis and sympathetic nervous system. Interestingly, CSC-induced colonic inflammation could at least be partially blocked by ADX prior to CSC exposure. Therefore, the mechanisms underlying the colonic damage are likely to include stress-induced maladaptations, particularly in the HPA axis at the level of the adrenal glands.

Establishment of CSC as model of chronic psycho-social stress in male mice

In agreement with previous studies in the rat [137], the data presented in this chapter provide evidence that the CSC procedure is an adequate model of chronic psycho-social stress for male mice. All subordinate mice displayed typical signs of chronic stress, including a reduction in body weight gain [66, 81, 292, 293]. Interestingly, it has been shown in rats that the extent of body weight gain is related to the rank of the animal within a social group [327]. Another indicator of acute or chronic stress is an elevated level of state anxiety [313, 314], which has been described both in rats [81, 315, 316] and in mice [82, 317]. In the present study, mice exposed to CSC displayed increased anxiety-related behaviour on the EPM compared with unstressed, single-housed control mice further validating the CSC model. Importantly, the increase in anxiety-related behaviour of CSC mice is not due to single housing for 15 h before plus-maze testing, as CSC mice directly transferred from colony housing to the plus-maze were even more anxious (see Chapter 5). As additional indicators of stress, CSC-exposed mice showed robust thymus atrophy and adrenal hypertrophy [81, 139, 295, 328]. With the notable exception of the strong influence of CSC on anxiety-related behaviour, this model of chronic psycho-social stress is comparable to the previously described SD/OC (social defeat/overcrowding) paradigm (see Chapters 2 and 5) [279]. However,

further testing with the SD/OC model may reveal an anxiety-like phenotype that was not observable on the EPM. Additionally, the experimental procedure of the CSC paradigm is simple compared with SD/OC and has the further benefit of avoiding daily manipulation of the animals [279].

On account of these stress-induced differences found between CSC and control mice that have been singly housed for the same time span I abstained from sacrificing single-housed controls at day 2, 4, 7, 14, and 20 of experiment 2. Indeed, the comparable organ weights, corticosterone levels, and histological scores between the above mentioned control mice of experiment1 and the basal mice of experiment 2 provide evidence, that single housing itself poses no stressor for male mice in the set-up of these studies. Remarkably, 24 h after colony formation (day 2), relative adrenal weight had doubled in comparison to basal values and remained significantly increased on days 3, 7, 14, and 20 of CSC exposure. Thymus involution was also seen at all time-points measured during CSC exposure. This is in agreement with thymus atrophy seen in defeated rats after 24 h of resident - intruder confrontations [139, 329]. The reduction in thymus mass is likely related to a decrease in the absolute cell numbers of all thymocyte subpopulations, most substantially within immature $CD4^+CD8^+$ cells [139]. The thymus exhibits a high density of GC type-II receptors, and the immature $CD4^+/CD8^+$-cell population are particularly sensitive to GCs [330, 331]. Therefore, the elevation of GCs observed during the CSC procedure may have induced apoptosis and inhibited cell proliferation of immune cells [332, 333]. Additionally, the increased relative thymus weight determined in non-stressed control mice after ADX indicates a basal role of endogenous corticosterone in regulating thymus size. However, in the studies described in this chapter, increased concentrations of plasma corticosterone were only found on day 2 of CSC exposure, which returned to baseline thereafter despite the maintenance of elevated relative

adrenal weight. Therefore, and because of the decreased relative thymus weight found in ADX mice after 19-day exposure to CSC, an additional factor must be taken into account contributing to stress-induced loss in thymus mass. In this respect, it is important to note that the medullary part of the thymus expresses high densities of β-adrenergic receptors [334], which are involved in cAMP-mediated thymocyte apoptosis and the consequent decrease in thymocyte numbers [335-337]. Thus, the stress-induced activation of the sympathetic-adrenomedullary system, as shown previously [338], and reflected in the present studies by increased plasma NE concentrations even 15 h following the termination of CSC could be causally involved in the loss of thymus mass in CSC mice. Recent observations in socially defeated rats, treated with the β-adrenergic antagonist propranolol confirmed the hypothesis that thymic atrophy as a result of social stress might, at least partially, be mediated by catecholamines [139]. With respect to CSC-induced alterations of the sympathetic nervous system, TH expression in the LC was found to be significantly decreased on day 2 of CSC. This might be due to an acute rise, within the first hours of colony formation, in LC neuronal activity and a rapid decrease thereafter by negative feedback regulation via NE and epinephrine binding to presynaptic a_2-adrenoceptors [339, 340]. This would be in agreement with the general knowledge, that stressor exposure acutely triggers elevated TH mRNA [31, 341, 342] and protein [343] levels in the brain stem. Therefore, it is probable that a combination of these mechanisms is involved in the decreased TH expression in the LC observed 24 h after colony formation in the studies described in this chapter.

Plasma corticosterone concentrations were significantly increased, but only on day 2 of CSC exposure, and returned to baseline afterwards. Various adaptive processes concerning the HPA axis are likely to occur during a long-lasting stressor exposure, in order to protect the body from an immunosuppressive, and therefore deleterious,

chronic exposure to increased corticosterone concentrations [74, 77]. Thus, at the level of hypothalamic CRH neurons, CRH mRNA expression was significantly increased in the same temporal fashion observed for plasma corticosterone, i.e. only on day 2 of CSC exposure. Thereby, the initial increase in CRH mRNA expression may reflect the acute activation of the HPA axis, as it is generally described in rats and mice after various acute stressors [120, 344-347]. In contrast, the similar CRH mRNA levels in the PVN between CSC and control mice after prolonged stressor exposure are hard to interpret, because hypothalamic CRH mRNA levels are known to vary depending to the type and duration of the stressor (for review see [5]; see also Chapter 1, Sec. 2.3). Therefore, the CRH mRNA content of the PVN might not be a reliable indicator of chronic stress in the present study. Severe stress-related changes of HPA axis reactivity are also likely to occur at the level of the adrenal glands. Despite an elevated relative adrenal weight during the CSC procedure, plasma corticosterone concentrations measured at the beginning of the light phase were found to be similar between CSC and control mice (except for the rise seen on day 2 of CSC). Furthermore, moderate stimulation of GC secretion during its diurnal rise [297, 321] seen in control mice was found to be attenuated in chronically stressed mice after 19 days of CSC. As a result, dark phase plasma corticosterone concentrations were significantly lower in CSC compared with control mice. Finally, in addition to the abolished *in vivo* adrenal response upon stimulation, adrenal cells of CSC mice were also found to insufficiently respond to an ACTH challenge *in vitro*. At all doses tested, ACTH-induced corticosterone secretion from isolated adrenal cells was significantly lower in CSC compared with control mice. Thus, adrenal cells seem to become insufficient to synthesize and/or secrete appropriate amounts of GCs during or after prolonged stressor exposure.

Effects of CSC exposure on colonic inflammation

As outlined in the introduction of this chapter, exposure to an acute stressor, and the consequently elevated corticosterone levels, have been shown to impair various parameters of intestinal barrier functions[276, 310-312], resulting in increased colonic permeability. The degree of permeability was positively correlated with the severity of the stressor exposure [276, 348]. Consequently, the presentation of luminal antigens to the mucosal and non-mucosal immune system is enhanced [349]. Although impaired barrier functions have been directly linked to the development of intestinal inflammation [192, 232, 350], pathological changes in colon histology have never been described immediately after stressor exposure. Thus, the data of the present study provide the first evidence in rodents that chronic exposure to a psycho-social stressor induces an increase in colonic inflammation. Since histological damage was not found during the first two weeks of CSC exposure, it is likely that in other studies the duration of stress was too short to reveal similar histological effects.

Additionally, secretion of pro- and antiinflammatory cytokines by mesenteric lymph node cells was found to be increased after exposure to CSC, also reflecting the increased inflammatory state in colonic tissue after chronic stress. Interestingly, CSC-induced effects on colonic histology and proinflammatory cytokine secretion by mesenteric lymph node cells were diminished or even completely abolished by ADX prior to stressor exposure. Therefore, the initial increase in basal plasma corticosterone concentrations and the increased sympathetic activity described after social defeat [138, 351, 352], and found on day 2 of CSC are likely to increase intestinal permeability and bacterial translocation to mesenteric lymph nodes, liver and spleen [349]. Further studies will be performed in order to confirm this hypothesis (see also Chapter 4). The commencement of an immune response requires acute activation of the HPA axis as well as the sympathetic nervous system for

mobilization of immune cells and their redistribution to relevant body compartments [353]. Indeed, with respect to the activation of the sympathetic nervous system, the present data demonstrate an elevated TH expression directly at the site of bacterial invasion in colonic tissue on day 2 of CSC. Thus, in addition to the hypothesized increased exposure to luminal antigens, CSC mice might also exhibit an acutely improved immune function resulting from the initial activation of these systems.

The initial rise in plasma corticosterone levels and the inability of the HPA axis to adequately respond thereafter, could partly contribute to the development of spontaneous colonic inflammation after prolonged CSC exposure. With the progression of chronic stress exposure, the HPA axis becomes unable to respond to stress, leading to insufficient production and secretion of antiinflammatory GCs. This may contribute to the increased epithelial damage score and the increased cytokine secretion found after prolonged CSC exposure. Indeed, a blunted activation of the HPA axis during stressor exposure makes the animals more vulnerable for chemically induced inflammations [108, 273-275]. Moreover, the diminished GC sensitivity found in LPS-stimulated immune cells of CSC mice may additionally contribute to the increased colonic inflammation found on days 14 and 20 of CSC. The development of GC resistance has been suggested as one of the mechanisms by which a hyper-inflammatory state may be induced under stressful conditions. Additionlly, uncoupling of the activity of the HPA axis and sympathetic nervous system during the CSC procedure may promote proinflammatory processes, since the synergism of steroid hormones and neurotransmitters of the sympathetic nervous system will be dissipated [319].

In conclusion, this study shows that a subordinate status within a group of male mice over three weeks is a potent chronic psycho-social stressor which induces a spontaneous colonic inflammation. The inflammatory process is, at least partially, mediated by the initial activation of the HPA axis and rise in plasma corticosterone followed by the inefficiency of the adrenals to secrete corticosterone.

Chapter 4

Chronic psycho-social stress (subordinate colony housing) increases the severity of a DSS-induced colitis in mice – implications and mechanisms

[adapted from: **Reber SO, Obermeier F, Straub RH, Veenema AH, Neumann ID; 2006; J Clin Invest, submitted]**

Abstract

In chapter 3, CSC was shown to lead to spontaneous colonic inflammation in male mice. In the studies described in this chapter it was investigated whether CSC exposure also influences the severity of a subsequent DSS-induced colitis. In addition, it was the aim to reveal possible neuroendocrine mechanisms involved, thereby focusing on the role of GCs.

After 19-day exposure to CSC, male C57BL/6 mice were subsequently treated with 1% DSS from day 20 to 27 wherby on day 2, 4, and 8 of DSS treatment the severity of the colitis was assessed in a subgroup of the mice. Already on day 2 of DSS treatment the secretion of the proinflammatory cytokines TNF-α, IFN-γ, IL-6 as well as the antiinflammatory cytokine IL-10 by mesenteric lymph node cells was increased in CSC compared with control mice. In contrast, reduced colon length and an increased histological score were found in CSC compared with control mice only after 8 days of DSS treatment. At this time point, the secretion of proinflammatory cytokines was already down-regulated again in CSC mice, paralleling the increase in plasma corticosterone first detected on day 8 of DSS treatment. Interestingly, in control mice elevation in cytokine secretion was only found on day 8 of DSS treatment, and was thereby associated with a rise in plasma corticosterone levels.

In order to investigate the role of corticosterone in CSC-induced effects on the severity of DSS colitis, mice were adrenalectomized prior to the exposure of CSC and subsequent DSS treatment. CSC-exposed ADX mice showed a significant reduction in the severity of DSS-induced colitis as indicated by a diminished body weight loss, less reduction in colon length, and a lower histological damage score compared with CSC-exposed SHAM mice on day 8 of DSS treatment.

In conclusion, exposure to chronic psycho-social stress increases the severity of an acute DSS colitis, an effect which is likely mediated by adrenal mechanisms.

Introduction

After first reports in the 1970s [105], stress has been recognized as one of the key factors in modulating the onset and the severity of spontaneous colitis in human [103, 104, 269, 270, 281] and nonhuman primates [106, 107]. As a consequence, there is a growing number of animal studies investigating the relationship between exposure to stress and its effects on an experimentally-induced colitis [109-111]. The described stress effects mainly depend on the quality and duration of the stressor and are therefore partially controversial [108, 273-275]. In chapter 3 it was shown that chronic exposure of male mice to a psycho-social stressor, CSC for 19 consecutive days, resulted in the development of a spontaneous colonic inflammation [302]. In CSC mice, an increased cytokine secretion by mesenteric lymph node cells and an increased histological score was found after prolonged CSC exposure compared with control mice. Interestingly, in CSC mice, an adrenal insufficiency both *in vivo* and *in vitro* could be revealed at the end of stressor exposure [302]. In this respect it is important to mention that there are studies providing evidence for a blunted responsiveness of the HPA axis making the animals more prone to a chemically induced inflammation [108, 273-275]. This may be due to the fact that adequate secretion of immunosuppressive GCs is important for the suppression of immune responses and for preventing them from overshooting [298, 300].

Additionally, ADX prior to CSC exposure was able to block, at least partially, the CSC-induced increase in the histological score and the secretion of proinflammatory cytokines by mesenteric lymph node cells [302]. This indicates that the rise in corticosterone during the acute phase of chronic stressor exposure also contributed to the increased spontaneous colonic inflammation after prolonged stressor exposure [302].

Not only CSC-exposure, but also exposure to another model of chronic stress, a chronic intermittent psycho-social stressor (19 days of social defeat/overcrowding; SD/OC; see Chapter 2) resulted in adrenal insufficiency [279] (see also Chapter 2). Additionally, SD/OC mice which were subsequently treated with DSS showed an increased body weight loss, increased inflammatory reduction of colon length, increased secretion of proinflammatory cytokines by mesenteric lymph node cells, and an increased histological score compared with controls [279]. Interestingly, the increased severity of the colitis was paralleled by a rise in plasma corticosterone. However, in that study colitis severity and plasma corticosterone levels were assessed only on day 8 of DSS treatment [279]. Therefore, the possibility exists that a blunted responsiveness of the adrenals may at least persist during the first days of DSS treatment and contributes to the chronic stress-induced increase in colitis severity [279]. Furthermore, the initial increase of plasma corticosterone during CSC exposure [302] may also contribute to the increased severity of a subsequent DSS colitis.

The first experiment of this chapter was designed to investigate, whether exposure to CSC increases the severity of a subsequent DSS-induced colitis in male mice. Furthermore, it was investigated whether the CSC-induced adrenal insufficiency persists during subsequent DSS treatment and therefore may contribute to the CSC-induced effect on colitis severity. Therefore, CSC and control mice were sacrificed on days 2, 4, or 8 of DSS treatment, and body weight loss, inflammatory reduction of colon length, histological damage score, cytokine secretion by mesenteric lymph node cells, and plasma corticosterone were estimated.

In the second experiment it was the aim to determine the influence of the initial rise in plasma corticosterone levels during CSC exposure [302] on the severity of a

subsequent DSS colitis. Therefore, mice were adrenalectomized before CSC exposure and subsequent DSS treatment.

Overall, the present study was designed to determine whether chronic psycho-social stress prior to a colitis induced by DSS treatment affects the severity of this chemically-induced intestinal inflammation and to which extend these effects are mediated by adrenal mechanisms.

Materials and Methods

Animals

Male C57BL/6 mice (Charles River, Sulzfeld, Germany) weighing 19-22 g (experimental mice) or 30-35 g (dominant mice) were individually housed in standard polycarbonate mouse cages (16 x 22 x 14 cm) for at least one week before the experimental procedure started. All mice were kept under standard laboratory conditions (12-h light/dark cycle, lights on at 0600 h, 22 °C, 60% humidity) and had free access to tap water and standard mouse diet. All experimental protocols were approved by the Committee on Animal Health and Care of the local government, and performed according to international guidelines on the ethical use of animals. All efforts were made to minimize the number of animals used and their suffering.

Chronic subordinate colony housing (CSC; Fig. 19)

One week after arrival, experimental mice were randomly assigned to the control or the CSC group. Controls were singly housed and remained undisturbed in their home cages except for change of bedding once a week. As described in chapter 3, four experimental mice were housed together with a larger dominant male in a polycarbonate observation cage (38 x 22 x 35 cm) for 19 consecutive days [302]. Before

starting the chronic stress procedure all male mice selected for becoming the dominants during the CSC paradigm were tested for their aggressive behaviour. Males that started to injure their opponents by harmful bites were not used. To avoid habituation, each dominant male was replaced by a novel dominant male on days 8 and 15. During the first 30 min of formation of the colonies on day 1, 8, and 15, the mice were videotaped for behavioural analyses. In all colonies, the larger male mouse established a "dominant" status while it was chasing and attacking all four experimental mice. The four experimental mice were considered as 'subordinates' based on their defensive behaviour, including flight, retreat and submissive upright as described before [302] and according to a recent report in rats [137].

Experimental procedures

Experiment 1: In order to investigate whether chronic psycho-social stress influences the severity of a subsequent DSS colitis and whether adrenal insufficiency determined after 19 days of CSC exposure [302] persists during DSS treatment, mice were randomly assigned to the control and the CSC group. At 1800 h on day 19 of CSC exposure all mice were singly housed. On the next day (day 20), CSC and unstressed control mice were tested on the EPM to confirm the effects of chronic psycho-social stress on anxiety-related behaviour [302]. Mice were then treated with 1% DSS in their drinking water from day 20 to 27, and were sacrificed on either day 21, 23 or 27, i.e. on days 2, 4, or 8 of DSS treatment, for quantification of colon length, histological assessment of the colon (histological score, see below), and plasma corticosterone concentrations. Additionally, cytokine secretion by mesenteric lymph node cells was assessed (see below) [279, 285, 302]. On day 27, additional groups of CSC and control mice, which were not treated with DSS (no DSS) were sacrificed

for assessment of direct effects of CSC exposure on colonic inflammatory parameters.

Experiment 2: In order to investigate the role of GCs in mediating the effects of CSC-exposure on the severity of a DSS-induced colitis, mice underwent either ADX or SHAM one week before the CSC procedure started. Respective unstressed controls of the ADX and SHAM group remained single-housed. At 1800 h on day 19, CSC mice were singly housed and from the next day an all mice were treated with 1% DSS from day 20 to 27. On day 8 of DSS treatment, mice were sacrificed for assessment of body weight, colon length, histological abnormalities of the colon (histological score, see below), and plasma corticosterone concentrations.

Elevated plus-maze test

To assess the effect of CSC on anxiety-related behaviour, both controls and CSC mice were transported to the plus-maze test room in the evening of day 19 of CSC exposure. The next day, they were tested for 5 min between 0800 h and 1100 h [324, 325]. The EPM adapted for mice 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. The open arm edges were 0.3 cm in height to avoid falling. Each mouse was placed on the central platform facing a closed arm. 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 onto open arms of the maze. The maze was cleaned thoroughly before each test.

Induction of acute colitis

An acute colitis was induced by administering 1% DSS (36-50 kDa; ICN Biomedicals, cat.no. 160110, Eschwege, Germany) in the drinking fluid (experiment 1: water; experiment 2: 0.9% saline; see below), *ad libitum* from day 20 to 27, as previously described [285].

Blood sampling and radioimmunoassay for corticosterone

To determine the effect of CSC on plasma corticosterone concentrations, mice were rapidly killed by decapitation under CO_2 anaesthesia within 3 min after entering the animal room. Trunk blood was collected in EDTA-coated tubes on ice (Sarstedt Nümbrecht, Germany) containing 10 µl aprotinin (Trasylol, Bayer Corp. AG, Leverkusen, Germany) and centrifuged at 4 °C (5000 rpm, 10 min). Plasma samples were stored at -20 °C until assayed using a commercially available radioimmunoassay for corticosterone (MP Biomedicals GmbH, Eschwege, Germany; detection limit: 10 ng/ml).

Determination of colonic length and histological score

The reduction of colonic length was used as a parameter to assess colonic inflammation [287, 288]. The colon was removed, mechanically cleaned, and measured to 0.1 cm precision. 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. Three 3-µm haematoxylin-eosin stained sections taken 100-µm apart were evaluated by histological scoring performed by an investigator blinded to treatment. For statistics, each individual score represented the mean of the three sections. Histology was scored as follows based on [289, 290, 302]:

Epithelium. 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

Infiltration. 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.

The total histological score represents the sum of the epithelium and infiltration score and ranges from 0 to 8.

Isolation and incubation of mesenteric lymph node cells

Mesenteric lymph nodes (pooled from each experimental group) 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)]. 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 200 µl of 2.5 µg/ml anti-CD3 antibody in the presence of IL-2 (final concentration 100 U/ml). Eight wells were transferred with the respective number of cells of each experimental group. After incubation for 24 h (37 °C, 5% CO_2), cytokine concentrations were measured in the supernatants by ELISA (all from Endogen, Woburn, MA) using four wells per experimental group.

Surgical procedure/ADX

ADX was performed under isoflurane anaesthesia. A 2-cm skin incision was performed on the back of the mice at the level of the kidneys (midline), and adrenals were removed bilaterally via two peritoneal incisions performed on the left and right side of the abdomen of the mouse. SHAM mice underwent the same procedure except removal of the adrenals. Following surgery, ADX and SHAM mice received 0.9% saline in drinking water (until they were sacrificed) and were housed single for one week until the CSC procedure started. Saline helped ADX mice to compensate for loss of mineralocorticoids.

Statistics

For statistical comparisons, the software package SPSS (version 12) was used. Data of two experimental groups were compared by using the Mann-Whitney *U*-test. All other comparisons were done using a two-way ANOVA (experiment 1: factor CSC, factor DSS; experiment 2: factor ADX, factor CSC) and following *post hoc* Tukey-HSD test. Correlation analysis of plasma corticosterone with body weight loss during DSS treatment was carried out using simple regression analysis. Data are presented as means + S.E.M. Significance was taken at $p < 0.05$.

Results

Effects of CSC on body weight and anxiety-related behaviour (experiment 1)

CSC mice gained significantly less body weight during the 19 days of CSC exposure compared with controls (Tab. 4). In addition, exposure to CSC significantly increased anxiety-related behaviour as reflected by a decrease in the percentage of time spent on the open arms, and in the percentage of open arm entries compared with control mice (Tab. 4). The number of entries into the closed arms, indicative of locomotor activity, was not altered by CSC exposure (Tab. 4).

Tab. 4. Effects of a chronic psycho-social stressor (chronic subordinate colony housing; CSC) on body weight gian and anxiety-related behaviour on the EPM. Increased anxiety of CSC mice is reflected by a reduced percentage of time spent on the open arms (% time) and reduced percentage of entries onto the open arms (% entries). The number of entries into closed arms (entries CA) reflects the motor activity and was not affected by CSC exposure.

		controls	**CSC**
body weight gain [g]		2.4 ± 0.1 (n = 37)	1.7 ± 0.2 ** (n = 39)
elevated plus-maze (EPM)	% time	43.2 ± 4.5 (n = 37)	11.9 ± 1.9 *** (n = 39)
	% entries	45.7 ± 3.2 (n = 37)	22.0 ± 1.9 *** (n = 39)
	entries CA	10.7 ± 0.9 (n = 37)	10.4 ± 0.7 (n = 39)

Mice were exposed to the CSC stress procedure for 19 days, or were singly housed (control) and were tested on the plus-maze on day 20. Numbers in parentheses indicate group size. Data represent means ± S.E.M.; ** < 0.01; *** $p < 0.001$ vs. unstressed controls.

Effects of CSC on the severity of a DSS-induced colitis and on plasma corticosterone concentrations (experiment 1)

Prior exposure to CSC significantly increased the severity of an acute DSS-induced colitis, as indicated by an elevated body weight loss, shorter colon length, higher histological damage score of the colon, and increased secretion of pro- and antiinflammatory cytokines by draining mesenteric lymph node cells compared with non-stressed controls.

Body weight (Fig. 28). The body weight gain of CSC mice did not differ from non-stressed mice between days 1 and 2, and between days 1 and 4 of DSS treatment, but between days 1 and 8 of DSS treatment, the body weight gain was found to depend on prior CSC exposure and DSS treatment (factor CSC x DSS: $F_{1, 33} = 21.5$; $p < 0.001$).

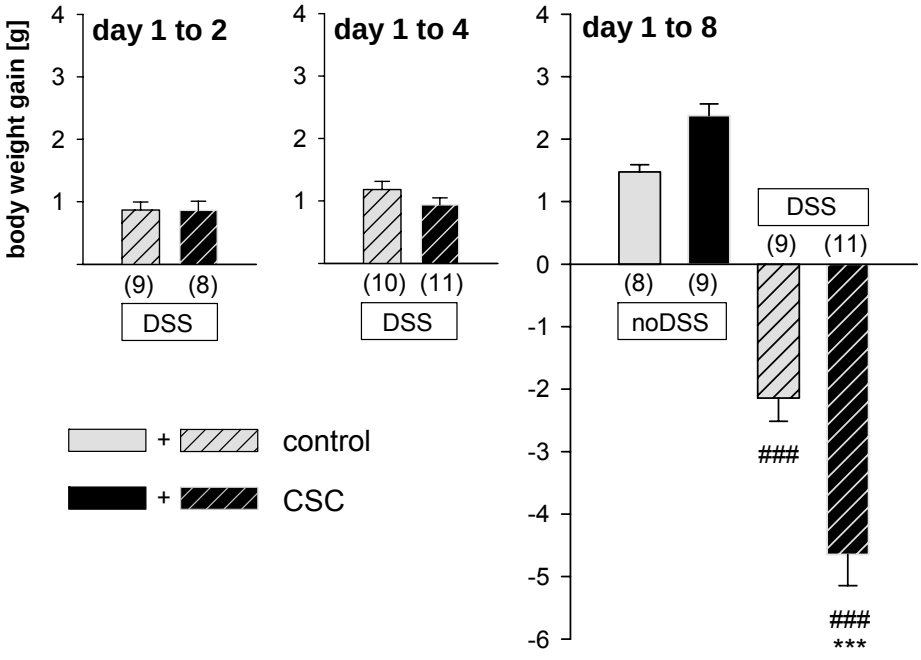


Fig. 28: Effects of prior exposure to chronic subordinate colony housing (CSC) on body weight gain from day 1 to 2 [control/DSS: n = 9; CSC/DSS: n = 8], day 1 to 4 [control/DSS: n = 10; CSC/DSS: n = 11], and day 1 to 8 [control/noDSS: n = 8; CSC/noDSS: n = 9; control/DSS: n = 9; CSC/DSS:

n = 11] of DSS treatment. CSC exposure prior to DSS treatment had no effect on body weight gain from day 1 to 2 and day 1 to 4 of DSS application. Enhanced body weight loss in CSC mice was found from day 1 to 8 of DSS treatment. Numbers in parenthesis indicate group size. Data represent mean ± S.E.M.; *** $p < 0.001$ vs. respective controls; ### $p < 0.001$ vs. respective group without DSS.

DSS treatment resulted in a decrease in body weight in both CSC and control mice, but the effect was more pronounced in CSC mice compared with unstressed controls.

Colon length (Fig. 29A). The colon length of CSC and control mice was not statistically different on day 2 or 4 of DSS treatment, but on day 8 of DSS treatment, a significant interaction between CSC and DSS treatment was found (factor stress x DSS: $F_{1, 33} = 7.51$; $p = 0.01$). Specifically, colon length was significantly reduced by DSS application, in both control and CSC mice, compared with respective mice treated with tap water. However, the effect of DSS on colon length reduction was more severe in CSC compared with control mice.

Histological score (Fig. 29B). A trend towards an increased histological score was detected in CSC mice compared with controls already on day 2 ($p = 0.064$) of DSS treatment. On day 8 of DSS treatment the histological score was dependent on prior CSC exposure and DSS treatment (factor CSC x DSS: $F_{1, 32} = 14.2$; $p = 0.001$). The low histological score of CSC and control mice receiving no DSS indicated no colonic inflammation. In contrast, DSS treatment increased the histological score in both CSC and control mice. Importantly, CSC mice receiving DSS showed a significantly increased histological score compared with respective controls, reflecting a more severe inflammatory infiltration and increased epithelial damage of the colon.

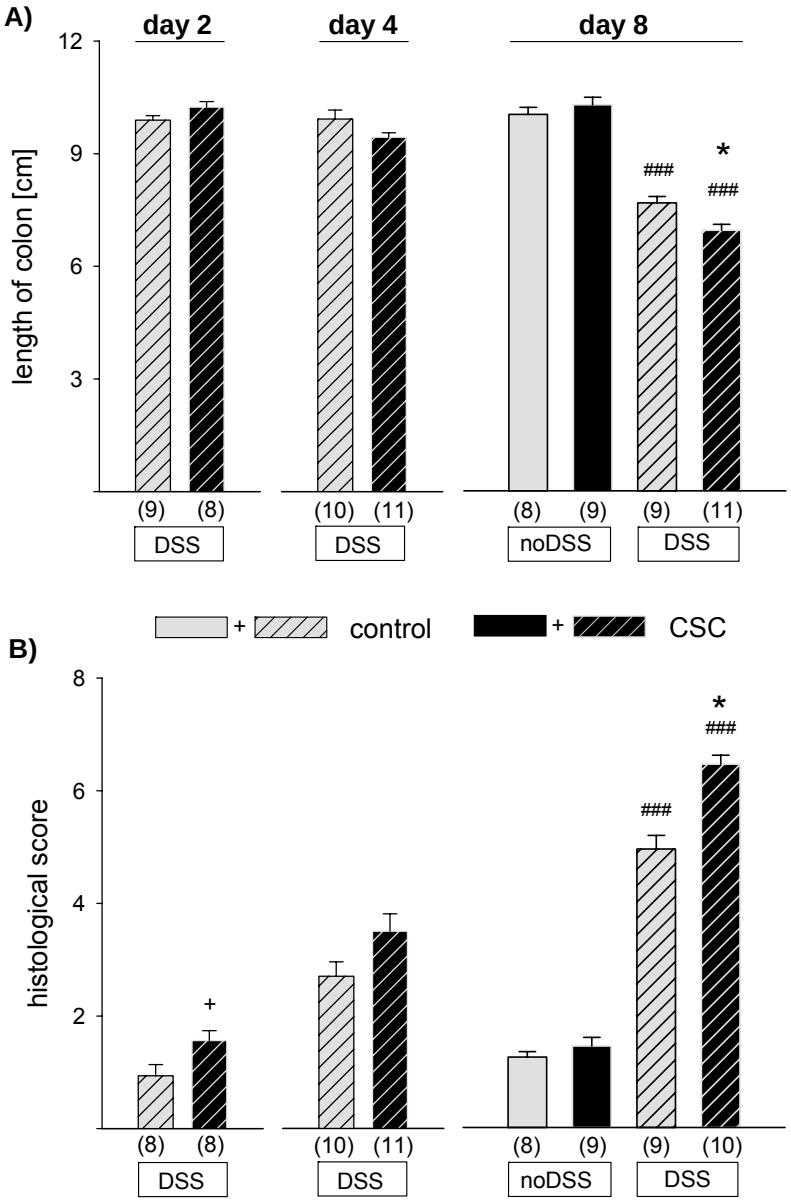


Fig. 29: Effects of prior exposure to chronic subordinate colony housing (CSC) on the length (A) and the histological damage score (B) of the colon estimated on day 2, day 4, and day 8 of DSS treatment. CSC exposure prior to DSS treatment further reduced colon length on day 8 of DSS treatment. The DSS-induced increase in the histological damage score was more pronounced in CSC mice on day and 8 of DSS treatment. Numbers in parenthesis indicate group size. Data represent mean ± S.E.M.; + $p = 0.064$; * $p < 0.05$ vs. respective controls; ### $p < 0.001$ vs. respective group without DSS.

Secretion of cytokines from draining mesenteric lymph node cells (Fig. 30).

In DSS-treated mice, the secretion of the proinflammatory cytokines TNF-α, IFN-γ, IL-6, and the antiinflammatory cytokine IL-10 from mesenteric lymph node cells

was significantly higher in CSC compared with control mice both on days 2 and 4 of DSS treatment.

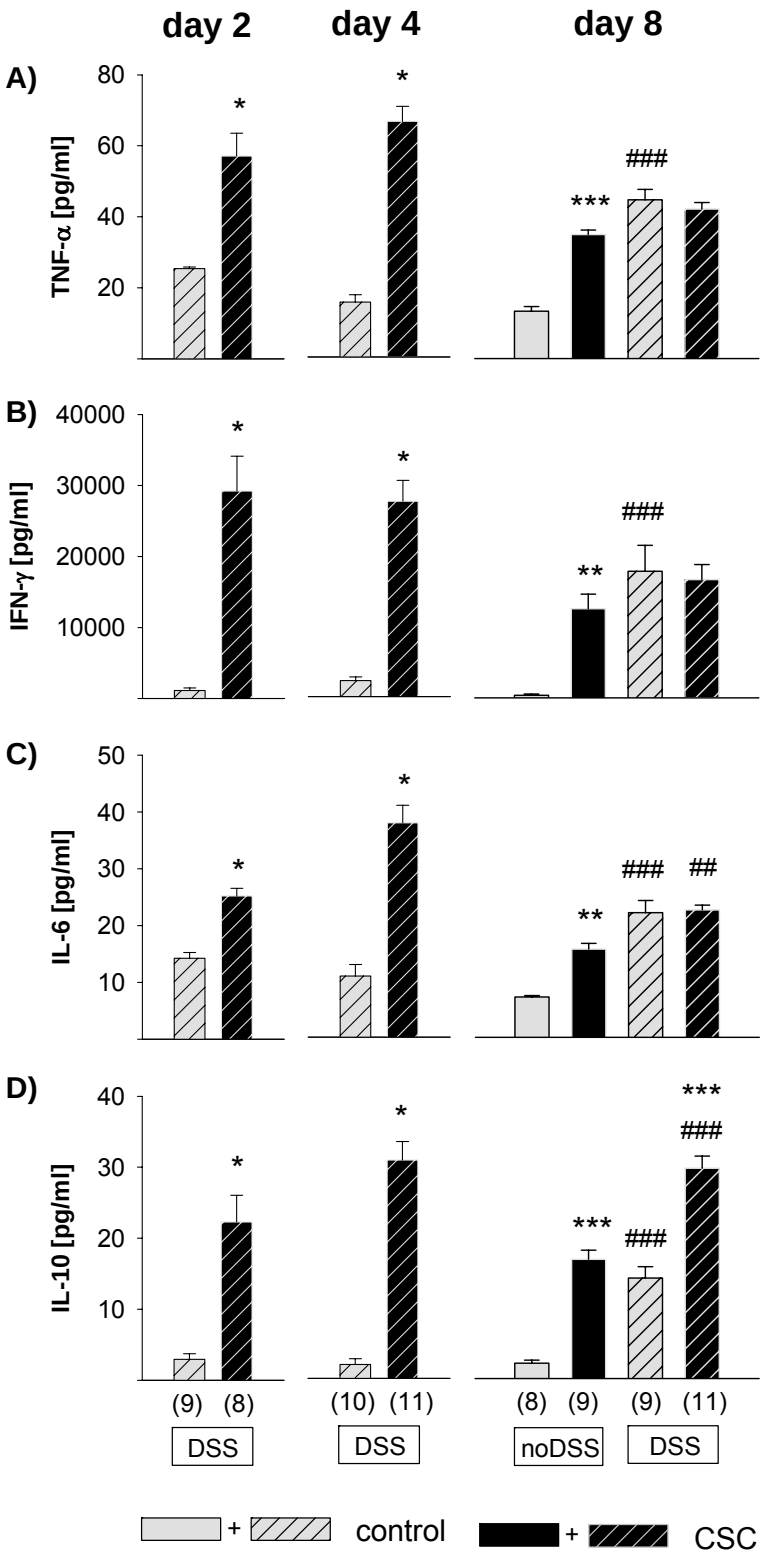


Fig. 30: Effects of prior exposure to chronic subordinate colony housing (CSC) on the secretion of proinflammatory [TNF-α (A), IFN-γ (B), IL-6 (C)] and antiinflammatory [IL-10 (D)] cytokines by

mesenteric lymph node cells on day 2, day 4, and day 8 of DSS treatment. CSC exposure prior to DSS treatment increased secretion of all cytokines on day 2 and 4 of DSS treatment. On day 8 after CSC exposure cytokines secretion was also increased in CSC mice treated with tap water only. Numbers in parenthesis indicate group size. Data represent mean ± S.E.M.; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. respective controls; ## $p < 0.01$; ### $p < 0.001$ vs. respective group without DSS.

On day 8 of DSS treatment, an interaction between CSC and DSS treatment was found for all proinflammatory cytokines (TNF-α: $F_{1,\ 12} = 38.7$; $p = 0.001$; IFN-γ: $F_{1,\ 12} = 8.03$; $p = 0.015$; IL-6: $F_{1,\ 12} = 10.1$; $p = 0.008$). In detail, DSS treatment from day 20 to 27 resulted in an increased secretion of TNF-α, IFN-γ, and IL-6 in controls compared with respective tap water-treated controls. DSS treatment also resulted in an increased secretion of IL-6 within the CSC group. Moreover, in CSC mice not treated with DSS after stressor exposure an increased secretion of proinflammatory cytokines was found compared with respective controls on day 27.

Finally, on day 8 of DSS treatment, a CSC effect ($F_{1,\ 12} = 123.0$; $p < 0.001$) and a DSS treatment effect ($F_{1,\ 12} = 83.8$; $p < 0.001$) were found for the antiinflammatory cytokine IL-10. In detail, IL-10 secretion was increased by prior CSC exposure in both the DSS and the tap water group compared with respective unstressed controls. In addition, DSS treatment resulted in an increased secretion of IL-10 in CSC and control mice.

Plasma corticosterone concentrations (Fig. 31).

Plasma corticosterone concentrations were similar between CSC and control mice on day 2 and 4 of DSS treatment. On day 8, corticosterone concentrations were dependent on prior CSC exposure and DSS treatment (factor CSC x DSS: $F_{1,\ 31} = 4.67$; $p < 0.038$). Plasma corticosterone was similar in CSC and control mice, which did not receive DSS, 8 days after termination of the chronic stressor (day 27).

In contrast, DSS treatment significantly increased plasma corticosterone concentrations in CSC and control mice, with the effect being more pronounced in CSC mice.

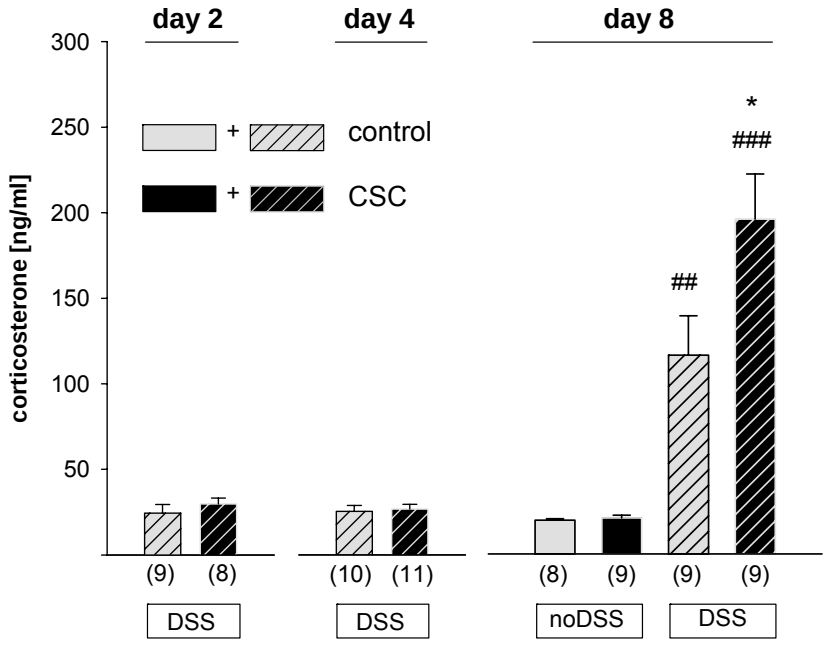


Fig. 31: Effects of prior exposure to chronic subordinate colony housing (CSC) on plasma corticosterone concentrations on day 2, day 4, and day 8 of DSS treatment. Corticosterone concentrations of CSC and control mice were increased on day 8 of DSS treatment, with the effect being more pronounced in CSC mice. Numbers in parenthesis indicate group size. Data represent mean ± S.E.M.; * $p < 0.05$ vs. respective controls; ## $p < 0.01$; ### $p < 0.001$ vs. respective group without DSS.

Effects of ADX on CSC-induced exacerbation of DSS colitis (experiment 2)

ADX, performed one week before CSC exposure, significantly reduced the CSC-induced exacerbation of an acute DSS colitis, as indicated by reduced body weight loss, less reduction in colon length, and lower histological damage score of the colon on day 8 of DSS treatment in CSC mice of the ADX compared with respective mice of the SHAM group.

Body weight gain (Fig. 32A).

The body weight gain during DSS treatment (from day 20 to 27) was dependent on ADX and CSC exposure (factor ADX x CSC: $F_{1, 25} = 7.23$; $p = 0.013$). DSS treatment resulted in a decrease in body weight gain in CSC mice of both the ADX and SHAM group. However, this effect was less pronounced in ADX compared with SHAM mice.

Colon length (Fig. 32B). A significant ADX ($F_{1, 25} = 7.40$; $p = 0.012$) and CSC ($F_{1, 25} = 54.2$; $p < 0.001$) effect was found for the length of the colon on day 8 of DSS treatment. More specifically, colon length was significantly reduced in CSC mice of both the ADX and SHAM group compared with respective control mice. However, the effect of DSS was less severe in the ADX group compared with the SHAM group.

Histological score (Fig. 32C). The histological damage score of colonic tissue was found to depend on ADX ($F_{1, 25} = 8.57$; $p = 0.007$) and CSC exposure ($F_{1, 25} = 61.6$; $p < 0.001$). Exposure to CSC increased the histological score compared with controls in both the ADX and SHAM group on day 8 of DSS treatment. However, there was a trend towards a less severe CSC-induced increase in the histological score in ADX compared to SHAM mice ($p = 0.087$). This effect reached statistical significance when performing a Mann-Whitney *U*-comparison between the histological score of these two groups ($p = 0.014$).

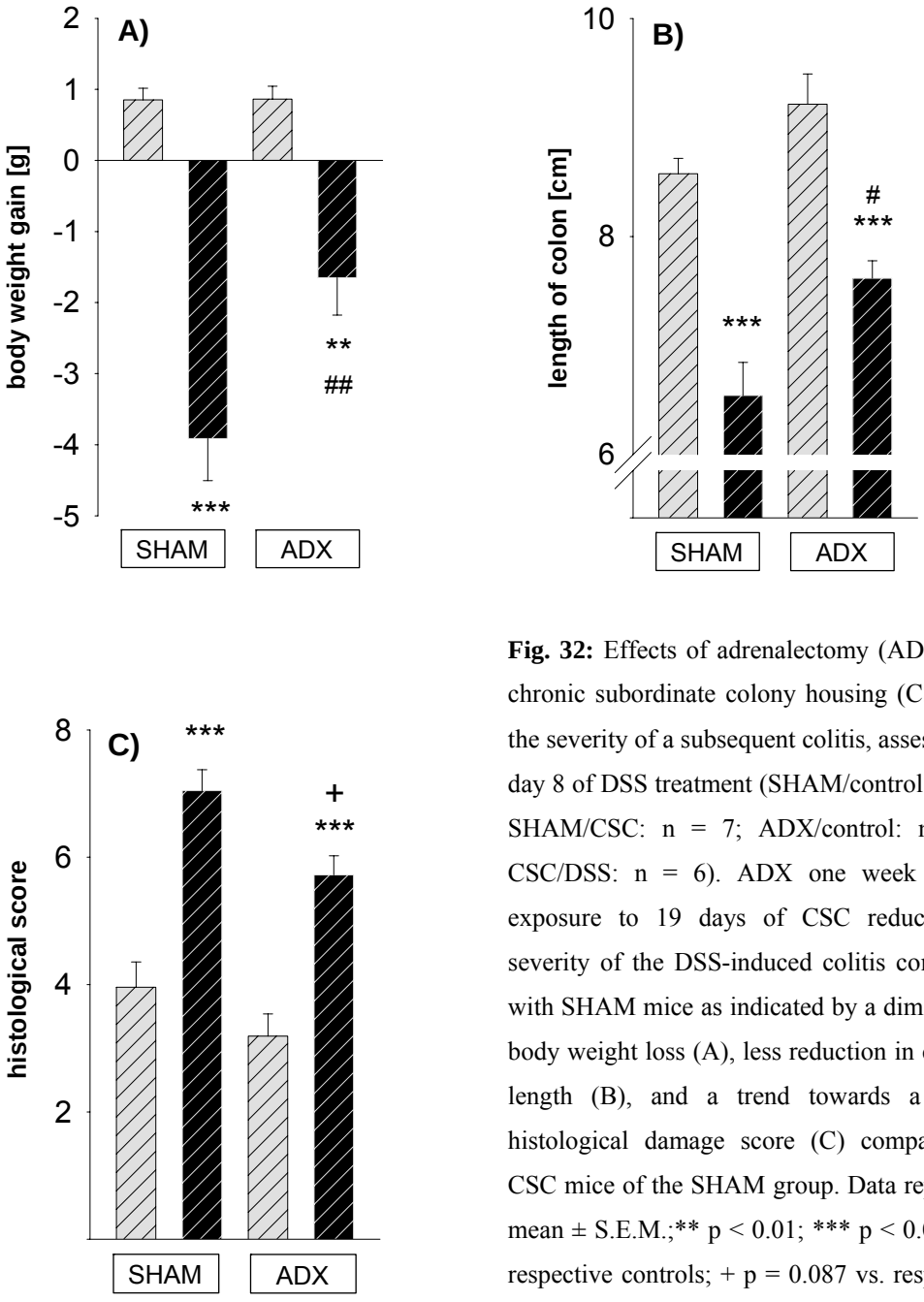


Fig. 32: Effects of adrenalectomy (ADX) and chronic subordinate colony housing (CSC) on the severity of a subsequent colitis, assessed on day 8 of DSS treatment (SHAM/control: n = 8; SHAM/CSC: n = 7; ADX/control: n = 8; CSC/DSS: n = 6). ADX one week before exposure to 19 days of CSC reduced the severity of the DSS-induced colitis compared with SHAM mice as indicated by a diminished body weight loss (A), less reduction in colonic length (B), and a trend towards a lower histological damage score (C) compared to CSC mice of the SHAM group. Data represent mean ± S.E.M.;** $p < 0.01$; *** $p < 0.001$ vs. respective controls; + $p = 0.087$ vs. respective SHAM group.

Plasma corticosterone (Fig. 33A). Plasma levels of corticosterone were found to depend on prior ADX and CSC exposure (factor ADX x CSC: $F_{1, 24} = 28.9$; $p < 0.001$). Plasma corticosterone was significantly increased in CSC compared to control mice in the SHAM group on day 8 of DSS treatment. Furthermore, CSC exposed ADX mice showed significantly decreased corticosterone levels compared

to respective SHAM mice and were not different compared with control-ADX mice. When performing a Mann-Whitney *U*-comparison between plasma corticosterone levels of the CSC and control mice of the ADX group, a significant increase in CSC compared to control mice was found ($p = 0.007$).

In addition, a positive correlation was found between the plasma levels of corticosterone and the body weight loss during DSS treatment ($r^2 = 0.742$, $p = 0.013$; Fig. 33B).

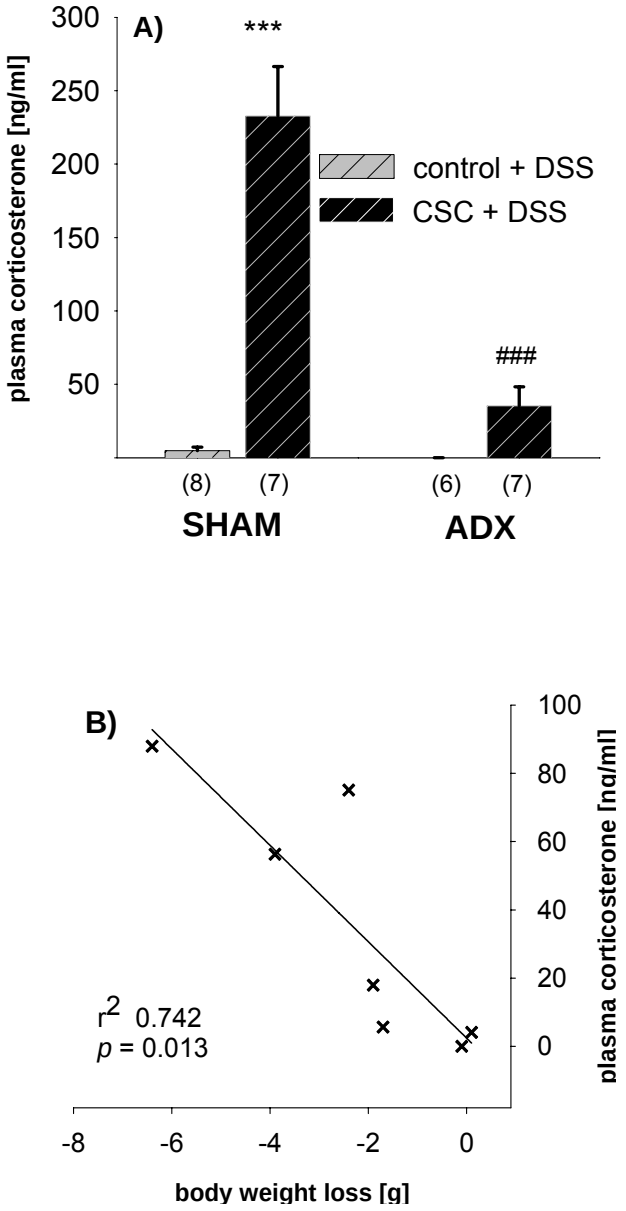


Fig. 33: A) Effects of ADX one week before CSC exposure on plasma corticosterone levels on day 8 of subsequent DSS treatment. Plasma corticosterone was significantly increased in CSC compared with control mice in the SHAM group. CSC-exposed ADX mice showed significantly decreased corticosterone levels compared with respective SHAM mice and were not different from control-ADX mice. **B)** Simple regression analysis revealed a positive correlation between plasma corticosterone and body weigth loss during DSS treatment. Data represent means ± S.E.M.; *** $p < 0.001$ vs. respective controls; ### $p < 0.001$ vs. respective SHAM group.

Discussion

The experiments described in this chapter were designed to investigate the effects of 19-day exposure to chronic psycho-social stress on the course and severity of a subsequent DSS-induced colitis and the underlying mechanisms involved. The results indicate that exposure to CSC fastens the onset and increases the severity of a subsequent DSS-induced colitis. Furthermore, adrenal insufficiency seen after CSC exposure is present at least until day 4 of DSS treatment. Finally, ADX prior to CSC exposure attenuates the CSC-induced increase in the severity of a DSS-induced colitis, indicating that the initial increase in plasma corticosterone during CSC exposure significantly contributes to the CSC-induced increase in the severity of a colitis subsequently induced by DSS.

After exposure to 19 days of CSC, mice showed a reduced body weight gain and an increased anxiety-related behaviour as reflected by a decreased time spent on the open arms and a decreased number of entries onto the open arms of the EPM compared with non-stressed controls. These findings are in agreement with the effects of CSC stress on body weight gain and anxiety in male mice described in chapter 3 [302]. Therefore, the present data indicate that the effects of CSC on established indicators of chronic stress [66, 81, 292, 293, 313] are robust and reproducible, underlining the validity of this chronic psycho-social stress paradigm.

After termination of the 19-day exposure to CSC, mice were treated with DSS to study the effects of prior exposure to CSC on the severity of a chemically-induced colitis, and to what extent adrenal mechanisms are involved. Within two days of DSS treatment, CSC mice showed an increased secretion of the proinflammatory Th1

cytokines IFN-γ, TNF-α, and IL-6 and of the antiinflammatory Th2 cytokine IL-10 by mesenteric lymph node cells. In contrast, in controls a comparable rise in the secretion of these cytokines was only found on day 8 of DSS treatment. Additionally, there was a trend towards an increased histological damage score in CSC mice already on day 2 of DSS treatment. These emerging signs of an acute colitis in CSC mice early on day 2 of DSS treatment suggest that exposure to CSC itself rather than DSS treatment induced spontaneous colonic inflammation. Indeed, in chapter 3 an increased secretion of IFN-γ, TNF-α, and IL-10 by mesenteric lymph node cells and an increased histological score after prolonged exposure to CSC was demonstrated [302]. Moreover, even 8 days after termination of CSC exposure, CSC mice of the present study not treated with DSS showed an increased cytokine secretion.

In general, an increased inflammatory state in colonic tissue results in peripherally generated inflammatory mediators and cytokines, and is supposed to rapidly activate the HPA axis. Activation of the HPA axis can occur at various levels, including the hypothalamic CRH neurons, the pituitary corticotrophs, and the adrenal cortex [108, 354]. Secreted glucocorticoids are important inhibitors of inflammatory processes, due to their ability to block the production and action of several lymphokines, such as IL-2 and IFN-γ [108, 298-300]. Indeed, in control mice the increased cytokine secretion by mesenteric lymph node cells on day 8 of DSS treatment was paralleled by high plasma corticosterone concentrations. In contrast, the increased inflammatory state in CSC mice after 2 days of DSS treatment was not paralleled by increased levels of plasma corticosterone. This finding suggests that the HPA axis reactivity is blunted as a result of CSC during the first days of a DSS-induced colitis. Indeed, 19-day exposure to CSC resulted in an insufficiency of the adrenals to adequately respond to ACTH *in vivo* as well as *in vitro* [302].

However, on day 8 of DSS treatment (i.e. after 8 days of termination of CSC exposure) CSC mice showed high levels of plasma corticosterone, suggesting that the adrenals of CSC mice may have recovered from CSC-induced insufficiency. This increase in immunosuppressive plasma corticosterone concentrations in CSC mice was paralleled by a down-regulated secretion of the proinflammatory Th1 cytokines TNF-α, IFN-γ, and IL-6, resulting in cytokine secretion not different from those of the respective non-stressed control mice. As the intense secretion of the antiinflammatory Th2-cytokine IL-10 is still maintained on day 8 of DSS treatment, an imbalance of the Th2/Th1 cytokine profile is likely to result in the generally known glucocorticoid-induced bias towards an antiinflammatory Th2 milieu [355-357]. Thus, these findings suggest a delayed, but efficiently working down-regulation of the inflammation by the secretion of antiinflammatory glucocorticoids on day 8 of DSS treatment.

However, on day 8 of DSS treatment, CSC mice showed a more pronounced body weight loss, an increased inflammatory reduction of colon length, and an increased histological damage score of the colon compared with controls. Therefore, the signs of subtle regulatory processes concerning the cytokine secretion could not be confirmed by macroscopic parameters analyzed on day 8 of DSS treatment, suggesting that these macroscopic changes may take longer to establish [272].

Taken together, the results from mice chronically exposed to SD/OC and subsequently treated with DSS (see Chapter 2) [279] were confirmed by the CSC-induced changes in macroscopic parameters on day 8 of DSS treatment. In addition, the information about the time course of the DSS-induced colitis provided in the present study further extend the knowledge of the effects of chronic psycho-social stress on the pathogenesis of a subsequent DSS colitis and its underlying mechanisms.

The finding of increased cytokine secretion of mesenteric lymph node cells in CSC mice already on day 2 of DSS treatment and in CSC mice not treated with DSS on day 8 after termination of CSC strongly suggest the occurence of an event during CSC exposure, triggering spontaneous inflammation. In support, spontaneous colonic inflammation was indeed seen after 19 days of CSC exposure (see Chapter 3) [302]. Interestingly, ADX performed one week prior to chronic stress ameliorated CSC-induced increase in spontaneous colonic inflammation (see Chapter 3) [302], suggesting an important regulatory role of the initial CSC-induced rise in plasma corticosterone during CSC exposure in the onset of colonic inflammation [302]. Increased levels of glucocorticoids were described to disrupt colonic barrier functions and, thus, to trigger the penetration of luminal antigens into the colonic tissue. In support, a decreased proinflammatory cytokine secretion by mesenteric lymph node cells and a lower histological score were found after 19 days of CSC exposure in ADX compared with SHAM mice (see Chapter 3) [302]. These findings are in agreement with the results described in the present chapter showing that ADX also ameliorated the CSC-induced increase in the severity of a DSS-induced colitis. This was reflected by a minor body weight loss, a diminished inflammatory reduction of colon length, and a lower histological score in stressed ADX mice compared with stressed SHAM mice on day 8 of DSS treatment.

In this context, the acute stress response during the first days of CSC exposure, has to be considered in more detail [302]. The initial rise in plasma corticosterone seen until day 2 of CSC exposure [302] may have resulted in the activation of the immune system [77, 353], and furthermore, reduced the epithelial barrier function in the colon [310, 312]. Therefore, the coincidence of an enhanced uptake and presentation of luminal antigens [349] to an activated intestinal immune system, at least during the initial acute phase of CSC, may be the key factors triggering the induction of

spontaneous colonic inflammation [302]. These inflammatory processes may be reinforced by the lack of immunosuppressive corticosterone due to CSC-induced adrenal insufficiency [302] after prolonged stressor exposure. Thus, it is likely that ADX and lack of the initial rise in corticosterone, attenuated the spontaneous inflammatory processes triggered by exposure to CSC and made the colonic tissue less vulnerable to subsequent DSS treatment. However, ADX also prevented the antiinflammatory actions of increased levels of glucocorticoids, as they were found in CSC mice on day 8 of subsequent DSS treatment, which could have limited the "beneficial effect" of ADX in the present study.

Significantly decreased plasma corticosterone concentrations in CSC-exposed ADX mice compared with respective SHAM mice suggested that the ADX procedure was mostly successful. Furthermore, these data confirm the above described recovery from CSC-induced adrenal insufficiency after 8 days of subsequent DSS treatment. However, plasma corticosterone levels of CSC mice were slightly increased compared with control mice within the ADX group. This effect became even significant when performing a Mann-Whitney *U*-comparison only between these two groups. This might indicate that in some of these mice the adrenals have been either not completely removed during ADX or that adrenal tissue has regenerated afterwards. Interestingly, there is a study by Hummel performed in 1958 that describes the presence of accessory adrenal cortical nodules in the mouse which are, in addition to the adrenal glands, able to produce less corticosterone [358]. In a situation of strong environmental challenge, as CSC exposure and DSS treatment are supposed to be, and in the absence of GC-secreting adrenals this might also be a possible source of slightly increased plasma corticosterone levels. However, a significant correlation between plasma corticosterone levels of CSC-exposed ADX mice and their body weight loss during DSS treatment indicated that mice which

were able to show an at least slight initial increase in corticosterone during CSC exposure showed an increased severity of subsequent colitis induced by DSS (Fig. 33B).

In summary, prior CSC exposure increased the severity of a subsequent DSS-induced colitis which was likely mediated by CSC-induced changes in HPA axis function. This is based on the findings that ADX prior to CSC attenuated the increased severity of a subsequent DSS colitis. Therefore, the initial rise in plasma corticosterone levels determined on day 2 of CSC [302] seems to be a key factor for the induction of spontaneous colonic inflammation and increased vulnerability of colonic tissue to subsequent DSS treatment. In addition, on day 2 and 4 of DSS treatment there was a lack of plasma corticosterone secretion in CSC compared with control mice due to CSC-induced adrenal insufficiency [279, 302]. The lack of the rise of this antiinflammatory factor, may further contribute to the increased severity of DSS-induced colitis after chronic psycho-social stress. The CSC paradigm used in the present study has been proved to be a valuable model for studying further effects of chronic psycho-social stress on distinct immunological parameters.

Chapter 5

General Discussion

Summary of results

The experiments of the present thesis were designed to investigate the effects of two different models of chronic psycho-social stress (SD/OC; CSC) on the severity of and the regeneration after an experimentally induced colitis and the development of spontaneous colonic inflammation in male mice. In addition, underlying mechanisms were investigated, thereby mainly focusing on the HPA axis as a possible mediator of chronic psycho-social stress effects on colonic inflammation. Further knowledge about brain-gut interactions during chronic psycho-social stress might be helpful to understand the pathogenesis of IBD and other chronic inflammatory diseases in more detail, and therefore might provide new targets for intervention of disease outcome

To investigate whether both the SD/OC and CSC paradigm represent adequate models for chronic psycho-social stress in male mice, their effects on different physiological, neuroendocrine, and behavioural parameters were characterized (Chapters 2, 3, 4). Both SD/OC and CSC mice showed typical signs of chronic stress, including reduced body weight gain, adrenal hypertrophy, and thymus atrophy. Interestingly, an increased level of anxiety, which is another indicator of acute and chronic stress, was found only after 19 days of CSC exposure. In addition, chronically stressed mice of both paradigms showed unchanged plasma corticosteone concentrations during the light phase, a diminished reactivity of their adrenal cells after either *in vivo* or *in vitro* stimulation, unchanged plasma ACTH and hypothalamic CRH mRNA levels after 19 days of stressor exposure compared to control mice. These findings clearly indicate that the effects of both the SD/OC and CSC paradigm are robust and reproducible, underlining the validity of these chronic psycho-social stress paradigms.

The lack of evidence describing the effects of chronic psycho-social stressors on the pathogenesis of IBD in rodents (Chapter 1, Sec. 4) first prompted the development and validation of the SD/OC paradigm in the present dissertation. Although the SD/OC paradigm induced typical signs of chronic stress and, thus, represented an adequate model for the investigation of the aims of this thesis as described in section 5 of chapter 1, a second model was developed in order to confirm the ability of chronic stress in rodents to influence IBD. Another reason for the establishment of the CSC paradigm was to assess the effects of a continuous chronic psycho-social stressor in comparison to the more intermittent stressor employed in the SD/OC paradigm. Furthermore, since the CSC model relies on less factors and avoids daily handling of the mice for the development of the chronic stress effects it provides greater inter- and intra-experimental reliability than the SD/OC. Therefore, the CSC paradigm is more likely to be successfully employed by other laboratories interested in chronic psycho-social stress than the SD/OC, which would require more validation.

As both the SD/OC and CSC paradigms have been shown to be adequate models for chronic psycho-social stress in male mice, their effects on a subsequent colitis induced by DSS were investigated (Chapters 2 and 4). Exposure to either SD/OC or CSC resulted in an increased severity of DSS colitis, as indicated by a stress-induced increase in body weight loss, inflammatory reduction of colon length, histological score, and cytokine secretion by mesenteric lymph node cells (found only in CSC animals; for detailed explanation see below) after 8 days of DSS treatment. Furthermore, the regeneration after colitis was found to be negatively affected by the exposure to SD/OC.

In chapter 2 it was hypothesized that adrenal insufficiency, found after 19 days of SD/OC exposure, might be a possible mediator of the stress-induced increase in the severity of the DSS colitis. Interestingly, it could indeed be shown by using the CSC paradigm that stress-induced adrenal insufficiency is still present at least until day 4 of subsequent DSS treatment (Chapter 4). This further supports the hypothesis that a lack of antiinflammatory corticosterone during subsequent DSS treatment may be involved in the stress-induced increase in colonic inflammation. Addionally, the findings of increased cytokine secretion by mesenteric lymph node cells in CSC mice on day 2 and 4 of DSS treatment and in CSC mice, not treated with DSS, on day 8 after termination of CSC (Chapter 4) strongly suggest the occurrence of an event during CSC exposure triggering the onset of spontaneous colonic inflammation.

In order to adress this question I used the CSC paradigm and investigated the time course of several physiological, neuroendocrine, and immunological parameters during exposure to chronic psycho-social stress (Chapter 3). It could be shown that CRH mRNA in the hypothalamic paraventricular nucleus, light phase corticosterone and TH expression in colonic tissue were increased, whereas TH expression in the locus coeruleus was decreased on day 2 of CSC; all these parameters returned to baseline levels thereafter. Importantly, in CSC mice significant histological abnormalities in the colonic tissue were found soonest after 14 days of CSC exposure, supporting the above mentioned hypothesis that spontaneous colonic inflammation is caused by the prolonged exposure to chronic psycho-social stress itself. In addition, this first evidence of spontaneous colonic inflammation was further supported by the finding of increased cytokine secretion by mesenteric lymph node cells after exposure to 19 days of CSC (SHAM mice). Concerning the underlying mechanisms, it was hypothesized that the initial rise in corticosterone

during CSC exposure might be involved in the mediation of the proinflammatory effect of chroinc psycho-social stress in colonic tissue.

This question was again adressed by using the CSC paradigm (Chapter 3). Interestingly, ADX one week before the start of CSC exposure was indeed able to attenuate the CSC-induced increase in both the histological score and the cytokine secretion by mesenteric lymph node cells. Additionally, ADX prior to CSC was also able to attenuate the CSC-induced increase in the severity of a subsequent colitis induced by DSS (Chapter 4). These findings suggest an important regulatory role of the intitial CSC-induced rise in plasma corticosterone in the onset of spontaneous colonic inflammation.

In conclusion, the results of the present thesis demonstrate that exposure to chronic psycho-social stress causes the onset of spontaneous colonic inflammation, which is at least partially mediated by the initial increase in corticosterone during stressor exposure. The hence induced inflammatory processes in the colon are reinforced by the lack of immunosuppressive corticosterone after prolonged stressor exposure due to adrenal insufficiency. Therefore, at the onset of DSS treatment chronically stressed mice already show a higher inflammatory state in colonic tissue and consequently might be more vulnerable to the proinflammatory effects of DSS. The lack of immunosuppressive GCs, due to adrenal insufficiency, during the first days of DSS treatment further enhances these initial differences in colonic inflammation between chronically stressed and control mice.

Single-housed controls – an adequate control group for both SD/OC and CSC mice?

As described in chaper 2, SD/OC mice were exposed to either social defeat or overcrowding, but were housed single during the rest of the experiment and therefore, choosing single-housed mice as unstressed controls seems to be adequate.

The situation is different in the CSC paradigm. Here, the experimental mice were group-housed with a large dominant male for 19 consecutive days (for details see Chapters 3 and 4) and the question arised whether single-housed or group-housed (without the large dominant mouse) mice would be adequate controls. However, I decided to compare the CSC mice with single-housed mice, because preliminary data indicated that group-housed mice were already in a state of mild stress and, thus, more comparable to CSC mice than to controls. This was indicated by a reduced body weight gain (Fig. 34A) of both CSC and group-housed compared with single-housed mice.

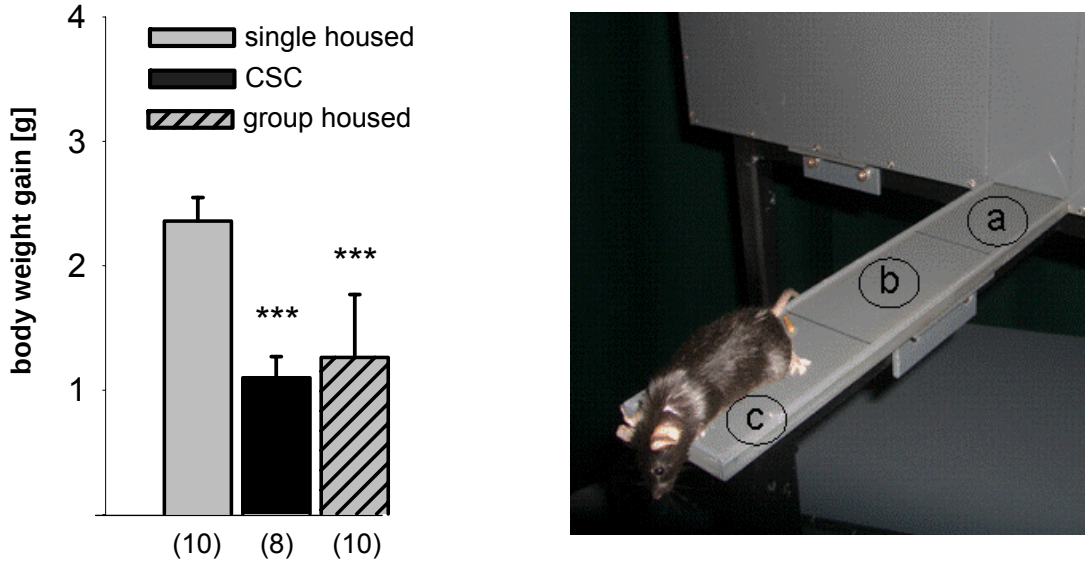


Fig. 34: A) Exposure to 19 days of either chronic subordinate colony housing (CSC) or group housing resulted in a decreased body weight gain compared to single-housed mice [one-way ANOVA (factor housing condition: $F_{2, 40} = 14.22$; $p < 0.001$) followed by *post hoc* Tukey HSD test;]. Numbers in parentheses indicate group size. Data represent means ± S.E.M.; *** < 0.001 vs. single-housed mice. **B)** Anxiety-related behaviour can be measured during exposure of one open arm of the EPM

which is divided in a proximal (a), middle (b), and distal (C) part. The number of total headdips, the time of total headdips, and the number of distal headdips were significantly decreased in CSC and group-housed compared with single-housed mice.

In addition, state anxiety, which was measured during exposure to one open arm of the EPM (Fig. 34B) as previously validated by Salome *et al.* (2006) [359], was found to be increased in both CSC and group-housed compared to single-housed mice [360]. Thus, CSC and group-housed mice displayed less headdips (distal as well as total headdips) [360]. The number of headdips is used as a paramteter related to exploratory behaviour and risk assessment [361] and is lower, e.g. in rats bred for high anxiety-related behaviour (HAB) compared to rats bred for low anxiety related behaviour (LAB).

Interestingly, both CSC and group-housed mice showed reduced neuronal activation (c-fos mapping) in response to open arm exposure in the intermediate lateral septum and in the ventral region of the lateral septum compared with single-housed controls [360]. This effect was more pronounced in CSC compared with group-housed mice [360]. However, it seems likely that the decrease in neuronal activation found in the lateral septum of CSC and group-housed mice after open arm exposure may contribute to their anxious phenotype, as it is known that stress-induced c-fos-like immunoreactivity is reduced in the LS of learned helpless rats [362], stimulation of LS neurons reduces fear, and that anxiety can be induced following the inhibition of LS neurons [363].

Further evidence for single housing representing a non-stress housing condition for male mice provides the finding that the relative adrenal and relative thymus weights as well as the plasma corticosterone concentrations were not affected by prolonged single housing. This is indicated by the finding that these parameters were comparable between mice that were singly housed for 19 days (plus one week of

single housing after arrival of the mice; controls in Chapters 2 and 4) and mice that were singly housed for just one week after arrival (basal mice in Chapter 3). These findings confirmed that it was really not necessary to sacrifice single-housed controls at days 2, 4, 7, 14, and 20 of CSC exposure (see Chapter 3).

Effects of 19-day exposure to chronic psycho-social stress on physiological, neurological, and behavioural parameters

Body weight gain, relative adrenal weight, relative thymus weight (Tab.4)

Exposure to 19 days of either SD/OC or CSC resulted in typical signs of chronic stress, including a reduction in body weight gain [66, 81, 292, 293]. Interestingly, both SD/OC and CSC mice showed an enhanced body weight gain in the time span between day 20 and 27 after termination of the respective chronic psycho-social stress paradigm compared with controls. This probably represented a compensatory effect for their reduced body weight gain during the time of stressor exposure. As an additional indicator of stress, SD/OC as well as CSC mice showed robust thymus atrophy and adrenal hypertrophy [81, 139, 295, 328]. Therefore, the present data demonstrate that both the SD/OC and the CSC paradigm are valid and clinically relevant mouse models for chronic psycho-social stress.

Basal plasma corticosterone concentrations and adrenal insufficiency (Tab.4)

Despite the elevated relative adrenal weights of SD/OC and CSC mice, plasma corticosterone concentrations of both groups were not different from the respective control mice during the light phase. These findings are at the first glance contrary to studies that show highly increased levels of GCs after exposure to chronic stress [138, 295, 349, 364]. However, this discrepancy may be explained by the fact that in these studies blood is collected right after termination of the last stress session. Therefore, these plasma corticosterone concentrations do not represent the basal homeostatic

state of an organism [138, 295, 349, 364] and, consequently, can not be compared to the findings of the present studies. As outlined in the introduction (Chapter 1, Sec. 2.3), it is generally accepted that the acute stress response to a repeated homotypic challenge decreases after several exposures. However, it is not surprising that repeatedly stressed animals still show higher levels of plasma corticosterone right after the last exposure to the homotypic stressor than completely unstressed mice. Interestingly, Zelena *et al.* (2003) found increased basal plasma corticosterone concentrations in repeatedly restrained mice after 7-8 days of stressor exposure [295]. In contrast, light-phase plasma levels of corticosterone in both SD/OC and CSC mice after 19 days of chronic psycho-social stress did not differ from those of unstressed controls. One possible explanation for this phenomenon might be the different duration of stressor exposure. This is further supported by the finding that basal plasma corticosterone levels were found to be increased during the first weeks of chronic stress, but returned to baseline afterwards despite continued stressor exposure [116-118]. Therefore, unchanged basal corticosterone levels are not contradicting the validity of the two chronic psycho-social stress models used in the present experimental series. The downregulation of plasma GCs to baseline levels during chronic stressor exposure, may prevent the organism from prolonged and deleterious exposure to increased levels of corticosterone, and could, therefore, be due to adaptation processes on different levels of the HPA axis (Chapter 1, Sec. 2.3). In detail, these adaptations are not well understood [5], but thought to permit an organism to remain responsive to novel or severe threats on the one hand, while being able to habituate to familiar or milder threats. Surprisingly, at the beginning of the dark phase basal plasma corticosterone concentrations were even lower in SD/OC and CSC animals compared to the respective controls.

Tab. 4: Overview of the effects of 19-day exposure to either SD/OC or CSC on different physiological, neuroendocrine, and behavioural parameters. Exposure to chronic psycho-social stress resulted in either an increase (↑), or decrease (↓), or had no effect (↔) on the distinct readout parameters.

effects of chronic psycho-social stress on:	**SD/OC**	**CSC**
body weight gain	↓	↓
relative adrenal weight	↑	↑
relative thymus weight	↓	↓
plasma corticosterone (light phase)	↔	↔
plasma corticosterone (dark phase)	↓	↓
plasma ACTH (dark phase)	↔	↔
CRH mRNA in the PVN	↔	↔
adrenal responsiveness (in vitro)	↓	↓
anxiety-related behaviour on the EPM	↔	↑

This suggests equal levels of corticosterone during light phase being rather due to insensitive adrenal cells of chronically stressed mice, and loss of their synthetic/secretory capability to appropriately respond to the diurnal rhythm [297, 321], than to beneficial adaptations. This is further supported by a similar expression of hypothalamic CRH mRNA (in SD/OC mice determined during the dark phase; in CSC mice determined during the light phase), as well as plasma ACTH concentrations during the dark phase (unpublished observation for CSC mice) in control and chronically stressed mice. Hypothalamic and adenohypophyseal corticotroph cells seem to be unaffected by either SD/OC or CSC exposure. Thus, the observed corticosterone deficiency at the end of chronic stress reflects an adrenal rather than a hypothalamic/adenohypophyseal dysfunction. Moreover, the finding of

an attenuated corticosterone secretion of adrenal cells of both SD/OC and CSC mice in response to acute ACTH challenge *in vitro* supports the hypothesis that chronic stress results in a loss of functional responsiveness of adrenal cells, which is necessary to mount an appropriate corticosterone response. Interestingly, Albeck *et al.* (1997) showed that chronically stressed animals did also not respond with an adequate increase in plasma levels of corticosterone after an acute challenge [114]. However, in contrast to the findings of the present thesis, this phenomenon was due to HPA axis dysfunctions on the level of the hypothalamus [114]. This indicates that, depending on the chronic stress procedure, dysfunctions at various levels of the HPA axis may occur during chronic stressor exposure, due to the permanent activation of these systems.

Anxiety-related behaviour on the elevated plus-maze (Tab.4)

Although an elevated level of state anxiety [313, 314] is a generally accepted indicator of acute and chronic stress in rats [81, 315, 316] and mice [82, 317], in the present theses only mice exposed to 19 days of CSC showed increased levels on anxiety-related behaviour on the EPM. This was reflected by a decrease in the percentage of time spent on the open arms and of entries onto the open arms compared with controls. The locomotor activity, indicated by the number of closed arm entries, was not affected by CSC exposure. In this respect it is important to note that CSC mice were housed single for 15 h before testing. One might, therefore, argue that the increased anxiety-related behaviour of CSC mice found after 19 days of CSC exposure is only due to the single housing procedure before plus-maze testing. However, a possible anxiogenic effect of single housing could be excluded, because the increase in anxiety-related behaviour of CSC mice was even more pronounced, when mice were directly transferred from coloncy housing to the plus-maze (Fig. 35).

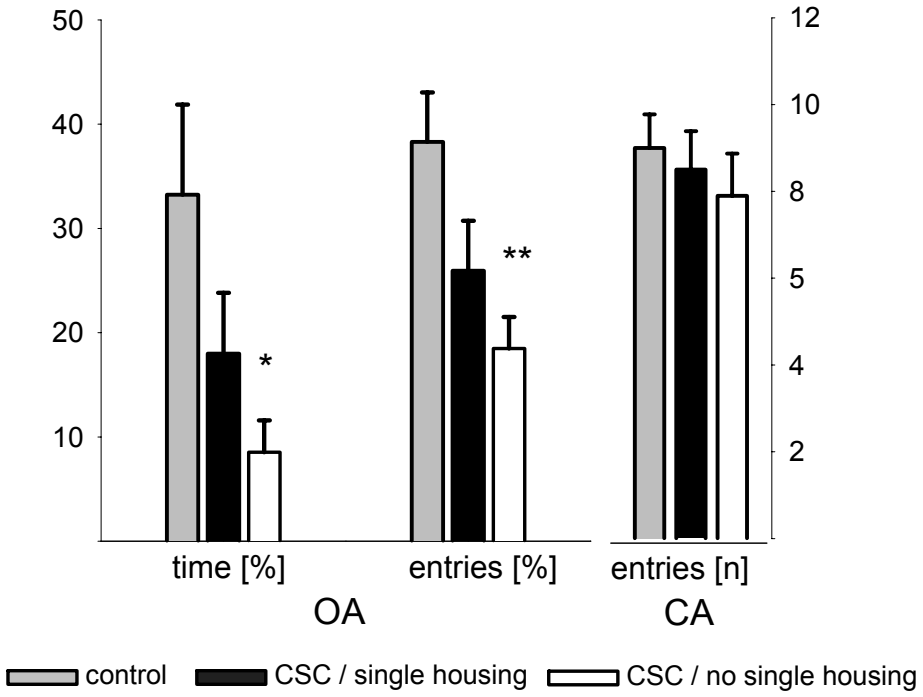


Fig. 35: Effects of exposure to 19 days of CSC on the anxiety-related behaviour on the EPM. CSC mice were either housed single (day 19; 1800 h) for 15 h before the testing (CSC/single housing; n = 8) or were transferred to the plus-maze directly out of the colony (CSC/no single housing; n = 10) and compared with single-housed control mice (control; n = 10). All mice were transported to the plus-maze test room on day 19 (1830 h) and tested on day 20 (0800 h – 1100 h). Exposure to CSC reduced the percentage of time spent on the open arms (time [%]; factor housing condition: $F_{2, 27} = 4.09$; $p = 0.029$) and of entries onto the open arms (entries [%]; factor housing condition: $F_{2, 27} = 6.06$; $p = 0.007$) of the maze compared to controls. This effect reached significance for both readout parameters only in the CSC/no single housing group [one-way ANOVA (factor housing condition) followed by *post hoc* Tukey HSD test]. The number of entries onto closed arms (CA) was not different in all three groups, indicating that locomotor activity was not affected by the housing conditions. Data represent means ± S.E.M.; * $p < 0.05$; ** < 0.01 vs. controls.

As already mentioned, anxiety-related behaviour on the EPM was not affected by 19-day exposure to SD/OC (Fig. 36). However, given that chronic stress increased the general locomotor activity in SD/OC mice, as indicated by increased number of closed arm entries, the hypothesized anxiogenic effects of SD/OC may have been masked. Therefore, a non-locomotor based anxiety test may reveal differences in anxiety-related behaviour in future studies.

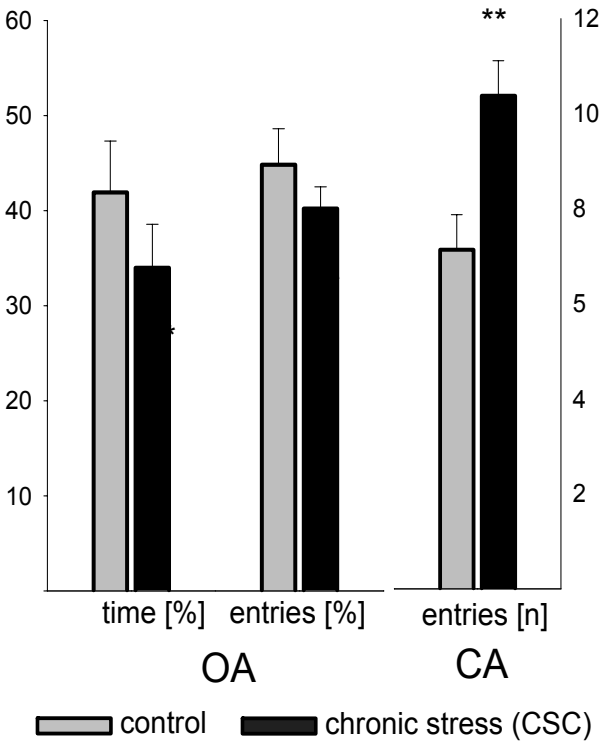


Fig. 36: Effects of exposure to 19 days of SD/OC on the anxiety-related behaviour on the EPM. All mice were transported to the plus-maze test room on day 19 (1830 h) and tested on day 20 (0800 h – 1100 h). Exposure to SD/OC (n = 43) had no effect on the time spent on the open arms (OA; time [%]) and the entries onto the OA (entries [%]) of the maze compared with controls (n = 44). The number of entries onto closed arms (CA) was significantly increased in SD/OC mice, indicating increased locomotor activity compared to controls [Mann-Whitney *U*-test]. Data represent means ± S.E.M.; ** < 0.01 vs. controls.

Effects of 19-day exposure to chronic psycho-social stress on a subsequent colitis induced by DSS

After termination of the 19-day exposure to chronic psycho-social stress, mice were treated with DSS from day 20 to 27 to study the effects of prior exposure to either SD/OC or CSC on the severity of the chemically-induced colitis.

Day 8 of DSS treatment (in SD/OC and CSC mice; Tab. 5)

Prior exposure to 19 days of either SD/OC or CSC resulted in an increased severity of a subsequent colitis induced by DSS. This was indicated by an increased body weight loss, inflammatory reduction of colon length, and histological score of SD/OC and CSC mice compared to respective controls on day 8 of DSS treatment. Furthermore, SD/OC mice showed an increased secretion of the proinflammatory cytokines IFN-γ, IL-6, and TNF-α on day 8 of subsequent DSS treatment, whereas no differences were found in the secretion of these cytokines in CSC mice after 8

days of DSS treatment, both compared to respective controls. In this context it is important to note that cytokine secretion was significantly increased in CSC mice after 2 and 4 days of subsequent DSS treatment compared to controls. A comparison of the absolute concentrations of secreted cytokines between day 4 and day 8 of subsequent DSS treatment indicated that equal cytokine levels, secreted by CSC and control mice on day 8 of DSS treatment, resulted from both an increased secretion in controls and a decreased one in CSC mice compared to day 4 of DSS treatment.

Although the effects of stress on the severity of an experimentally-induced colitis are controversial [108, 273-275], there is evidence that a blunted responsiveness of the HPA axis makes the animals more vulnerable for a chemically induced inflammation [108, 273-275]. Therefore, it is likely that adrenal insufficiency and the consequent lack of corticosterone secretion in response to an inflammatory stimulus (DSS) [108, 298, 354] may be involved in the stress-induced increase in colitis severity.

However, both SD/OC and CSC mice showed increased levels of plasma corticosterone on day 8 of DSS treatment, indicating that the adrenals may have recovered from chronic stress-induced insufficiency. As already indicated above, these high levels of plasma corticosterone were paralleled by a down-regulated secretion of the proinflammatory cytokines IFN-γ, TNF-α, and IL-6 by mesenteric lymph node cells in mice exposed to the CSC, but not in mice exposed to the SD/OC paradigm. This finding suggests that the immunosuppressive effect of corticosterone on inflammatory processes might be attenuated in SD/OC mice, probably promoting the development of systemic inflammation. This is supported by the decreased survival rate on day 4 after termination of the DSS treatment in prior SD/OC-exposed mice. In contrast, impaired regeneration after a subsequent DSS-induced colitis was not found in CSC mice (unpublished observation), although isolated

splenocytes of mice exposed to 19 days of CSC were found to show a diminished *in vitro* reactivity range to different concentrations of corticosterone. This could be either due to the fact that the determined insensitivity to GCs found after CSC exposure was either tissue specific [138], and therefore not prominent in mesenteric lymph node tissue at all, or that it was a reversible process [365] with the insensitivity in mesenteric lymph node cells not being maintained until day 8 of DSS exposure.

Tab. 5: Overview of the effects of 19-day exposure to either SD/OC or CSC on the severity of a subsequent colitis on day 8 of DSS treatment. Exposure to chronic psycho-social stress resulted either in an increase (↑), or decrease (↓), or had no effect (↔) on the distinct readout parameters.

effects of chronic psycho-social stress on:	**SD/OC**	**CSC**
body weight loss	↑	↑
inflammatory reduction of colon length	↑	↑
histological score	↑	↑
proinflammatory cytokine secretion by mesenteric lymph node cells	↑	↔

Further support for the hypothesis that the immunosuppressive effect of high levels of GCs is not attenuated in CSC mice, as it is in SD/OC mice, provides the finding of unaffected high secretion of the antiinflammatory Th2-cytokine IL-10 on day 8 of DSS treatment. The resulting imbalance in the Th2/Th1 cytokine profile reflects the GC-induced bias towards an antiinflammatory Th2 mileu, as it is known from several other studies [355-357]. However, as described in chapter 4, the signs of subtle regulatory processes concerning the cytokine secretion could not be confirmed by macroscopic parameters analyzed on day 8 of DSS treatment, suggesting that these macroscopic changes may take longer to establish [272].

In general, increased levels of cytokines precede visible macroscopic damages during inflammatory responses, because their secretion by immune cells after antigen contact is important for the onset and maintenance of inflammatory processes. As a consequence, during antiinflammatory processes, the cytokine secretion is initially found to be down-regulated, before effects on macroscopic parameters could be detected. Therefore, the lack of further antiinflammatory signs, next to the downregulated cytokine secretion described in chapter 4 on day 8 of DSS treatment, suggestes that the regeneration of chronic stress-induced adrenal insufficiency and the resulting GC-mediated immunosuppression appeared only during the last days of DSS treatment. This implicates that a delayed GC response might be involved in the enhanced severity of DSS colitis in chronically stressed mice due to the lack of immunosuppression during the first days of DSS treatment (this hypothesis is further addressed below).

Interestingly, CSC mice not treated with DSS also showed increased cytokine secretion by mesenteric lymph node cells after 8 days of termination of the chronic psycho-social stressor, suggesting the occurrence of an event during CSC exposure triggering spontaneous inflammation.

Day 2 and 4 of DSS treatment (in CSC mice)

Within two days of subsequent DSS treatment, CSC mice showed an increased secretion of the proinflammatory Th1 cytokines IFN-γ, TNF-α, and IL-6 and of the antiinflammatory Th2 cytokine IL-10 by mesenteric lymph node cells. In contrast, in controls a rise in the secretion of these cytokines was only found on day 8 of DSS treatment (see chapter 4).

In general, an increased inflammatory state, for instance in colonic tissue, results in peripherally generated inflammatory mediators and cytokines, and is supposed to

rapidly activate the HPA axis. This activation of the HPA axis caused by inflammatory stimuli can occur at various levels, including hypothalamic CRH neurons, pituitary corticotrophs, and the adrenal cortex [108, 354]. The consequently secreted GCs are important inhibitors of inflammatory processes, due to their ability to block the production and action of several lymphokines, such as IL-2 and IFN-γ [108, 298-300]. Indeed, in control mice the increased cytokine secretion by mesenteric lymph node cells on day 8 of DSS treatment was paralleled by high plasma corticosterone concentrations (see chapter 4). In contrast, the increased inflammatory state in CSC mice after 2 days of DSS treatment was not paralleled by any increased levels of plasma corticosterone. These findings provide evidence that the HPA axis reactivity is still blunted as a result of CSC during the first days of DSS-induced colitis.

Concerning the macroscopic readout parameters such as body weight loss, inflammatory reduction of colon length, and histological score, no differences were found between CSC and control mice on either day 2 or 4 of subsequent DSS treatment, exept a trend towards an increased histological score in CSC mice on day 2 of colitis induction. This is in confirmation with the above mentioned general finding that visible macroscopic damages may take longer to establish [272].

In addition to the above mentioned increased cytokine secretion by mesenteric lymph node cells in CSC mice (not treated with DSS) on day 8 after termination of the chronic psycho-social stressor, the finding of emerged signs of an acute colitis in CSC mice aleady on day 2 of DSS treatment further supports the above mentioned hypothesis that a key event during CSC exposure may trigger spontaneous inflammation.

Effects of 19-day exposure to chronic psycho-social stress (CSC) on various physiological, neuroendocrine, and immunological parameters

To address the question whether spontaneous colonic inflammation is triggered during CSC exposure itself, and which mechanisms might be involved, the time course of various physiological, neuroendocrine, and immunological parameters during CSC exposure were investigated. Based on these findings and what is generally known from the literature, a hypothesis on the underlying mechanisms arised and is discussed in detail below.

Physiological and neuroendocrine parameters: As mentioned above, exposure to 19 days of CSC resulted in severe adrenal hypertrophy and thymus atrophy compared with controls. Remarkably, already after 24 h of CSC (day 2) relative adrenal weight had doubled in comparison to basal values and remained significantly enlarged on days 3, 7, 14, and 20 of CSC exposure. Therefore, it is at the first glance surprising that plasma corticosterone levels were significantly increased only on day 2 of CSC exposure, and returned to baseline afterwards. One possible explanation for the unchanged corticosterone levels between CSC and control mice after prolonged stressor exposure might be the occurrence of adaptive processes, in order to protect the body from an immunosuppressive, and therefore deleterious, chronic exposure to increased corticosterone concentrations [74, 77] (Chapter 1, Sec. 2.3). This is supported by the finding that CRH mRNA expression at the level of hypothalamic CRH neurons was significantly increased in the same temporal fashion observed for plasma corticosterone, i.e. only on day 2 of CSC exposure. Thereby, the initial increase in CRH mRNA expression may reflect the acute activation of the HPA axis as it is generally described in rats and mice after various acute stressors [120, 344-347]. In contrast, the similar CRH mRNA levels in the PVN between CSC and control mice after prolonged stressor exposure are hard to interpret, because hypothalamic CRH mRNA levels are known to vary depending on the type and duration of the stressor (for review see [5]; see also Chapter 1, Sec. 2.3).

Another possible explanation for similar corticosterone levels between CSC and control mice after prolonged stress might be a decreased responsiveness of the adrenals to environmental challenges, due to its persistent activation during CSC exposure. This is suggested by the finding that corticosterone levels were increased on day 2, but started to decline already on day 3 of CSC exposure, although the adrenals were significantly enlarged at that time point.

In addition, unpublished data of Kiank *et al.* (2006) provide further evidence that repeated/prolonged stressor exposure may already induce adrenal dysfunctions after a few days and consequently results in inadequate corticosterone secretion during various challenges [366]. They showed that after 9 exposures to combined acoustic and restraint stress (2h/day over 4 days) ACTH injection induced a less pronounced increase in plasma corticosterone in repeatedly stressed compared with control mice. Interestingly, these repeatedly stressed mice showed a hypertrophy of the zona fasciculate, but reduced size of steroid-storage vacuoles within the adrenal glands, suggesting that, despite enlargement, the adrenals were unable to produce adequate amounts of corticosterone, due to a lack of the substrate cholesterol.

Thymus involution was also already seen after 24 h of CSC exposure and was maintained on all further time points measured during CSC. This is in agreement with a decreased relative thymus weight seen in defeated rats after 24 h of resident-intruder confrontations [139, 329]. The reduction in thymus mass is likely related to a decrease in the absolute cell numbers of all thymocyte subpopulations, most substantially within immature $CD4^+CD8^+$ cells [139]. The thymus exhibits a high density of GC type-II receptors, and the immature $CD4^+/CD8^+$-cell population is particularly sensitive to GCs [330, 331]. Therefore, the initial elevation of GCs, observed during the CSC procedure, may have induced apoptosis and inhibited cell proliferation of immune cells [332, 333]. In addition, the increased relative thymus

weight determined in non-stressed control mice after ADX indicates a basal role of endogenous corticosterone in regulating thymus size. However, in the experiments described in chapter 3, increased concentrations of plasma corticosterone were only found on day 2 of CSC exposure, which returned to baseline thereafter despite the maintenance of an elevated relative adrenal weight. Therefore, and because of the decreased relative thymus weight also found in ADX mice, lacking of corticosterone anyway, after 19-exposure of CSC exposure an additional factor must be taken into account contributing to stress-induced loss in thymus mass. In this respect, it is important to note that the medullary part of the thymus expresses high densities of β-adrenergic receptors [334], which are involved in a cAMP-mediated thymocyte apoptosis and the consequent decrease in thymocyte numbers [335-337]. Thus, the stress-induced activation of the sympathetic-adrenomedullary system, as shown previously [338], and reflected in the present thesis (see Chapter 3) by increased plasma NE concentrations even 15 h following the termination of CSC could be causally involved in the loss of thymus mass of CSC mice. Recent observations in socially defeated rats, treated with the β-adrenergic antagonist propranolol confirmed the hypothesis that thymic atrophy as a result of social stress might, at least partially, be mediated by catecholamines [139].

With respect to CSC-induced alterations of the sympathetic nervous system, TH expression in the LC was found to be significantly decreased only on day 2 of CSC exposure. In general it is known that stressor exposure acutely triggers elevated TH mRNA [31, 341, 342] and protein [343] levels in the brain stem. Therefore, it is likely that LC neuronal activity and TH expression were acutely increased within the first hours of colony formation, which could not be found in the experiments of the present thesis because investigation started only 24 h after colony formation. This initial activation of LC neurons thereafter may have caused a rapid decrease in neuronal activity by

negative feedback regulation. This downregulation is due to NE and epinephrine binding to presynaptic a_2-adrenoceptors [339, 340] and likely to result in an overshooting reduction of TH expression, as it is found on day 2 of CSC exposure. In contrast, TH expression in colonic tissue was increased on day 2 of DSS treatment and returned to baseline levels afterwards. Interestingly, increased activation of sympathetic nerves in the tissue during the onset phase of an inflammation was shown to have proinflammatory effects, whereas its effects during the late phase of inflammation are rather antiinflammatory [320]. Therefore, the increased TH expression in colonic tissue on day 2 of DSS treatment, as described in chapter 3 of the present thesis, might have profound effects on inflammatory parameters and is therefore discussed further below.

Immunological parameters: Interestingly, the above described changes in physiological and neuroendocrine parameters in the early CSC phase were followed by an increase in the histological damage score, first detected after 14 days of CSC exposure. Thus, the data of the present studies provide the first evidence in rodents that chronic exposure to a psycho-social stressor induces spontaneous colonic inflammation. Since histological damage was not found during the first two weeks of CSC exposure, it is likely that in other studies the duration of stress was too short to reveal similar histological effects [276, 310-312].

Speculations on the underlying mechanisms: During IBD an excessive penetration of antigens through the epithelial layer, due to barrier disturbances, results in an inappropriate immune stimulation, leading to chronic gastrointestinal inflammation [204-206]. The data that support a link between increased epithelial permeability and IBD are derived from both animal models and humans. In the latter there is also evidence implicating increased epithelial permeability playing a role in the onset [367-369] and relapses of CD [204]. In animals, the link between a disturbed

barrier function and IBD is supported by the finding that bypassing the epithelial barrier and injecting luminal bacterial wall extracts directly into the submucosa initiates a chronic relapsing inflammatory syndrom similar to CD [276, 350]. Furthermore, simply decreasing epithelial barrier function in mice, by altering intracellular adhesion molecule expression, prompts the spontaneous generation of intestinal inflammation [232, 276] and alters luminal constituents by housing these animals in a germ free environment can prevent or delay disease [192]. Interestingly, the different compartments of intestinal barrier function, i. e. physical diffusion barriers, regulated physiological and enzymatic barriers, and immunological barriers, are under neurohormonal control and therefore possible targets for the influence of stress [370].

Therefore, it is not surprising that both animal and human studies demonstrate that exposure to various stressors affects the functional integrity of the gastrointestinal tract resulting in an altered mucin production [110, 303, 371], secretion of ions and water [305-307], and intestinal permeability [310-312, 372-374]. The exact mechanisms are not completely understood, but severel studies investigating the influence of stress on colonic permeability suggest an important role for mast cells, peripheral CRH, and cholinergic parasympathetic nervous mechanisms in the pathophysiology of stress-mediated barrier disturbances [303, 311, 372-374]. It is hypothesized that mast cells may be activated via neurons releasing CRH and/or acetylcholine, whereas the magnitude of the stress-induced mast cell activation can be modulated by various other factors [370]. Stress-induced opening of colonic epithelial tight juctions was hypothesized to be also due to the increased secretion of IFN-γ by $CD4^+$/$CD8^+$ T cells after stressor exposure, which in turn might activate myosin light chain kinase [310]. Furthermore, Meddings and Swain (2000) recently confirmed that 3 h of restraint stress or 20 min of forced swimming increased intestinal permeability of all segments of the

gastrointestinal tract and that endogeneous GCs might mediate this effect [276]. Interestingly, no histological abnormalities were found in these animals. In a previous study, this group was already able to show that increased intestinal permeability to sucrose was observable after 20 minutes of swim stress [375]. In addition, barrier defects have also been induced by high doses of dexamethasone [376], further supporting the involvement of GCs in stress-induced barrier dysfunction. Furthermore, administration of GCs to rats also has been shown to reduce biliary immunoglobulin secretion and increase bacterial adherence to the intestinal mucosa [377].

Due to the above mentioned hints that GCs might be possible mediators of the stress-induced increase in intestinal permeability, and because it is known that many of the end organ effects of stress are adrenally mediated [370], it was hypothesized in the present thesis that the initial increase in plasma corticosterone during CSC exposure might also result in a breakdown of the colonic barrier function in CSC mice. One might now argue that the finding of slightly increased corticosterone levels on day 2 of DSS exposure is not comparable to the above mentioned study [276] in which acute high levels of corticosterone were hypothesized to be important for mediating the stress-induced increase in epithelial permeability [276]. However, the finding of an about 5-fold higher corticosterone concentration in CSC compared to control mice after 8 h of CSC exposure in the studies of this dissertation suggests that plasma corticosterone levels might have been even higher immediately after starting of the CSC paradigm (Fig. 37). Therefore, the acute stress response after starting CSC exposure might have been indeed accompanied by a strong rise in GCs, affecting intestinal barrier function. According to previous reports stress-induced barrier dysfunction might result in increased bacterial translocation from the gastrointestinal

tract to mesenteric lymph nodes, liver, and spleen [349, 378-381]. This hypothesis is further supported by unpublished data of Kiank *et al.* (2006) in which a single exposure to combined acoustic and restraint stress (2 h) was shown to cause an increased permeability of the gut barrier due to the induction of apoptosis in intestinal epithelial cells [382]. This was associated with a transcient bacterial translocation into mesenteric lymph nodes and liver, and was found to be mediated by TNF-α.

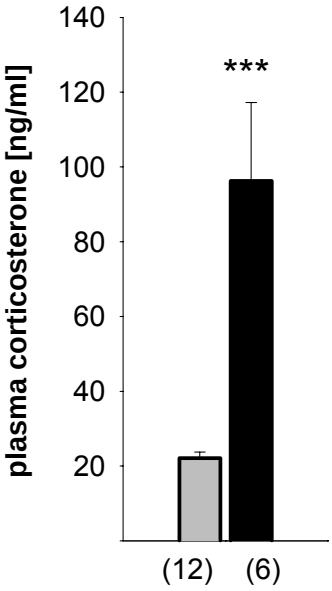


Fig. 37: Effects of 8 h of exposure to CSC on plasma corticosterone concentrations. Mice exposed to CSC for 8 h showed an about 5-fold increase in plasma corticosterone levels [Mann-Whitney *U*-test; $p < 0.001$]. Data represent means ± S.E.M.; * $p < 0.05$; ** < 0.01 vs. single-housed controls.

In addition, Dhabhar *et al.* proposed a biphasic model in which acute stress enhances, and chronic stress suppresses, the immune response [74, 75, 77, 78, 353, 383-386]. They also showed that GCs play an important role in mediating these effects. The transfer of their findings to the present study promted me to hypothesize that the acute stress response after starting CSC might have resulted in a mobilization of immune cells and their redistribution to relevant body compartments, including the intestinal tract. Furthermore, the increased expression of TH in the colonic tissue might have contributed to an improved immune function at the site of later assessed inflammation. This is supported by the findings of Härle *et al.* [301, 320] who showed

that at the initial stage of an inflammatory reaction, the SNS mediates an increase in plasma extravasation, vasodilatation, and vessel leakage as important stimuli for inflammation. In addition, the SNS is a supportive factor for the redistribution, migration, and directed chemotaxis of leukocytes to the site of inflammation and the creation of a proinflammatory cytokine milieu in local draining lymph nodes [301, 320].

In conclusion (Fig. 38), it was hypothesized that the acute stress response after starting CSC exposure may have resulted in an enhanced immune function, especially in the colonic tissue. Therefore, a stress-induced increase in epithelial permeability might have resulted in an exaggerated immune response towards translocated antigens.

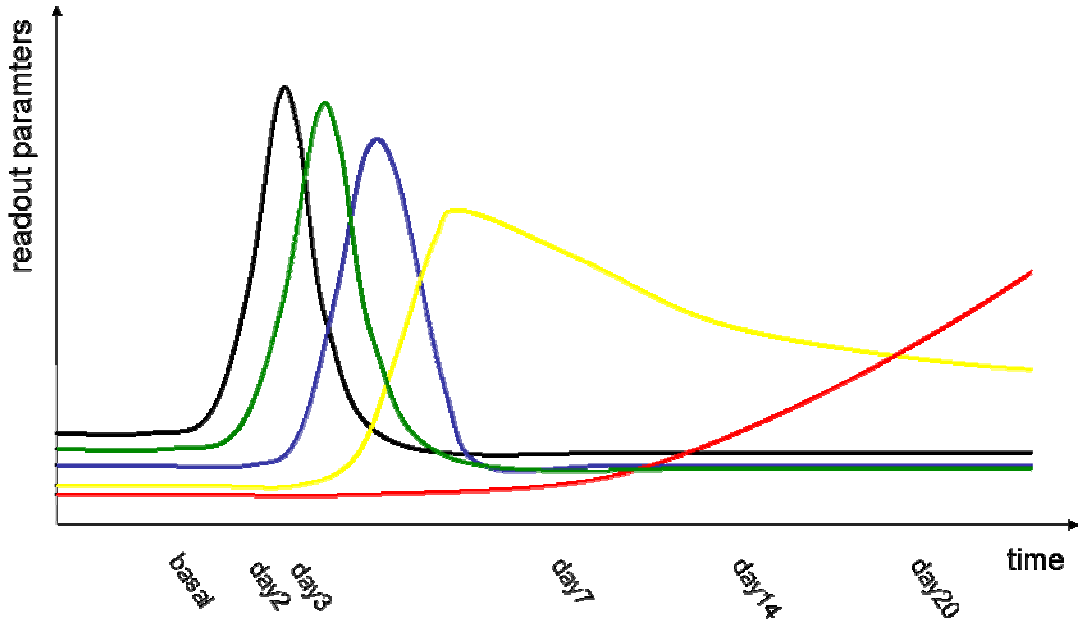


Fig. 38: Schematic representation of the hypothesis on the mechanisms behind the development of spontaneous colonic inflammation during CSC exposure. The initial rise in plasma corticosterone [black line] may have caused a redistribution of immune cells resulting in an activation of an organism's immune status [green line, representing for example the peripheral blood mononuclear cells (PBMCs)]. Additionally, the initial rise in GCs may have resulted in an increased epithelial permeability [blue line] and consequently caused an increased antigen translocation from the gut lumen into the colonic tissue [yellow line]. This may have led to the onset of colonic inflammation, further potentiated by inadequate secretion of antiinflammatory GCs due to CSC-induced adrenal insufficiency. Finally, these inflammatory processes may have resulted in the histological abnormalities, detected after 2 weeks of CSC exposure [red line].

With progression of stressor exposure, the HPA axis became insufficient and therefore unable to respond to different stimuli as stress and/or inflammatory markers, leading to an insufficient production and secretion of antiinflammatory GCs. This may further potentiate the inflammatory processes initiated by acute stress and finally led to histological disturbances of the colonic epithelium. Moreover, the dimished GC sensitivity found in LPS-stimulated immune cells of CSC mice may have additionally contributed to the increased colonic inflammation found after prolonged CSC exposure, because development of GC resistance has been suggested as one of the mechanisms by which a hyper-inflammatory state may be induced under stressful conditions. The finding of increased plasma NE levels after CSC exposure suggests that in contrast to the HPA axis the SNS is still able to responde when challenged. This uncoupling of the activity of the HPA axis and SNS during CSC exposure may also promote proinflammatory processes, since the synergism of steroid hormones and neurotransmitters of the sympathetic nervous system will be dissipated [319].

Effects of ADX prior to 19-day exposure to chronic psycho-social stress (CSC) on colonic inflammation

To investigate the above hypothesized key-role of the initial increase in plasma corticosterone during chronic psycho-social stress exposure in mediating CSC-induced development of spontaneous colonic inflammation ADX was performed one week before CSC exposure.

Thereby, exposure to 19 days of CSC also resulted in an increased histological damage score in SHAM mice, as described before in respective non-operated mice. In addition, an increased cytokine secretion by mesenteric lymph node cells in

SHAM mice exposed to CSC provided further evidence for CSC exposure itself causing the development of spontaneous colonic inflammation. Interestingly, CSC-induced effects on colonic histology and proinflammatory cytokine secretion by mesenteric lymph node cells were diminished or even completely abolished by ADX prior to stressor exposure. These findings provide the first support for the above described hypothesis (Fig. 38) that the initial increase in basal plasma corticosterone during CSC exposure plays a key-role in the induction of spontaneous colonic inflammation.

Furthermore, ADX performed one week prior to chronic psycho-social stress ameliorated the CSC-induced increase in the severity of a subsequent colitis induced by DSS. This is indicated by a minor body weight loss, a diminished inflammatory reduction of colon length, and a lower histological score in CSC-exposed ADX mice compared to respective SHAM mice on day 8 of DSS treatment. Plasma corticosterone concentrations were significantly increased in CSC compared to control mice in the SHAM group, confirming the above described recovery from CSC-induced adrenal insufficiency until day 8 of subsequent DSS treatment. Furthermore, slightly increased corticosterone levels in some of the CSC-exposed ADX mice indicated that either ADX was not completely successful or accessory adrenal cortical nodules [358] secreted little amounts of corticosterone during this strong environmental challenge (i.e. CSC exposure and DSS treatment). Interestingly, a significant correlation between plasma corticosterone levels of CSC-exposed ADX mice and their body weight loss during DSS treatment indicated that mice which were in principle able to show an at least slight initial increase in corticosterone during CSC exposure show an increased severity of subsequent colitis induced by DSS.

In conclusion, ADX prior to exposure to CSC probably attenuated the spontaneous inflammatory processes triggered by exposure to CSC itself, and therefore made the tissue less vulnerable for subsequent DSS treatment.

Summarizing conclusions and outlooks

In summary, the data of the present thesis provide evidence that chronic psycho-social stress causes the development of spontaneous colonic inflammation. This effect is likely to be mediated by the interplay of the proinflammatory HPA axis activation during the very early (initial corticosterone increase) and the lack of antiinflammatory HPA axis activation during the late phase (adrenal insufficiency) of chronic psycho-social stress. The differences in the inflammatory state of colonic tissue between chronically stressed and control mice are further increased during DSS treatment, again mediated by the lack of immunosuppressive corticosterone due to chronic stress-induced adrenal insufficiency, which persists at least during the first days of DSS treatment.

Consequently, the present dissertation provides evidence that exposure to both SD/OC and CSC represent adequate models of chronic psycho-social stress for male mice. In particular the CSC paradigm seems to be very promising because it is quite heuristic and, thus, resembles closely the natural situation within a colony of rodents. In addition, it is also comparable to the human situation, where mostly continuous psycho-social challenges were shown to be most detrimental. Furthermore, the immunmodulatory effects of CSC-induced chronic psycho-social stress and the straightforward experimental design of this model make the CSC paradigm interesting for the investigation of various questions in the field of Psychoneuroimmunology, on a national as well as an international level. As far as chronic stress is also discussed as possible risk factor in the development of

psychological diseases, the CSC paradigm could also help to elucidate the role of chronic psycho-social stress in the development of e.g. depression.

Therefore, a number of studies are planned following this dissertation which should on the one hand elucidate the mechanisms behind the CSC-induced colonic inflammation in more detail, and on the other reveal the role of chronic psycho-social stress in the development of psychological diseases. To investigate the proinflammatory effects of GCs during the early phase of CSC exposure in more detail, it is first planned to catheterize the mice for obtaining an even more detailed time course of plasma corticosterone during CSC exposure, as described in the present thesis. The imitation of this time course in ADX mice by the injections of corticosterone during CSC exposure should verify and further characterize the important role of GCs in the onset of colonic inflammation. In addition, the role of luminal bacteria in the development of the CSC-induced colonic inflammation should be assessed by the application of antibiotics before and during CSC exposure. Furthermore, it is planned to investigate the effects of chronic psycho-social stress on the number and function of various leukocyte subpopulations within the intestinal tract. Thereby, the intestinal mast cells should be focused on because this subpopulation is known to be involved in the stress-induced decrease of intestinal barrier function, due to the secretion of TNF-α. As another important subpopulation regulatory T cells and their role in stress-induced development of spontaneous colonic inflammation should be investigated. Concerning the role of chronic psycho-social stress in the development of psychological diseases, the aim is to assess the effects of CSC exposure on depression-like behaviour of the mice in distinct and well-established tests (tail suspension test, forced swim test, sucrose preference test) and to reveal the underlying mechanisms.

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Summary in German

Zusammenfassung auf Deutsch

In der Literatur wird die Rolle von psychosozialen Faktoren bei der Pathogenese von chronisch entzündlichen Darmerkrankungen (CED) kontrovers diskutiert. Es gibt zahlreiche Hinweise darauf, dass Stress im Hinblick auf die Entstehung und den Krankheitsverlauf von CED sowohl in humanen, als auch in nicht-humanen Primaten eine modulatorische Rolle einnimmt. Allerdings gibt es auch Studien, die keinen Zusammenhang zwischen erhöhten Stresswerten oder größerer Depressivität und einem Krankheitsrezidiv finden. Diese gegensätzlichen Ergebnisse sind angesichts der einzigartigen und sehr detailiert erforschten Interaktionen zwischen dem Gehirn und dem Verdauungstrakt und angesichts der allgemeinen klinischen Erfahrung, dass bestimmte stressassoziierte Lebensereignisse oft einem akuten Schub nach Krankheitsremission bei CED vorausgehen, etwas überraschend.

Im Gegensatz dazu gibt es in vielen Studien, die sich mit einer experimentell induzierten Colitis bei Nagetieren beschäftigen, klare Hinweise darauf, dass Stressexposition sowohl den Schweregrad als auch die erneute Schubauslösung einer bereits abgeheilten Darmentzündung beeinflussen kann. Kontrovers sind hier allerdings die Befunde, was die Richtung der Stresseffekte angeht. Es gibt einerseits Befunde, die einen entzündungsfördernden Effekt von Stress auf die Pathogenese einer induzierten Colitis zeigen, andererseits wird auch von entzündungshemmenden Effekten berichtet. Allgemein muss man aber feststellen, dass diese Studien eine stressinduzierte Erhöhung von immunsuppressiven Plasmaglucocorticoiden eher mit einer Verminderung des Entzündungsschweregrades in Verbindung bringen als mit einer Erhöhung des selbigen (z.B. durch permeabilitätssteigernde Effekte von Corticosteron). Somit könnte eine unterschiedlich starke stressbedingte Aktivierung der HPA Achse und die daraus resultierende unterschiedliche Verfügbarkeit von

immunsuppressiven Glucocorticoiden, eine mögliche Erklärung für die entgegengesetzten Effekte von verschiedenen Stressoren auf den Schweregrad einer experimentell induzierten Colitis sein. Mögliche Gründe dafür können einerseits die Diversität von akuten Stressoren und andererseits die unterschiedlichen Adaptation während wiederholter Stressexsposition sein. Es gibt in der Literatur aber auch Hinweise darauf, dass der Effekt von Stress auf den Schweregrad einer Entzündung sehr stark davon abhängt, in welcher Phase sich die Entzündung zum Zeitpunkt der Stressexposition befindet. Fraglich allerdings erscheint die Übertragbarkeit dieser Ergebnisse, welche nur die Effekte von relativ kurzweiligen und unnatürlichen Stressoren auf eine experimentell induzierte Colitis beschreiben, auf die menschliche Situation, in der hauptsächlich chronische psychosoziale Stressoren als Risikofaktoren für die Pathogenese von CED diskutiert werden.

Das Ziel der vorliegenden Arbeit war es nun, an männlichen Mäusen (C57BL/6) den Einfluss von zwei unterschiedlichen chronischen psychosozialen Stressoren auf den Schweregrad und die Abheilung einer experimentell induzierten bzw. auf die Enstehung einer spontanen Darmentzündung zu untersuchen und die verantwortlichen Meachanismen offenzulegen. Das Hauptaugenmerk wird dabei auf die Involvierung von adrenalen Mechanismen und die regulatorische Wirkung von Glucocorticoiden gerichtet.

Chronischer psychosozialer Stress wurde dabei auf zweierlei Arten induziert. Im sogenannten SD/OC („social defeat/overcrowding“) Paradigma wurden die Versuchsmäuse über 19 Tage unterschiedlichen Stressoren ausgesetzt. Nach einem festgelegten Schema wurden sie entweder für zwei Stunden in einen fremden Käfig mit einem konfrontationserfahrenen Männchen gesetzt, wobei beide nach der ersten Attacke („social defeat“) des Residents durch ein Gitter getrennt wurden, oder

zusammen mit 15 weiteren Versuchsmäusen für 24 Stunden in einem Käfig gehalten („overcrowding“). Im sogenannten CSC („chronic subordinate colony housing“) Paradigma wurden 4 Versuchsmäuse in den Käfig eines älteren, schwereren und konfrontationserfahrenen Männchens gesetzt, was innerhalb kurzer Zeit zu Kämpfen um die Rangordung führte, wobei der Resident die dominante und die Versuchstiere die subordinierten Positionen einnahmen. Diese subordinierte Koloniehaltung wurde 19 Tage aufrechterhalten, wobei am Tag 8 und 15 die dominante Maus in jeder Kolonie ersetzt wurde, um Gewöhnungseffekte auszuschließen.

Um festzustellen, ob die beiden 19-tägigen Paradigmen SD/OC und CSC adequate Modelle für chronischen psychosozialen Stress im Tiermodell Maus darstellen, wurden deren Effekte auf verschiedene physiologische, neuroendokrine und ethologische Parameter untersucht. Hierbei zeigten sowohl SD/OC also auch CSC Mäuse typische Anzeichen von chronischem Stress, einschließlich einer verminderten Zunahme an Körpergewicht, einer Hypertrophie der Nebennieren und einer Atrophie des Thymus. Interessanterweise konnte eine erhöhte Ängstlichkeit, die im Allgemeinen ebenfalls als Indikator für akuten und chronischen Stress gilt, nur in CSC Mäusen nachgewiesen werden. Obwohl die Corticosteron-konzentrationen im Plasma von chronisch gestressten und Kontrollmäusen während der Hell-Phase vergleichbar waren, zeigten sowohl SD/OC als auch CSC Mäuse einen verminderten Corticosteronanstieg zu Beginn der Dunkelphase (Tag-Nacht-Rhythmus). Eine dabei unveränderte Konzentration an ACTH im Plasma und CRH mRNA im PVN deuteten darauf hin, dass diese Fehlregulationen wohl auf der Ebene der Nebennieren stattfinden würden. Dies konnte anschließend durch eine verminderte Reaktivität der Nebennierenzellen bezüglich *in vitro* Stimulation mit ACTH bestätigt werden.

Zusammenfassend ist davon auszugehen, dass sowohl das SD/OC als auch das CSC Paradigma ein geeignetes Modell für chronischen psychosozialen Stress in der Maus darstellen. Die Effekte auf stressrelevante Parameter sind robust und reproduzierbar, was die Validität beidere Stressmodelle unterstreicht.

Nach Etablierung der Stressoren, wurden chronische Stresseffekte auf eine sich anschließende DSS-induzierte Colitis untersucht. Dabei hat sich gezeigt, dass sowohl SD/OC als auch das CSC Exposition zu einer Erhöhung des Schweregrades der DSS Colitis führen, was man an einem Anstieg des Körpergewichtsverlusts, der entzündlichen Verkürzung des Colons, des histologischen Scores und der Zytokinsekretion aus mesenterialen Lymphknotenzellen erkennen konnte. Ausserdem wurde die Regeneration von der akuten DSS Colitis durch vorherige SD/OC Exposition stark beeinträchtigt. Im Laufe der vorliegenden Arbeit hat sich die Hypothese bestätigt, wonach die stressinduzierte Insuffizienz der Nebennieren ein möglicher Mediator für den Anstieg im Schweregrad der DSS Colitis nach chronischem psychosozialem Stress sein könnte. In CSC-Mäusen konnte gezeigt werden, dass die Nebennieren zumindest während der ersten 4 Tage der DSS Applikation weiterhin insuffizient waren und somit keine immunsuppressiven Glucocorticoide als Reaktion auf eine voranschreitende Darmentzündung produzieren konnten.

Weiterhin deutete eine bereits am zweiten Tag der DSS Applikation erhöhte Zytokinsekretion aus mesenterialen Lymphknotenzellen von CSC Mäusen darauf hin, dass es während der 19-tägigen Stressexposition zu einem Schlüsselereignis kam, welches eine spontane Darmentzündung triggerte. Eine erhöhte Zytokinsekretion am Tag 8 nach Stress in CSC Mäusen, die nicht mit DSS behandelt wurden, stützte diese Hypothese zusätzlich.

Um das erwähnte ursächliche Schlüsselereignis offenzulegen, wurde erneut das CSC Modells verwendet und der zeitlichen Verlauf einiger physiologischer, neuroendokriner und immunologischer Parameter wärend der 19-tägigen Stressexposition untersucht. Am Tag 2 der CSC-Exposition waren die CRH mRNA Expression im PVN, die Plasmakonzentration von Corticosteron und die Expression von TH im Kolongewebe signifikant erhöht, wohingegen die Expression von TH im LC am selben Tag signifikant erniedrigt war. Danach kehrten diese Werte wieder auf basales Niveau zurück. Die Hypothese einer spontanten Darmentzündung, die allein durch chronischen psychosozialen Stress hervorgerufen wird, konnte durch einen erhöhten histologischen Score nach 14-tägiger Stressexposition bestätigt werden. Zusätzlich wurde dieser erste Hinweis auf eine spontane CSC-induzierte Darmentzündung durch eine erhöhte Zytokinsekretion aus mesenterischen Lymphknotenzellen bei CSC Mäusen nach 19 Tagen Stressexposition gestützt (in SHAM Mäusen). Hinsichtlich des involvierten Mechanismus wurde die Hypothese aufgestellt, wonach der initiale Anstieg im Corticosteron während der akuten Stressreaktion nach dem Start der CSC-Exposition das entzündungsauslösende Ereignis darstellen könnte. Diese Annahme beruhte darauf dass ein akuter Anstieg im Corticosterone in vielen Studien einerseits mit einer Zerstörung der Darmbarriere und andererseits mit einer Aktivierung des Immunsystems durch die Redistribution von immunkompetenten Zellen in Verbindung gebracht wird.

Um diese Hypothese zu bestätigen wurde eine Woche vor Beginn der 19-tägigen CSC-Exposition bei der Hälfte der Mäuse eine Nebennierenektomie vorgenommen. Tatsächlich konnte dadurch die CSC-induzierte Erhöhung des histologischen Scores und auch der Zytokinsekretion aus mesenterialen Lymphknotenzellen reduziert werden. Zusätzlich konnte auch die CSC-induzierte Erhöhung des Schweregrads

einer im Anschluss daran mittels DSS induzierten Colitis durch eine der chronischen Stressexposition vorangehende Nebennierenektomie abgeschwächt werden. Diese Ergebnisse deuten an, dass der initiale Anstieg im Corticosteron eine wichtige regulatorische Rolle bei der Auslösung einer spontanen Darmentzündung einnimmt, wobei weitere Studien nötig sind, um die Mechanismen im Detail zu verstehen.

Zusammenfassend lässt sich feststellen, dass chronischer psychosozialer Stress eine spontane Darmentzündung auslösen kann und dass dabei der initiale Anstieg im Plasmacorticosteron eine wichtige Rolle spielt. Die auf diese Weise hervorgerufenen entzündlichen Vorgänge im Darm werden durch die fehlende Sekretion von immunsuppressivem Corticosteron während der stressinduzierten Insuffizienzphase der Nebennieren noch weiter verstärkt, was dann letztenendes in den beschriebenen Anzeichen einer spontanen Darmentzündung resultiert. Somit zeigen chronisch gestresste Mäuse bereits einen erhöhten Entzündungszustand, wenn die DSS Applikation beginnt und sind folglich anfälliger für die entzündungsfördernde Wirkung von DSS. Dieser anfängliche Unterschied im Schweregrad der Darmentzündung wird durch die stressinduzierte Nebenniereninsuffizienz, die mindestens während der ersten 4 Tage der DSS Applikation anhält, zusätzlich verstärkt.

Somit kann man abschließend bemerken, dass sich beide, in dieser Dissertation verwendeten, Paradigmen als relevante Modelle für chronischen psychosozialen Stress in der Maus erwiesen haben. Insbesondere das CSC Paradigma, welches einerseits der natürlichen Situation in Nagetierkolonieen sehr nahekommt und andererseits wegen der tatsächlich ununterbrochenen psychosozialen Belastungssituation gut auf die menschliche Situation übertragen werden kann, stellt ein sehr vielversprechendes Modell für chronischen psychosozialen Stress dar.

Ausserdem machen die immunmodulatorischen Effekte von CSC-induziertem chronischem psychosozialem Stress und das unkomplizierte experimentelle Design dieses Modell äußerest interessant für die Untersuchung verschiedenster Fragestellungen aus dem Bereich der Psychoneuroimmunologie, sowohl auf nationaler als auch auf internationaler Ebene. Ebenfalls sei hier angeführt, dass chronischer psychosozialer Stress auch als Einflussfaktor bei der Entstehung von psychischen Erkrankungen diskutiert wird. Somit könnte das hier etablierte CSC-Modell auch in diesem Forschungsgebiet zum Einsatz kommen und wichtige Erkenntnisse im Hinblick auf die stressinduzierte Entstehung von z.B. Depressionen liefern.

Im Anschluss an diese Promotion sind deswegen bereits weitere Studien geplant, die einerseits die Mechanismen detailierter beleuchten sollen, die für die CSC-induzierte Darmentzündung verantwortlich sind, und andererseits den Einfluss von chronischem psychosozialem Stress auf die Entstehung von psychischen Erkrankungen offenlegen sollen. Um die entzündungsauslösende Wirkung von Glucocorticoiden in der frühen Phase einer chronischen Stressexposition genauer zu untersuchen, soll anfangs durch Katheterisierung der Mäuse ein noch exakterer Verlauf der Glucocorticoidkonzentrationen im Blut erhalten werden. Durch die anschließende Imitation dieses Verlaufs durch Verabreichung von Corticosteron in adrenalektomierten Mäusen während der CSC-Exposition kann dann der Einfluss von Glucocorticoiden bei der Auslösung einer stressinduzierten Darmentzündung bestätigt bzw. deutlicher charakterisiert werden. Ausserdem soll die Rolle der Darmflora bei der Enstehung einer stressinduzierten Darmentzündung mittels Antibiotikaapplikation, vor und während chronischem psychosozialem Stress, beleuchtet werden. Ebenfalls geplant sind Studien, die die Effekte von chronischem

psychosozialem Stress auf die Anzahl und Funktion von verschiedenen Immunzellsubpopulationen im Darm untersuchen. Ein besonderes Augenmerk soll hier auf Darm-Mastzellen gelegt werden, da es Hinweise in der Literatur gibt, dass diese Leukozytensubpopulation durch die stressinduzierte Freisetzung von TNF-α die Zerstörung der Darmbarriere bewirkt. Unter anderem ist dabei auch der Einsatz von Mastzelldefizienten und TNF-α Knockoutmäusen geplant. Weiterhin soll auch verstärkt die Rolle von regulatorischen T-Zellen bei der Entstehungen einer stressinduzierten Darmentzündung untersucht werden, da in der Literatur ein gestörtes Gleichgewicht zwischen immunaktiven Effektorzellen und Regulatorzellen oft in Zusammenhang mit der Entstehung oder Pathogenese von CED gebracht werden. Im Hinblick auf die Tatsache, dass chronischer psychosozialer Stress möglicherweise Einfluss auf die Entwicklung von psychischen Erkrankungen haben könnte, sollen CSC-Mäuse außerdem in verschiedenen etablierten Tests (tail suspension test; forced swim test; sucrose preference test) auf depressionsähnliche Verhaltensweisen untersucht werden, um anschließend die beteiligten zentralen und peripheren Mechnismen offenzulegen.

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Abbreviations

Abbreviations

ACTH	Adrenocorticotropin Hormone
ADX	Adrenalectomy
Ag	Antigen
AMP	Adenosine Monophosphate
ANOVA	Analysis Of Variance
ANS	Autonomic Nervous System
AVP	Arginin Vasopressin
BSA	Bovine Serum Albumin
CA	Closed Arms
CD	Crohn's Disease
CED	Chronisch Entzündliche Darmerkrankungen
CNS	Central Nervous System
CRH	Corticotropin Releasing Hormone
CSC	Chronic Subordinate Colony housing
DC	Dendritic Cells
DNBS	Dinitrobenzenesulfonic acid
DOPA	Dihydroxyphenylalanin
DSS	Dextran Sulphate Sodium
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzym Linked Immuno Sorbent Assay
ENS	Enteric Nervous System
EPM	Elevated Plus-Maze
ES	Electric Shock
GC	Glucocorticoid
G-Protein	Guanosine triphosphate -binding proteins
GR	Glucocorticoid Receptor
HAB	High Anxiety-related Behaviour
HEV	High Endothelial Venules
HPA	Hypothalamo-Pituitary-Adrenal
HSD	Honestly Significant Difference
HSP	Heat Shock Protein
HSV	Herpes Simplex Virus

IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrom
IEG	Immidiate-Early Gene
IEL	Intraepithelial Leukocytes
IFN	Interferone
Ig	Immunglobulin
IL	Interleukin
KRBB	Krebs-Ringer Bicarbonate-glucose Buffer
LAB	Low Anxiety-related Behaviour
LC	Locus Coeruleus
LP	Lamina Propria
lpC	light phase Corticosterone
LPS	Lipopolysaccharide
LS	Lateral Septum
MALT	Mucosa Associated Lymphoide Tissue
MHC	Major Histocompatibility Complex
MPO	Myeloperoxidase
MR	Mineralocorticoid Receptor
mRNA	messenger Ribonucleic Acid
NCNA	Non-Cholinergic-Non-Adrenergic
NE	Norepinephrine
NF	Nuclear Factor
Nod	Nucleotide-binding oligomerization domain
OA	Open Arms
OC	Overcrowding
OHSD	Hydroxysteroid Dehydrogenase
PAMP	Pattern Associated Molecular Patterns
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PC	Plasma Cells
PNS	Parasympathetic Nervous System
PP	Peyer`s Patches
PRS	Partial Restraint Stress
PVN	Paraventricular Nucleus

RAG	Recombination-Activating Gene
RNA	Ribonucleic Acid
RT	Room Temperature
SCID	Severe Combined Immunodeficiency
SD	Social Defeat
SD/OC	Social Defeat/Overcrowding
SHAM	Sham operated
SNS	Sympathetic Nervous System
TBS	Tris Buffered Saline
TCR	T-Cell Receptor
TGF	Transforming growth factor
Th	T helper
TH	Thyrosine Hydoxylase
TLR	Toll-Like-Rezeptor
TNBS	Trinitrobenzene Sulphonic acid
TNF	Tumor Necrosis Factor
UC	Ulcerative Colitis
V1A	Vasopressin 1A
VIP	Vasoactive Intestinal Peptide

Curriculum Vitae

List of publications

Curriculum Vitae

21. August 1978	Geboren als Sohn von Berthold und Gerlinde (geb. Gleißner) Reber in Weiden i. d. Oberpfalz
09/85 – 07/89	Besuch der Grundschule in Neustadt an der Waldnaab
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1998	Erlangen der **allgemeinen Hochschulreife** (Note 1,7).
10/98 – 06/03	**Studium für das Lehramt an Gymnasien** mit der Fächerkombination Biologie/Chemie an der Universität Bayreuth. (Gesamtnote 1,7)
04/01 – 02/02	**Zulassungsarbeit** mit dem Thema "*Sozialer Stress und Verteilungsmuster von T-Lymphozyten bei männlichen Laborratten: Wanderung aus dem Blut in lymphatische Organe und die Haut*" in der Arbeitsgruppe von Herrn Prof. Dr. V. Stefanski am Lehrstuhl für Tierphysiologie von Herrn Prof. Dr. D. von Holst (Note 1,0)
10/03 – heute	**Doktorarbeit** mit dem Thema *"Chronic psycho-social stress & colitis: Neuronal, neuroendocrine, and immunological studies with male C57/BL6 mice"* am Institut für Zoologie von Frau Prof. Dr. I. D. Neumann

List of publications

Stefanski V, Peschel A, Reber SO 2003 Social stress affects migration of blood T cells into lymphoid organs. J Neuroimmunol 138:17-24

Reber SO, Obermeier F, Straub HR, Falk W, Neumann ID 2006 Chronic intermittent psychosocial stress (social defeat/overcrowding) in mice increases the severity of an acute DSS-induced colitis and impairs regeneration. Endocrinology 147:4968-4976

Reber SO, Birkeneder L, Veenema AH, Obermeier F, Falk W, Straub RH, Neumann ID 2006 Adrenal insufficiency and colonic inflammation following a novel chronic psycho-social stress paradigm in mice: implications and mechanisms. Endocrinology doi:10.1210/en.2006-0983

Reber SO, Obermeier F, Straub HR, Veenema AH, Neumann ID 2006 Chronic psycho-social stress (subordinate colony housing) increases the severity of a DSS-induced colitis in mice - implications and mechanisms. J Clin Invest., submitted

Singewald G, Reber SO, Singewald N, Neumann ID Chronic subordinate colony housing changes anxiety-related behaviour and brain immediate early gene expression. In preparation

Veenema AH, Reber SO, Selch S, Obermeier F, Neumann ID Effects of early life stress on chronic stress responsivity and on subsequent DSS-induced colitis in adult mice. In preparation

Eidesstattliche Erklärung

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

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

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

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(Reber Stefan)

Endlich
hat diese unterwürfige
Strammsteherei ein
Ende!!!