Chronic psychosocial stress - induced pathologies in male C57BL/6 mice – impact of the central oxytocinergic system

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Sebastian Thomas Peters

aus Landshut

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

Gewidmet meiner Familie

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Chapter 1

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General Introduction

1 Introduction

In the modern, fast moving society stress is an omnipresent phenomenon and a concept everybody can identify with. The elevated activity of the hypothalamo-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS) upon stressor exposure mediates a broad variety of functions within an organism by the secretion of hormones and neurotransmitters (Dhabhar and McEwen 1996). Although stress is often perceived as negative, an acute exposure to stressors has profound positive effects on an organism, since it results in enhanced attention and allocation of additional energy to increase the physical capability. From an evolutionary point of view, those stress-induced alterations are important, because they increase the fitness of an organism and, therefore, its chances to survive. In contrast, chronic stress exposure and in particular chronic psychosocial stress is a major burden of modern societies and an accepted risk factor for the development of a variety of disorders including somatic disorders like inflammatory bowel disease (IBD) (Duffy et al. 1991), cancer (Reiche et al. 2004), cardiovascular diseases (Dimsdale 2008), as well as affective disorders like anxiety and major depression (Reiche et al. 2004, McEwen et al. 2012), but also substance abuse disorders (Koob 2008). Due to the omnipresence of psychosocial stressors and a rising number of people suffering from stress-related diseases, a deeper understanding of stress-related symptoms at physiological, behavioural and cellular levels is needed for a sufficient treatment of those pathologies. Concerning the lack of sufficient medications to treat stress-induced disorders, the development of new therapeutics is urgently required. The chronic subordinate colony housing (CSC) paradigm represents an established and clinically relevant animal model of chronic psychosocial stress in male mice (Reber et al. 2007). Mice exposed to CSC develop various symptoms including increased anxiety-related behaviour and colonic inflammation, which reflect the human situation following psychosocial stressor exposure nicely. During the last years neuropeptides got in the focus of interest to serve as possible new treatment options for stress-induced pathologies (Griebel and Holsboer 2012) with the nonapeptide oxytocin (OXT) being one of those candidates. Animal as well as human studies demonstrate that OXT exerts anxiolytic

and stress-protective effects following acute administration (Meyer-Lindenberg et al. 2011, Neumann and Landgraf 2012). In addition, the oxytocinergic system is often discussed to be a possible target for treating substance abuse disorders (McGregor and Bowen 2012).

2 Stress systems

The French physiologist Claude Bernard (1813-1878) postulated the importance of a relative constancy of the internal environment for the functional integrity and the independent existence of an organism. The internal environment defined Bernard as the “internal milieu”, which we call “homeostasis” today (Bartolomucci 2007).

Our understanding of the stress response and the physiological and behavioural reactions of an organism to maintain the homeostasis began during the Great American Depression. In the year 1935, the physiologist Walter Cannon described the extraordinary flexibility of an organism and its ability to respond to stressful situations or “accidents of existence” (Sorrells et al. 2009). The focus of his work was on exploring the sympathetic adrenal response to an immediate threat, and he found that an organism prepares itself by the release of the adrenal hormones, which subsequently activate the body´s energy reserves and, therefore, primes the organism for a fight-or-flight (Bartolomucci 2007, Thiel and Dretsch 2011) or freeze response (Bracha et al. 2004, Thiel and Dretsch 2011). In the year 1936, Hans Selye gave a more precise definition of the terminus stress. According to him, stress is the “non-specific reaction of the body to any demand for change”. In his eyes, stress is a typical defensive reaction of an organism exposed to stressors, and he further described this stereotypic reaction to different stressors as the “General Adaption Syndrome” comprising three different phases: (1) the alarm stage (i.e., physiological activation of the HPA axis and the SNS in preparation to deal with the stressor), (2) a resistance stage (i.e., the period following the initial reaction to the threat whereby the body mediates ongoing stress and attempts to return to steady-state levels), and (3) an exhaustion stage (i.e., when a prolonged stress response overexerts the body´s defence system, thus draining it of its reserve sources and leading to

illness) (Selye 1956, Thiel and Dretsch 2011). A more recent definition explains stressors as internal or external threats that disturb the homeostasis of an organism, leading to specific reactions (stress response) of the body to restore the homeostasis. However, the stress response has a degree of specificity depending on the particular stressor, the organism´s perception of the stressor and the ability to cope with it (McEwen and Stellar 1993, Goldstein and Kopin 2007).

2.1 The acute stress response

Stressor exposure results in an acute stress response which involves a variety of physiological as well as behavioural alterations including increased attention, cardiovascular activity, respiration and allocation of additional energy on the one hand and decreased digestion, growth and sexual activity on the other, to restore the homeostasis (Tsigos and Chrousos 2002, Carrasco and Van de Kar 2003). The HPA axis and the SNS act in concert to enable those alterations, although the two systems act with different velocity. The nerve fibres of the SNS allow a rapid neuronal regulation of vital functions compared to the relatively slow hormonal responses of the HPA axis, whose products have to be transported to their respective target tissues via the blood stream (Kyrou and Tsigos 2009).

2.1.1 The sympathetic nervous system (SNS)

Exposure to stressors results in the disruption of the homeostatic balance of an organism, which, in turn, activates the SNS and the HPA axis. The autonomic nervous system comprising the SNS, the parasympathetic nervous system and the enteric nervous system responds rapidly to stressor exposure and controls a broad range of functions. The cardiovascular, respiratory, gastrointestinal, renal, endocrine and other systems are under the control of the SNS and the parasympathetic nervous system with the latter assisting the SNS functions either by withdrawing or antagonizing and therefore increasing its activity

(Chrousos 1998). The major regulatory region of the SNS is the locus coeruleus (LC), an area located in the brain stem comprising a cluster of noradrenalin-containing neurons (Berridge and Waterhouse 2003). The LC represents the major noradrenergic nucleus within the brain, innervating almost the entire neuroaxis and getting activated by many different stressors, including hypotension, forced swim, shock, immune challenge, but also social stressors (Valentino and Van Bockstaele 2008). Interestingly, this stressor-induced activation of the LC might be due to the monosynaptical innervations of catecholamine-containing dendrites by afferents of the paraventricular nucleus (PVN) (Reyes et al. 2005) whereas LC neurons were found to project to the PVN (Aston-Jones et al. 1986). That PVN-LC circuitry represents an important mechanism to restore the homeostasis upon stressor exposure. Moreover, the LC directly projects to the sympathetic preganglionergic neurons (Valentino and Van Bockstaele 2008) and regulates the activity of the SNS by the secretion of noradrenalin that binds to α_1-adrenoreceptors (Lewis and Coote 1990). The cell bodies of the preganglionergic neurons originate in the intermediolateral column (lateral horn) at the thoracic and lumbar region ("thoracolumbar system") of the spinal cord. Preganglionergic neurons of the sympathetic nervous system synapse with the postganglionergic neurons within the sympathetic trunk, representing an area of ganglia which are located on each side of the spinal cord. Postganglionergic neurons innervate the peripheral organs (Abboud 2010). However, sole exception represents the adrenal gland, with the adrenal medulla being directly innervated by the great splanchnic nerve, triggering the release of catecholamines, mainly adrenalin from chromaffin cells, into the blood stream (Holgert et al. 1998). Acteylcholine (ACh) represents the major neurotransmitter of the preganglionergic neurons whereas for the postsynaptic neurons the main neurotransmitter is noradrenalin to activate the target organs (Holgert et al. 1998). Noradrenalin as well as adrenalin binds to their respective α- and β-adrenoreceptors (α_1, α_2, β_1, β_2, β_3) that belong to a family of seven-transmembrane domain receptors, also known as G-protein coupled receptors (GPCR), which stimulate or inhibit intracellular pathways (Molinoff 1984). Due to different expression

patterns of the adrenoreceptors, the catecholamine-mediated effects are tissue- and organ-specific.

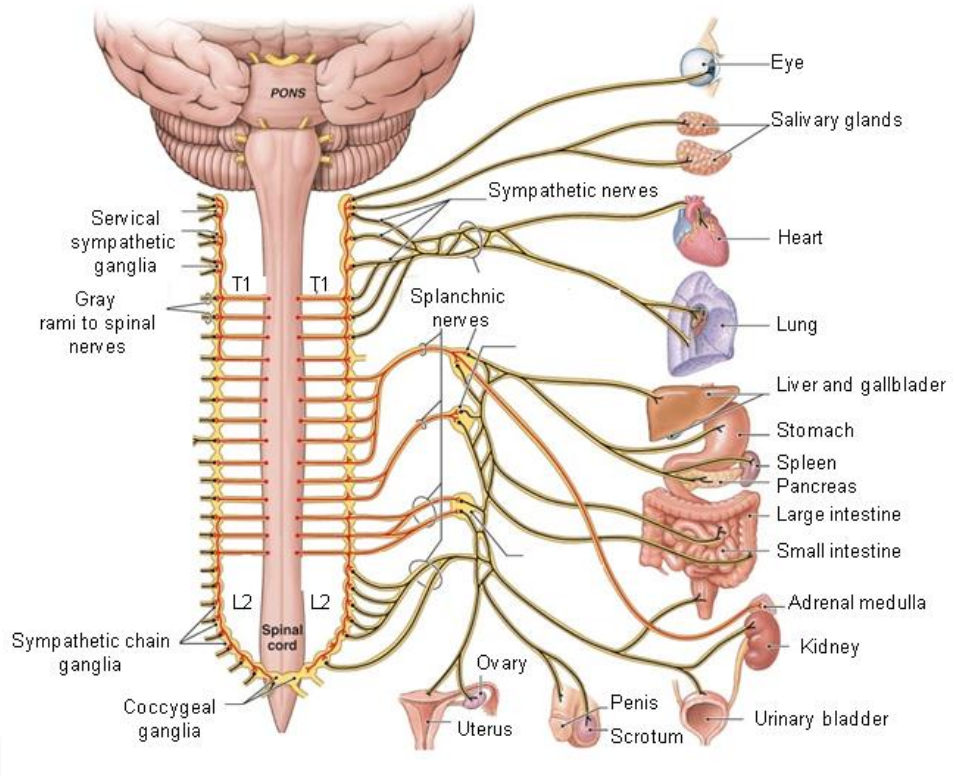

Figure 1: Schematic illustration of the autonomic nervous system. Preganglionergic neurons originating from the first thoracic segment (T1) and extending to the second lumbar segment (L2) of the spinal cord synapse with postganglionic neurons lying in small sympathetic ganglia, named the sympathetic trunk and innervating the peripheral organs. As an exception, the greater splanchnic nerve, originating from the thoracic spinal cord, directly innervates chromaffin cells of the adrenal medulla, without synaptic contact in the sympathetic ganglia. [taken and adapted from: http://www.highlands.edu/academics/divisions/scipe/biology/faculty/harnden/2121/images/sympathetic.jpg]

2.1.2 The hypothalamo-pituitary-adrenal (HPA) axis

The HPA axis (Fig. 2) plays a major role in mediating the body`s response to a physical or emotional stressor (Papadimitriou and Priftis 2009). As the name implies, the HPA axis consists of several parts including the hypothalamus, the adenohypophysis (pituitary) and the adrenal gland. The hypothalamus as part of the diencephalon located next to the third

ventricle consists of several nuclei (hypophyseotropic areas) with neurons producing neuropeptides that regulate various functions of the pituitary (Moyes 2008). When the homoeostasis of an organism is disrupted, the PVN gets stimulated. Each PVN comprises both parvicellular and magnocellular subdivisions. Parvocellular neurons of the medial part of the PVN mostly produces corticotropin-releasing hormone (CRH), a 41-amino-acid peptide, upon stimulation, the intermediate part synthesizes the nonapeptide arginine-vasopressin (AVP), and neurons of the lateral area primarily produce CRH and innervate noradrenergic and other neurons of the stress system in the brain stem (Saper et al. 1976, Chrousos 1992, Chrousos and Gold 1992). Axons of parvocellular PVN neurons project to the *Eminentia mediana* of the hypophyseal stalk and secrete CRH (and AVP) into the thalamic-hypophyseal portal vein system. Via these portal blood vessels CRH obtains the anterior lobe of the pituitary and binds to its respective receptors located in the plasma membrane of the corticotroph cells (Aguilera 2011) that produce proopiomelanocortin (POMC), a precursor of adrenocorticotropic hormone (ACTH).

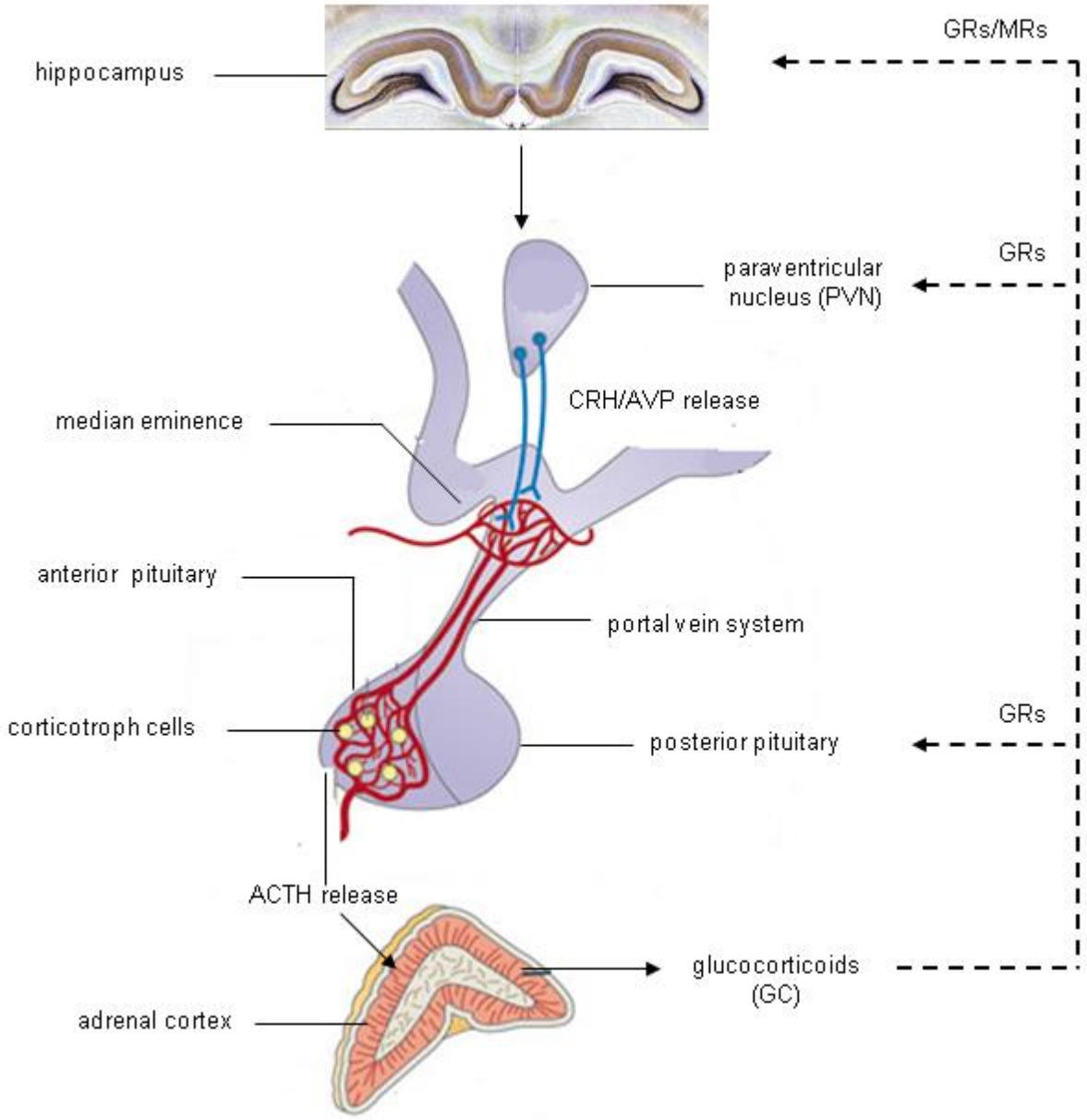

Figure 2: Schematic illustration of HPA axis activation. Upon stimulation, the paraventricular nucleus (PVN) of the hypothalamus releases corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP). Neurons from the PVN reach the *Eminentia mediana* and trigger the release of CRH and AVP into the portal vein circulation. Both peptides bind to their receptors within the anterior pituitary and corticotroph cells secrete adrenocorticotropic hormone (ACTH) into the blood stream. After binding to its receptors ACTH triggers the release of glucocorticoids into the blood stream (GC, cortisol in humans and corticosterone in rodents) which inhibit their own release via negative feedback loops to the pituitary, the PVN and the hippocampus. [adapted from (Lightman and Conway-Campbell 2010)]

AVP is the second regulator of the HPA axis, especially during chronic stressor exposure. It is transported to the anterior pituitary on the identical route like CRH and stimulates the production and secretion of ACTH through binding to vasopressin type 1b (V1b) receptors (Aguilera 2011). CRH was originally thought to be the major stimulus for ACTH secretion, and AVP was thought to only stimulate ACTH functions as a co-activator with additive effects (Kyrou and Tsigos 2009). However, recent studies assume that the two peptides act in concert and that AVP is a strong synergistic partner of CRH to stimulate ACTH-secretion (Aguilera et al. 2008). After its secretion into the general circulation, the major target of ACTH is the adrenal cortex. Here, ACTH binds to melanocortin-2-receptors (Mc2r) and thereby stimulates the synthesis and secretion of glucocorticoids (GCs; cortisol in humans and corticosterone (CORT) in rodents) (Gorrigan et al. 2011). GCs play a major role in the allocation of energy by enhancing blood glucose and lipid levels, the modulation of the immune response and the cardiovascular functions, but they are also known to affect emotional responses and cognitive processes (Sapolsky et al. 2000). Before binding to their respective receptors, GCs are regulated at a variety of different stages. In blood, about 90% of GCs are bound to corticosterone-binding globulin, but only unbound GCs are able to cross the blood-brain barrier and cell membranes due to their lipophilic character as steroids. Once in the cytoplasm, GCs bind to two different types of receptors, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR), which are bound to heat shock proteins (HSPs) while being unoccupied. After activation by GC-binding the receptors homodimerize, shed their HSP chaperones and translocate to the nucleus, where they regulate gene transcription (Sorrells et al. 2009). Those receptors play a major role in mediating the effects of GCs on basal HPA axis activity, including also the coordination of circadian rhythms, food intake and the sleep/wake cycle. Further, MRs and GRs play an important role in the termination of the stress response. The two receptors display different primary structures, localisations, and affinities to bind GCs. The major expression sides of the MRs are limbic structures like the hippocampus, the lateral septum (LS) and, to a lower extend, the amygdaloid nuclei, the hypothalamus and the pituitary (Moguilewsky and Raynaud 1980, Reul and de Kloet

1985).The GR is expressed all over the brain, including the LS, hippocampus, nucleus tractus solitarii, amygdala and the PVN (Reul and de Kloet 1985). Binding studies were able to demonstrate that the MR has a 10-fold higher affinity ($K_d \approx 0.5$) to bind GCs compared to the GR ($K_d \approx 5.0$) resulting in an occupation of the MR already at low levels of circulating GCs (Reul et al. 2000). In contrast, the GRs become activated at higher GC levels, e.g. at the circadian peak and during stressor exposure (Reul and de Kloet 1985). Those facts suppose the MR to be involved in the tonic inhibition of the HPA axis under basal conditions and the GR is thought to play a major role in the termination of the stress response, during recovery from stressor exposure and the preparation to following stressors. Termination of the HPA axis activity is mediated via negative feedback loops of the activated MRs and GRs at various levels axis like the pituitary, the hypothalamus and also the hippocampus (see Fig. 2) (De Kloet et al. 1998, Ladd et al. 2004). Altogether, the activation of the SNS and the release of the catecholamines lead to the "fight or flight" response with the main effects representing an enhanced heart rate, respiration, blood pressure, and allocation of additional energy to increase the biological fitness of an organism.

2.2 Acute vs. chronic stress

As already stated above, "stress" consists of a chain of physiological and behavioural alterations that reinstate homeostasis after disruption of the internal milieu of an organism. Thereby, stressful life events called stressors can be defined as "good", "tolerable", or "toxic", depending of the ability of an organism to control the stressor and whether there are enough resources left to cope with the stressor (McEwen and Gianaros 2011). The brain is the main organ in stress processing. It determines, what is threatening during stressor perception and therefore stressful for an individual and regulates all physiological, behavioural, emotional and immunological responses an organism needs for coping with the stressor. Independent of the nature of the stressor, the orchestrated interplay of all of those responses leads to the activation of the cardiovascular, respiratory, neuroendocrine, but also the immune system

facilitating the “fight or flight” response (Dhabhar 2009, McEwen and Gianaros 2011). The sum of all active processes of the body to return to homeostasis was defined as allostasis by Sterling and Eyer in 1988, literally meaning “maintaining stability through change”. The stress hormones, mainly catecholamines and GCs secreted by the adrenal medulla and adrenal cortex, respectively, act as key players for the achievement of the allostasis (McEwen and Seeman 1999). Acutely, these alterations are beneficial, since they enhance the biological fitness of an individual and therefore its chances to survive. Acute stress is also often referred to as “eustress” (Dhabhar 2000). On the contrary, whether an individual gets exposed to immoderate stressors or for a prolonged period, referred to as “distress” (Dhabhar 2000), those beneficial adaptations exert deleterious effects and are the origin of a variety of different affective as well as somatic pathologies (Chrousos 2009). For this circumstances McEwen and Stellar introduced the term “allostatic load” describing the wear and tear of the body and brain that results from chronic dysregulation, either overactivity or inactivity, of mediators of the stress response (McEwen and Stellar 1993). Therefore, important characteristics for the discrimination of stressors are its duration, but also its intensity. An acute stressor exposure lasts minutes to hours, whereas chronic stressor exposure continues for days or months. The strength or magnitude of a given stressor can be estimated by peak levels of stress hormones or neurotransmitters, but also by the determination of the heart rate and the blood pressure (Dhabhar 2000). Rodent, but also human studies demonstrated a collapse in the regulation of the circadian CORT rhythm as one significant indicator for the deleterious effects of chronic stressor exposure (Dhabhar 2000, Sephton et al. 2000). Accordingly, blood GC levels are shown to peak after 15 to 30 min following acute stress and to decline to baseline levels after 60 to 120 min depending on the nature and magnitude of the stimulus (de Kloet et al. 2005). However, whether a chronic exposure to stressors is accompanied by sustained exaggerated plasma CORT levels is controversially discussed within the literature and strongly depends on the nature of the stressor, the endurance of stressor exposure but also individual characteristics like genetic predisposition, education and personal experiences. Animal studies demonstrated enhanced

basal plasma CORT levels in male rats after a 14-day exposure to the visible burrow system paradigm, representing an animal model of social stress (Albeck et al. 1997), but also an exposure to restraint stress (1.5h/day) for 7 consecutive days was shown to result in enhanced basal plasma CORT levels in rats (Zelena et al. 1999). Accordingly, male mice exposed to repeated social defeat for 3 consecutive weeks displayed similar sustained elevated plasma CORT levels compared to respective unstressed animals (Keeney et al. 2006). As stated above, persistent high levels of circulating GCs may exert deleterious effects on an individual leading to several pathologies (Sorrells et al. 2009). On the other hand, there are also animal studies displaying an unaffected level of circulating CORT upon chronic stressor exposure or a decline to basal levels after an originally rise (Armario et al. 1986, Djordjevic et al. 2012), representing a possible mechanism of adaption enabling an organism to acclimatise to consistent stressors and preventing the negative effects of hypercorticism. It has to be emphasized that for habituation processes, the stressor must be of homotopic nature, since individuals are unable to adapt to heterotopic stressors (Bartolomucci 2007). Those adaptive mechanisms might be due to changes in the secretion of hypothalamic regulators like CRH and AVP, alterations of the CRH/AVP receptor density, but also an altered GC feedback signalling during chronic stressor exposure (Aguilera 1994). As stated above, the effects of chronic stressor exposure not only depend on the duration and the nature of the stressor, but also on individual characteristics, like the genetic predisposition and life experiences including trauma or abuse (McEwen and Gianaros 2011). In animal studies, Dhabhar and colleagues demonstrated that hairless SKH1 mice with an increased anxiogenic phenotype display an increased stress burden and are more prone to develop UVB-induced skin cancer compared to mice with normal anxiety-related behaviour (Dhabhar et al. 2012). In line, mice selectively bred for different levels of anxiety show a diverse susceptibility for developing affective and somatic consequences induced by chronic psychosocial stressor exposure. Individuals with a high anxious phenotype were shown to be more vulnerable to chronic psychosocial stress compared to animals with a low anxiety-related behaviour, giving evidence for the anxiety-related phenotype to mediate resilience to

stress-induced pathologies (Liebsch et al. 1998, Liebsch et al. 1998, Füchsl et al. 2013). Regarding early life experiences, Levine was the first to demonstrate a reduction in emotional as well as neuroendocrine responses upon stressor exposure in adulthood in rats after maternal separation (Levine 1957). In contrast, various other studies showed an increased vulnerability for developing stress-related pathologies after repeated daily maternal separation (3h/day) during postnatal day 2 to 14 in rats (Ladd et al. 2004) and mice (Veenema et al. 2008).

2.3 Psychosocial stress

The General Adaption Syndrome introduced by Hans Selye and mentioned in chapter 1.2 describes the occurring adaptations of an organism during stressor exposure, but is only referred to physical stressor like exposure to cold, surgical injury and intoxication. More recent studies demonstrate different physiological and behavioural effects of stressful events, depending on the type of the stressor. For example, water deprivation produced a duration-dependent anxiolytic effect in rats tested on the elevated plus maze (EPM) whereas 1 h of restraint stress induced an anxiogenic phenotype in the same behavioural paradigm, although both stressors triggered increased circulating plasma CORT levels (McBlane and Handley 1994). In addition, social defeat was shown to decrease various cardiac parameters compared to non-social stressors such as restraint, shock and forced swimming, with the latter leading to an increased or unaffected variability (Sgoifo et al. 1999). Further studies demonstrated a reactivation of a latent herpes simplex virus type 1 in mice after disrupting the social hierarchy, but not following restraint stress (Padgett et al. 1998). Given the diverse responses to different stressors, ongoing research is supposed to focus on stressful stimuli with varying qualities that serve as stressors across mammalian species, including humans (Blanchard et al. 2001), for the development of adequate strategies to treat stress-induced pathologies. For the human situation, social or psychological stressors are the most important and naturalistic threats with psychosocial stress combining those two important

aspects of stress. Psychological stressors are a product of cognitive assessment of environmental claims that are thought to exceed the individual´s capability to deal with (Cohen et al. 2007). These stressors reflect a learned response to previously experienced adverse stimuli, whereas social stressors originate from disturbed interactions among different individuals (Pacak and Palkovits 2001). Based on the literature chronic exposure to psychosocial stressors is a well described risk factor for the development of stress-related disorders (Reiche et al. 2004, Reber 2010). Therefore, animal models of chronic psychosocial stress are clinically relevant, since they reflect powerful paradigms to investigate the underlying mechanisms of stress-derived pathologies in humans. According to that, the CSC paradigm (for details see chapter 1.6) represents a pre-clinically relevant and well-established murine model to induce chronic psychosocial stress by housing four small male mice together with a larger dominant male mouse for 19 consecutive days. CSC was shown to induce a broad variety of physiological, neuroendocrine, immunological and behavioural changes. Exposure to CSC has been repeatedly shown to induce thymus atrophy as well as adrenal hypertrophy (Reber et al. 2007, Slattery et al. 2012, Uschold-Schmidt et al. 2012, Füchsl et al. 2013, Uschold-Schmidt et al. 2013). Further important alterations seen after 19 days of CSC are the induction of a spontaneous colonic inflammation (Reber et al. 2007), aggravation of a chemically induced colitis (Reber et al. 2008) and the enhancement of anxiety-related behaviour (Reber and Neumann 2008, Slattery et al. 2012). Therefore, the CSC paradigm seems to provide a powerful and relevant tool for investigating the the underlying mechanisms of the development and maintenance of stress-related pathologies in humans.

3 Stress and carcinogenesis

3.1 Inflammation-mediated colon carcinogenesis

3.1.1 Clinical relevance

The term tumour or neoplasia describes a disease with an excessive or uncontrolled growth of structurally and biological abnormally cells that can originate from any tissue of the body. Thereby, benign and malign neoplasm can be differentiated with the benign neoplasm showing close morphological similarity to the tissue of origin, displaying slow growth patterns and forming circumscribed and encapsulated masses. Further, benign tumours do not infiltrate their surrounding tissue and do not metastasize. In contrast, malign neoplasms resemble their adjacent tissue less closely and are characterized by more abnormal cells regarding their cellular form and function. The majority of malign tumours grow rapidly and extend gradually throughout their surrounding tissue and metastasize to different sites within the organism (Tanaka 2009). The progression of colon carcinogenesis can be subdivided into three sections namely the initiation, promotion, and progression phase, which are known as adenoma-carcinoma-sequence, which represents the progress from the first mutation within the epithelium of the colon to the invasive growing colon carcinoma (Fearon and Vogelstein 1990).

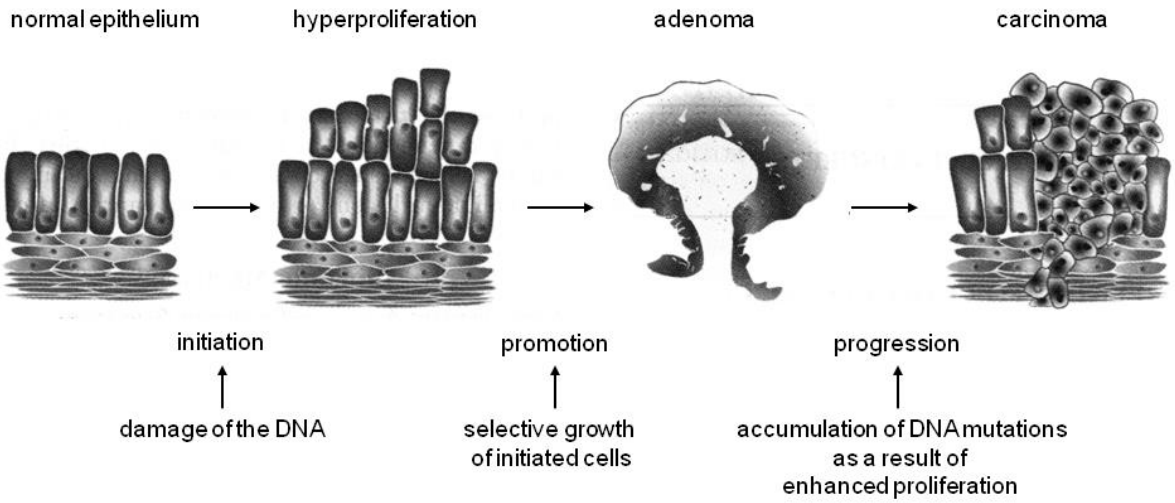

Figure 3: Schematic illustration of the adenoma-carcinoma sequence. [taken and adapted from: http://ediss.sub.uni-hamburg.de/volltexte/1999/161/html/1_%20Einleitung.htm

During the last century there was an increase of the cancer-related death rate, which is the most frequent cause of death within the Western countries (Karim-Kos et al. 2008, Jemal et al. 2009). Epidemiological studies demonstrate rather personal life-style and dietary habits as the cause of the increased colorectal cancer (CRC) rate than the genetic predisposition (Hollman 2000). This aspect became evident in the Japanese population developing more distal CRC after adapting to a more Western high fat diet (Tajima and Tominaga 1985). Another important factor supporting tumour development within an organism is inflammation. In 1863, Rudolf Virchow noted leukocytes in neoplastic tissue and postulated a connection between inflammation and cancer. He further suggested the “lymphoreticular infiltrate” reflecting the origin of cancer at the sides of inflammation with certain cytokines and chemokines in combination with tissue damage and the resulting inflammatory processes leading to an increased proliferation (Balkwill and Mantovani 2001). Although, an enhanced proliferation of cells is not the only reason to cause cancer, a sustained proliferation in an environment rich of inflammatory cells, growth factors and DNA-damage promoting agents, certainly potentiates and/or promotes neoplastic risk (Coussens and Werb 2002). For example, patients who longlastingly suffer from *Crohn´s disease* (CD) or *ulcerative colitis* (UC) have an increased risk to develop pre-cancerogenic dysplastic alterations or colon

cancer. For the latter 8-10 years after diagnosis the risk for CRC increases by 0.5-1% per year (Munkholm 2003, Herszenyi et al. 2007). Studies reporting the risk for developing CRC for patients suffering from UC to be at 2% after 10 years, 8% after 20 years and 18% after 30 years of disease (Eaden et al. 2001). Compared to sporadic colorectal carcinomas, CRC developing in IBD patients affects individuals at younger age more often than the general population and progresses to invasive adenocarcinoma from flat and nonpolypoid dysplasia.

3.1.2 Immunological and cellular issues

The hallmarks of inflammation -related cancer include the presence of inflammatory cells and mediators like chemokines, cytokines and prostaglandins in tumour tissues. These factors are also involved in tissue remodelling and angiogenesis similar to that seen in chronic inflammation (Mantovani et al. 2008). They have also been detected in breast cancer without any ongoing inflammation (Balkwill et al. 2005). In addition, cancer cells are able to produce a variety of inflammation-related mediators by their own leading to the recruitment and infiltration of leukocytes that are known to secrete cytokines and cytotoxic substances like reactive oxygen and nitrogen compounds, but also interleukins (IL) and interferons (IFN) (Wahl and Kleinman 1998, Kuper et al. 2000). Enhanced levels of circulating reactive oxygen and nitrogen compounds as a result of infectious stimuli can increase the amount of DNA damages in tissues with an increased proliferation ratio (Maeda and Akaike 1998). Peroxinitrite for example, acts as a mutagen by interacting with the DNA on sides of an enhanced proliferation resulting in permanent genomic alterations like point mutations, deletions or reorganisations (Coussens and Werb 2002). According to this, mutations of the gene encoding for the protein p53, which is known to exert tumour suppressive properties, are almost as frequent in tumours as in chronic inflammation (Yamanishi et al. 2002). In addition, there are various essential factors being involved in the processes of inflammation-mediated colon carcinogenesis, resembling transcription factors like nuclear factor kappa-light-chain-enhancer of activated B cells (NF-$_K$B), activators of signal transducer and activator

of transcription 3 (STAT3) as well as proinflammatory cytokines like tumour necrosis factor (TNF) and IL-6 (Marazziti and Catena Dell'osso 2008, Colotta et al. 2009). NF-$_K$B resembles a key regulator of inflammatory processes mediated by the innate immune system. Unactivted, NF-$_K$B forms inactive dimers within the cytoplasm and gets activated by signal-transduction-cascades, initiated by toll-like-receptors or TNF and IL-1β, after binding to their respective receptors. Upon activation NF-$_K$B affects the expression of genes, which can be arranged into three different groups: anti-apoptotic genes, genes regulating immune response and proliferation, and genes encoding for their own negative regulation (Naugler and Karin 2008). Therefore, activation of this transcription factor is closely linked to different types of cancer including breast cancer, prostate cancer, Hodgkin´s lymphoma and colon cancer (Li et al. 2005). Lee and colleagues demonstrated that for a consistent NF-$_K$B-activity within carcinomas STAT3 is essentially required (Lee et al. 2009). STAT3 itself also affects the cell cycle and apoptosis by regulating the expression of different genes e.g. encoding for the antiapoptotic B cell lymphoma (Bcl-2). One major effector molecule downstream of NF-$_K$B-activation represents the cytokine IL-6, which is secreted by activated T-lymphocytes, epithelial cells but also by a variety of different tumour cells, and again activates STAT3. The transcription of IL-6 is regulated by bacterial endotoxines like lipopolysacharide, proinflammatoriy cytokines as TNF, but also IL-6 itself (Holländer 2006). In addition, recent studies were able to demonstrate that a process called *Il-6-trans-signalling* is essentially involved in proliferation processes. Thereby, the membrane bound IL-6 receptor transfers by a metalloprotease-mediated process into a soluble form, achieves its target cells and stimulates tumour growth (Becker et al. 2004). Becker and colleagues demonstrated a decelerated adenoma development in late stages of colon carcinogenesis by inhibition of the IL-6 mediated signal cascade (Becker et al. 2004). After injection of hyper-IL-6, a designer cytocine consisting of IL-6 and a covalently linked soluble IL-6 receptor, for 1 week, mice displayed an enhanced proliferation of dysplastic epithelial cells and also an increased amount of phosphorylated STAT3. In addition, a reduced inflammation-induced colon carcinogenesis was observed in mice after blocking the *Il-6-trans-signalling* (Becker et al.

2004). Grivennikov and colleagues were able to confirm these results by using IL-6$^{-/-}$ knockout mice with the wild-type animals displaying an enhanced carcinogenesis after the AOM-DSS-model compared to the knockout mice (Grivennikov et al. 2009). In addition to IL-6, TNF - another proinflammatory cytokine - is mainly involved in the development of tumours. TNF, originally thought to hold neoplasia-destroying properties, was found to increase carcinogenesis instead. After administration of dimethylbenzanthrancen, a strong carcinogen, TNF-deficient mice displayed resistance for developing skin cancer compared to wild-type animals (Moore et al. 1999). A possible mechanism for TNF to promote tumour development as well as progression is the activation of cyclooxigenase II (COXII), an enzyme known to be involved in processes like apoptosis, angiogenesis and invasion of tumour cells into the surrounding tissue. In detail, human colonic cancer cells, overexpressing COX II were shown to be resistant to apoptosis (Sun et al. 2002). Regarding cancer progression, COX II is known to increase the invasiveness of colon cancer cells by activating metalloproteinase-2 (Li et al. 2002), whereas COX II suppression resulted in reduced levels of metalloproteinase-2 and -9 in human prostate cancer (Attiga et al. 2000). Further, in relation to angiogenesis, expression levels of COX II in colon cancer cells correlate with high levels of vascular endothelial growth factor, basic fibroblast growth factor and endothelin-1, which stimulate endothelial migration and endothelial tube formation (Tsujii et al. 1998). Finally, an enhanced concentration of circulating TNF leads to the inhibition of tyrosinephosphatase, resulting in an attenuated expression of MHC-I-antigens on the cell surface. Therefore, it is impossible for the immune system to detect degenerated cells facilitating their development into solid tumours by continuous replication (Reiche et al. 2004).

3.1.3 The azoxymethane (AOM) - dextran sulphate sodium (DSS) model of chemically-induced inflammation-related CRC

The AOM-DSS- model established by Tanaka and colleagues is based on the enhanced risk to develop CRC following inflammatory processes (Tanaka et al. 2003). The principle of this animal model consists of one single ip injection of the carcinogen AOM followed by repeated chemical inductions of colitis via several cycles of DSS administrations in mice. Here, the order of administering the chemicals is important for a successful induction of colon carcinogenesis with the best result occurring with a single AOM injection followed by one DSS administration for seven days via the drinking water (Tanaka et al. 2003). The genetic background of the animals is the second important factor, since there are mouse strain differences in the susceptibility to AOM-DSS treatment (Suzuki et al. 2006, Rosenberg et al. 2008). Suzuki and colleagues were able to demonstrate different tumour ratios in diverse mouse strains, beginning with the most fragile: Balb/c > C3H/HeN > C57BL6/N > DBA/2N (Suzuki et al. 2006).

Azoxymethane (AOM)

AOM is a metabolite of the colon carcinogen 1, 2-dimethylhydrazine (DMH) with both being precursors of methylazoxymethanol (MAM). This carcinogen was identified by a study about the inhabitants of Guam (largest and southernmost of the Mariana Islands) consuming cycad and developing colorectal cancer at a high percentage. The carcinogen *cycasine* being a metabolic precursor of MAM reflects the carcinogen localized within the cycad (Laqueur 1964). Compared to DMH and MAM, AOM is known to exert a higher effectiveness and stability (Papanikolaou et al. 1998, Neufert et al. 2007). Both, DMH and AOM are pre-carcinogens that require a metabolic activation to produce reactive products which are able to alter the DNA. One key player for the production of the reactive MAM is the cytochrome – isoform *CYP2E1*. Within the colon MAM decays into an alkyl radical and due to its strong electrophilic character, is able to alkylate the DNA and forms 6-O-methulguanine. If not being eliminated by the 6-O-methylguanine-DNA-methyltransferase it establishes a mismatch pair

with thymine during the process of replication resulting in adenine-thymine pair instead of a guanine-cytosine pair (Fiala 1977, Fiala et al. 1984, Pegg 1984, Sohn et al. 2001).

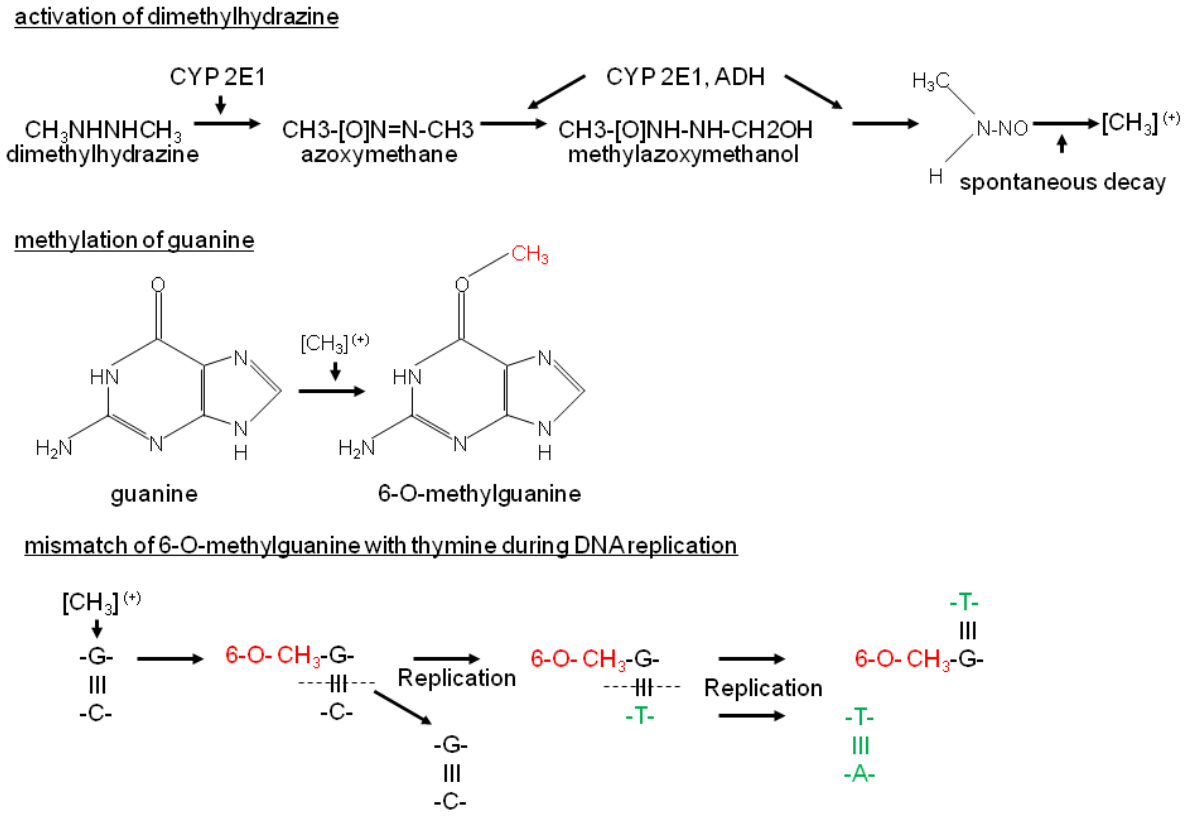

Figure 5: Schematic illustration of the GC-AT transition induced by azoxymethane (AOM). The carcinogens dimethylhydrazine (DMH) and azoxymethane (AOM) build free radicals within the colon that methylate guanine. The methylated guanine (6-O-methylguanine) pairs with a thymine instead of a cytosine resulting in a GC-AT transition during the cell cycle [taken and adapted from (Fähndrich 2005)]

Dextran-sulphate-sodium (DSS)

The risk for developing colorectal cancer is closely linked to IBDs like CD and UC. As stated above, the risk for developing CRC increases with severity and duration of the colonic inflammation which can be reliably induced by DSS administration (Okayasu et al. 1990, Eaden et al. 2001, van Hogezand et al. 2002). Animals treated with DSS display marked loss of body weight, rectal bleeding, reduction of colonic length and destruction of the epithelial

layer and glandular architecture of the large intestine (Okayasu et al. 1990, Cooper et al. 1993, Kitajima et al. 1999) and due to the restriction to the large intestine DSS colitis is considered to be a useful and relevant animal model for UC (Okayasu et al. 1990, Kitajima et al. 1999).

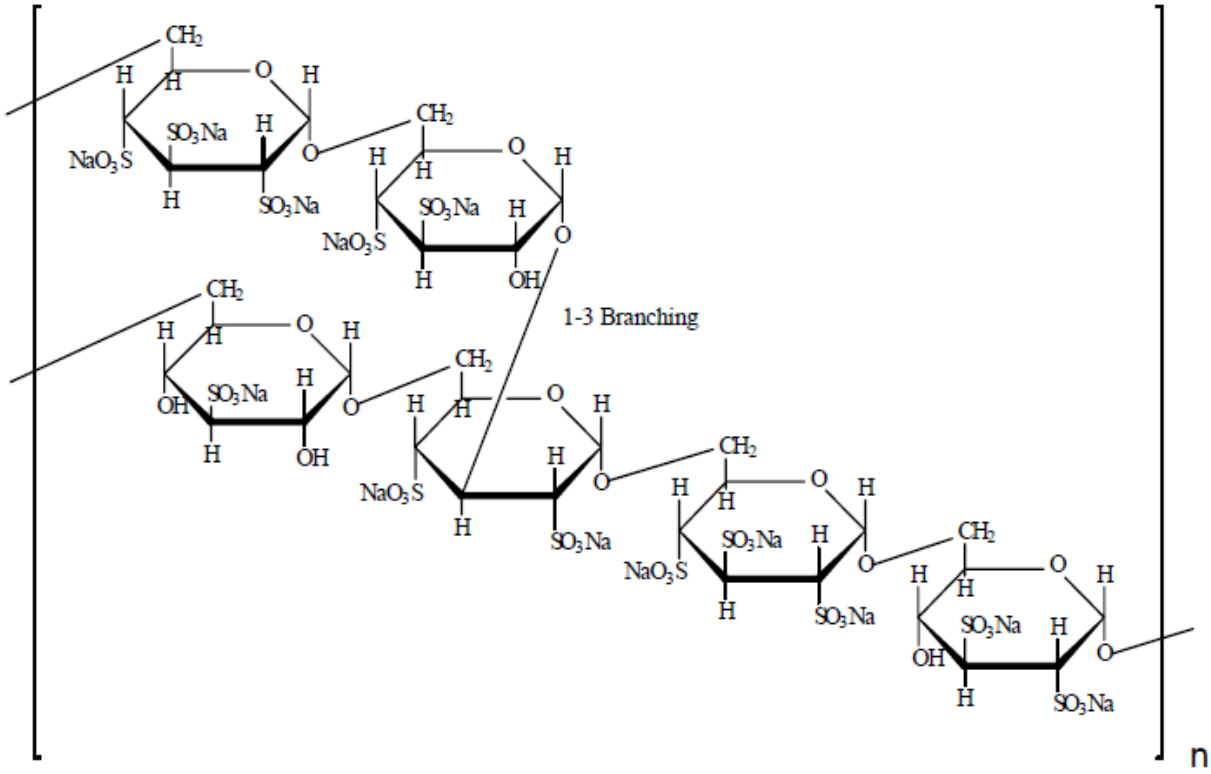

Figure 6: Chemical structure of dextran sulphate sodium (DSS). [adapted from http://www.sigmaaldrich.com/content/dam/sigmaaldrich/docs/Sigma/Product_Information_Sheet/d8906pis.pdf]

3.2 Stress and carcinogenesis

The hypothesis that psychological states affect the development of diseases is an old one. About 200 AD the Greek physiologist Galen postulated “melancholic” women being more susceptible to develop “swellings” of the breast than “sanguine” woman (Reiche et al. 2004). Stressors can affect the proliferation, the growth of tumours, development of metastases as well as the immune response via the endproducts of the stress response, the catecholamines and GCs. Animal studies demonstrated that the catecholamine-mediated

effects on carcinogenesis are mediated via β-adrenergic receptors. Thaker and colleagues demonstrated that mice receiving an injection of a HeyA8-ovarian-tumour cell line followed by a daily administration of isoproterenol (nonspecific β-receptor agonist), terbatuline (specific β_2-receptor agonist), xamoterol (specific β_1-receptor agonist), or isoproterenol (nonspecific β-receptor antagonist) differed in the amount of solid tumours, but also the weight of the tumours varied. In more detail, administration of unspecific β-receptor agonists and specific β_2-receptor agonists led to an increase in the number of tumours and also their weight, whereas unspecific β-receptor antagonists resulted in an opposite outcome (Thaker et al. 2006). The catecholamine-mediated stimulation of β-adrenergic receptors activates the proteinkinase C-/-Erk1/2-/-cyclooxygenase II cascade, which is known to be involved in cell proliferation (Thaker and Sood 2008, Armaiz-Pena et al. 2009). Interestingly, it has to be mentioned that acute and chronic stress exerts different effects on cancerogenisis. Acute stressor exposure induced a redistribution of immune cells and increased the concentration of leukocytes at sides of wounds and an activated immune system and, thus, strengthens the innate and adaptive immune response (Viswanathan and Dhabhar 2005). In line daily acute restraint stress (2.5h, 2 weeks) activated the immune system resulting in a reduced development of skin cancer in mice. In contrast, chronic stress suppressed the innate as well as the adaptive immune response by reducing the levels of circulating immune cells, but also activated immune-suppressive mechanisms like the activation of regulatory T-cells (Dhabhar 2009). Therefore, stressors affect also the production and secretion of cytokines derived by those immune cells (Reiche et al. 2004). Dhabhar and colleagues demonstrated an increased susceptibility of mice, exposed to chronic restraint stress (6h, 2 weeks) to develop UV-induced skin cancer (Saul et al. 2005). Similar results namely an enhanced tumour development and accelerated growth, was demonstrated in studies with mice receiving an injection of a T-cell lymphoma in combination with chronic restraint stress (6h, 3 weeks) (Frick et al. 2009). It has to be emphasized that most of the studies are not about cancer development but rather investigate the effects of stressor exposure on injected tumour cells and therefore on the progression of cancer. In addition, there are only a few studies about

the development or the progression of colonic cancer during or after stressor exposure, although colon cancer is one of the most common cancers worldwide and therefore possesses a high clinical and social relevance.

Morphologically, the intestinal tract comprises three different compartments. The small intestine, including *duodenum*, *jejunum*, and *ileum*, the *caecum*, and the large intestine comprised of the *colon* and *rectum* (Moyes 2008). The colonic surface contains invaginations/crypts with continuously dividing stem cells on their base, which are responsible for the regeneration and renewal of the colonic epithelial layer. The new cells migrate from the base of the crypt to the tip and compensate via apoptosis the continuous proliferation (D'Errico and Moschetta 2008) (see Fig. 7).

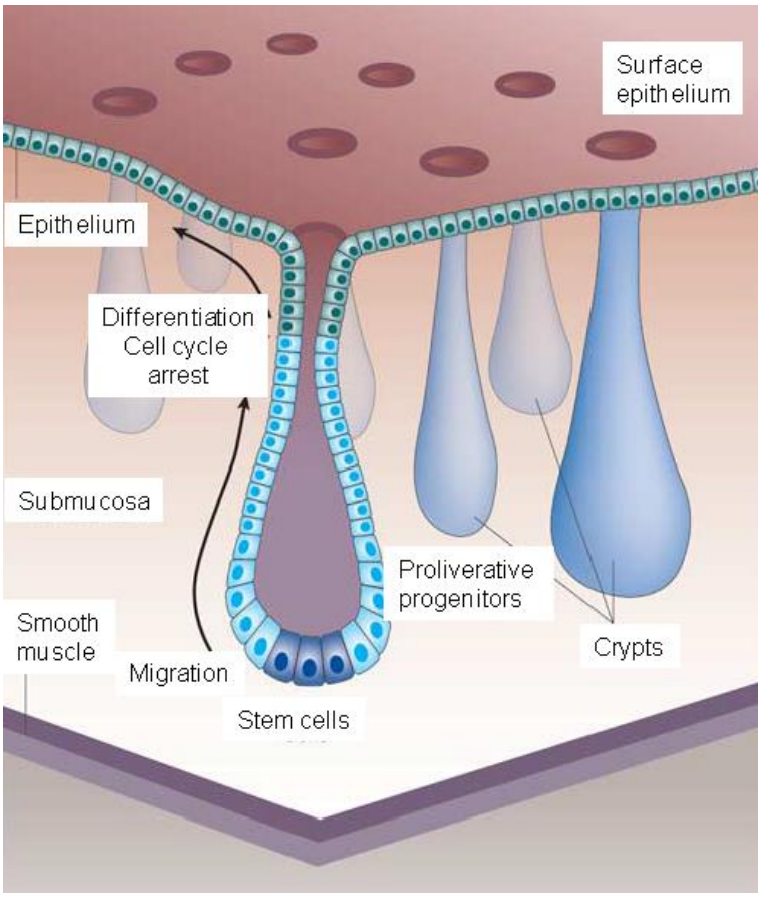

Figure 7: Consistently dividing stem cells on the base of the crypts form proliferative progenitor cells that migrate from the base of the crypt to the tip, regenerate and renew the colonic surface epithelial layer, and compensate via apoptosis the continuous proliferation. [taken and adapted from (D'Errico and Moschetta 2008)

One major control element of this colonic cell renewal is the WNT/β-catenin cascade with the main effector molecule β-catenin. At inactivated WNT receptors, β-catenin is bound to the two cytoplasmatic proteins of the "destruction" complex, the adenomatous polyposis coli-protein and axin, and inactive. Mutations in the genes encoding for those two proteins can result in a continuous activity of β-catenin and an enhanced proliferation (Reya and Clevers 2005). Recent studies demonstrate the nuclear receptor *liver-receptor-homolog-1* (LRH-1) that is mainly expressed in omnipotent embryonic stem cells and is involved in early developmental and differentiation stages, to be also involved in processes of cell renewal within the intestinal tract (Botrugno et al. 2004). In the adult organism LRH-1 is predominantly produced within the liver, the exocrine pancreas and the intestinal crypts and

controls cascades regulating the homeostatsis of cholesterol and bile acid (D'Errico and Moschetta 2008). With respect to cell renewal processes, LRH-1 is able to affect the cell cycle *via* two different cascades. First, LRH-1 acts as a co-activator of the β-catenin/Tcf4-cascade with stimulating the expression of cyclin D1 and c-Myc. Cyclin D1serves as a cell cycle regulatory switch in actively proliferating cells with regulating the continuation of the cell cycle. Thereby, cycline D1 is required in G1 phase for the initiation of DNA synthesis and for the entry into S phase (Stacey 2003). With respect to the cell cycle, c-Myc is known to regulate cell cycle duration with high levels of c-Myc leading to a more rapid cycle (Karn et al. 1989). For the second possibility a conserved LRH-1 element binds to the promotorregion of cyclin E1 with an excess cyclin E1 activity causing cells to progress through G 1 phase more quickly (Hwang and Clurman 2005). Therefore, combination of those two cascades, result in an enhanced and accelerated cell cycle.

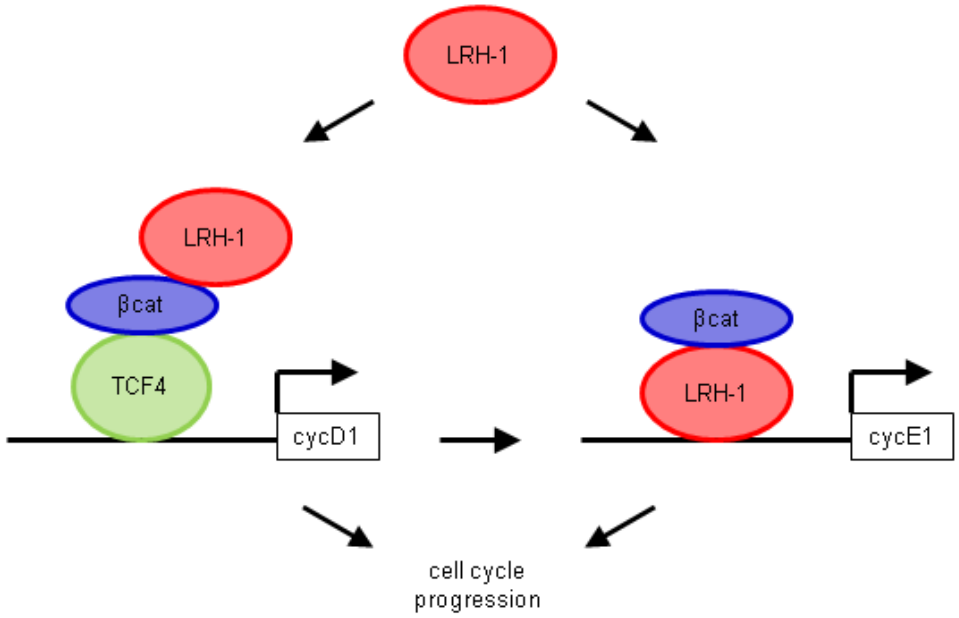

Figure 8: Schematic illustration of the dual mechanism of LRH-1 mediated cell proliferation. LRH-1 promotes proliferation via two mechanisms. First, inducing cyclinD1 expression by co-activating the β-catenin/Tcf4 cascade and second by directly binding to the promoter of cyclinE1 with β-catenin co-activating LRH-1 transcription. The combination of both DNA-dependent and –independent transcriptional events leads to an accelerated cell cycle progression. [taken and adapted from (Botrugno et al. 2004)]

In addition, LRH-1 is also involved in mediating inflammatory processes within the intestine. Studies with heterocygote LRH-1 knockout mice demonstrate a more severe inflammation and a reduced capability to re-built the colonic epithelium of the knockout mice compared to wild type animals. In addition, LRH-1 regulates the enterocyte-dependent local production of GCs which is decreased for the haploinsufficient LRH-1 mice (Coste et al. 2001). In combination, those results reveal an enhanced risk for developing CRC due to an increased LRH-1 expression. Accordingly, the LRH-1 haploinsufficient mice are less susceptible for developing AOM-induced CRC (Schoonjans et al. 2005).

4 Stress and drug addiction

Addiction to drugs of abuse reflects a chronically relapsing disorder of the central nervous system (CNS) including the hallmarks of compulsive drug use and loss of control over drug intake. Addictive behaviour is described to consist of three different phases: (1) preoccupation and anticipation, (2) binge consumption and intoxication and (3) withdrawal with its negative effects. The characteristics of those phases are impulsive behaviour, which often dominates the early stages of addiction, whereas compulsive behaviour is more prominent for the later phases. The shift from impulsivity to compulsivity induces the changes from positive reinforcement driven motivated behaviour into negative reinforcement driven motivated behaviour. Those three stages are thought to interact with each other, thus becoming more and more intense and finally resulting in the pathological state known as addiction (Koob and Volkow 2010). A more clinical definition states the term addiction as a consumption of high doses of psychoactive substance for a prolonged period, craving for the drug but also unsuccessful attempts to discontinue drug intake, accompanied by consumption of the substance of abuse despite negative effects on the social and professional life, and increasing tolerance and withdrawal symptoms and therefore an elevated intake to reduce or alleviate those symptoms (Filip and Frankowska 2008). Cursory examinations display only a few similarities among all drugs of abuse with some exerting sedative effects like barbiturates, alkohol (EtOH), opiates and benzodiazepines, whereas nicotine, amphetamine, and cocaine display more stimulant effects. Further, EtOH and opiates are known to produce striking degrees of physical dependence while others like cocaine produce little or any signs of physical dependence. There are some similarities of central actions of all kind of drugs of abuse. They are pleasurable, reinforcing, and all of them activate the mesolimbic dopaminergic reward system (Gardner 2011). These effects are due to the fact that all of them resemble functional direct, indirect or transsynaptic dopamine (DA) agonists (Gardner 2011). The mesolimbic dopaminergic reward system was first discovered by Olds and Milner in 1950 by electrical brain stimulation (Olds and Milner 1954). The reward system is formed by dopaminergic cell bodies within the ventral tegmental

area (VTA) and their projections systems to the nucleus accumbens-olfactory tubercle complex. First, the posteriomedial VTA selectively project to the ventromedial striatum, which includes the medial olfactory tubercle and the medial NAc shell. Second, the lateral VTA projects largely to the ventrolateral striatum, which includes the NAc core, the medial NAc shell and the lateral olfactory tubercle. (George et al. 2012). A population of GABAergic neurons located within the VTA supplies inhibitory effects on dopaminergice neurons, but also affect other structures like the pedunculopontine tegmental nucleus and glutamatergic neurons (Dobi et al. 2010). The VTA receives the major excitatory glutamatergic and cholinergic inputs from the ventromedial prefrontal cortex, ventral subiculum, subthalamic nucleus, pedunculopontine tegmental nucleus, laterodorsal tegmental nucleus (Kalivas 1993), but also prominent inputs from the nucleus accumbens (NAc) shell and the ventromedial ventral pallidum (Oades and Halliday 1987). Following acute administration, drugs of abuse trigger the release of DA within the NAc. As a result, DA receptors, comprising two receptor families, become activated and trigger different intracellular signal cascades. The DA D1-like receptors, comprising D1 and D5 receptors, were shown to enhance the activity of the adenylyl cyclise, whereas the DA D2-like receptors including D2 and D4 receptor exert inhibitory effects (Spanagel 2009). Due to the brain reward enhancing properties of drugs of abuse, Blum and colleagues hypothesized a chronic basal brain reward deficiency to be causally involved in addiction (Blum et al. 1996). In line, animal studies with Lewis and Fisher 344 rats, a strain selectively bred for different vulnerabilities to drug addiction, demonstrate differences in the dopaminergic reward circuitries (Guitart et al. 1992). Nestler and colleagues found that Lewis rats, which display a behavioural phenotype of huge addictive drug acceptance and an escalated drug seeking behaviour, have a pathological reduction in the neurofilamentous transport system for the dopamine-synthesizing enzyme tyrosine hydroxylase compared to Fisher 344 animals not displaying such excessive drug-seeking behaviour. Further, Lewis rats reveal a deficiency of tyrosine hydroxylase in the dopaminergic axon terminals of the NAc and reduced extracellular DA

levels within the NAc leading to pathological aberrations in the postreceptor signal transduction mechanisms (Beitner-Johnson et al. 1991, Guitart et al. 1992).

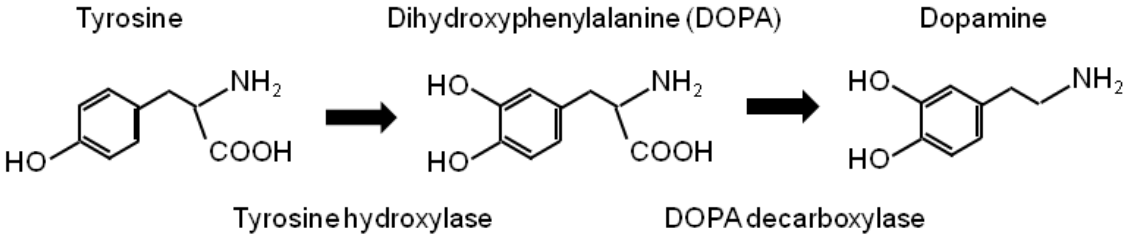

Figure 9: Schematic illustration of dopamine synthesis. Dopamine synthesis starts with the tyrosine hydroxylase mediated conversion of tyrosine to dihydroxyphenylalanine (DOPA), which represents the rate-limited step in the production of dopamine. Next, dopamine is formed by the DOPA decarboxylase. [taken and adapted from http://www.vivo.colostate.edu/hbooks/pathphys/endocrine/adrenal/medhormones.html]

For the human situation, family, adoption and twin studies were able to demonstrate a genetic influence on the development of drug addiction disorders. It has to be emphasized that also individual characteristics including, sensation and novelty seeking, trait impulsivity, antisocial conduct disorder, depression but also attention deficit/hyperactivity disorder are known to enhance the susceptibility to develop substance abuse disorders. (Uhl 2004, Uhl et al. 2008)

4.1 Alcohol addiction

The consumption of alcoholic beverages is a major global burden and an extremely common feature of social gatherings throughout many cultures. Therefore, EtOH is one of the most frequent substances of abuse worldwide with recent statistics revealing that EtOH consumption increases the risk for developing adverse health and social consequences that are related to its noxious and dependence producing properties (World Health Organization

2012). In fact, EtOH abuse is related to more than 60 different pathologies, including cancer, cardiovascular disorders, injuries but also neuropsychiatric diseases (Spanagel et al. 2010).

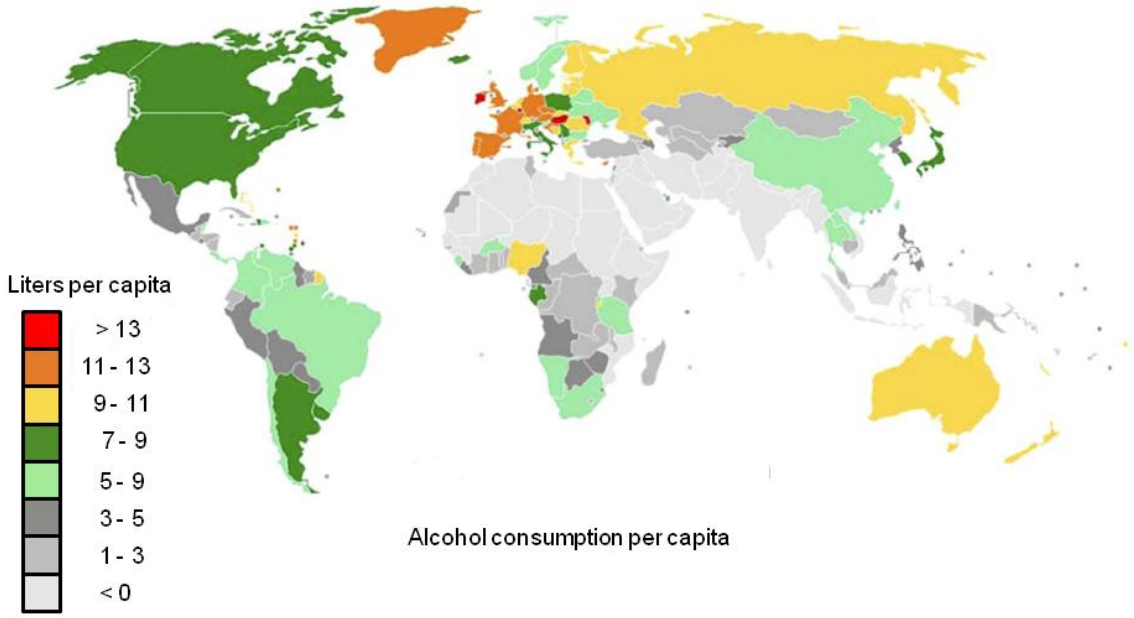

Figure 10: Alcohol consumption per capita in the year 2006. [taken and adapted from (Spanagel 2009)

Despite the enormous health and socioeconomic problems of the use and abuse of alcoholic beverages, EtOH also exerts some positive effects, at least in light to moderate doses. EtOH is known to improve mood, to attenuate stress and to reduce the risk for developing coronary heart disease, type-2 diabetes mellitus as well as cancer (Spanagel 2009). In addition to the social component mentioned above, developing EtOH abuse disorders is also promoted by a number of risk factors including personal history of alcohol usage, genetic factors like alterations in EtOH-pharmacokinetics, -pharmacodynamics and genes modulating neurophysiological responses (Buscemi and Turchi 2011) as well as mood and anxiety disorders (Kessler et al. 1996, Hines et al. 2005, Barrenha et al. 2011). Furthermore, chronic stress, and in particular chronic psychosocial stress, which is very common in modern societies, has been shown to represent a high risk for developing substance abuse and addiction in general, alcoholism in particular, as well as causing relapse in abstinent

individuals (Brown et al. 1990, Litt et al. 1990, Brown et al. 1995, Cooney et al. 1997, Sinha 2008) (see chapter 4.2 and 4.3). During the last decades different theories about the possible binding sides within the CNS were discussed. Until the 1990s different lipid theories hypothesized that EtOH acts via some perturbation of the membrane lipids of CNS neurons. Recent studies mostly describe membrane proteins like receptors and ion channels as the major side of action of EtOH. Several studies demonstrate EtOH to inhibit NMDA receptor activity (Weight et al. 1991), to potentiate $GABA_A$ receptor functioning and to directly affect glycine receptors (Mihic et al. 1997). Furthermore, EtOH potentiates neuronal ACh (Narahashi et al. 1999) and serotonin receptor functioning (Lovinger and White 1991, Machu and Harris 1994) and directly interacts with non-ligand ion channels by either inhibition (Wang et al. 1994) or activation (Kobayashi et al. 1999). In addition, it has to be mentioned that the inhibitory or excitatory effects of EtOH on receptors depend on a variety of variables including EtOH concentration and the subunit composition of the respective channel or receptor (Spanagel 2009).

4.2 Stress and addiction in general

Exposure to stressors is often related to the development of substance abuse disorders with a variety of different hypothesises trying to explain the connection of stressor exposure and addiction. The stress-coping model of addiction described by Shiffman postulates that consumption of drugs of abuse reduces the negative effects of stressor exposure and additionally enhances the positive effects. Thus, this theory predicts drug taking as a maladaptive, but effective strategy for better stress coping (Shiffman 1982). In line, Marlatt and Gordon described individuals with poor coping resources in addition to other risk factors including parental drug use and peer pressure to be more prone to use addictive substances (Marlatt GA and JR 1985). Finally, the reinforcement theory (Conger 1956), the tension reduction theory (Sher and Levenson 1982) and self-medication hypothesis (Khantzian 1985) predict that stressed individuals being in a drug naive state are more prone to consume

substances of abuse due to their mood-enhancing and emotional distress-reducing properties (Sinha 2001). Affective pathologies like anxiety-related disorders are induced by chronic exposure to stressors and are associated with dys-regulation of several brain circuitries (Arborelius et al. 1999), which might lead to an enhanced sensitivity to the reinforcing properties of substances of abuse and, therefore, to an increase in the amount of drugs consumed in those patients (Sinha 2001). In line with these hypothesises several animal studies were able to demonstrate a stress-induced increase in the self-administration of drugs of abuse. Chronic immobilization stress (15 min/day; 50 days) exaggerated the self-administration of morphine or fentanyl in rats compared to unstressed animals (Shaham 1993). Additionally, Long Evans rats displayed an enhanced cocaine self-administration following exposure to repeated restraint stress (Covington and Miczek 2001) or repeated social defeat (25 min on days 1, 4, 7, 10) (Cruz et al. 2011). Further, human studies were able to reveal an increased consumption of drugs of abuse including nicotine, EtOH and cocaine (Maddahian et al. 1988) after exposure to chronic stressful life events like physical and sexual abuse (Harrison et al. 1997) with the susceptibility for developing substance abuse disorders correlating with the exposure to stressors (i.e. amount/duration of abuse during childhood) (Lo and Cheng 2007).

As already stated above, all drugs of abuse exert their rewarding properties via activation of the mesolimbic dopaminergic reward pathways, which were shown to be also responsive to exposure to stressors (Cleck and Blendy 2008). For example, microdialysis studies demonstrated an increased DA release within the shell of the NAc after an acute stressor exposure (mild footshock) (Kalivas and Duffy 1995) or (10-minute tail pinch) (Rouge-Pont et al. 1998) in rats. Further, drugs of abuse including cocaine, EtOH and nicotine as well as acute stressor exposure (cold forced swim; 6°C, 4-6 min) lead to an increased excitatory synaptic transmission in dopaminergic neurons within the VTA, as evidenced by an increase in glutamate receptor activation (Saal et al. 2003) in mice. Finally, repeated injections of cocaine and amphetamine increase the number dendritic branches and the density of dendritic spines within the NAc and prefrontal cortex (PFC) (Robinson and Kolb 1999),

whereas repeated injections of morphine were shown to reduce dendritic branching within the NAc and the neocortex in rats (Robinson and Kolb 1999), an effect, which is also described for the medial PFC of rats exposed to repeated restraint stress (6 h/day, 21 days) (Liston et al. 2006). These findings give evidence for stressors as well as substances of abuse to affect the neurochemistry, electrophysiology and morphology of neurons involved in the rewarding pathways in a similar manner.

The mechanisms that increase the intake of drugs of abuse due to stressor exposure include dysregulation of the activity of the HPA axis and the sympathoadrenomedullary system and their products interacting with the mesolimbic dopaminergic reward system and its glutamatergic and GABAergic modulation (Kalivas and Volkow 2005, Hyman et al. 2006, Koob and Kreek 2007). CORT plays a crucial role in the acquisition of drug intake. Goeders and colleagues demonstrated that the inhibition of CORT release by adrenalectomy attenuates cocaine self-administration in rats (Goeders and Guerin 1996). In line, rats displaying an enhanced locomotor activity and increased circulating CORT levels (high responders) upon exposure to a novel environment displayed an exaggerated cocaine intake compared to animals with low CORT levels and a low locomotor activity (low responders) (Piazza et al. 1991). Moreover, a daily CORT administration switched the low-responding phenotype into the high-responding phenotype indicated by an induction and maintenance of comparable levels of amphetamine self-administration (Piazza et al. 1991). The CORT-mediated effects of drug intake are mediated via GRs located on neurons of the mesolimbic dopaminergic reward system (Harfstrand et al. 1986). Adrenalectomy results in a reduced DA release from the NAc shell in response to a subcutaneous (sc) morphine or an intraperitoneal (ip) cocaine injection (Barrot et al. 2000), and also after acute stressor exposure (10 min tail pinch) (Rouge-Pont et al. 1998) in rats, which can be prevented by CORT replacement (Barrot et al. 2000).

Another prominent candidate linking stress and addictive behaviour is the neuropeptide CRH. In addition to its HPA axis activating properties, CRH is known to mediate neurotransmission within the CNS. The expression patterns of CRH and its respective

receptors, namely CRH receptor 1 (CRHR1) and CRHR2, throughout the brain suggest a fundamental role in affective disorders and addiction (Sarnyai et al. 2001). The CRH system is known to be involved in mediating hormonal effects of drugs of abuse, including the drug-induced activation of the HPA axis, but also adaptation to chronic administration. Further, CRH mediates conditioned as well as unconditioned behavioural effects of substances of abuse and is involved in drug self-administration and rewarding processes. In addition, exposure to drugs of abuse and also drug withdrawal is known to alter the central CRH mRNA and protein levels (Sarnyai et al. 2001). Studies using CRHR antagonists demonstrate the involvement of the CRH system in the initial behavioural and biochemical effects of cocaine. Non-specific (α-helical CRH) as well as specific CRHR1 antagonists (CP-154,526) but not CRHR2 antagonists (anti-sauvagine-30) decreased cocaine-induced DA release from the NAc and VTA and reduced the rewarding and locomotor activating properties of cocaine (Lu et al. 2003). Further, animal studies indicate the CRH system as an attractive target for addiction pharmacotherapy. Blocking CRH activity by CRHR1 antagonism was shown to attenuate stress-induced relapse to drug consumption (Cleck and Blendy 2008).

Recent studies indicated another peptidergic system to be involved in the stress response as well as addiction-related behaviours (Schank et al. 2012). Besides its potent anxiolytic properties (Jungling et al. 2008, Leonard et al. 2008, Rizzi et al. 2008, Vitale et al. 2008), neuropeptide S (NPS) is known to induce arousal and stress-responsive mechanisms (Smith et al. 2006, Schank et al. 2012). Regarding its involvement in addiction processes, neurochemical studies demonstrated a facilitated corticomesolimbic DA neurotransmission in rats following central NPS infusion (Mochizuki et al. 2010, Si et al. 2010). In addition, NPS given intracerebroventricular (icv) potentiated relapse to EtOH in rats (Cannella et al. 2009) and also reinstated extinguished lever pressing for cocaine in mice (Paneda et al. 2009). These behavioural effects are likely to be mediated due to a downstream activation of the central CRH system, since they were prevented by administration of a CRHR1 antagonist and completely absent in CRHR1 knockout mice (Paneda et al. 2009).

4.3 Stress and alcohol addiction

As a risk factor for developing EtOH abuse disorders, the influence of stress on EtOH drinking is complicated by a host of EtOH-related factors. These include the personal history, level and pattern of consumption, and accessibility of EtOH in combination with stress-related factors like type of stressor, chronicity, intermittency, predictability and controllability. These factors interact with a number of biological variables including genetics, age, and sex (Becker et al. 2011). Despite this complexity of the interactions between stressor exposure and EtOH reflected in a variety of different study outcomes, it is generally acknowledged that chronic stress and in particular chronic psychosocial stress represents a high risk factor for developing substance abuse disorders, including alcoholism, and causing relapse in abstinent individuals with former drinking history (Brown et al. 1990, Litt et al. 1990, Brown et al. 1995, Cooney et al. 1997, Sinha 2001, Weiss and Porrino 2002). It is hypothesized that one of the main reasons for this link is the potential of EtOH to act anxiolytic and/or to have anti-stress effects; at least acutely and in moderate doses (Broadbear et al. 2005, Kameda et al. 2007, Varlinskaya and Spear 2010, Fukushiro et al. 2012, Gilpin and Roberto 2012, Varlinskaya and Spear 2012). Thus, it is postulated that stressed, or anxious, individuals are more predisposed to the psychogenic effects of EtOH, which in turn leads to an enhanced inclination for consuming EtOH as summarized in Bale's theory (Cappell and Herman 1972, Bowen et al. 1984). However, despite this knowledge and substantial research interest, the etiology of EtOH dependence remains poorly elucidated with only a few treatment options available (Buonopane and Petrakis 2005, Garbutt 2009).

One reason for this is the lack of appropriate animal models. While several rodent studies have demonstrated increased EtOH intake following repeated stress, like restraint (Chester et al. 2004), forced swim (Lowery et al. 2008), immobilization (Nash and Maickel 1985), and foot shock (Volpicelli et al. 1990), these stressors have low etiological validity and clinical relevance, when compared with the identified risk factors in humans, e.g. high anxiety and chronic psychosocial stress. More akin to the human situation, it has been demonstrated that innate anxiety levels correlate with EtOH intake in naive animals (Ellison 1987, Blanchard et

al. 1993) and that dominant animals living in hierarchic organized groups consume less EtOH in comparison to their subordinated counterparts (Ellison 1987, Blanchard et al. 1993). However, the effects of intermittent social defeat on EtOH consumption are controversial with some studies showing an increase, and others no effect on EtOH intake (Ellison 1987, Blanchard et al. 1993, van Erp and Miczek 2001, van Erp et al. 2001).

Therefore, research assessing both, the etiology of, and potential novel treatments for, EtOH dependence in animal models more relevant to human situations are urgently required.

5 Stress and oxytocin

5.1 The oxytocinergic system

The neurohypophyseal nonapeptide OXT was discovered by Sir Henry Dale in the year 1906. Because of the uterine-contracting properties of extracts from the human pituitary gland he coined OXT from the Greek words ωκωνξ and τοκοχξ, meaning “quick birth” (Viero et al. 2010). Almost 50 years later, the nine amino acid sequence was sequenzed and synthesized by Vincent du Vigneaud, and for this achievement he was awarded with the Nobel Prize in 1955 (du Vigneaud Science 1956). OXT is composed of nine amino acids with a disulfide bridge connecting the two cysteines (see Fig. 11).

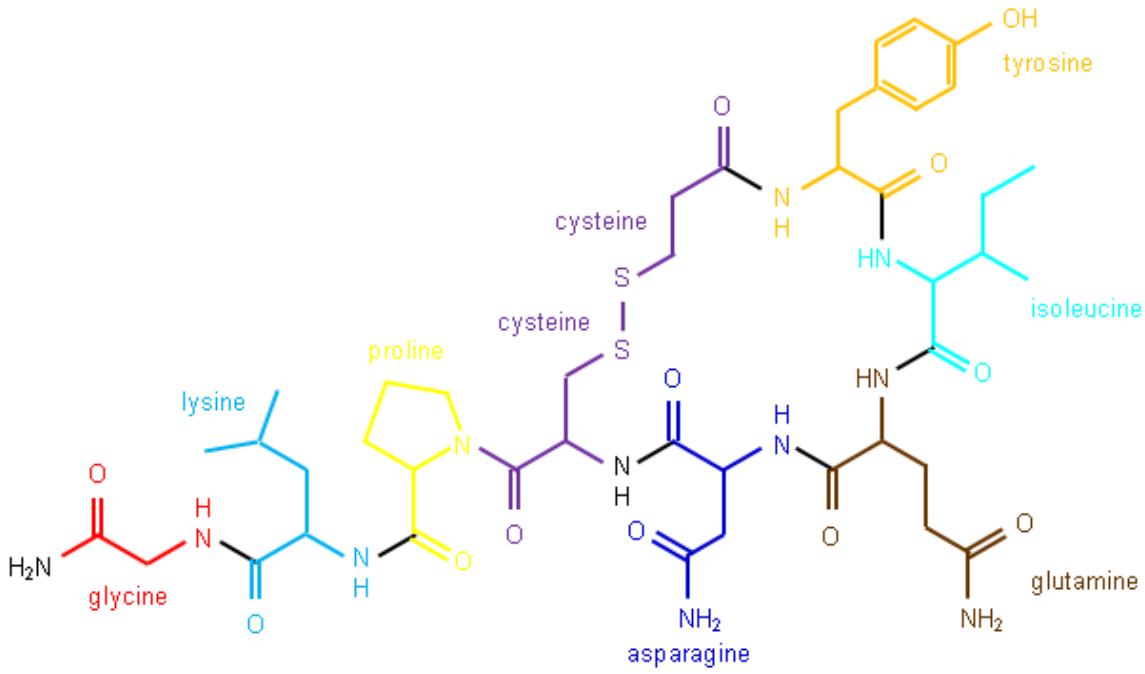

Figure 11: Chemical structure of oxytocin. The nonapeptide oxtocin (OXT) is composed of nine amino acids: Cyr-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-GlyNH_2 with a disulfide bridge connecting the two cysteins. [taken and adapted from: http://nauka.in.ua/en/news/short/article_detail/8024]

As a result of gene duplication 500 Mio years ago, OXT is very similar to its sister peptide AVP, the other neurohypophyseal neuropeptide. The genes for the two peptides are located on the same chromosome, but transcriptionally orientated in the opposite direction (Lee et al. 2009). OXT is mainly synthesized in the magnocellular neurons of the PVN and the supraoptic nucleus (SON) of the hypothalamus. These nuclei project to the neurohypophysis release OXT *via* exocytosis into the blood (Neumann and Landgraf 2012). Compared to AVP, which has three different subtypes of receptors (Caldwell et al. 2008), OXT is known to exert its actions only via one receptor (OXTR). The OXTR is a member of the rhodopsin-type (class I) GPCR family and is coupled to phospholipase C through $G_{\alpha q11}$. Activation of the receptor results in the generation of diacylglycerol and inositol trisphosphate with the latter triggering the release of Ca^{2+} from thapsigargin-sensitive intracellular stores, and additionally from effects on the Ca^{2+} influx similarly to the V_{1A} receptors (Dayanithi et al. 2000). Studies

using [d(CH2)5,Tyr(Me)2,ORN8]-vasotocin demonstrate this OXTR antagonist to selectively block the OXT mediated Ca^{2+} influx whereas selective antagonists of AVP receptors display no effect, allocating the specificity of OXT (Viero et al. 2010). The OXTR is widely spread throughout the brain. Most prominent sides for rodents include the olfactory bulb, tubercle, neocortex, endopiriform cortex, hippocampus, subregions of the amygdala, the bed nucleus of the stria terminalis, the NAc and the ventromedial hypothalamus (Insel 1992, Veinante and Freund-Mercier 1997, Gimpl and Fahrenholz 2001). For the human situation, the brain areas with the highest OXTR density are the basal nucleus of Meynert, the nucleus of the vertical limb of the diagonal band of borca, the ventral part of the LS, the reoptic/anterior and posterior hypothalamic area, the substantia nigra pars compacta of the caudal spinal trigmental nucleus and of the dorsal horn of the upper spinal cord (Loup et al. 1989, Loup et al. 1991).

Originally considered as the major female reproductive hormone, when released into the blood stream, OXT has been implicated to be involved in a broad variety of different behaviours in both females and males including social behaviours like social memory, social recognition, affiliation, sexual behaviour, but also aggression (Gimpl and Fahrenholz 2001, Donaldson and Young 2008, Neumann and Landgraf 2012, Lukas and Neumann 2013). These effects are based on the brain OXT system. As stated above, OXT is released as a neurohormone from magnocellular hypothalamic neurons within the posterior pituitary upon stimulation. Moreover, these neurons were shown to project to limbic brain regions *via* axon collaterals (Knobloch et al. 2012). Studies also demonstrate OXT to act as a neuromodulator, with a central release from dendrites and perikiarya (Ludwig et al. 2002, Landgraf and Neumann 2004). In combination with the OXTR distribution throughout the brain, the centrally released OXT levels determine the activity of the OXT endogenous OXT system, thus being involved in the regulation of complex social as well as emotional behaviour (Neumann 2009, Neumann and Landgraf 2012). Further, this neuropeptide regulates non-social behaviour including learning and memory processes and exerts also effects on the stress response/HPA axis functionality, stress-induced disorders like anxiety-related

disorders and depression but also substance abuse disorders (Gimpl and Fahrenholz 2001, Lee et al. 2009, Lukas et al. 2011, Sarnyai 2011, McGregor and Bowen 2012).

5.2 Oxytocin, stress and anxiety

In addition to its role in the regulation of reproductive functions and maternal behaviour, recent animal as well as human studies reveal a strong link of OXT with stress-buffering and anxiolytic effects. It was demonstrated that OXT neurons respond to physical, psychological as well as social stressors with an enhanced synthetic activity and increased release of the peptide into the blood (Slattery and Neumann 2008) and into brain areas strongly involved in stress-responsivity including the PVN (Wigger and Neumann 2002, Bosch et al. 2004) and the central amygdala (CeA) (Bosch et al. 2005). Animal studies with male and virgin female rats associated the endogenous oxytocinergic system with the regulation of the HPA axis (re)activity. Thus, central administration of a selective OXTR antagonist demonstrated an attenuated release of ACTH as well as CORT under basal and stressed conditions (Neumann et al. 2000, Neumann et al. 2000). However, these effects of OXT are dependent on the sex, duration of treatment and brain region studied. Moreover, an enhanced availability of endogenous OXT, e.g. in lactating females or in males after mating results in the establishment of stress resilience and a reduced anxiety-related behaviour. In contrast, a reduced availability of endogenous OXT, e.g. studies with OXT knockout mice demonstrated their lack of adaptation to chronic homoptypic stressor exposure (Babygirija et al. 2011), an enhanced stress response as well as increased anxiety-related behaviour, which could be reversed by central OXT administration (Mantella et al. 2003, Mantella et al. 2004). Also, genetic variations in the OXT system were linked to differences in both stress susceptibility as well as social behavior (Tost et al. 2010, Bartz et al. 2011).

In humans, OXT has been implicated in mediating the positive effects of social support by buffering the response to stress with the greatest dampening effects on circulating CORT levels and anxiety, when social support and exogenous OXT were given in combination

(Heinrichs et al. 2003). In rodents synthetic OXT has been applied intracerebroventricularly (icv) or locally into a specific brain target region – in addition to intranasally – the common thing with most human studies. Surprisingly, despite the acute beneficial effects of OXT on stress-responsivity and anxiety, there have been only a few studies examining the consequence of chronic psychosocial stress on the OXT system or chronic OXT administration on the stress response with some reporting beneficial and others more negative effects. In detail, chronic central OXT administration 10ng/h; 100ng/h) for five consecutive days dose-dependently reduced noise stress-induced (114 dB; 10 min) CORT release and anxiety in female rats (Windle et al. 1997). Further, chronic icv OXT (1ng/h; 10ng/h) given for five consecutive days was shown to attenuate the release of ACTH and CORT as well as the increased CRH mRNA within the PVN and the enhanced c-fos mRNA expression in brain regions involved in the modulation of the HPA axis in response to acute restraint stress (30 min) in female rats (Windle et al. 2004). In line, a chronic central OXT (10 ng/h) infusion for six following days was shown to reduce the high levels of state anxiety of selectively bred female but not male Wistar rats (Slattery and Neumann 2010). Finally, repeated systemic OXT (20 µg/50 µl) injections for the last two weeks during a four week social isolation were shown to buffer the autonomic response to a resident intruder test (5 min) in female prairie voles. In contrast to those beneficial effects of central OXT, Bales and colleagues report an impaired partner preference formation in male prairie voles after a daily intranasal OXT application for three weeks (Bales et al. 2012). Overall, there is evidence from animal and clinical studies for a potential role of the oxytocinergic system as a possible target to treat stress-related disorders, but more studies need to be done to investigate the behavioural, physiological and molecular effects of a chronic administration.

5.3 Oxytocin and addiction

The idea that the oxytocinergic system is involved in drug effects and addiction-relevant behaviours has been discussed for many years (Kovacs et al. 1998, Sarnyai 2011). Over the last years a variety of studies demonstrated both direct effects of OXT on drug-taking behaviour and addiction and indirect effects of OXT on key systems involved in addictive processes including the mesolimbic dopaminergic system, the HPA axis, the serotonergic system, glia and the peripheral immune system as well as the vagus nerve (for review see: (Buisman-Pijlman et al. 2013). Kovacs and colleagues were able to demonstrate that centrally acting OXT inhibits the establishment of morphine tolerance and reduces withdrawal symptoms in mice, while reducing the self-administration of heroin in rats (Ibragimov and Kovacs 1987, Ibragimov et al. 1987, Kovacs et al. 1998). Regarding methamphetamine-induced conditioned place preference, icv administrations of OXT were able to inhibit the establishment and also to facilitate the extinction of the latter and also prevented its stress-induced reinstatement in mice (Qi et al. 2009). In addition, studies with local injections of OXT into the NAc displayed an inhibition of cocaine-induced stereotypic behaviour in rats (Sarnyai et al. 1991, Baracz and Cornish 2013) with a decrease of cocaine-induced hyperlocomotion and stereotyped grooming behaviour (Kovacs et al. 1998). Also ip administered OXT affected drug-seeking behaviour reducing the intravenous self-administration of methamphetamine (0.3 – 1 mg/kg; (Carson et al. 2010) and the EtOH consumption (1 mg/kg ip; (Bowen et al. 2011) in rats. Due to its interaction with the mesolimbic dopaminergic system, the HPA axis, the serotonergic system and the vagus nerve, OXT might mediate its effects on drug-induced or drug-taking behaviour *via* those systems. Studies observing the maternal behaviour of lactating rats demonstrate that OXT regulates the dopamine levels of the NAc at the level of the VTA. Rats displaying variations in maternal care (licking, grooming) were shown to have different levels of the dopamine signals within the NAc, whereas those dopamine signals could be altered by local infusions of OXT or an OXTR antagonist into the VTA leading to an improvement or an aggravation in maternal care (Shahrokh et al. 2010). Further, studies by Young and colleagues using prarie

voles gave further evidence of an interaction between the oxytocinergic and dopaminergic systems in mediating both, social as well as drug reward (Young et al. 2008, Young et al. 2011).

As described in detail in sections 4.2 and 4.3, stress and a dysregulated HPA axis can be involved in addiction-related behaviour. Further OXT is known to interact with the HPA axis and modulate the stress response (see 5.2). In addition, with the effects of OXT described above and the known interaction with the serotonergic system and the vagus nerve, the oxytocinergic system is possible target for a successful treatment of addiction-related pathologies.

6 Effects of CSC on physiological, immunological and behavioural parameters

CSC paradigm

The CSC paradigm developed by Reber and colleagues (2007) was employed in all experiments of the present thesis. CSC is a well-established and pre-clinically relevant animal model to induce chronic psychosocial stress in male C57BL/6 mice. Therefore, four experimental mice (subordinates) are housed together with a slightly larger and more aggressive male mouse (resident; offspring of high anxiety-related behaviour female mice and male C57BL/6 mice) for 19 consecutive days to induce chronic psychosocial stress. Every single week, the subordinates are exposed to a new resident to avoid any habituation (see Fig.12).

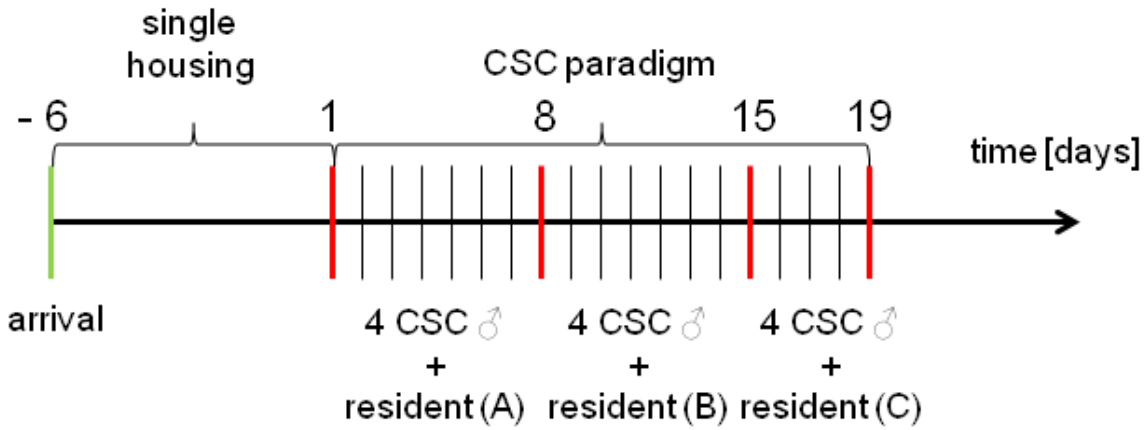

Figure 12: Schematic illustration of the time course of the experimental design of the chronic subordinate colony housing (CSC) paradigm. To induce chronic psychosocial stress, four experimental male C57BL/6 mice (subordinates) are exposed to a larger, dominant and more aggressive male mouse (resident) for 19 consecutive days. To avoid habituation, the resident is exchanged on days 8 and 15.

For the successful induction of chronic psychosocial stress the subordinate position of the four experimental mice is essential, which is ensured by behavioural observations during the first 30 to 60 min after starting the CSC paradigm. In general, the resident displays offensive-like behaviour like chasing, mounting and attacking towards its four cage-mates, whereas the four experimental mice show defensive-like behaviour like flight or defensive upright (Reber and Neumann 2008). The experimental mice are exposed to this chronic psychosocial stress paradigm for 19 consecutive days and after that period they develop typical behavioural, physiological, neuroendocrine as well as immunological alterations induced by chronic stressor exposure compared to their respective single-housed control (SHC) mice (Reber et al. 2007, Reber and Neumann 2008, Veenema et al. 2008, Schmidt et al. 2010, Slattery et al. 2012). SHC mice were considered to be the most appropriate controls (Singewald et al. 2009) which is in line with the literature demonstrating that single housing is less stressful in male mice compared to group housing (Bartolomucci et al. 2003, Gasparotto et al. 2005).

A B

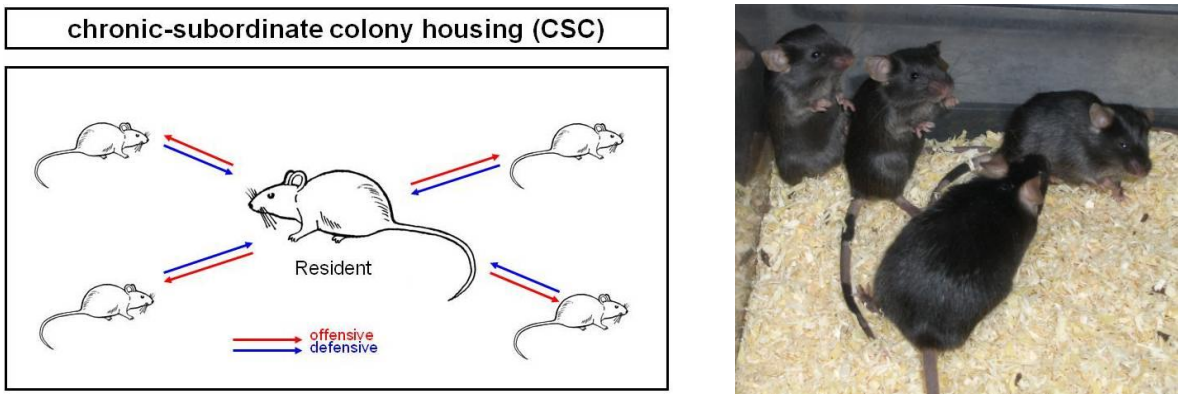

Figure 13: Schematic illustration (A) and representative image (B) of the chronic subordinate colony housing (CSC) paradigm. The subordinates display defensive-like behaviour (defensive upright position or flight response) while the resident shows offensive-like behaviour and attacks the mouse that tries to escape. [taken and adapted from http://epub.uni-regensburg.de/10558/1/Version_Final_Offiziell.pdf]

CSC-induced physiological, neuroendocrine and immunological alterations

Anyalysis of physiological parameters after 19 days of CSC revealed that stressed mice develop typical hallmarks of chronic stressor exposure like reduced body weight gain, thymus atrophy as well as adrenal hypertrophy. Further, assessment of neuroendocrine parameters indicated effects on the functionality of the HPA-axis after a 19-day CSC exposure. Regardless of adrenal hypertrophy, basal plasma morning CORT levels are exclusively elevated after the initial 24 h of CSC exposure and remain unchanged after 19 days of stressor exposure, whereas assessment of plasma CORT in the evening indicated a reduction in circulating GC levels in CSC compared to SHC mice. These results suggest an inability of the stressed animals to mount the circadian rise in plasma CORT (Reber et al. 2007). Moreover, adrenal explants of CSC mice revealed a reduced responsiveness upon ACTH stimulation *in vitro* compared to SHC animals, further supporting the hypothesis of adrenal dysfunction (Reber et al. 2007, Uschold-Schmidt et al. 2012). Interestingly, CSC mice displayed an exaggerated CORT release upon exposure to an acute heterotypic stressor (5 min elevated platform (EPF)) despite they did not differ in their ACTH secretion

compared to SHC animals (Uschold-Schmidt et al. 2012). This increased *in vivo* CORT response during acute heterotypic stressor exposure might be due to an enhanced scavenger receptor class B type 1 protein expression and increased levels of plasma low-density lipoprotein-cholesterol of CSC compared to SHC animals, indicating in combination with the pronounced adrenal hyperthrophy an enhanced adrenal availability of and capacity to mobilize cholesterol in chronically-stressed mice (Füchsl et al. 2013). Taken together, unchanged basal plasma morning CORT levels indicate a possible mechanism of adaption to protect the body from exaggerated circulating GC levels during chronic stressor exposure while a sensitization process enables the organism to respond to a new challenge with an adequate GC response (Uschold-Schmidt et al. 2012).

Furthermore, a 19-day exposure to CSC demonstrated CSC mice to develop a GC resistance in target cells. *In vitro* lipopolysaccharide–stimulation of splenocytes as well as Th2 cells isolated from the peripheral lymph nodes of CSC mice showed a reduced sensitivity to a variety of physiological and pharmacological doses of GCs (Reber et al. 2007, Schmidt et al. 2010).

CSC-induced immunological and behavioural alterations

Assessment of immunological parameters after 19 days of CSC indicated an enhanced amount of secreted of IFN-γ by anti-CD3-stimulated mesenteric lymph node cells (mesLNCs) after termination of the stress paradigm. In addition, stressed mice develop a spontaneous colonic inflammation (Reber et al. 2007) due to an adrenal hormone-mediated local immune suppression, paralleled by impaired intestinal barrier functions, resulting in enhanced bacterial load in the stool and colonic tissue in the initial phase of the CSC paradigm (Reber et al. 2011). Further, a chemically-induced colitis by administration of DSS, results in a more severe inflammation in CSC mice (Reber et al. 2008, Veenema et al. 2008).

Regarding behavioural alterations, CSC was shown to induce an anxiogenic phenotype, with stressed animals spending less time on the open arm of the EPM (Reber et al. 2007, Slattery

et al. 2012), in the lit zone of the light-dark box (LDB) (Reber and Neumann 2008), in the the distal part of the open arm (OA) (Singewald et al. 2009), in the outer zone of the elevated platform (EPF) (Uschold-Schmidt et al. 2012) and exploring a novel object (Veenema et al. 2008) compared with unstressed control animals The increased anxiety-related behaviour of stressed mice might be induced by altered neuronal activity patterns of brain regions involved in emotionality, like the PVN, the LS, the dorsal CA3 subregion of the hippocampus as well as different subregions of the periaqueductal grey and of the NAc (Singewald et al. 2009). Surprisingly, 19 days of CSC did not affect depressive- and anhedonic-like behaviours as revealed in the tail suspension, forced swim and saccharine preference tests (Slattery et al. 2012).

Taken together, the CSC paradigm reliably induces profound physiological, neuroendocrine, immunological and behavioural alterations. Therefore, this animal model represents an adequate and pre-clinically relevant paradigm to investigate the mechanisms underlying the development of stress-related somatic as well as affective disorders. Is has to be emphasized that the CSC paradigm represents a sufficient model for studying the environmental effects as well as the genetic predisposition on the development of stress-related pathologies, since early life stress increases the vulnerability to develop stress-induced disorders (Veenema et al. 2008) and animals bred for different levels of innate anxiety display different stress-coping styles and stress resilience (Füchsl et al. 2013)

7 Aims of the present thesis

As already described, chronic exposure to psychosocial stress represents a high risk factor for the development of somatic, affective as well as substance abuse disorders. Although stress-related pathologies like cardiovascular diseases, cancer, burn-out syndrome, major depression, anxiety-related disorders and alcoholism became more and more prominent in our society. However, successful and effective medications are largely lacking. The central oxytocinergic system represents, in particular, a prominent target system for successful

medication of stress-induced pathologies. As mentioned above the CSC paradigm reflects a clinically relevant model to mimick chroinic stress effects and to study the underlying mechanisms in great detail. Thus, the initial aims of the present thesis were to explore in more detail the involvement of chronic psychosocial stress on the development of stress-related pathologies including colorectal cancer and alcohol abuse. The major goal of the current thesis was to investigate whether a chronic central OXT administration during psychosocial stress exposure is able to prevent/attenuate the establishment of physiological and behavioural alterations induced by CSC.

In **chapter 2** of my thesis, I aimed to investigate the effects of 19 days of CSC exposure on the development of a chemically-induced and inflammation-mediated colon carcinogenesis. Therfore, morpholocial alterations of the colon during repeated cycles of a DSS-indued colitis were analysed in SHC and CSC mice *via* colonoscopy. Proliferation and Apoptosis representing two hallmarks of carcinogenesis, those ratios were investigatet using immunohistochemistry as well as TUNEL labelling. In addition, expression levels of molecular markers known to be involved in proliferative and apoptotic prosesses were investigated by Western Bolt and TaqMan Realtime PCR. Finnaly, immunological alterations known to promote carcinogenesis were assessed.

The aim of **chapter 3** was to test the hypothesis whether 15 days of CSC increase the voluntary EtOH self-administration in mice, maybe due to an increased anxiety-related behaviour. In addition, due to anti-stress and anti-addictive properties of OXT following acute administration, the effects of an acute ip injection as well as a single central infusion on the EtOH intake was assessed.

The major goal of **chapter 4** was to investigate whether a chronic central OXT infusion during 19 days of CSC prevents or attenuates stress-induced physiological as well as behavioural changes. Therefor, the initial aim was to explore the effects of the implantation of an osmotic minipump (OMP) on stress-related parameters in SHC and CSC mice. Further, I aimed to investigate whether a chronic central OXT administration affects stress-related

parameters in a dose-dependent manner. To study the effects of a chronic central OXT administration on the endogenous OXT system, the OXT mRNA expression as well as the OXTR binding was assesed in different brain regions including the PVN, septal and amygdaloid subregions and the MeRN.

Chapter 2

Effects of chronic psychosocial stress on inflammation-related colon carcinogenesis

Sebastian Peters: Study design, performance of experiments and data analysis, writing the first draft of the manuscript

Nicole Grunwald: Protein expression analysis via Western Blot, mRNA expression analysis via TaqMan-qPCR, *in vitro* stimulation of mesLNCs

Petra Rümmele: Determination of colonic morphology, revision of manuscript

Esther Endlicher: Colonoscopy, revision of manuscript

Anja Lechner: Flow cytometrie, revision of manuscript

Inga D. Neumann: Study design, revision of manuscript

Florian Obermeier: Revision of manuscript

Stefan O. Reber: Study design, determination of histological damage score, revision of manuscript

[taken and partly adapted from Sebastian Peters, Nicole Grunwald, Petra Rümmele, Esther Endlicher, Anja Lechner, Inga D. Neumann, Florian Obermeier & Stefan O. Reber, 2012. Chronic psychosocial stress increases the risk for inflammation-related colon carcinogenesis in male mice. *Stress*. 15, 403-415]

Abstract

Patients with IBDs have a higher risk of developing CRC than general population. Furthermore, chronic psychosocial stress increases the likelihood of developing IBD and multiple types of malignant neoplasmas, including CRC. The aim of the study described in the following chapter was to investigate the effects of chronic psychosocial stress in male mice on an artificially induced CRC, by employing the CSC paradigm in combination with the reliable AOM/DSS/CRC model. Colonoscopy revealed that CSC mice showed accelerated macroscopic suspect lesions. In addition, more CSC mice developed low-grade dysplasia (LGD) and/or high-grade dysplasia (HGD) in the colonic tissue compared to the SHC animals. CSC mice showed an increased number of $Ki67^+$ and a decreased number of terminal deoxynucleotidyl transferase dUTP nick end labelling epithelial cells in colonic tissue. Colonic LRH-1, COXII, TNF, forkhead box 3 (FoxP3) mRNA as well as colonic β-catenin, COXII, and LRH-1 protein expression were also increased in CSC compared with SHC mice. Although the number of $CD4^+$ Th cells was increased, a tendency toward a decreased colonic IFN-γ mRNA expression was observed. Furthermore, despite an increased percentage of $CD3^+$ cells and $CD3^+$/$FoxP3^+$ double-positive cells within mesenteric lymph node cells of CSC mice, IFN-γ secretion from these cells was unaffected. Altogether, these results suggest that chronic psychosocial stress increases the risk for AOM/DSS-induced and, thus, inflammation-related CRC. Finally, assessment of additional time points may test whether the shift from tumor-protective Th1 cell to regulatory T-cell immunity represents a consequence of increased carcinogenesis or a causal factor involved in its development.

Introduction

Chronic stress, in particular chronic psychosocial stress, is a general burden of modern societies and an acknowledged risk factor for numerous disorders, including IBD (Bernstein et al. 2010) and cancer (Levav et al. 2000, Reiche et al. 2004). The concept of stress-promoted carcinogenesis and the hypothetical link between stress-induced suppression of cellular immunity and tumour progression is also suggested by animal studies. For example, a reduction in blood $CD4^+$ and $CD8^+$ T cells and in T-cell and natural killer cell activities following prolonged social (7-48 h) stress was paralleled by a significantly lower clearance of injected MADB 106 tumour cells (Stefanski and Ben-Eliyahu 1996, Stefanski 2000). In addition to these studies, there is also evidence indicating that stress exposure can promote spontaneous cancer development. Thus, repeated immobilization (6 h/day; 3 weeks) increased the susceptibility to UV radiation-induced development of squamous cell carcinoma (Saul et al. 2005). Importantly, this was again paralleled by a decrease in the number of protective $CD4^+$ Th1 cells and Th1 cytokine expression, both measured in dorsal skin tissue, as well as an increase in the number of blood regulatory T cells. CRC represents one of the most common types of cancer worldwide (Tanaka 2009). As no less than one-third of all human cancer is associated with inflammation, it is not surprising that the overall risk of CRC is 10-fold higher in IBD patients, a figure which rises to a 38-fold increase if IBD is diagnosed before the age of 30 (Yang et al. 2009). This is also supported by indirect evidence gathered from human (Ekbom et al. 1990, Choi and Zelig 1994, Rutter et al. 2004) and mouse studies (Chulada et al. 2000, Pouyet et al. 2010), showing that dampening colonic inflammation ultimately reduces tumour incidence. CRC, in most cases, is only diagnosed at an advanced stage (O'Shaughnessy et al. 2002) and poses one of the most serious complications in patients with IBD (Eaden and Mayberry 2000, Eaden et al. 2001, van Hogezand et al. 2002). These observations are most likely due to a lack of appropriate biomarkers and insight into CRC pathogenesis (O'Shaughnessy et al. 2002). Using a novel colitis-related mouse model (Tanaka et al. 2003), in which CRC is initiated with AOM and promoted by repeated cycles of DSS administration, initial steps toward a better

understanding of the pathogenesis of IBD-related CRC have been taken (Tanaka 2009). CRC is initiated by abnormal growth patterns, called LGD or HGD, and/or uncontrolled growth of the initiated (either naturally occurring or AOM-induced) cryptal cells, followed by the formation of adenomatous polyps (neoplasms). After a prolonged time period, these eventually evolve into cancer (malignant neoplasm; (Xie and Itzkowitz 2008)). However, although it is known that IBD increases the risk for CRC and that perceived life stress is involved in the development/progression of both IBD and cancer in general, to the best of your knowledge the influence of environmental factors, such as chronic psychosocial stress, on the development of inflammation-related CRC has not been addressed to date. Exposure to CSC a clinically relevant animal model of psychosocial stress (Reber et al. 2007, Reber and Neumann 2008, Reber et al. 2008, Veenema et al. 2008, Singewald et al. 2009, Schmidt et al. 2010, Reber et al. 2011) results in spontaneous colitis (Reber et al. 2007, Reber 2011) and aggravates DSS-induced (Reber et al. 2008, Veenema et al. 2008) colitis. Moreover, DSS colitis has been shown to promote AOM-induced colon carcinogenesis depending on its severity (Suzuki et al. 2005). Therefore, the aim of the study described in this chapter was (i) to test the hypothesis that 19 days of CSC increase the risk for inflammation-related CRC and (ii) to dissect the underlying mechanisms. In terms of aim (i), development of HGD and/or LGD was assessed, as dysplasia occurring in adenomas is considered to be the selective marker for the increased risk for colorectal malignancies (Tanaka 2009). With respect to aim (ii), β-catenin signalling pathway causes gastrointestinal tumor development (Oshima et al. 1995) and as LRH-1 has been shown to be involved in the control of intestinal cell renewal (Botrugno et al. 2004) and in the promotion of CRC (Schoonjans et al. 2005). Furthermore, the number of F4/80$^+$ colonic macrophages as well as TNF and COXII mRNA and/or protein expression were quantified, as macrophage-derived TNF has been shown to promote tumour development in gastric mucosa (Oguma et al. 2008) and as COXII is considered to contribute to tumour development by modulating apoptosis, angiogenesis, and tumour invasiveness (McConnell and Yang 2009). As an impaired clearance of abnormal cells by immune competent cells might also be involved in the promotion of AOM/DSS-

induced CRC, we assessed the number, and cytokine profile (FoxP3 and IFN-γ), of Th cells in colonic and mesenteric lymph node (mesLN) tissue in CSC and SHC mice.

Material and Methods

Animals

Male C57BL76 mice (Charles River, Sulzfeld, Germany) weighing 19-22 g (5–6 weeks of age; 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 CSC procedure started. All mice were kept under standard laboratory conditions (12-h light/dark cycle, lights on at 06:00 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 the international guidelines on the ethical use of animals. All efforts were made to minimize the number of animals used and their suffering.

Experimental procedure and AOM/DSS model of CRC

In order to investigate whether chronic psychosocial stress influences the severity of AOM/DSS- induced CRC, mice were split into two weight-matched groups, a control (n = 11) and a chronic stress group (n = 12). Mice of both groups were injected (ip) with 10 mg/kg AOM (74.08 kDa, Fa. Sigma-Aldrich, Steinheim, Germany) 6 days prior (day -6) to the start (day 1) of chronic psychosocial stress induced by 19 days of CSC. On day 20, CSC mice were single housed. Both groups received DSS (36-50 kDa, ICN Biomedicals, cat. No. 160110, Eschwege, Germany) via drinking water (Obermeier et al. 1999): on days 50-57 (1% DSS), 121-131 (0.5% DSS), and 163-167 (1% DSS). Importantly, three CSC mice died during the first DSS cycle. The progression of colon carcinogenesis was monitored in the same two mice per group, employing colonoscopy on days 80, 117, and 160. These mice

were not included in the analysis of any other readout parameter assessed in the study. On day 186, mice of both groups were killed by decapitation following CO_2 anaesthesia for assessment of colon carcinogenesis and immunological status at the level of the colonic and draining mesLN tissue. CSC has been shown to cause and to aggravate DSS-induced colitis. As AOM-induced carcinogenesis has been shown to be promoted by subsequent inflammation, CSC and DSS were given subsequent to AOM application.

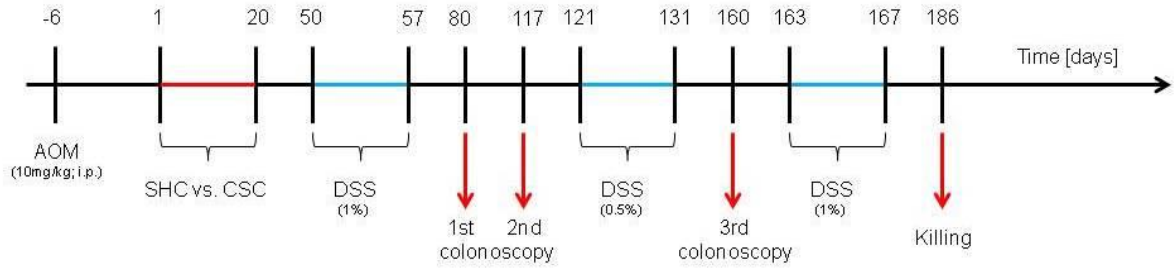

Figure 14: Schematic illustration of the experimental procedure. Following AOM injection (10mg/kg); i.p.) on day -6, mice were either exposed to CSC housing (n = 12) or kept as SHCs (n = 11) from day 1 to 20. Subsequently, both SHC and CSC mice were treated with 1% DSS from day 50 to 57, with 0.5% DSS from day 121 to 131, and with 1% DSS from day 163 to 167. Importantly, three CSC mice died during first DSS cycle. Macroscopic changes were monitored by three colonoscopies performed on day 80, 117, and 160 in the same two narcotized mice of each group. On day 186, all mice were killed. Mice that underwent colonoscopy were not included in the analysis of any other readout parameter.

CSC housing

The CSC paradigm was conducted as described previously (Reber et al. 2007, Reber and Neumann 2008, Reber et al. 2008, Veenema et al. 2008, Singewald et al. 2009). Briefly, four experimental CSC mice were housed together with a larger dominant male for 19 consecutive days as a chronic stressor. Before the CSC procedure, the future dominant males were tested for their aggressive behaviour. Males that started to injure their opponents by harmful bites were not used. To avoid habituation during the chronic stressor exposure, each larger male was replaced by a novel larger male on days 8 and 15. Following prior

studies, SHC were considered as most appropriate controls in this paradigm (Singewald et al. 2009). This finding is in agreement with others showing that in the male mouse, single housing is less stressful than group housing (Misslin et al. 1982, Bartolomucci et al. 2003, Gasparotto et al. 2005).

Colonoscopy

The progression of colon carcinogenesis was monitored by colonoscopy employing the Coloview miniendoscope (STORZ GmbH & Co. KG, Tuttlingen, Germany) on days 80, 117, and 160, performed as previously described (Becker et al. 2004). To avoid exposure of all mice to the additional stressor of anaesthesia and colonoscopy, the same two mice of each group were narcotized by a ketamin-xylacin (100 mg/kg ketamin and 7 mg/kg xylacin; Betapharm, Augsburg, Germany) injection (i.p.; 120 µl/mouse) 10-15 min prior to the start of each colonoscopy. These mice were randomly selected. During colonoscopy an experienced gastroenterologist blind to treatment group assessed whether macroscopic suspect lesions, i.e. granular mucosa and/or flat polypoid lesions/polyps were present in none (indicated by “0”), one (indicated by “1”), or both (indicated by “2”) mice per treatment group. Importantly, mice that underwent repeated colonoscopy were not included in analysis of any other readout parameter assessed in the study described in this section.

A) B)

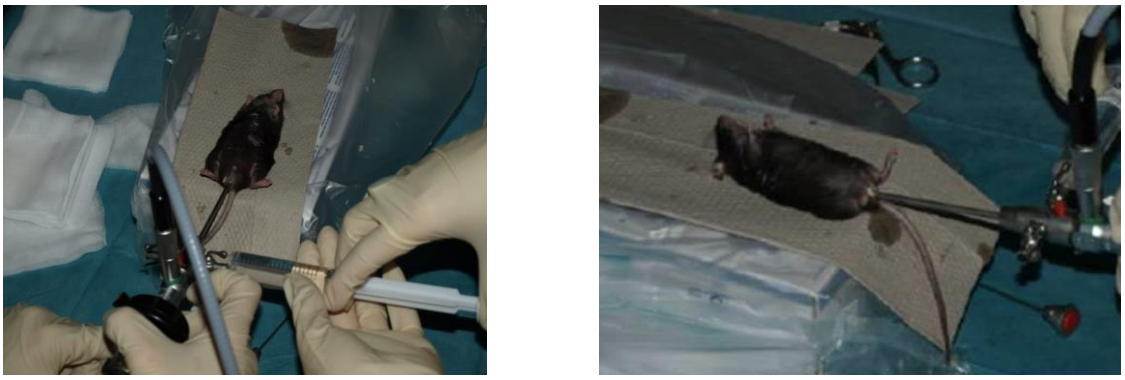

Figure 15: Representative images of C57BL/6 mouse during colonoscopy.

Immunohistochemistry/immunofluorescence

After killing (by decapitation following CO_2 anesthesia), the mesLNs and the colon were removed. The mesLNs were separated from fat and one lymph node (LN) per mouse was embedded in histological tissue-freezing medium and snap frozen in liquid nitrogen. The other LNs were used for mesLNC stimulation. The colon was mechanically cleaned and rinsed with phosphate buffered saline (PBS). Then a segment 0.5 cm from the distal colon was cut longitudinally, embedded in histological tissue-freezing medium, and snap frozen in liquid nitrogen. The following day the frozen mesLNs and the colonic tissues were cryosectioned (longitudinally for the colon). After further tissue collection for mRNA and western blotting, the rest of the colon was spirally coiled, laid on a filter paper, and fixed in 10% formalin overnight. The next day the fixed tissue was embedded in paraffin and cut longitudinally. Ki67 immunohistochemistry (IHC) was performed in paraffin sections (one 6-µm section) and frozen sections (three 6-µm sections taken 100µm apart). F4/80 and CD4 IHC were performed in frozen mesLN tissue only. Paraffin sections (colon) were deparaffinised and demasked by heating in citrate buffer (10 mM, 40 min) in a microwave oven, and frozen sections (colon and mesLN) were fixed with acetone [10 min, room temperature (RT)]. Afterwards, for IHC, deparaffinised and frozen sections were incubated with 0.3% H_2O_2 (30 min, RT), washed with PBS (10 min, RT; F4/80, CD4) or phosphate buffered saline tween 20 (PBS-T) (10 min, RT; Ki67), and blocked with 5% goat serum for 30

min. For immunofluorescence, this was followed by incubation with 150 µl of a mixed 5 µg/ml hamster anti-mouse CD3 (BD Biosciences) and 2.5 µg/ml rat anti-mouse FoxP3 (BD Biosciences) solution (in 20% FCS) overnight at 4°C. After washing with PBS (three times, 5 min, RT), IHC slices were incubated with the respective biotynilated goat anti-rat secondary antibodies (1:500 in 5% goat serum; Jackson ImmunoResearch; Suffolk, UK) for 1 h at RT and washed with PBS (three times, 5 min, RT). Immunofluorescence slices were incubated with biotinylated goat anti-hamster secondary antibodies (1:2500 in PBS; Jackson ImmunoResearch). Respective positive cells were visualized either by the use of a Vectastain ABC Kit followed by Vector NovaRed Substrate Kit (Vector Laboratories, Leorrach, Germany) for IHC or by the use of a mixture of streptavidin-coupled Alex-Flour 594 (1:500 in PBS; Invitrogen GmbH, Darmstadt; Germany) and donkey anti-rat Alexa Flour 488 antibodies (1:200 in PBS; Invitrogen GmbH) for immunofluorescence. Afterwards, immunofluorescence slices were washed again with PBS (three times, 5 min, RT) and mounted using ProLong Gold antifade reagent with 4´,6-Diamidin-2-phenylindol (DAPI) (Invitrogen GmbH). In frozen colon slices the number of $Ki67^+$ (absolute proliferation), $CD4^+$, and $F4/80^+$ cells per slice (averaged from three cross-sectioned slices per mouse) and in paraffin colon slices the number of $Ki67^+$ cells per crypt in LGD/HGD and normal tissue (relative proliferation; averaged from 10 cross-sectioned crypts per mouse) were determined. In frozen mesLN slices, the absolute number of double-stained $CD3^+/FoxP3^+$ cells per 25.600µm^2 of the T-cell-rich interfollicular area of each LN was assessed and averaged from three slices per mouse (taken 100 µm apart). Isotope controls were used in each approach to verify specificity.

Terminal deoxynucleotidyl transferase dUTP nick end labelling

Three 6-µm cross sections from frozen colon tissue per mouse (taken 100 µm apart) were used for Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL; Promega, Mannheim, Germany) according to the manufacturer´s recommendation. Labeled apoptotic

epithelial nuclei were counted per mm^2 in three fields of view per cross section (fluorescein filter, 465-495 nm) and averaged per mouse. The mean + SEM per group was calculated and presented in the graphs. Positive controls (DNase I; Roche Diagnostics GmbH, Mannheim, Germany) and negative controls [no TdT enzyme] were used in each approach to verify specificity.

Quantitative real-time polymerase chain reaction using TaqMan technology

Quantitative real-time polymerase chain reaction (qRT-PCR; (Bustin et al. 2009)) was performed as described previously (Blaas et al. 2009). Briefly, after killing (by decapitation following CO_2 anesthesia) about 0.5 cm (segment proximal to colon tissue used for ICH and immunofluorescence) of the removed, PBS rinsed, and mechanically cleaned colon was placed in RNA later (Applied Biosystems, Foster City, CA, USA) and stored at -20°C until assayed. Total RNA was isolated using the RNeasy MiniKit (Qiagen, Hilden, Germany) and reverse transcribed into first-strand cDNA (Reverse Transcription System; Promega). Expression levels of IFN-γ, TNF, FoxP3, LRH-1, β-catenin, and COXII were then quantified by TaqMan®-qPCR (ABI PRISM 7900 HT Sequence Detection System; Applied Biosystems), as single-tube reactions (20 µl) in 384-well plates. IFN-γ forward: TGCTGATGGGAGGAGATGTCT, IFN-γ reverse: TGCTGTCTGGCCTGCTGTTA; TNF forward: CACAAGATGCTGGGACAGTGA, TNF reverse: TCCTTGATGGTGGTGCATGA; Foxp3 forward: GTGGGCACGAAG GCAAAG; Foxp3 reverse: CCTTGT TTTGCGCTGAGAGTCT; LRH-1 forward: TTGAGTGGGCCAGGAGTAGT, LRH-1 reverse: ATCAAGAGCTCACTCCAGC AGTT; ß-catenin forward: GGTCCGAGCTGCCATGTTCC, ß-catenin reverse: CGTCAAACTGCGTGGATGGG; COX II forward: GGCAAAGGCCTCCATTGACC, COX II reverse: GAGAAGCTGTTGCGGTACTCA. The probes were labelled 5′ with 6-carboxy-fluorescein (FAM) and 3′ with 6-carboxytetramethyl-rhodamine (TAMRA). TaqMan®-qPCR was performed using 1 µl cDNA, 1 µl forward and reverse primer each (18 µM), 1 µl probe (5 µM), 10 µl *Taq*Man Mastermix (Applied

Biosystems, Foster City, CA, USA), 1 µl glyceraldehyde-3-phosphatedehydro-genase (GAPDH)-Mix (served as reference; Applied Biosystems, Foster City, CA, USA), and made up to the final volume of 20 µl with sterile H_2O. Cycling was as follows: 50°C for 2 min, 95 °C for 10 min followed by 40 repeats: 95 °C for 15 s and 60 °C for 1 min. mRNA expression for each gene was quantified for each individual mouse in triplets and averaged per mouse.

Western Blotting

After killing (decapitation following CO_2 anesthesia) about 0.5 cm (proximal to colon tissue used for qRT PCR) of the removed, PBS rinsed, and mechanically cleaned colon was immediately shock-frozen in liquid nitrogen and stored at -80 °C until assayed. For protein extractions frozen colonic tissue was homogenized in ethylendiamin tetraacetic acid (EDTA) lysis buffer (50mM EDTA, 250 mM NaCl, 0.5 mM 2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethansulfonsäure (HEPES), 0.5% Igepal, 10% Complete Mini Protease Inhibitor (Roche Diagnostics GmbH, Mannheim Germany)) and total protein concentration was determined using a commercial kit (Bicinchoninic Acid Protein Assay Kit, Thermo Scientific, Rockford, USA). Western blot analysis was carried out using 60 µg of protein per animal. Samples were loaded on sodium dodecyl sulfate-polyacrilamide gels (7.5%) and transferred on nitrocellulose membranes. The membranes were then blocked for 2 h in 5% milk (COX II, ß-catenin) or for 1.5 h in 5 % bovine serum albumin (BSA)(LRH-1), both diluted in Tris-buffered saline (TBS) with 0.05% Tween 20 (TBST, Sigma-Aldrich, Milan, Italy), at 22–24°C before being probed with primary anti-ß-catenin antibody, anti-COX II antibody, (1 : 400; Santa Cruz Biotechnology, Inc., Heidelberg, Germany), or anti-LRH-1 antibody (1 : 200; Santa Cruz Biotechnology, Inc., Heidelberg, Germany), applied overnight at 4°C. Visualisation was performed using horseradish peroxidase-conjugated whole donkey anti-rabbit antibodies (1 : 1000; GE Healthcare, Freiburg, Germany) followed by ECL Western Blotting Detection Reagents (GE Healthcare, Freiburg, Germany). Immunoblots were digitized using Molecular Imager® ChemiDoc™ XRS+ system and analysed using Image Lab™ software (Bio Rad

Laboratories GmbH, München, Germany). Afterwards each membrane was stripped using Re-Blot Plus Mild Antibody Stripping Solution (Millipore GmbH, Schwalbach, Germany), blocked twice with 5 % milk in TBST for 5 min, probed with primary rabbit anti-ß-tubulin antibodies (1 : 1000, Cell Signaling Technology, New England Biolabs GmbH, Frankfurt am Main, Germany) for 2 h. Visualization, digitization, and analysis was performed as described above. ß-catenin (92 kDa), COX II (61 kDa), and LRH-1 (70 kDa) protein expression of each mouse was normalized to ß-tubulin (50 kDa) expression and averaged per group.

Determination of morphological changes in the colon

After tissue collection for mRNA and western blotting and IHC, the rest of the colon was spirally coiled up, laid on a filter paper, and fixed in 10% formalin overnight. The next day the fixed tissue was embedded in paraffin and cut longitudinally (6µm sections). Haematoxylin-eosin stained sections were evaluated by an experienced pathologist with respect to the development of LGD and HGD. For statistics, the percentage of mice developing either LGD and/ or HGD was compared between the SHC and CSC group.

Isolation/incubation of mesLNCs

Isolation/incubation of mesLNCs was performed as previously described (Reber et al. 2007, Veenema et al. 2008). Briefly, mesLNs from SHC and CSC mice were collected, separated from fat, and after freezing one LN of each mouse mouse in histological tissue-freezing medium for immunofluorescence, the remainder were pooled (three pools per treatment group), respectively, from two to three mice of the same treatment group. From all three pools (per treatment group), cells were isolated and incubated in quadruplicates for 24 h (200.000 cells, 37°C, 5%) in anti-CD3-coated wells in the presence of IL-2 as described previously (Reber et al. 2006, 2007). After incubation, supernatants of all four wells were harvested; IFN-γ levels were measured by ELISA (Endogene, Woburne, MA, USA); and the

values were averaged for each individual pool. Consequently, for each group, the mean + SEM was calculated from these averaged pool data and presented in the graphs.

Flow cytometrie

Flow cytometrie analysis was performed as described earlier (Obermeier et al. 2006) using a Beckman Coulter EPICS® XL-MCL flow cytometer (Coulter Immunotech, Hamburg, Germany). Briefly, isolated and pooled mesLNC (all mice per treatment group) were surface stained with fluorescein isothiocyanate (FITC)-conjugated rat IgG2a anti-CD3 nd respective isotype control (rat IgG2a) antibodies (BD Biosciences) to quantify the percentage of $CD3^+$ cells within all gated cells.

Statistics

For statistical comparisons, the software package SPSS (version 12) was used. All parameters depending on just one factor were analyzed using the two-tailed Student´s *t*-test, with the exception of the morphological changes in colonic mucosa (Fisher´s exact test) and IFN-γ secretion from mesLNC (Mann-Whitney *U* test). Epithelial cell proliferation was analyzed using a two-way analysis of variance (ANOVA; factor treatment group and factor dysplasia), followed by Bonferroni *post hoc* pairwise comparisons if appropriate. Repeated colonoscopy was done in only two mice (always the same) per treatment group, so no statistical analysis of the macroscopic suspect lesions was performed. Data are presented as mean + SEM. Significance was taken at $p \leq 0.05$.

Results

Effects of CSC on body weight development

Following AOM treatment, CSC mice (2.09 + 0.20 g, n = 12) gained significantly less body weight during the 19 days of CSC exposure (p = 0.017; two-tailed Student´s *t*-test) compared with respective SHC control mice (2.91 + 0.24 g, n = 11). No differences in the body weight change between SHC and CSC mice were observed during any of the three subsequent DSS cycles. However, three CSC mice died during the first cycle of DSS application and have therefore been included only in the analysis of body weight development during CSC.

Effects of CSC on the development of macroscopic suspect lesions in the colon

Data from three colonoscopies repeatedly performed in two mice of each treatment group indicated that CSC mice develop earlier granular mucosa and flat polypoid lesions/polyps compared with SHC mice.

A)

Day of colonoscopy	Day 80		Day 117		Day 160	
Treatment group	SHC	CSC	SHC	CSC	SHC	CSC
Granular mucosa	0	2	0	2	2	2
Flat polypoid lesions /polyps	0	2	0	2	0	2

B) C) D)

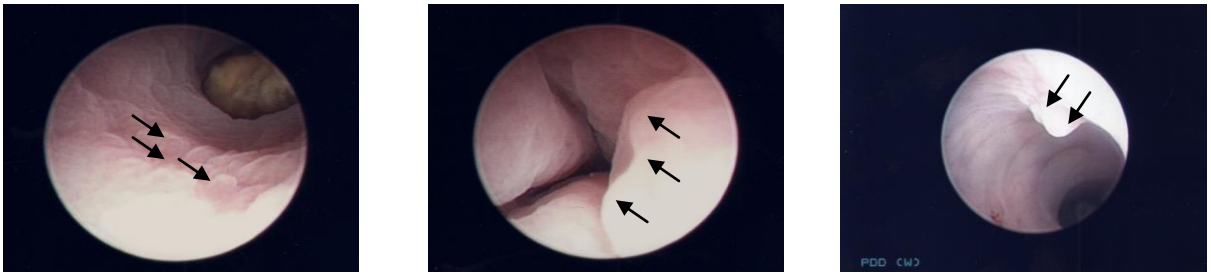

Figure 16: Effects of chronic psychosocial stress on the development of macroscopic lesions in the colon. Following injection of AOM (10mg/kg; i.p.) on day -6, mice were either exposed to CSC housing or kept as SHCs from day 1 to 20. Subsequently, both SHC and CSC mice were treated with three cycles of DSS, and macroscopic changes were monitored by three colonoscopies performed on day 80, 117, and 160 in the same two narcotized mice of each group (n = 2 per group). An experienced gastroenterologist assessed whether granular mucosa (indicated by black arrows in B) and/or flat polypoid lesions (indicated by black arrows in C)/polyps (indicated by black arrows in D) were present in none (0), one (1), or both (2) mice of each group (no statistical analysis was performed to the low number of mice per group). As these mice were exposed to the additional stressor of repeated anesthesia and colonoscopy, they were not included in the assessment of any other readout parameter in the current study.

Effects of CSC on colonic morphology and relative epithelial cell proliferation

AOM/DSS-treated CSC mice tended to develop more often LGD and/or HGD in colonic tissue compared with respective SHC mice ($p = 0.069$; Fisher´s exact test; Fig. 17A). In addition, a significantly increased number of $Ki67^+$ epithelial cell proliferation in dysplastic compared with normal tissue (factor dysplasia $F_{1,22} = 363.997$; $p < 0.001$; two-way ANOVA, factor treatment group and factor dysplasia; Fig. 17F).

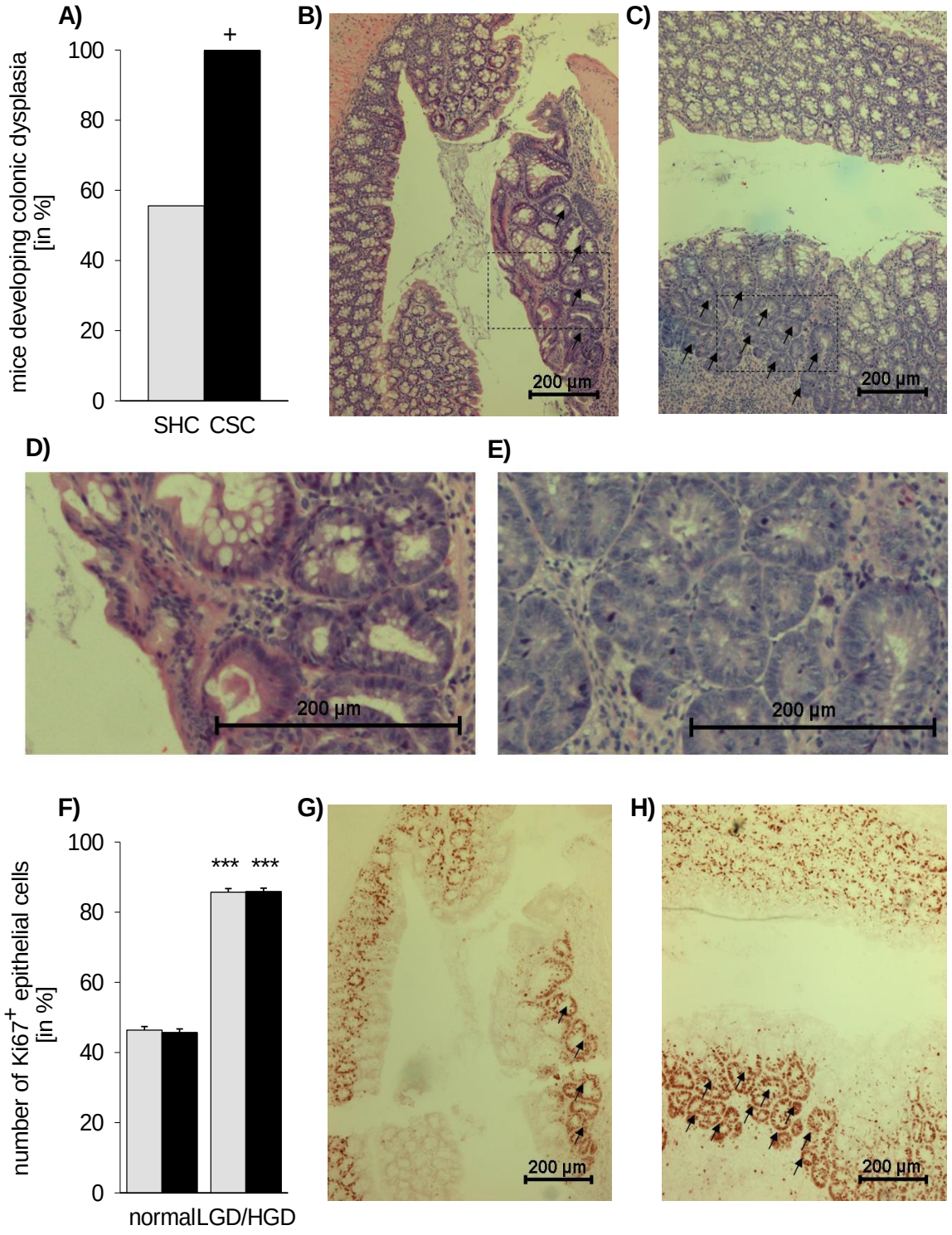

Figure 17: Effects of chronic psychosocial stress on colonic morphology and relative epithelial cell proliferation. Both SHC (n = 9, gray bars) and CSC housing (n = 7, black bars) mice were killed on day 186 following three cycles of DSS administration. (A) Hematoxylin-eosin stained paraffin sections of the distal colon were evaluated by ab experienced pathologist with respect to the development of low-grade dysplasia (LGD; black arrows in B; section surrounded by the rectangle in (C) is shown in higher

magnification in €. Data represent percentage of mice developing colonic dysplasia (HGD and/or LGD; $^{+}p = 0.069$ vs. respective SHC mice; Fisher´s exact test). (F) Relative epithelial cell proliferation was assessed in Ki67-stained paraffin sections by determining the percentage of $Ki67^{+}$ cells per cross-sectioned crypt in non-dysplastic tissue (normal, G&H) and LGD (indicated by arrows in H). Data represent mean + SEM. $^{***}p < 0.001$ vs. respective non-dysplastic (normal) tissue mice (two-way ANOVA, factor treatment group and factor dysplasia).

Effects of CSC on colonic mRNA expression patterns

Compared with AOM/DSS-treated SHC mice, colonic mRNA expression for COXII ($p = 0.025$; two-tailed Student´s *t*-test; Fig. 18E), LRH-1 ($p = 0.027$; two-tailed Student´s *t*-test; Fig. 18F), FoxP3 ($p = 0.038$; two-tailed Student´s *t*-test; Fig. 18B), and TNF ($p = 0.040$; two-tailed Student´s *t*-test; Fig. 18C), were significantly increased in respective CSC mice. Furthermore, IFN-γ mRNA expression in the colonic tissue of AOM/DSS-treated CSC mice tended to be reduced ($p = 0.063$ vs. SHC; two-tailed Student´s *t*-test; Fig18A). Colonic mRNA expression of β-catenin was not affected by CSC (Fig. 18D).

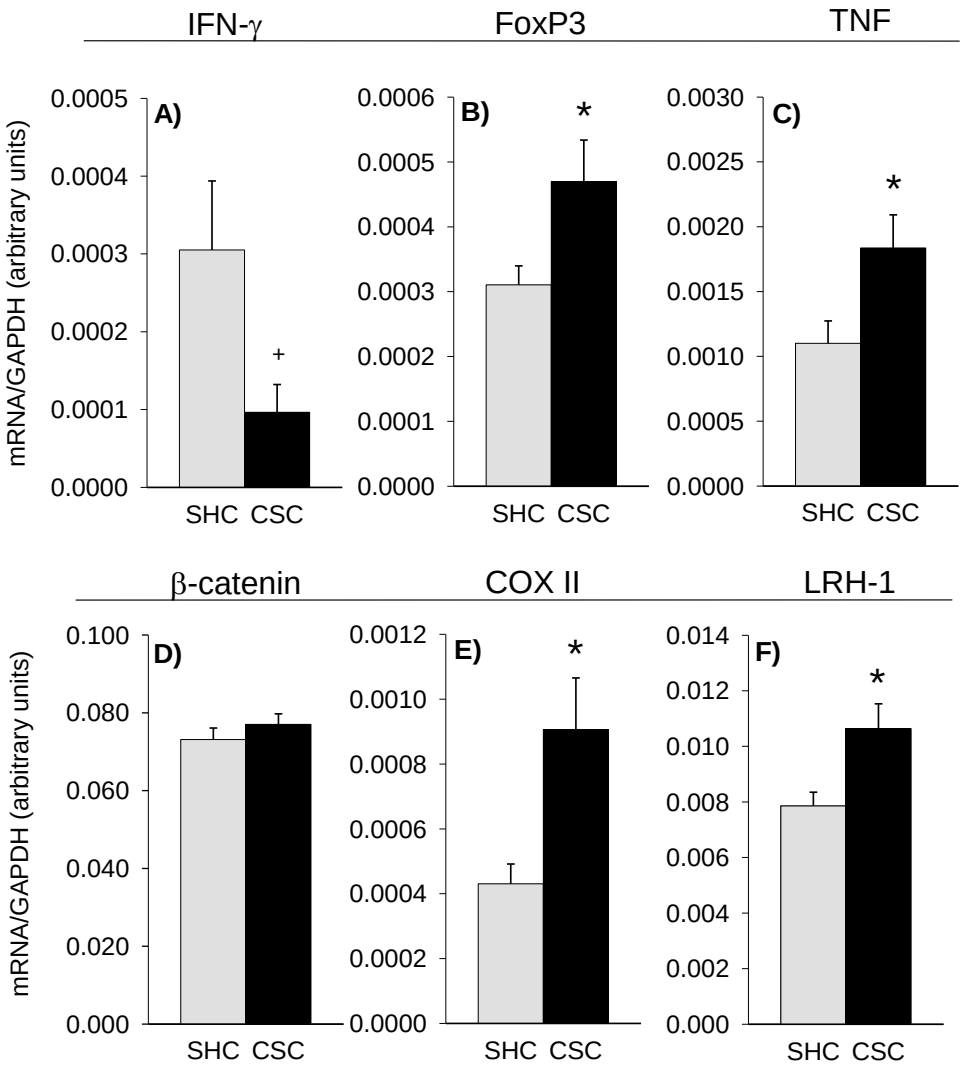

Figure 18: Effects of chronic psychosocial stress on colonic IFN-γ, FoxP3, TNF, β-catenin, COXII, and, LRH-1 expression patterns. Both SHC (n = 9, grey bars) and CSC housing (n = 7, black bars) mice were killed on day 186 following three cycles of DSS administration. After killing mRNA expression levels IFN-γ (SHC: n = 9, CSC: n = 7; A), FoxP3 (SHC: n = 8, CSC: n = 6; B), TNF (SHC: n = 7, CSC: n = 6; C), β-catenin (SHC: n = 7, CSC: n = 6; D), COXII (SHC: n = 7, CSC: n = 6; E), and LRH-1 (SHC: n = 9, CSC: n = 6; F) were quantified by TaqMan®-qPCR (relative to the expression of the housekeeping gene GAPDH). The mRNA expression for each gene was quantified for each individual mouse in triplets and averaged per mouse. Data represent mean + SEM; $^{*}p < 0.05$, $^{+}p = 0.063$ vs. respective SHC mice (two-tailed Student´s *t*-test).

Effects of CSC on colonic protein expression patterns

Colonic protein expression of β-catenin (p = 0.028; two-tailed Student´s *t*-test; Fig. 19A); COXII (p = 0.022; two-tailed Student´s *t*-test; Fig19B), and LRH-1 (p = 0.016; two-tailed Student´s *t*-test; Fig19C) were significantly increased in CSC compared with SHC mice, both treated with AOM/DSS.

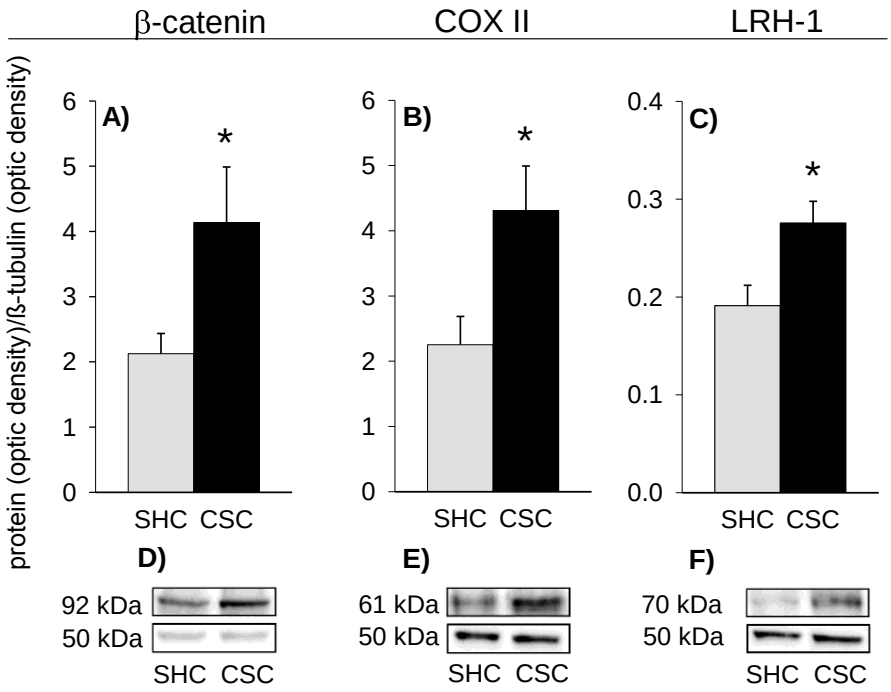

Figure 19: Effects of chronic psychosocial stress on colonic β-catenin, COXII, and LRH-1 protein expression patterns. Both SHC (n = 9, grey bars) and CSC housing (n = 7, black bars) mice were killed on day 186 following three cycles of DSS administration. After killing protein (tissue was collected proximal to tissue used for qRT-PCR) expression levels of β-catenin (SHC: n = 9, CSC: n = 7; A,D), COXII (SHC: n = 8, CSC: n = 6; B,E), and LRH-1 (SHC: n = 7, CSC: n = 6; C,F) were quantified by western blotting (relative to the expression of the housekeeping gene product β-tubulin). Figures D-F show representative Western blot images of β-catenin (D, upper bands; 92kDa), COXII (H, upper bands; 61kDa), LRH-1 (F, upper bands; 70kDa), and the corresponding β-tubulin (D-F, lower bands) protein expression of one SHC and one CS mouse per treatment group. Data represent mean + SEM; *p < 0.05 vs. respective SHC mice (two-tailed Student´s *t*-test),

Effects of CSC on epithelial apoptosis, absolute epithelial cell proliferation and the number of CD4$^+$ and F4/80$^+$ cells in the colonic tissue

The number of TUNEL$^+$ (p = 0.050; two-tailed Student´s *t*-test; Fig. 20A) epithelial cells per cross-sectioned slice was significantly decreased, whereas the number of Ki67$^+$ (p = 0.001; two-tailed Student´s *t*-test; Fig. 20B) epithelial cells per cross-sectioned colon slice was significantly increased in AOM/DSS-treated CSC compared with respective SHC mice, indicating decreased epithelial apoptosis and enhanced absolute epithelial cell proliferation in CSC mice. In addition, AOM/DSS-treated CSC mice showed a significantly increased number of CD4$^+$ Th cells (p = 0.038; two-tailed Student´s *t*-test; Fig. 20C) per cross-sectioned slice, whereas the number of colonic F4/80$^+$ macrophages cells was not different between CSC and SHC mice (Fig. 20D), both treated with AOM/DSS.

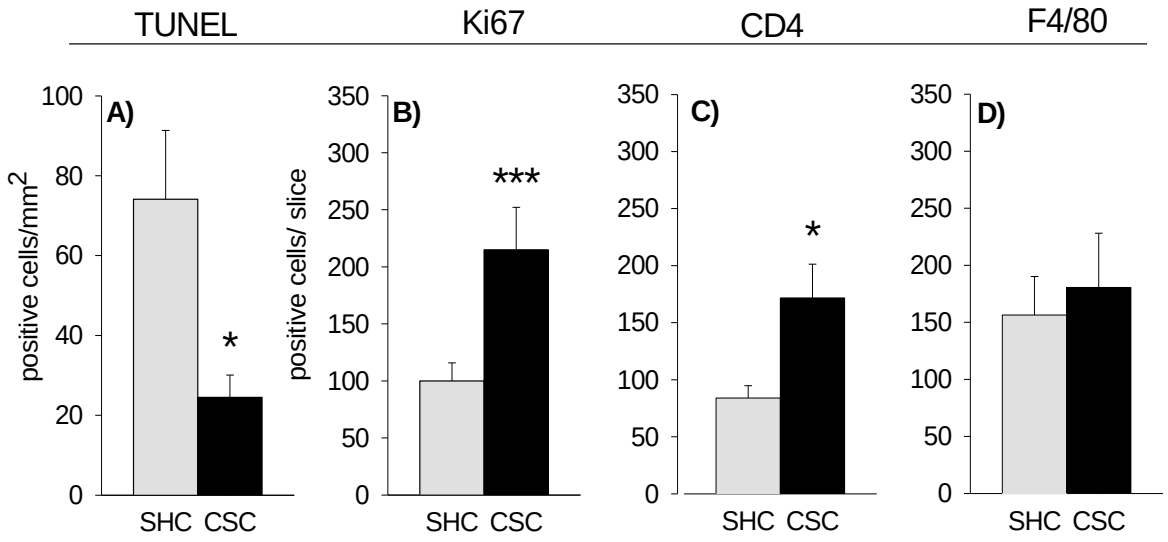

Figure 20: Effects of chronic psychosocial stress on epithelial apoptosis, absolute epithelial cell proliferation, and the number of CD4$^+$ and F4/80$^+$ cells in the colonic tissue. Both SHC (n = 9, grey bars) and CSC housing (n = 7, black bars) mice were killed on day 186 following three cycles of DSS administration. After killing, the colon was removed, mechanically cleaned, rinsed, embedded in histological tissue-freezing medium, snap-frozen, and cut on a cryostat. Three 6µm cross sections taken 100 µm apart were acetone fixated and afterwards stained for epithelial apoptosis (TUNEL; SHC: n =8, CSC: n = 5; A), absolute epithelial proliferation (Ki67; SHC: n = 9, CSC: n = 7; B); and the

number of $CD4^+$ (SHC: n = 7, CSC: n = 5; C) or $F4/80^+$ (SHC: n = 8, CSC: n = 6; D) cells. Respective positive cells were counted and averaged from the three cross-sectioned slices per mouse (Ki67, CD4, F4/80) or counted per mm^2 of colon tissue in three fields of views in each cross section and averaged per mouse (TUNEL). Data represent mean + SEM; $^+p < 0.05$ vs. respective SHC controls (two-tailed Student´s *t*-test).

Effects of CSC on the percentage of $CD3^+$ mesLNC, the in vitro IFN-γ secretion from isolated mesLNC, and the number of $CD3^+/FoxP3^+$ mesLNC

The percentage of $CD3^+$ cells within all pooled (per treatment group) cells isolated from mesLNs was found to be increased by 20% in AOM/DSS-treated CSC compared with respective SHC mice (Fig. 21A.; no statistical analysis was done as all mice per group were pooled). However, IFN-γ secretion from isolated and anti-CD3-stimulated mesLNC did not differ between SHC and CSC mice (Fig. 21B), both treated with AOM/DSS. Importantly, the number of regulatory $CD3^+FoxP3^+$ mesLNC was significantly higher in AOM/DSS-treated CSC (Fig. 21D) compared with respective SHC (Fig. 21E) mice ($p = 0.043$; two-tailed Student´s *t*-test; Fig. 21C.).

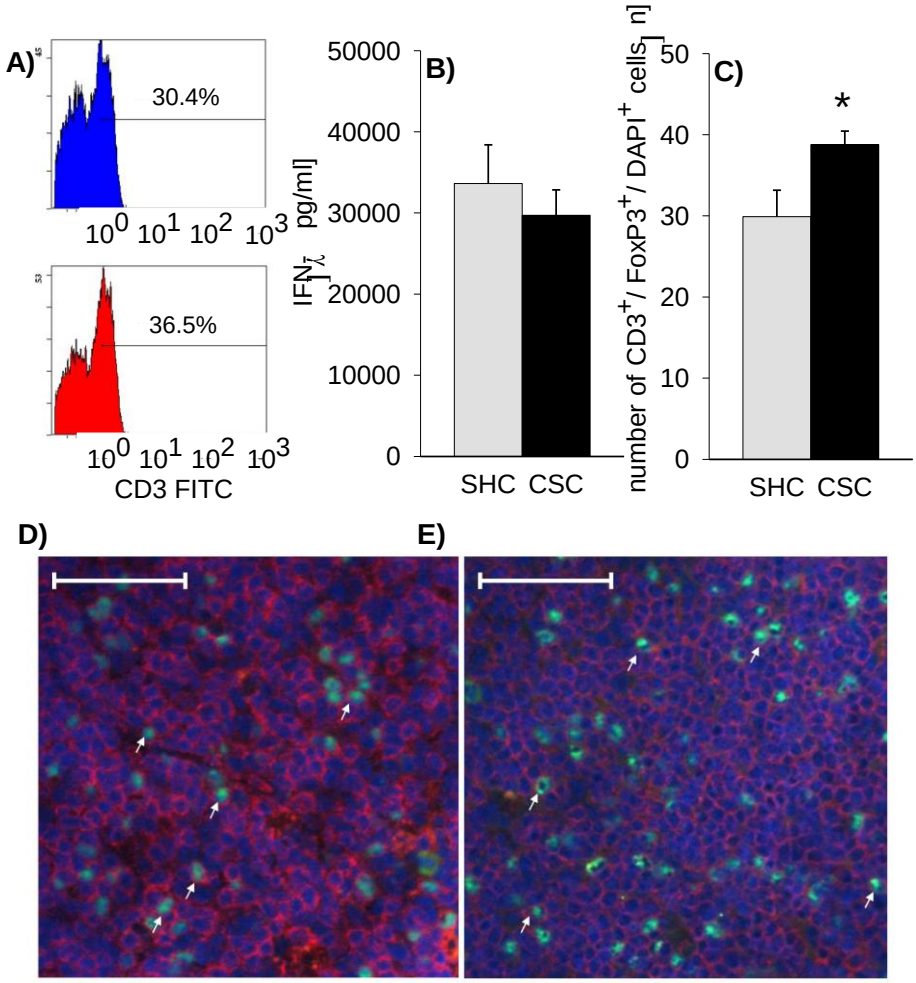

Figure 21: Effects of chronic psychosocial stress on the percentage of $CD3^+$ mesLNC, the *in vitro* IFN-γ secretion from isolated mesLNC, and the number of $CD3^+$/$FoxP3^+$ mesLNC. Both SHC (n = 9, grey bars) and CSC housing (n = 7, black bars) mice were killed on day 186 following three cycles of DSS administration. After killing, mesLNC were either double-stained for $CD3^+$ (red)/$FoxP3^+$ (green) using immunofluorescence (C; SHC, D; CSC, E; white arrows indicate representative double-positive cells in each slice) or mesLNC were isolated and pooled (three pools per treatment group containing 2-3 mice each) for assessment of anti-CD3-induced IFN-γ secretion (B). Remaining cells of all three pools were again pooled and surface stained with FITC-conjugated rat IgG2a anti-CD3 and respective isotype control (rat IgG2a) antibodies to quantify the percentage of $CD3^+$ cells within all gated cells (A; no statistical analysis was done as all mice per group were pooled) using flow cytometry. IFN-γ data represent mean + SEM of three pools (2 – 4 mice per pool; Mann-Whitney *U* test) per treatment group. Numbers of $CD3^{+/}FoxP3^+$ mesLNC represent mean + SEM of individual mice ($^*P < 0.05$; two-tailed Student´s *t*-test). Scale bars represent 50 µm (SHC, D; CSC, E).

Discussion

In the present study we demonstrate that chronic psychosocial stress increases the risk for inflammation-related CRC triggered by AOM/DSS. This was indicated by more frequent colonic dysplasia and enhanced expression of colonic β-catenin, LRH-1, COXII, and TNF. Moreover, absolute epithelial cell proliferation was increased in the colonic tissue of chronically stressed mice. However, assessment of further time points will be essential to test whether the shift from tumor-protective Th1 cell to regulatory T-cell immunity represents a consequence of increased CRC or a causal factor in its development. In our study, all mice were injected with AOM, a mutagen commonly used to induce carcinogenesis. One week later, mice were either exposed to 19 days of CSC or were kept as SHCs in order to study the effect of chronic psychosocial stress on inflammation-related CRC. CSC has previously been established as a clinically relevant model of chronic psychosocial stress (Reber et al. 2007, Reber and Neumann 2008, Reber et al. 2008, Veenema et al. 2008, Singewald et al. 2009, Schmidt et al. 2010, Reber 2011, Reber et al. 2011). In line with previous findings, CSC mice in the present study showed a reduced body weight gain during stressor exposure. As DSS colitis promotes AOM-induced CRC, depending on its severity (Suzuki et al. 2005), and as CSC itself induces a slight colitis but heavily exaggerates DSS colitis (Reber et al. 2007, Reber et al. 2008, Reber et al. 2011), SHC and CSC mice received three cycles of DSS to amplify the hypothesized stress effects on AOM-induced CRC. During the second cycle, DSS concentration was reduced as three CSC mice died during the first DSS cycle recapitulating previous results showing that CSC mice develop a more severe DSS colitis (Reber et al. 2008). Importantly, an exacerbating effect of chronic psychosocial stress on AOM/DSS-induced CRC was suggested by several findings. Repeated colonoscopy (in two mice per treatment group) revealed that macroscopically abnormal lesions, for instance granular mucosa and/or flat polypoid lesions/polyps, developed earlier in CSC mice. Although only a small fraction of these polyps may finally become malignant, there is evidence indicating that the large majority of colorectal carcinomas develop from these adenomatous polyps (Konishi and Morson 1982). In line with our data, chronic immobilization

(6 h/day for 3 weeks) also resulted in a faster development of UVB exposure-induced skin tumors (Saul et al. 2005). Furthermore, assessment of HE-stained colonic slices revealed that CSC mice tended ($p = 0.069$) to develop more often LGD and/or HGD compared with SHC mice (CSC: 7 out of 7; SHC: 5 out of 9). The lack of a statistically significance in dysplasia development between SHC and CSC mice probably due to the fact that three CSC mice died from severe colitis during the first cycle of DSS treatment, resulting in insufficient statistical power. Humans who develop severe dysplasia in adenomas are considered to be at increased risk for developing cancer (Konishi and Morson 1982). Our stressor-independent finding of increased $Ki67^+$ epithelial cells in dysplastic compared with non-dysplastic tissue, indicating increased, possibly uncontrolled, cell proliferation, further strengthens this idea. Although we did not detect differences in relative epithelial cell proliferation (per crypt) between CSC and SHC mice, either in dysplastic- or non-dysplastic-colonic tissue, the absolute number of $Ki67^+$ epithelial cells per colonic cross section was increased in CSC mice. Together with the decreased epithelial apoptosis found in the colon of CSC compared with SHC mice, this clearly indicates abnormal patterns of cell replication, as detected in several clinical conditions associated with an increased risk for colorectal malignancies (Tanaka 2009). The recently described reduction in epithelial cell apoptosis following 10 h of CSC (Reber et al. 2011) further shows that this effect is of rapid onset and, thus likely to be causally involved in CSC-induced promotion of AOM/DSS-induced CRC. Quantification of proliferating colonic epithelial cells as a common marker for the assessment of CRC severity has also been used in other studies employing the AOM model (Komatsu et al. 2001). At present we can only speculate about the mechanism underlying the increased epithelial cell proliferation in CSC mice. Under normal conditions degradation of the proliferation-inducing factor β-catenin translocates to the nucleus and influences most basic cellular functions including proliferation, cell-fate determination, and survival (Moon et al. 2002). Uncontrolled activation of the β-catenin signaling pathway has further been shown to cause gastrointestinal tumour development (Oshima et al. 1995), and the β-catenin protein is one of the key determinants in the pathogenesis of colon cancer (Kinzler and Vogelstein

1996). Therefore, the increased colonic β-catenin protein expression in CSC mice in the present study is likely to be involved in upregulation of epithelial cell proliferation. However, Western blot results from the study described in this chapter do not allow any conclusions to be drawn on whether nuclear β-catenin is also increased in developing cancer lesions. This important information needs to be assessed in future studies employing IHC. In addition to β-catenin, LRH-1 is also involved in the control of intestinal cell renewal (Botrugno et al. 2004) and promotion of CRC (Schoonjans et al. 2005). LRH-1 acts as a potent co-activator of β-catenin via the cyclinD1 promotor (Botrugno et al. 2004) or by binding directly to the cyclinE1 promotor, resulting in increased cell proliferation. In the study described in this chapter, both colonic LRH-1 mRNA and protein expression were significantly elevated in CSC compared with SHC mice indicating also a role for LRH in the enhancement of absolute epithelial cell proliferation in CSC mice. Besides dysplasia formation and increased epithelial cell proliferation, impaired clearance of abdominal cells might also be involved in the promotion of AOM/DSS-induced CRC in CSC mice. However, an increased number of $CD4^+$ Th cells in the colonic tissue and an increased percentage of $CD3^+$T cells within polled isolated mesLNC were found in CSC compared with SHC mice, suggesting an upregulation of tumor-protective Th1 cell immunity. This may seem to be contrary to a CSC-induced impairment of tumor cell clearance. However, the tendency to reduced colonic IFN-γ mRNA coupled with the unaltered IFN-γ secretion from mesLNC, in CSC mice compared with non-stressed control mice, suggests that the increased numbers of intestinal T cells in CSC mice do not represent tumour-eliminating Th1 cells. In contrast, an increased colonic FoxP3 mRNA expression and an increased number of $CD3^+$/$FoxP3^+$ mesLNC indicate a regulatory and, thus, rather immunosuppressive quality of gut-residing T cells, which would promote tumour progression rather than clearance. In support, an increased susceptibility to UV-induced skin cancer by suppressing type-1 cytokines, protective T cells, and increasing regulatory T cell numbers (Saul et al. 2005) has been found following repeated immobilization (6 h/day over 3 weeks). Interestingly, several studies have reported that increased regulatory T-cell infiltration in tumor bed predicted reduced survival in cancer-bearing patients (Martin et al. 2010). In a

recent study, we showed that Th2, but not Th1, cell subpopulations develop GC resistance following 19 days of CSC (Schmidt et al. 2010). Moreover, CSC mice overcome stress-induced adrenal insufficiency at least 8 days following termination of CSC, and the inflammatory episodes induced by DSS treatment increase plasma CORT levels in both SHC and CSC mice (Reber et al. 2008). Therefore, a specific downregulation of tumour-protective Th1 immune responses during repeated cycles of DSS in the study described in this chapter might have promoted a gradual shift toward regulatory T-cell immunity and, consequently, the development of CRC. Importantly, the latter would also be in line with the observation that CSC mice showed a body weight development comparable with SHC mice during the second and third DSS cycles, indicative of an equally severe colitis, whereas they developed a more severe colitis during the first cycle. The latter is indicated by three CSC mice dying of severe colitis and is in line with an aggravated colonic inflammation in CSC compared with SHC mice when DSS is administered immediately after stressor termination (Reber et al. 2008). Together with recent findings showing that the numbers of regulatory T cells were significantly reduced in peripheral LN tissue immediately following termination of CSC (Schmidt et al. 2010), these data indicate that regulatory T cell numbers increase gradually following CSC, ameliorating the effects of chronic stress on DSS colitis but promoting those on CRC development. Most organ-related carcinomas are associated with high levels of local TNF. TNF has been shown to inhibit the activity of tyrosine phosphatases and to diminish class-I major histocompatibility complex (MHC) antigen expression on the cell surface, permitting malignant cells, which enables them to escape immune surveillance. Furthermore, macrophage-derived TNF has been shown to promote signaling via the β-catenin pathway, thereby contributing to tumor development in the gastric mucosa ((O'Callaghan et al. 2005)). As high levels of perceived life stress are associated with increased expression of TNF, released from the macrophage/monocyte lineage, this might be a general mechanism underlying stress-induced promotion of tumourogenesis (Reiche et al. 2004). Although the number of colonic F4/80^{+} macrophages was not affected by CSC in the present study, an increased colonic TNF mRNA expression indicated an increased activity of these or other

TNF-producing cells maybe contributing to the CSC promoting effects on CRC development. Interestingly, recent evidence indicates that TNF is able to activate and expand regulatory T cell number by activating their TNFR2 receptors, especially in the tumour microenvironment (Chen et al. 2010, Chen and Oppenheim 2011). Therefore, increased TNF mRNA expression in the colonic tissue of CSC mice might be involved in mediating the shift toward regulatory T cell immunity. However, future studies are needed to clarify this hypothesis. Finally, the increased expression of COXII in CSC mice further supports our stress-promoted carcinogenesis concept. COXII is considered to contribute to tumour development by modulating apoptosis, angiogenesis, and tumour invasiveness (McConnell and Yang 2009), and is overexpressed in approximately 80% of CRC and 40% of colorectal adenomas relative to normal mucosa (Eberhart et al. 1994). Indeed, as already discussed above, epithelial apoptosis was decreased in CSC compared with SHC mice in the study described in that chapter. In conclusion, our findings indicate for the first time that chronic psychosocial stress clearly increases the risk for inflammation-related CRC. Chronically stressed mice more frequently developed colonic LGD and/or HGD, showed an enhanced colonic expression of TNF and COXII, and an increase in epithelial cell proliferation, likely mediated by an enhanced β-catenin and LRH-1 signaling. However, further time points need to be assessed to clarify in detail whether the detected shift from tumour-protective Th1cell to regulatory T cell immunity represents cause or consequence of increased CRC found in CSC mice.

Chapter 3

Effects of baclofen and oxytocin on ethanol consumption following chronic psychosocial stress

Sebastian Peters: Study design, performance of experiments and data analysis, writing the first draft of the manuscript

David A. Slattery: Study design, ICV surgery, central injections, revision of manuscript

Peter J. Flor: Study design, revision of manuscript

Inga D. Neumann: Study design, revision of manuscript

Stefan O.Reber: Study design, revision of manuscript

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Abstract

Chronic stress is known to enhance the susceptibility for addiction disorders including alcoholism. While these findings have been recapitulated in animal models, the majority of these studies have utilized non-social rather than social stress paradigms; the latter of which are believed to be more relevant to the human situation. Therefore, the major aim of the study described in this chapter was to investigate, if 14 days of CSC, a pre-clinically validated psychosocial stress paradigm relevant for human psychiatric and somatic disorders, enhances EtOH consumption in male mice. To assess this, the well-established two-bottle free-choice paradigm where mice were given access to water and 2, 4, 6 and 8% EtOH solutions (with the concentrations increasing each fourth day) following termination of the stress procedure was employed. After 14 days of CSC, stressed mice consumed significantly more EtOH at all concentrations tested and displayed increased EtOH preference at concentrations of 6 and 8%. This effect was not due to an altered taste preference in CSC mice as assessed by saccharine- and quinine-preference tests, but was accompanied by increased anxiety-related behaviour. Systemic administration of baclofen (2.5 mg/kg) or OXT (10 mg/kg) reduced the EtOH intake in SHC (baclofen, OXT) and CSC (baclofen) mice, whereas icv OXT (0.5 µg/2 µl) was ineffective in both groups. Taken together, these results suggest that (i) chronic psychosocial stress enhances EtOH consumption, and (ii) baclofen and OXT differentially affect EtOH intake in mice.

Introduction

EtOH is one of the most commonly abused substances with recent statistics revealing that the European Union is the heaviest drinking region in the world. Over one-fifth of the European population aged 15 years and above, report heavy episodic drinking, at least once a week, dramatically increasing their risk for developing health and social problems (World Health Organization 2012). The consequences of profound alcohol intake are related to the noxious and dependence-producing properties of the active component, ethanol (EtOH). In addition to peer pressure and social drinking, EtOH dependence is promoted by a number of risk factors, including personal history of EtOH use, genetic factors that alter EtOH-pharmacokinetics and neurophysiological responses (Buscemi and Turchi 2011), as well as mood and anxiety disorders (Kessler et al. 1996). Furthermore, chronic stress, and in particular chronic psychosocial stress, represents a high risk factor for developing substance abuse disorders, including alcoholism, and causes relapse in abstinent individuals with a former drinking history (Sinha 2001). It is hypothesised that one of the main reasons for this link is the potential of EtOH to act as an anxiolytic and/or to have anti-stress effects, at least acutely and in moderate doses (Varlinskaya and Spear 2010). Thus, it is postulated that stressed, or anxious, individuals are more predisposed, or sensitive, to the psychogenic effect of EtOH, which in turn leads to an enhanced inclination for consuming EtOH (Cappell and Herman 1972). However, despite this knowledge and substantial research interest, the aetiology of EtOH dependence remains poorly elucidated with only a few treatment options available (Heilig and Egli 2006). One reason for this is the lack of appropriate animal models. While several rodent studies have demonstrated increased EtOH intake following a variety of repeated stressors, like restraint, forced swim or immobilization (Becker et al. 2011), these stressors have low etiological validity and clinical relevance when compared with the identified risk factors in humans, e.g. anxiety and chronic psychosocial stress. More akin to the human situation, it has been demonstrated that innate anxiety levels correlate with EtOH intake in naive animals (Spanagel et al. 1995) and that dominant animals consume less EtOH in comparison with their subordinated counterparts (Ellison 1987). However, the effects

of intermittent social defeat on EtOH consumption are controversial with some studies showing an increase, and others no effect on EtOH intake (Becker et al. 2011). Therefore, research assessing both, the aetiology of, and potential novel treatments for, EtOH dependence in animal models more relevant to the human situations are urgently required. We have developed such a model in male mice, called the chronic CSC paradigm, which represents a persistent, continuous psychosocial stressor and, thus, allows a better translation of rodent findings to the human situation (Reber et al. 2007). Indeed, both 14 and 19 days of CSC have been repeatedly demonstrated to result in psychological, behavioural and immunological alterations, including increased state anxiety (Reber et al. 2007, Reber and Neumann 2008), reflecting the situation observed in humans exposed to chronic stressful life events. As mentioned above, there are few approved treatments for EtOH dependence (Heilig and Egli 2006). A growing number of clinical studies have demonstrated that the gamma-aminobutyric acid ($GABA_B$) receptor agonist baclofen decreases the voluntary consumption of different drugs of abuse, including EtOH (Cousins et al. 2002), as well as craving in EtOH-dependent individuals (Addolorato and Leggio 2010). Further support comes from pre-clinical studies, showing that baclofen reduces EtOH consumption in rats (Janak and Gill 2003, Walker and Koob 2007) and mice (Tanchuck et al. 2010, Orru et al. 2012). In keeping, $GABA_B$ receptor positive allosteric modulators, like BHF177, have recently been shown to reduce EtOH intake in mice (Orru et al. 2012). Interestingly, baclofen attenuated withdrawal-induced deficits in social interaction in rats subjected to multiple EtOH withdrawal periods during which they were repeatedly stressed by immobilization (Knapp et al. 2011). While these studies show that baclofen is a promising treatment for EtOH dependency (Edwards et al. 2011), it can also result in the occurrence of unfavourable side effects in some patients (Macaigne et al. 2011). Another emerging opportunity in terms of treating substance-abuse disorders including alcoholism is the neuropeptide OXT (Sarnyai 2011, McGregor and Bowen 2012). Both endogenous and exogenous central OXT have been shown to act in an anxiolytic fashion and to attenuate the physiological and psychological effects of stressor exposure (Neumann and Landgraf 2012). Some of these

effects could be replicated after peripheral administration of synthetic OXT (Ring et al. 2006, Grippo et al. 2009). Importantly, peripheral and central OXT treatment has been shown to reduce EtOH intake and the development of EtOH tolerance in rats (Bowen et al. 2011), as well as the neurochemical response to drugs of abuse, in rodents (McGregor and Bowen 2012, Qi et al. 2012). In this context, it is of note that chronic EtOH exposure causes a decrease in the number of OXT-containing neurons in the SON in humans (Sivukhina et al. 2006) and in the paraventricular PVN in rats (Silva et al. 2002). Interestingly, baclofen modulates oxytocinergic neuronal firing *in vitro* (Jourdain et al. 1996), and $GABA_B$ receptors act as modulators for excitatory synaptic transmission within the SON of rats (Kombian et al. 1996). Moreover, systemic baclofen administration causes increased c-Fos expression within the PVN (van Nieuwenhuijzen et al. 2009, McGregor and Bowen 2012) and local administration of $GABA_B$ receptor antagonist phaclofen into the PVN of rats led to a reduction of plasma OXT levels (Marques de Souza and Franci 2008). These findings, taken together with those mentioned earlier, have lead to the speculation that baclofen may mediate its anti-abuse properties, at least in part, *via* an oxytocinergic mechanism (van Nieuwenhuijzen et al. 2009, McGregor and Bowen 2012). However, to the best of our knowledge it has not been investigated to date, whether or not an acute central or peripheral OXT-injection can reduce stress-induced EtOH consumption. Therefore, the major aims of the study described in this chapter were to determine, if 14 days of CSC result in (i) increased anxiety-related behaviour, as it has been shown that the physiological and immunological effects at this timepoint match those of 19 days of CSC; (ii) increased EtOH intake and preference, and (iii) if this could be attenuated by acute baclofen (ip) or OXT (icv; ip) administration. OXT was applied both ip and icv to determine, if it exerts *via* peripheral and/or central mechanisms because of the fact that systemic administration of neuropeptides shows poor brain penetration (Neumann & Landgraf 2012).

Material and Methods

Animals

Male C57BL/6N 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 1 week before the CSC paradigm started. All mice were kept under standard laboratory conditions (12-hour light/dark cycle, lights on at 06:00 am, 22°C, 60% humidity) and had free access to tap water (H_2O) 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

One week after arrival of the mice, the CSC paradigm was performed for 14 days in all experiments. Based on prior studies, SHC mice were considered to be the most appropriate controls in this paradigm (Singewald et al. 2009). After termination of the stress paradigm, all further experimental procedures commenced on day 15 (see Fig. 22). In the first experiment (exp. 1), mice (SHC: n = 8; CSC: n = 8) were tested in the LDB for anxiety-related behaviour (day 15; described later) and subsequently underwent the two-bottle free-choice paradigm (days 15-28; described later), followed by 3 days of saccharine administration (days 28-31) and 3 days of quinine administration (days 32-34) to detect any alterations in their taste preference (described later; Fig. 22).

In the second experiment (exp. 2), the effects of an acute ip injection of either baclofen or OXT on EtOH intake were tested. Thus, mice (SHC n = 8; CSC n = 8) underwent the two-bottle free-choice paradigm. After displaying a stable 3-day mean for their EtOH consumption at the concentration of 8% (described later), they received a single ip injection of either

vehicle (VEH), baclofen or synthetic OXT, using a counterbalanced design (between days 36 and 50; Fig. 22).

To determine the effect of a single icv injection of OXT on EtOH intake, mice (SHC n = 23; CSC n = 24) in the third experiment (exp. 3) were stereotactically implanted with an icv-directed indwelling cannula on day 15 (described later), and then underwent the two-bottle free-choice paradigm. After displaying a stable 3-day mean for their EtOH consumption at the concentration of 8%, mice were injected icv with either VEH or OXT using a counterbalanced design (between days 32 and 37; Fig. 22).

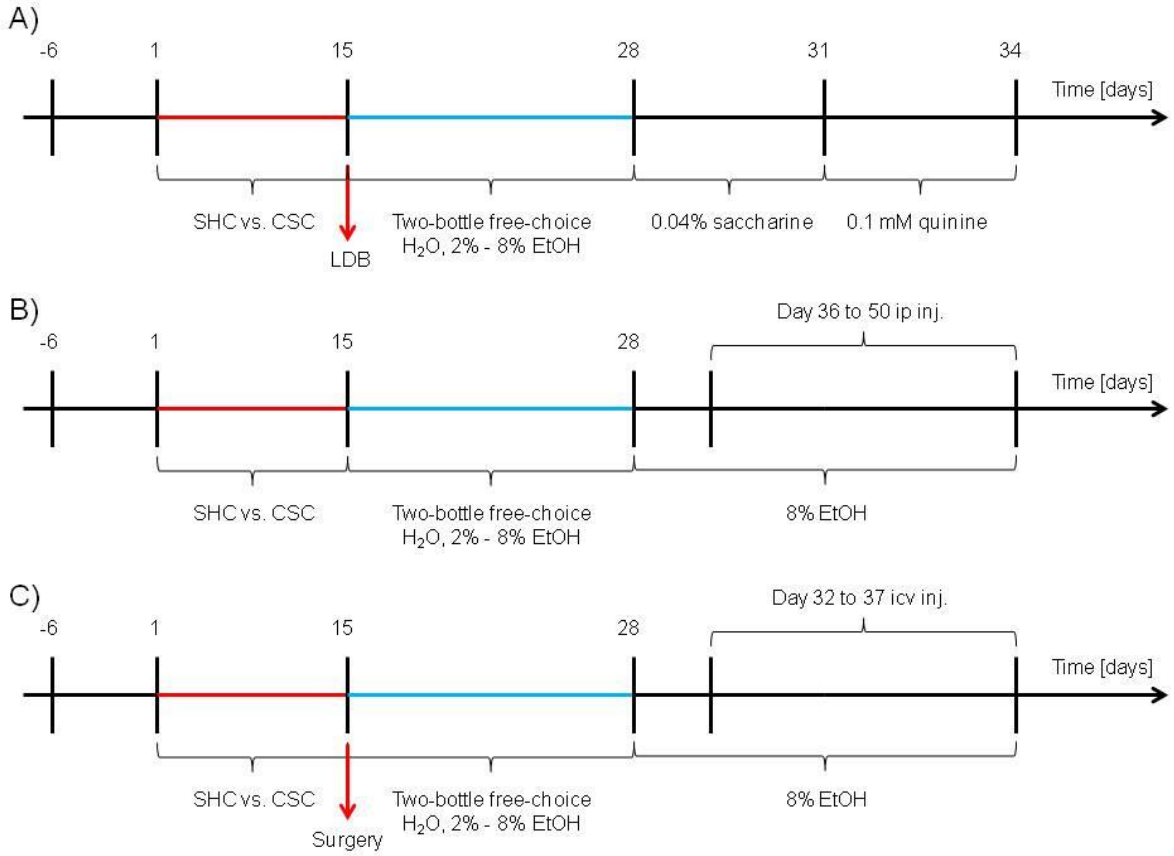


Figure 22: Schematic illustration of the experimental procedure. After 1 week of single housing (arrival on day -6) the chronic subordinate colony housing (CSC) paradigm (14 days) was performed in all three experiments. In experiment 1 (A), on day 15 the light-dark box was performed to determine differences in anxiety-related behaviour between single housed control (SHC) mice (n = 8) and CSC mice (n = 8). Subsequently, the two-bottle free-choice paradigm was performed (days 15-28), to reveal CSC-induced differences in ethanol (EtOH) intake. To determine any CSC-induced differences in taste preference, both SHC and CSC mice underwent the saccharine (days 28-31)/quinine (days 31-34) test. In experiment 2 (B) and 3 (C), after termination of the two-bottle free-choice paradigm, SHC (n = 8, experiment 2; n = 23, experiment 3) and CSC (n = 8, experiment 2; n = 24, experiment 3) mice received 8% EtOH solution until they displayed a stable 3-day mean baseline. During days 36-50 they received intraperitoneal (ip) injections of either vehicle (VEH), baclofen or oxytocin (OXT) (experiment 2) or during days 32 to 37 intracerebroventricular (icv) injections of VEH or OXT (experiment 3), in a counterbalanced design. Icv surgery in experiment 3 took place on day 15.

CSC housing

The CSC paradigm was conducted as described previously (Reber et al. 2007), but for a duration of 14 days. Body weight was assessed prior to, and immediately after CSC exposure. In all experiments, no weight differences were observed between the SHC and CSC mice as recently described (Slattery et al. 2012).

LDB (exp. 1)

To assess anxiety-related behaviour mice were transported to the test room in the evening of day 14 of CSC exposure to be tested on the next day between 08:00 and 11:00 h. The LDB consists of a brightly lit (27 x 27 x 27 cm, 350 lux) and a dark (18 x 27 x 27 cm, 50 lux) compartment, separated by a partition wall that had a small opening (6 x 7 cm) at floor level. Mice were individually placed in the dark compartment to habituate (with the opening being closed). After 30 s the opening between the light and the dark compartment was opened and mice were allowed free exploration for 5 min. The time spent in the light box (as a measure of general anxiety) and the total distance moved (as a measure of general activity), were analyzed using EthoVision XT (Version 5.0.216, Noldus Information Technology, Wageningen, the Netherlands) as previously described (Slattery et al. 2012).

EtOH consumption (exps. 1-3)

In order to determine whether CSC alters EtOH consumption, a two-bottle free-choice experiment was employed as described previously (Tanaka et al. 2010). On day 15 (in exp. 1 directly after LDB testing) all mice were single-housed and received two 10 ml pipettes containing tap H_2O for the first 2 days to acclimatize to the two pipettes and to determine the existence of any side preference. Thereafter, one pipette was replaced by another containing 2% EtOH. Every fourth day the EtOH concentration was increased by 2% up to a maximum of 8% and the position of the EtOH was changed every day to rule out any influence of side

preference on the EtOH intake (see Fig. 22). EtOH solutions were prepared freshly every afternoon by diluting 96% EtOH (Fortior PrimaspritNeutralalkohol, Brüggemann Alcohol, Heilbronn, Germany) with tap H_2O (v/v). After weighing the animals the EtOH and H_2O pipettes were refilled immediately before lights off (i.e. 18:00). EtOH intake was calculated as described previously (Thiele et al. 2000). Briefly, to obtain a body weight-corrected measure of EtOH consumption, the volume (ml) of EtOH consumed per 24 hours was measured for each mouse. Subsequently, the mass of consumed EtOH (g) per kg bodyweight and per 24 hours was calculated for each mouse and computed as 3-day mean for each given concentration. In addition, EtOH preference ratios were calculated for each mouse by dividing the consumed EtOH solution in 24 hours by the total fluid (EtOH plus H_2O) consumed in 24 hours and displayed as 3-day means for each given concentration. Moreover, 24 hour H_2O consumption (ml) was calculated per kg bodyweight for each mouse and computed as 3-day mean (the calculation was performed as described earlier for the EtOH intake).

Test for preference (saccharine/quinine test) (exp. 1)

In order to determine whether the CSC-induced increase in EtOH consumption was due to a general alteration in taste preference, two-bottle free-choice tests for saccharine and quinine were performed. As previously described, subsequent to the EtOH intake paradigm (see Fig. 22), experimental mice were presented both a sweet [saccharine 0.04% (Bahi et al. 2011); saccharine sodium salt, Sigma Aldrich GmbH, Deisenhofen, Germany] or a bitter (quinine 0.1 mM (Tanaka et al. 2010); Alfa Aesar GmbH & Co KG, Karlsruhe, Germany) solution together with water. Each taste preference test was conducted for three consecutive days and the positions of the pipettes were changed each day (Tanaka et al. 2010). The volume (ml) of fluid consumed was measured and the preference for each solution was calculated as described above for the EtOH consumption.

Effect of baclofen and OXT on EtOH consumption (exp. 2-3)

For exps. 2 and 3, the animals received the 8% EtOH solution until they showed a stable 3-day mean baseline (less than 10% standard deviation from previous 3-day mean and comparable with baseline without any injections) before and between each drug administration. Animals that needed more than 8 days to re-establish a stable 3-day mean were excluded from the study. Subsequently, the drugs were administered using a within-subject counterbalanced design, whereby each animal received the drugs in different order to ensure that the response was not due to the sequence of administration. The effects of drugs on EtOH intake were calculated and presented in the graphs as consumption compared with the 3-day mean in baseline EtOH drinking in percent.

Acute ip injections of baclofen or OXT (exp. 2)

Each subject received an ip injection of VEH (0.01 ml/g body weight, Ringer solution; pH 7.4; Serumwerk Bernburg AG, Bernburg, Germany), baclofen [2.5 mg/kg (Tanchuck et al. 2010); Sigma Aldrich GmbH, Deisenhofen, Germany] or OXT [10 mg/kg (Ring et al. 2006)]; Sigma Aldrich GmbH, Deisenhofen, Germany) 15 min before the dark phase, as previous studies (Rhodes et al. 2005) and exp. 1 of the current study showed that animals consume EtOH mainly during the dark phase. EtOH intake was then monitored during the 24 hours after drug application. To exclude non-specific stress responses to the injections, all animals were handled daily during establishment of the EtOH preference. The baclofen dose was selected based on previous studies showing baclofen to effectively reduce EtOH consumption at doses ranging from 2 to 3 mg/kg (Janak and Gill 2003, Walker and Koob 2007, Tanchuck et al. 2010, Orru et al. 2012), while the ip dose of OXT was chosen based on its anxiolytic effects in mice in previous studies (Ring et al. 2006).

Surgical and icv infusion procedures (exp. 3)

On day 15 of CSC, mice were implanted with a guide cannula to determine if, icv OXT could influence EtOH intake (Fig. 27). All stereotactic surgeries were performed as described previously (Toth et al. 2012). After development of a stable 3-day mean baseline to 8% EtOH, mice received an acute infusion of either VEH (2 µl, Ringer solution; pH 7.4; Serumwerk Bernburg AG, Bernburg, Germany) or OXT (0.5 µg/2 µl, Sigma Aldrich GmbH, Deisenhofen, Germany) 15 min before the dark phase. The substances were slowly infused over 1 min using a 27-G infusion cannula, which extended 2 mm beyond the end of the guide cannula and remained in place after infusion for 30 s to allow diffusion. The icv OXT dose was selected based on previous studies showing anxiolytic effects of this dose in mice (Ring et al. 2006, Toth et al. 2012) and prosocial effects of comparable doses in mice and rats (Lukas et al. 2011).

Verification of cannula placement

After the experiment, mice were killed by decapitation following inhalation anaesthesia and blue dye was injected *via* the infusion system to verify correct placement of the cannula. No animals were removed from the study.

Statistics

Statistical analyse were carried out using PASW statistics (version 18; SPSS inc., Chicago, IL, USA) statistics (version 18). All data represent mean + standard error of the mean (SEM). Animal numbers are provided in the legends of each figure. Data were compared using either a two-way analysis of variance (ANOVA) followed by a Bonferroni *post hoc* pairwise comparison, if appropriate (factor stress: SHC versus CSC; factor treatment: drug administration) or ANOVA for repeated measures (rmANOVA; factor EtOH concentration

from 2 to 8%) followed by Bonferroni *post hoc* test, if appropriate or a Student´s *t*-test (factor CSC). Significance was taken at $p < 0.05$.

Results

Exp. 1: Effects of CSC on anxiety-related behaviour and EtOH consumption

Increased anxiety-related behaviour after 14 days of CSC

Statistical analysis of anxiety-related behaviour revealed that CSC mice spent significantly less time in the lit box of the LDB ($p = 0.024$; Fig. 23A) compared with SHC mice, indicating increased levels of anxiety-related behaviour. General motor activity, reflected by the total distance moved, was unaffected by CSC ($p = 0.191$; not significant; Fig. 23B).

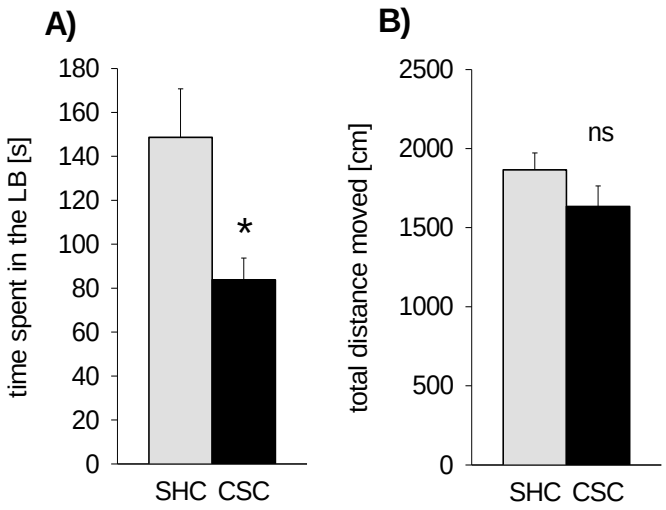


Figure 23: Effects of chronic subordinate colony housing (CSC) on anxiety-related behaviour in the light-dark box (LDB). Fourteen days of CSC exposure increase anxiety-related behaviour, reflected by a decreased time spent in the lit compartment of the LDB in CSC compared with single-housed control (SHC) mice. CSC exposure has no effect on general activity reflected by an unchanged total distance moved. Data represent mean + SEM; $^{*}p < 0.05$ vs. SHC (two-tailed Student´s *t*-test) SHC (n = 8, grey bars); CSC (n = 8, black bars)

Increased 24-h EtOH consumption/EtOH preference in CSC mice

Statistical analysis indicated a significant effect of factors EtOH concentration ($F_{3,42}$ = 48.26; $p < 0.001$) and stress ($F_{1,14}$ = 9.79; $p = 0.007$). *Post hoc* analysis revealed that CSC mice consumed more EtOH compared with SHC mice at all concentrations tested (2%: $p < 0.01$; 4%: $p < 0.05$; 6%: $p < 0.05$; 8%: $p < 0.01$; Fig. 24A). Furthermore, EtOH consumption increased in both CSC and SHC mice with increasing EtOH concentration (CSC: 4, 6 and 8%, all $p < 0.001$ compared with 2%; SHC: 6% ($p < 0.05$) and 8% ($p < 0.05$) compared with 2%, Fig. 24A). EtOH preference was found to be dependent on the interaction of EtOH concentration and stress ($F_{3,42}$ = 4.483; $p = 0.008$). *Post hoc* analysis revealed a higher EtOH preference in CSC compared with SHC mice at EtOH concentrations of 6% ($p < 0.05$) and 8% ($p < 0.05$; Fig. 24B). Statistical analysis indicated a significant interaction effect of EtOH concentration and stress on 24-h H_2O consumption ($F_{3,42}$ = 5.79; $p = 0.002$). *Post hoc* analysis revealed that CSC, but not SHC, mice consumed less H_2O with increasing EtOH concentrations (CSC: 6% compared with 2, 4 and 8% compared with 4%; all $p < 0.01$, Fig. 24C). Importantly, total fluid intake did not differ at any concentration between the groups.

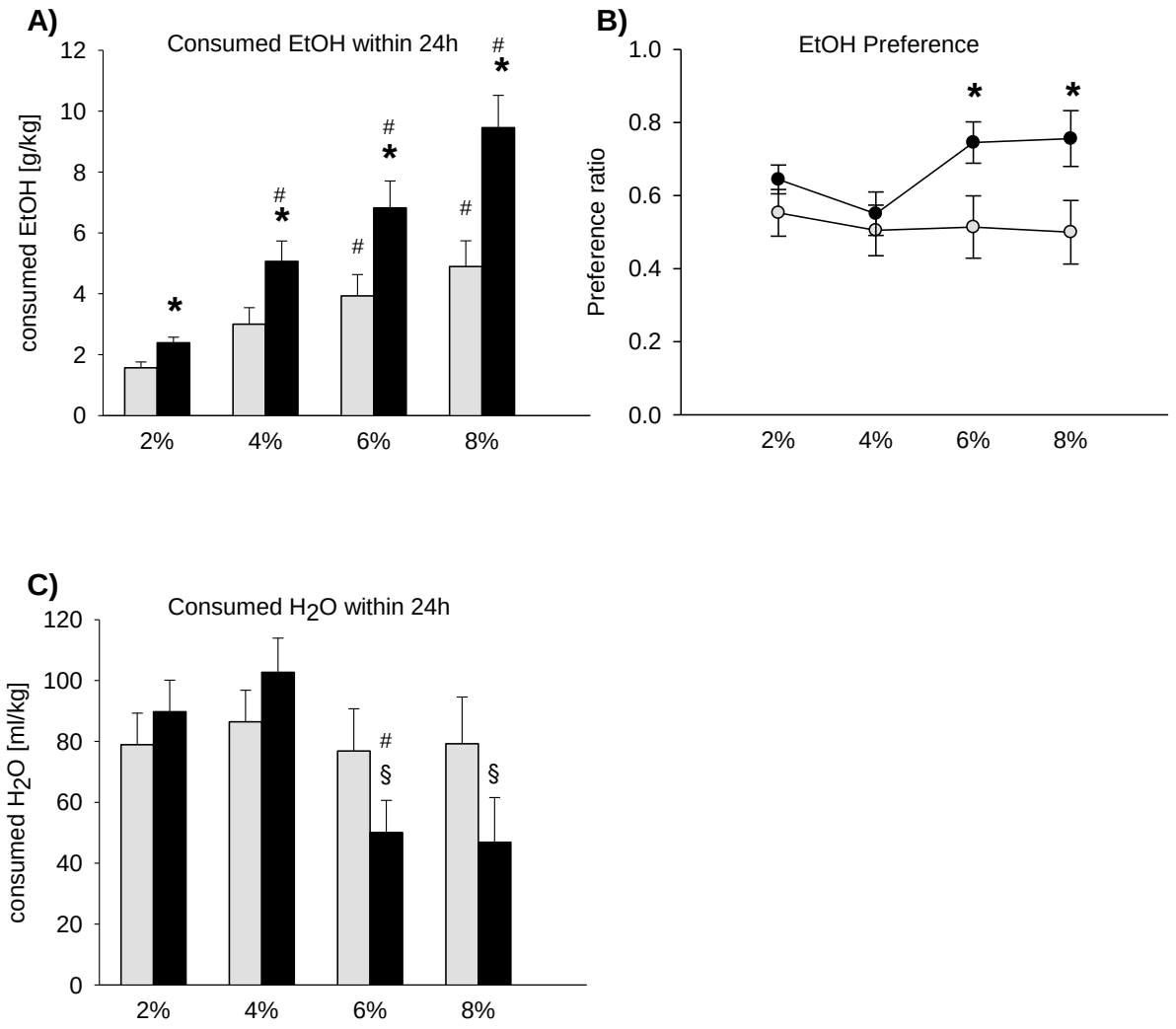


Figure 24: Effects of chronic subordinate colony housing (CSC) on ethanol (EtOH) consumption/ preference, water (H_2O) consumption, and taste preference. CSC exposure increases EtOH self-administration compared with single-housed control (SHC) mice at all EtOH concentrations tested (A). CSC compared with SHC mice display an increased EtOH preference at concentrations 6 and 8% (B). With increasing EtOH concentrations (6 and 8%), CSC mice display a reduced H_2O consumption compared with 2 and 4% (C). Data represent mean + SEM; $^{*}p < 0.05$ vs. SHC; $^{\#}p < 0.05$ vs. 4% (rmANOVA with adjacent Bonferroni *post hoc* test). SHC (n = 8, grey dots/bars):; CSC (n = 8, black dots/bars)

No effects of CSC on 24-h saccharine or quinine consumption/ preference

Statistical analysis revealed no differences in saccharine (Fig. 25A) or quinine consumption (Fig. 25B) or preference (Fig. 25C/D) between CSC and SHC mice.

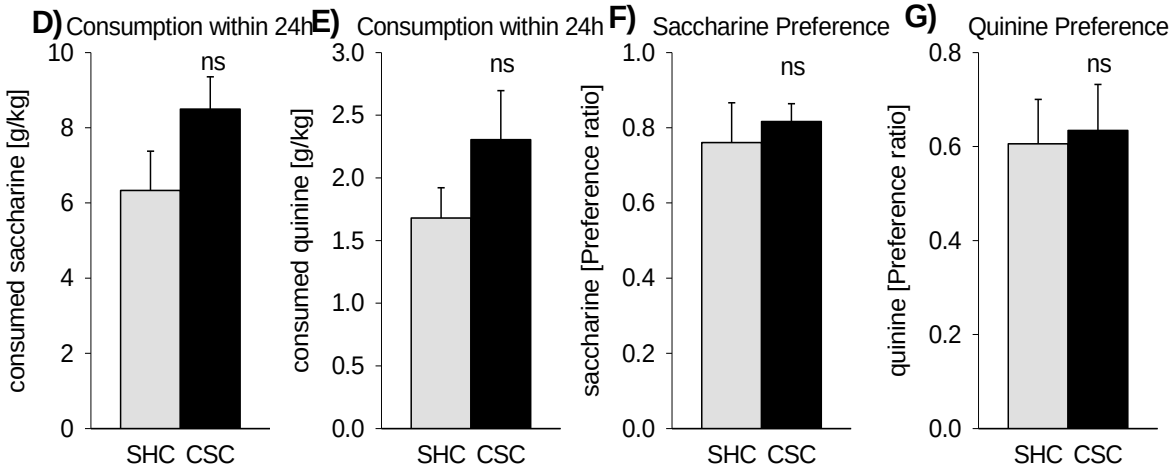


Figure 25: Effects of chronic subordinate colony housing (CSC) on taste alterations. CSC exposure does not cause taste alterations, reflected by no differences in the consumption of/ preference for saccharine and quinine (A -D). Data represent mean + SEM; $^{*}p < 0.05$ vs. SHC; (Two-tailed Student´s t-test) SHC (n = 8, grey bars); CSC (n = 8, black bars)

Exp. 2: Effects of systemic baclofen and OXT on EtOH consumption

As an important prerequisite for exp. 2, CSC mice displayed similar alterations in their EtOH and H_2O consumption and EtOH preference as described in exp. 1 (data not shown).

Acute ip baclofen or OXT injections decrease EtOH consumption

Statistical anylysis indicated a significant effect of treatment ($F_{2,34} = 11.164$; $p < 0.001$). *Post hoc* analysis revealed that both SHC and CSC mice treated with baclofen consumed less EtOH compared with respective VEH-injected mice ($p < 0.05$; Fig. 26A.). Furthermore, and in contrast to CSC, SHC ice consumed less EtOH following ip OXT injection compared with respective VEH-injected mice ($p < 0.05$; Fig. 26A). Moreover, neither drug altered 24-h H_2O consumption in CSC or SHC mice (Fig. 26B).

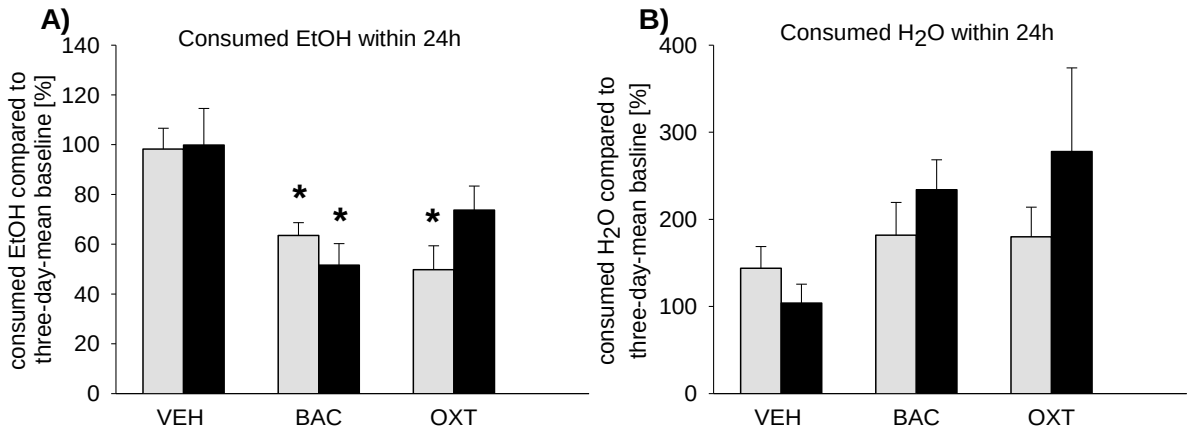


Figure 26: Effects of an acute intraperitoneal (ip) vehicle (VEH), baclofen (BAC) or oxytocin (OXT) injection on ethanol (EtOH) and water (H_2O) consumption. An acute ip BAC injection reduces EtOH self-administration in both single-housed control (SHC) and CSC mice, whereas ip OXT has this effect exclusively in SHC mice, all compared with respective VEH-injected mice (A). Neither an acute ip BAC nor OXT injection affected total H_2O consumption (B). Data represent mean consumption compared with three-day-mean baseline + SEM; $^{*}p < 0.05$ vs. respective VEH-injected animals (Two way ANOVA with adjacent Bonferroni *post hoc* pairwise comparisons). SHC (n = 7, 7, 6, grey bars); CSC (n = 7, 6, 6, black bars):

Exp. 3: Effect of icv OXT on EtOH consumption

An important prerequisite for exp. 3 was to show that icv surgery immediately after termination of CSC and prior to EtOH consumption does not affect stress-induced changes in EtOH and H_2O consumption/preference in CSC or SHC mice (Fig. 27A.) Statistical analysis indicated a significant effect of factor EtOH concentration ($F_{3,14} = 95.37$; $p < 0.001$) and factor stress ($F_{1,45} = 11.95$; $p = 0.001$; Fig. 27A). CSC mice consumed more EtOH at 4, 6 and 8% (all $p < 0.001$) compared with SHC mice. Furthermore, EtOH consumption increased in both SHC and CSC mice with increasing concentrations of EtOH, i.e. at 4, 6 and 8% compared with 2% (all $p < 0.001$; Fig. 27A.). The preference for EtOH was found to be dependent on the interaction of both factors EtOH concentration and stress ($F_{3.34} = 3.38$; $p = 0.02$) with an increased EtOH preference of CSC compared with SHC mice at concentrations of 4% ($p < 0.001$), 6% ($p < 0.01$) and 8% ($p < 0.01$; Fig. 27B). Statistical analysis indicated a significant

interaction effect between factors EtOH concentration and treatment ($F_{3,14}$ = 4.817; p = 0.003). Although there was no difference in H_2O intake between CSC and SHC mice at 2, 4 and 6% of EtOH, CSC mice consumed less water at a concentration of 8% compared with SHC mice ($p < 0.05$). Furthermore, CSC, but not SHC mice, consumed less H_2O with increasing EtOH concentrations, i.e. at 4, 6 (both $p < 0.001$) and 8% ($p < 0.01$) compared with 2% (Fig. 27C.). Importantly total fluid intake did not differ between the groups at any EtOH concentration tested (data not shown).

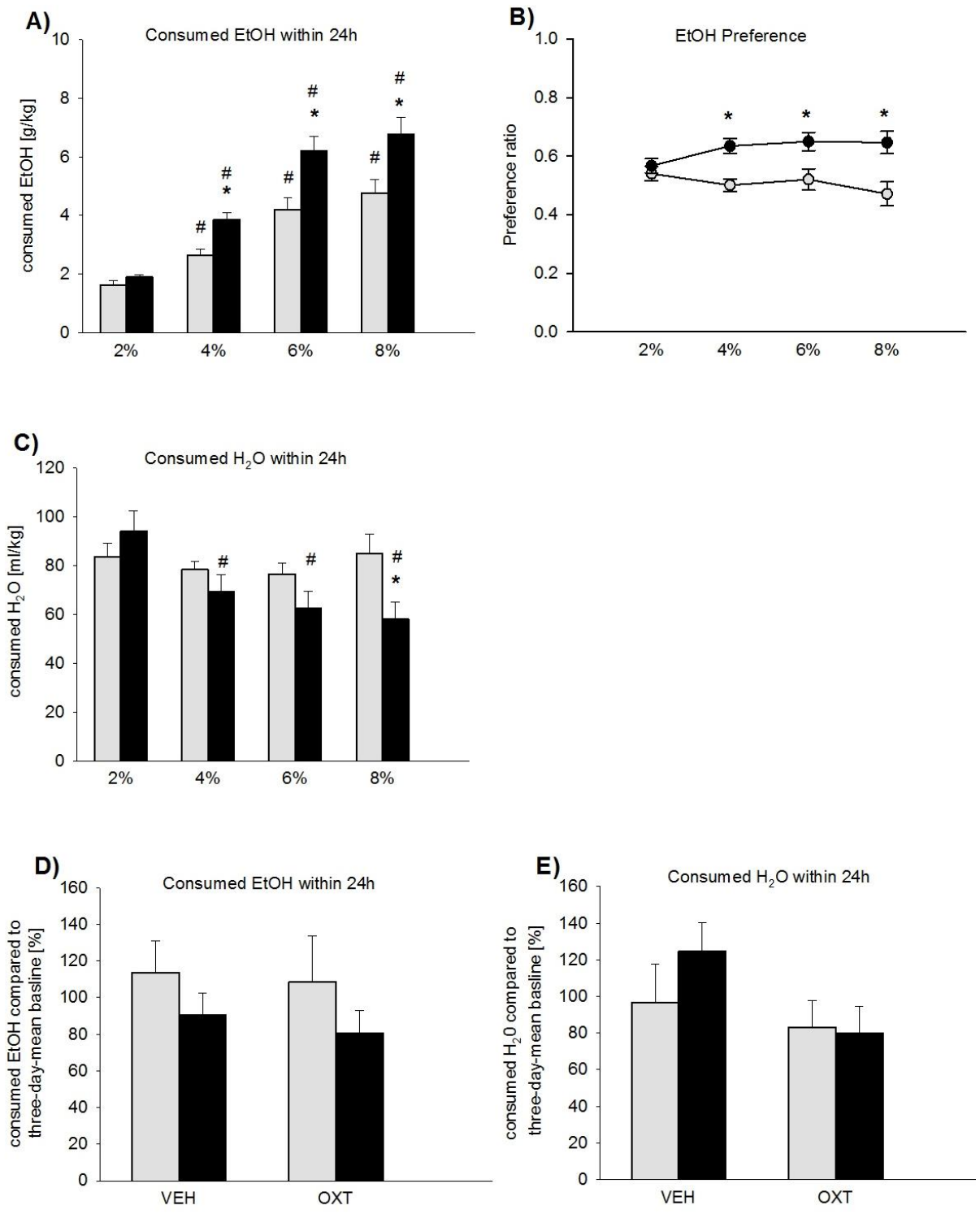


Figure 27: Effects of an acute intracerebroventricular (icv) vehicle (VEH) or oxytocin (OXT) injection on ethanol (EtOH) consumption/ preference and water (H_2O) consumption. Icv surgery following CSC and prior to EtOH self-administration did not affect the CSC-induced increase in EtOH consumption/ preference. CSC compared with single-housed control (SHC) mice consumed more EtOH (A) and showed an increased EtOH preference (B) at concentrations 4, 6 and 8%. With increasing EtOH

concentrations (4,6 and 8%9 CSC mice consumed less H_2O, first compared with the respective 2% EtOH solution and second compared with respective SHC mice at 8% EtOH (C). Acute icv OXT injection did neither affect EtOH self-administration (D) nor total H_2O consumption €. Data in D) and E) represent mean consumption compared with three-day mean baseline + SEM; $^{+}p < 0.05$ vs. respective SHC; $^{\#}p < 0.05$ vs. respective 2% EtOH (rmANOVA or two way ANOVA with adjacent Bonferroni post hoc test). SHC (n = 23, grey dots/bars); CSC (n = 24, black dots/bars)

Acute icv administration of OXT did not affect fluid intake in SHC or in CSC mice

Statistical analysis revealed no differences in the EtOH consumption after an acute icv OXT administration in SHC mice or in CSC animals (Fig. 27D). Moreover, acute icv OXT did not alter 24-h H_2O consumption of any of the groups (Fig. 27E.).

Discussion

In the present study, we could demonstrate for the first time that 14 days of chronic psychosocial stress in mice (CSC) induces an anxiogenic-like phenotype and enhances EtOH consumption and preference without any effect on taste preference. Moreover, while baclofen administration was able to reduce intake of 8% EtOH in both SHC and CSC mice, OXT was only effective in SHC mice; and only following peripheral administration. These drug-induced reductions in EtOH consumption were not the result of a general decrease in fluid intake, revealing their specificity. Taken together these results suggest that baclofen and OXT differentially affect EtOH intake and that the CSC model represents an appropriate animal model to study the aetiology and treatment of EtOH-related diseases. We have repeatedly demonstrated that a 19 days of CSC result in increased anxiety-related behaviour (Reber and Neumann 2008, Veenema et al. 2008) and that 14 days of CSC cause the same physiological and immunological alterations than 19-day exposure to this paradigm (Reber et al. 2007). Increased anxiety-related behaviour is a hallmark of chronic stressor exposure in rodents and is a known risk factor for developing EtOH dependence in humans (Cappell and

Herman 1972). Therefore, the initial aim was to demonstrate that 14 days of CSC exposure also lead to the development of an anxious phenotype. Indeed, CSC mice spent less time in the lit compartment during LDB testing on day 15 of CSC exposure, indicating increased levels of anxiety-related behaviour compared with SHC mice. In addition, and in line with our own recently published data (Slattery et al. 2012), 14 days of CSC in the present study did not cause any alterations in body weight gain. Taken together with the previous findings, these results demonstrate that a 14-day CSC exposure is sufficient to lead to the characteristic physiological, behavioural and immunological alterations observed following a 19-day CSC exposure. Having established that a 14-day CSC exposure is sufficient to induce an anxiogenic phenotype, I next determined its effects on subsequent EtOH consumption/preference. The results demonstrate that chronic psychosocial stress increases intake of, and preference for, EtOH, in male mice. The increased consumption was shown for all EtOH concentrations tested (2-8%), indicating that 14 days of CSC poses a severe chronic stressor, as it increases the EtOH intake even for low EtOH concentrations. This is in line with human studies, demonstrating a strong correlation between stressor exposure and the amount of EtOH consumed. For instance, it was shown that individuals with increased numbers of stressful life events consume more EtOH and exhibit more indicators of EtOH dependence (Linsky et al. 1985). Furthermore, rodent studies have indicated that 5 min of daily social defeat for five consecutive days has the potential to increase EtOH consumption in male Long-Evans rats (Caldwell and Riccio 2010) and male C57Bl/6 mice (Croft et al. 2005). However, there have also been studies failing to detect a link between social stressor exposure (5 days of social defeat or exposure to the residents home cage) and EtOH consumption (van Erp and Miczek 2001, Becker et al. 2011). These inconsistencies lead us to consider our CSC model to be more relevant for the human situation, as it reliably induces an increase in EtOH consumption for a wide range of EtOH concentrations. Importantly, the stress-induced enhancement of EtOH consumption in the current study is not due to any alterations in taste preference, as CSC mice showed no differences in their intake of/ preference for saccharine or quinine or total fluid intake. In support, previous studies

described an unaffected preference for saccharine following 19 days of CSC (Slattery et al. 2012). Therefore, the next goal was to determine whether pharmacological treatments could reverse this CSC-induced increase in EtOH consumption. Alterations in the GABAergic system after chronic EtOH consumption are well described in the literature (Dahchour and De Witte 2000). Indeed, the $GABA_B$ receptor agonist baclofen is currently undergoing extensive trails for the treatment of EtOH disorders in humans with promising results (Cousins et al. 2002, Addolorato and Leggio 2010). In the present study, we were able to demonstrate that a single ip injection of baclofen reduces EtOH consumption in both SHC and CSC mice. These results are in agreement with current studies using rodents to determine the dampening effects of baclofen on the consumption of EtOH in unstressed animals (Janak and Gill 2003, Walker and Koob 2007, Tanchuck et al. 2010, Orru et al. 2012). However, to the best of our knowledge, this is the first study to demonstrate that baclofen can reduce chronic stressor-induced EtOH intake, which suggests that baclofen may be a potential medication for the treatment of stress-induced alcohol disorders in humans. With regards to potential mechanisms of action, *in vitro* and *in vivo* studies have demonstrated that $GABA_B$ receptors modulate the activity of the OXTergic system (Jourdain et al. 1996, Kombian et al. 1996, Marques de Souza and Franci 2008), a neuropeptide, which displays anxiolytic and stress-reducing properties (Neumann and Landgraf 2012). Given that anxiety-related behaviour and elevated hypothalamic-pituitary-adrenal (HPA) axis activity are linked with elevated EtOH intake (Boschloo et al. 2011), it can be postulated that alterations in central OXT release may play a role in the baclofen-induced reduction in EtOH intake observed in the current study. In support of this hypothesis, chronic EtOH intake reduces OXT-containing neurons in the hypothalamus in humans and rats (Silva et al. 2002, Sivukhina et al. 2006), and peripheral administration of synthetic OXT has been shown to reduce consumption of a number of drugs of abuse (McGregor and Bowen 2012). In the study described in that chapter, I were able to demonstrate that a single injection of OXT reduced EtOH consumption in SHC mice; an effect that was only detectable after peripheral administration. A possible reason for the discrepancy between the effectiveness of the ip and

icv treatments are the doses employed, suggesting that higher icv doses of OXT could be effective in reducing EtOH consumption. However, given that the ip and icv doses chosen have been shown to be behaviourally active (see above), this explanation seems unlikely. In contrast to SHC mice, the CSC-induced increase in EtOH intake could not be significantly attenuated by OXT. A potential explanation for the lack of OXT effect in CSC mice is that both chronic stress and EtOH have been shown to alter some parameters of the OXTergic system (Silva et al. 2002, Grippo et al. 2007, Litvin et al. 2011). Therefore, in the stressed mice, a single acute administration of OXT may be insufficient to overcome these deficits, and future studies have to reveal, if repeated or chronic OXT treatment can reduce EtOH consumption after chronic stress. Moreover, these results suggest that it is unlikely that baclofen mediates its effects on EtOH intake in CSC mice primarily via the OXTergic system: although further studies employing baclofen in combination with, for example, an OXT receptor antagonist would be required to confirm this. As it was demonstrated that manipulation of other neuropeptide systems that directly interact with the HPA axis, for example via CRH and AVP receptor antagonism, influence the EtOH intake (Heilig and Egli 2006), further studies are required to investigate the ability to affect CSC-induced EtOH intake. Although central OXT did not affect EtOH intake in any group, it is important to note that the increased EtOH consumption/preference following 14 days of CSC could even be detected in a set of SHC and CSC mice undergoing icv surgery on the last day of CSC (day 15), underlining the robustness and potency of this effect of CSC. In conclusion, I demonstrate that a 14-day exposure to CSC leads to an anxiogenic phenotype and a concomitant elevation in EtOH consumption and preference. Interestingly, our results show that a single administration of the $GABA_B$ receptor agonist baclofen is sufficient to acutely attenuate EtOH consumption in both control and chronically-stressed mice. In contrast, OXT was only able to reduce EtOH consumption in control mice and only via peripheral administration. Taken together, these results support the employment of the CSC paradigm to gain a better understanding of the aetiology of and potential novel treatments for alcohol-related disorders. Moreover, the findings suggest that while both acute baclofen and OXT

can reduce basal EtOH consumption, only baclofen reduces a stress-induced increase in EtOH consumption. Further studies are required to reveal whether repeated or local infusions of OXT into relevant brain regions are capable of reducing such a stress-induced increase in EtOH consumption.

Chapter 4

Effects of chronic oxytocin on behaviour, physiology and the oxytocin system

Sebastian Peters: Study design, performance of experiments and data analysis, writing the first draft of the manuscript

David A. Slattery: Study design, minipump surgery, revision of manuscript

Nicole Uschold-Schmidt: Performing of experiments and data analysis, *in vitro* adrenal gland stimulation

Stefan O. Reber: Study design, minipump surgery, revision of manuscript

Inga D. Neumann: Study design, revision of manuscript

[taken and partly adapted from Sebastian Peters, David A. Slattery, Nicole Uschold-Schmidt, Stefan O. Reber, Inga D. Neumann. Dose-dependent effects of chronic central infusion of oxytocin on anxiety, oxytocin receptor binding and chronic stress-related parameters in mice. *Psychoneuroendocrinology.* http://dx.doi.org10.1016/j.psy-neuen.2014.01.021. in press]

Abstract

Chronic psychosocial stress is a recognized risk factor for various affective and somatic disorders. In an established murine model of chronic psychosocial stress, exposure to chronic subordinate colony housing (CSC) results in an alteration of physiological, behavioral, neuroendocrine and immunological parameters, including a long-lasting increase in anxiety, adrenal hypertrophy and thymus atrophy. Based on the stress-protective and anxiolytic properties of OXT after acute administration in rodents and humans, the major aims of our study were to assess whether chronic administration of OXT dose-dependently affects the behavior and physiology of male mice, as for therapeutic use in humans, mostly chronic treatment approaches will be used. Further, I studied, whether chronic administration during CSC prevents stress-induced consequences. The results indicate that chronic icv infusion of OXT (15 days) at high (10 ng/h), but not at low (1 ng/h) dose, induces an anxiogenic phenotype with a concomitant reduction of OXT receptor (OXTR) binding within the septum, the basolateral and medial amygdala, as well as the median raphe nucleus. Further, I demonstrate that chronic icv infusion of OXT (1 ng/h) during a 19-day CSC exposure prevents the hyper-anxiety, thymus atrophy, adrenal hypertrophy, and decreased *in vitro* adrenal ACTH sensitivity. Thus, given both negative, but also beneficial effects seen after chronic OXT treatment, which appear to be dose-dependent, a deeper understanding of long-lasting treatment effects is required before OXT can be considered for long-term therapeutic use for the treatment of psychopathologies such as autism, schizophrenia or anxiety-disorders.

Introduction

In modern societies, chronic psychosocial stress has repeatedly been related to the etiology of numerous somatic diseases, such as IBD (Duffy et al. 1991, Levenstein et al. 2000) or cancer (Reiche et al. 2004), and depression- and anxiety-related (Shalev 2009) disorders. In clinically relevant animal models a link between repeated or chronic psychosocial stress and emotional, physiological, and immunological adaptations has been demonstrated (Stefanski et al. 2001, Berton et al. 2006, Reber et al. 2007). CSC of male mice represents such a validated mouse model of chronic psychosocial stress. Exposure to CSC reliably affects stress-related parameters (Reber et al. 2011, Peters et al. 2012, Slattery et al. 2012, Peters et al. 2013), reflecting the situation in humans who experience chronic stressful life events (Levenstein et al. 2000, Reiche et al. 2004). Due to the omnipresence of psychosocial stress on the one hand and limited treatment options on the other, the development of new therapeutics for stress-related pathologies is highly needed. In this context, neuropeptides have emerged as such promising novel targets (Slattery and Neumann 2010, Griebel and Holsboer 2012, Macdonald and Feifel 2012, Neumann and Landgraf 2012).

One such candidate is the nonapeptide OXT, which is synthesized in the PVN and SON nuclei of the hypothalamus. Following acute application, OXT has been shown to exert anxiolytic and stress-protective effects in rodents (Bale et al. 2001, Blume et al. 2008, Viviani et al. 2011, Jurek et al. 2012) and humans (Heinrichs et al. 2001, Kirsch et al. 2005, Guastella et al. 2009, Meyer-Lindenberg et al. 2011, Neumann and Landgraf 2012). In this context, pharmaco-genetic manipulation of the brain OXT system in rats and mice revealed a direct inhibitory effect on the (re)activity of the HPA-axis and on anxiety, and promotion of an active stress-coping style (Windle et al. 1997, Neumann et al. 2000, Amico et al. 2004, Sala et al. 2011, Neumann and Landgraf 2012).The areas *via* which OXT exerts these effects could be localized within amygdala and septal regions, the raphe nucleus and the hypothalamic PVN (Neumann et al. 2000, Yoshida et al. 2009, Viviani et al. 2011, Jurek et al. 2012, Knobloch et al. 2012, Guzman et al. 2013). Moreover, OXT promotes social

behaviours in rodents and humans including social bonding, social recognition and social preference (Donaldson and Young 2008, Lukas and Neumann 2013). The effects described above make OXT a promising candidate for preventing, or attenuating, the negative consequences of chronic psychosocial stress.

In humans, there is growing interest in the intranasal application of OXT due to a series of promising effects on emotionality, stress responsiveness and social behaviour (Heinrichs et al. 2003, Kirsch et al. 2005, Guastella et al. 2009). Therefore, intranasal OXT has become an attractive treatment option for anxiety- and stress- related disorders, autism and schizophrenia, which are likely to require repeated or chronic treatment. However, in most studies to date, only acute application of OXT has been studied. Thus, it is essential to investigate the behavioural, physiological and molecular effects of long-term OXT administration, especially chronic OXT effects on the endogenous OXT system. The few rodent studies assessing such repeated or chronic administration have reported inconsistent results, with some reporting beneficial (Windle et al. 1997, Windle et al. 2004, Slattery and Neumann 2010, Grippo et al. 2012) and others more negative (Bales et al. 2012) effects. For example, icv OXT infusion using OMP at either 1, 10 or 100 ng/h for 5 consecutive days dose-dependently reduced anxiety and HPA axis responsiveness to acute stress including hypothalamic CRH expression in female rats (Windle et al. 1997) (Windle et al. 2004). In line, chronic icv OXT (10 ng/h) infusion for six days was shown to reduce anxiety in female Wistar rats selectively bred for high anxiety-related behaviour (HAB) (Slattery and Neumann 2010). In contrast to those beneficial effects of chronic or repeated central OXT, Bales and colleagues report impaired partner preference formation in male prairie voles after a daily intranasal OXT application over 3 weeks (Bales et al. 2012). However, none of these studies have examined the impact of chronic up-regulation of central OXT availability on the endogenous neuropeptide system, which is of particular importance given the potential long-lasting or even chronic therapeutic use of OXT in various psychopathological disorders.

Thus, the major aims of the current study were to investigate, whether chronic icv infusion of OXT using OMP dose-dependently affects physiological and behavioral parameters, and the endogenous OXT system under basal conditions, and whether chronic OXT can reduce chronic stress-induced alterations in male mice.

Material and methods

Animals

Male C57BL/6 mice (Charles River, Sulzfeld, Germany) weighing 19-22 g (experimental mice) or 30-35 g (dominant mice) were individually housed in standard polycarbonate mouse cages (16 x 22 x 14 cm) for one week before the start of CSC exposure. All mice were kept under standard laboratory conditions (12 h light/dark cycle, lights on at 06:00 h, 22°C, 60% humidity) with 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.

Experimental protocols

Experiment 1: Effects of implantation of an osmotic minipump (OMP) on CSC-induced changes

In order to investigate the impact of an OMP implantation and chronic icv infusion of VEH during 19 days of CSC exposure (see below) on CSC-induced physiological and behavioural changes, mice were randomly assigned into four groups: a single-housed control (SHC) group without surgery (SHC-NS), a SHC group with OMP surgery (SHC-OMP), a chronic stress group without surgery (CSC-NS) and a chronic stress group with OMP surgery (CSC-OMP). We have previously shown SHC to be less stressful than group-housing and the most

appropriate control group for the mouse CSC paradigm (Reber et al. 2007, Singewald et al. 2009, Reber et al. 2011, Slattery et al. 2012). Further, previous studies demonstrate that single housing per se neither affects immunological nor endocrine stress parameters in male mice (Bartolomucci et al. 2003, Chourbaji et al. 2005, Gasparotto et al. 2005). For OMP surgery, mice underwent stereotaxic implantation of an icv cannula attached to an Alzet® OMP filled with Ringer's solution. On day 19 of CSC their anxiety-related behavior was assessed on the EPM. After testing, the stressed mice were placed back into their respective CSC-colony and SHC mice remained single-housed. On day 20, mice were rapidly decapitated between 08:00 and 10:00 h, trunk blood was collected for quantification of plasma CORT, adrenal and thymus weights, and the histological damage score of the colon were assessed. The placement of the icv cannula was verified on cryocut brain slices.

Experiment 2: Dose-dependent effects of chronic icv OXT

To investigate whether chronic icv OXT (1 ng/h, 10 ng/h) affects physiological and behavioral parameters and the endogenous OXT system, SHC-VEH, SHC-OXT_{low} (1 ng/h), and SHC-OXT_{high} (10 ng/h) mice were implanted with an OMP filled with one of the substances (see below). The 10 ng/h dose was chosen based on previous studies performed in rats (Windle et al. 1997, Slattery and Neumann 2010) and of behavioural effects of comparable doses in mice and rats (Lukas et al. 2011), a lower dose (1 ng/h) (Windle et al. 2004) was added for comparison. Given the literature demonstrating that two weeks of CSC exposure are enough to induce CSC-related changes (Reber et al. 2007, Peters et al. 2013), mice were tested on the EPM or in the light-dark box (LDB) on day 15 of OXT treatment to measure their anxiety-related behavior. Since the high dose was already shown to induce an anxiogenic phenotype, mice were decapitated on day 16, between 0800 and 1000 h and underwent the same assessments as described for experiment 1. In addition, for determination of OXT mRNA expression, OXTR binding, and cannula verification, brains were removed, shock-frozen in 2-methylbutane (Sigma Aldrich GmbH, Germany) and stored at -80°C.

Experiment 3: Effects of chronic icv OXT (1 ng/h) on CSC-induced changes after 19 days

To investigate whether chronic icv OXT_{low} (1 ng/h) during 19 days of CSC alleviates or even prevents CSC-induced changes and influences the central oxytocinergic system, mice were randomly implanted with an OMP filled with either saline (VEH) or OXT (1 ng/h) to provide the following groups: SHC-VEH, SHC-OXT_{low}, CSC-VEH, and CSC-OXT_{low}. Since the CSC phenotype is more robust and characterized following 19 days, anxiety-related behavior was tested in the LDB on day 19, and mice were returned to their respective CSC or SHC cage to allow recovery from the acute testing procedure. 24 h later, mice were decapitated between 0800 and 1000 h and processed as described above. In addition, the *in vitro* reactivity of adrenal explants to ACTH was measured (see below).

CSC paradigm

For the CSC paradigm (Reber et al. 2007), four experimental male mice were housed together with a slightly larger resident mouse for 19 consecutive days. To avoid habituation, each dominant male was replaced by a novel one on days 8 and 15. To ensure the dominant position of the resident and the subordinated position of the four experimental mice within the CSC group, behaviors of all mice were observed during the first 30 min of colony formation on days 1, 8 and 15 as also described previously (Reber and Neumann 2008). During colony formation and in the course of the CSC paradigm the resident displays offensive behaviours including chasing, mounting and attacking the subordinate mice resulting in chronic psychosocial stress. To avoid habituation and to ensure a persistent stressor exposure the dominant mice were exchanged on days 8 and 15. Moreover, the amount of water and food was checked and refilled on a regular basis to ensure that all mice have access throughout the entire stress paradigm. It is the subordinate position of the four CSC mice which determine their chronic stress level consistently resulting in typical signs of chronic stress (Reber et al. 2007, Reber and Neumann 2008, Reber et al. 2008, Reber et al. 2011,

Uschold-Schmidt et al. 2012). SHC mice were used as appropriate controls (Singewald et al. 2009) being in line with previous studies demonstrating single housing to be less stressful in male mice compared to group housing (Bartolomucci et al. 2003, Chourbaji et al. 2005, Gasparotto et al. 2005). After surgery, all mice were single-housed and remained undisturbed for one week of recovery.

Surgical procedure

For chronic infusion an icv cannula, attached to an Alzet® OMP (infusion rate: 0.11 µl/h, Alzet®, Model 1004, Cupertino, USA), was stereotaxically implanted under isoflurane anesthesia (Baxter, GmbH, Germany) and semi-sterile conditions (Slattery and Neumann 2010). Each OMP was implanted subcutaneously in the abdominal region *via* a 1-cm long skin incision at the neck of the mouse and connected with the icv cannula by a silicone tubing. Animals were placed into a stereotaxic frame, and the icv cannula (23G, 3mm length) was lowered into the right lateral ventricle (posterior 0.3 mm, lateral 1 mm, depth 3 mm; (Paxinos and Franklin 1997). The cannula was fixed with two stainless steel screws using dental cement (Kallocryl, Speiko-Dr. Speier GmbH, Münster, Germany). The skin of the neck was closed with sutures. During surgery, the body temperature was maintained by a heating pad. To avoid post-surgical infections, mice were locally treated with betaisodona (Mundipharma GmbH, Limburg, Germany) and received 0.1 ml antibiotics (SC, Baytril® 2.5 % Bayer Vital GmbH, Leverkusen, Germany). OMPs were filled with OXT (OXT_{low}: 9.09 ng/µl for 1 ng/h; OXT_{high}: 90.9 ng/µl for 10 ng/h; Sigma Aldrich, Steinheim, Germany) or VEH (Ringer`s solution, Braun AG, Germany). The tubing was filled with VEH and its length calculated (90.46 mm) to ensure that all animals only received VEH infusion during the 1-week recovery period.

EPM and LDB

To assess the effects of CSC and icv OXT on anxiety-related behaviour, mice were tested on the EPM (Reber and Neumann 2008) or in the LDB (Peters et al. 2013) between 0800 and 11:00 h. 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 latency to the first open arm entry, the number of entries into the open and closed arms, and the time spent on the respective arms were recorded by means of a video/computer setup to allow calculation of the percentage of time spent on, and the percentage of entries performed into the open arms of the maze.

The LDB consisted of a brightly lit (27 x 27 x 27 cm; 350 lux) and a dark (18 x 27 x 27 cm; 50 lux) compartment, separated by a partition wall that had a small opening (6 cm in length and 6 cm high) at floor level. For habituation, mice were individually placed in the dark box, with the opening of the partition wall closed, for 30 s. After this time period the partition wall was opened and mice were allowed to freely explore the arena for 5 min. The time spent in the light box (as a measure of anxiety) and the distance moved (as a measure of general activity) were analyzed using EthoVision XT (Version 5.0.216, Noldus Information Technology, Wageningen, the Netherlands). The LDB was cleaned thoroughly before each test. CSC mice were directly taken from the colony cages without single housing prior to LDB testing.

Body, adrenal and thymus weight

To assess the effects of OMP surgery, chronic icv OXT and/or CSC on body weight gain, mice were weighed on treatment day 1 and prior to decapitation to calculate body weight gain. After decapitation, the adrenals and the thymus were removed, pruned of fat and

weighed. The left and right adrenals were pooled for each animal. Values reported represent relative (mg/g body weight) adrenal and thymus weights.

ELISA for plasma CORT

Quantification of plasma CORT was performed using ELISA (Bartlang et al. 2012). SHC and CSC mice were rapidly decapitated following brief inhalation anesthesia. Approximately 500 µl trunk blood were collected in EDTA-coated tubes on ice (Sarstedt, Nümbrecht, Germany), centrifuged at 4 °C (2000 g, 10 min), and stored at -20 °C until assayed using a commercially available ELISA for CORT (sensitivity < 1.63 nmol/l, intra-assay and inter-assay coefficients of variation ≤ 6.35%, IBL International, Hamburg, Germany).

ACTH stimulation of adrenal explants in vitro

In vitro stimulation of adrenal explants with ACTH was performed as previously described (Bartlang et al. 2012, Uschold-Schmidt et al. 2012). Briefly, left and right adrenals were separately weighed and stored in ice-cold DMEM/F-12 (Life Technologies, Inc.) containing 0.1 % BSA until all mice were killed and adrenals removed. Afterwards, each left and right adrenal gland was cut into two halves each containing cortical and medullary tissue. The halves were then weighed and pre-incubated in 200 µl DMEM/F-12 for 4 h (37 °C, 95 % O_2, 5 % CO_2) before any further treatment. Culture medium was then replaced, and each half of one adrenal was incubated with medium containing either 0.9% saline (basal) or 0.9% saline plus ACTH (100 nM) for 6 h at 37 °C (95 % O_2, 5 % CO_2). After incubation, supernatants were carefully removed and stored at -20 °C until ELISA analysis of corticosterone. Basal as well as ACTH-induced corticosterone concentrations (ng/ml) were summed up afterwards for left and right adrenal per mouse respectively, and expressed in relation to respective adrenal explants weight (sum of respective left and right adrenal explants) (ng/ml per mg).

Determination of histological damage score of the colon

The histological damage score of colonic tissue was assessed as described previously (Reber et al. 2007, Reber et al. 2008). Briefly, 1 cm of the distal third of the cleaned colon was cut longitudinally, laid on filter paper, and fixed in 10 % formalin over night. The next day, the fixed tissue was embedded in formalin and cut longitudinally. Three 3-µm hematoxylin-eosin-stained sections taken 100 µm apart were histologically scored by an investigator blind to treatment. For statistics, each individual score represented the mean of the three sections. Histology was scored as follows (Reber et al. 2007, Reber et al. 2008): 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) and infiltration: (0, no infiltration; 1, infiltrate around crypt basis; 2, infiltrate reaching the 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.

In situ hybridization of hypothalamic OXT mRNA

Assessment of hypothalamic OXT mRNA expression was performed as described previously (Reber and Neumann 2008). Briefly, after decapitation, brains were rapidly removed, snap-frozen in methylbutane cooled on dry-ice, and stored at -80 °C for subsequent *in situ* hybridization. A series of 16-µm cryocut sections of the hypothalamus including the SON) and PVN nuclei was thaw-mounted onto slides at -20 °C and used to quantify hypothalamic OXT mRNA expression. As described before (Reber and Neumann 2008), hybridization was performed using specific 48-mer, 35S-labeled oligonucleotide probes for OXT mRNA (5'CTCGGAGAAGGCAGACTCAGGGTCGCAGGCGGGGTCGGTGCGGCAGCC3').

Hybridized slices were exposed to BioMax MR film (Kodak, Cedec, France). OXT mRNA expression in the PVN and SON were measured as optical density using NIH ImageJ 1.31

program (http://rsb.info.nih.gov/ij/). Bilateral measures were taken from two to four PVN and SON sections for each brain, which were pooled to provide individual means per mouse. For tissue background, the optical density of a non-hybridized region outside the PVN and SON was measured.

Receptorautoradiography for OXT receptors

The distribution of OXTR binding sites within the dorsolateral septum (DLS), ventrolateral septum (VLS), central amygdala (CeA), medial amygdala (MeA), basolateral amygdala (BLA), and median raphe nucleus (MeRN) was performed using receptorautoradiography (Lukas et al. 2010). Briefly, brains were cut into 16-µm coronal cryostat sections and mounted on slides. The receptor autoradiography procedure was performed using a linear OXTR antagonist [^{125}I]-d(CH_2)$_5$[Tyr(Me)$_2$-Tyr^9-NH_2] (Perkin Elmer, USA) as tracer. The slides were thawed and dried at room temperature followed by a short fixation in paraformaldehyde (0.1%). The slides were washed two times in 50 mM Tris (pH 7.4), exposed to tracer buffer (50 pM tracer, 50 mM Tris, 10mM $MgCl_2$, 0.01% BSA) for 60 min, and washed four times in Tris + 10 mM $MgCl_2$ (room temperature, 3 x 7 min, 1 x 30 min on a shaker). The slides were then shortly dipped in pure water, air-dried, and exposed to Biomax MR films (Kodak, Cedex, France). The exposure time varied between 16h and 21 d depending on the receptor density in the region of interest. To ensure a constant exposure time all slides comprising the same brain area of each group were processed on the same film. Exposure time varied between 16 h and 21 d depending on the receptor density in the region of interest. Optical density was calculated per mouse by taking the mean of bilateral measurements of four to six brain sections per region of interest. For tissue background, the optical density of a non-specific region outside the respective brain area was measured. After background subtraction, the data were converted into alteration in per cent.

Verification of cannula placement

The correct placement of the icv cannula was verified on Nissl-stained cryostat slices (40 µm). One mouse (CSC-OXT_{high} group) was excluded from statistical analysis due to incorrect cannula location.

Statistics

Statistical analyses were performed using SPSS for Windows (Version 18; SPSS Inc, Chicago, IL, USA). One-way (experiment 2: factor icv treatment) or two-way (experiment 1: factors OMP surgery x CSC exposure; experiment 3: factors icv treatment x CSC exposure or factors adrenal stimulation x CSC exposure) analysis of variance (ANOVA) followed by *post hoc* Bonferroni pairwise comparisons, when appropriate, was used. All data represent mean + SEM. Significance was set to $p \leq 0.05$.

Results

Experiment 1: Effects of OMP surgery on CSC-induced anxiety and physiological parameters

There was a main effect of CSC on EPM anxiety ($F_{1, 26}=21.8$; $p<0.001$), body weight gain ($F_{1,26}=15.8$; $p<0.001$), relative adrenal ($F_{1,26}=19.8$; $p<0.001$) and thymus ($F_{1,26}=20.1$; $p<0.001$) weight, and histological damage score of the colon ($F_{1,26}=7.85$; $p<0.01$).

Post-hoc Bonferroni pairwise comparisons revealed an increased anxiety-related behaviour in CSC compared with SHC mice independent of prior OMP surgery. In detail, both CSC-NS and CSC-OMP mice spent less time on the open arms of the EPM compared with respective SHC-NS ($p=0.001$) and SHC-OMP ($p<0.01$) mice (Fig. 28A).

Also established physiological parameters of CSC were confirmed in mice implanted with an OMP. In detail, a reduced body weight gain was found in CSC-NS ($p<0.05$) and CSC-OMP ($p<0.01$) mice compared with respective SHC mice (Fig. 28B).

An increased relative adrenal weight was found in both CSC-NS and CSC-OMP mice compared with respective SHC-NS and SHC-OMP (both $p<0.01$) mice (Fig. 28C), and a reduced relative thymus weight was seen in CSC-NS compared with SHC-NS ($p<0.05$) and CSC-OMP compared with SHC-OMP ($p=0.001$) mice (Fig. 28D).

Histological damage score of the colon was found to be increased in CSC-NS animals ($p<0.05$ vs. SHC-NS; Fig. 28E).

Importantly, in the SHC-group OMP did not affect any parameter assessed, although a trend towards increased histological damage score was found. Additionally, neither CSC nor OMP surgery affected plasma morning CORT levels (SHC-NS: 29.1±5.03 ng/ml; CSC-NS: 46.6±13.8 ng/ml; SHC-OMP: 35.6±9.03 ng/ml; CSC-OMP: 53.2±11.2 ng/ml).

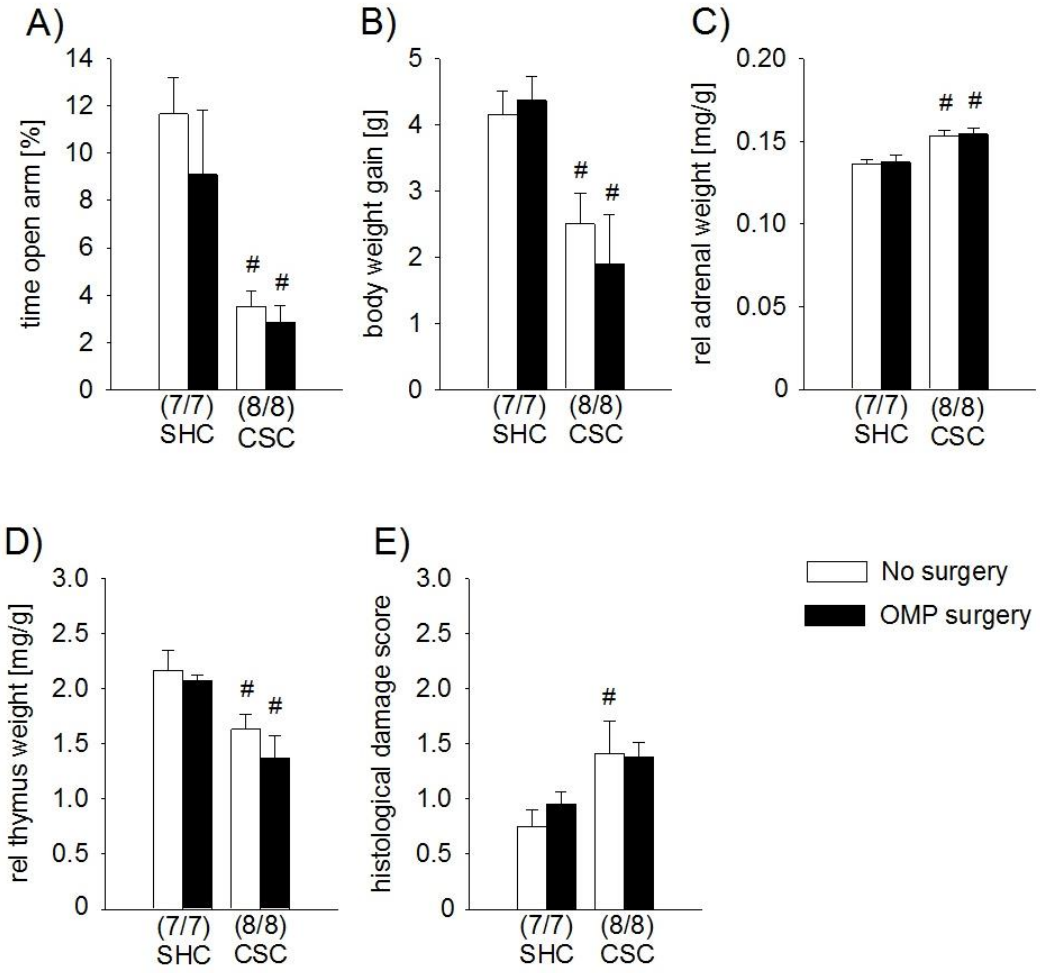


Figure 28: No effects of implantation of an osmotic minipump (OMP) and chronic icv infusion of vehicle (VEH) on anxiety and physiological parameters altered by 19 days of exposure to chronic subordinate colony housing (CSC) in male mice. Anxiety-related behaviour is reflected by a reduced percentage of time spent on the open arms of the elevated plus-maze. Data represent mean + SEM; # $p<0.05$ vs. SHC; animal numbers are given in brackets; two-way ANOVA followed by Bonferroni *post hoc* pairwise comparisons.

Experiment 2: Dose-dependent effects of chronic icv OXT (1 ng/h, 10 ng/h; 15 days) on anxiety and physiology

Anxiety: There was a main effect of chronic icv treatment on anxiety-related behaviour in the LDB ($F_{2,33}=7.67$; $p<0.01$) and on the EPM ($F_{2,33}=4.62$; $p<0.05$). OXT_{high}-treated mice (10 ng/h) spent less time in the lit compartment ($p<0.05$ vs. VEH; $p=0.001$ vs. OXT_{low}; Fig. 29A) and on the open arms ($p<0.05$ vs. VEH and OXT_{low}; data not shown), respectively, indicating

increased anxiety. Further, OXT_{low}-treated mice (1 ng/h) displayed an increased locomotor activity in the LDB ($p<0.001$ vs. VEH, OXT_{high}), but not in the EPM (data not shown).

Physiology: There was an effect of chronic icv treatment on body weight gain ($F_{2,53}=4.03$; $p<0.05$) with an increased body weight gain found in OXT_{high}-treated mice ($p<0.05$ vs. VEH-treated mice; Fig. 29B). However, chronic icv OXT did not affect adrenal weight (Fig. 29C), thymus weight (Fig. 29D), histological damage score of the colon (Fig. 29E) or plasma CORT (data not shown). The animal numbers given in Fig. 29B-E comprise both the animals tested for their anxiety-related behavior in the LDB on day 15 and on the EPM on day 15 whereas Fig. 29A comprises only the LDB animals.

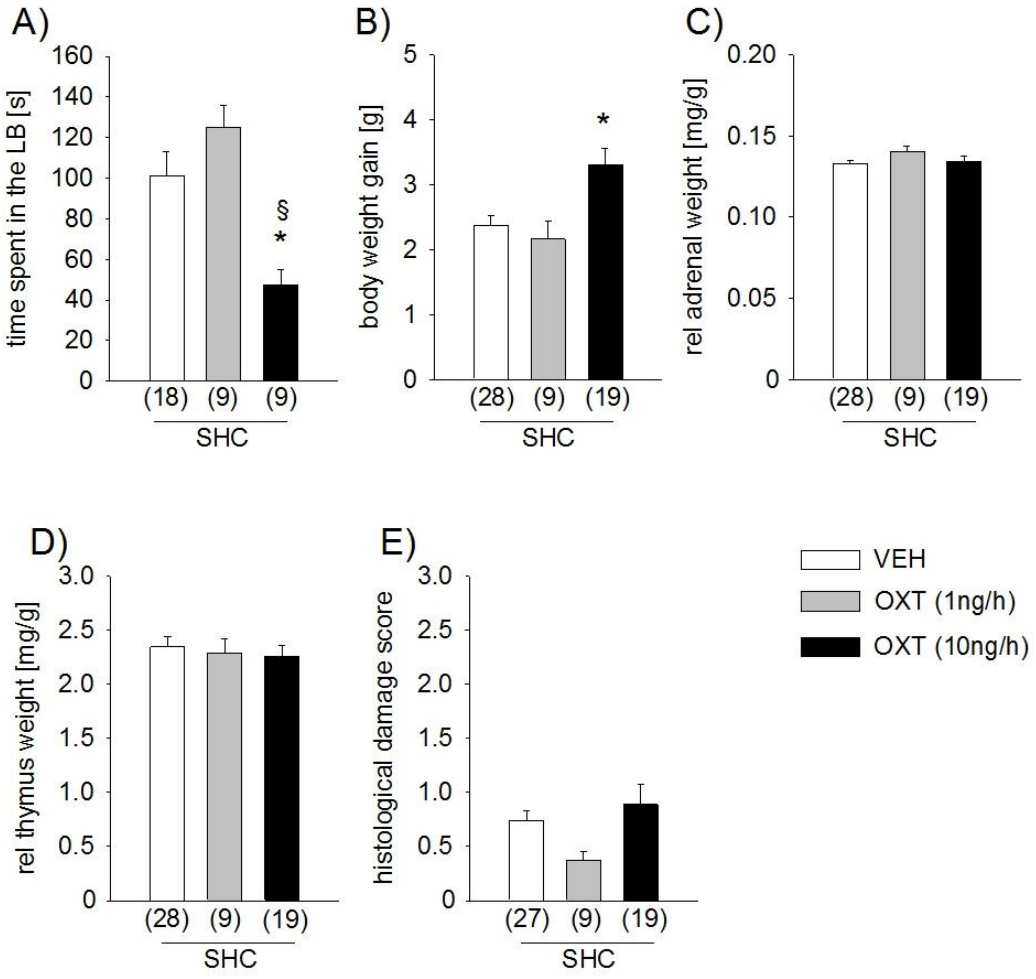


Figure 29: Effects of icv infusion of oxytocin (OXT, 1 ng/h, 10 ng/h *via* OMP, 15 days) on anxiety, body, relative (rel) adrenal and thymus weight, as well as histological damage score of the colon. Anxiety-related behaviour is reflected by a reduced time spent in the lit compartment of the light-dark

box. Data represent mean + SEM; * p<0.05 vs. VEH; § p<0.05 vs. OXT (1ng/h;); animal numbers are given in brackets; one-way ANOVA followed by Bonferroni *post hoc* pairwise comparisons.

Experiment 3: Effects of chronic icv OXT_{low} (1 ng/h, 19 days) and CSC Anxiety: There was an interaction of CSC exposure and icv treatment ($F_{1,38}$=4.21; p<0.05). An increase in anxiety-related behaviour could be confirmed in CSC-VEH mice (p<0.05 vs. SHC-VEH), which was prevented in CSC-OXT_{low}-treated mice; thus, CSC-OXT_{low} mice were less anxious than CSC-VEH mice (p<0.01; Fig. 30A). There was no group difference in locomotor activity (Fig. 30G).

Physiology: Whereas body weight gain did not differ between the 4 treatment groups (Fig. 30B), CSC and icv treatment affected relative adrenal weight ($F_{1,37}$=4.15; p<0.05), which was found to be increased in CSC-VEH mice (p<0.001 vs. SHC-VEH). Adrenal hypertrophy was found to be prevented by chronic OXT_{low} infusion (p<0.01 CSC-OXT_{low} vs. CSC-VEH; Fig. 30C).

Also, there was a main effect of CSC ($F_{1,38}$=8.69; p<0.01) and icv treatment ($F_{1,38}$=11.9; p=0.001) on thymus weight, which was found to be reduced in CSC-VEH mice (p<0.01 vs. SHC-VEH); chronic OXT_{low} prevented this CSC-induced effect (p<0.001; Fig. 30D).

Further, there was an effect of CSC on the histological damage score of the colon ($F_{1,36}$=4.54; p<0.05), which was increased in CSC-VEH-treated mice (p=0.01 vs. SHC-VEH; Fig. 30E).

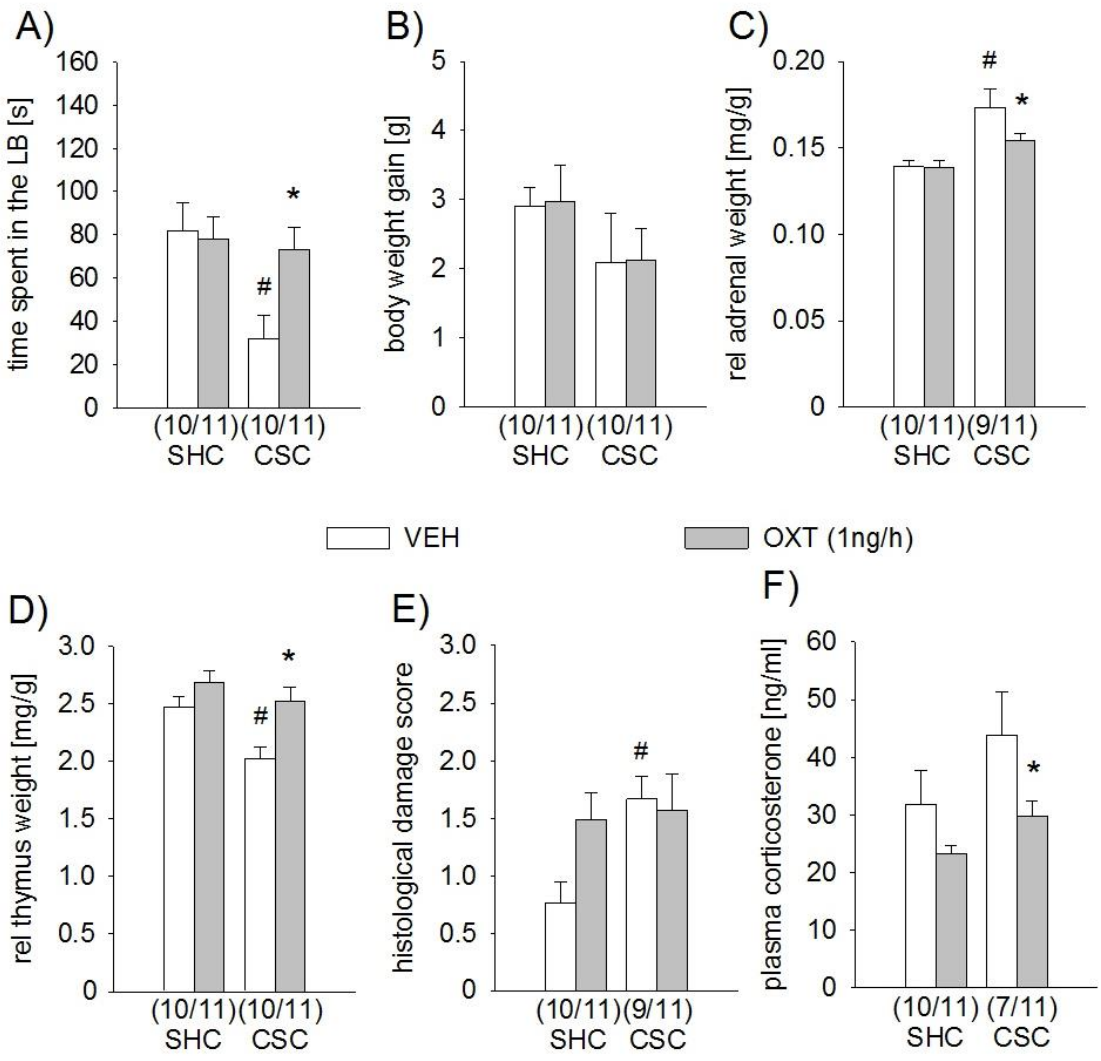
Plasma CORT concentrations in trunk blood were dependent on CSC exposure ($F_{1,35}$=4.46; p<0.05) and icv treatment ($F_{1,35}$=6.23; p<0.05). While CSC exposure did not alter plasma CORT independent of icv treatment, we found reduced CORT levels in CSC-OXT_{low}-treated mice (p<0.05 vs. CSC-VEH; Fig. 30F).

In vitro CORT response to ACTH

There was a main effect of ACTH ($F_{1,30}$=42.5; p<0.001) and CSC ($F_{1,30}$=4.87; p<0.05) on *in vitro* reactivity of adrenal explants of icv VEH-treated animals. In both SHC and CSC mice

ACTH stimulated adrenal CORT secretion (SHC: p<0.001; CSC: p=0.05 vs. basal). However, CSC mice displayed a decreased adrenal reactivity upon ACTH stimulation (p=0.05 vs. SHC; Fig. 30H).

In ICV OXT-treated mice (1 ng/h) only a main effect of ACTH ($F_{1,40}$=23.4; p<0.001) on *in vitro* adrenal reactivity was found. In both SHC and CSC mice, ACTH-stimulated CORT secretion from adrenal explants was increased compared with respective basal values ($p \leq 0.001$) with no difference between stressed and unstressed OXT_{low}-treated mice (Fig. 30H).



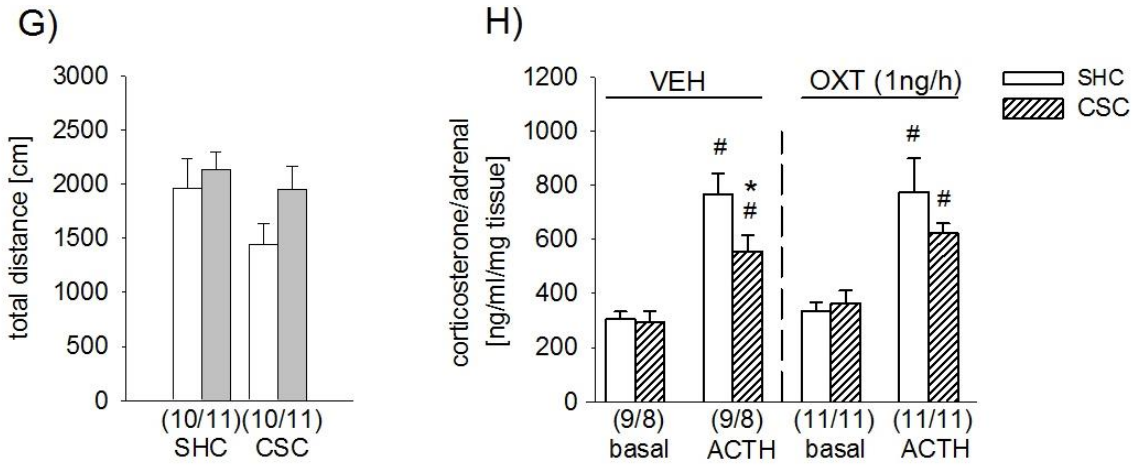


Figure 30: Effects of chronic icv infusion of oxytocin OXT_{low} (1 ng/h) or vehicle (VEH) during 19 days of chronic subordinate colony housing (CSC) exposure on CSC-induced hyper-anxiety (A), body weight gain (B), adrenal hypertrophy (C), thymus atrophy (D), colonic inflammation (E), basal morning plasma CORT concentrations (F), locomotor activity in the light-dark box (G) and *in vitro* adrenal responsiveness to ACTH (H) in single-housed mice (SHC) and CSC mice concomitantly infused with either OXT_{low} (1 ng/h) or VEH. Adrenal responsiveness to ACTH (100 nM) is indicated by *in vitro* secretion of CORT. Data represent mean + SEM; # $p<0.05$ vs. SHC; * $p<0.05$ vs. VEH; animal numbers are given in brackets; two-way ANOVA followed by Bonferroni *post hoc* pairwise comparisons.

Experiments 2 and 3: Effects of chronic icv OXT (on OXT mRNA expression and OXTR binding

In experiment 2, icv infusion of OXT over 15 days did not alter OXT mRNA expression within the PVN and the SON (Fig. 31A). Similarly, in experiment 3, there was no effect of exposure to CSC for 19 days or of chronic icv infusion of OXT over 19 days on OXT mRNA expression within both PVN and SON (Fig. 31B).

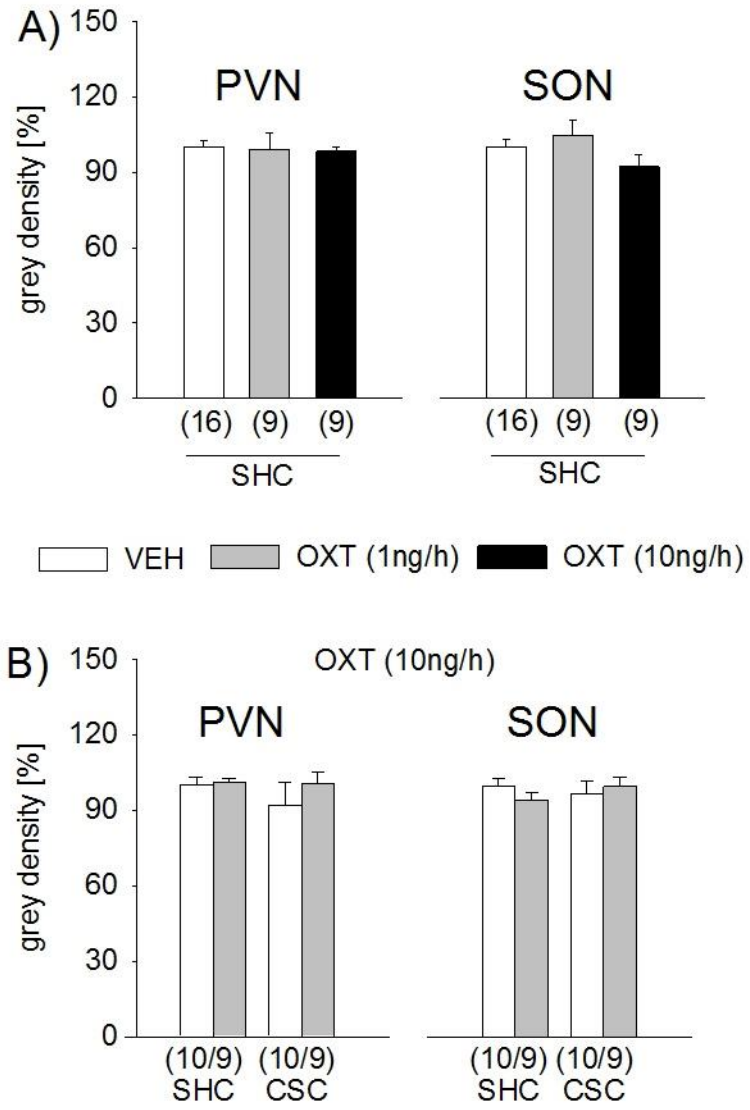


Figure 31: Oxytocin (OXT) mRNA expression within the paraventricular (PVN) and supraoptic (SON) nucleus after 15 days of chronic icv infusion with OXT (1 ng/h, 10 ng/h) or vehicle (VEH) (A), or after 19 days of CSC and concomitant infusion of either OXT (1 ng/h) or VEH; B). Animal numbers are given in brackets; one-way ANOVA (A) or two-way ANOVA (B) followed by Bonferroni *post hoc* pairwise comparisons.

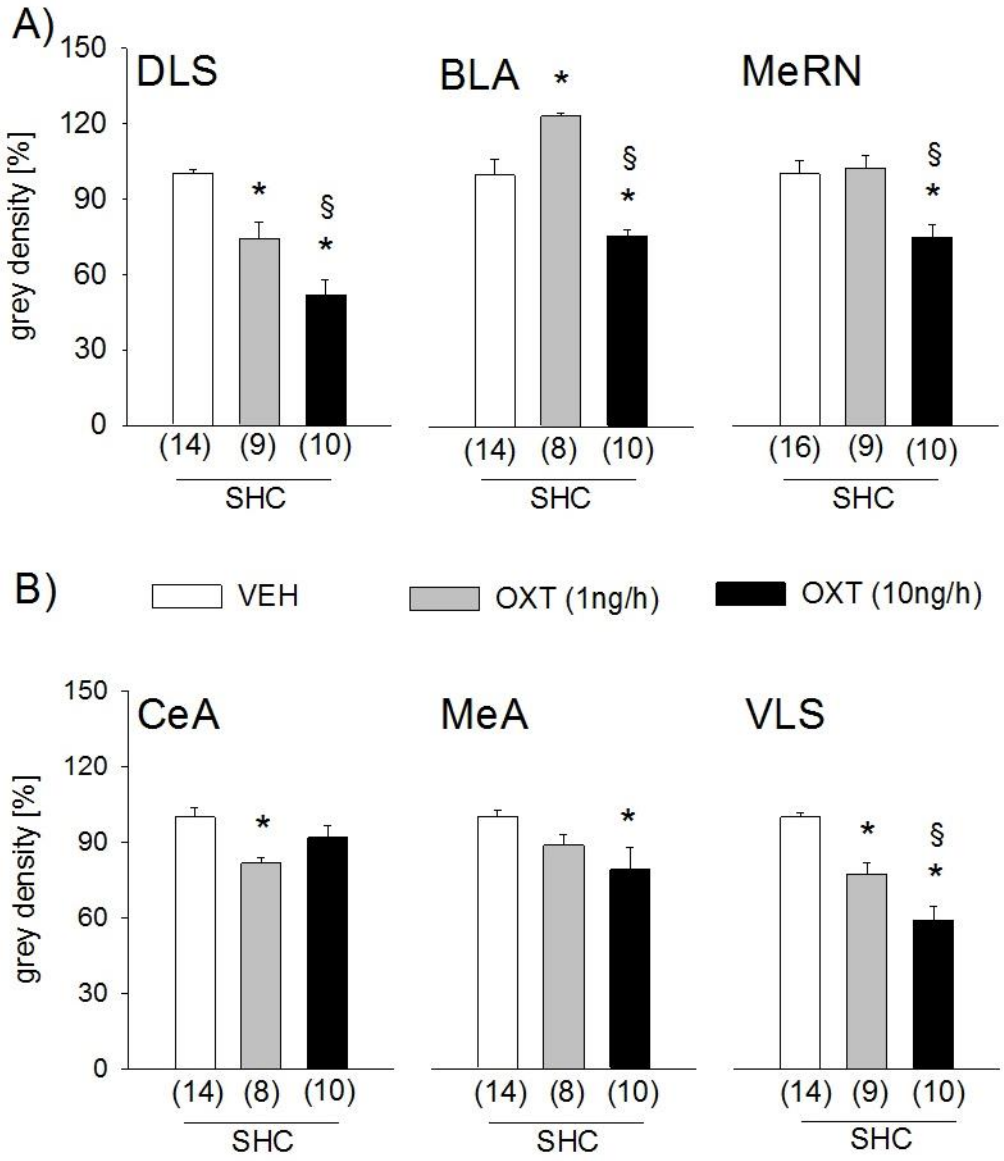
With respect to OXTR binding, in experiment 2, we found an effect of chronic icv treatment (15 days, 2 doses of OXT) on OXTR binding in the DLS ($F_{2,30}$=30.6; p<0.001), VLS ($F_{2,30}$=29.16; p<0.001), BLA ($F_{2,29}$=18.9; p<0.001), MeRN ($F_{2,32}$=7.42; p<0.01), CeA ($F_{2,29}$=5.10; p<0.05), and MeA ($F_{2,29}$=4.19; p<0.05). Specifically, reduced OXTR binding was found in the DLS and VLS of both $OXT_{low/high}$-treated animals (DLS, VLS: p<0.001 vs. VEH),

with an even lower OXTR binding found in OXT_{high}- compared with OXT_{low}- treated mice (DLS: $p<0.01$, VLS: $p<0.05$). Whereas within the MeRN, BLA and MeA OXTR binding was reduced in OXT_{high} mice (MeRN, BLA: $p<0.01$ vs. VEH and OXT_{low},; MeA: $p<0.05$ vs. VEH), it was reduced within the CeA ($p<0.05$ vs. VEH) and increased within the BLA ($p<0.01$ vs. VEH) after OXT_{low}-treatment (Fig. 32A).

In experiment 3, OXTR binding was affected by 19-days exposure to CSC and/or icv infusion of OXT_{low} in the DLS (ICV treatment: $F_{1,33}=32.6$; $p<0.001$), VLS ($F_{1,33}=33.9$; $p<0.001$) and MeRN (CSC x ICV treatment: $F_{1,33}=4.20$; $p<0.05$). *Post hoc* Bonferroni pairwise comparisons revealed a region-specific reduction in OXTR binding induced by CSC and/or chronic icv treatment. In detail, in both DLS and VLS, lower OXTR binding was found as a result of icv OXT in both SHC-OXT_{low} and CSC-OXT_{low} mice compared to respective VEH-treated animals (DLS and VLS: SHC: $p<0.001$; CSC: $p<0.01$; Fig. 32B).

In the MeRN, we found a reduction in OXTR binding upon CSC exposure ($p<0.01$ CSC-VEH vs. SHC-VEH), which was prevented by icv OXT_{low} resulting in an increased OXTR binding in CSC-OXT_{low}-treated compared with CSC-VEH-treated mice ($p<0.05$; Fig. 32B).

Within the CeA, BLA and MeA , neither CSC nor icv treatment did not affect local OXTR binding (Fig. 32B).


A)
DLS
BLA
MeRN
grey density [%]
150
120
90
60
30
0
(14) (9) (10)
(14) (8) (10)
(16) (9) (10)
SHC
B)
VEH
OXT (1ng/h)
OXT (10ng/h)
CeA
MeA
VLS
(14) (8) (10)
(14) (9) (10)

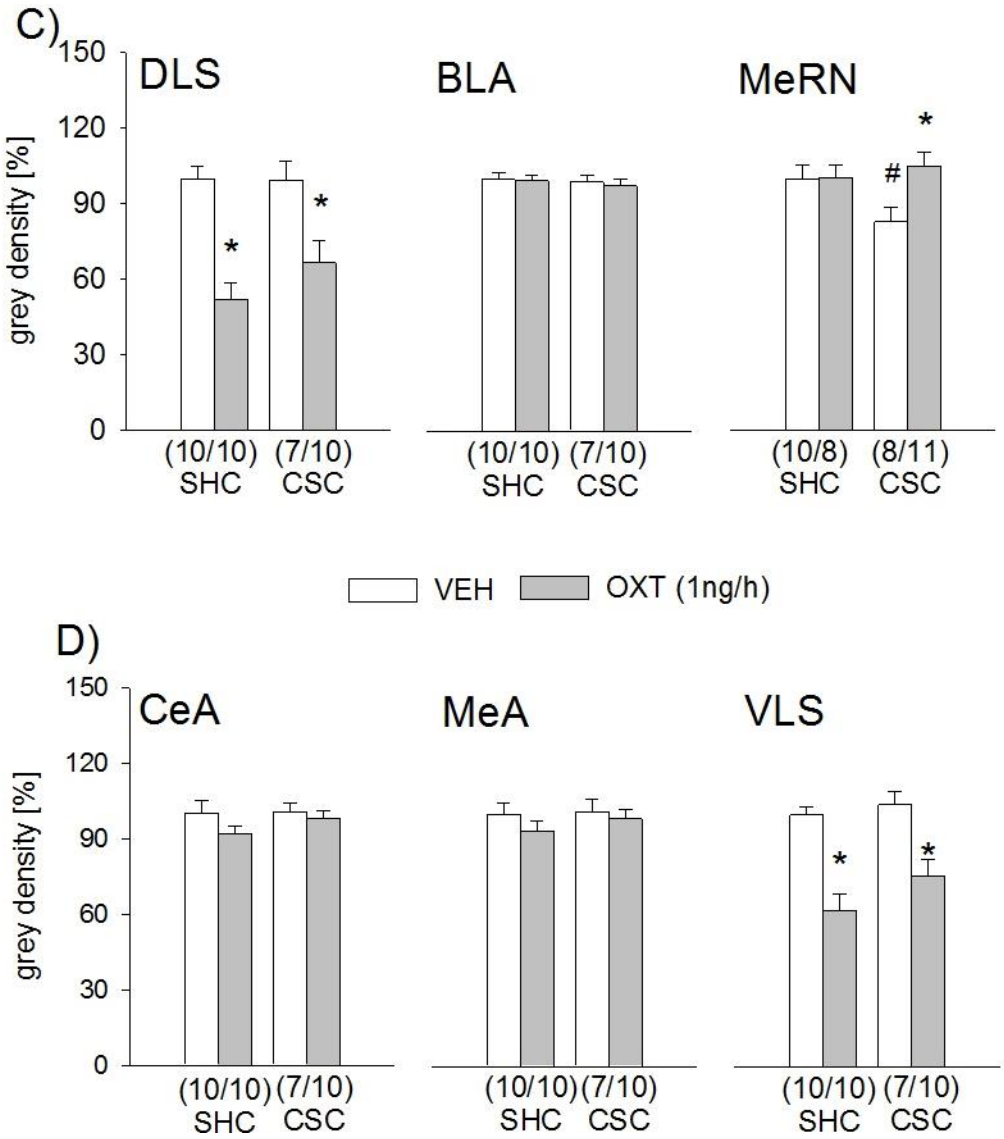


Figure 32: Effects of chronic icv of oxytocin (OXT) or vehicle (VEH) on OXT receptor binding within the dorsolateral septum (DLS), basolateral amygdala (BLA), median raphe nucleus (MeRN), central amygdala (CeA), medial amygdala (MeA) and ventrolateral septum (VLS) in (A) single-housed mice (SHC) after 15 days of infusion at either 1 ng/h or 10ng/h and (B) in SHC and CSC mice infused with OXT (1 ng/h) or VEH. Data represent mean + SEM; * $p<0.05$ vs. VEH (A, B); # $p<0.05$ vs. SHC (B); § $p<0.05$ vs. OXT (1ng/h) (A); animal numbers are given in brackets; one-way ANOVA (A) or two-way ANOVA (B) followed by Bonferroni *post hoc* pairwise comparisons.

Discussion

In the study described in that chapter, I demonstrate that chronic icv infusion of OXT in mice i) increases anxiety and reduces OXTR binding in several stress-and anxiety-related brain regions at high dose, but ii) can also be beneficial and protect against chronic stress-induced behavioural and physiological alterations at a low dose. In more detail, continuous central infusion of a high (10 ng/h), but not low (1 ng/h), dose of OXT *via* OMP over 15 days increased anxiety-related behaviour on the EPM and in the LDB, and body weight gain. Additionally, we found a reduction in OXTR binding within septal (DLS, VLS) and amygdala subregions (MeA, CeA, BLA), as well as the MeRN, with the lowest binding seen in OXT_{high}-treated mice. In contrast, exposure to CSC exclusively decreased OXTR binding in the MeRN with the expected concomitant increase in anxiety. Importantly, both of these CSC-induced effects were prevented by chronic OXT_{low} infusion throughout the stress paradigm. Our findings support bi-directional and dose-dependent consequences of chronically increased availability of intracerebral OXT, with a high dose being rather detrimental, and a low dose being able to prevent the consequences of chronic psychosocial stress exposure.

Increased anxiety-related behaviour, thymus atrophy, adrenal hypertrophy and, to a lesser extent, reduced body weight gain are established characteristics of chronic exposure to psychosocial stressors (Berton et al. 1998, Engler and Stefanski 2003, Reber et al. 2007, Schmidt et al. 2010, Slattery et al. 2012). In the present study I could confirm these alterations after 19 days of CSC exposure in male mice, which underwent surgical implantation of a vehicle-filled OMP one week prior to the onset of the CSC paradigm. Crucially, surgery and implantation of an OMP did not affect anxiety-related behaviour, basal plasma CORT levels or body, adrenal and thymus weights in SHC mice. These results laid the foundation to study dose-dependent effects of chronic icv infusion of OXT on anxiety and physiology, and whether chronic OXT can prevent the CSC-induced phenotype.

These results demonstrate that chronic icv OXT at low dose (1 ng/h) did not alter emotional or physiological parameters after 15 days, as anxiety-related behaviour on the EPM and in the LDB, thymus and adrenal weight, and basal plasma CORT levels remained unchanged. The lack of an anxiolytic effect is especially remarkable given the high anxiolytic potential of acute OXT administration in rodents (Blume et al. 2008, Jurek et al. 2012, Mak et al. 2012). Further, and in contrast to our prediction, we observed that a 10-fold higher dose of chronic icv OXT was anxiogenic. This OXT_{high}-induced phenotype was accompanied by a significant reduction in OXTR binding in various brain regions including the DLS, VLS, CeA, MeA, BLA and MeRN; findings in line with rapid desensitization of receptors following persistent agonist stimulation (Kelly et al. 2008). Such changes may provide a mechanistic insight behind the anxiogenic phenotype observed by this treatment. In further support, a link between down-regulation of the endogenous brain OXT system and anxiety is also suggested by studies in female OXT KO mice (Mantella et al. 2003) and male OXTR KO mice (Peters et al., unpublished data), whereas activation of the endogenous OXT system (Caughey et al. 2011, Hillerer et al. 2011) is associated with anxiolysis and stress hypo-responsiveness (Neumann et al. 2000, Waldherr and Neumann 2007, Slattery and Neumann 2008, Nyuyki et al. 2011). In further experimental corroboration, chronic icv infusion of OXT over 5 days (10 ng/h, 100 ng/h) reduced anxiety in ovariectomized, sex steroid-primed virgin female rats (Windle et al. 1997). An anxiolytic effect of chronic icv OXT (10 ng/h) over 6 days using OMP was also found in non-primed virgin female, but not male, rats with high innate anxiety (Slattery and Neumann 2010). Moreover, daily sc injection of OXT (20 µg/50µl; 14 days) attenuated social isolation stress-induced symptoms in female voles (Grippo et al. 2009). However, treatment-induced changes in the brain OXT system were not investigated in these studies. In the only human study employing repeated intranasal application of OXT over 3 weeks, reduced anxiety symptoms, especially social anxiety levels, and antipsychotic properties were described in schizophrenic patients (Feifel et al. 2010). Finally, while OXT_{low} treatment was found to increase OXTR binding in the BLA after 15 days (Fig. 4A) this effect was not observed after 19 days of treatment (Fig 4B). Although it has previously been shown that

social defeat across 10 days can increase OXTR mRNA (Litvin et al., 2011) mRNA and protein levels do not always follow the same pattern. Therefore, the relevance and mechanism underlying the observation after 15 days is presently unknown. However, these findings suggest that alterations in both the dose and timeframe of chronic exogenous OXT can lead to different outcomes at the receptor level. Taken together, it is obvious from these findings that more chronic studies are required to determine whether chronic OXT in humans can be used as an adequate treatment option. Just recently, our findings of reduced OXTR availability within distinct brain regions were confirmed in male mice, which were treated twice daily with OXT via the intranasal route for up to 21 days. After chronic OXT treatment, reduced OXTR binding was detected in the lateral septum, amygdala and nucleus accumbens (Huang et al. 2013), thus confirming the changes in OXTR binding observed in the present study While our study focused on anxiety and stress-related changes, chronic intranasal administration was shown to impair social interaction, suggesting that the detrimental effect of chronic OXT may extend to several psychiatric domains.

Due to the similarity of OXT to the related neuropeptide vasopressin, which exerts anxiogenic properties (Neumann and Landgraf 2012), we cannot rule out the possibility that central OXT at high concentrations cross-reacts with vasopressin receptors. Thus, future studies using a selective vasopressin receptor antagonist are needed in order to demonstrate receptor specific effects of chronic OXT infusion.

Although the lack of an anxiolytic effect after OXT_{low} and the anxious phenotype after OXT_{high} treatment seems, at first glance, to conflict with the literature, we are not aware of any study demonstrating anxiolytic and stress-protective effects of chronic administration of OXT in male rodents. In support, although robust local anxiolytic effects of synthetic OXT, e.g. within the PVN, were found in male and female rats (Blume et al. 2008, Jurek et al. 2012), acute icv infusion of OXT or the OXTR antagonist does not alter anxiety levels in male and virgin female rats under basal and non stressed conditions (Neumann et al., 2000). Further, the OXTR agonist carbetocin has also not been able to modulate anxiety state in mice withdrawn

for seven days from saline (i.e.non-stressed condition) implicating OXT to be only beneficial under conditions of stress (Zanos et al.; 2013). In addition, recent studies have revealed sex-dependent negative effects of chronic OXT. After 21 days of daily intranasal administration of OXT male, but not female voles displayed deficits in the formation of partner preference (Bales et al. 2012). Thus, it seems that chronic OXT infusion under non-stress conditions exerts sex-specific effects, which are, in addition, dependent on the physiological state of the individual.

The interpretation of increased body weight gain after chronic ICV OXT at high dose in male mice being accompanied by a reduction in central OXTR binding is consistent with the general agreement that brain OXT importantly regulates food intake (Blevins and Ho 2013). For example, mice with global- or anatomically-selective loss of OXT or OXTR show increased body weight gain and may develop adult-onset obesity without alterations in daily food intake (Blevins and Ho 2013).

As chronic icv chronic infusion of OXT at 1ng/h did not alter the anxiety phenotype, weight gain or OXTR binding in most brain regions, this dose was used for subsequent experiments to study, whether OXT_{low} can prevent CSC-induced changes (Reber et al. 2007, Slattery et al. 2012). Indeed, chronic OXT_{low} throughout exposure to 19 days of CSC prevented stress-induced anxiogenesis, adrenal hypertrophy and thymus atrophy. While CSC did not reduce body weight gain in this experiment, we have previously shown this parameter to be inconsistent across studies (Reber et al. 2007, Slattery et al. 2012). Furthermore, CSC-induced colonic inflammation, as indicated by an increased histological damage score and adrenal insufficiency, was prevented by OXT_{low} treatment. Interestingly, CSC-induced colitis is causally related to the initial increase in plasma CORT (Reber et al. 2007, Reber et al. 2011), and activation of the central OXT system, e.g. *via* social contact, can suppress HPA axis activity and, consequently, attenuate the stress-induced delay in wound healing (Detillion et al. 2004). Thus, these findings suggest that the ameliorating effect of chronic icv

OXT_{low} on CSC-induced peripheral alterations may be due to an efficient reduction in circulating plasma CORT levels during the initial phase of CSC exposure.

Taken together, the findings of the study described in that chapter demonstrate that chronic OXT_{low} prevented the CSC-induced increase in anxiety, whereas chronic OXT_{high} infusion resembled more a stress-like phenotype. Therefore, I assessed the effect of CSC on OXTR binding and whether OXT_{low} also prevented any alterations at this level. CSC exposure was only found to decrease OXTR in the MeRN, akin to the local decrease observed in this region after OXT_{high} treatment. The association between reduced OXTR binding and increased anxiety, confirmed by two different manipulations, suggests that this region may be critically involved in anxiety regulation (Yoshida et al. 2009). In support, chronic OXT_{low} infusion prevented the CSC-induced reduction in MeRN OXTR binding. Therefore, it would be of interest to determine whether chronic and/or acute OXT administration into the MeRN would be sufficient to reverse the behavioural and physiological consequences of CSC exposure. It is perhaps surprising that CSC did not affect OXTR in the DLS or MeA, as 10 days of social defeat were found to enhance local OXTR mRNA expression (Litvin et al. 2011). However, as mentioned above, mRNA and protein levels do not always correlate, and differences in experimental timing and quality of the stressors may explain this apparent discrepancy.

In summary, this study is the first to demonstrate that chronic icv infusion of OXT at high dose induces an anxiogenic phenotype and a reduction in OXTR binding in various relevant brain regions. Further, I can show that OXT infused at low dose during ongoing chronic psychosocial stress exerts stress-protective effects and prevents CSC-induced anxiety and physiological changes. These bi-directional consequences of chronic OXT treatment reveal the requirement of studies, in both males and females, to further improve our understanding of the effect of chronic manipulation of the OXT system with respect to the dose-dependent behavioral, neuronal and molecular adaptations, before chronic OXT can be considered as a possible treatment option of stress-related disorders.

Chapter 5

General Discussion

5.1 Summary of results

Chronic psychosocial stress is known to cause a variety of diseases including somatic, affective and substance abuse disorders. In this cotneext, the aims of the present thesis were to investigate the role of chronic psychosocial stress in the development of inflammation-related colorectal cancer as well as in the voluntary self-administration of EtOH. Another major goal of the experiments was to determine the involvement of the central OXT system on the development of stress-related pathologies and whether exogenous OXT, either following a single acute injection (EtOH consumption) or after a chronic administration can attenuate or block the establishment of stress-related behavioural and physiological alterations.

In chapter 2, I describe the effects of a 19-day exposure to our established model of chronic psychosocial stress, i.e. CSC, on the development of inflammation-related colon cancer. I was able to demonstrate that stressed mice developed more rapidly morphological alterations including a granular mucosa, flat dysplasias and solid tumours compared to unstressed mice after AOM-DSS treatment. Moreover, despite these macroscopic changes of the colonic surface, changes on the molecular level were found that are closely related to carcinogenesis, namely, a shift towards a regulatory T-cell mediated immune response, increased expression patterns of proliferation promoting proteins and, in addition, an increased proliferation with a concomitant reduced apoptosis. Therefore, I propose the combination of the CSC paradigm with the AOM-DSS model as an important and attractive tool to study the underlying mechanisms of stress- and inflammation-induced colon carcinogenesis.

In chapter 3, as an important prerequisite for possible anxiety-induced EtOH consumption, I could demonstrate that a 15-day CSC exposure was sufficient to induce an anxiogenic phenotype in male mice. In fact, I was able to show that 15 days of CSC enhanced the consumption of, and the preference for EtOH compared to unstressed animals at EtOH doses ranging from 2 – 8%. Importantly, the increased consumption and preference was not

due to any alterations in taste preference. Moreover, surgery for an icv guide cannula did not disrupt the differences in EtOH consumption of stressed and unstressed animals. The major goal of that study was to investigate, whether an acute peripheral or central OXT administration could reduce the 24-h EtOH intake. I demonstrated that the $GABA_B$ receptor agonist baclofen, taken as a control, reduced the EtOH consumption in stressed and unstressed animals, whereas OXT was only affective in SHC mice and only after peripheral injection. These results demonstrate the therapeutic efficacy of baclofen for the treatment of EtOH abuse, but also reveal the need for more studies investigating OXT as a possible medication to treat alcoholism.

In chapter 4, the major goal was to investigate whether chronic central OXT administration during 19 days of CSC could attenuate or prevent the establishment of stress-induced anxiety as well as physiological changes. As an important prerequisite, I could demonstrate that the implantation of an OMP and the central administration of VEH did neither influence the CSC-induced alterations nor induce behavioural or physiological stress symptoms in unstressed male mice. Moreover, I could show that chronic central infusion of OXT dose-dependently reduced OXTR binding within septal (DLS, VLS) and amygdaloid (MeA, CeA, BLA) subregions as well as the MeRN. Surprisingly, after 15 days of chronic treatment, OXT was anxiogenic at the high dose (10ng/h), whereas the low dose did not alter anxiety-related behaviour of mice. On the other hand, infused chronically during 19 days of CSC at low dose, OXT was able to prevent the stress-induced anxiety-related behaviour and also the physiological alterations induced by CSC. Since the only brain region showing a reduced OXTR binding after both CSC and chronic OXT_{high} administration was the MeRN, and because of the fact that chronic OXT_{low} was able to prevent this stress-induced downregulation of the OXTR, it is likely that the serotonergic and oxytocinergic systems are involved in the establishment and the prevention of the CSC-induced anxious phenotype. Therefore, manipulation of the central OXT system reflects an encouraging target for the treatment of stress-related pathologies. Due to dosage and temporal dynamics a greater

understanding of the chronic effects is needed before the OXT system can be considered as possible therapeutic target for a successful medication of stress-induced disorders.

In summary, the results of the present thesis demonstrate that chronic psychosocial stress is linked to diverse pathologies. In combination with other studies demonstrating acute anxiolytic, anti-stress, and anti-addiction properties in rodents and humans, the central OXT system ranges as a possible target for the successful treatment of those pathologies. Due to the current lack of chronic administration studies and the few existing studies demonstrating positive as well as negative effects after a chronic OXT administration, more research is needed for a better understanding of the involvement of the OXT system in mediating stress effects and a successful treatment of patients.

5.2 Effects of CSC on inflammation-mediated colon carcinogenesis

Tumour development represents a dynamic and constant ongoing process already described as adenoma-carcinoma-sequence with morphological alterations of the colonic surface that can be monitored *via* colonoscopy. Under healthy conditions, the colonic epithelium has a smooth surface. In the course of colon carcinogenesis, the mucosa develops a granular crown, followed by the appearance of flat polypoid dysplasia which can be the origin of polyps. The stressed mice developed all of these alterations at an earlier stage of the experiment which gives a first evidence for chronic psychosocial stress to promote inflammation mediated colon carcinogenesis.

Two fundamental hallmarks of carcinogenesis are an enhanced proliferation combined with a reduced apoptosis (Colotta et al. 2009). Further, studies demonstrated an increased proliferation at sides of inflammation or wounds until the tissue is completely re-established. Therefore, if DNA mutations accumulate at sides of an enhanced proliferation the risk for developing cancer dramatically increases. I could demonstrate that stressed mice have an increased proliferation ratio within the colon. This could be due to a more severe colonic inflammation, since CSC was shown to result in the development of spontaneous colitis

(Reber et al. 2007) and to aggravate DSS-induced colitis (Reber et al. 2008). On the other hand, several molecular factors are known to influence the proliferation cycle within the colonic tissue. One possibility reflects the enhanced expression of LRH-1 in stressed mice compared to unstressed controls. As already described in chapter 1, LRH-1 leads to an increased proliferation by acting as a co-activator of β-catenin and therefore, enhancing the Cycline D1 expression. On the other hand LRH-1 directly binds to the promotorregion of Cyclin E1. In that case, β-catenin acts as a co-activator of LRH-1 (Botrugno et al. 2004). Therefore, enhanced LRH-1 levels are able to increase the proliferation ratio also with unchanged levels of β-catenin. The results of the present thesis correlate with other studies associating an enhanced LRH-1 expression with an increased cell cycle progression (Benod et al. 2011) and cancer tissue (Wang et al. 2008, Chand et al. 2013). Further, the unchanged β-catenin levels of stressed and unstressed animals are in line with a recent study demonstrating no differences in the expression of that protein in tumour and healthy tissue of patients suffering of gastric adenoma (Wang et al. 2008).

As already mentioned, despite an increased proliferation, a reduced apoptosis is also an important mechanism for carcinogenesis. From the results of the present thesis I can only speculate about the underlying mechanisms. The trend towards a decreased IFNγ expression might give evidence for the decreased apoptosis. IFNγ can activate transduction mechanisms that comprise activated Stat1-complexes which initiate the apoptosis of cells. These processes result in an IFNγ/Stat1-dependent activation of genes, which encode for membrane proteins like caspase 1, leading to an acceleration of apoptotic processes (Ikeda et al. 2002).

Despite morphological alterations, increased epithelial cell proliferation and a reduced apoptosis, an impaired clearance of abnormal cells might be involved in the stress-mediated progression of inflammation-related CRC. For a successful clearance of tumour cells the presence or absence of cytotoxic T cells, T helper cells or regulatory T cells is essential. For example, Th1 cells activate $CD8^+$ cytotoxic T cells which incorporate perforins, granzymes, proteoglycans and lysosymes. Upon stimulation, the cytotoxic T cells release their contents

via exocytosis leading to lysis, necrosis or apoptosis of the target cell (Holländer 2006). In contrast, Th2 cells, but also regulatory T cells, promote carcinogenesis by suppressing the Th1 cell -mediated immune response. At first glance, the increased amount of $CD4^+$ Th cells within the colonic tissue and the enhanced percentage of $CD3^+$ T cells of isolated and pooled mesLNCs of stressed mice compared to unstressed animals suggested an upregulated tumour-protective Th1 cell profile. However, the tendency towards a decreased colonic IFNγ mRNA expression and an unchanged IFNγ secretion from mesLNCs in combination with an enhanced colonic FoxP3 mRNA expression gives evidence for the $CD4^+$ and $CD3^+$ cells to be rather regulatory T cells than tumour-eliminating Th1-cells. This hypothesis is strengthened by an increased number of $CD3^+$/$FoxP3^+$ T cells within the mesLNs. In support, an increased susceptibility to UV-induced skin cancer by suppressing type-1 cytokines, protective T cells, and increasing regulatory T cell numbers has been found following repeated restraint stress (6h/day, 3 weeks) (Saul et al. 2005). Moreover, a variety of studies demonstrated that increased regulatory T cell infiltration in tumour bed predicted reduced survival patients suffering from cancer (Martin et al. 2010). A recent study demonstrated that Th2 but not Th1 cells develop a glucocorticoid resistance after 19 days of CSC (Schmidt et al. 2010) representing a shift from a Th1-mediated towards a Th2-mediated immune response. Several studies demonstrate that this shift is typical for chronic stress exposure (Dhabhar and McEwen 1999, Elenkov and Chrousos 2006) but also the reason for an ineffective cellular immune response during carcinogenesis (Cui and Florholmen 2008). Further, inflammatory episodes of repeated DSS cycles were shown to increase plasma CORT levels (Reber et al. 2008) leading to a specific downregulation of tumour protective Th1 immune suppression. These data indicate an enhancement of regulatory T cells after termination of CSC promoting a development of CRC. Another very important factor with respect to carcinogenesis reflects TNF. I could demonstrate an increased TNF mRNA expression within the colonic tissue which is in line with a variety of studies demonstrating enhanced TNF expression patterns within adenomatous tissue. High amounts of TNF were reported to reduce immune-mediated tumour cell elimination (Reiche et al. 2004), to promote signalling

and proliferation *via* the β-catenin pathway (Oguma et al. 2008) and to activate COXII (McConnell and Yang 2009). As already described, COX II is involved in mediating processes including apoptosis, angiogenesis and tissue invasiveness (McConnell and Yang 2009). In detail, human colonic cancer cells, overexpressing COX II were shown to be resistant to apoptosis (Sun et al. 2002). Regarding cancer progression, COX II is known to increase the invasiveness of colon cancer cells by activating metalloproteinase-2 (Li et al. 2002), whereas COX II suppression resulted in reduced levels of metalloproteinase-2 and -9 in human prostate cancer (Attiga et al. 2000). Further, in relation to angiogenesis, expression levels of COX II in colon cancer cells correlate with high levels of vascular endothelial growth factor, basic fibroblast growth factor and endothelin-1, which stimulate endothelial migration and endothelial tube formation (Tsujii et al. 1998). In line with those studies, our results of an increased mRNA as well as protein expression of colonic COX II support the stress-promoted carcinogenesis concept. Interestingly, a recent study was able to show that individual differences in anxiety correlate with chronic stress burden and cancer progression. High anxious animals were reported to have increased plasma CORT levels, enhanced amounts of regulatory T cells and an exaggerated tumour burden (Dhabhar et al. 2012). Therefore, it might be interesting to investigate the effects of chemotherapy combined with the administration of anxiolytic drugs on carcinogenesis. With respect to the acute anti-stress and anxiolytic effects of OXT and the findings described in chapter 4 of this thesis, a chronic OXT administration during CSC might be able to reduce the stress-induced increase in CRC.

5. 3 Effects of CSC on voluntary EtOH self-administration

Over several decades the effects of stress on EtOH drinking have been studied by a variety of animal models and experimental procedures. These studies often link stress exposure to an enhanced EtOH consumption, but the association of stress and EtOH abuse is multifaceted and poorly understood.

As already reviewed in chapter 1, EtOH exerts anti-stress and anxiolytic effects which are well described in rodents and humans and serve as the cornerstone of the tension (stress)-reduction hypothesis (Cappell and Herman 1972, Pohorecky 1991, Brady and Sonne 1999, Sayette 1999). A previous study demonstrated that 15 days of CSC are sufficient to induce stress-related physiological symptoms (Reber et al. 2007). In addition, the data of the current thesis show that 15 days of CSC induced an anxiogenic phenotype in male mice. An increased anxiety-related behaviour following chronic stress exposure is in line with a variety of animal studies and is known to serve as a major risk factor for EtOH abuse in humans (Cappell and Herman 1972). Indeed, studies demonstrate a strong correlation of EtOH intake and stress exposure (Becker et al. 2011) (Spanagel et al. 2010), with increased anxiety enhancing the EtOH intake in rodents (Becker et al. 2011)(Spanagel et al. 1995) and humans (Cappell and Herman 1972) (Spanagel et al. 2010). These findings support the hypothesis that some alcoholics consume EtOH as a self-medication to reduce the anxiety and stress symptoms (Bolton et al. 2006). Therefore, investigating the role of anxiety on EtOH consumption might give important evidence on the link of an escalated EtOH consumption and chronic stress exposure (Addolorato et al. 2009). The data of the current thesis are in line with studies correlating a more anxious phenotype with enhanced EtOH consumption. Stressed mice displayed an increased consumption of, and preference for, EtOH compared to unstressed animals. This enhanced intake was present for all EtOH concentrations tested (2 – 8%). Importantly, the escalation in EtOH intake following chronic psychosocial stress was not due to alterations in taste preference of the male mice, since CSC animals did not differ from SHC mice in their preference for consuming sweet (saccharine) or bitter (quinine) solutions or their total fluid intake. This data indicate the CSC paradigm to act as a severe psychosocial stressor since it increased the EtOH consumption even at low doses. Further, an implantation of a guide cannula targeting the lateral ventricle was not able to disrupt the CSC effects on the voluntary self-administration of EtOH in male mice, which is a very important prerequisite for the effects of an acute central OXT injection on the stress-induced EtOH intake (see 5.4.1).

5.4 Effects of chronic central OXT on CSC-induced behavioural and physiological alterations

It is commonly reported in the literature that increased anxiety-related behaviour, enhanced consumption of substances of abuse, reduced body weight gain, thymus atrophy and adrenal hypertrophy are general characteristics of chronic exposure to stressors (Berton et al. 1998, Engler and Stefanski 2003, Sinha 2008, Schmidt et al. 2010). The data of the current thesis demonstrate that these alterations are present after CSC exposure, as previously reported (Reber et al. 2007). As we intended to reverse CSC-induced effects with chronic icv infusion of OXT, I had to develop a suitable experimental approach without interference of the health of the animals or the CSC effects. Importantly, the implantation of an OMP suitable to deliver OXT for 4 weeks the week prior to CSC onset did not affect any of these CSC effects in comparison with non-operated CSC mice. In addition, in SHC mice OMP implantation had no effect on anxiety-related behaviour, body, thymus, and adrenal weight as well as colonic inflammation reflected by an identical histological damage score. This important result enabled me to proceed to the major goal, namely whether behavioural and physiological parameters are affected by a chronic central OXT administration in a dose-dependent manner and whether chronic central OXT reverses or attenuates the stress-induced changes of a 19-day CSC exposure. As already reviewed in chapter 1, OXT is known to exert anti-stress and anxiolytic properties in rodents and humans after acute administration. With respect to the endogenous brain OXT system animal studies demonstrated that an up-regulation of the endogenous OXT system results in hypo-responsiveness to stressor exposure, an effect that is present in lactating female rats (Slattery and Neumann 2008), in male rats after sexual contact (Waldherr and Neumann 2007), and rats after social attachment (Babygirija et al. 2010). However, regarding repeated or chronic central or peripheral OXT injections, there are only a few studies with some of them demonstrating positive effects on anxiety-related behaviour and stress-related parameters (Windle et al. 1997, Windle et al. 2004, Grippo et al. 2009) and others more negative effects (Bales et al. 2012, Huang et al. 2013).

5.4.1 Effects of exogenous OXT on stress-induced physiological alterations

Having established the implantation of an OMP without affecting the stress-induced symptoms including the behaviour and physiology of SHC mice, I assessed the efficacy of chronic central OXT on stress-related physiological alterations. In more detail, I could demonstrate that chronically central administered OXT (1ng/h, 10ng/h) for 15 days exerts no effects on thymus weight, adrenal weight and histological damage score of the colon. Regarding body weight, I demonstrated an increased body weight gain following an icv OXT_{high} administration period of 15 days. The interpretation of this finding of increased body weight gain after chronic icv infusion of OXT_{high} in male mice accompanied by a reduction in central OXTR binding is consistent with the general agreement that the brain OXT system importantly regulates food intake and inhibits body weight gain. For example, administration of OXT reduced, and an OXTR antagonist increased body weight gain, respectively. Further, mice with global or anatomically selective loss of OXT or OXTR show increased body weight gain and may develop adult-onset, without alterations in daily food intake (Blevins and Ho 2013). Interactions of brain OXT with the sympathetic nervous system and with other factors regulating the body's energy balance including leptin are likely after its chronic infusion, but need to be studied in detail.

As chronic icv infusion of 1ng OXT did not alter the stress-related physiological symptoms and anxiety-related behaviour (see 5.4.2) after 15 days, this dose was used for subsequent experiments to study whether OXT_{low} can prevent CSC-induced changes reported before (Reber et al. 2007, Slattery et al. 2012). Indeed, I could reveal that chronic icv OXT_{low} infusion throughout the 19-day CSC paradigm prevents the stress-induced thymus atrophy, and adrenal hypertrophy. Furthermore, in contrast to mice that did not undergo OMP surgery at all or were implanted with a OMP containing VEH only, CSC did not increase the histological damage in chronically icv OXT_{low}-treated mice. Interestingly, social contact prevents the stress-induced delay in wound healing through a mechanism that involves central OXT-induced suppression of the HPA axis (Detillion et al. 2004). Therefore, it is not

unlikely that the ameliorating effect of chronic icv OXT_{low} on CSC-induced colitis is due to an efficient reduction in circulating CORT during the initial phase of CSC exposure which was shown to be causally involved in inflammatory processes developing in the colon later on (Reber et al. 2007, Reber et al. 2011). Given that chronic icv OXT_{low} application might also increase peripheral OXT levels, another possible explanation for the lack of a pro-inflammatory CSC effect in OXT–treated mice could be that peripheral OXT has been shown to ameliorate a chemically-induced colitis (Iseri et al. 2005) and, thus, in general is considered to have anti-inflammatory properties.

However, it has to be mentioned that in contrast to these findings, chronic icv OXT_{low}-compared with VEH-treatment in SHC resulted in development of a mild colitis; in its severity comparable to what I saw in CSC mice that did not undergo OMP surgery at all or were implanted with an OMP containing VEH only. As we only assessed basal morning plasma CORT levels in the present study, it might very well be that chronic icv OXT_{low}-treatment, like it was shown for CSC mice developing a spontaneous colitis (Reber et al. 2007), results in basal plasma evening hypocorticism, promoting a overall pro-inflammatory milieu and development of spontaneous colitis. In this context it is of interest that OXT_{low} was also found to prevent the development of adrenal insensitivity to ACTH shown in an *in vitro* preparation after 19 days of CSC in VEH-treated mice. A blunted stressor perception and attenuated HPA-axis response (Neumann et al. 2000, Heinrichs et al. 2001, Windle et al. 2004) have been described under conditions of elevated OXT system activity. Further, shifting the neuropeptide balance to an enhanced activity of the central oxytocinergic system leads to an increased resilience to stressor exposure, which may further contribute to the attenuated effects of CSC exposure (Neumann and Landgraf 2012). Therefore, chronic low doses of OXT may be a beneficial approach for preventing and/or treating stress-related stress-induced physiological changes.

5.4.2 Effects of exogenous OXT on stress-induced anxiety-related behaviour

Having established the implantation of an OMP without affecting the stress-induced symptoms including the behaviour and physiology of SHC mice, I assessed the efficacy of chronic central OXT on anxiety-related behaviour in unstressed mice in two different doses. In more detail, I could demonstrate that chronically central administered OXT_{low} for 15 days exerts no effect on anxiety-related behaviour of male mice tested on the EPM or in the LDB which is remarkable given the high anxiolytic potential of acute OXT administrations in rodents (Ring et al. 2006, Blume et al. 2008, Jurek et al. 2012, Mak et al. 2012). Even more surprising was the fact that a 10-fold increase of the chronically administered OXT dose induced an anxiogenic phenotype. At first glance this result is conflictive with the literature since chronic exogenous OXT administration was shown to reduce anxiety-related behaviour. A chronic central OXT infusion (10ng/h, 100ng/h) for 5 consecutive days was shown to reduce anxiety-related behaviour in ovariectomized sex steroid-primed female rats. Moreover, a chronic OXT (10ng/h) icv administration for 6 consecutive days reduced anxiety-related behaviour in virgin non-primed female but not male rats bred for high innate anxiety (Slattery and Neumann 2010). The only human study so far about chronic OXT administration reported also beneficial effects on schizophrenic patients with a reduction of social anxiety symptoms following 3 weeks of daily intranasal application (Feifel et al. 2010).

Interestingly, I was able to demonstrate that this anxiogenic phenotype was accompanied by a downregulation of the OXTR binding within stress- and anxiety-related brain regions including the VLS, DLS, CeA, BLA, MeA and MeRN. These results are in line with a recent study demonstrating a downregulation of the OXTR binding in various brain regions of male mice after intranasal application of OXT (21 days) which was accompanied by a decreased social exploration of same-sex novel stimulus male mice (Huang et al. 2013). In confirmation of these results, one hour of restraint stress stimulated OXT release within the PVN, which was accompanied by a downregulation of local OXTR (Smith and Wang 2012). Those studies support a rapid desensitation and internalisation of GPCRs following prolonged activation (Kelly et al. 2008), mimicking a knockout phenotype. With respect to the

endogenous OXT system, behavioural studies on a downregulated OXT system did not display unambiguous effects (Kormos and Gaszner 2013). DeVries and colleagues described a reduced aggression in OXT knockout mice, but there was no difference in their anxiety-related behaviour (DeVries et al. 1997). In contrast, female OXT knockout mice displayed an increased anxiety-related behaviour compared to wildtypes, and the anxious phenotype of the knockout mice could be rescued by central administration of OXT, whereas central administration of an OXTR antagonist increased the anxiety-related behaviour of wildtype animals. My own data on OXTR knockout mice confirm a more anxious phenotype accompanied by a downregulated OXT system (Fig. 33). Further, an upregulation or activation of the endogenous OXT system, including increased OXTR binding, is associated with anxiolysis and stress hypo-responsiveness, as seen in lactating females (Neumann et al. 2000, Slattery and Neumann 2008, Caughey et al. 2011, Hillerer et al. 2011), in males after sexual contact (Waldherr and Neumann 2007) and after social attachment (Babygirija et al. 2010).

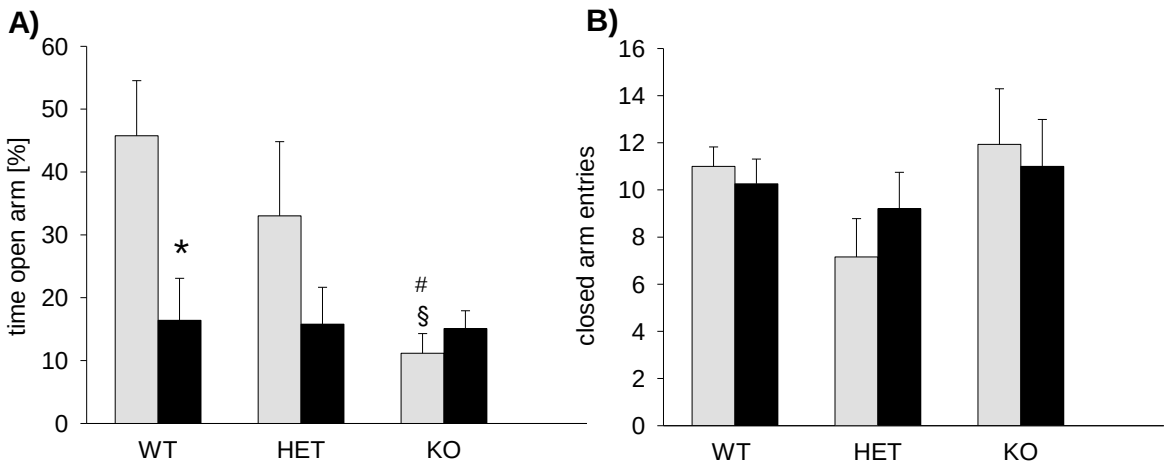


Figure 33: Effects of chronic subordinate colony housing (CSC) on anxiety-related behaviour of OXT receptor (OXTR) knockout mice on the elevated plus maze (EPM). 19 days of CSC increased anxiety-related behaviour reflected by a reduced time spent on the open arm of the EPM in CSC wildtype mice compared with single housed control (SHC) wildtype animals (A). Knockout of the OXTR induced an anxious phenotype comparable to the CSC wildtype and heterozygote animals (A). There was no effect of CSC exposure or genotype on general activity reflected by unchanged total arm entries (B). Data represent mean + SEM; $^{*}p = 0.01$ vs. SHC; $^{\#}p < 0.01$ vs. respective WT; $^{\$}p < 0.05$ vs. respective HET; (Two way ANOVA followed by Bonferroni *post hoc* pairwise comparisons). SHC (n = 6, 10, 13, grey bars); CSC (n = 8, 9, 16, black bars).

As chronic central OXT_{low} infusion did not alter anxiety-related behaviour, body weight gain, adrenal and thymus weight or OXTR binding in most brain regions, this dose was chosen to study whether OXT_{low} dose can prevent or attenuate CSC-induced anxiety-related behaviour. Indeed, the data of the current thesis demonstrate that chronic OXT_{low} administration throughout a 19-day exposure to the CSC paradigm prevented the establishment of an anxious phenotype. As both 15 days of chronic OXT_{high} administration and 19 days of CSC increased anxiety-related behaviour I assessed the effect of both manipulations on the central OXT system to determine if there were similar changes. While chronic central $OXT_{low/high}$ had no effect on OXT mRNA expression within the PVN or SON, chronic *icv* OXT dose-dependently decreased OXTR binding in the DLS, VLS, MeA, and CeA, whereas CSC

only affected OXTR binding in the MeRN. This effect was also observed following OXT_{high} administration but importantly, OXT_{low} infusion, while not altering OXTR binding alone, reversed the CSC-induced decrease in OXTR binding. This effect is of interest as the raphe nuclei are the main synthesis and projection site of serotonin in the brain and serotonin is known to be an important modulator of a range of social and emotional processes (Heisler et al. 1998, Miczek et al. 2002). An acute OXT infusion (5mg/ml) into the MeRN has been shown to facilitate serotonin release from that region and further result in decreased anxiety-related behaviour in mice. Importantly, this OXT-mediated effect was reversed by an additional systemic injection of ritanserin, a serotonin receptor ($5\text{-}HT_{2A/2C}$) antagonist (Yoshida et al. 2009). Further evidence for a close interaction of the oxytocinergic system and the serotonergic system in modulating stress-related adaptations was given by animal studies demonstrating that both, basal and restraint stress-induced plasma OXT levels were shown to be mediated via serotonin (Uvnas-Moberg et al. 1996, Marazziti and Catena Dell'osso 2008). A very interesting aspect that needs further elucidation represents the close similarity of OXT to AVP. Shifting the balance from a more active OXT system towards a more active AVP system is known to result in a more anxious phenotype (Neumann and Landgraf 2012). Therefore, chronic elevated levels of central OXT accompanied by a downregulation of OXTR binding might result in cross-reactions with OXT binding to AVP receptors and inducing an increased anxiety-related behaviour. Therefore, giving an AVP-antagonist during CSC and chronic central OXT might block the OXT-induced anxiety.

5.4.3 Effects of exogenous OXT on stress-induced EtOH intake

As reviewed in chapter 1, acute central or peripheral OXT injections are known to reduce anxiety-related behaviour, the self-administration of substances of abuse and to attenuate the effects of drugs on the physiology as well as the behaviour of an organism. Therefore, I assessed, whether an acute ip (10 mg/kg) or icv (0.5 µg/2µl) OXT injection blocks or attenuates the stress-induced EtOH intake in male mice. As a second pharmacological

manipulation, I investigated the therapeutic efficacy of an acute ip baclofen (2.5 mg/kg) injection on the EtOH intake in mice. This $GABA_B$ receptor agonist represents a promising treatment option for EtOH dependency (Edwards et al. 2011), due to its property to reduce EtOH intake in rats (Janak and Michael Gill 2003, Walker and Koob 2007), mice (Tanchuck et al. 2010) and humans (Addolorato et al. 2002, Cousins et al. 2002, Addolorato and Leggio 2010). Interestingly, Baclofen was shown to modulate oxytocinergic neuronal firing *in vitro* (Jourdain et al. 1996). Further, $GABA_B$ receptors act as modulators for excitatory synaptic transmission within the SON of rats (Kombian et al. 1996) and systemic administration of baclofen induced an increased c-Fos expression within the PVN of rats (van Nieuwenhuijzen et al. 2009). Moreover, a local injection of the $GABA_B$ receptor antagonist phaclofen into the PVN led to a reduction of plasma OXT levels in rats (Marques de Souza and Franci 2008). These findings lead to the speculation that baclofen may mediate its anti-abuse properties, at least in part, *via* interacting with the OXT system (van Nieuwenhuijzen et al. 2009, McGregor and Bowen 2012). Indeed, I was able to demonstrate that an acute ip baclofen injection could reduce the EtOH intake in SHC as well as CSC mice. Moreover, I could show that a single OXT injection reduced EtOH consumption in SHC mice; an effect that was only detectable after ip injection. Given the efficacy of baclofen to reduce EtOH intake in both, SHC and CSC mice, and the fact that OXT was only affective in SHC animals and only after ip injection, it is unlikely that baclofen mediates its effects on EtOH intake in CSC mice primarily *via* the OXT system. A possible reason for the discrepancy between the effectiveness of the ip and icv administrations are the doses employed, suggesting that higher icv doses of OXT could be effective in reducing EtOH consumption. However, given that the ip and icv doses have been shown to be behaviourally active and due to the fact that in another experiment I could demonstrate a reduced EtOH intake and EtOH preference following acute icv administration of OXT in rats (see Fig. 34), this explanation seems unlikely.

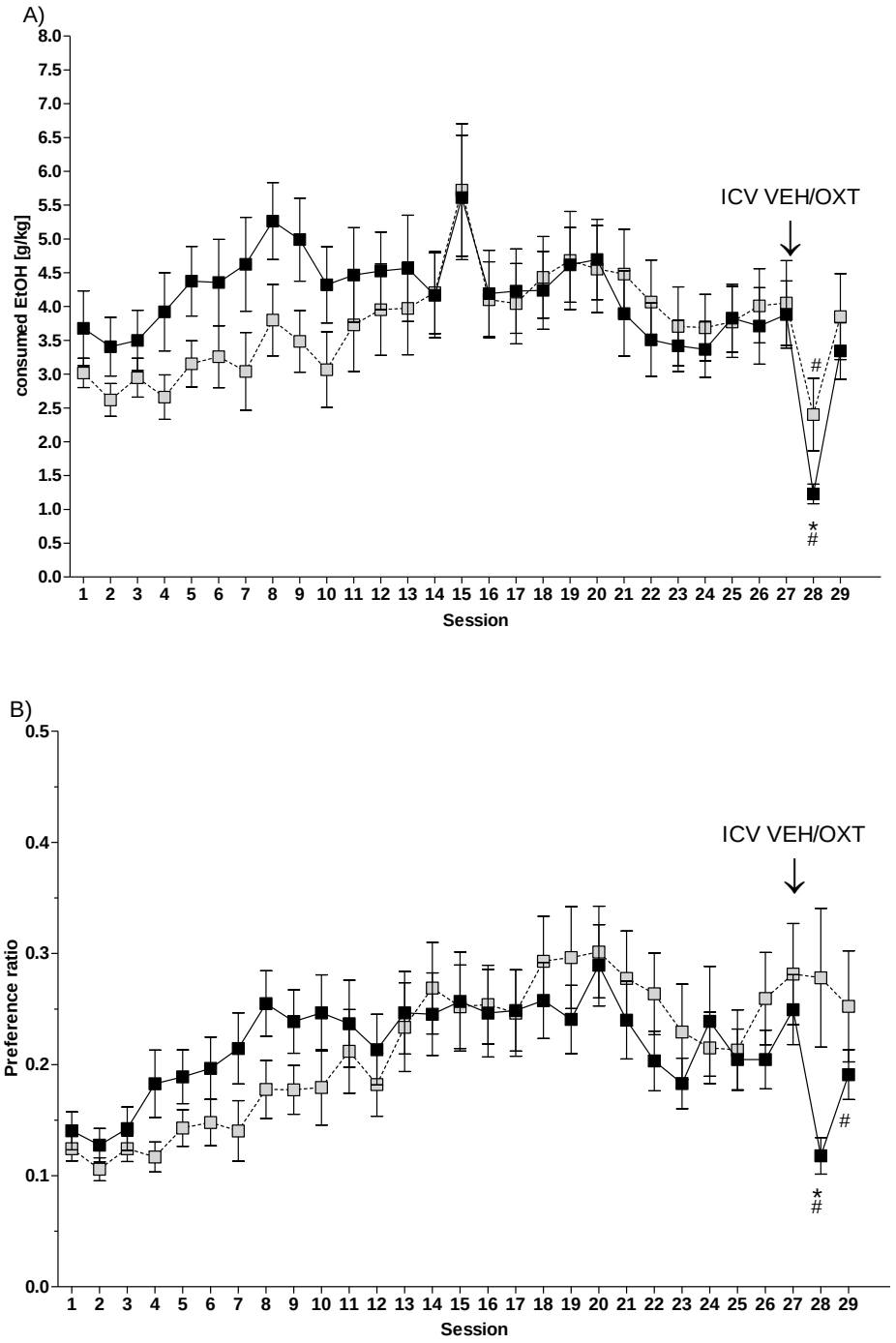


Figure 34: Effects of acute intracerebroventricular (icv) vehicle (VEH) or oxytocin (OXT) infusion on the voluntary EtOH intake. An acute icv VEH or OXT reduces voluntary EtOH intake after in rats, whereas in addition, icv OXT also reduces the preference for consuming EtOH. Data represent mean + SEM; $^{*}p = 0.01$ vs. VEH; $^{\#}p < 0.01$ vs. prior session; (rm ANOVA followed by Bonferroni *post hoc* pairwise compairisons). VEH (n = 10, grey squares); OXT (n = 10, black squares).

With regard to the effects following ip injection, the EtOH intake reducing properties of OXT are in line with the literature demonstrating positive effects of peripheral OXT on the self-administration of drugs of abuse (McGregor and Bowen 2012). Carson and colleagues demonstrated that ip OXT (0.3 – 1 mg/kg) inhibits intravenous methamphetamine self-administration in rats (Carson et al. 2010) an effect that was also shown for EtOH intake (Bowen et al. 2011). Due to poor penetration of neuropeptides of the blood brain barrier (Neumann and Landgraf 2012), a possible mechanism for peripheral OXT to reduce the self administration of substances of abuse is by interacting with the vagus nerve (Buisman-Pijlman et al. 2013). A recent study demonstrated that vagal nerve stimulation resulted in an inhibition of heroin- or heroin cue-induced relapse in rats, which was accompanied by a reduced c-Fos and an enhanced p-CREB expression within the NAc (Liu et al. 2011). Since the vagus nerve is known to contain OXT receptors (Welch et al. 2009) and to be involved in social behaviour (Carter et al. 2008), there is mounting evidence for the vagus nerve to mediate addiction and the effects of OXT on addiction (Buisman-Pijlman et al. 2013). Therefore, the peripheral effects of OXT may be transmitted to the CNS *via* the vagus nerve. A potential explanation for the lack of an OXT effect in CSC mice is that both chronic stress and EtOH have been shown to alter some parameters of the OXT system (Silva et al. 2002, Grippo et al. 2007, Litvin et al. 2011). Therefore, in the stressed mice, a single acute administration of OXT may be insufficient to overcome these deficits. Due to the lack of an acute icv OXT administration on EtOH intake and the stress- and anxiety-reducing effects of chronic OXT described in paragraph 5.4.1 and 5.4.2, I administered OXT chronically during 19 days of CSC and determined the 24h intake for rising EtOH concentrations (2 – 8%) following termination of the stress paradigm. Indeed, I was able to show a reduced 24h voluntary self-administration for EtOH doses ranging from 4 – 8% for the stressed animals that received OXT (see Fig. 35).

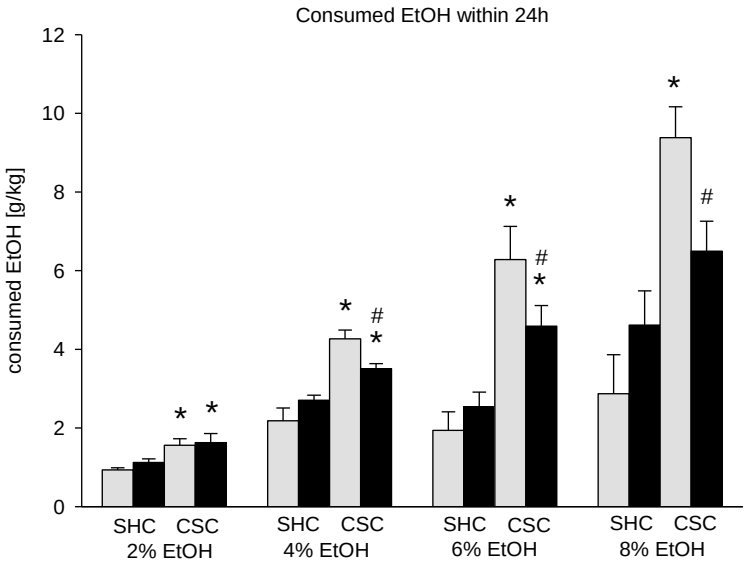


Figure 35: Effects of chronic intracerebroventricular (icv) vehicle (VEH) or oxytocin (OXT) administration on chronic subordinate colony housing (CSC)-induced voluntary EtOH self-administration. A chronic icv OXT administration reduces voluntary EtOH intake in mice for EtOH doses ranging from 4 -8%. Data represent mean + SEM; $^{*}p = 0.01$ vs. SHC; $^{\#}p < 0.01$ vsVEH; (rm ANOVA followed by Bonferroni *post hoc* pairwise compairisons). VEH (n = 8, 8, grey bars); OXT (n = 8, 8, black bars).

The underlying mechanism of OXT on the attenuation of stress-induced EtOH intake still needs further investigation. One possible mechanism might be due to the anxiety-reducing properties of chronic central OXT during CSC exposure which would confirm the tension (stress)-reduction theory. Another possibility reflects the acute effects of central OXT on the DA release from the NAc. OXT-containing axons from the PVN contact mesolimbic neurons and likely display an inhibitory effect as exogenous OXT was shown to reduce amphetamine-evoked DA turnover in the NAc (Qi et al. 2008) and methamphetamine-induced Fos expression (Carson et al. 2010). Interestingly, lithium, known for its OXT releasing properties in rodents, also prevents methamphetamine-induced Fos expression in the NAc (McGregor and Bowen 2012). In line, I was able to demonstrate that an acute central OXT injection reduced the increased DA release from the NAc upon a single ip EtOH injection (see Fig. 36).

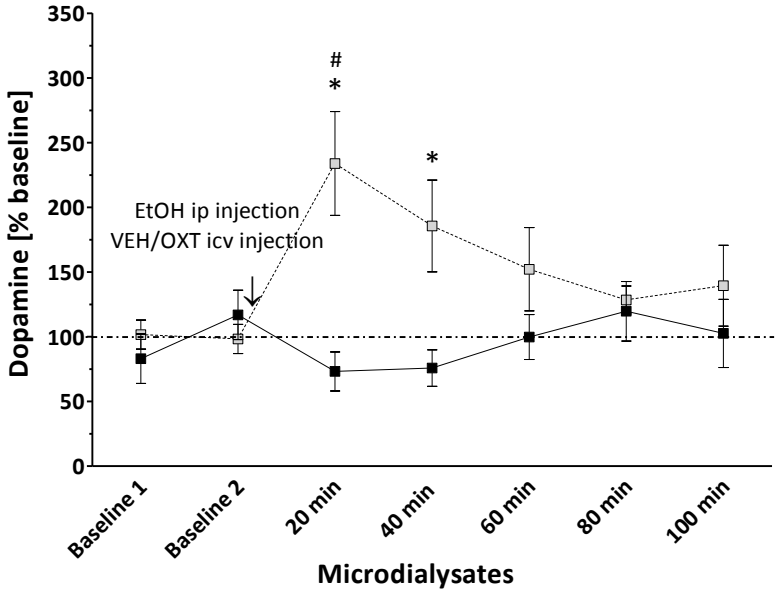


Figure 36: Effects of chronic intracerebroventricular (icv) vehicle (VEH) or oxytocin (OXT) administration on the release of dopamine (DA) in the nucleus accumbens (NAc) shell after intraperitoneal (ip) ethanol (EtOH) injection. An acute icv OXT injection blocks the DA release within the NAc following an acute ip EtOH administration. Data represent mean + SEM; $^{*}p = 0.05$ vs. OXT; $^{\#}p < 0.05$ vs. baseline 2; (rm ANOVA followed by Bonferroni *post hoc* pairwise compairisons). VEH (n = 7, grey squares); OXT (n = 9, black squares).

Whether OXT can reduce DA release upon chronic activation of the mesolimbic reward system is still under investigation. Therefore, the data of the current thesis confirm the literature for OXT to be a possible medication for substance abuse disorders and provide important findings on the potential underlying mechanisms, dosage and temporal effects.

5.5 Conclusion

Stress-induced pathologies comprising somatic disorders like cancer, affective disorders including anxiety-related diseases, but also substance abuse disorders like alcoholism gain more and more relevance in modern societies. Unfortunately, the underlying molecular and

neurobiological mechanisms remain poorly understood, leading to an insufficient medication of these stress-related disorders. Here, I could demonstrate that exposure to 19 days of CSC result in an enhanced risk to develop colorectal cancer, likely *via* shifting from a Th 1 tumour-protective towards a more regulatory T cell-mediated immune response. Further, a 15-day CSC exposure increases the anxiety-related behaviour of male mice and leads to an escalation of voluntary EtOH-self-administration. One likely explanation for this effect is the self-medication to reduce anxiety and stress effects as described in the tension (stress)-reduction theory.

Rodent as well as human studies give evidence for the highly conserved neuropeptide OXT to be a possible target for the treatment of stress-induced pathologies. However, due to the limitation in administering and investigating the molecular effects of central OXT on stress-related diseases, further studies on the involvement of this central neuropeptide system in rodent models of chronic stress exposure is highly needed.

Finally, I am able to give further evidence for the OXT system to be involved in the regulation of stress-related pathologies. The results of the current thesis reveal administration-dependent effects (peripheral vs. central) of a single, acute OXT injection, but also dose-dependent and temporal effects of a chronic OXT treatment. Thus, before the OXT system can be considered as a possible target for the treatment of stress-induced pathologies, a greater understanding of its chronic effects is needed with respect to dosage, temporal dynamics of treatment and adaptations of the endogenous OXT and related brain system.

However, I am convinced that the results of this thesis are important to improve the understanding of possible underlying mechanisms of stress-induced pathologies and the OXT system as a possible target for a successful medication in humans.

Summary in German

Deutsche Zusammenfassung

Chronischer Stress, insbesondere chronisch psychosozialer Stress, ist in unserer heutigen Gesellschaft allgegenwärtig und stellt einen hohen Risikofaktor für die Entstehung somatischer Erkrankungen, wie Herz-Kreislaufstörungen, entzündliche Darmerkrankungen sowie Krebs dar. Darüber hinaus gilt chronischer Stress als etablierter Risokofaktor für die Entstehung affektiver Pathologien, wie Depressions- und Angsterkrankungen, wird aber auch mit Suchterkrankungen, wie beispielsweise Alkoholismus in Verbindung gebracht. Wegen dieser allgegenwärtigen Präsenz von Stress und einer stetig steigenden Zahl an stressinduzierten Pathologien ist ein besseres Verständnis der Effekte einer chronischen Stressexposition auf emotionaler, physiologischer, immunologischer sowie molekularer Ebene dringend nötig. Ebenso ist auf Grund mangelnder erfolgreicher Behandlungsmöglichkeiten eine Weiterentwicklung bzw. Neuentwicklung geeigneter Therapien zwingend erforderlich.

Adäquate und hilfreiche Werkzeuge in der Beantwortung dieser Fragestellungen stellen geeignete Tiermodelle dar. Das in den Experimenten dieser Arbeit verwendete Modell der chronisch subordinierten Koloniehaltung (chronic subordinate colony housing (CSC)) repräsentiert ein präklinisch gut etabliertes Modell für chronisch psychosozialen Stress in männlichen Mäusen. Nach 19 Tagen entwickeln die CSC Tiere typische Symptome einer chronischen Stressexposition einschließlich einer reduzierten Körpergewichtszunahme, Atrophie der Thymusdrüse, sowie Hypertrophie der Nebennieren. Überdies hinaus entwickeln die gestressten Tiere eine spontane Colitis, welche sich auf eine lokale Immunsuppression in Kombination mit einer reduzierten Barrierefunktion des Darms zurückführen lässt. Auch eine chemisch induzierte Colitis nimmt einen deutlich schwereren Verlauf als in ungestressten Tieren. Desweiteren konnte in früheren Studien gezeigt werden, dass das CSC Paradigma ein gesteigertes Angstverhalten induziert, während das depressionsähnliche Verhalten der gestressten Tiere nicht verändert ist. Aufbauend auf diesen bereits publizierten Daten, die das CSC Paradigma als ein verlässliches Tiermodell

für chonischen psychosozialen Stress ausweisen, war das Ziel dieser Promotionsarbeit das Wissen über den Einfluss von chronisch psychosizialem Stress auf die Entwicklung von entzündungsvermitteltem Darmkrebs sowie den Alkoholkonsum zu erweitern und das Potential des Neuropeptids Oxytozin als mögliche Medikation für stress-induzierte Pathologien zu erörtern.

Das Hauptziel der in Kapitel 2 beschriebenen Studie war die Untersuchung der Effekte von chronisch psychosozialem Stress auf eine chemisch induzierte, entzündungsvermittelte Coloncarzinogenese. Bezüglich der Morphologie des Darmepithels konnte ich zeigen, dass getresste Mäuse deutlich früher makroskopische Veränderungen der Mucosa aufweisen als nichtgestresste Kontrolltiere. Desweiteren war es mir mittels Immunhistochemie sowie TUNEL-Färbung möglich, eine gesteigerte Proliferationsrate mit einer zeitgleich verringerten Apoptoserate nachzuweisen. Diese zellulären Veränderungen konnte ich auch auf molekularer Ebene mit veränderter mRNA sowie Proteinexpression bekannter Proliferationsmarker (LRH-1; β-Katenin) bestätigen. Über dies hinaus, kam es zu einem „Shift“ von einer Th1-Zell-vermittelten, tumorprotektiven Immunantwort zu einer regulatorischen T-Zell-vermittelten Immunantwort. Dies konnte ich anhand von Immunhistochemie sowie Immunfluoreszenz aber auch der mRNA Expression entsprechender Zytokine sowie der FACS-Analyse der T-Zellzusammensetzung von mesenterialen Lymphknoten belegen. Diese Ergebnisse bestätigen einen begünstigenden Effekt von chronisch psychosozialem Stress auf das Tumorwachstum.

In Kapitel 3 wurden männliche C57BL/6 Mäuse 15 Tage dem CSC Paradigma ausgesetzt, um zu zeigen, dass zwei Wochen CSC, neben den beschriebenen physiologischen, stress-relevanten Symptomen ebenso das Angstverhalten zu steigern. Nach der Stress-Prozedur und dem Verhaltenstest wurde das Alkoholtrinkverhalten der Mäuse untersucht, um die Hpothese zu bestätigen, dass chronisch psychosozialer Stress die freiwillige Alkoholzufuhr steigert. Ich konnte einen erhöhten Alkoholkonsum für alle getesteten Konzentrationen zeigen (2 – 8%). Bezüglich des Hauptziels der in diesem Kapitel beschriebenen Studie, einer möglichen Reduktion der Alkoholaufnahme durch eine akute periphere oder zentrale

Oxytozininjektion war ich in der Lage zu demonstrieren, dass das Neuropeptid nur in ungestressten Tieren und zusätzlich nur nach peripherer Administration, den Alkoholkonsum in Mäusen reduzierte. Nach peripherer Injektion des als Positivkontrolle verwendeten $GABA_B$ Rezeptor Agonist Baclofen, zeigten sowohl ungestresste als auch chronisch gestresste Tiere eine reduzierte Alkoholzufuhr. Diese Ergebnisse unterstreichen das Potential von Baclofen als mögliche Medikation für die Behandlung von Alkoholismus, verdeutlichen aber auch den Bedarf weiterer Studien bezüglich des oxytozinergen Systems als potentielles Behandlungsziel.

Das Hauptziel der in Kapitel 4 dieser Promotionsarbeit beschriebenen Studie, war die Untersuchung, in wie weit eine kontinuierliche, zentrale Oxytozininfusion während chronischer Stressexposition, die Entstehung von stressinduzierten Veränderungen der Physiologie sowie des Verhaltens blocken oder zumindest lindern kann. Nach erfolgreicher Etablierung einer osmotischen Minipumpen-Implantation, war es mir möglich zeit-, aber auch dosisabhängige Effekte einer chronischen Oxytozinadministration nachzuweisen. In diesem Zusammenhang war eine niedrige Oxytozindosis in der Lage die physiologischen Veränderungen sowie die Ausbildung eines gesteigerten Angsverhltens nach 19-tägiger CSC Exposition zu unterdrücken. Zusätzlich konnte ich demonstrieren, dass eine kontinuierliche, zentrale Oxytozininfusion eine dosisabhängige Herabregulation des zentralen, oxytozinergen Systems zur Folge hat, was bei chronisch hohen Dosen von einem gesteigerten Angstverhalten begleitet wird.

Zusammenfassend konnte ich in der vorliegenden Dissertation das Wissen über den Einfluss von chronisch, psychosozialem Stress auf verschiedenste Pathologien erweitern. Bezüglich einer Medikation stressinduzierter Krankheiten mittels Oxytozin konnte ich wichtige temporale aber auch dosisabhängige Effekte darlegen, was den Bedarf an weiteren Studien bezüglich der Wirkungsweise dieses Neuropeptides verdeutlicht, bevor es beim Menschen angewendet werden kann.

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Abbreviations

µl	Microlitre
µM	Micromolar
ACTH	Adrenocorticotropic hormone
am	Ante meridem
ANOVA	Analysis of variance
AOM	Azoxymethane
AVP	Arginine-vasopressin
BLA	Basolateral amygdala
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CD	Crohn´s disease
cDNA	Complementary deoxyribonucleic acid
CeA	Central amygdala
CM	Centimetre
CNS	Central nervous system
CO_2	Carbon dioxide
CORT	Corticosterone
CRC	Colorectal cancer
CRH	Corticotropin-releasing hormone
CRHR	CRH receptor
CSC	Chronic subordinate
DA	Dopamine
DAPI	4´,6- Diamidin-2-phenylindol
DLS	Dorsolateral septum
DMH	1, 2- dimethylhydrazin
DNA	Deoxyribonucleic acid
DOPA	Dihydroxyphenylalanine
DSS	Dextran sulphate sodium

EDTA	Ethylendiamintetraaceticacid
ELISA	Enzyme-linked immunosorbent assay
EPF	Elevated platform
EPM	Elevated plus maze
EtOH	Alcohol
FAM	6-carboxy-fluorescin
FITC	Fluorescin isothiocyanate
FoxP3	Forkhead box 3
g	Gram
GABA	Gamma-aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Glucocorticoids
GPCR	G protein-coupled receptor
h	Hour
H_2O	Water
HE	Hematoxilin-eosine
HEPES	2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethansulfonsäure
HGD	High grade dysplasia
HPA axis	Hypothalamo-pituitary-adrenal axis
HSPs	Heat schock proteins
IBD	Inflammatory bowel disease
icv	Intracerebroventricular
IFN	Interferone
IHC	Immunohistochemistry
IL	Interleukine
ip	Intraperitoneal
kDa	Kilodalton
kg	Kilogram

LC	Locus coeruleus
LDB	Light-dark box
LGD	Low grade dysplasia
LS	Lateral septum
MAM	Methylazoxymethanol
Mc2r	Melanocortin-2-receptor
MCH I	Class-I major histocompatibility complex
MeA	Medial Amygdala
mesLN	Mesenteric lymph node
mesLNC	Mesenteric lymph node cell
mg	Milligram
min	Minute
ml	Mililitre
mM	Millimolar
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
n	Number
NAc	Nucleus accumbens
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
nm	Nanometer
OA	Open arm
OMP	Osmotic minipump
OXT	Oxytocin
OXTR	OXTR receptor
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline twee 20
PFC	Prefrontal cortex
POMC	Proopiomelanocortin

PVN	Paraventricular nucleus
qRT-PCR	Quantitative real time polymerase chain reaction
RNA	Ribonucleic acid
RT	Room temperature
s	Second
sc	Subcutaneous
SEM	Standard error of the mean
SHC	Single housed control
SNS	Sympathetic nervous system
SON	Supraoptic nucleus
STAT3	Signal transducer and activator of transcription 3
TAMRA	6-carboxytetramethylrhodamine
TBS	Tris-buffered saline
TNF	Tumour necrosis factor
TUNEL	Terminal deoxynucleotidyl transferase d UTP nick end labeling
UC	Ulcerative Colitis
UV	Ultraviolet
V1b	Vasopressin type 1b
VEH	Vehicle
VLS	Ventrolateral septum
VTA	Ventral tegmental area

CV and publications

Curriculum vitae

Dipl. Biol. Sebastian Thomas Peters

Verdunstraße 5, 93053 Regensburg

Personal details

* June 25th, in Landshut, Niederbayern, Germany

married, no children

1995 – 1999

Secondary school, Donau-Gymnasium, Kelheim, Germany

1999 – 2002

Secondary school, Goethe-Gymnasium, Regensburg, Germany

Scientific education:

2010 to present

PhD student in neurobiology in Prof. Neumann´s group at the University of Regensburg

2009 to 2010

MSc (Diplom) in biology in Prof. Neumann´s group at the University of Regensburg (Diploma/Master thesis: Effects of chronic psychosocial stress on the colon cancerogenesis in male C57BL/6 mice)

2008 to 2009

Graduate studies in biology at the University of Regensburg with focus on Zoology/Neurophysiology, Cellbiology and Biochemistry

2004 to 2008

Undergraduate studies in biology at the University of Regensburg, Germany

Publications

Reber SO, Peters S, Slattery DA, Hofmann C, Schölmerich J, Neumann ID, Obermeier F. 2011. Mucosal immunosuppression and epithelial barrier defects are key events in murine psychosocial stress-induced colitis. *Brain, Behavior, and Immunity*, 25(6): 1153-116.

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Author`s declaration – Eidesstattliche Erklärung

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

Weitere Personen 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 mittelbar noch unmittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit der vorliegenden Dissertation stehen.

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

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Ort, Datum Sebastian Peters