

Dissertation

**Impact of stress and colitis on neuronal plasticity in the
amygdala-hippocampus network**

submitted by

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Declaration:

I hereby declare that this dissertation is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this dissertation. Due acknowledgement has been made in the text to all other material used. Throughout this dissertation and in all related publications I followed the guidelines of "Good Scientific Practice".

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Table of contents

Acknowledgements	3
Table of contents	5
Abbreviations	9
Kurzzusammenfassung.....	12
Abstract.....	14
1 Introduction.....	16
1.1 The gut-brain axis	16
1.2 Inflammatory bowel diseases	18
1.3 The role of stress in inflammatory bowel disease	20
1.4 Modelling inflammatory bowel disease in animals	21
1.5 The role of stress in animal models of colitis	22
1.6 Stress and adaptation	25
1.7 Detection and processing of stress.....	26
1.8 Neuropeptide Y - a key neuropeptide in the course of stress.....	28
1.9 Neurochemical basis of stress resilience	30
1.10 Post-traumatic stress disorder	32
1.11 Aims of the project.....	33
2 Material and Methods.....	35
2.1 Experimental animals	35
2.2 Experimental protocols	35
2.2.1 Protocol 1	35
2.2.2 Protocol 2	36
2.2.3 Protocol 3	36
2.2.4 Protocol 4	36
2.2.5 Protocol 5	37

2.2.6 Protocol 6	37
2.3 Water avoidance stress	37
2.4 Standard and enriched housing conditions	38
2.5 Induction of experimental gastritis or colitis	39
2.6 Disease activity score	40
2.7 Assessing food and liquid intake	41
2.8 Myeloperoxidase levels in the stomach and colon	41
2.9 Circulating corticosterone	41
2.10 Immunohistochemistry	42
2.11 Cell counting and quantification of c-Fos and FosB/ Δ FosB expressing cells	45
2.12 Colon histology	47
2.13 Molecular cloning	47
2.14 DNA template and riboprobe production	50
2.15 Radioactive neuropeptide Y in situ hybridization	50
2.16 Grain and cell counting of the neuropeptide Y in situ hybridization	51
2.17 Mouse forebrain microdissection	52
2.18 Real-time PCR	52
2.19 Electron microscopy	53
2.20 Light/dark box test	55
2.21 Sucrose preference test and circadian activity	55
2.22 Tail suspension test	55
2.23 Statistics	56
3. Results	57
3.1 Effects of water avoidance stress on brain c-Fos expression	57
3.2 Effects of environmental enrichment and gastrointestinal inflammation on stress-induced neuronal activation	59

3.2.1 Environmental enrichment enhanced the stress-induced c-Fos expression in the dentate gyrus and unmasked an inhibitory effect of gastritis and colitis	59
3.2.2 Environmental enrichment inhibited the stress-induced c-Fos expression in the central amygdala and prevented the inhibitory effect of gastritis and colitis.....	63
3.2.3 Environmental enrichment inhibited the stress-induced c-Fos expression in the infralimbic cortex and attenuated the inhibitory effect of gastritis and colitis but had little effect in the cingulate cortex and hypothalamus	65
3.3 Interactions between gastrointestinal inflammation and environmental enrichment	67
3.3.1 Environmental enrichment increased the susceptibility to dextran sulfate sodium-induced colitis, but not iodoacetamide-induced gastritis	67
3.3.2 Colitis enhanced the stress-induced rise of plasma corticosterone under enriched, but not standard housing	70
3.4 Effects of environmental enrichment on brain structures of the hippocampal formation	71
3.4.1 Environmental enrichment increased neuropeptide Y expression in the polymorph layer of the dentate gyrus	71
3.4.2 Environmental enrichment decreased stress-induced FosB/ Δ FosB expression within the CA1 and CA3 fields of the hippocampus	74
3.4.3 Environmental enrichment did not induce ultrastructural changes within the CA1 field of the hippocampus and the polymorph layer of the dentate gyrus	77
3.5 Effects of colitis and water avoidance stress on behaviour and brain neuropeptide Y expression.....	79
3.5.1 Dextran sulfate sodium treatment induced colonic inflammation and altered daily food and water intake	79
3.5.2 Dextran sulfate sodium treatment modified behaviour during the water avoidance stress session and enhanced corticosterone levels	83

3.5.3 Water avoidance stress enhanced locomotor activity and time spent in the light compartment of the light/dark box	84
3.5.4 Colitis and water avoidance stress did not disturb the circadian pattern of activity and exploration of mice and did not modify sucrose preference	85
3.5.5 Water avoidance stress increased curling behaviour during the tail suspension test	87
3.5.6 Colitis enhanced hypothalamic but blunted amygdalar neuropeptide Y expression	88
4 Discussion	90
4.1 Rationale of the project	90
4.2 Influence of water avoidance stress on c-Fos expression in the brain	91
4.3 Influence of environmental enrichment on stress-induced c-Fos and FosB/ Δ FosB expression in the brain.....	94
4.4 Influence of environmental enrichment on stress-induced neuropeptide Y expression in the brain	96
4.5 Influence of environmental enrichment on hippocampal synaptic ultrastructure	98
4.6 Influence of gastrointestinal inflammation on stress-induced c-Fos expression in the brain under standard and enriched housing	99
4.7 Influence of environmental enrichment and water avoidance stress on gastrointestinal inflammation.....	101
4.8 Influence of gastrointestinal inflammation, environmental enrichment and water avoidance stress on circulating corticosterone levels	103
4.9 Influence of water avoidance stress and colitis on behaviour of mice.....	104
4.10 Influence of water avoidance stress and colitis on neuropeptide Y expression in the brain	107
4.11 Key findings and implications	109
Bibliography	111

Abbreviations

ABE	Acetic acid n-butyl ester
ACTH	Adrenocorticotropic hormone
AD	Antibody diluent
AIC	Agranular insular cortex
ANS	Autonomic nervous system
ARC	Arcuate hypothalamic nucleus
ARCC	Arcuate hypothalamic nucleus, caudal
ARCr	Arcuate hypothalamic nucleus, rostral
BDNF	Brain-derived neurotrophic factor
BLA	Basolateral amygdala
Bp	Base pair
BSTMA	Bed nucleus of stria terminalis; medial division, anterior part
CA1	CA1 field of the hippocampus
CA3	CA3 field of the hippocampus
CB	Cacodylate buffer
CC	Cingulate cortex
CD	Crohn's disease
CeA	Central amygdala
CL	Centrolateral thalamic nucleus
CM	Centromedial thalamic nucleus
CRF	Corticotropin-releasing factor
DAB	3,3-diaminobenzidine substrate
DAS	Disease activity score
DGgl	Granular cell layer of the dentate gyrus
DGpl	Polymorph cell layer of the dentate gyrus
DSS	Dextran sulfate sodium
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EE	Environmental enrichment
ENS	Enteric nervous system
EtOH	Ethanol

fMRI	Functional magnetic resonance imaging
H ₂ O ₂	Hydrogen peroxide
HPA axis	Hypothalamic-pituitary-adrenal axis
IAA	Iodoacetamide
IBD	Inflammatory bowel disease
ILC	Infralimbic cortex
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ISH	In situ hybridization
LC	Locus coeruleus
L/D	Light/dark box test
LHb	Lateral habenula
LPAG	Lateral periaqueductal grey
LPS	Lipopolysaccharide
LS	Lateral septum
LSV	Lateral septal nucleus, ventral part
MeA	Medial amygdala
MPO	Myeloperoxidase
NPY	Neuropeptide Y
NTS	Nucleus tractus solitarii
PBS	Phosphate-buffered saline
PC	Paracentral thalamic nucleus
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
Pir	Piriform cortex
PN	Pontine nuclei
PVH	Paraventricular nucleus of the hypothalamus
PVT	Paraventricular nucleus of the thalamus
RLi	Rostral linear of the raphe nucleus
ROI	Region of interest
SOC	Super optimal broth with catabolite repression
SP	Sucrose preference test
SSC	Saline-sodium citrate buffer
TNBS	Trinitrobenzene sulfonic acid
TST	Tail suspension test

UC
WAS
WB

Ulcerative colitis
Water avoidance stress
Washing buffer

Kurzzusammenfassung

Medizinischer Hintergrund und Zielsetzung: Chronisch entzündliche Darmerkrankungen („IBDs“) sind ein häufiges Krankheitsbild. Trotzdem ist die Pathophysiologie dieser Erkrankungen nicht vollständig geklärt. Für den Krankheitsverlauf scheint Stress ein wichtiger Faktor zu sein, da er IBDs exazerbieren kann. Welche Rolle dabei die Darm-Gehirn-Kommunikation spielt, ist allerdings zum großen Teil unbekannt. In Tierversuchen, welche „environmental enrichment“ (EE), eine alternative Käfighaltung von Mäusen, verwenden, konnte gezeigt werden, dass diese Haltungsart die Fähigkeit mit Stress umzugehen verbessert. Welche Gehirnareale und welche molekularen Mechanismen für diesen Effekt zuständig sind, wurde allerdings nur unzureichend erforscht. Ob EE den Verlauf von gastrointestinalen Entzündungen beeinflusst, ist ebenfalls unbekannt. Auf Grund der komplexen Interaktion zwischen Stress und viszeraler Entzündung, stellte sich diese Dissertation der Aufgabe, die Rolle von Stress und vor allem die Stressverarbeitung im Gehirn im Verlauf gastrointestinaler Entzündungen zu charakterisieren.

Methoden: In den Versuchen verwendete ich zwei Tiermodelle für viszerale Entzündung: „Dextran Sulfate Sodium“ (DSS)-induzierte Kolitis und „Iodoacetamid“ (IAA)-induzierte Gastritis. Ein Teil der Tiere wurde dem psychologischen Stressor „Water Avoidance Stress“ (WAS) ausgesetzt und/oder unter EE gehalten. Nach den Behandlungen quantifizierte ich die Anzahl der c-Fos- und FosB/ Δ FosB-exprimierenden Zellen in verschiedenen Gehirnarealen, was als Maß für neuronale Aktivierung gilt. Weitere Messungen inkludierten Neuropeptid Y (NPY)-Expression, Struktur und Anzahl von hippocampalen Synapsen und verschiedene Tests für emotional-affektives Verhalten. Zur Überprüfung der Behandlungseffizienz von DSS und IAA wurde der Myeloperoxidasegehalt des Kolons sowie ein Krankheitsaktivitäts-Score bestimmt.

Ergebnisse: Die wichtigsten Ergebnisse dieser Arbeit sind: 1) WAS erhöht die neuronale Aktivität im Gehirn. 2) Die WAS-induzierte neuronale Aktivierung wird durch viszerale Entzündung gehemmt. 3) EE modifiziert die WAS-induzierte neuronale Aktivierung des Hippocampus und der Amygdala in entgegengesetzter Art und Weise. 4) EE erhöht die hippocampale NPY-Expression und modifiziert die Anzahl der FosB/ Δ FosB-exprimierenden Zellen, verändert aber nicht die Struktur

oder Anzahl von Synapsen im Hippocampus. 5) EE verschlechtert den Krankheitsverlauf einer Kolitis und beeinflusst die WAS-induzierte neuronale Aktivität im Gehirn unter entzündlichen Bedingungen. 6) WAS, aber nicht Kolitis, verändert emotional-affektives Verhalten. 7) WAS und Kolitis modifizieren die NPY-Expression im Hippocampus, Amygdala und Hypothalamus auf unterschiedliche Art.

Fazit: Es konnte gezeigt werden, dass viszerale Entzündung mit signifikanten molekularen Veränderungen im Gehirn einhergeht. Dies legt nahe, dass eine veränderte Darm-Gehirn-Kommunikation im Verlauf gastrointestinaler Entzündungen die Gehirnfunktion beeinträchtigt. Aus translationaler Sichtweise liefern diese Daten weitere Erklärungsansätze, wie Stress den Verlauf von IBDs negativ beeinflusst. Vor allem die beobachteten Veränderungen im limbischen System erklären, warum die Verarbeitung von Emotionen bei IBD-Patienten verändert ist.

Außerdem erweitern diese Daten die Vorstellung, wie EE die negativen Konsequenzen von Stress reduziert. Im Einklang mit dem derzeitigen Wissensstand wurde der Hippocampus als die Struktur identifiziert, welche die meisten Veränderungen nach EE zeigt. Speziell der Befund, dass hippocampales NPY durch EE erhöht wird, bietet eine Erklärung, wie EE den Umgang mit Stress verbessert. Überraschenderweise verschlechtert EE den Krankheitsverlauf einer Kolitis, was im Gegensatz zu positiven Effekten von EE in anderen Krankheitsmodellen steht. Dieser Befund könnte durch die bekannten immunmodulatorischen Eigenschaften von EE erklärt werden, muss jedoch noch weiter untersucht werden.

Abstract

Background and Aim: Inflammatory bowel diseases (IBDs) are a major health concern, but the pathophysiology of these diseases is still incompletely understood. It was suggested that stress is a crucial factor in the development of IBDs, able to exacerbate disease course and to trigger flares. While a large body of evidence showed that stress negatively affects parameters of inflammation, the role of the gut-brain communication in this process remains largely elusive. Increasing evidence in animal experiments using environmental enrichment (EE), an alternative housing condition for mice, demonstrated that environmental factors may influence stress resilience, the ability to cope with stress. However, brain sites and molecular events producing this effect need further investigation. Whether EE influences the course of visceral inflammation is also unknown. In view of the complex interaction between stress and visceral inflammation, the current thesis project was designed to elucidate the role of psychological stress and particularly the brain response to stress in mouse models of chronic gastrointestinal inflammation.

Methods: Gastrointestinal inflammation was modelled by dextran sulfate sodium-induced colitis and iodoacetamide-induced gastritis. Subgroups of animals were exposed to water avoidance stress (WAS), a psychological stress paradigm, and/or kept under EE. Readouts included neuronal activation in the brain, as measured by the quantification of the number of c-Fos- and FosB/ Δ FosB-expressing cells, brain neuropeptide Y (NPY) expression, structure and number of hippocampal synapses and various tests of emotional-affective behaviour. The efficacy of the treatments to induce visceral inflammation was assessed by colonic myeloperoxidase content and a disease activity score.

Results: Several key findings arose from this project. 1) WAS produces strong neuronal activation in the brain. 2) WAS-induced neuronal activation is markedly blunted by gastrointestinal inflammation. 3) EE modifies WAS-induced neuronal activation of the hippocampus and the amygdala in an opposite manner. 4) EE-induced modifications of c-Fos levels are accompanied by an increase of hippocampal NPY expression and altered FosB/ Δ FosB levels, but not by a change of the hippocampal synaptic ultrastructure. 5) EE increases the susceptibility to colitis and alters stress-induced neuronal activation in the brain under

inflammatory conditions. 6) WAS, but not colitis, influences emotional-affective behaviour. 7) WAS and colitis differentially modify NPY expression within the hippocampus, amygdala and hypothalamus.

Conclusions: My data contribute to the growing literature that the brain is an important factor in the course of visceral inflammation. The molecular changes observed in the brain indicate that altered gut-brain signalling in response to gastrointestinal inflammation can impair brain function. In a translational view, these data add further information on how stress can negatively affect the course of IBD. Especially the alterations observed in the limbic system provide an additional explanation why emotional processing is impaired in IBD.

Furthermore, these data expand the knowledge as to how EE reduces negative consequences of stress exposure. In line with the current literature, the hippocampus was identified as the structure showing the most pronounced EE effects. Particularly the finding of increased hippocampal NPY expression points to a signal pathway whereby EE confers stress resilience. Surprisingly, EE increased the susceptibility to colitis, which is in contrast to positive effects of EE in other disease models. This effect may be explained by the immunomodulatory properties of EE, but needs further investigation.

1 Introduction

1.1 The gut-brain axis

The term gut-brain axis refers to the close interconnections between the gut and the brain. For many years now it has been recognized that gut-brain communication is bidirectional and not only responsible for the regulation of digestive functions. Indeed, accumulating evidence suggests that emotional states, motivation and affect are influenced by signals from the gut (1).

Gut-brain communication is mediated by two major signal pathways connecting these organ systems. On the one hand, brain and gut communicate via neuronal signals (autonomic nervous system (ANS) and sensory neurons) and, on the other hand, communication occurs via the blood stream. As depicted in figure 1, multiple components are involved in the neuronal communication between the gut and the brain. Efferent signals (signals from the brain to the gut) are conveyed by the ANS, which can be divided in a sympathetic branch, a parasympathetic branch and the enteric nervous system (ENS) (2). Because of the intensive neuronal innervation, the huge number of neurons and the complexity of the ENS, this network is also called the “brain of the gut” (3). The sympathetic innervation of the gut originates from the thoracolumbar spinal cord, while parasympathetic signals are derived from the sacral spinal cord and the brain stem (vagal efferents). The interplay of sympathetic and parasympathetic efferents and the ENS is essential to regulate motility and secretion in the gastrointestinal tract (2). The close proximity of vagal nerve endings and neurons of the ENS allows a crosstalk between these systems (1). Afferent signals (from the gut to the brain) are conveyed to the spinal cord via spinal afferents and to the brain stem via vagal afferents. It is believed that these signals are subsequently transmitted to subcortical brain structures like the limbic system, which allows the modification of brain functions such as motivation and emotions (1).

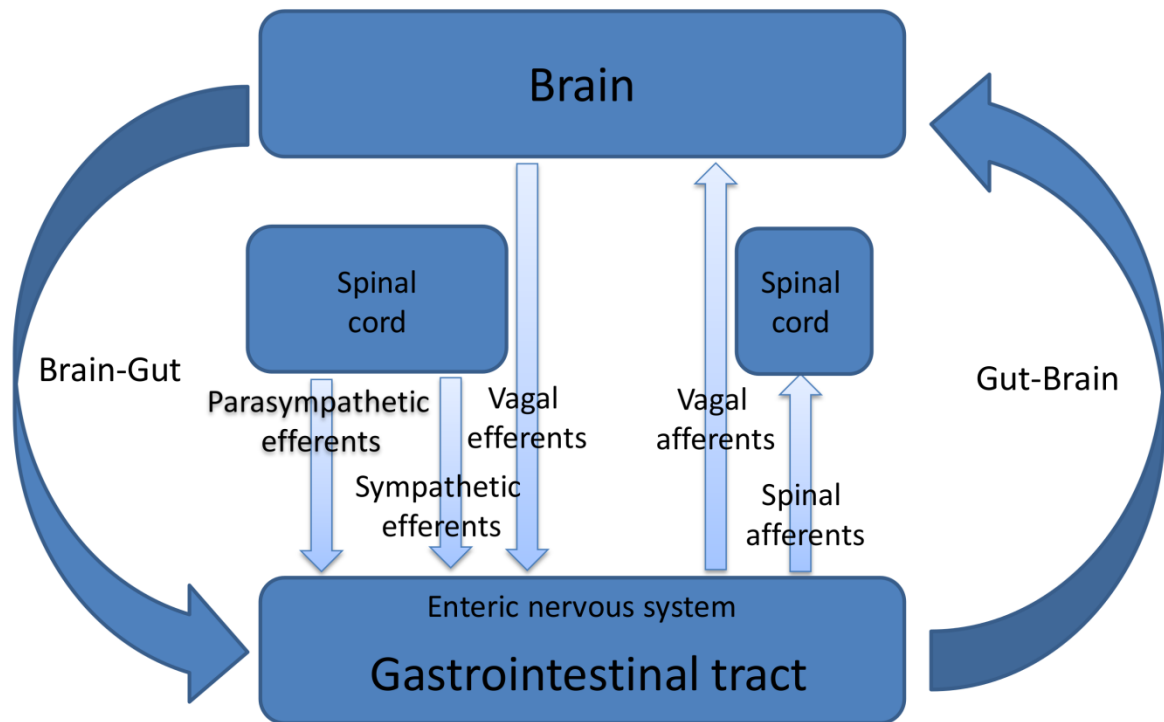


Figure 1. Neuronal gut-brain communication. Gut-brain communication is bidirectional. Neuronal pathways from the brain to the gut (efferents) are depicted on the left side and neuronal pathways from the gut to the brain (afferents) are depicted on the right side. Efferent pathways can be divided into parasympathetic efferents (spinal and vagal) and spinal sympathetic efferents. Afferents are divided into spinal and vagal afferents. The close proximity between neurons of the enteric nervous system and vagal nerve endings allow a crosstalk between these systems.

Besides neuronal signals, gut-brain communication also occurs via the bloodstream. Thus, if stressful stimuli are perceived by the brain, the hypothalamic-pituitary-adrenal axis (HPA axis) and the sympatho-adrenal axis are activated. As described in detail below, HPA axis activation results in the secretion of cortisol/corticosterone into the blood stream, and activation of the sympatho-adrenal axis enhances circulating adrenaline and noradrenaline levels, which can reach all organs of the gastrointestinal tract (2,4). Vice versa hormones released from enteroendocrine cells, inflammatory mediators including proinflammatory cytokines released from immune cells or microbial products derived from the microbiota can reach the brain via the circulation (5,6). These molecules can cross the blood-brain barrier and thus also influence behaviour.

There are also considerable interactions between gut-derived molecules and the neuronal innervation of the gut. Vagal and spinal afferent nerve terminals are chemosensitive and can thus, depending on receptor expression, be activated

by hormones, neuropeptides or inflammatory mediators (7,8). Hormones and biologically active peptides within the intestinal tract are released from enteroendocrine cells, which respond to different luminal stimuli including mechanical activation, stimulation by vagal efferents or activation by microbial organisms via pattern recognition receptors (1,9,10). This paracrine signalling is very important for activation of afferent nerve endings and the neuronal component of gut-brain signalling. However, afferent nerve endings can also be activated by inflammatory mediators. Thus, in response to luminal antigens, lymphocytes and mast cells can release histamine, serotonin and cytokines, which can modify afferent neuronal signalling (11). It is important to note that both gut-brain communication routes are closely interrelated comprising a dynamic system. Chronic perturbations of this system can result in altered behaviour and disease processes.

1.2 Inflammatory bowel diseases

Evidence suggests that a disturbance of the gut-brain axis is an important factor in IBDs. A modern view of these diseases considers the major role of psychoneuroimmunomodulation of IBDs. Both organic and psychological factors are crucial for the disease outcome (12). IBDs can be divided into two major entities, namely ulcerative colitis (UC) and Crohn's disease (CD). There are many similarities between these diseases, which poses a challenge to the diagnosing clinician. Most importantly, both disorders are chronic inflammatory diseases of the intestinal tract and patients suffering from either of these diseases report symptoms like bloody stool, chronic diarrhoea or pain. The onset of both diseases is typically in young adulthood (peak between 20-40 years) and for both diseases no causal therapy is available to date. Incidence and prevalence rates are similar around 6-15/100000 and 50-200/100000, respectively, with higher rates in westernized countries (13). However, there are also clear differences between these diseases, which allow a proper diagnosis.

Anatomically, UC typically starts in the distal colon and progresses proximally to the caecum and eventually the terminal ileum (backwash ileitis). The disease affects mainly the colonic mucosa, but not deeper structures of the colon

wall. The most important risk factor for UC is a positive family history of IBD. Another risk factor is previous gastrointestinal infections, while smoking and appendicectomy are protective (14). Pathophysiological changes during UC include alterations of the immune response, a disturbed mucosal barrier function and alterations of the microbiome (15-17). In the course of the disease, patients experience periods of active disease processes and periods of remission. Bloody diarrhoea is the most prominent symptom of the disease. Current treatment options are mesalazine as a first-line treatment for mild to moderate UC. If mesalazine is not effective, glucocorticoids, immunosuppressants and anti-TNF treatment are prescribed. Colectomy is used as an emergency treatment in very severe active disease or as an elective treatment in case of refractoriness or intolerance of maintenance treatment with mesalazine (13,14).

The second major entity of IBD is CD. In contrast to UC, CD often starts in the terminal ileum or the colon. CD does not spread continuously from the terminal ileum into either direction of the intestinal tract. In fact, CD can be present discontinuously in all parts of the digestive tract starting from the oral cavity to the anus. Typically, CD is a transmural inflammation, affecting all layers of the tubular organs of the digestive tract. This can result in complications like abscesses, fistulas and strictures (13). Like in UC, genetic predisposition is the major risk factor for CD. CD shows familial aggregation and genome-wide association studies found around 70 susceptibility loci for CD (18,19). Other risk factors are tobacco use and previous gastrointestinal infections. Disturbances of the epithelial barrier function, dysregulation of the immune system and alteration in gut microbial composition are important pathophysiological processes in CD (20-23). Like in UC, patients with CD undergo active phases of the disease and periods of remission. Symptoms vary from abdominal pain, symptoms of bowel obstruction to diarrhoea with or without blood and mucus. Treatment of CD aims to achieve clinical and endoscopic remission. For this purpose, patients can be treated initially with anti-TNF treatment or steroids to achieve disease control. This medication is combined with immunosuppressive drugs to achieve long-term remission. Complications like complex fistulas, abscesses, strictures and cancer require surgery (23).

1.3 The role of stress in inflammatory bowel disease

Monitoring and follow-up of patients with UC and/or CD identified stress as an important factor in the course of these diseases (12,24-26). The majority of studies conclude that stress can exacerbate IBDs and cause relapses, but does not influence disease onset (25,27). However, it is complicated to evaluate the effects of stress on the course of IBD due to the problem of measuring the individual stress load accurately, which is subjective and depends heavily on individual coping strategies of patients. Also methodological differences in assessing individual stress levels makes a comparison between studies difficult and may explain conflicting results. For example, with regard to the effects of stress on disease course, two studies reported that adverse life events can trigger IBD, while another study did not confirm this notion (28-30). A large population-based study evaluated recently the factors inducing flares (changes from inactive to active disease state) within an IBD patient cohort (31). High perceived stress and negative mood were associated with increased risk for disease exacerbation. The study also found that patients with chronically active disease report higher levels of stress more frequently than patients with inactive disease, indicating a bidirectional relationship between stress and disease activity (31). A role of stress in inducing relapses has also been suggested by others (28,32,33).

Some mechanistic explanation how stress affects the digestive tract is provided by studies in humans using acute mild experimental stressors. It was found that stress increases levels of interleukin 6 and tumour necrosis factor α in response to immune stimulation in healthy individuals and in UC patients in remission (34,35). Another study reported increased interleukin 6 levels in UC patients after a mental calculation stress test, an effect absent in control subjects (36). Furthermore, experimental stress enhances colonic motility and decreases rectal pain threshold (37,38). Thus, it may be surmised that acute stress modifies immune system activity and gastrointestinal function. Also the functionality of the HPA axis and the ANS is altered in IBD patients (39,40).

Given the negative consequences of stress in the course of IBD, it is reasonable to advocate anti-stress or stress management therapy in the setting of IBD. However, studies addressing the effects of such a treatment approach are sparse and face methodological difficulties. For example, it is hard to conduct well

designed, controlled studies examining the effects of anti-stress therapy in a blinded manner. From the available studies it appears that the effects of psychological therapy on disease course are weak (41). Recent studies reported that psychosomatic therapy reduces days in hospital of CD patients, while a comprehensive life style modification programme for UC patients had effects on quality of life 3 months, but not 12 months after therapy, with no effect on clinical disease variables at any time point during the observational period (42,43).

1.4 Modelling inflammatory bowel disease in animals

Since IBDs are a major health concern and the pathophysiology of the disease is still incompletely understood, efforts have been taken to develop appropriate disease models. In this regard, mainly two animal models are currently used to study IBD nowadays. Both are chemically-induced colitis models that produce colitis either by treatment with dextran sulfate sodium (DSS) or with trinitrobenzene sulfonic acid (TNBS).

The use of DSS to induce colitis in mice was introduced by Okayasu et al. (44) in 1990. Based on the clinical phenotype and histological findings of DSS-treated mice, DSS colitis is considered as a model for UC (44). DSS reliably induces colitis in mice and is easy to handle as DSS can be applied via the drinking water. Typically, mice are treated for 5-7 days with 2-5 % DSS in the drinking water (45). It has been found that different strains of mice are differentially sensitive to DSS, which may be related to their genetic background and differences in their microbiome (46,47). Kitajima et al. (48) described that the molecular weight of DSS is crucial for colitis development. DSS with a molecular weight of 40 kDa produces strong colitis, while DSS of other molecular weights produces mild colitis or no colitis at all. After several days of treatment with DSS, mice develop symptoms such as bloody diarrhoea and weight loss. However, the pathophysiological processes whereby DSS produces colitis are still incompletely understood. It has been proposed that DSS exerts a toxic effect on the colon epithelium, enhances colonic permeability and thus increases interactions of the microbiota with the colonic immune system; in addition, DSS can also alter the composition of the gut microbiome (47,49,50). After ingestion, DSS is taken up

into the circulation and accumulates in certain tissues after several treatment days. Specifically high levels of DSS are present in the colon, mesenteric lymph nodes and the liver, while no DSS is present in brain and heart tissue (51). Histological analysis of the intestinal tract revealed that the colon is most affected by DSS treatment, but inflammation is also present in the upper gastrointestinal tract (52). Routine histological staining techniques consistently showed leukocyte infiltration into mucosal and submucosal layers of the colon and loss of crypts and goblet cells (53-55). The type of inflammation after DSS treatment resembles therefore inflammatory processes present in both UC (colonic localization) and CD (transmural inflammation) (45).

Another way to model IBD in rodents is the use of TNBS. In 2002, TNBS was introduced as a substance to induce colitis in mice (56). Based on the resulting type of inflammation, TNBS colitis was proposed as a model for CD (57). Like in DSS-induced colitis, the susceptibility of mice to TNBS depends on the mouse strain used, also suggesting a genetic component in disease development (58). In contrast to DSS, TNBS is administered only once, rectally, at doses between 0.5-6 mg/mouse in 35-50 % ethanol (EtOH), which produces a severe colitis in mice (57). Pathophysiological changes in TNBS colitis are poorly understood. The EtOH is used to break the epithelial barrier, while TNBS is believed to haptinize colonic autologous or microbiota-derived proteins, which renders them immunogenic to the mucosal immune system (59). This results in a local inflammatory reaction with a peak cytokine production 2-3 days after TNBS instillation (60). Symptoms of TNBS colitis include body weight loss and a poor clinical state. Histologically, colonic mucosal ulceration, transmural leukocyte infiltration and crypt loss can be detected, which resembles the inflammatory processes of CD (61).

1.5 The role of stress in animal models of colitis

Investigating the role of stress in animal models of colitis allows a more detailed analysis as to how stress affects visceral inflammation, compared to human studies. Studies assessing the effect of stress during visceral inflammation can be divided into (a) studies assessing whether stress is a risk factor for colitis

development, (b) studies investigating whether stress can exacerbate colitis and (c) studies examining whether stress can reactivate quiescent colitis.

As mentioned above, studies of IBD patients do not support a role of stress as a risk factor for colitis development, which may be related to the difficulty to identify all relevant risk factors in a retrospective study and the lack of prospective studies investigating this issue. In contrast, animal studies do suggest a role of stress in colitis development. For example, a study in rats reported that 4 days of restraint stress before treatment with DSS shortens the latency to colitis development (62). A study in mice showed that maternal separation and chronic subordinate colony housing enhances the vulnerability to DSS-induced colitis in adulthood, indicating that both early life stress and stress during adulthood are risk factors for colitis (63). Furthermore, another study from the same group reported that chronic subordinate colony housing leads to colitis development itself (64). Recently, the ability of chronic stress to induce colitis has also been shown for repeated social stress and for overcrowding stress (65,66).

A number of observations suggest that stress exacerbates colitis. Both studies in mice and rats using either DSS or TNBS reported an effect of stress on disease activity. For example, chronic psychosocial stress increases the severity of DSS-induced colitis in mice (54). Another group found that a different psychological stressor likewise exacerbates DSS-induced colitis (55). Chronic water avoidance stress (WAS) worsens both DSS- and TNBS-induced colitis (67,68). All these animal studies are thus in good accordance with human data, suggesting a role of stress in disease exacerbation. There are, however, also contradicting studies, suggesting an amelioration of colitis by stress exposure. Accordingly, acute WAS 6 hours after colitis induction by acetic acid as well as acute controllable electric shock before colitis induction by TNBS reduced colitis severity (69,70). The beneficial effects of stress in these studies may be explained by the rise of anti-inflammatory glucocorticoids after acute stress.

Finally, studies investigated whether stress can reactivate a quiescent colitis. In a translational view, these studies investigate whether stress can cause relapse (switch from inactive disease to active disease). Regarding this point, Qiu et al. (71) demonstrated that a combination of restraint and sonic stress, together with a subthreshold dose of dinitrobenzene sulfonic acid reactivated colitis in mice. Colitis reactivation by chronic WAS was also seen in the DSS colitis model (72).

As described above, studies in humans using experimental stressors have suggested that stress enhances colonic permeability, produces enhanced levels of circulating cytokines and alters the functionality of the HPA axis and the ANS (35,37,39,40). Animal studies expanded these findings, by using animal models of colitis and experimental stressors, revealing additional mechanisms of stress-induced exacerbation of chronic gut disease. It was suggested that stress exacerbates colitis also by alterations of peripheral and central corticotropin-releasing factor (CRF) signalling and by modifying microbial-host interactions (24). Specifically, a study using TNBS showed that colitis in Lewis rats, which have a hyporesponsive HPA axis, is especially aggravated by stress (67). Thus, a genetic abnormality of the activity of the CRF system is a risk factor for stress-induced worsening of colitis. This is supported by a recent study using CRF knockout mice, which have higher levels of colonic inflammation after DSS treatment and exhibit an impaired recovery from colitis relative to wild-type mice (73).

As stress enhances colonic permeability, it also increases interactions of bacteria with the mucosal immune system. It was demonstrated that chronic subordinate colony housing promotes bacterial translocation into the colonic wall (74). Chronic WAS promotes bacterial adhesion and penetration into the surface epithelial cells, and bacterial cells were even detected in mesenteric lymph nodes, potentially triggering immune responses and inflammation (75).

Another study using interleukin 18 knockout mice suggested that this cytokine has a special role in stress-induced exacerbation of colitis (55). In this study, psychological stress exacerbated colitis and increased colonic expression of interleukin 18 in wild-type mice. In interleukin 18 knockout mice, however, psychological stress did not worsen colitis (55).

Finally, cellular components of the immune system also have a role in the interaction between stress and colitis. For example, an early study demonstrated that stress-induced relapse of colitis is mediated by CD4-positive immune cells and that transplantation of these cells into other animals “transplants” colitis susceptibility (71). Another study found an increased number and activity of mast cells within the intestinal tract after stress exposure (65).

1.6 Stress and adaptation

Stress is a term deeply rooted and used frequently in our everyday language. In the 1930s Hans Selye was the first to systematically investigate stress reactions. According to him, acute nonspecific noxious agents affect the body resulting in a syndrome which he called “general adaptation syndrome”, later simply termed stress (76). Since then, stress has been classified in multiple ways. For example, acute stress (single short-term exposure to a stressor) can be differentiated from chronic stress (long-term and/or multiple exposure to a stressor). Another classification divides chronic stressors into homotypic (multiple exposure to the same stressor) or heterotypic stressors (exposure to different kinds of stressors). Finally, stressors can also be classified as internal (osmotic stress, inflammatory stress) or external stressors (psychological stress, physical stressors such as restraint stress).

In a modern view, stressors are defined by their ability to disturb the homeostasis of the body (77). These disturbances are detected by the sensory systems of the body and the information is transmitted to the brain. Depending on the magnitude of homeostatic alterations, the perception of stress initiates the activation of adaptation systems to restore homeostasis. The best described signal pathway to achieve this aim is the HPA axis. It has been demonstrated that exposure of animals to stress leads to the production of CRF, a 41-amino acid neuropeptide, within the paraventricular nucleus of the hypothalamus (PVH) (4). This adaptation process is fast as the peak of CRF heteronuclear RNA, which reflects gene transcription, is already reached 30 minutes after stress exposure (78). CRF is then released into the hypophyseal portal circulation where it can reach the anterior pituitary gland to stimulate the production of adrenocorticotrophic hormone (ACTH). ACTH is subsequently secreted into the systemic circulation to drive the production of glucocorticoids (cortisol in humans and corticosterone in rodents) in the adrenal cortex. Glucocorticoids are regarded as stress hormones and act on many cells expressing the glucocorticoid receptor. Upon activation by glucocorticoids, this receptor complex is internalized and acts as a transcription factor to influence gene expression. This is believed to be the major effect whereby glucocorticoids can influence homeostasis (79).

The second major signal pathway activated by stress is the ANS. This system is relevant to the regulation of many organ functions essential for life, such as respiration, osmotic regulation, cardiovascular control, thermoregulation, digestion and excretion (2). ANS adaptation processes in response to stress are even quicker than HPA axis adaptations and occur within seconds (2). The ANS can be divided in a sympathetic branch, a parasympathetic branch and the ENS. Stress leads to enhanced sympathetic nerve activity. Subsequently, sympathetic nerve endings release noradrenaline, which is also considered as a stress transmitter. Furthermore, activation of the sympathetic system stimulates the production of noradrenaline and adrenaline within the medulla of the adrenals and their secretion into the blood stream (2). Based on the localization of sympathetic nerve endings and the respective adrenoceptors, stress-induced neurotransmitter release increases heart rate and contraction, decreases visceral blood flow and increases blood flow in the skeletal muscles of the limbs (2). As described by Cannon et al. in 1915 (80), this signal pathway is crucial in an evolutionary perspective as it initiates the “fight or flight” response in the face of threats.

Besides these important and well-characterized signal systems, many other molecules and processes have been implicated in the stress response. For example, stress alters the activity of neuropeptide systems (NPY, calcitonin gene-related peptide, galanin), neurotrophic factors (brain-derived neurotrophic factor (BDNF), nerve growth factor) and immediate early genes (fos genes) (81-85). Furthermore, stress modifies neurogenesis and induces structural alterations within the brain (86,87).

1.7 Detection and processing of stress

The recognition of a stimulus as a stressor is made by the central nervous system. This process involves stimulus detection by sensory neurons and complex stimulus processing within the brain. A basic idea as to how stimuli are identified as stressors may be given by the example of restraint stress. One way to apply this type of stress is putting animals into special designed tubes, which severely impair body movements of mice or rats (88). The inability to move and the tightness of the restraining tube is detected by the sensory systems of the body.

The primarily visual and tactile experience of the restraining tube is then encoded as a neuronal signal and transmitted to the brain via afferent nerve fibres. On the way to its final destination, the primary somatosensory cortex, the tactile information is transmitted via axons of the first-order sensory neurons in the ipsilateral dorsal column of the spinal cord to the lower medulla (89). From there, the axons of the second-order sensory neurons cross the midline of the brainstem, and the information is conveyed to the thalamus. The thalamus is particularly important as it has a gating and modulatory role for the sensory information on its way to the sensory cortex. Finally, thalamic neurons receiving this information send axons to the primary somatosensory cortex, responsible for the processing of somatosensory information (89). Visual information is conveyed via the optical nerves, the optical chiasm and the optical tract to the lateral geniculate nucleus of the thalamus and from there to the primary visual cortex. Multimodal sensory association areas are then responsible to integrate sensory information of different modalities. To judge the emotional relevance of the incoming signal, information from the limbic system is integrated (89). Major components of the limbic system are the amygdala, the hippocampus and the medial prefrontal cortex. Roughly said, the hippocampus adds information about previous situations comparable to the current one as this structure plays an important role in memory (90). The amygdala evaluates the emotional relevance of the stimuli, potentially induces the feeling of fear and influences ANS and HPA activity via efferent connections to the hypothalamus and the brainstem (91). The medial prefrontal cortex has a coordinating role in this process, suggested by the bidirectional connections between this region, the amygdala and the hippocampus (92). Thus, finally, the limbic system is the essential component to evaluate nature and intensity of a stimulus and to produce, if necessary, an appropriate behavioural response.

Interestingly, recent evidence suggests that the physiological processing of stressful or emotional stimuli may be impaired in IBD patients. Specifically, a study in UC patients using functional magnetic resonance imaging (fMRI) detected a reduced blood oxygen level-dependent signal in the thalamus, cerebellum and amygdala in response to viewing positive pictures compared to control patients, indicating a deficit in emotional processing in the course of UC (93). Another study found reduced gray matter volumes within the frontal and the cingulate cortex (CC), a part of the medial prefrontal cortex, of patients with CD (94). These

findings of alterations within the limbic system may also help to explain the increased incidence and prevalence of primary psychiatric diseases in patients with IBD (95,96).

1.8 Neuropeptide Y - a key neuropeptide in the course of stress

As described above, besides the ANS and the HPA axis, also neuropeptides have a central role in the stress response. In contrast to neurotransmitters, neuropeptides have no specific reuptake mechanisms. This instance and the kinetics of their action explain why neuropeptides have longer lasting actions on target neurons. One of the most abundant neuropeptides within the brain is NPY (97). NPY is a 36-amino acid peptide that belongs to the so-called NPY family of biologically active peptides, together with two other members, peptide YY and pancreatic polypeptide (98). Originally NPY was isolated from brain extracts (99). Since then, studies reported a pivotal role of NPY in many physiological functions including food intake, energy homeostasis, circadian rhythm, and cognition (100-103). Besides these functions, NPY has a crucial role in the stress response, anxiety and depression (81,97).

Within the brain, NPY is transcribed in specific brain nuclei. Mapping studies investigating NPY mRNA production within the rodent brain identified 4 regions as the main sources of brain NPY synthesis (104). These regions include the hypothalamic arcuate nucleus (ARC), the locus coeruleus (LC), the nucleus tractus solitarius (NTS) and the septohippocampal nucleus (104). NPY is additionally present in many cortical interneurons, amygdala, hippocampus, nucleus accumbens, periaqueductal gray, basal ganglia and thalamus (98,104). An important fibre tract containing NPY exists between the ARC, the major source of NPY, and the PVH, the major source of CRF, allowing a crosstalk between these two neuropeptide systems (105).

Already in 1994, a still valid hypothesis about the interactions of CRF and NPY in the course of stress was proposed by Heilig et al. (106). Accordingly, NPY counteracts the biological effects of CRF. As described above, neurons of the PVH respond rapidly to stress exposure with the transcription of CRF (78). Functionally it has been demonstrated, that stress-induced CRF expression, CRF

overexpression as well as central CRF application produce anxiety in rodents (107). NPY, the functional counter player of CRF, is also regulated by stress exposure. Depending on type and duration of stress as well as brain region examined, stress modifies the expression of NPY (108-111). NPY has behavioural effects opposite to those of CRF. Early studies showed that intracerebroventricular application of NPY renders mice less anxious (112). However, not only intracerebroventricular application of NPY has this effect, it can also be seen after NPY injection directly into the amygdala or the hippocampus, indicating that these regions are key components in the anxiolytic effect of the peptide (113,114). The findings that NPY knockout mice are more anxious, and hippocampal or amygdalar NPY overexpression renders animals less anxious, confirmed the idea of the anxiolytic properties of NPY and the importance of the limbic system for this effect (115-118).

The behavioural effects of NPY depend on the receptor activated. NPY exerts its effects via four receptors: Y1, Y2, Y4 and Y5 (98). Studies using selective receptor agonists or antagonists demonstrated that the anxiolytic effect of NPY is mediated by the Y1 receptor. Thus, Y1 receptor blockade is anxiogenic, while stimulation of this receptor with a selective agonist mimics the anxiolytic effects of NPY (117,119). Furthermore, administration of a selective Y1 receptor antagonist into the amygdala abolished the anxiolytic effect of co-administered NPY (120). In contrast to the Y1 receptor, stimulation of the Y2 receptor has an anxiogenic effect, which is related to the presynaptic localization of this receptor and its function as an autoreceptor reducing NPY release (121,122). As expected, blocking this receptor is anxiolytic (123). To date no study investigated the effects of a selective Y4 agonist or antagonist on anxiety-like behaviour. A study using Y4 knockout mice demonstrated, however, that these mice are less anxious, indicating anxiogenic properties of the Y4 receptor (124). The data about the role of the Y5 receptor in anxiety are controversial. Thus, one study reported an anxiolytic effect of a selective Y5 receptor agonist, while another study reported an anxiolytic effect of a selective Y5 receptor antagonist (119,125).

Little information is available as to how brain NPY is affected by the internal stress of visceral inflammation. As described in Holzer et al. (98), TNBS-induced colitis increases the NPY concentration in brain and plasma (61) whereas the expression of NPY mRNA in the brain remains unaltered by *Helicobacter pylori*

infection (126). An important interaction between visceral inflammation and the NPY system is suggested by a study using NPY knockout mice. Behavioural analysis with the open field test revealed that female NPY knockout mice spend less time in the central area of the open field after DSS treatment, while female wild-type mice do not show this effect (127). In contrast DSS treatment enhanced the immobility time of female wild-type mice in the forced swim test, an effect not seen in female NPY knockout mice (127). Thus, deletion of NPY signalling leads to a distinct behavioural phenotype of mice when challenged with DSS treatment.

1.9 Neurochemical basis of stress resilience

The importance of NPY in stress processing and anxiety raised the question whether NPY has a role in the ability to cope with stress. An initial study in transgenic rats with hippocampal NPY overexpression found that these animals are protected from the anxiety-producing effects of restraint stress (116). A subsequent study demonstrated that repeated administration of NPY into the BLA produces resilient mice that are protected from the negative consequences of restraint stress (114). Recently, a study by Cohen et al. (128) described a correlation between behavioural disruption of animals after predator scent stress and brain NPY levels. Animals whose behaviour was extremely disrupted had the lowest brain NPY levels. Treatment of animals with NPY shortly after stress exposure significantly reduced the prevalence of extremely disrupted behaviour (128). These data support a role for NPY in stress resilience.

In this context, the term “stress resilience” denotes the ability of individuals to maintain physiological functioning after exposure to extreme stress (129). Individual coping strategies determine how humans react to a stressful situation, which can be quite different even to the same adverse life event. After such events, some people develop diseases like post-traumatic stress disorder (PTSD), while other people stay healthy (130). This finding suggests that individual genetic, molecular or environmental factors favour or protect from the development of disease after trauma exposure.

Some insight regarding these issues was gained from PTSD animal models. To model PTSD in animals, a behavioural paradigm based on repeated

social defeat has been developed (131). In brief, mice are exposed to repeated social defeat stress for 10 days. Social defeat is accomplished by putting experimental mice into the home cage of a larger, more aggressive mouse, which results in a submissive phenotype of the intruder mouse. After this period of social defeat, the defeated mice are submitted to a social interaction test. Based on the time spent in the interaction zone, they are classified as either susceptible or resilient to stress (131). Using this approach it has been shown that susceptible and resilient mice have certain neurobiological differences within their brains. For example, susceptible mice have increased BDNF levels within the nucleus accumbens compared to unstressed animals, a change not present in resilient mice (131). Another study found that resilient animals have a higher expression of Δ FosB, a transcription factor of the Fos family, within the same brain region (132). This suggests that both BDNF and Δ FosB are important mediators of stress resilience within the nucleus accumbens. Resilient mice are also characterized by a different methylation pattern of the *crf* gene compared to susceptible mice (133). Thus, epigenetic mechanisms appear relevant for stress susceptibility. In humans, some genetic resilience factors have been identified with a candidate gene approach. Thus, polymorphisms of HPA axis genes, serotonergic system-associated genes and NPY-related genes may contribute to stress resilience although genome-wide association studies have not been conducted so far (129).

Increasing evidence in animal experiments suggests that environmental factors may influence stress resilience. This is based on findings that environmental enrichment (EE), an alternative housing condition for mice, reduces the impact of stress on animals (134). At the behavioural level, EE-housed mice are less affected by the negative behavioural consequences of stress and show a better performance in several behavioural tests after stress exposure (135-138). EE-housed mice also have a different neuroendocrine response to stress, since their stress-induced corticosterone levels are lower than in conventionally housed mice (136,138,139). The stress-resilient phenotype of EE-housed mice may be mediated by certain molecular and structural brain changes. Specifically, EE increases the expression of neurotrophic factors, enhances adult neurogenesis, alters the activity of neurotransmitter systems and increases the number of synapses and spines (140-144).

1.10 Post-traumatic stress disorder

As mentioned above, PTSD is a disease related to a deficit in stress coping strategies in response to a severe trauma. Accordingly, an essential criterion for the PTSD diagnosis is the occurrence of illness after a traumatic experience such as sexual or physical violence, war trauma, actual death or fear of imminent death (145). A recent systematic review described prevalence rates of 28 % and 17 % 1 and 12 months after trauma exposure, respectively (146). Essential symptoms of PTSD are the re-experiencing of the traumatic event (distressing memories or dreams), strategies to avoid confrontation with the trauma (avoidance of conversations about the incident, avoidance of places associated with the trauma) and symptoms of increased arousal such as sleep disturbances (145).

Since the majority of people exposed to a severe physical or emotional event stay healthy, individual resilience or vulnerability factors appear crucial for the development of the disorder. Family studies revealed an increased disease risk for first-degree relatives of PTSD patients and twin studies support the view of a genetic component in PTSD development (147,148). Identified risk genes for PTSD development include the serotonin transporter gene, the serotonin 2A receptor gene and the CRF type 1 receptor gene (149). Besides genetics, human research has focused on stress-related neuroendocrine pathways (HPA axis and ANS), neurotransmitters and neuropeptides. Surprisingly, most studies reported serum cortisol concentrations within the normal range in PTSD patients, which speaks against the hypothesis that cortisol levels predict the magnitude of the stress response. This finding and reports of higher CRF levels in PTSD patients points to an enhanced responsiveness of peripheral and central glucocorticoid receptors (150). Indeed, a study demonstrated that leukocytes of PTSD patients are more sensitive to glucocorticoids (151). ANS activity, as reflected by noradrenaline levels within the cerebrospinal fluid, is increased in PTSD, which may explain the hyperarousal status of these patients (152). Some studies also linked the glutamatergic, GABAergic and opioidergic system to the pathophysiology of PTSD (145). Accumulating evidence suggests that NPY is a key player in PTSD (130). As in rodent studies, increased NPY levels have been associated with stress resilience in humans, an argument mainly based on studies with combat veterans. Thus, combat veterans suffering from PTSD have lower

plasma and cerebrospinal NPY levels (153,154). A role of NPY as a resilience factor in these patients is suggested by the finding that combat veterans without PTSD have higher plasma NPY levels than combat veterans with PTSD (155).

The two main strategies to treat PTSD are psychotherapy and pharmacological treatments. Beneficial effects were demonstrated for exposure-based cognitive behavioural therapy and for eye movement desensitization and reprocessing therapy (156,157). According to the American Psychiatric Association's practice guidelines 2004, selective serotonin reuptake inhibitors are the first-line pharmacological treatments for PTSD (145). Indeed, a large review confirmed treatment efficacy of these drugs (158). Furthermore, some benefit has been reported for prazosin, an α -adrenoceptor antagonist, in the treatment of sleep disturbances and trauma-related nightmares of PTSD patients (159). Finally, atypical antipsychotic drugs may enhance the response to selective serotonin reuptake inhibitors (145,160).

1.11 Aims of the project

In view of the complex interaction between stress and visceral inflammation, the current project was designed to elucidate the role of psychological stress and particularly the brain response to stress in mouse models of chronic gastrointestinal inflammation. Given that stress is a crucial factor in the development and course of IBD (24,25,36,161) and that chronic gastrointestinal diseases are associated with primary psychiatric diseases (95,162,163), it is crucial to conduct studies investigating brain function in the course of visceral inflammation.

To address this issue, the first aim of the project was to establish a psychological stress paradigm, which has translational value. Among a wide variety of stressors used during animal studies, the WAS paradigm appeared to be an ideal psychological stressor for mice (72,164,165). Previous studies showed already that WAS can alter neuronal activation in certain brain areas, as measured by the quantification of cells expressing the immediate early gene product c-Fos (165-168). There is however no systematic study investigating WAS-induced c-

Fos changes within the limbic system. Therefore, I screened the mouse brain for WAS-induced c-Fos alterations with particular emphasis on the limbic system.

The second aim of the project was then to elucidate if WAS-induced brain c-Fos or NPY levels, a neuropeptide crucial for the processing of stress (81), are modified by visceral inflammation. For this purpose I selected iodoacetamide (IAA)-induced gastritis and DSS-induced colitis as animal models of visceral inflammation. Both paradigms are well validated as translational models for dyspepsia and UC, respectively (45,169,170). Additionally I investigated whether EE, an alternative housing condition for mice, can influence the brain response to WAS in both health and disease. This is particularly interesting as EE modifies the neuroendocrine response to stress and alters stress coping (136-138,171). It is not known, however, whether inflammatory treatments or differential housing conditions can modulate the number of stress-induced c-Fos expressing cells or modify the activity of the NPY system.

The third aim of the project was to analyse if EE modifies FosB/ Δ FosB expression, another marker of neuronal activation, or induces ultrastructural changes within subregions of the hippocampus.

Finally, the fourth aim was to evaluate the effects of colitis and/or WAS on anxiety-like and depression-related behaviour.

2 Material and Methods

2.1 Experimental animals

Animal experiments were carried out with either female C57BL/6J mice obtained from the Division of Laboratory Animal Science and Genetics of the Department of Biomedical Research of the Medical University of Vienna (Himberg, Austria) used for experimental protocol 1 (see below), female adult C57BL/6N mice obtained from Charles River (Sulzfeld, Germany) used for experimental protocol 3 (see below) or male adult C57BL/6N mice obtained from Charles River (Sulzfeld, Germany) used for all other experiments. Mice were kept in the in-house animal facility under controlled conditions of temperature (set point 21 °C) and air humidity (set point 50 %) and under a 12 h light/dark cycle (lights on at 5:30 h, lights off at 17:30 h) (172). All experiments were approved by an ethical committee at the Federal Ministry of Science and Research of the Republic of Austria (BMWF-66.010/0037-II/10b/2008, BMWF-66.010/0073-II/10b/2009, BMWF-66.010/0119-II/3b/2011 and BMWF-66.010/0037-II/3b/2013) and conducted according to the Directive of the European Communities Council of 24 November 1986 (86/609/EEC). The number of animals used was reduced as far as possible and group sizes were chosen in a way to detect statistically meaningful differences. Before the start of all experiments, animals were allowed to habituate to the animal facility for at least 2 weeks, and during the experiments the health status of the animals was closely monitored. Therefore the experiments are in agreement with the “3Rs” (Replace, Refine and Reduce) of animal use in research.

2.2 Experimental protocols

2.2.1 Protocol 1

The aim of protocol 1 was to identify the effects of WAS on the expression of c-Fos, a marker of neuronal activation, in the mouse brain. Therefore mice were submitted to a 1-hour session of WAS and sacrificed 2 hours after the beginning of

stress. Collected mouse brains were used for c-Fos immunohistochemistry to identify brain regions affected by stress exposure.

2.2.2 Protocol 2

The first aim of this experiment was to assess whether stress-induced c-Fos and/or NPY expression can be modified by visceral inflammation and/or EE. The second aim was to investigate whether environment influences the course of visceral inflammation. Therefore mice were kept under standard or enriched housing for 9 weeks (week 1-9). During the last week of differential housing (week 9) animals were allocated to different treatment groups. Mice were treated either with vehicle (drinking water), IAA (0.1 %; added to the drinking water) or DSS (2 %; added to the drinking water). One day after this 7-day treatment period (week 10, day 1) the animals were exposed to WAS for 30 minutes. Following a 90-min stress-free interval in the home cage, the animals were euthanized. Trunk blood was collected for corticosterone measurements, colons and stomachs were extracted for myeloperoxidase (MPO) determination and their brains were removed for immunohistochemical visualization of c-Fos and analysis of NPY expression by in situ hybridization (ISH) (172).

2.2.3 Protocol 3

Experiment 3 evaluated whether EE can change synaptic morphology within the hippocampus. Therefore after a 5 or 9-week period of differential housing, mice were euthanized and their brains were analysed by electron microscopy.

2.2.4 Protocol 4

Protocol 4 was designed to investigate the effects of colitis and acute stress on depression-like behaviour. Thus, stressed and unstressed mice with and without colitis were tested in the tail suspension test (TST), an established test to assess depression-like behaviour in mice (173). Therefore mice were treated for 7 days with either vehicle (drinking water) or DSS (2 %, added to the drinking water) and submitted afterwards either to WAS for 30 minutes or left in their homecage

(unstressed controls). After a 90-minute interval in their homecage WAS-treated animals and their controls were tested in the TST.

2.2.5 Protocol 5

To assess the effects of colitis and acute WAS on anxiety and anhedonia, stressed and unstressed mice with and without colitis were subjected to the light/dark box (L/D) and sucrose preference (SP) test. Similarly to protocol 4, mice were treated for 7 days with either vehicle or DSS and were either submitted to WAS for 30 minutes or not. In contrast to protocol 4, they were tested in the L/D after a 90 minute interval in their homecage. After another 30 minutes in their homecage mice were placed in the Labmaster system to measure SP for 48 h.

2.2.6 Protocol 6

In this protocol, mice were submitted to the DSS/WAS paradigm as mentioned in protocols 4 and 5. This time, however, consumed food and liquid amount/cage were assessed by weighing food and drinking bottles during the treatment period, respectively. Furthermore, stressed mice were closely monitored for their behaviour during the WAS session and two hours after the beginning of WAS brains were extracted without any behavioural testing to investigate the role of stress and colitis in brain NPY expression. Colons were collected to measure the effects of stress and DSS on MPO content, colon length and colon histology.

2.3 Water avoidance stress

The WAS procedure is considered a psychosocial stressor for rodents (67,174). As described in Reichmann et al. (172), at the beginning of the procedure mice were placed on a small plastic block (6 x 3 x 6 cm, length x width x height) in the centre of a water-filled tank (61 x 40 x 22 cm, length x width x height), the level of the water (25 °C) in the tank being just beneath the block. During the WAS procedure escape trials (jumping from the block towards the wall of the tank or swimming in the water) were recorded. Following exposure to WAS for 60 (protocol 1) or 30 minutes (protocols 2,4,5,6) the animals were returned to their home cage.

2.4 Standard and enriched housing conditions

In protocol 2 and 3, animals were housed under either standard or enriched housing conditions for 9 or 5 weeks, respectively. As described in Reichmann et al. (172) under standard conditions, the mice were housed in groups of 5 in polycarbonate cages of size II L measuring 36.5 x 20.7 x 14.0 cm (length x width x height, floor area: 530 cm²). EE was achieved by housing the mice in groups of 5 in polycarbonate cages of size IV (175-177) measuring 59.0 x 38.0 x 20.0 cm (length x width x height, floor area: 1815 cm²). Thus, under standard conditions an average floor area of 106 cm² was available to each animal, compared with a floor area of 363 cm² per animal under enriched conditions. The following enrichment items were provided (178-180): nesting material (standard paper towels), a running wheel (diameter: 14 cm; Dehner, Graz, Austria), a tunnel made of hay (length: 20 cm, inner diameter: 5 – 8 cm; Dehner), a tunnel made of timber with 6 side holes (length: 25 cm, inner diameter: 4 cm; Dehner), a tunnel made of cardboard (length: 13 cm, inner diameter: 9 cm; Scanbur, Karlslunde, Denmark), mouse houses with two openings made of red transparent polycarbonate measuring 10 x 9 x 5.5 cm (length x width x height; Ehret, Tulln, Austria), mouse houses with 8 openings made of cardboard measuring 17 x 17 x 7 cm (length x width x height; Scanbur), and a tunnel made of red transparent polycarbonate (length: 10 cm, inner diameter: 5.5 cm; Scanbur) which during certain weeks of the enriched housing procedure was hung on the cage lid. Table 1 describes the items used during each week of the enrichment protocol. The enriched housing procedure used here incorporated elements of published protocols (178-180), and the duration of enriched housing (9 weeks) was modeled along the studies of Marashi et al. (177) and Amaral et al. (181) who found that 8 weeks of enriched housing have a particularly profound impact on behaviour. This methodological description was published in an original article (172). The 5-week protocol consisted of the enforced enrichment protocol only (Table 1; week 5-9). The effectiveness of the current protocols to foster spatial learning and memory through increasing neuronal plasticity in the brain (175,182,183) was confirmed by the improved Morris Water Maze performance of the mice kept under EE (172).

Week and experimental phase	Basic items	Additional mouse houses	Additional tunnels
Week 1: Basic Enrichment	Nesting material Hay tunnel Running wheel	2 polycarbonate	1 timber
Week 2: Basic Enrichment	Nesting material Hay tunnel Running wheel	2 polycarbonate	1 timber
Week 3: Basic Enrichment	Nesting material Hay tunnel Running wheel	2 polycarbonate	1 timber
Week 4: Basic Enrichment	Nesting material Hay tunnel Running wheel	2 polycarbonate	1 timber
Week 5: Enforced Enrichment	Nesting material Hay tunnel Running wheel	1 polycarbonate 1 hard paper	1 hard paper
Week 6: Enforced Enrichment	Nesting material Hay tunnel Running wheel	2 hard paper	1 timber 1 hard paper
Week 7: Enforced Enrichment	Nesting material Hay tunnel Running wheel	2 polycarbonate	1 timber 1 polycarbonate*
Week 8: Enforced Enrichment	Nesting material Hay tunnel Running wheel	2 hard paper	1 timber 1 polycarbonate
Week 9: Enforced Enrichment	Nesting material Hay tunnel Running wheel	1 polycarbonate 1 hard paper	1 polycarbonate 1 polycarbonate*

Table 1: Environmental enrichment protocol. For the environmental enrichment housing procedure, animals were housed in polycarbonate cages of size IV for either 9 or 5 (enforced enrichment only) weeks. The table describes the items placed in the cage during the different phases of the procedure. This table has also been published in an original article (172). * Tunnel hung on cage lid.

2.5 Induction of experimental gastritis or colitis

To induce a mild gastritis in mice, 0.1 % (w/v) IAA (Sigma, Vienna, Austria) was added to the drinking water for 7 days (172,184), while control animals received normal tap water. Mild colitis was induced by adding 2 % (w/v) DSS (molecular weight 36000 - 50000; MP Biomedicals, Illkirch, France) to the drinking water for 7 days (172,185). The control animals received normal tap water.

2.6 Disease activity score

At the end of the 7-day treatment period with either IAA or DSS a disease activity score (DAS) was taken to obtain information about the clinical status of the animals and the effectiveness of the treatments. As described in Reichmann et al. (172), for experimental protocol 2 the score was calculated from the parameters fur appearance (score 0: normal fur, score 1: disturbed fur), stool consistency (score 0: normal stool, score 1: soft but formed stool, score 2: loose stool) and presence of blood in the perianal region (score 0: no traces of blood, score 1: traces of blood in the perianal region, score 2: bloody perianal region). Thus the minimum score is 0 and the maximum score is 5. For protocol 4-6 the score was refined (Table 2) and calculated from weight change (score 0: weight increase ≥ 1 g, score 1: weight increase < 1 g, score 2: weight decrease < 1 g, score 3: weight decrease ≥ 1 g), stool consistency (score 0: normal stool, score 1: soft but formed stool, score 2: loose stool) and presence of blood in the perianal region (score 0: no traces of blood, score 1: traces of blood in the perianal region, score 2: bloody perianal region). For this refined score the minimum score is 0 and the maximum score is 7.

Parameters	Score	Description
Weight	0	Increase ≥ 1 g
	1	Increase < 1 g
	2	Decrease < 1 g
	3	Decrease ≥ 1 g
Stool	0	Normal
	1	Soft but formed
	2	Loose
Perianal Blood	0	No blood
	1	Traces of blood
	2	Bloody perianal region

Table 2: Disease activity score. The table describes the parameters and the scoring system used for calculating the disease activity score.

2.7 Assessing food and liquid intake

To assess whether DSS treatment affects food and liquid intake, the weight of the food pellets and the drinking bottles of each cage was measured. Each cage contained 2 mice and the measurements were made daily between 8 and 9. For statistics, the consumed food and drinking amount/cage in gram per day was divided by the combined pretreatment-weight of the 2 mice within a given cage.

2.8 Myeloperoxidase levels in the stomach and colon

The tissue levels of MPO were used to quantify inflammation-associated infiltration of neutrophils and monocytes into the tissue (186). As previously described (172,185), after euthanasia, full-thickness pieces of the gastric corpus and distal colon were excised, shock-frozen in liquid nitrogen and stored at -70 °C until assay. After weighing, the frozen tissues were placed, at a ratio of 1 mg per 20 µl, in MPO lysis buffer. The composition of this buffer was: 200 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 10 mM trishydroxymethylaminomethane, 10 % glycine, 0.1 mM phenylmethylsulphonyl fluoride, 1 µg/ml leupeptide, 28 µg/ml aprotinin, pH 7.4. The samples were homogenized on ice with an Ultraturrax (IKA, Staufen, Germany) and then subjected to two centrifugation steps at 6000 x g (7000 rpm) and 4 °C for 15 min. The MPO content of the supernatant was measured with an enzyme-linked immunosorbent assay kit specific for the rat and mouse protein (Hycult Biotechnology, Uden, The Netherlands). The sensitivity of this assay is 1 ng/ml at an intra- and inter-assay variation of around 10 %. This methodological description was also published in an original article (172).

2.9 Circulating corticosterone

Trunk blood was collected from unstressed mice or from stressed mice 2 hours after the begin of the 30-min exposure to WAS. Since the concentration of circulating corticosterone is subject to circadian variations, blood was sampled between 10:00 h and 12:00 h. To this end, the mice were deeply anaesthetized with intraperitoneal pentobarbital (150 mg/kg) before they were decapitated. Within 2 min after the injection of the anaesthetic, trunk blood was collected into vials coated with EDTA (Greiner, Kremsmünster, Austria) kept on ice. Following

centrifugation for 10 min at 4 °C and 1200 x g, blood plasma was collected and stored at -20 °C until assay. The plasma levels of corticosterone were determined with an enzyme immunoassay kit (Assay Designs, Ann Arbor, Michigan, USA). According to the manufacturer's specifications, the sensitivity of the assay is 27 pg/ml, and the intra- and inter-assay coefficient of variation amounts to 7.7 and 9.7 %, respectively. This methodological description was also published in an original article (172).

2.10 Immunohistochemistry

The activation of neurons in selected nuclei and cortical areas of the brain was visualized by c-Fos or FosB/ Δ FosB immunohistochemistry. Sixty minutes after a 1-hour exposure of mice to WAS (experimental protocol 1) or 90 minutes after a 30-min exposure of mice to WAS (experimental protocol 2) brains were processed for c-Fos or FosB/ Δ FosB immunohistochemistry. In both protocols the 2 hour post-stress time point was selected as c-Fos expression is maximal 1-3 hours after stimulus exposure (83). The stress exposure time in protocol 2 was reduced from 60 to 30 minutes as preliminary experiments showed that the brain c-Fos response is similar using both protocols. In protocol 1, mice were euthanized with an overdose of pentobarbital (150 mg/kg) and transcardially perfused with 0.1 M phosphate-buffered saline (PBS) of pH 7.4 followed by 4 % paraformaldehyde (PFA; Sigma-Aldrich, Vienna, Austria) in PBS. Brains were postfixed overnight in PFA, cryoprotected for 48 hours in 20 % sucrose solution and stored at -70 °C until use. For immunohistochemistry, brains were cut at a Microm HM 560 cryostat (Microm, Walldorf, Germany) and free floating coronal brain sections (40 μ m thickness) were collected in PBS. To demonstrate c-Fos, sections were washed three times for 10 minutes in PBS containing 0.3 % Triton X 100 (Carl Roth, Karlsruhe, Germany). Then sections were put in 0.3 % hydrogen peroxide (H₂O₂) in PBS for 30 minutes and after 3 further washes incubated with the primary antibody (rabbit polyclonal anti-c-Fos SC-52, 1:20000, Santa Cruz Biotech, Santa Cruz, USA) for 40 hours at 4 °C. After three washes in PBS/Triton X 100 sections were incubated with the biotinylated secondary antibody (goat anti-rabbit IgG 1:200, Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, USA) at room temperature for 45 minutes. After another 3 washes, sections were incubated for 1

hour in avidin-biotin complex (Vectastain Elite ABC Kit, Vector Laboratories) and after 3 final washes sections were developed with 3,3-diaminobenzidine substrate (DAB; DAB substrate kit for peroxidase, Vector Laboratories). After stopping the reaction in distilled water, sections were mounted onto slides, air-dried overnight, cleared in xylene (Merck-Millipore, Darmstadt, Germany) and coverslipped with Entellan (Merck-Millipore).

For protocol 2 the brain extraction procedure as well as the immunohistochemistry was refined and modified (172). Therefore, following euthanasia by intraperitoneal injection of an overdose of pentobarbital (150 mg/kg) the brains were removed, frozen in 2-methylbutane (Roth) on dry ice and stored at -70°C until use. Immunohistochemistry was performed according to a slightly modified version of the protocol provided by Sundquist et al. (187). As described in Reichmann et al. (172), serial coronal sections of 20 μm thickness were cut from each forebrain with the cryostat. Every sixth section was used for c-Fos immunohistochemistry and mounted on Superfrost Plus slides (Menzel, Braunschweig, Germany). The sections were surrounded with a hydrophobic barrier pen (ImmEdge Pen, Vector Laboratories) and then incubated for 10 minutes in 4 % PFA in PBS of pH 7.4. Afterwards the slides were washed three times for 5 minutes in washing buffer (WB; 0.05 % Tween 20 (Roth) in 0.1 M PBS) and incubated in 0.3 % H_2O_2 in methanol for 15 minutes. After three further washes in WB, the tissues were incubated with 10 % normal goat serum in antibody diluent (AD; 0.1 M PBS containing 0.05 % Tween 20 and 1 % bovine serum albumin) for 5 minutes and then with the primary antibody in AD (rabbit polyclonal anti-c-Fos SC-52, 1:2000 or rabbit polyclonal anti-FosB SC-48, 1:5000, Santa Cruz Biotech) overnight at 4°C . On the next day the sections were washed three times in WB and incubated for 30 minutes in AD containing the biotinylated secondary antibody (goat anti-rabbit IgG 1:200) at room temperature. After three further washes in WB they were incubated for 30 minutes in avidin-biotin complex. Subsequently the tissues were rinsed three times in WB and developed with DAB. Finally the sections were washed three times for 5 minutes in distilled water, air-dried overnight, cleared in xylene and coverslipped with entellan. Antibody specificity was tested by pre-absorbing the primary antibody with an excess amount of c-Fos or FosB/ Δ FosB blocking peptide (SC-52P or SC-48P, Santa Cruz Biotech). Following this procedure no specific staining was detectable in any brain

region under study (Figure 2) Furthermore, omission of the primary and secondary antibodies resulted in a loss of specific staining product. This method description was also published in an original article (172).

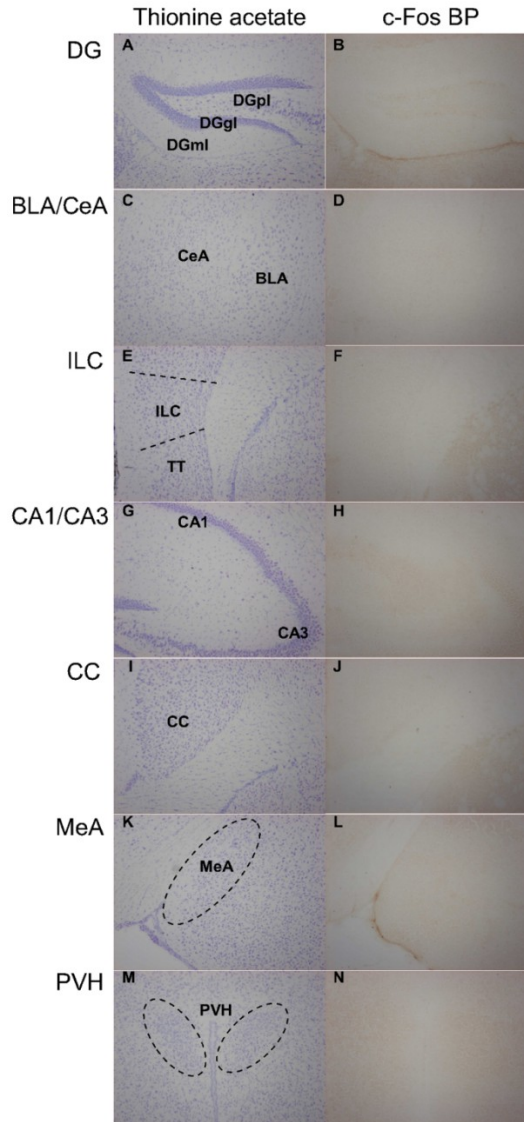


Figure 2. Pre-absorption of the c-Fos antibody with c-Fos blocking peptide prevents c-Fos staining in all brain regions under study. The left column (panels A, C, E, G, I, K, M) shows representative micrographs of the brain regions stained with thionine acetate to reveal their morphology. Parallel sections processed for c-Fos immunohistochemistry in which the c-Fos antibody was pre-absorbed with excess c-Fos blocking peptide (BP) are shown in the right column (panels B, D, F, H, J, L, N). No specific c-Fos staining was detected in any of the brain regions when the pre-absorbed antibody was used. Abbreviations: BLA = Basolateral amygdala; CA1 = CA1 field of the hippocampus; CA3 = CA3 field of the hippocampus; CC = Cingulate cortex; CeA = Central amygdala; DGgl = Dentate gyrus, granular cell layer; DGml = Dentate gyrus, molecular cell layer; DGpl = Dentate gyrus, polymorph cell layer; ILC = Infralimbic cortex; MeA = Medial Amygdala; PVH = Paraventricular nucleus of the hypothalamus; TT = Tenia tecta. These findings have been published in an original article (172)

2.11 Cell counting and quantification of c-Fos and FosB/ Δ FosB expressing cells

The immunohistochemically processed brain sections were examined with a light microscope (Axiophot, Zeiss, Oberkochen, Germany) coupled to a computerized image analysis system (MCID Basic, version 7.0, Imaging Research Inc., Brock University, St. Catharines, Ontario, Canada) (172). The slides were coded such that the investigator was blind to the treatment group under investigation. Brain regions of interest (ROIs) were identified with the help of adjacent Nissl-stained sections and the mouse brain stereotaxic atlas of Paxinos and Franklin (188).

For experimental protocol 1 a manual counting method was applied. The number of c-Fos expressing cells was quantified in 23 ROIs. A representative section showing c-Fos expression within the ROI was selected and cell counts of the whole ROI were made bilaterally. Selected brain regions included the agranular insular cortex (AIC; Bregma: +0.98), piriform cortex (Pir; Bregma: +0.98), CC (Bregma: +0.14), bed nucleus of stria terminalis/medial division/anterior part (BSTMA; Bregma: +0.26), lateral septal nucleus/ventral part (LSV; Bregma: +0.26), paraventricular nucleus of the thalamus (PVT; Bregma: -0.34), centromedial thalamic nucleus (CM; Bregma: -1.06), paracentral thalamic nucleus (PC; Bregma: -1.06), centrolateral thalamic nucleus (CL; Bregma: -1.06), lateral habenula (LHb; Bregma: -1.06), PVH (Bregma: -0.70), arcuate hypothalamic nucleus/rostral part (ARCr; Bregma: -1.46), arcuate hypothalamic nucleus/caudal part (ARCc; Bregma: -2.30), basolateral amygdala (BLA; Bregma: -1.58), central amygdala (CeA; Bregma: -1.58), medial amygdala (MeA; Bregma: -1.58), CA1 field of the hippocampus (CA1; Bregma: -2.92), CA3 field of the hippocampus (CA3; Bregma: -2.92), granular cell layer of the dentate gyrus (DGgl; Bregma: -2.92), lateral periaqueductal grey (LPAG; Bregma: -3.80), rostral linear of the raphe nucleus (RLi; Bregma: -3,80), pontine nuclei (PN; Bregma: -4.16) and LC (Bregma: -5.52).

For experimental protocol 2 c-Fos and FosB/ Δ FosB expression was quantified with the help of MCID Basic. As described in Reichmann et al. (172), in order to count all c-Fos or FosB/ Δ FosB positive cells (cells containing a brown/black reaction product of sufficient intensity within the nucleus) with the

computerized image analysis system, an intensity-based background threshold was determined for each brain ROI. Based on the mean intensity value of three background measurements/region, the background threshold was defined such that the maximum number of c-Fos labelled cells was counted without inclusion of any background staining. While in the DGgl and the PVH all c-Fos or FosB/ Δ FosB positive cells were counted, the number of c-Fos or FosB/ Δ FosB labelled cells in the other ROIs was quantitated within a square of 200 x 200 μ m. Three consecutive sections were counted bilaterally to quantify the number of c-Fos or FosB/ Δ FosB positive cells in the CC (Bregma +1.10 to +0.86), BLA, CeA and MeA (all Bregma -1.06 to -1.34) as well as in the CA1, CA3 and DGgl (all Bregma -1.34 to -1.58). Two consecutive sections were counted bilaterally to evaluate the expression of c-Fos or FosB/ Δ FosB in the infralimbic cortex (ILC) at Bregma +1.54 to +1.42 and the PVH at Bregma -0.58 to -0.70 (Figure 3). The cell counts obtained for each ROI in the different sections of each animal were averaged to quantify the mean number of c-Fos positive cells within a particular brain region of that animal. These average values/brain region of each animal were used for statistical analysis (172).

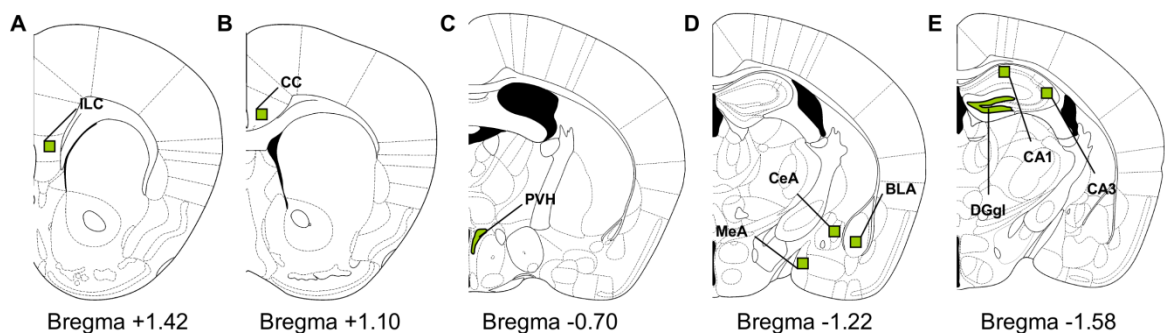


Figure 3. Mouse brain atlas figures (modified from Paxinos and Franklin (188)) showing brain regions used for c-Fos quantification. The size and localization of the counting area within each region of interest is shown in green. Abbreviations: BLA = Basolateral amygdala; CA1 = CA1 field of the hippocampus; CA3 = CA3 field of the hippocampus; CC = Cingulate cortex; CeA = Central amygdala; DGgl = Dentate gyrus, granular cell layer; ILC = Infralimbic cortex; MeA = Medial Amygdala; PVH = Paraventricular nucleus of the hypothalamus.

2.12 Colon histology

Full thickness pieces of the distal colon were processed for histology. Following excision the colon was opened longitudinally and a 1 cm² piece of the distal colon was stapled on cardboard with the mucosal side up. The colon was then placed in a 4 % PFA solution for 72 hours. Afterwards the tissue was washed in tap water for 1 hour and dehydrated in an ascending EtOH row. Then colons were incubated in a 1:2 dilution of acetic acid n-butyl ester (ABE, Lactan, Graz, Austria) in 100 % EtOH for 30 minutes followed by two 30-minute incubations in fresh ABE, a 30-minute incubation in a 1:2 dilution of ABE in paraffin and finally a paraffin incubation at 70 °C overnight. On the next day, the tissues were embedded in paraffin. Free floating colon sections (5 µm) were cut on a Microm HM 335E microtome (Microm) and mounted on Superfrost Plus slides. The slides were deparaffinized in xylol for 40 minutes, incubated in a 1:2 dilution of xylol in 100 % EtOH and rehydrated in a descending EtOH row and distilled water. Then sections were stained with hematoxylin (Roth) for 8 minutes, cleaned in distilled water for 30 seconds and running tap water for 15 minutes and then stained with eosin (Sigma-Aldrich) for 1 minute. After two 10-minute washes in running tap water, sections were dehydrated in an ascending EtOH row, cleared in xylol and coverslipped with entellan.

2.13 Molecular cloning

A prerequisite for the NPY ISH was to generate plasmids containing the appropriate NPY base pair (bp) sequence. Therefore, primers were designed to amplify a NPY-specific 288 bp product from mouse brain cDNA (Table 3). For primer design, NPY gene sequence information was obtained from Pubmed Nucleotide, and primers were generated with the software Primer Designer 2.0. Appropriate primers were defined as having a length of at least 20 bps, a guanine-cytosine content of 50-60 %, similar melting temperatures and a low probability of primer dimer formation. Target specificity of primers was tested with the online available Primer BLAST (Basic Local Alignment Search Tool) software.

Gene	Sequence forward primer (5'→3')	Sequence reverse primer (5'→3')	Product size (bp)
NPY	CCGCCACGATGCTAGGTAACAA	GGTCTTCAAGCCTTGTTCTGG	288

Table 3: NPY-specific primers used for amplification of NPY cDNA during PCR.

Next, the target sequence was amplified using polymerase chain reaction (PCR). Therefore, RNA was extracted from mouse brain tissue using the RNeasy Lipid Tissue Mini kit (Qiagen, Vienna, Austria) and 1.13 µg RNA was reverse transcribed using the High capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, USA). Then NPY forward and reverse primers (Table 3) were used at a concentration of 0.4 µM each and mixed with 100 ng mouse brain cDNA, double distilled water and the peqLab PCR-Master-Mix S (peqLab, Erlangen, Germany). The PCR run consisted of an initial 3-minute denaturation step at 95 °C followed by 40 cycles of 1) 30 seconds at 95 °C 2) 1 minute at 60 °C and 3) 1 minute at 72 °C. After a final 10-minute elongation step at 72 °C, the PCR amplicon was visualized using a 1.5 % agarose gel and ethidium bromide staining (Figure 4A). The band corresponding to the predicted amplicon size of NPY was excised from the agarose gel with a scalpel. The DNA within the excised gel piece was extracted and purified using the MinElute Gel Extraction Kit (Qiagen). DNA yield was 75.21 ng/µl measured with a Nanodrop ND 1000 spectrophotometer (peqLab).

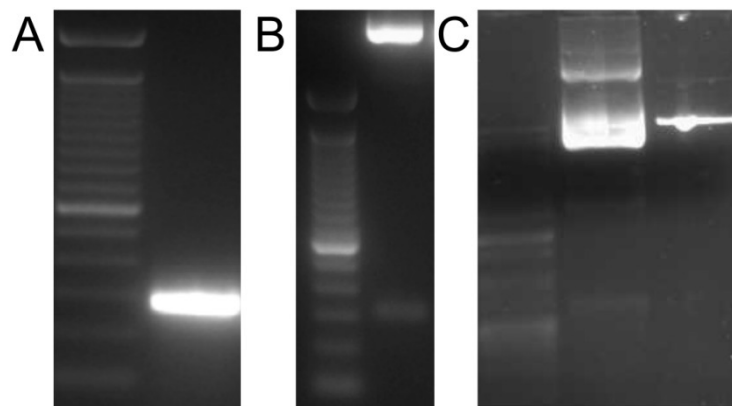


Figure 4. Quality controls during molecular cloning and after NPY template preparation for *in vitro* transcription. Panels A-C show images of 1.5 % agarose gels containing DNA stained with ethidium bromide. On the left lane of each gel image a DNA ladder is shown. Panel A illustrates the NPY amplicon after PCR. In the right lane of the

panel a clear band was visualized corresponding to the predicted NPY amplicon size of 288 bps. Panel B shows the presence of NPY within the vector used for molecular cloning. After restriction digest the vector DNA appears on the upper end of the right lane and the NPY insert appears at the expected position (288 bps) in the lower part of the right lane. Panel C demonstrates plasmid DNA containing the NPY insert used as template for NPY riboprobe production. The middle lane shows the uncut plasmid and the right lane shows the linearized plasmid used for in vitro transcription.

The purified NPY DNA was then ligated into the multiple cloning site of ampicillin resistance gene carrying pDrive Cloning Vectors (Qiagen) using the PCR cloning kit (Qiagen). According to the manufacturer's instructions and the equation

$$\text{ng PCR product required} = \frac{50\text{ng} \times \text{PCR product size (bp)} \times \text{molar ratio}}{3851 \text{ bp}}$$

37.39 ng NPY DNA was used in the ligation reaction together with 100 ng pDrive Cloning Vector DNA representing a 5 times molar excess of PCR product. After ligation, vectors were transformed into Subcloning Efficiency DH5 α Competent Cells (Invitrogen) expressing the marker lacZ Δ M15, which allows blue/white screening of colonies. In this process 50 μ l bacterial cells were gently mixed with 1 μ l of the NPY ligation reaction and incubated on ice for 30 minutes. Afterwards cells were heat-shocked in a 42 $^{\circ}$ C water bath for 45 seconds and again put on ice for another 2 minutes. Then 950 μ l of pre-warmed SOC (super optimal broth with catabolite repression) medium was added to the cells and they were incubated on a shaker at 37 $^{\circ}$ C for 1 hour. After this incubation, the transformation product (100 μ l) was added onto a pre-warmed agar plate containing 100 μ g/ml ampicillin, 100 μ l IPTG (Isopropyl β -D-1-thiogalactopyranoside; 100 mM stock solution) and 50 μ l X-gal (20 mg/ml stock solution) and incubated at 37 $^{\circ}$ C overnight. The next day 4 white clones (= clones containing the vector) were selected, each put into a reaction tube containing 4 ml Lysogeny broth medium and 4 μ l ampicillin (50 mg/ml) and incubated again on a shaker at 37 $^{\circ}$ C overnight. The next day bacterial cells were lysed and plasmid DNA was purified using the QIAprep Spin Miniprep kit (Qiagen). The presence of the NPY insert within the plasmid DNA was checked by restriction digest (Figure 4B) using the restriction enzyme EcoRI (Promega, Madison, USA). Clones actually containing an insert were selected and the correct amino acid sequence of the

insert was confirmed by DNA sequencing (CEQ 8000; Beckman Coulter, Brea, USA) and BLAST software.

2.14 DNA template and riboprobe production

To produce riboprobes for ISH, DNA templates from plasmid DNA containing the desired NPY insert were generated. An appropriate RNA polymerase and restriction enzyme to produce DNA templates for antisense riboprobes were identified by sequencing of the NPY insert and BLAST search. The templates for antisense riboprobes were produced by linearizing 20 µg plasmid DNA with the restriction enzyme XhoI (Promega). Linearized plasmid DNA was purified using the MinElute Reaction Cleanup Kit (Qiagen). Complete digestion of the template was confirmed by gel electrophoresis (Figure 4C).

³⁵S NPY riboprobes from DNA templates were synthesized by in vitro transcription. Therefore antisense riboprobes were produced using 1µg linearized plasmid DNA, ³⁵S-labelled UTP (Uridine triphosphate; Perkin Elmer, Waltham, USA), dithiothreitol (DTT), RNasin, transcription buffer, unlabelled deoxynucleotide triphosphates (ATP+GTP+CTP), sterile distilled water and T7 RNA polymerase (Riboprobe Systems, Promega) according to the manufacturer's instructions. After running the reaction at 37 °C for 60 minutes, the reaction was stopped by adding 1 µl RQ1 DNase, which was followed by another incubation at 37 °C for 15 minutes. Afterwards RNA was extracted using the phenol-chloroform method. RNA was then precipitated using sodium acetate and 100 % EtOH. Precipitated RNA was resuspended in TE-buffer (10 mM Tris, 1 mM EDTA; pH 8.0). Specific activity of the riboprobes was measured with a liquid scintillation counter and riboprobe quality was checked by running a urea polyacrylamide gel.

2.15 Radioactive neuropeptide Y in situ hybridization

For ISH fresh frozen, 20 µm thick brain sections were brought to room temperature, fixed in 4 % PFA, washed in 0.5X saline-sodium citrate buffer (SSC) and treated with proteinase K. After another washing step in 0.5X SSC, the sections were postfixated in 4 % PFA, dehydrated in an ascending EtOH row, delipidated in chloroform, again rinsed in EtOH and put into 1X PBS. Sections were then covered with hybridization solution (50 % deionized formamide, 0.3 M

sodium chloride, 20 mM tris-hydroxymethyl-aminomethane buffer (Tris; pH 8.0), 5.0 mM EDTA, 1X Denhardt's solution, 10 % dextran sulfate, and 10 mM DTT) and prehybridized for 2 hours at 55 °C. ³⁵S-labelled antisense RNA probes (3 x 10⁵ cpm/slides) plus tRNA (10 mg/ml; 5 µl/slide) were added to the hybridization solution, and the incubation continued for 12-18 h at 57 °C. After hybridization, the sections were washed for 20 min in 2X SSC containing 10 mM β-mercaptoethanol and 1 mM EDTA, treated with RNase A (10 µg/ml) for 30 minutes at room temperature, and washed at high stringency (0.1X SSC, 10 mM β-mercaptoethanol, 1 mM EDTA) for 2 h at 60 °C and 20 minutes in 0.5X SSC at room temperature. Tissue sections were then dehydrated in graded EtOH containing 0.3 M ammonium acetate, air dried and dipped in photographic emulsion NTB-2 (Kodak, Rochester, USA). Slides were stored at 4 °C in light tight boxes. After 6 weeks of exposure, sections were developed and counterstained with thionine.

2.16 Grain and cell counting of the neuropeptide Y in situ hybridization

Brain sections used for ISH were examined with a light microscope (Axiophot) coupled to a computerized image analysis system (MCID Basic). As in the immunohistochemical analysis of c-Fos expression, the slides were coded such that the investigator was blind to the treatment group under investigation. Brain ROIs were identified with the mouse brain stereotaxic atlas of Paxinos and Franklin (188) and thionine counterstaining of the slides. To quantify NPY expression within the polymorph layer of the dentate gyrus (DGpl) and the BLA, the number of NPY-positive neurons and the number of grains/neuron within these ROIs were measured. NPY-positive neurons were defined as cells containing three times the amount of grains compared to adjacent background areas. Background grain density was calculated as the mean of three measurements in cell-free areas within each ROI. Measurements of grain density/neuron were performed with a circle like tool (20 µm diameter) placed over each NPY-positive cell. The intensity threshold for counting was set in such a way that only grains and no background were counted by the image analysis program. In cells containing clumps of grains, the number of grains/grain clump was estimated by

dividing the grain clump area by the average grain size, which was determined to be 0.3 μm . To quantify NPY-positive neurons/brain region the mean number of NPY-positive neurons/section in the dentate gyrus and the mean number of NPY-positive neurons/ mm^2 in the BLA were determined. Per animal, three sections of the DGpl (Bregma: -1.34 to -1.58) and two sections of the BLA (Bregma: -0.82 to 0.94) were counted bilaterally. For statistical analysis the average number of neurons/ROI and the average number of grains/neuron were used.

2.17 Mouse forebrain microdissection

Mouse brains used for microdissection were frozen in a petridish containing 2-methyl butan at $-70\text{ }^{\circ}\text{C}$ for 7 seconds. The petridish was kept on dry ice to maintain the liquid at $-70\text{ }^{\circ}\text{C}$. After freezing, brains were wrapped with aluminum foil and stored at $-70\text{ }^{\circ}\text{C}$ till further processing. For the microdissection, brains were put into a cryostat at $-20\text{ }^{\circ}\text{C}$ for 30 minutes. Afterwards the forebrain was cut into approximately seven slices from which ROIs were isolated. For this purpose the sliced brain was placed on a cold plate (Weinkauf Medizintechnik, Forchheim, Germany) set at $-20\text{ }^{\circ}\text{C}$, on which amygdala, hippocampus and hypothalamus were microdissected under a stereomicroscope. The amygdalar tissue taken ranged from the anterior edge of the optical tract (Bregma: -0.58) to the posterior part of the basolateral and the basomedial amygdala (Bregma: -1.26), the hippocampal tissue taken extended from the limit of the hippocampal formation (Bregma: -0.94) to the caudal end of the dentate gyrus (Bregma: -4.04) and the hypothalamic tissue taken stretched from the preoptical area (Bregma: +0.26) to the end of the mammillary bodies (Bregma: -2.92). Isolated brain areas were collected in tubes kept on dry ice and stored at $-70\text{ }^{\circ}\text{C}$ till further processing.

2.18 Real-time PCR

Brain tissues used for real-time PCR were homogenized with a Precellys 24 homogenizer (PeqLab Biotechnologie, Polling, Austria) and RNA was extracted using the RNeasy Lipid Tissue Mini kit (Qiagen). After extraction, the RNA concentration was measured and 1 μg RNA/sample was inserted in a reverse transcription reaction using the high capacity cDNA reverse transcription kit (Applied Biosystems). RNA integrity was confirmed using the 2100 Bioanalyzer

(Agilent Technologies, Santa Clara, USA). For PCR measurement of NPY mRNA levels within a given brain area, the cDNA was diluted 1:50, 3 ng cDNA being subjected to the PCR reaction, together with NPY forward and reverse primers at a concentration of 1 μ M each (Table 4) and the Fast Sybr Green Mastermix (Applied Biosystems). The PCR was run on a Bio-Rad CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, USA) and consisted of the following protocol:

- 95 °C for 20 seconds
- 40 cycles of 95 °C for 3 seconds and 60 °C for 30 seconds
- 95 °C for 15 seconds
- 65 °C for 15 seconds
- Melting curve from 65 °C to 95 °C with a 0.5 °C increment every 5 seconds

Gene	Sequence forward primer (5'→3')	Sequence reverse primer (5'→3')	Product size (bp)
NPY	CAGATACTACTCCGCTCTGCG ACACTACA	TTCCTTCATTAAGAGGTCT GAAATCAGTGTC	106

Table 4: NPY-specific primers used for amplification of NPY cDNA during real-time PCR.

DNA sequencing and BLAST search confirmed the amplification of the correct bp sequence. Group differences were expressed as fold changes according to the $2^{-\Delta\Delta Ct}$ method using the mean value of the control group as the calibrator (189).

2.19 Electron microscopy

To prepare tissues for electron microscopy, brains were fixed for two days in 2 % PFA / 2.5 % glutardialdehyde in 0.1 M cacodylate buffer (CB) pH 7.4 at 4 °C. Afterwards brains were stored in CB for 1 day at 4 °C and the CA1 region of the hippocampus (9-week EE protocol) or the dentate gyrus of the hippocampal formation (5-week EE protocol) was microdissected. The brain regions were then postfixed in 2 % osmium tetroxide in CB for 2 hours and washed in CB at 4 °C overnight. The next day the specimens were dehydrated in an ascending EtOH

series and incubated in propylenoxide at room temperature for 1 hour. Afterwards sections were incubated for 3 hours in a 1:2 dilution of propylenoxide in TAAB embedding resin (TAAB, Berkshire, UK) at room temperature followed by a 1:3 dilution of propylenoxide in resin at 4 °C overnight. The next day sections were incubated 2 times in fresh resin at 45 °C for 60 minutes. Finally sections were stored in embedding forms filled with fresh resin and the resin was cured at 60 °C for 3 days.

For the analysis of synapses and synaptic parameters 55 nm thick sections were cut with a Leica Ultracut UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany) and contrasted with 2 % uranyl acetate and lead citrate. To determine the number of synapses and the synaptic morphology within the CA1 and the DGpl region a stereological counting method was applied (190-192)(190-192). For this, 20 image pairs were taken from two serial sections per animal with a Tecnai G2 20 electron microscope (FEI, Hillsboro, USA) equipped with a Gatan US 1000 digital camera (Gatan, Pleasanton, USA). ROIs were identified by comparing low magnification transmission electron micrographs with toluidine blue stained semi-thin parallel sections. The ROIs were manually outlined and 20 positions within the boundaries of the outlined area were randomly calculated by custom-made software. At each calculated position an image was taken. Serial section software permitted to go from the position on the first section, the reference section, to the same position on the second section, the lookup section. For quantifying synapses only those were counted which were present within a 5.5 x 5.5 μm^2 counting frame on the reference section image, but not on the lookup section image. This allowed unbiased assessing of the number of synapses/ μm^2 and avoids overestimation of the number of synapses. Furthermore, the average diameter of the presynaptic membrane and of the postsynaptic density, the average width of the synaptic cleft as well as the average number of docked vesicles (vesicles with a distance from the presynaptic membrane of less than two times the average vesicle diameter) and undocked vesicles were measured. Synapse counts were derived from all 20 image pairs and the mean synapse count/counting frame of each animal was used for statistical analysis. For the other synaptic parameters seven synapses/animal were randomly selected and the mean of the measurements from these synapses was used for statistics.

2.20 Light/dark box test

The L/D test assesses anxiety-like behaviour of mice (193). For this test each mouse was placed in an illuminated (300 lux) box at the entrance of a small passage (4.5 x 6 cm; width x height) connecting this box (18.5 x 21 x 20.5 cm; length x width x height) to a dark box of equal size. Over a period of 10 minutes the mouse behaviour was recorded with the LabMaster system (TSE systems, Bad Homburg, Germany). As previously described (185) this system consists of six recording units, each unit comprising a metal frame containing several infrared beams and their respective detectors. This setting allows recording of the exact movement and position of the mouse during the 10-min test period. The recording units are connected to a personal computer that was used to collect and analyse the data with the LabMaster software. For the analysis of anxiety and locomotor behaviour during the test, the time spent in the light box, latency to re-enter the light box (first reappearance in the light after disappearing in the dark compartment), transitions between the compartments and total distance travelled were assessed by a blinded observer.

2.21 Sucrose preference test and circadian activity

The SP test assesses anhedonia, one aspect of depression-like behaviour, of mice (194). For this test, the mice were placed in homecages (42.5 x 26.5 x 15 cm; length x width x height), which fit into the metal frames of the LabMaster system. Mice were offered the choice to drink from two bottles, one filled with tap water and the other filled with 1 % sucrose in tap water. The circadian pattern of locomotion, exploration and liquid consumption was assessed by the system for 48 hours. SP was expressed as percentage and calculated as consumption of sucrose-containing water/g bodyweight of each mouse divided by the total liquid consumption/g bodyweight of each mouse.

2.22 Tail suspension test

The tail suspension test assesses depression-like and stress coping behaviour of mice (173). The test was performed according to the analysis method of Berrocoso et al. (195). Mice were fixed by their tail with an adhesive tape to the

edge of a custom-made box for 6 minutes at a light intensity of 20 lux. Each session was video recorded and manually scored with a tracking software (VideoMot 2, TSE Systems) by a blinded investigator. The total duration of immobility, curling and swinging behaviour was scored during the 6-minute testing period.

2.23 Statistics

Statistical data analysis was performed on SPSS 20.0 (SPSS Inc., Chicago, USA). For all analyses the normal distribution of the data was assessed with the Shapiro-Wilk test. Data not normally distributed were log-transformed or ranked to achieve normal distribution. If log or rank transformation did not result in normally distributed data, a non-parametric test was used. Student's t-test was used to analyse 2 normally distributed groups and Chi-square test to analyse 2 not normally distributed groups. Data consisting of one variable and 2 factors (e.g. stress and inflammation) were analysed by two-way analysis of variance (ANOVA). In case of a significant interaction of the 2 factors or a significant main effect of a factor with more than 2 groups without a significant interaction term, post-hoc tests were performed (t-test for 2 groups and one-way ANOVA for 3 groups). Post-ANOVA analysis of group differences was performed with the Tukey HSD (honestly significant difference) test, when the variances were equal, and with the Games-Howell test, when the variances were unequal. Consumed food and drink amounts over 7 days (factor 1 treatment day, factor 2 treatment) and data generated by the Labmaster system were analysed using repeated-measures ANOVA. If the interaction was significant, t-tests or one-way ANOVA followed by Tukey HSD or Games-Howell test for each time-point was used for post-hoc testing, respectively. Bonferroni correction was used to adjust for multiple testing. Probability values of $p < 0.05$ were regarded as statistically significant and p -values < 0.1 were regarded as trends. All data are presented as means \pm SEM or + SEM, n referring to the number of mice in each group.

3. Results

3.1 Effects of water avoidance stress on brain c-Fos expression

Various stressors are known to alter the number of c-Fos expressing cells within the limbic system (83,196-198). In contrast, the effect of WAS on the number of c-Fos expressing cells within this system has not been described. Thus, this experiment was designed to evaluate whether a 1-hour exposure to WAS modifies the level of c-Fos in the brain and which nuclei express this marker of neuronal activation. The data showed that basal c-Fos expression is low throughout the brain and that WAS dramatically increases c-Fos expression in almost all brain regions investigated (Table 5). As described in Table 5, the largest WAS-induced increases in c-Fos expression were observed in the AIC, a part of the insular cortex (15 fold, $t_{(6.808)} = -7.893$, $P < 0.001$) and the PVH, recognised as the key nucleus initiating a HPA-axis response to stress (14.6 fold, $t_{(6.131)} = -7.248$, $P < 0.001$). However, large alterations in the number of c-Fos expressing cells were also observed in the CA1 (10 fold, $t_{(6.689)} = -7.211$, $P < 0.001$) and CA3 (8 fold, $t_{(7.483)} = -8.364$, $P < 0.001$), parts of the hippocampus, an important component of the limbic system. The LSV (9.3 fold, $t_{(6.813)} = -13.088$, $P < 0.001$), a part of the septum and the LC (9.3 fold, $t_{(12)} = -5.872$, $P < 0.001$), responsible for regulating arousal levels during stress exposure, showed similar stress-induced c-Fos changes. More than fivefold but less than eightfold stress-induced c-Fos elevations were seen in ARCr ($t_{(12)} = -5.111$, $P < 0.001$) and ARCc ($t_{(10)} = -5.192$, $P < 0.001$), two levels of the ARC, another hypothalamic nucleus, which has also close connections to the PVH. Stress-induced alterations of a similar magnitude were found in the BSTMA ($t_{(6.704)} = -8.648$, $P < 0.001$), Pir ($t_{(6.880)} = -5.526$, $P < 0.001$) and PC ($t_{(7.433)} = -8.481$, $P < 0.001$). Stress-induced increases of c-Fos expressing cells in the range of 2.2 to 4.7 fold were present in BLA ($t_{(12)} = -5.435$, $P < 0.001$), CeA ($t_{(12)} = -2.457$, $P < 0.05$) and MeA ($t_{(7.755)} = -6.488$, $P < 0.001$), three subnuclei of the amygdala and essential components regulating the behavioural and neuroendocrine response to stress. The number of c-Fos expressing cells was also increased in the CC ($t_{(12)} = -5.753$, $P < 0.001$), a part of the medial prefrontal cortex. Furthermore, subregions of the thalamus namely the

PVT ($t_{(7.984)} = -8.619$, $P < 0.001$), CM ($t_{(7.303)} = -4.978$, $P < 0.01$), CL ($t_{(12)} = -6.678$, $P < 0.001$) and LHb ($t_{(7.460)} = -3.374$, $P < 0.05$) showed increased c-Fos levels after stress exposure, which indicates a large somatosensory input to the thalamus in the setting of WAS. Finally, the brainstem regions PN ($t_{(8.007)} = -6.289$, $P < 0.001$), LPAG ($t_{(12)} = -5.172$, $P < 0.001$) and RLi ($t_{(12)} = -3.719$, $P < 0.01$), which take part in fine-tuning of the behavioural response to WAS, showed a strong stress-induced increase of c-Fos expressing cells. However, in the DGgl, another part of the hippocampus, which is closely connected to CA1 and CA3, WAS did not change c-Fos expression.

Brain region	Number of c-Fos positive cells		
	Control	WAS	P-value
<i>Telencephalon</i>			
<i>Cortex</i>			
Agranular insular cortex	1±0.5	15±1.8	P<0.001
Cingulate cortex	9±1.9	41±5.2	P<0.001
Piriform cortex	7±1.5	38±5.5	P<0.001
<i>Striatum/Pallidum</i>			
Bed nucleus of stria terminalis; medial division, anterior part	10±1.6	68±6.5	P<0.001
Lateral septal nucleus; ventral part	21±3.4	196±13.0	P<0.001
<i>Hippocampus</i>			
CA1 field of the hippocampus	3±0.9	30±3.6	P<0.001
CA3 field of the hippocampus	2±0.6	16±1.6	P<0.001
Granular cell layer of the dentate gyrus	12±3.0	13±1.2	P=0.848
<i>Amygdala</i>			
Basolateral amygdala	9±1.8	34±4.4	P<0.001
Central amygdala	9±1.9	20±4.0	P<0.05
Medial amygdala	13±2.2	53±5.8	P<0.001
<i>Diencephalon</i>			
<i>Thalamus</i>			
Paraventricular nucleus of the thalamus	168±11.9	437±28.8	P<0.001
Centromedial thalamic nucleus	19±3.6	76±10.8	P<0.01
Paracentral thalamic nucleus	10±1.6	50±4.5	P<0.001
Centrolateral thalamic nucleus	10±1.3	36±3.7	P<0.001

Lateral habenula	6±1.4	20±3.9	P<0.05
<i>Hypothalamus</i>			
Paraventricular nucleus of the hypothalamus	13±2.5	190±24.1	P<0.001
Arcuate hypothalamic nucleus, rostral	15±1.2	81±12.9	P<0.001
Arcuate hypothalamic nucleus, caudal	28±6.0	144±21.7	P<0.001
<i>Mesencephalon/Rhombencephalon</i>			
Rostral linear of the raphe nucleus	3±1.2	14±2.8	P<0.01
Lateral periaqueductal grey	16±3.9	45±3.9	P<0.001
Locus coeruleus	3±0.7	28±4.0	P<0.001
Pontine nuclei	72±11.2	255±26.9	P<0.001

Table 5: WAS increases c-Fos expression in the mouse brain. The table shows the average number of c-Fos positive cells in different brain areas of control and WAS-treated animals. Data are presented as means ± SEM, n = 6-7.

3.2 Effects of environmental enrichment and gastrointestinal inflammation on stress-induced neuronal activation

3.2.1 Environmental enrichment enhanced the stress-induced c-Fos expression in the dentate gyrus and unmasked an inhibitory effect of gastritis and colitis

Visceral inflammation can be exacerbated by stress (54,55,68). The contribution of specific brain areas to this effect as well as the benefits or disadvantages of environmental stimulation in this setting is largely unknown. Given that WAS massively altered the number of c-Fos expressing cells, this experiment was designed to investigate whether EE and visceral inflammation can modify the stress-induced c-Fos response. The experiment showed that the number of c-Fos expressing cells in the DGgl (Figures 5A,B and 5A) and in the CA1 region (Figures 5C,D and 6B) of the hippocampus following exposure to WAS was markedly changed by housing and treatment, whereas the stress-induced expression of c-Fos in the CA3 region (Figures 5C,D and 6C) remained unaltered by housing and treatment. These findings have been published in an original article (172).

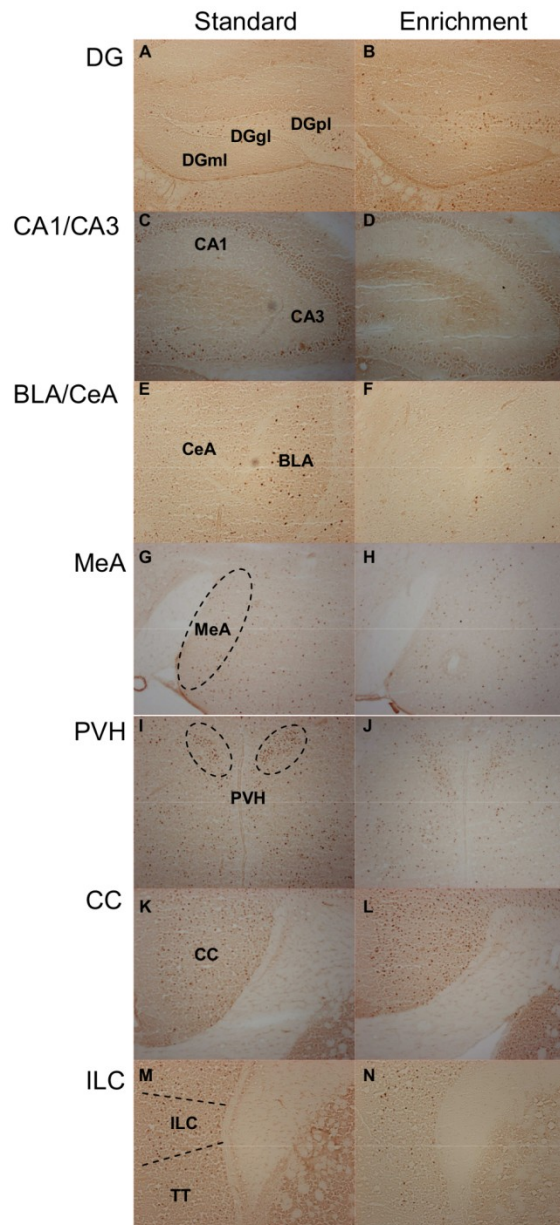
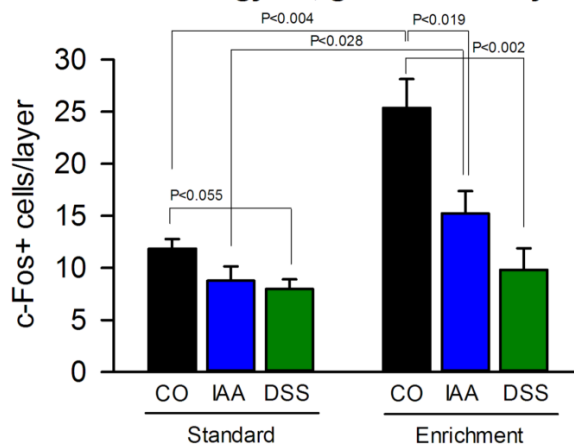


Figure 5. Environmental enrichment alters stress-induced c-Fos expression in the forebrain. The effect of water avoidance stress (30 min) on the cerebral c-Fos expression was examined in mice kept for 9 weeks under standard (SE) or enriched environment (EE). The left column panels (A,C,E,G,I,K,M) show representative micrographs of 9 forebrain regions taken from SE mice while the right column panels (B,D,F,H,J,L,N) depict micrographs of the same brain regions taken from EE mice. Relative to SE animals, EE increased stress-induced c-Fos expression in the granular cell layer of the dentate gyrus (A,B), but decreased it in the CA1 region of the hippocampus (C,D), the central amygdala (E,F) and the infralimbic cortex (M,N). In contrast, EE failed to modify stress-induced c-Fos expression in the CA3 region of the hippocampus (C,D), the basolateral and medial amygdala (E,F,G,H), the paraventricular nucleus of the hypothalamus (I,J) and the cingulate cortex (K,L). Abbreviations: BLA = Basolateral amygdala; CA1 = CA1 field of the hippocampus; CA3 = CA3 field of the hippocampus; CC = Cingulate cortex; CeA = Central amygdala; DGgl = Dentate gyrus, granular cell layer; DGml = Dentate gyrus, molecular cell layer; DGpl = Dentate gyrus, polymorph cell layer; ILC = Infralimbic cortex; MeA = Medial Amygdala; PVH = Paraventricular nucleus of the hypothalamus; TT = Tenia tecta. These findings have been published in an original article (172).

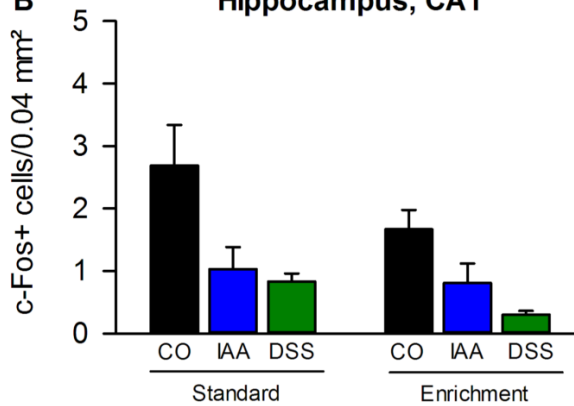
The most remarkable effects of the experimental interventions under study were seen in the DGgl, given that housing ($F_{(1,35)} = 25.198$, $P < 0.001$) and treatment ($F_{(2,35)} = 15.324$, $P < 0.001$) interacted ($F_{(2,35)} = 5.408$, $P = 0.009$) with each other (172). On the one hand, enriched housing increased the stress-induced expression of c-Fos in control animals, an effect that was also seen in IAA-treated mice (Figure 6A). On the other hand, enriched housing unmasked a strong inhibitory effect of IAA and DSS treatment on the stress-evoked activation of the DGgl (Figure 6A). In contrast, IAA and DSS treatment under standard housing attenuated the c-Fos expression in the DGgl due to WAS only by a small margin (Figure 6A).

The stress-induced c-Fos expression in the CA1 region of the hippocampus (Figure 6B) also varied with housing ($F_{(1,35)} = 4.055$, $P = 0.052$) and treatment ($F_{(2,35)} = 10.812$, $P < 0.001$), without a significant interaction between these factors. Main effect analysis revealed that the WAS-evoked expression of c-Fos in the CA1 region was depressed by enriched housing independently of treatment. Treatment with IAA (Games-Howell test: $P = 0.026$) and DSS (Games-Howell test: $P = 0.003$) blunted the stress-evoked activation of the CA1 region both under standard and enriched housing (Figure 6B). These findings have been published in an original article (172).

A Dentate gyrus, granular cell layer



B Hippocampus, CA1



C Hippocampus, CA3

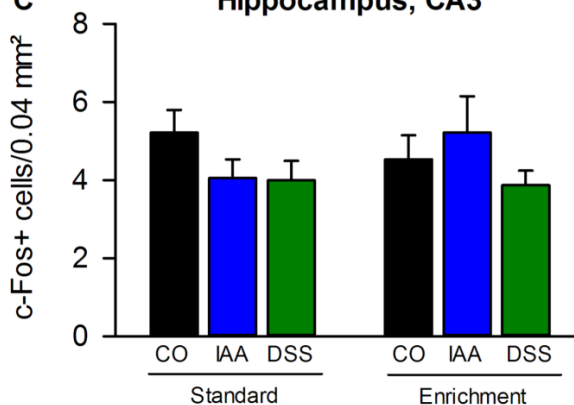


Figure 6. Environmental enrichment increases stress-induced expression of c-Fos in (A) the granular cell layer of the dentate gyrus, decreases c-Fos expression in (B) the hippocampal CA1 region, but does not alter c-Fos levels in (C) the hippocampal CA3 region. Gastritis evoked by iodoacetamide (IAA) and colitis evoked by dextran sulfate sodium (DSS) dampened stress-induced c-Fos expression in the dentate gyrus and CA1 region of mice kept both under standard (SE) or enriched environment (EE). Mice were maintained for 9 weeks under SE or EE and then treated for 7 days with IAA (0.1 % added to the drinking water) or DSS (2 % added to the drinking water), while control (CO) mice drank plain water. Expression of c-Fos was visualized 2 h after the beginning of a 30-min exposure to water avoidance stress at the end of the treatment period. The values represent means + SEM, $n = 6 - 7$. These findings have been published in an original article (172).

3.2.2 Environmental enrichment inhibited the stress-induced c-Fos expression in the central amygdala and prevented the inhibitory effect of gastritis and colitis

The number of cells expressing c-Fos in the BLA, CeA and MeA in response to WAS differed with treatment and/or housing conditions (Figures 5E,F,G,H and 7A,B,C). In the BLA, the stress-induced expression of c-Fos was unrelated to housing but differed with treatment ($F_{(2,37)} = 4.753$, $P = 0.015$), without an interaction between these factors (Figure 7A). Post-ANOVA analysis of the treatment effect showed that treatment with DSS (Tukey HSD: $P = 0.01$), but not IAA decreased c-Fos expression independently of housing condition. These findings have been published in an original article (172).

The stress-induced expression of c-Fos in the CeA (Figures 5E,F and 7B) varied with housing ($F_{(1,37)} = 3.178$, $P = 0.083$) and treatment ($F_{(2,37)} = 13.080$, $P < 0.001$), with a significant interaction between these factors ($F_{(2,37)} = 8.676$, $P = 0.001$) (172). Under standard housing, the stress-evoked activation of the CeA was markedly depressed by IAA and DSS treatment. This treatment effect was absent under EE which in control animals itself depressed stress-induced c-Fos expression by about 50 % (Figure 7B).

The number of cells expressing c-Fos in the MeA in response to WAS did not differ when the animals were housed under standard or enriched conditions, while treatment ($F_{(2,37)} = 8.357$, $P = 0.001$) reduced the stress-induced expression of c-Fos independently of housing condition (Figure 5G,H and 7C). Main effect analysis revealed that both DSS (Tukey HSD: $P < 0.001$) and IAA (Tukey HSD: $P = 0.033$) reduced c-Fos levels. These findings have been published in an original article (172).

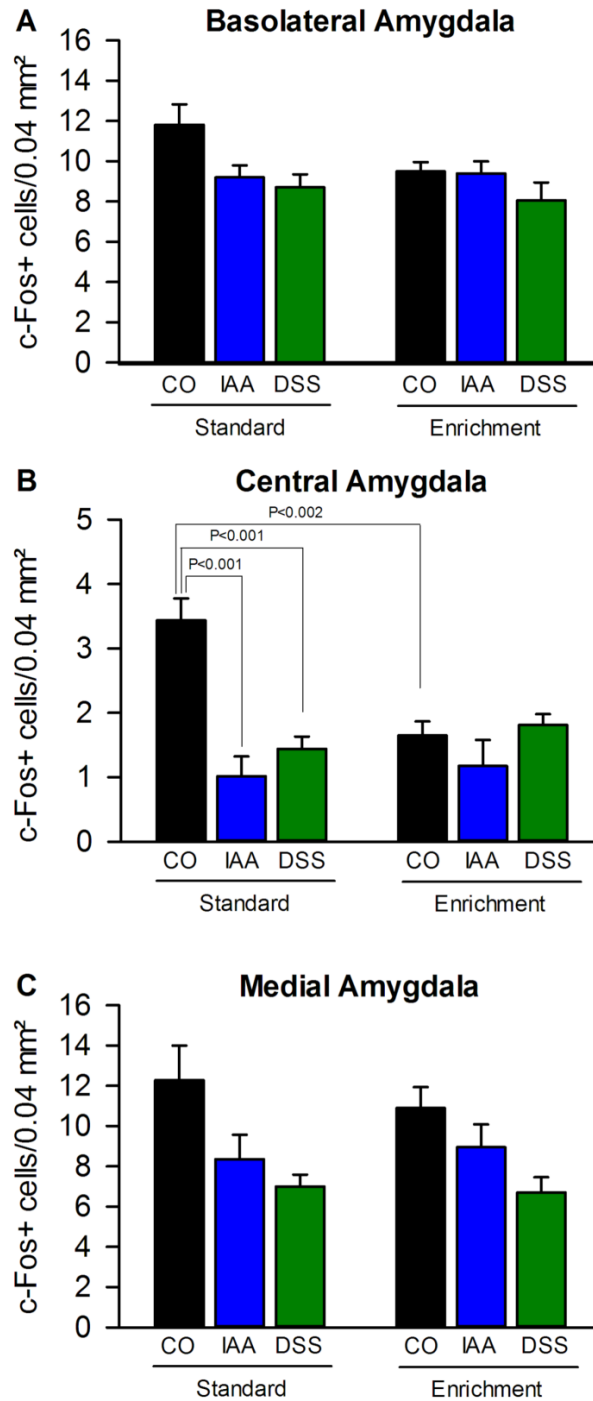


Figure 7. Environmental enrichment decreases stress-induced c-Fos expression in (B) the central amygdala but does not alter stress-induced c-Fos levels in (A) the basolateral and (C) medial amygdala. Gastritis evoked by iodoacetamide (IAA) and colitis evoked by dextran sulfate sodium (DSS) dampened stress-induced c-Fos expression in all 3 amygdalar nuclei of mice kept under standard environment (SE), an effect that is largely absent in mice kept under enriched environment (EE). Mice were maintained for 9 weeks under SE or EE and then treated for 7 days with IAA (0.1 % added to the drinking water) or DSS (2 % added to the drinking water), while control (CO) mice drank plain water. Expression of c-Fos was visualized 2 h after the beginning of a 30-min exposure to water avoidance stress at the end of the treatment period. The values represent means + SEM, n = 7 – 8. These findings have been published in an original article (172).

3.2.3 Environmental enrichment inhibited the stress-induced c-Fos expression in the infralimbic cortex and attenuated the inhibitory effect of gastritis and colitis but had little effect in the cingulate cortex and hypothalamus

The stress-induced c-Fos expression in the PVH did not significantly depend on housing conditions but was attenuated by IAA and DSS treatment ($F_{(2,36)} = 4.742$; $P = 0.015$) without a significant interaction between the factors (Figures 5I,J and 8A). Main effect analysis revealed that both DSS (Tukey HSD test: $P = 0.016$) and IAA (Tukey HSD test: $P = 0.027$) reduced c-Fos independently of housing condition. Likewise, the WAS-evoked activation of the CC (Figures 5K,L and 8B) was unaffected by housing conditions, but altered by treatment ($F_{(2,37)} = 4.278$, $P = 0.021$). Specifically, DSS treatment (Tukey-HSD: $P = 0.017$) but not IAA treatment decreased stress-induced c-Fos expression independently of housing condition. There was no significant interaction between these factors. These findings have been published in an original article (172).

As also described in that article (172), appreciable treatment effects ($F_{(2,37)} = 19.324$, $P < 0.001$) but no housing effects, with a significant interaction between both factors ($F_{(2,37)} = 5.454$, $P = 0.008$), were observed in the ILC. Thus, the number of cells expressing c-Fos in response to WAS in control mice was reduced by enriched housing (Figures 5M,N and 8C). Moreover, treatment with IAA and DSS reduced the stress-evoked activation of the ILC under standard housing, whereas under enriched housing only treatment with DSS blunted the stress-induced c-Fos response (Figure 8C).

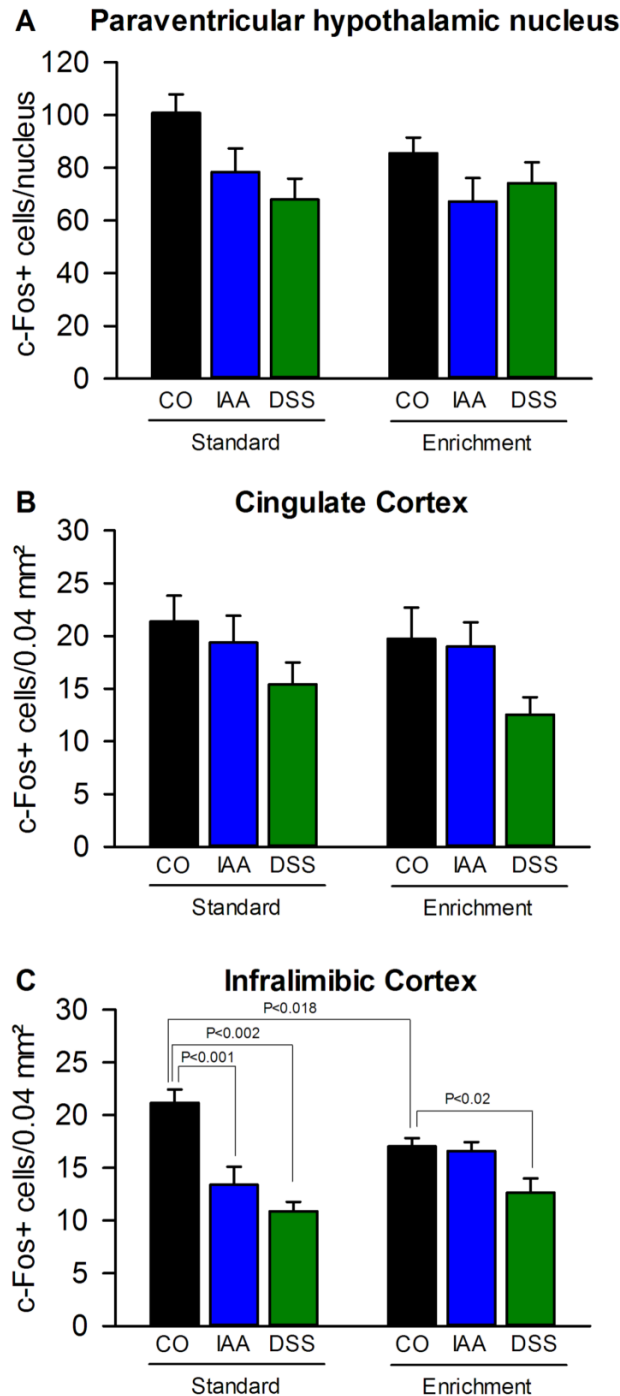


Figure 8. Environmental enrichment decreases stress-induced c-Fos expression in (C) the infralimbic cortex but does not alter stress-induced c-Fos levels in (B) the cingulate cortex and (A) paraventricular nucleus of the hypothalamus. Gastritis evoked by iodoacetamide (IAA) and colitis evoked by dextran sulfate sodium (DSS) dampened stress-induced c-Fos expression in mice kept under standard environment (SE) in a region-dependent manner, an effect that is largely absent in mice kept under enriched environment (EE). Mice were maintained for 9 weeks under SE or EE and then treated for 7 days with IAA (0.1 % added to the drinking water) or DSS (2 % added to the drinking water), while control (CO) mice drank plain water. Expression of c-Fos was visualized 2 h after the beginning of a 30-min exposure to water avoidance stress at the end of the treatment period. The values represent means + SEM, n = 7 – 8. These findings have been published in an original article (172).

3.3 Interactions between gastrointestinal inflammation and environmental enrichment

3.3.1 Environmental enrichment increased the susceptibility to dextran sulfate sodium-induced colitis, but not iodoacetamide-induced gastritis

Given that EE and inflammation altered the stress-evoked expression of c-Fos in distinct brain nuclei, the question arises as to whether EE affects the magnitude of gastrointestinal inflammation and in this way impacts on the cerebral expression of c-Fos. Therefore, at the end of the 7-day treatment with IAA or DSS, the health status of the animals was evaluated by measuring body weight and DAS, while gastrointestinal inflammation was assessed by the MPO content of the stomach and colon, respectively. The design of this experiment using both standard housing conditions and EE, allowed to assess additionally whether EE can modify the course of visceral inflammation (172). The parameters measured differed with housing and were influenced by treatment with DSS and IAA. Thus, the loss of body weight over the treatment period depended on treatment ($F_{(2,54)} = 33.215$, $P < 0.001$) but not housing, with a significant interaction between these factors ($F_{(2,54)} = 6.741$, $P = 0.002$). Specifically, animals treated with DSS lost significantly more weight than the respective control animals under both housing conditions. Furthermore, DSS-treated animals kept under EE lost significantly more weight than DSS-treated mice under standard housing ($t_{(11.912)} = -2.195$; $P < 0.05$), while IAA-treated animals kept under EE lost less weight ($t_{(18)} = 2.347$; $P < 0.05$) than IAA-treated mice under standard housing (Figure 9A). At the end of the treatment period the body weight varied with treatment ($F_{(2,54)} = 9.546$, $P < 0.001$) and housing ($F_{(1,54)} = 13.597$, $P < 0.001$) without a significant interaction between these factors (Table 6) These findings have also been published in an original article (172). Main effect analysis revealed that DSS (Games-Howell test: $P < 0.05$) but not IAA treatment reduced body weight at the end of the treatment period independently of housing condition and that animals housed under EE had less weight than mice under standard housing independently of treatment ($F_{(1,54)} = 13.597$, $P < 0.001$).

	Standard Environment	Enriched Environment
Control	28.2 ± 0.26	26.9 ± 0.44
Iodoacetamide	27.5 ± 0.35	26.9 ± 0.38
Dextran sulfate sodium	26.8 ± 0.71	24.0 ± 0.78

Table 6. Body weight (g) at the end of the treatment period. Means ± SEM, n=10 per group. These findings have been published in an original article (172).

Treatment with DSS, but not IAA, was associated with symptoms of colonic inflammation such as a bloody perianal region and soft to loose stool consistency. These symptoms were quantified by the DAS which varied with housing conditions ($F_{(1,54)} = 3.941$, $P = 0.052$) and treatment ($F_{(2,54)} = 193.086$, $P < 0.001$), with a significant interaction between these factors ($F_{(2,54)} = 3.941$, $P = 0.025$). Figure 9B shows that the DAS in DSS-treated mice tended to be larger under enriched housing than under standard housing. These findings have been published in an original article (172).

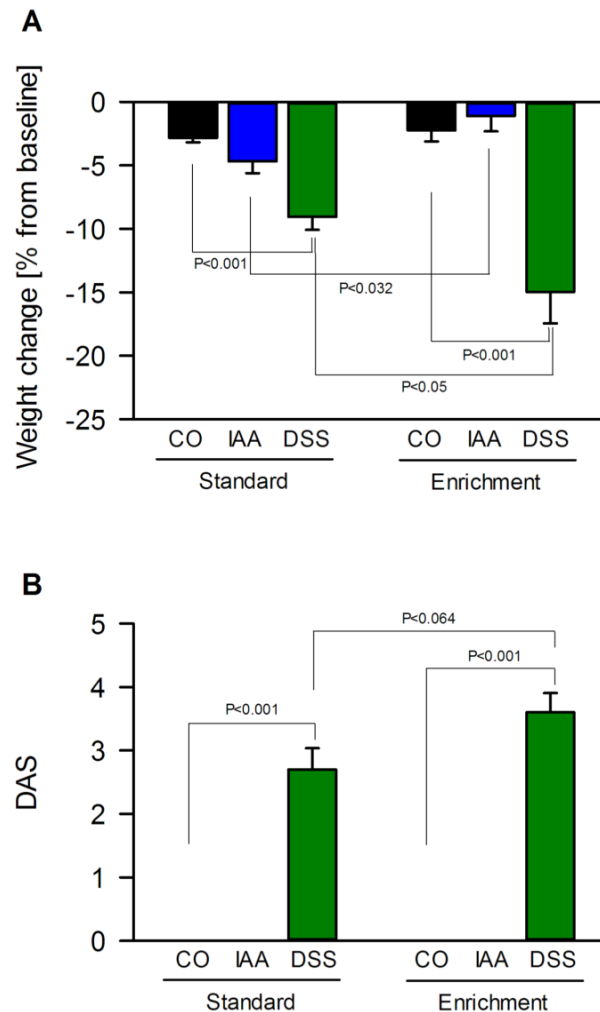


Figure 9. Environmental enrichment increases dextran sulfate sodium (DSS)-induced weight loss (A) and disease severity (B). Mice were treated for 7 days with iodoacetamide (IAA, 0.1 %, added to the drinking water) or DSS (2 %, added to the drinking water) or kept under control conditions (CO, plain drinking water) after standard or enriched housing for 9 weeks. Body weight was measured before and after the treatment period, and the weight loss (panel A) during the treatment period was expressed as a percentage of the body weight measured pre-treatment. The disease activity score (DAS, panel B), a measure of the animal's health status, was assessed at the end of the treatment period. The values represent means -SEM (A) and means +SEM (B), $n = 10$. These findings have been published in an original article (172).

The gastric and colonic MPO content was used as a measure of inflammation induced by IAA and DSS, respectively. IAA treatment increased the gastric MPO content under standard and enriched housing conditions to a similar extent (Figure 10A). As shown by two-way ANOVA, the gastric MPO content varied with treatment ($F_{(1,36)} = 14.411$, $P = 0.001$), but not with housing conditions, without a significant interaction between these factors (Figure 10A). In contrast, the DSS-evoked increase of the colonic MPO content tended to be larger under enriched ($t_{(10.799)} = 2.156$, $P=0.055$) than under standard housing (Figure 10B). Specifically, the colonic MPO content varied with housing ($F_{(1,35)} = 5.256$, $P = 0.028$) and treatment ($F_{(1,35)} = 80.107$, $P < 0.001$), with a significant interaction between these factors ($F_{(1,35)} = 5.319$, $P = 0.027$). These findings have been published in an original article (172).

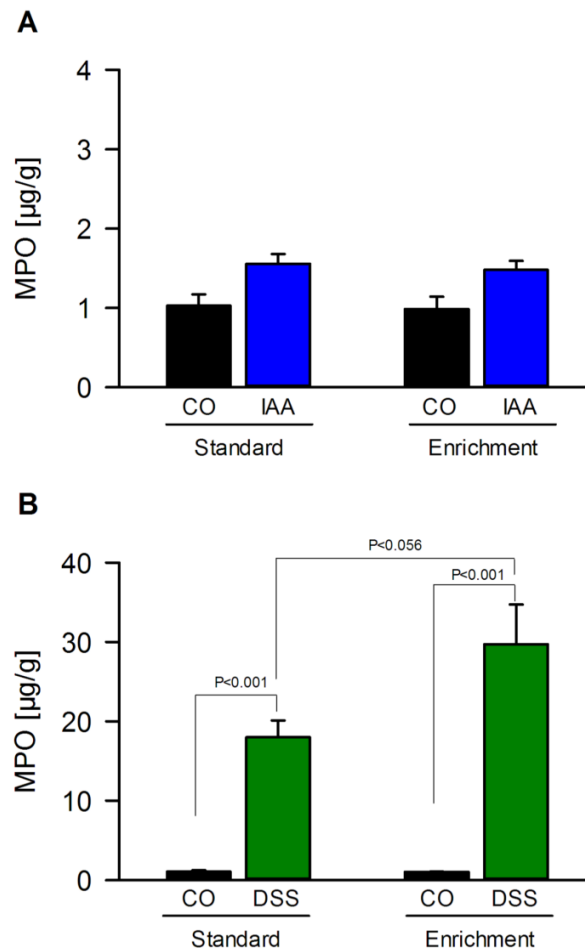


Figure 10. Environmental enrichment increases the dextran sulfate sodium (DSS)-induced rise of colonic myeloperoxidase (MPO) content (B), but does not influence the iodoacetamide (IAA)-induced rise of gastric MPO content (A). Mice were treated for 7 days with IAA (0.1 %, added to the drinking water) or DSS (2 %, added to the drinking water) or kept under control conditions (CO, plain drinking water) after standard or enriched housing for 9 weeks. The tissue MPO content was determined at the end of the treatment period. Note that the scale of the ordinate is different between panel A and B. The values represent means + SEM, n = 10. These findings have been published in an original article (172).

3.3.2 Colitis enhanced the stress-induced rise of plasma corticosterone under enriched, but not standard housing

Plasma corticosterone was determined 2 h after the beginning of a 30-min exposure to WAS, i.e., at the time when the brain was removed for visualization of c-Fos expression. Two-way ANOVA showed that post-stress corticosterone depended on treatment ($F_{(2,54)} = 9.955$, $P < 0.001$) but not housing, with a significant interaction ($F_{(2,54)} = 4.609$, $P = 0.014$) between both factors. Post-hoc analysis disclosed that, relative to the values measured in control animals, treatment with DSS caused a significant rise of the plasma corticosterone levels

under enriched, but not standard housing, whereas IAA had no effect. Furthermore DSS-treated enriched-housed animals tended to have higher corticosterone levels than DSS-treated standard-housed mice (Figure 11). These findings have been published in an original article (172).

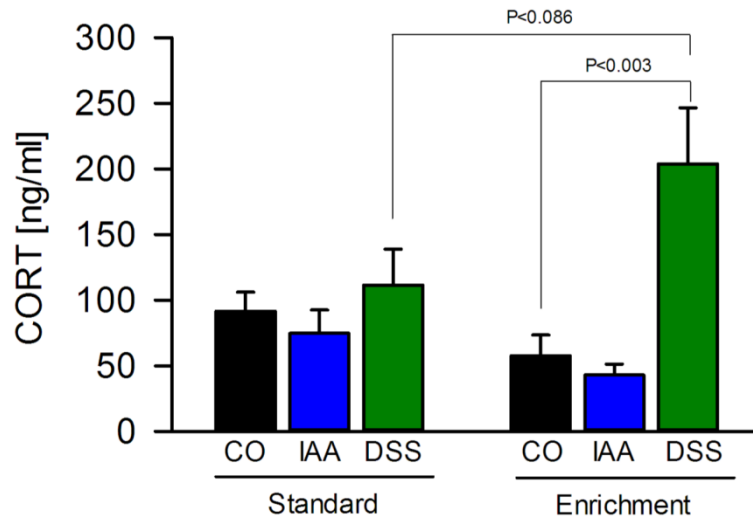


Figure 11. Environmental enrichment increases the dextran sulfate sodium (DSS)-induced rise of post-stress corticosterone (CORT) levels. Mice were treated for 7 days with IAA (0.1 %, added to the drinking water) or DSS (2 %, added to the drinking water) or kept under control conditions (CO, plain drinking water) after standard or enriched housing for 9 weeks. Plasma CORT was measured 2 h after the beginning of a 30-min exposure to water avoidance stress at the end of the treatment period. The values represent means + SEM, $n = 10$. These findings have been published in an original article (172).

3.4 Effects of environmental enrichment on brain structures of the hippocampal formation

3.4.1 Environmental enrichment increased neuropeptide Y expression in the polymorph layer of the dentate gyrus

Microscopy of brain sections processed for NPY ISH revealed a dense expression of NPY within the hippocampal and amygdalar brain regions. Particular high expression levels were found in the polymorph layer of the dentate gyrus (DGpl) and in the BLA (Figure12).

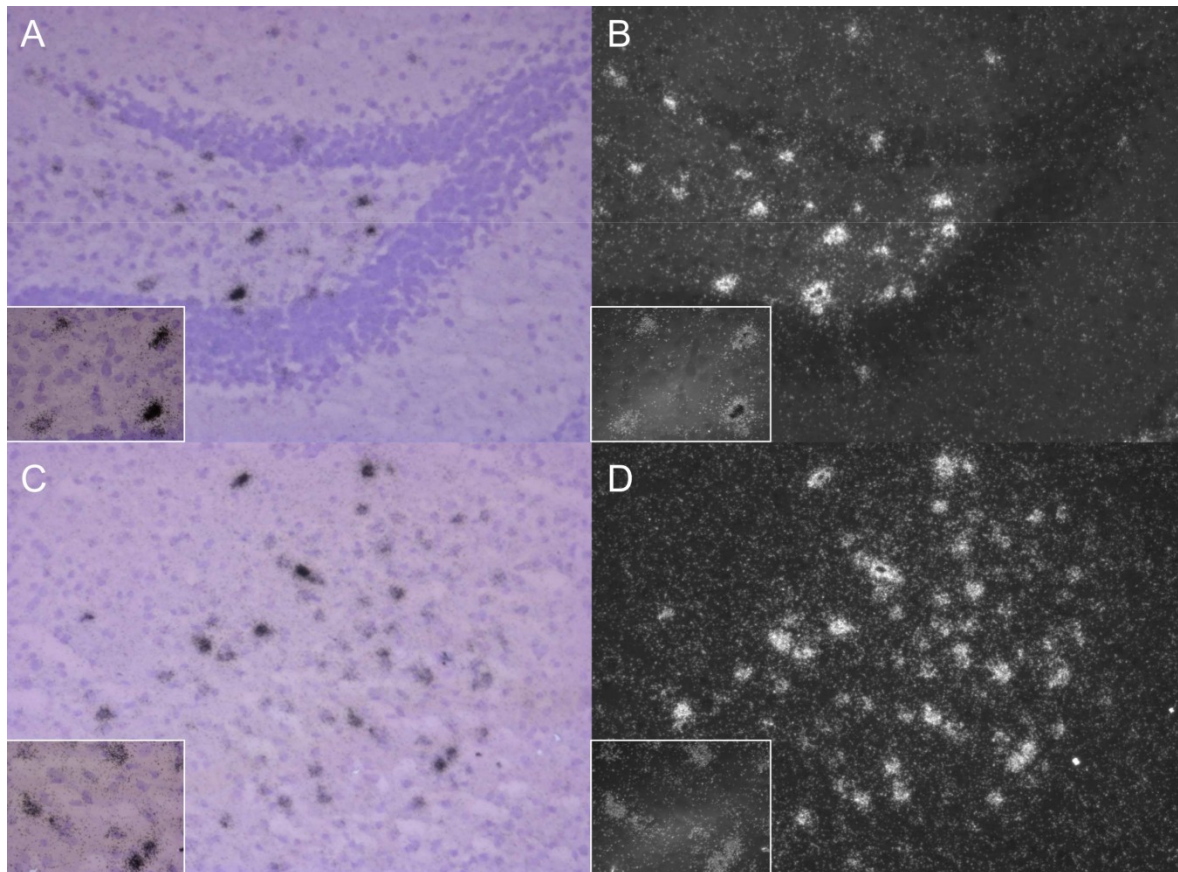


Figure 12. Representative bright and dark field images depicting NPY expression within the dentate gyrus and the amygdala. NPY expressing cells were mainly located in the polymorph layer of the dentate gyrus (A,B) and in the basolateral amygdala (C,D). Images were taken using a 10x objective. Images shown in the inserts were taken using a 40x objective. All sections processed for NPY *in situ* hybridization were counterstained with thionine acetate.

Quantitation of the number of grains/neuron within the DGpl showed that EE increased the number of grains/neuron ($F_{(1,36)} = 11.748$, $P = 0.002$) independently of treatment conditions. Furthermore, two-way ANOVA revealed a significant treatment effect ($F_{(2,36)} = 6.204$, $P = 0.005$) independently of housing conditions. In detail, IAA-treated mice tended to have a higher NPY expression/neuron than control mice (Tukey HSD test: $P = 0.073$). There was no interaction between the factors housing and treatment for this parameter (Figure 13A).

The number of NPY expressing cells within the DGpl remained unaltered by IAA or DSS treatment and by the housing condition (Figure 13B).

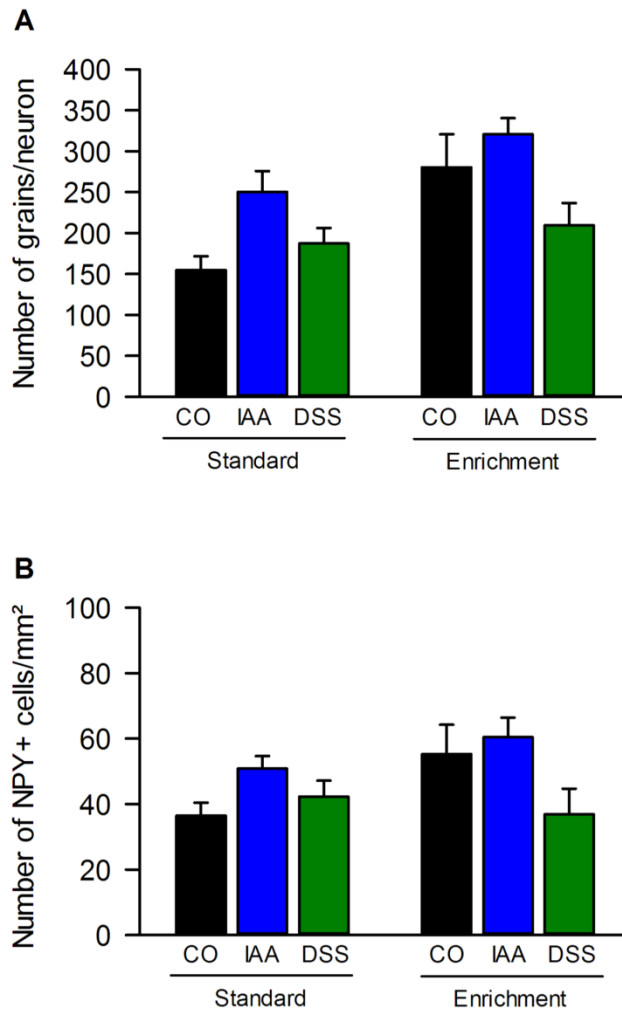


Figure 13. Environmental enrichment increases neuropeptide Y (NPY) expression/neuron (A) but does not influence the number of NPY expressing cells (B) within the polymorph layer of the dentate gyrus. Mice were maintained for 9 weeks under standard or enriched environment and then treated for 7 days with iodoacetamide (IAA; 0.1 % added to the drinking water) or dextran sulfate sodium (DSS; 2 % added to the drinking water), while control (CO) mice drank plain water. Expression of NPY was visualized 2 h after the beginning of a 30-min exposure to water avoidance stress at the end of the treatment period. The values represent means + SEM, $n = 7$.

In contrast to the situation in the DGpl, neither visceral inflammation nor EE influenced grain density/neuron (Figure 14A) or the number of NPY-positive cells/mm² (Figure 14B) within the BLA.

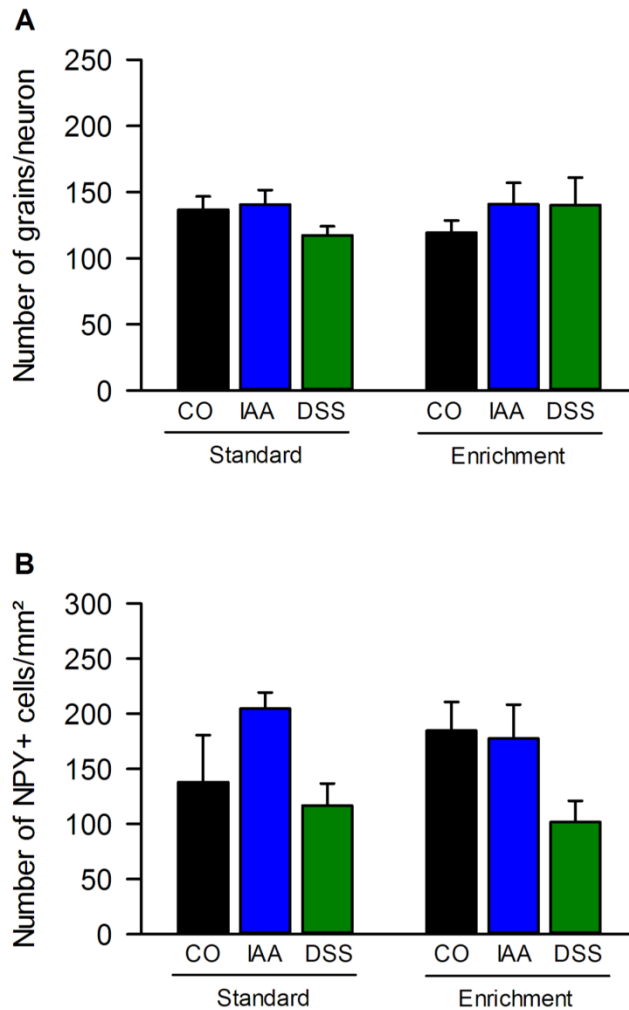


Figure 14. Environmental enrichment and visceral inflammation do not modify neuropeptide Y (NPY) expression/neuron (A) and the number of NPY expressing cells (B) within the basolateral amygdala. Mice were maintained for 9 weeks under standard and enriched environment and then treated for 7 days with iodoacetamide (IAA; 0.1 % added to the drinking water) or dextran sulfate sodium (DSS; 2 % added to the drinking water), while control (CO) mice drank plain water. Expression of NPY was visualized 2 h after the beginning of a 30-min exposure to water avoidance stress at the end of the treatment period. The values represent means + SEM, $n = 5 - 7$.

3.4.2 Environmental enrichment decreased stress-induced FosB/ Δ FosB expression within the CA1 and CA3 fields of the hippocampus

EE had an effect not only on the stress-induced expression of c-Fos but also on that of FosB/ Δ FosB, another marker of neuronal activation. Similarly to the stress-induced c-Fos expression, EE decreased the number of FosB/ Δ FosB positive cells ($t_{(11)} = 2.486$, $P = 0.03$) within the CA1 field of the hippocampus (Figures 15A,B and 16A). Additionally, EE decreased the number of FosB/ Δ FosB

expressing cells ($t_{(11)} = 4.348$, $P = 0.001$) in the CA3 region of the hippocampus (Figures 15A,B and 16A). Within the DGgl, FosB/ Δ FosB levels were nominally higher in EE-housed animals, but this change failed to reach significance (Figures 15C,D and 16A).

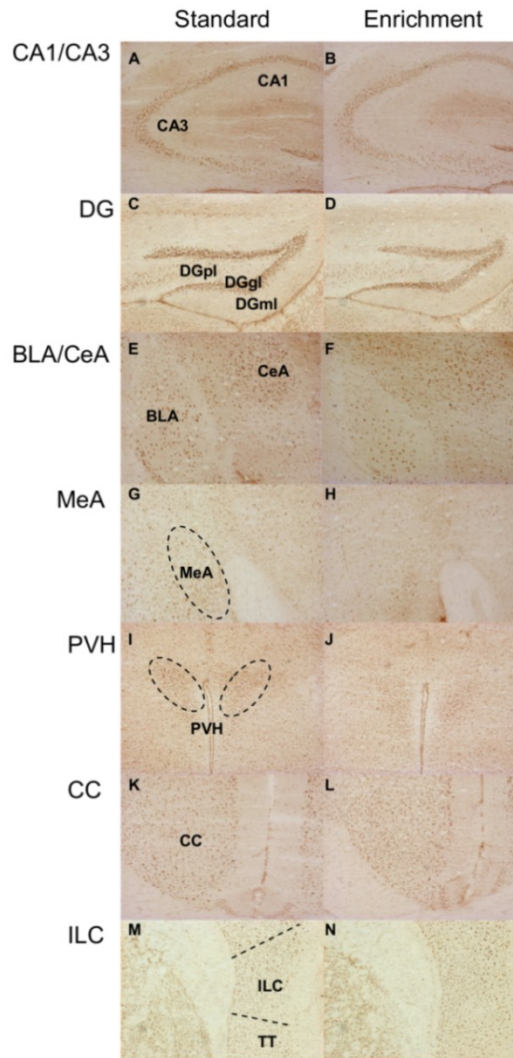


Figure 15. Environmental enrichment alters stress-induced FosB/ Δ FosB expression in the forebrain. The effect of water avoidance stress (30 min) on the cerebral FosB/ Δ FosB expression was examined in mice kept for 9 weeks under standard (SE) or enriched environment (EE). The left column panels (A,C,E,G,I,K,M) show representative micrographs of 9 forebrain regions from SE mice while the right column panels (B,D,F,H,J,L,N) depict micrographs of the same brain regions from EE mice. Relative to SE animals, EE decreased stress-induced FosB/ Δ FosB expression in the CA1 and CA3 region of the hippocampus (A,B). In contrast, EE failed to modify stress-induced FosB/ Δ FosB expression in the granular cell layer of the dentate gyrus (C,D), the basolateral, central and medial amygdala (E,F,G,H), the paraventricular nucleus of the hypothalamus (I,J), the cingulate cortex (K,L) and the infralimbic cortex (M,N). Abbreviations: BLA = Basolateral amygdala; CA1 = CA1 field of the hippocampus; CA3 = CA3 field of the hippocampus; CC = Cingulate cortex; CeA = Central amygdala; DGgl = Dentate gyrus, granular cell layer; DGml = Dentate gyrus, molecular cell layer; DGpl = Dentate gyrus, polymorph cell layer; ILC = Infralimbic cortex; MeA = Medial amygdala; PVH = Paraventricular nucleus of the hypothalamus; TT = Tenia tecta.

In contrast to the hippocampus, EE failed to alter stress-induced FosB/ Δ FosB expression within the CeA, BLA and MeA (Figures 15E,F,G,H and 16B).

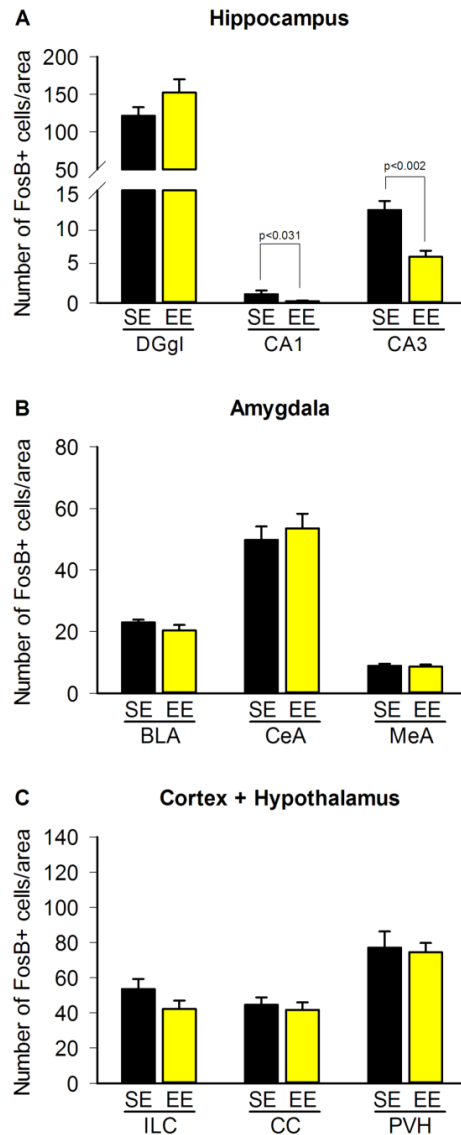


Figure 16. Environmental enrichment decreases the number of stress-induced FosB/ Δ FosB expressing cells in (A) the hippocampus but does not alter the number of stress-induced FosB/ Δ FosB expressing cells in (B) the amygdala, the hypothalamus and cortical brain areas (C). Within the hippocampus environmental enrichment (EE) dampened stress-induced FosB/ Δ FosB expression in the CA1 and CA3 region of the hippocampus, but not in the dentate gyrus compared to mice kept in standard environment (SE). Mice were maintained for 9 weeks under SE or EE. Expression of FosB/ Δ FosB was visualized 2 h after the beginning of a 30-min exposure to water avoidance stress at the end of the differential housing period. The values represent means + SEM, n = 6-8. Abbreviations: BLA = Basolateral amygdala; CA1 = CA1 field of the hippocampus; CA3 = CA3 field of the hippocampus; CC = Cingulate cortex; CeA = Central amygdala; DGgl = Dentate gyrus, granular cell layer; ILC = Infralimbic cortex; MeA = Medial Amygdala; PVH = Paraventricular nucleus of the hypothalamus.

Similarly to the amygdala no significant differences could be detected in the cortical brain regions (ILC, CC) investigated and in the PVH (Figures 15I,J,K,L,M,N and 16C).

3.4.3 Environmental enrichment did not induce ultrastructural changes within the CA1 field of the hippocampus and the polymorph layer of the dentate gyrus

To investigate ultrastructural changes in response to EE, the number and morphology of synapses were assessed in the CA1 region of the hippocampus and the DGpl (Figure 17).

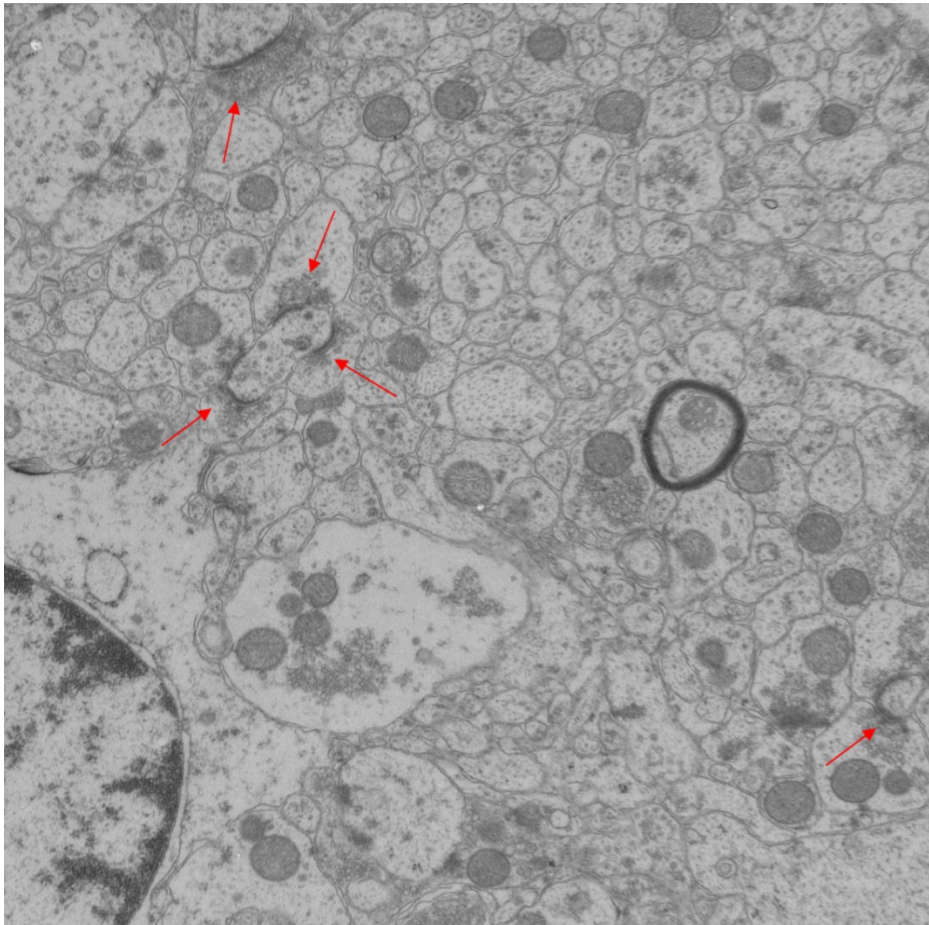


Figure 17. Representative electron micrograph showing synapses within the polymorph layer of the dentate gyrus. The red arrows indicate synapses within the region of interest. For quantification, images were taken from twenty 7x7 μm areas present on two consecutive sections (55nm section thickness). Only synapses found on the first but not on the second section were included in the analysis. All images were taken at 5000x magnification.

Statistical analysis disclosed that the number of synapses/ μm^3 , the width of the synaptic cleft, postsynaptic density length, presynaptic membrane length, number of vesicles/vesicle pool or number of docked vesicles within the CA1 did not differ between mice kept under SE or EE (Figure 18A-F).

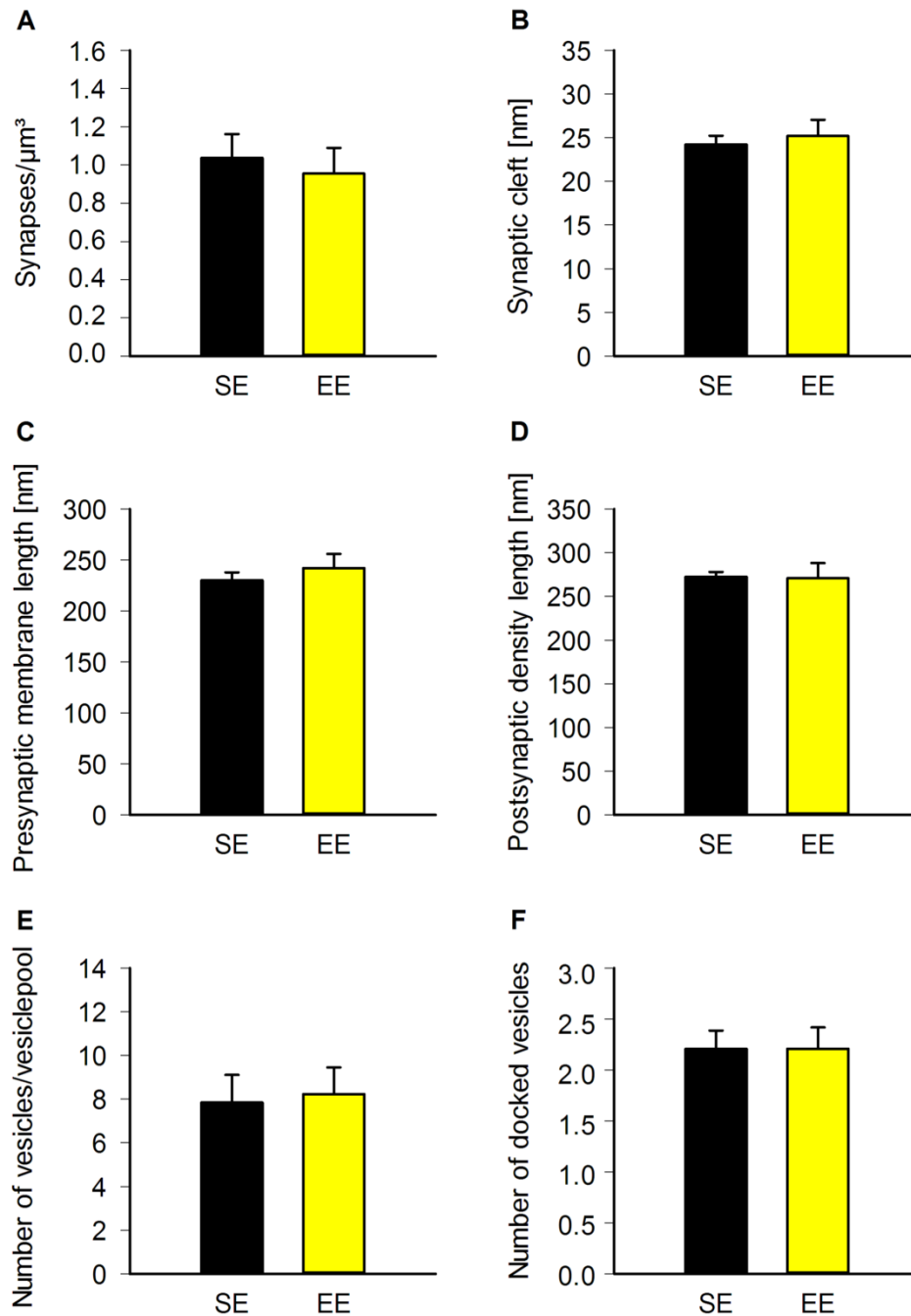


Figure 18. Environmental enrichment does not alter synaptic parameters within the CA1 region of the hippocampus (A-F). Mice were maintained for 9 weeks under standard (SE) or enriched environment (EE). After this differential housing period the number of synapses/ μm^3 , the width of the synaptic cleft, the post synaptic density length, the presynaptic membrane length, the number of vesicles/vesicle pool and the number of docked vesicles in the CA1 were assessed by electron microscopy. The values represent means + SEM, $n = 4-5$

Similarly, the number of synapses/ μm^3 in the DGpl was not altered by EE (Figure 19).

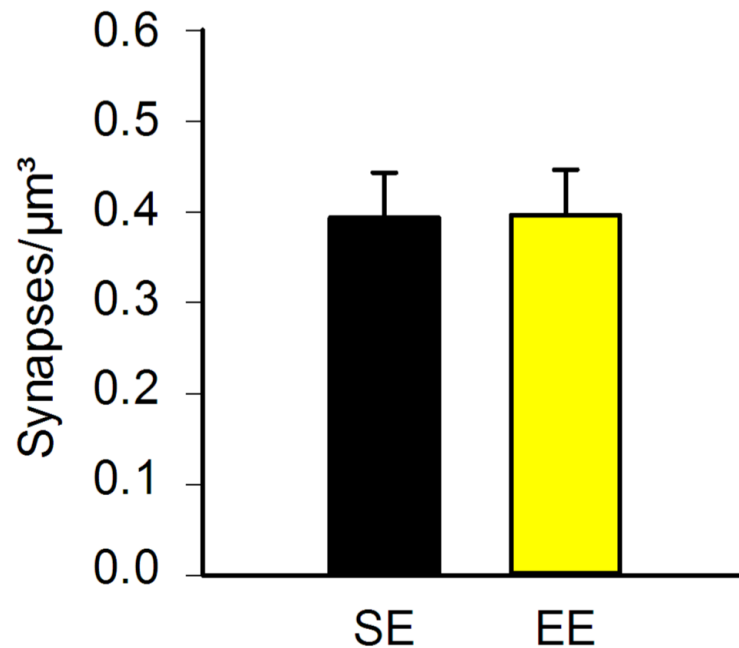


Figure 19. Environmental enrichment does not alter synapse density within the polymorph layer of the dentate gyrus (DGpl). Mice were maintained for 5 weeks under standard (SE) or enriched environment (EE). After this differential housing period the number of synapses/ μm^3 in the DGpl was assessed by electron microscopy. The values represent means + SEM, $n = 5$.

3.5 Effects of colitis and water avoidance stress on behaviour and brain neuropeptide Y expression

3.5.1 Dextran sulfate sodium treatment induced colonic inflammation and altered daily food and water intake

To monitor the effectiveness of DSS treatment in inducing colitis, several parameters were assessed at the end of the 7-day treatment period. DSS treatment increased DAS ($F_{(1,36)} = 230.769$, $P < 0.001$) and colonic MPO levels ($F_{(1,34)} = 77.559$, $P < 0.001$), an indirect marker of tissue leukocyte infiltration (Figure 20A,B). These parameters were also assessed in animals exposed to WAS, but WAS had no effect on DAS or MPO levels. Furthermore, WAS did not modify DAS or MPO in DSS-treated animals.

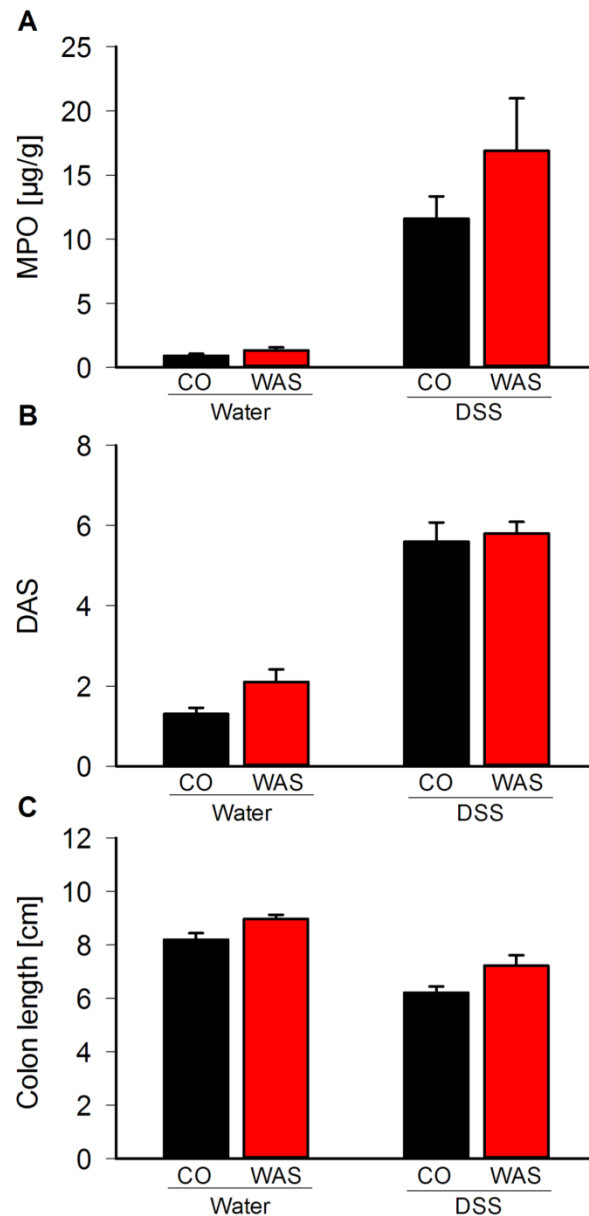


Figure 20. DSS treatment increases (A) colonic myeloperoxidase (MPO) levels and (B) the disease activity score (DAS), but (C) decreases colon length. WAS increased colon length, but did not influence MPO or DAS. Mice were treated for 7 days with dextran sulfate sodium (DSS; 2 % added to the drinking water), while control mice drank plain water (Water). At the end of the treatment period mice were exposed to water avoidance stress (WAS) for 30 minutes. Two hours after the beginning of WAS colonic MPO, DAS and colon length were determined. Non stressed mice (CO) were directly taken from their home cage at the end of the treatment period. The values represent means + SEM, n = 8-10.

Colonic leukocyte infiltration after 7-day DSS treatment was also confirmed by histology. As depicted in Figure 21, DSS treatment resulted in a loss of crypts and goblet cells as well as infiltration of leukocytes into the mucosal and submucosal layer of the colon.

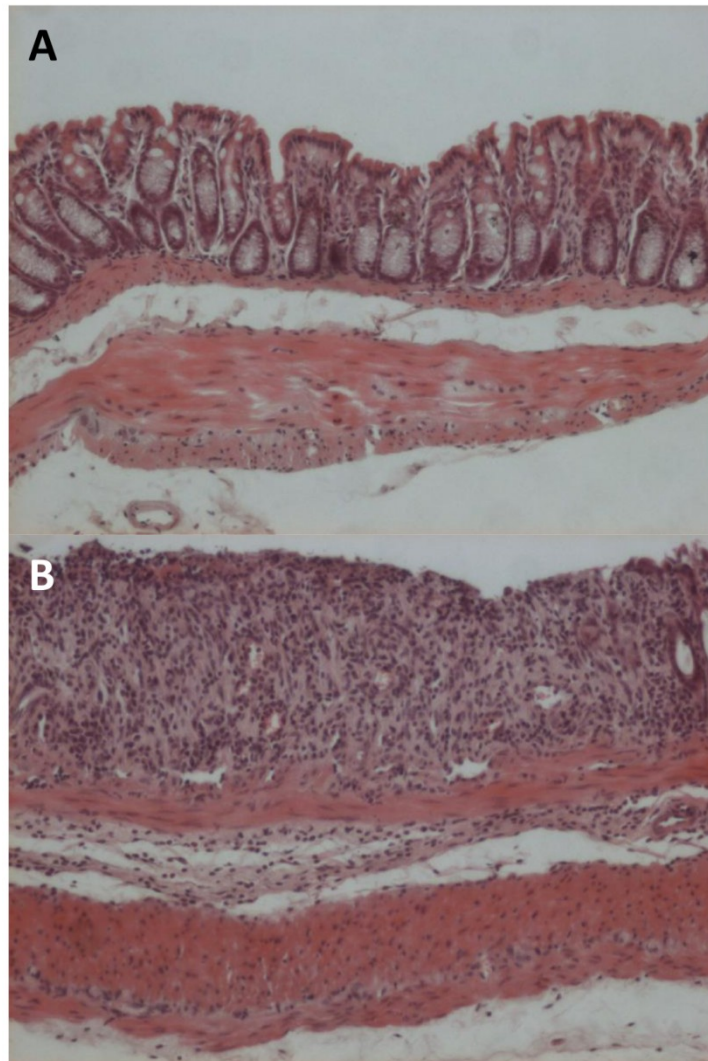


Figure 21. DSS treatment alters colon morphology. Colons of DSS-treated mice (B) revealed a loss of crypts and goblet cells and mucosal/submucosal leukocyte infiltration at certain levels of the colon compared to colons of control mice (A). Mice were treated for 7 days with DSS (2 % added to the drinking water), while control mice drank plain water. At the end of the treatment period full-thickness colon sections were excised and stained with hematoxylin and eosin.

Colon length, another parameter for assessing colonic inflammation, was decreased by colitis ($F_{(1,36)} = 47.765$, $P < 0.001$), but increased by WAS ($F_{(1,36)} = 11.000$, $P = 0.002$), without an interaction between the two factors (Figure 20C).

DSS treatment influenced the eating and drinking behaviour/g bodyweight of mice as measured by daily weighing of food pellets and drinking bottles, respectively. Repeated-measures ANOVA revealed that fluid intake varied with treatment ($F_{(1,17)} = 37.840$, $P < 0.001$) and treatment day ($F_{(1.582,26.886)} = 36.259$, $P < 0.001$) with a significant interaction between these factors ($F_{(1.582,26.886)} = 24.796$, $P < 0.001$). In detail, fluid intake/g was higher in DSS-treated mice during the first

four treatment days, but reached control levels afterwards (Figure 22A). Food intake/g also varied with treatment ($F_{(1,16)} = 45.213$, $P < 0.001$) and treatment day ($F_{(3,701,59,213)} = 86.468$, $P < 0.001$) with a significant interaction between the factors ($F_{(3,701,59,213)} = 61.235$, $P < 0.001$). In contrast to liquid intake/g, food intake/g dropped during the course of DSS treatment. Thus, food intake was higher in DSS-treated mice on treatment day 1, but lower on the treatment days 5, 6 and 7 (Figure 22B).

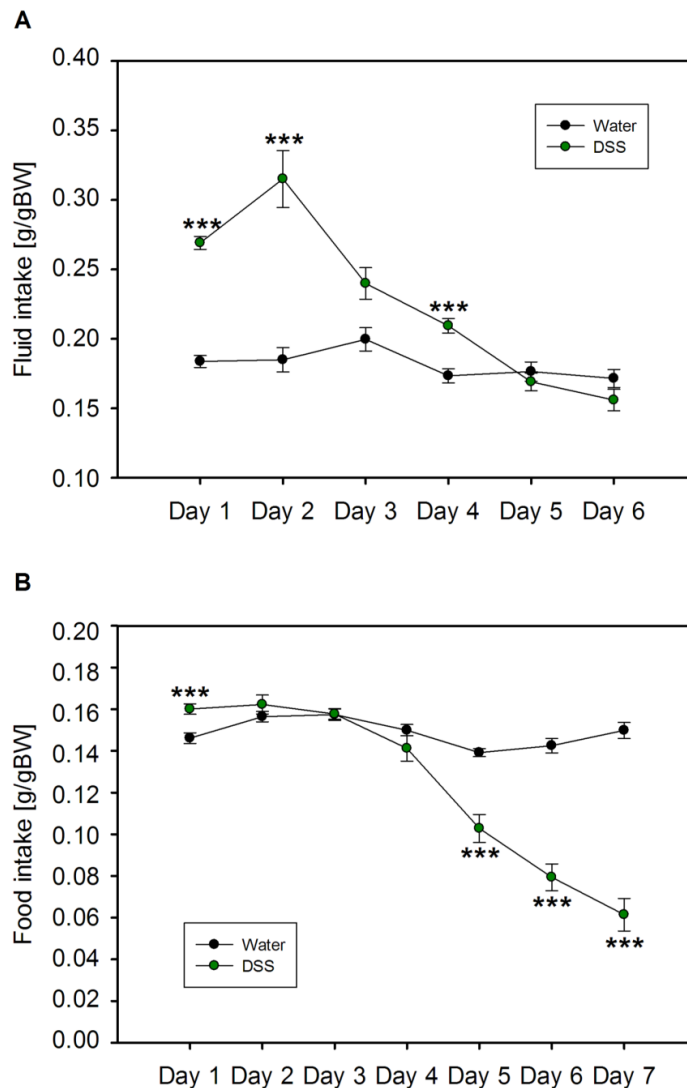


Figure 22. DSS treatment alters fluid and food intake. Fluid intake was higher in dextran sulfate sodium (DSS)-treated mice during the first treatment days, but reached control levels towards the end of the treatment period (A). Food intake was slightly higher in DSS-treated mice on the first treatment day, but dropped dramatically towards the end of the treatment (B). Mice were housed in groups of two and treated for 7 days with DSS (2 % added to the drinking water), while control mice drank plain water (Water). Fluid and food intake/cage was measured daily by weighing the respective water bottles and food pellets during the treatment period. The daily consumed food and liquid amount/cage in g was then divided by the combined pretreatment body weight (BW) of the mice within a given cage. The values represent means \pm SEM, $n = 8-10$. ***, $P < 0.001$ vs. control.

3.5.2 Dextran sulfate sodium treatment modified behaviour during the water avoidance stress session and enhanced corticosterone levels

To assess whether DSS treatment changes behaviour during the 30-minute WAS session, animals were closely monitored. As shown in Figure 23A, animals with colitis tried to escape more frequently from the platform than control animals, but this difference did not reach significance.

Analysis of corticosterone measurements revealed that 2 hours after WAS corticosterone levels of DSS-treated and control animals were similar to those of the respective unstressed controls (Figure 23B). It was clearly evident, however, that DSS treatment per se elevated basal corticosterone levels independently of stress exposure ($F_{(1,36)} = 24.212$, $P < 0.001$).

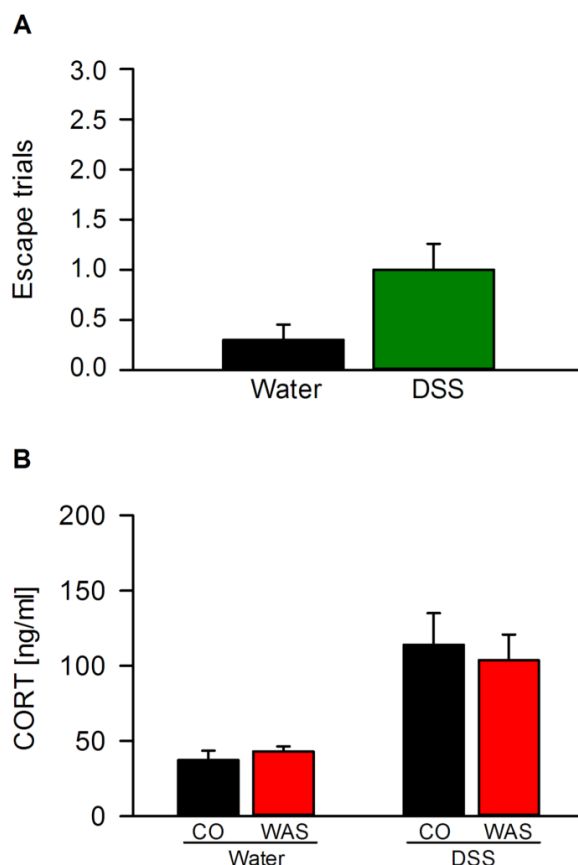


Figure 23. DSS treatment increases (A) the number of escape trials from WAS and (B) corticosterone (CORT) levels. Mice were treated for 7 days with dextran sulfate sodium (DSS; 2 % added to the drinking water), while control mice drank plain water (Water). At the end of the treatment period mice were exposed to water avoidance stress (WAS) for 30 minutes during which their escape behaviour was monitored. Plasma CORT was measured 2 h after the beginning of a 30-min exposure to WAS or from mice directly taken from their home cage (CO) at the end of the treatment period. The values represent means + SEM, $n = 10$.

3.5.3 Water avoidance stress enhanced locomotor activity and time spent in the light compartment of the light/dark box

Exposure to WAS altered the behaviour of mice in the L/D box test. In detail, it appeared that stressed mice spent more time in the light compartment (Figure 24A) of the test box ($F_{(1,28)} = 4.521$, $P = 0.042$) independently of DSS treatment. WAS-exposed mice also exhibited an enhanced locomotor activity ($F_{(1,28)} = 5.537$, $P = 0.026$) compared to non-stressed animals under both treatment conditions (Figure 24B). Animals with colitis tended to spend more time in the light compartment than healthy animals independently of stress exposure ($F_{(1,28)} = 3.798$, $P = 0.061$). No group differences were found for the latency to re-enter the light compartment (Figure 24C) or the number of transitions between the compartments (Figure 24D). There was also no interaction of the factors stress and colitis in any of the parameters measured.

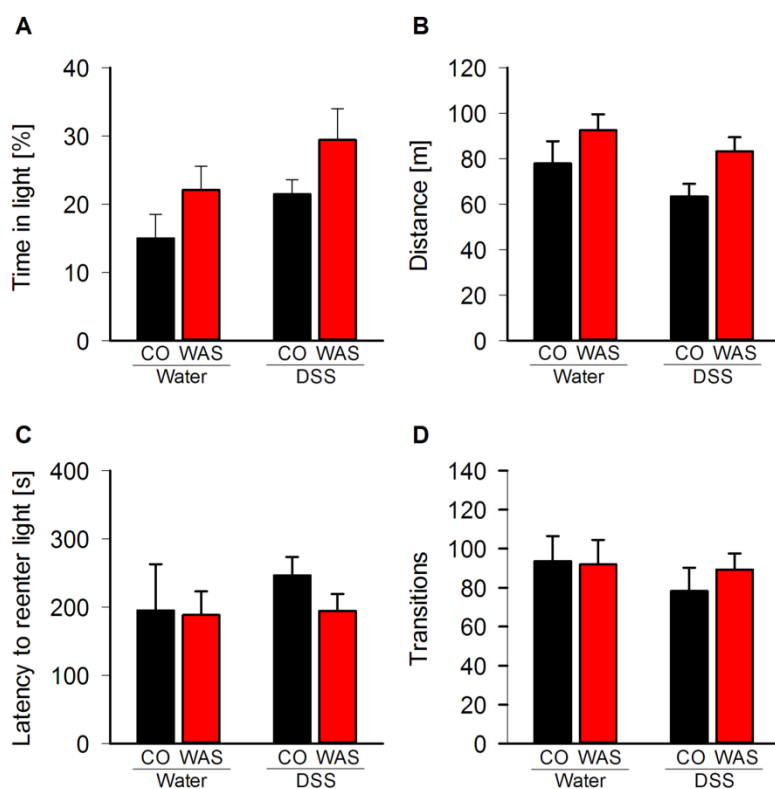


Figure 24. WAS increases locomotor behaviour (B) and the time mice spent in the light compartment of the light/dark box test (A). The latency to reenter the light compartment after leaving this compartment (C) as well as transitions between the two compartments (D) were not altered by the treatments. Mice were treated for 7 days with dextran sulfate sodium (DSS; 2 % added to the drinking water), while control mice drank plain water (Water). Mice were put into the light/dark box either directly from the homecage (CO) or 2 h after the beginning of a 30-min exposure to water avoidance stress (WAS) at the end of the treatment period. The values represent means + SEM, $n = 8$.

3.5.4 Colitis and water avoidance stress did not disturb the circadian pattern of activity and exploration of mice and did not modify sucrose preference

The recordings in the Labmaster system revealed a circadian variation of activity ($F_{(1.275,75)} = 49.988$, $P < 0.001$) and exploration ($F_{(1.327,75)} = 31.052$, $P < 0.001$) within each treatment group. All animals showed higher activity and exploration during the dark phase compared to the light phase (Figure 25A,B). Differences between the 4 treatment groups could not be detected at any time point.

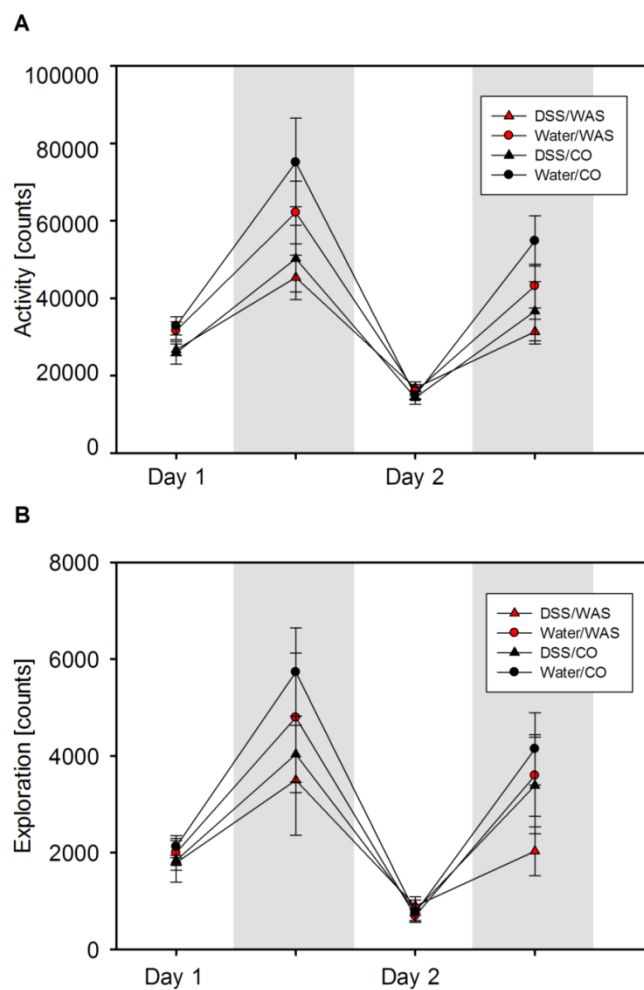


Figure 25. Mice show circadian variations in activity and exploration within each treatment group. Mice were treated for 7 days with dextran sulfate sodium (DSS; 2 % added to the drinking water), while control mice drank plain water (Water). Mice were put into the Labmaster system 30 minutes after testing in the light/dark box. The animals used for these experiments were taken either directly from the homecage (CO) or 2 hours after the beginning of a 30-min exposure to water avoidance stress (WAS) at the end of the treatment period. Each data point represents a 12-hour interval either during the light phase (white areas) or during the dark phase (grey areas) of the 48-hour testing period. The values represent means \pm SEM, $n = 5-8$.

Neither stress nor colitis had any effect on SP (Figure 26A,B), but this parameter was subject to circadian variation ($F_{(1,327,75)} = 44.342$, $P < 0.001$). Total liquid consumption was also influenced by the circadian rhythm ($F_{(1,838,75)} = 128.861$, $P < 0.001$) and treatment ($F_{(3,25)} = 3.118$, $P = 0.044$) with a significant interaction between these factors ($F_{(5,513,75)} = 3.909$, $P < 0.001$). However, after adjusting for multiple testing no significant treatment effect was present at any time point.

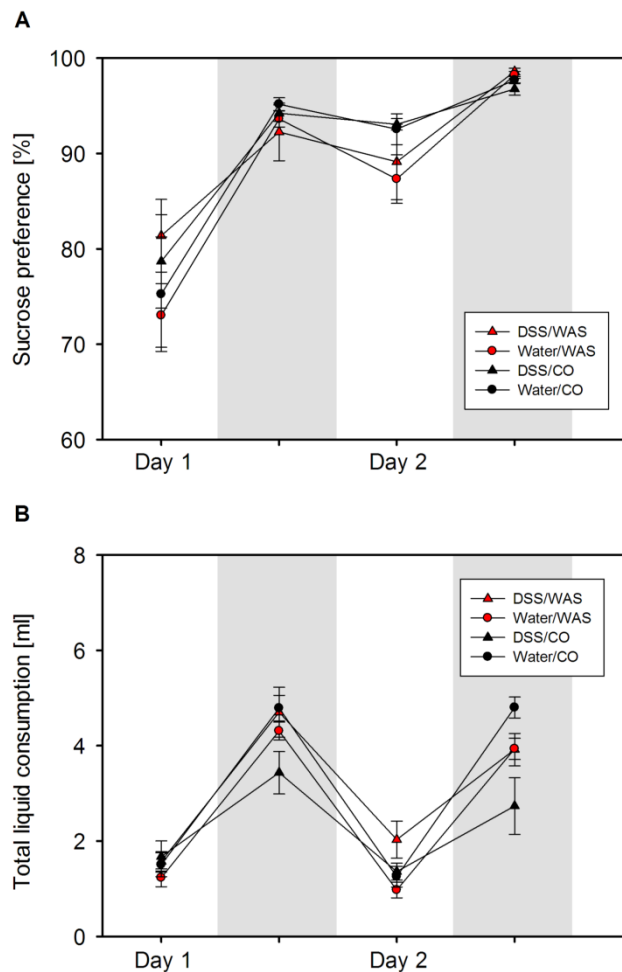


Figure 26. Mice show circadian variations in sucrose preference and liquid consumption within each treatment group. Mice were treated for 7 days with dextran sulfate sodium (DSS; 2 % added to the drinking water), while control mice drank plain water (Water). Mice were put into the Labmaster system 30 minutes after testing in the light/dark box. The animals used for this sequence of behavioural tests were taken either directly from the homecage (CO) or 2 h after the beginning of a 30-min exposure to water avoidance stress (WAS) at the end of the treatment period. Each data point represents a 12-hour interval either during the light phase (white areas) or during the dark phase (grey areas) of the 48h testing period. Sucrose preference was expressed as percentage and refers to the consumed amount of sucrose-containing water/g bodyweight of each mouse in relation to the total liquid consumption/g bodyweight of each mouse. The values represent means \pm SEM, $n = 5-8$.

3.5.5 Water avoidance stress increased curling behaviour during the tail suspension test

WAS, but not colitis, increased the time mice spent curling (Figure 27A) in the TST ($F_{(1,24)} = 8.208$, $P = 0.009$) independently of DSS treatment. Furthermore, stressed mice tended to spend less time swinging ($F_{(1,25)} = 3.434$, $P = 0.076$) than control animals (Figure 27C). However, WAS did not alter immobility time in this test (Figure 27B). No difference in any parameter measured could be detected between colitis and control animals and there was also no interaction between the factors stress and colitis (Figure 27A-C).

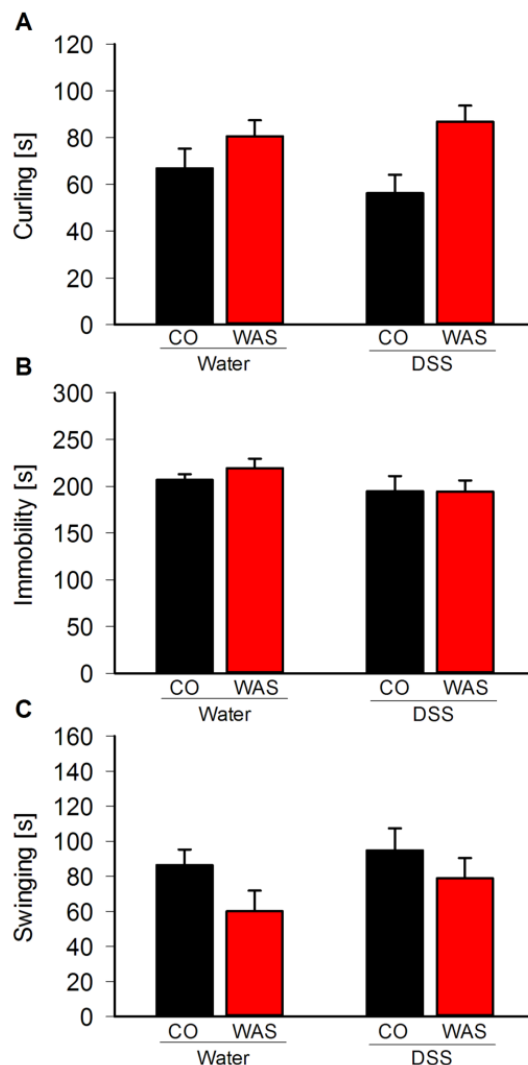


Figure 27. WAS increases curling time of mice during the tail suspension test. Mice were treated for 7 days with dextran sulfate sodium (DSS; 2 % added to the drinking water), while control mice drank plain water (Water). The mice used in the tail suspension test were taken either directly from the homecage (CO) or 2 h after the beginning of a 30-min exposure to water avoidance stress (WAS) at the end of the treatment period. The values represent means + SEM, $n = 6-8$.

3.5.6 Colitis enhanced hypothalamic but blunted amygdalar neuropeptide Y expression

As shown in Figure 28, DSS treatment and WAS had differential effects on NPY mRNA levels depending on the brain region investigated. The largest effects of DSS treatment were found in the hypothalamus (Figure 28A). Within this region DSS treatment increased NPY expression on average by 80 % ($F_{(1,16)} = 8.678$, $P = 0.009$) independently of stress exposure. There was no interaction of stress and colitis within this region.

Appreciable effects of colitis ($F_{(1,15)} = 12.743$, $P = 0.003$) were also seen in the amygdala (Figure 28B) with a significant interaction between colitis and WAS ($F_{(1,15)} = 8.391$, $P = 0.011$). Post-hoc analysis revealed that DSS treatment decreased NPY levels in non-stressed animals ($t_{(3.359)} = 3.654$, $P = 0.029$), but did not alter NPY in WAS-treated mice. Furthermore, WAS increased NPY expression in DSS-treated mice ($t_{(8)} = -3.911$, $P = 0.004$), but did not alter NPY expression in control mice. In the hippocampus neither WAS nor colitis changed NPY expression (Figure 28C).

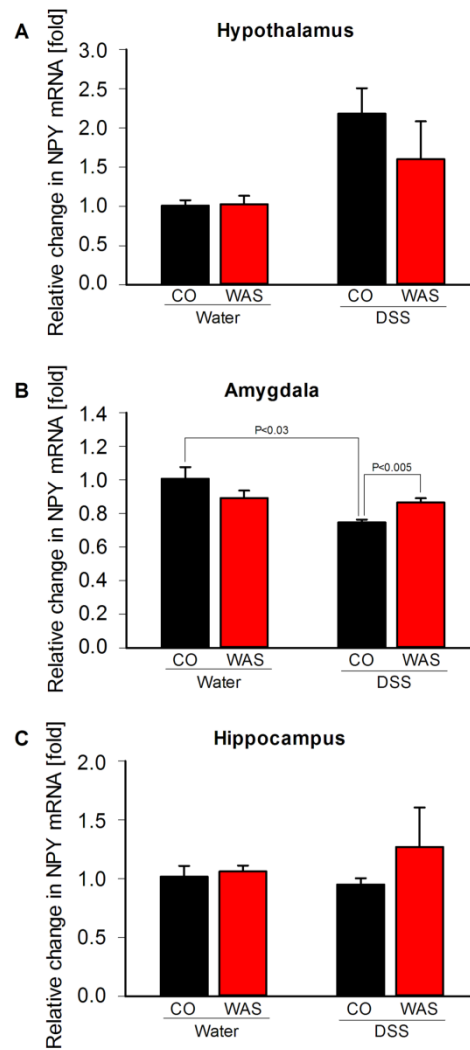


Figure 28. Colitis increases NPY mRNA levels within the hypothalamus, but decreases NPY levels within the amygdala. WAS elevated NPY expression within the amygdala in DSS-treated mice, but did not alter amygdalar NPY expression in control mice. Mice were treated for 7 days with dextran sulfate sodium (DSS; 2 % added to the drinking water), while control mice drank plain water (Water). At the end of the treatment period mice were taken for the analysis of NPY either directly from the homecage (CO) or 2 h after the beginning of a 30-min exposure to water avoidance stress (WAS). Group differences were expressed as fold changes according to the $2^{-\Delta\Delta C_t}$ method using the mean value of the control group as the calibrator. The values represent means + SEM, *n* = 4-5.

4 Discussion

4.1 Rationale of the project

Epidemiological data suggest that the incidence and prevalence of IBD are rising (13). It is also known that IBDs are often associated with primary psychiatric disorders such as depression or anxiety disorders and that stress exacerbates IBDs (95,161,162). Despite this knowledge little is known about the reason for this association and the underlying molecular changes. Conceptually two major pathways are connecting the brain with the gut. On the one hand, the vagal and spinal afferent neurons transmit information from the gut to the brain and, on the other hand, molecules released from the gut into the circulation may influence the brain. In the course of visceral inflammation, it has been suggested that signalling from the gut to the brain is altered (1). Although several fMRI-studies detected changes in brain area activation among irritable bowel syndrome or IBD patients (93,94,199-202), biochemical changes within the brain in the course of visceral inflammation are relatively unknown.

Chronic psychological stress worsens many diseases. This is especially true for IBD and irritable bowel syndrome, because stress can exacerbate these diseases/disorders or even be a causal factor for the development of these diseases (24,25,32,203). Stressors can be defined by their ability to disturb the homeostasis of the body. In this sense visceral inflammation can be regarded as a chronic internal stressor. Therefore, in the setting of IBD, an internal inflammatory stressor can coincide with an external stressor such as psychological stress. The chronic internal stress load in this setting may thus alter the perception and processing of external stress ultimately leading to more deleterious negative consequences of stress. The psychological stressor may be perceived or processed differentially within the brain and the inflammatory stressor may make the body more susceptible to negative consequences of psychological stress.

EE is enforced as a measure to increase the welfare of captive animals (172). By enabling them to express a variety of species-specific behaviours, EE is thought to enhance the translational value of animal models in the preclinical study of biological processes relevant to human health and disease (175). EE is also

considered to reduce the stress which impoverished maintenance under standard conditions imposes on the animals (204). In line with this contention, Belz et al. (205) report that the basal levels of circulating corticosterone in rats are lowered when the animals are kept under enriched housing, while other studies have failed to confirm this finding (176,177,206,207).

Against this background the current project aimed to better characterize the interactions between internal inflammatory stress and external psychological stress by investigating the cerebral stress response of mice. To study stress-induced molecular changes, I recorded the activation of stress-relevant brain nuclei, as measured by the expression of the immediate early genes c-Fos and fosB, in response to WAS, a psychological stress paradigm. In particular I examined whether the WAS-induced brain response is altered by EE or internal stressors such as inflammation which causes long-term alterations in pain sensitivity (208-210) and anxiety (127,184). Findings from this part of the thesis project have been published in an original article (172). Additionally I investigated how EE, visceral inflammation and WAS affect the NPY system, which plays a crucial role in the stress response (81). I also examined whether emotional-affective behaviour is altered by internal or external stress. Finally I evaluated whether alterations in the hippocampal synapse ultrastructure may be involved in EE-induced stress resilience (171).

I hypothesized that inflammation could modify the cerebral sensitivity to stress as well as the biochemical and behavioural response to psychological stress. Furthermore I hypothesized that EE can modulate the stress response and induce lasting changes in the amygdala-hippocampus network.

4.2 Influence of water avoidance stress on c-Fos expression in the brain

Visualization of neuronal activation in distinct brain nuclei by means of the immediate early gene c-Fos is a common approach in the field of neurosciences. It has previously been reported for several stress paradigms (166,196,198,211-213) that stress increases c-Fos expression in certain areas of the brain. However, only few studies investigated the effect of WAS on the brain. In the first study investigating the effect of WAS on the rat brain, Bonaz et al. (166) found increases

in the number of c-Fos expressing cells within the parvocellular part of the PVH, LC, bed nucleus of the stria terminalis, lateral septum (LS), dorsal raphe nucleus, NTS and in the A5 and A1 noradrenergic cell groups. The WAS-induced c-Fos increase in the PVH and NTS of rats was confirmed in another study of the same group (167), whereas the WAS-induced increase in the NTS was absent in another study (168). So far only one study investigated the effect of WAS on c-Fos expression in mice (165). In agreement with the study of Bonaz et al. (166), Goebel-Stengel et al. (165) reported WAS-induced c-Fos elevations in the PVH, LC and NTS. Additionally they found a WAS-induced c-Fos increase within the Edinger-Westphal nucleus, the rostral raphe pallidus nucleus and the ventrolateral medulla. To date, however, no study systematically examined WAS-induced c-Fos expression within the forebrain.

As described in the Results section, I found a strong rise of c-Fos expressing cells within many areas of the mouse brain. Similarly to previous studies (165-167) I showed that WAS increased c-Fos expression within the PVH, LC, BST and LS. For the first time I demonstrated, however, that WAS enhances c-Fos expression in the corticolimbic system and other brain regions. Specifically I found higher stress-induced c-Fos levels in the BLA, CeA, MeA, the CA1 and CA3 regions of the hippocampus and in the CC, a part of the medial prefrontal cortex. These structures belong to the corticolimbic system and are closely interconnected with each other (92,214,215). Functionally all of these structures are important coordinators and potential modulators of an appropriate stress response (216). WAS-induced elevations of c-Fos within these regions are in accordance with other studies using different types of stressors (197,217-219). Similarly to the study of Chowdhury et al. (197) I did not find stress-induced elevations of c-Fos within the DG while other studies reported such an increase in response to elevated plus maze exposure and injection stress (219,220). Furthermore, the WAS-induced increase of c-Fos within the CeA was not found in the study of Bonaz et al. (166) on rats. The reason for these diverse findings may be related to different species, stressors and quantification methods used.

Besides stress-induced changes in the corticolimbic system, I also found elevations in the number of c-Fos expressing cells in subregions of the thalamus, the ARC, the AIC, Pir, LPAG, RLi and within the PN. Stimulus-induced c-Fos increases in the PN, an integrative centre linking the cerebral and cerebellar

cortices, have also been seen in one of the first studies using c-Fos as a marker of neuronal activation (221). The same study also found increases in the number of c-Fos expressing cells within the thalamus, which was later confirmed by studies using immobilization stress (222,223). Furthermore, the pattern of WAS-induced c-Fos expression within the murine thalamus and the brainstem, i.e. the periaqueductal grey, raphe nuclei and LC, resembles immobilization stress-induced c-Fos expression in rats (222,223). Another study found foot shock stress-induced activation of the PVT and brain regions sending afferents to the PVT (224). Based on retrograde tract tracing in combination with the immunohistochemical detection of c-Fos, Bubser and Deutch (224) consider the PVT as a relay station modifying dopaminergic input from the ventral tegmental area to the mPFC and amygdala via glutamatergic afferents to these structures which in turn regulate the activity of the dopaminergic system (224). Finally activation of the insular cortex and Pir has also been demonstrated previously using injection stress (220). Enhanced activation of the insula by stress suggests changes in brain functions such as awareness and interoception (225).

Taken together I found that WAS enhances neuronal c-Fos expression in a way that is similar to previous studies using different types of stressors. In the field of neurosciences c-Fos mapping is mainly used to identify neurons that are “activated” by certain kinds of stimuli. An increase in c-Fos expression within a brain region is thought to represent increased afferent input to and/or increased external stimulation of this region rather than increased depolarization of neurons (83,172,226). Specifically, c-Fos is expressed in neurons receiving enhanced synaptic input by signals from the periphery or other brain regions (83,196). My findings demonstrate that in the setting of WAS many brain areas receive enhanced synaptic input or in other words are activated. Since this neuronal activation pattern of WAS is similar to other stressors, I conclude that WAS is a valid and potent psychological stress paradigm in mice.

4.3 Influence of environmental enrichment on stress-induced c-Fos and FosB/ Δ FosB expression in the brain

Based on the results of protocol 1, demonstrating large WAS-induced neuronal activation in the limbic system, I decided to investigate whether WAS-induced biochemical changes depend on environmental factors such as housing animals in EE before stress exposure. Thus, neuronal activation as visualized by c-Fos and FosB/ Δ FosB-like immunoreactivity was assessed in cerebral regions known to be involved in the general stress response, including the hypothalamus, hippocampus, amygdala, ILC and CC (196,198,211,212).

As described in Reichmann et al. (172), EE led to a significant change in the WAS-evoked c-Fos expression in 4 brain regions: the DGgl, the CA1 region of the hippocampus, the CeA and the ILC. While in the ILC, CeA and CA1 region EE attenuated the stress-induced expression of c-Fos, the opposite was true in the DGgl in which EE enhanced the number of c-Fos expressing neurons. I therefore hypothesize that changes in stress-evoked c-Fos expression reflect changes in stress-mediated stimulation of neurons caused by the housing conditions under study.

Changes of FosB/ Δ FosB expression within the hippocampus were similar to the observed changes of c-Fos expression. Specifically, the number of FosB/ Δ FosB-positive cells was decreased in the CA1 region of the hippocampus and nominally higher in the DGgl. In contrast to c-Fos, I detected a significant decrease of FosB/ Δ FosB-positive cells within the CA3 field of the hippocampus, but no change within the CeA and ILC. It is known that most fosB gene products reach expression maxima around 6 hours after acute stimulation (227). Nevertheless two hours after acute stress these proteins are increased compared to control conditions (228). As with c-Fos, enhanced fosB gene products may indicate increased afferent input to a certain brain region, while decreased fosB gene products may indicate the opposite. FosB gene derived proteins include full length FosB, Δ FosB and certain isoforms of these proteins (227-229). Recently, much attention has been paid to Δ FosB. Δ FosB is unique among the Fos family members since it accumulates after chronic but not acute stimulation, a consequence of the high stability of this protein (229). As a transcription factor Δ FosB modifies gene expression (230) and has been implicated in stress

resilience (132). In the current experiment the contribution of Δ FosB to the overall FosB signal is unclear. EE is a potential chronic stimulus, while WAS is a potent acute stimulus inducing all short-term FosB proteins. Therefore it cannot be told which portion of the observed signal is due to Δ FosB and which portion results from the other FosB proteins. Further work is needed to determine whether EE per se is a stimulus intense enough to promote the accumulation of Δ FosB.

As also discussed in (172), the effects of EE on c-Fos expression within the DGgl are consistent with other effects of EE in the dentate gyrus. For instance, various EE paradigms stimulate adult neurogenesis (144,231-233) which in the dentate gyrus takes place in the subgranular zone, the new neurons being subsequently integrated into the granular layer (234-236). EE-induced increases in neurotrophin levels, increases in dendritic growth and other morphological changes as well as alterations in neurotransmitter dynamics have also been observed in the dentate gyrus (182,237,238). Importantly, the current findings indicate that the structural and neurochemical effects of EE result in alterations of stress-induced activity in the dentate gyrus.

In contrast to what was seen in the DGgl, EE decreased the stress-induced c-Fos and FosB/ Δ FosB expression in the CA1 region of the hippocampus (172). EE also reduced FosB/ Δ FosB in the CA3 region while c-Fos expression was blunted in the CeA and the ILC region of the medial prefrontal cortex by EE. The similarity of the effect of EE in these regions (blunted expression of neuronal activation markers) may be explained by the close connections between the amygdala, the hippocampus and the medial prefrontal cortex (92,214-216,239). The CeA is a major output nucleus of the amygdala to autonomic brainstem centres and endocrine regions of the hypothalamus (91,240,241) and receives input from the CA1 region, while there are no monosynaptic connections with the dentate gyrus. Projections from the amygdala to the hippocampus terminate especially in the CA1 and also in the CA3 field of the hippocampus (214). Like the hippocampus, the amygdala is involved in the processing of stress and emotions such as fear (242). The attenuation of stress-induced c-Fos expression in the CeA under enriched housing may hence imply that EE reduced the fear perceived during the WAS procedure in a novel environment. This argument is supported by findings that animals kept under EE adapt more quickly to novel situations and can better cope with stress than animals kept under standard conditions

(135,139,206,243). These behavioural adaptations may also be related to the effect of EE to blunt the stress-induced stimulation of neurons in the ILC, an observation that is in line with the ability of EE to dampen stress-induced acetylcholine and dopamine release in the prefrontal cortex (244,245). This part of the discussion has also been published in an original article (172).

The divergent effects of EE on WAS-evoked c-Fos and FosB/ Δ FosB expression in the DGgl, on the one hand, and the ILC, CeA, CA1 and CA3 region, on the other hand, need to be seen in context with the reciprocal connections between amygdala, hippocampus and prefrontal cortex (211,214-216,246). The hippocampus and amygdala are essential components of the cerebral circuitry mediating the stress response. Importantly, the two structures issue opposing outputs to the stress effector system (211,216,247,248). The amygdala (including the CeA) activates the behavioural and endocrine centres of the stress response (91), while the hippocampus provides an important negative feedback on the stress effector system (4). Since EE reduced the stress-induced c-Fos expression of the CeA but enhanced it in the DGgl, it would appear that EE reduced the overall activity of the stress effector system by altering the impact of the amygdala-hippocampus network on this system. In other terms, EE improved stress resilience. This conclusion is in keeping with a number of observations that EE is able to counteract and prevent the behavioural and neuroendocrine effects of stress (136,144,171,176,205-207,231,249) This part of the discussion has also been published in an original article (172).

4.4 Influence of environmental enrichment on stress-induced neuropeptide Y expression in the brain

In addition to the evaluation of stress-induced Fos proteins, the expression of NPY, an important modulator of the stress response (81,97,98), was quantified in the amygdala and the hippocampus of EE- and standard-housed mice. In accordance with previous studies (104) I found a strong expression of NPY within cortical interneurons, the ARC, the DGpl and the BLA. Since I was most interested in the role of NPY within the limbic system, I analysed NPY expression in the DGpl and BLA by grain counting. I detected an increase of NPY mRNA expression per cell within the DGpl but not within the BLA of WAS-treated EE-housed animals. By

using real-time PCR I found that this elevation is due to EE and not WAS since WAS did not alter hippocampal NPY compared to unstressed controls.

So far very little has been known as to whether EE modifies NPY levels of rodents. The only study investigating this point reported that EE did not influence amygdalar NPY expression (250). This is in accordance with my finding since I also did not detect changes in amygdalar NPY expression related to EE. In contrast, the observed changes in hippocampal NPY expression have not been described previously. It is well established that NPY modifies the stress response (81) and a recent study suggests that NPY levels correlate with stress resilience, the ability to cope with stress (128). Cohen et al. (128) found that animals whose behaviour was extremely disrupted after stress exposure had lower levels of NPY in the hippocampus, amygdala, posterior cortex and periaqueductal grey. Furthermore stress-induced behavioural disturbances could be prevented by hippocampal administration of NPY (128). In line with these findings, other studies reported the absence of anxiogenic-like effects after stress exposure in animals with hippocampal NPY overexpression (116,251), indicating the importance of hippocampal NPY in stress coping. Previous studies also identified the amygdala as a crucial structure for the resilience-promoting actions of NPY. For example, repeated infusions of NPY into the BLA result in a stress-resilient behavioural phenotype (114), rats with a variable coping strategy in a chronic unpredictable stress paradigm have increased amygdalar NPY immunoreactivity (252) and overexpression of amygdalar NPY renders animals less anxious (117). Like NPY, EE also promotes stress resilience. Specifically, EE attenuated chronic stress-induced behavioural abnormalities in a radial water maze and corresponding reductions in CA3 dendritic length (253). EE also prevented social defeat stress-induced depressive-like and anxiety-like behaviour (137), while it did not prevent inescapable foot shock-induced anxiety (231), indicating that the beneficial effect of EE depends on the nature of the stressor.

Taken together, the behavioural data demonstrating that both NPY and EE promote stress resilience and my finding that EE increases hippocampal NPY expression suggest that EE promotes stress resilience via hippocampal, but not amygdalar NPY.

4.5 Influence of environmental enrichment on hippocampal synaptic ultrastructure

As described above, EE alters stress-induced c-Fos and NPY expression in the brain, with the largest effects seen in the hippocampus. This raised the question whether these hippocampal biochemical changes are accompanied by structural changes. I therefore investigated whether EE for 5 or 9 weeks changes the hippocampal ultrastructure, as measured by the structure and number of synapses, within the DGpl of female mice or the CA1 of male mice. Data analysis revealed, however, that EE did not result in any changes at the synaptic level.

Reports about EE-induced changes in either synapse density or other synaptic parameters are very diverse. An early study described that long-term EE for 30 months does not modify synaptic density in the rat frontal cortex, but changes the density of synaptic vesicles (254). One year later another study found that EE for 8 weeks enhances synapse density in the CA1 region of mice (140). This change was not seen by Xu et al. (192), who reported that EE for only 2 weeks failed to alter synapse density within the CA1 or the parietal cortex. They found, however, that EE decreased synaptic cleft width and increased postsynaptic density length in both areas, while another study did not find any changes of the synaptic ultrastructure in EE-housed animals (192,255). Other studies detected EE-induced synaptic alterations outside the hippocampus. For example, a 50-day EE paradigm enhanced synaptic density, but did not alter the architecture of synapses within the primary somatosensory cortex or the cerebellum, and an 8-week enrichment period in the wilderness increased the density of excitatory and inhibitory synapses within the C2 barrel cortex of mice (191,256). These diverse findings of studies measuring synaptic ultrastructure are complemented by conflicting results of electrophysiological studies in both the CA1 and the DG (257).

Given this divergent regulation of brain ultrastructure by environmental conditions, the current study set out to examine synaptic density, diameter of the presynaptic membrane and the postsynaptic density, width of the synaptic cleft and the number of docked and undocked vesicles within CA1 and DG. In previous experiments within this lab it was found that EE for 5 or 9 weeks enhances the performance of male and female mice within the Morris water maze, a behavioural

test for spatial learning and memory (172). It is also known that learning and memory depends on the hippocampus (258), which tempted me to investigate this structure. Surprisingly, no change in any of these parameters was detected. Comparing this result with other enrichment studies appears difficult as nearly every study used its own enrichment paradigm in terms of duration and cage setup. Taking this into account, the current results might be at best comparable to the study of Rampon et al. (140). Especially the region examined, the duration of EE, the age of the animals and the species used are similar to the current experiment. However the findings of Rampon et al. (140) are in conflict with the present results. Rampon et al. (140) describe that EE enhances synapse density in the CA1, while my experiment did not detect such an effect. These discrepancies could be explained by distinct differences between the two experimental paradigms used. For instance, Rampon et al. (140) used a different mouse strain; mice were exposed to EE only 3 hours daily and then put back into a standard cage, and EE involved a larger box (1.5 x 0.8 x 0.8 m) containing several other tools of a special design than the current paradigm. Especially the daily reexposure to EE for a limited period of time could be the decisive stimulus enhancing the number of synapses in their study, but not in my experiment. No comparison can be made for the DG, because my study is the first investigating synapse density within this brain structure.

Taken together, housing female or male C57Bl6/N mice under EE for 5 or 9 weeks did not alter synaptic ultrastructure in the DGpl or CA1, respectively. Data from this lab showed, however, that both 5-week EE of female mice (unpublished data) and 9-week EE of male mice (172) improves memory in the Morris water maze. This indicates that the EE-induced improvements in cognitive behaviour do not require structural alterations of synapses within the hippocampus.

4.6 Influence of gastrointestinal inflammation on stress-induced c-Fos expression in the brain under standard and enriched housing

In the paradigm I used to investigate whether housing animals under EE affects the brain response to WAS, an external psychological stressor, I also checked whether visceral inflammation, an internal stressor, may alter this brain response. Thus the same brain regions as described in chapter 4.3 were analysed

in animals with gastritis or colitis and compared to control animals. Furthermore I analysed whether stress-induced c-Fos expression of animals with visceral inflammation differs with their housing condition (i.e standard vs. enriched housing).

Under standard housing, experimental gastritis and colitis inhibited stress-induced c-Fos expression in several nuclei of the brain, most prominently in the CeA, the CA1 region of the hippocampus and the ILC. The WAS-induced expression of c-Fos in the BLA, MeA, DGgl, CC and PVH was also reduced by gastrointestinal inflammation, albeit to a smaller extent. The effect of DSS-evoked colitis was in general more pronounced than that of IAA-evoked gastritis, which may be related to different degrees of inflammation in the colon and stomach or to region-specific differences in signal transmission from the gut to the brain. The present observations are important inasmuch as they show that an internal (systemic) stressor can modify the cerebral response to an external (psychological) stressor. This finding is analogous to explaining experimental and clinical observations that gastrointestinal inflammation and stress can interact with each other in aggravating gastrointestinal disease, hyperalgesia and anxiety, which are prevalent in patients with IBD and irritable bowel syndrome (202,203). This part of the discussion was published in an original article (172).

Conceptually, the interaction between inflammation and stress can take place at several levels of the gut-brain axis. Within the gut, WAS has been found to enhance the permeability of the intestinal mucosa (259,260), which is likely to promote activation of the mucosal immune system and the development of mucositis. Accordingly, stress has been found to aggravate experimentally induced colitis (68) and to reactivate quiescent colitis (72). Stress and inflammation are also able to cause mechanical and chemical hypersensitivity in the gastrointestinal tract (170,174,185,261-263), but an interaction between stress and inflammation in the development of visceral hypersensitivity has been negated (164). Within the brain it has been shown that colitis upregulates the expression of CRF mRNA in the magnocellular part of the PVH but blunts the stress-induced increase in CRF transcription in the parvocellular part of the PVH (264). This part of the discussion was published in an original article (172).

EE did not alter the inhibitory effect of gastrointestinal inflammation, notably colitis, on WAS-induced c-Fos expression in the CC, MeA and CA1 region of the

hippocampus. In the ILC and CeA, however, the inflammation-evoked inhibition of the c-Fos response to WAS was blunted or abolished by EE, whereas in the DGgl it was greatly amplified. These observations further attest to the ability of environmental factors to modify the complex interactions between internal and external stressors in the prefrontal cortex – amygdala – hippocampus circuitry in a topographically specific manner. The divergent effects which EE exerts in the CeA and DGgl emphasize the opposing roles of the amygdala and hippocampus in the orchestration of the stress response (4,91,211,216,247,248). This part of the discussion was published in an original article (172).

4.7 Influence of environmental enrichment and water avoidance stress on gastrointestinal inflammation

As described in Reichmann et al. (172), the impact of gastrointestinal inflammation on the cerebral stress response to WAS under standard and enriched housing was studied by the use of IAA-induced gastritis (170,262) and DSS-induced colitis (185,261). Both models of inflammation are convenient to handle because IAA and DSS are added to the drinking water and the degree of inflammation can be titrated by the concentration of IAA and DSS in the drinking water. The severity of inflammation was estimated by the increase in the MPO levels in the gastric and colonic wall, respectively (127,185), which reflect inflammation-associated infiltration of neutrophils and monocytes into the tissue (186). Previous experiments had established that the gastritis evoked by 0.1 % IAA and the colitis evoked by 2 % DSS are mild and do not cause any substantial deterioration of mucosal architecture (184,185). The induction of colitis was, in addition, confirmed by a rise of DAS which relates to the overall health status of the animals and histological evaluation of the colon wall, which demonstrated a decreased number of crypts and mucosal leukocyte infiltration after DSS treatment. As judged from the increase in tissue MPO levels, the degree of gastric inflammation appeared to be lower than the degree of colonic inflammation.

EE failed to modify IAA-induced gastritis as judged from its lack of effect on DAS and gastric MPO content, whereas the susceptibility to DSS-evoked colitis was enhanced by EE as deduced from an increase in weight loss, DAS and colonic MPO content. The MPO data indicate that EE promotes DSS-induced

colitis by enhancing leukocyte infiltration in the colonic tissue (186). Although it was beyond the scope of my study to analyse this unexpected finding, some mechanistic explanation is offered by reports that EE might increase the activity of the immune system. Thus, there is evidence that EE enhances natural killer cell activity in the spleen (136), causes inconsistent changes in circulating cytokine levels and reduces circulating IgG1 levels (177). Moreover, EE can promote T-lymphocyte infiltration in a model of viral encephalitis (265). In view of these findings I hypothesize that DSS-induced colitis is particularly susceptible to the EE-evoked sensitization or stimulation of the immune system, which results in aggravation of inflammation, disease severity and weight loss. The increased weight loss of DSS-treated EE-housed mice may also be related to alterations in food intake. In this regard I found that DSS treatment decreases food intake over the 7-day treatment period. It is likely that the more intense colonic inflammation of EE-housed mice led also to a more pronounced reduction of food intake. It remains to be investigated, however, whether a propensity for inflammation or enhanced locomotion and exploration of EE-housed mice can contribute to this finding. This part of the discussion was published in an original article (172).

Other factors that could play a role in the EE-induced enhancement of DSS-evoked colitis include the gut microbiota and the ENS. Inflammatory conditions affect enteric neurons which, in turn, are able to modify intestinal immune responses and epithelial barrier function (266). DSS is known to enhance colonic mucosal permeability, increase the penetration of bacteria into the intestinal mucus layer, and change gut microbial diversity (50,267-270). Since these and other environmental factors have an impact on the severity of DSS-induced colitis, great care was taken to avoid contamination of the EE cages by external microbiota. It awaits to be examined whether EE per se modifies mucosal permeability or alters the gut microbiota in a way that increases mucosal vulnerability by DSS. This part of the discussion was published in an original article (172).

In a further experiment I investigated whether acute WAS alters DSS-induced colitis severity. Thus MPO, DAS and additionally colon length were assessed in stressed and unstressed mice with and without colitis. As in the above mentioned experiment, DSS treatment increased the colonic MPO content and DAS, while acute WAS did not affect these parameters in both controls and

animals with colitis. In addition I found that DSS treatment reduced colon length, which is in accordance with other studies (54,55,185,271). WAS, however, affected colon length in the opposite way. This may be explained by the observation that enhanced sympathetic nerve activity, which is the case during stress exposure, lowers smooth muscle tone of the distal colon resulting in colonic relaxation (272). Several other studies investigated how stress affects the development of colitis. An initial study showed that repeated restraint or WAS exacerbates TNBS-induced colitis (67). This was confirmed by several other studies with different chronic stress paradigms using the DSS colitis model (54,55,63,271). Furthermore Melgar et al. (72) reported that chronic WAS can reactivate quiescent DSS-induced colitis, while Larsson et al. (164) reported that chronic WAS before or after DSS treatment does not alter disease severity (72,164). Information about the effect of acute stress on colitis severity is limited. This is the first study investigating the effect of acute WAS in DSS-induced colitis. My finding that acute WAS does not influence DSS-induced colitis is consistent with the study of Kresse et al. (264) demonstrating the same result but in TNBS-induced colitis (264). Therefore it is speculated that one session of WAS is not enough to alter colitis severity.

4.8 Influence of gastrointestinal inflammation, environmental enrichment and water avoidance stress on circulating corticosterone levels

In experimental protocol 2 the circulating levels of corticosterone were measured when the stress-induced expression of c-Fos in the brain was visualized (i.e. 90 minutes after a 30-min exposure to WAS). The data revealed that the corticosterone levels of DSS-treated EE-housed animals were higher than the levels of the respective control group. Although circulating corticosterone is thought to be an index of HPA axis activity (136,177,273), the elevated corticosterone levels post-stress in mice under conditions of EE and colitis were not associated with an increased expression of c-Fos in the PVH. In assessing this finding it is appropriate to consider that colitis has differential effects on the magnocellular and parvocellular part of the PVH and can even blunt the corticosterone response to WAS (264). This part of the discussion has also been published in an original article (172).

Without knowing the basal (pre-stress) corticosterone concentrations it is not possible to deduce the magnitude of the corticosterone response to WAS (which usually is maximal within 20 min after exposure to stress (274)) from the post-stress levels of this glucocorticoid (172). To fill this gap I investigated in experimental protocol 6 whether the post-stress corticosterone levels (i.e. 90 minutes after a 30-min exposure to WAS) differ from basal levels in mice with colitis or control mice. I found that 2 hours after WAS corticosterone levels were similar to basal levels. This was true for both control and colitis animals, indicating that colitis did not impair the cessation of the stress response or prolong the corticosterone response to WAS. However I also found that the basal levels of corticosterone are enhanced in animals with DSS-induced colitis, a finding that was also seen in the TNBS colitis model (275,276). Enhanced corticosterone levels in the course of colitis support the notion that DSS-induced colitis is a chronic internal stressor elevating stress hormone levels. The severity of inflammation may correlate with corticosterone levels as suggested by the observation that the highest corticosterone levels were measured in animals with severe colitis as seen under EE. Given that an appropriate HPA axis response to internal and external threats, as investigated in the present experiments, is crucial for homeostasis (277), the enhanced levels of corticosterone under conditions of EE and colitis can be seen as an attempt of the body to counteract inflammation and restore homeostasis (91,216). The basal and post-stress levels of corticosterone measured here provide some information on the time course of the corticosterone response but further experiments need to complement these findings of stress-induced corticosterone levels in the setting of colitis. Especially the maximal amplitude of the corticosterone response may be altered and requires further investigation. These findings have been published in an original article (172).

4.9 Influence of water avoidance stress and colitis on behaviour of mice

The results of experimental protocol 2 suggested that visceral inflammation, in particular colitis, strongly alters stress-induced neuronal activation of the hippocampus, amygdala, medial prefrontal cortex and hypothalamus. All of these

brain structures are involved in the regulation of behaviours including memory, fear, anxiety and stress coping (90,242,278). In several behavioural experiments I tested therefore if the alterations in the cerebral c-Fos response to WAS under colitis translate into alterations in stress coping and emotional behaviour.

First, stressed and unstressed animals with and without colitis were tested in the L/D box test, a test to evaluate anxiety-like behaviour (193) followed by a test for anhedonia, the SP test (194). In both tests DSS treatment did not alter behaviour, while acute WAS changed the behaviour in the L/D box, but not in the SP. Specifically WAS-treated animals spent more time in the light compartment of the L/D box, which is usually an index for an anxiolytic phenotype. This has to be interpreted with caution, however, since locomotor activity was also increased in WAS-treated animals. Therefore, the observed anxiolytic effect is probably false-positive and produced by the enhanced locomotor activity (193), a result of a higher arousal state of WAS-treated animals. Enhanced locomotor activity following stress is in accordance with another study demonstrating increased wheel running in mice after acute exposure to footshock stress (279). The current result also suggests that DSS-induced colitis does not modify anxiety, which is in contrast to evidence from the literature demonstrating that visceral inflammation can induce anxiety (68,127,184,280-282). However, so far only one previous study detected an anxiogenic effect of visceral inflammation in the L/D box using *Trichuris muris*, a non-invasive parasite (281). In contrast to the DSS-induced colitis model used here, *T. muris* infection develops over a course of 4 weeks and primarily affects the caecum. Therefore the different inflammation model and mouse strain used may explain the conflicting result regarding colitis-induced anxiety in the L/D box. There are also some reports about anxiety-inducing effects of DSS. Data from this lab indicated that male, but not female mice with DSS-induced colitis are more anxious in the elevated plus maze and open field, while another study reported that male AKR mice tested after 3 cycles of DSS treatment are more anxious in the step down test (127,282). These contrasting results could be accounted for by the different mouse strains used, with the use of the L/D box as an anxiety test being another factor. In contrast to the other tests for anxiety, the L/D box test is based on the conflict of mice to avoid brightly lit spaces and their inherent trait to explore the environment (193). It could be that DSS-induced colitis does not influence this special form of anxiety.

After testing mice of the different treatment groups in the L/D box, they were subjected to the SP test. This test failed to reveal any differences between the treatment groups. There is evidence that chronic stress, i.e. maternal separation and chronic mild stress, reduces SP of rats (283,284). My data showed that WAS, an acute mild stressor, is not strong enough to alter SP. Regarding the effects of inflammation on anhedonia, a study in humans reported that systemic treatment with endotoxin increases depressed mood over time (285), while a study in rats failed to detect changes in SP after neonatal lipopolysaccharide (LPS) administration (286). In contrast, this lab and another group found that LPS treatment leads to a decrease in SP (287,288). LPS-induced and DSS-induced inflammation, however, produce different patterns of circulating cytokines, which are considered as mediators of depression-related behaviour, potentially explaining the differential effects of DSS and LPS in the SP test (289-291).

Next I tested whether DSS-treated and WAS-treated mice behave differentially in the TST, a test for depression-like behaviour and stress coping (173). Neither colitis nor stress altered the immobility parameter suggesting that the treatments do not alter depression-like behaviour, but WAS changed the active behaviour during the test. Specifically, it increased the time mice spent curling. Analysis of the TST as described allows subdividing the active movements of a mouse during the test into curling and swinging behaviour (195). A study using this paradigm reported that curling behaviour in the TST is increased in mice after opioid administration and reduced in μ -opioid receptor knockout mice (195). Given that WAS also modifies curling behaviour, it may be speculated that WAS modifies opioidergic neurotransmission within the brain. Regarding the negative finding in colitis animals, the current results are in accordance with previous work from this lab showing that DSS treatment does not modify depression-like behaviour of male mice in the forced swim test (127).

Finally I found that the behaviour of colitis mice is changed during the WAS session. Analysis of this period revealed that colitis animals tended to escape more often from the aversive stress situation than control animals. This change in behaviour may reflect elevated arousal levels and differences in stress perception and processing. Elevated levels of proinflammatory cytokines in the course of DSS colitis (289), modified gut-brain signalling (1) and the observed changes in glucocorticoid signalling may contribute to this finding.

Taken together, two hours after WAS exposure stressed mice showed several behavioural disturbances compared to non-stressed animals independent of DSS treatment, which may be explained by the arousal-producing effects of this stressor. Analysis of the number of c-Fos expressing cells within the limbic system revealed that WAS-induced neuronal activation differs between DSS-treated and control mice. However, this molecular change did not translate into behavioural alterations. Mice with DSS-induced colitis only behaved differently during the WAS session. This indicates that a single exposure to WAS produces short-term, but not long-term behavioural alterations in DSS-treated animals.

4.10 Influence of water avoidance stress and colitis on neuropeptide Y expression in the brain

Given that colitis alters stress-induced c-Fos expression and modifies circulating corticosterone levels, I compared the neuronal expression of NPY, a stress-relevant neuropeptide, between DSS-treated and WAS-treated animals and the respective control groups. First I quantified the stress-induced NPY expression in the DGpl and BLA from animals with visceral inflammation by grain counting. Analysis of these data failed to reveal any difference between the animals with visceral inflammation and the control group. However, from these data one cannot deduce the effect of WAS on NPY expression. Thus in another experiment I analysed NPY expression in the hippocampus, amygdala and hypothalamus of stressed and unstressed mice with and without colitis using PCR. I found that colitis increased hypothalamic NPY expression and decreases amygdalar NPY expression. WAS enhanced NPY in the amygdala of mice with colitis, but did not alter amygdalar NPY of control animals.

Besides an important role in the stress response, the hypothalamus is a crucial brain structure for initiating food intake. Studies have shown that hypothalamic NPY, mainly localized in the ARC, is a key peptide in this process (98). As described above I found that DSS treatment leads to a reduction in food intake during the course of colitis. Therefore the observed increase of hypothalamic NPY in colitis animals is probably a counterregulatory mechanism to restore energy homeostasis. Increased hypothalamic NPY expression together with decreased food intake has also been observed in other inflammation models

(292,293). The question remains, however, whether altered gut-brain signalling during the course of DSS colitis contributed to the observed NPY changes. As reviewed in Holzer et al. (98), information about the effects of visceral inflammation on NPY mRNA or protein levels within the brain is limited. Evidence suggests that TNBS-induced colitis increases the NPY concentration in brain and plasma (61) whereas the expression of NPY mRNA in the brain remains unaltered by *Helicobacter pylori* infection (126). Furthermore, NPY release in the PVH is increased in animals with colitis, but healthy animals pair-fed with the colitis group (same food intake/day) show the same increase in NPY release from the PVH as colitis animals (294). This suggests that reduced food intake and not inflammatory signals during colitis is the main drive for changes in hypothalamic NPY levels.

For the first time I discovered that DSS-induced colitis reduces NPY expression within the amygdala. In contrast to the hypothalamus the amygdala may play a role in food preference, but not in total food intake (295). Evidence suggests, however, that the amygdala plays an essential role in fear and stress processing (81). Less NPY within the amygdala of colitis animals indicates a deficit in stress coping or enhanced emotionality (81,114). Interestingly, WAS counteracted the DSS-induced decrease of amygdalar NPY expression. Additionally, both PCR and grain counting showed, that two hours after WAS, NPY expression did not differ between colitis and control animals. In other words, WAS increased NPY expression in animals with colitis, but not in controls. This indicates that the WAS session was differentially perceived and processed by the different treatment groups, which is in accordance with the above described altered behaviour of mice with colitis during the stress exposure. Within the hippocampus neither colitis nor stress altered NPY expression as shown by PCR and grain counting. This indicates that hippocampal NPY is not crucial for the processing of acute psychological stress or chronic inflammatory stress.

The current experiment suggests that WAS per se does not alter brain NPY expression. This is surprising as several studies reported that stress exerts region-specific effects on NPY expression within the brain. As reviewed in Holzer et al. (98), acute restraint stress decreases NPY expression in the amygdala, whereas intermittent restraint stress over a 10-day period increases NPY expression within this structure (108,109). Exposure to footshocks for as little as 15 min is able to enhance amygdalar NPY expression as measured 2 weeks after the intervention,

while chronic variable stress decreases amygdalar NPY levels (110,111). In my experiment a 30-min WAS session did not change amygdalar NPY expression in control animals. Together with the pertinent literature, this finding demonstrates that changes in amygdalar NPY expression depend on the type and duration of stress exposure as well as on the time point of NPY measurement. As regards hypothalamic NPY expression, a recent study failed to observe alterations of hypothalamic NPY mRNA up to 60 minutes after a 30-min restraint stress session, which is in accordance with the current observations and another study (108,296). This is in agreement with Conrad et al. (297) who suggested that NPY expression of the ARC increases 24 hours after stress stimulation, but not earlier (297). However, there is also contradictory evidence that NPY expression in the ARC increased 60 minutes after stress and that the NPY mRNA levels in this nucleus were enhanced two hours after 15-min foot shock stress (298,299). It appears, therefore, that stress-induced NPY mRNA levels, if measured across the whole hypothalamus, can be increased, unchanged or blunted, depending on the time point of NPY measurement. However, in the ARC stress always elevates NPY expression. The finding that WAS did not increase hippocampal NPY expression is in accordance with a previous study. Specifically, Sweerts et al. (298) found that prepro-NPY mRNA expression in the dentate gyrus did not change after acute and repeated restraint stress (298).

In summary, DSS colitis exerts region-specific effects on brain NPY expression. In contrast, WAS does not modify NPY expression of healthy animals, but changes amygdalar NPY in animals with colitis. Therefore it is proposed that an alteration of amygdalar NPY levels is a crucial factor for altered stress responsiveness and coping during visceral inflammation. This finding also supports the concept that an internal stressor (colitis) may alter the sensitivity to an external stressor.

4.11 Key findings and implications

In conclusion, my data contribute to the growing literature that stress is an important factor in the course of visceral inflammation (12,64,72). For the first time, I could demonstrate that stress-induced neuronal activation in the brain, as measured by the expression of the immediate early gene product c-Fos, is altered

by gastritis and colitis. In addition, colitis exerts brain region-specific effects on the NPY system. In a translational view, these data add further information on how stress can negatively affect the course of IBD and complement some fMRI studies demonstrating brain alterations in IBD (93,94). It appears that most regions of the limbic system are modified at a molecular level via altered gut-brain signalling in the course of visceral inflammation, resulting in differential stress responsiveness and probably in a deficit in emotional stimulus processing.

Furthermore, this is also the first report that EE, a stress-reducing housing condition, influences stress-induced neuronal activation and modifies the effects of visceral inflammation locally and within the brain. I also found that EE alters hippocampal NPY expression, but does not affect synaptic ultrastructure in this region. Thus, the stress resilience-promoting abilities of EE may be related to differential processing of stress, on the one hand, and altered activity of the NPY system, on the other hand. My findings support the idea that cognitive or social stimulation can protect from the negative consequences of stress (171). The data also reemphasize the notion that the hippocampus plays a key role for the biological effects of EE (175). My data, however, also demonstrate a downside of using EE as a housing condition. In contrast to studies reporting beneficial effects of EE in various brain disease models (300), my study is one of the few reports demonstrating negative effects of EE (i.e. increased susceptibility to colitis). This surprising finding may be explained by the immunomodulatory properties of EE, but still needs further investigation (136,177,265).

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