Diplomarbeit

Impact of colitis on stress-induced activation of central neurons

eingereicht von

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Geb.Dat.: 14.04.1981

zur Erlangung des akademischen Grades

Doktorin der gesamten Heilkunde

(Dr. med. univ.)

an der

Medizinischen Universität Graz

ausgeführt am

Institut für Experimentelle und Klinische Pharmakologie

Forschungseinheit für Translationale Neurogastroenterologie

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Graz, Juni 2014 *Julia Pauer*

Preamble

"Health is a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity."

(Preamble to the Constitution of the World Health Organization, New York, 19-22 June, 1946)

Acknowledgments

To begin, I would like to express my most sincere appreciation to Univ. Prof. Dr. Peter Holzer for giving me the great opportunity to write this thesis under his supervision, and for his heartfelt support and patience throughout the process. I would also like to thank my tutor, Dr. Florian Reichmann, from whom I have learned so much, and without whom I would have stumbled.

Special thanks go to the team of the Research Unit of Translational Neurogastroenterology, especially to Dr. Aitak Farzi, MSc. Piyush Jain and MSc. Ahmed Hassan for their support and active exchange of ideas. Thanks are also due to Malika Stuerznickel for proofreading.

I would like to express my gratitude to my dear friends for their companionship through the sometimes hard, but mostly happy times during our studies and in life.

Thank you to my parents, Johanna and Hans Pauer, my brother Janos, and my partner Daniel for their encouragement, their belief in me, and their calming presence when I juggled too many things at once. I could not have done it without you.

Zusammenfassung

Hintergund: Es ist bekannt, dass Stress negative Auswirkungen auf Krankheitsverläufe hat. So auch bei gastrointestinalen Entzündungen und psychiatrischen Erkrankungen. Bei PatientInnen mit entzündlichen Darmerkrankungen gibt es zudem eine höhere Prävalenz an Angststörungen und depressiven Erkrankungen.

Fragestellungen: Basierend auf diesen Prämissen ist das Ziel dieser Studie den Einfluss von Stress und Kolitis auf die neuronale Aktivierung in stressrelevanten Hirnarealen von Mäusen zu überprüfen.

Methoden: Die Mäuse wurden 7 Tage mit 2% dextran sulfate sodium (DSS) im Trinkwasser behandelt um eine Kolitis hervorzurufen während die Kontrollmäuse reines Trinkwasser erhielten. Nach der Behandlung wurde die Hälfte der Mäuse einer 30minütigen Einheit des water avoidance stress (WAS) ausgesetzt, die andere Hälfte stellt die ungestresste Kontrollgruppe dar. 90 Minuten nach dem WAS wurden die Gehirne entnommen, aufbereitet und der immunhistochemischen Analyse für c-Fos, einem Marker für neuronale Aktivierung, unterzogen. Für die statistische Auswertung wurden die c-Fos exprimierenden Zellen in Gehirnregionen des limbischen Systems computer-assistiert quantifiziert.

Ergebnisse: Stress erhöhte die Zahl der c-Fos exprimiernden Zellen im CA3 areal des Hippocampus ($P<0.05$), in der basolateralen ($P<0.001$), zentralen ($P<0.001$) und medialen Amygdala ($P<0.001$), dem paraventrikulären hypothalamischen Nukleus ($P<0.001$), dem cingulären Cortex ($P<0.001$) und dem infralimbischen Cortex ($P<0.001$) unabhängig von Kolitis. In der medialen Amygdala induzierte Stress in mit DSS behandelten Tieren stärker ($P<0.001$) als bei mit Trinkwasser versorgten ($P<0.05$) die Anzahl der c-Fos exprimierenden Zellen. In einigen Hirnarealen der Kontrolltiere führte DSS zu einer nicht-signifikanten, aber deutlichen Verringerung der Anzahl an c-Fos exprimierenden Zellen

Schlussfolgerung: Die Studie zeigt deutliche Veränderungen der c-Fos-Expression im limbischen System bei Stress. In der medialen Amygdala kam es zu einer besonders starken Erhöhung der Anzahl an c-Fos exprimierenden Zellen bei an Kolitis erkrankten Tieren. Zusammenfassend offenbaren diese Entdeckungen neue funktionale und neuroanatomische Aspekte der Interaktion zwischen externen psychologischen und internen physikalischen Stressoren im Gehirn.

Abstract

Background: Stress can aggravate various pathologies including chronic inflammatory and psychiatric disorders. A higher prevalence of anxiety and depressive disorders is documented in patients suffering from inflammatory bowel diseases.

Aims: Given this background, the aim of this study was to test the effects of acute stress and colitis on activation of stress-relevant neurons in the mouse brain.

Methods: Mice were treated with 2% dextran sulfate sodium (DSS, added to the drinking water) to induce mild colitis, whereas control mice drank normal tap water. After 7 days of treatment, the mice either underwent a 30-minute session of water avoidance stress (WAS) or remained unstressed. Ninety minutes after WAS brains were removed and processed for immunohistochemistry of c-Fos, a marker of neuronal activation. The number of c-Fos expressing cells in brain regions of the limbic system was quantified by computer-assisted cell counting.

Results: Stress increased the number of c-Fos expressing cells in the cingulate cortex ($P<0.001$), the infralimbic cortex ($P<0.001$), the CA3 region of the hippocampus ($P<0.05$), the basolateral ($P<0.001$), central ($P<0.001$) and medial amygdala ($P<0.001$) and the paraventricular hypothalamic nucleus ($P<0.001$) independently of colitis. In the medial amygdala, the stress-induced c-Fos expression in mice with colitis was larger ($P<0.001$) than in healthy animals ($P<0.05$). In control animals DSS reduced the number of c-Fos expressing cells in several areas to an insignificant amount.

Conclusion: The current experiments show the large impact of stress on central activation and that colitis enhanced stress-evoked stimulation of distinct neurons in the medial amygdala. These results provide functional and neuroanatomical evidence for a distinct interaction of external psychological stressors and internal physical stressors (colitis) in the limbic system of the brain.

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Table of abbreviations

ACTH	adrenocorticotropic hormone
AD	antibody diluent
ANS	autonomic nervous system
BGA	brain-gut axis
BLA	basolateral amygdala
CAS	computerized image analysis system
CC	cingulate cortex
CeA	central amygdala
CNS	central nervous system
CD	Crohn´s disease
CRD	colorectal distension
CRF	corticotropin-releasing factor
DG	dentate gyrus
DSS	dextran sulfate sodium
EE	enteroendocrine cells
ENS	enteric nervous system
GBA	gut-brain axis
GID	gastrointestinal diseases
GIT	gastrointestinal tract
HC	hippocampus
HPA	hypothalamus-pituitary-adrenal
IBS	irritable bowel syndrome
IHC	immunohistochemistry
ILC	infralimbic cortex
LC	locus coeruleus
MeA	medial amygdala
NA	noradrenaline
NTS	nucleus tractus solitarius
PAN	primary afferent neurons
PBS	phosphate-buffered saline
PFC	prefrontal cortex
PNS	parasympathetic nervous system

PVH	paraventricular hypothalamic nucleus
ROI	brain regions of interest
SNS	sympathetic nervous system
TLR	toll-like receptor
TNBS	trinitrobenzenesulfonic acid
UC	ulcerative colitis
WAS	water avoidance stress
WB	washing buffer

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1 Introduction

1.1 Topic and aim of the thesis

Acute and chronic stress, either physical or psychological, has an impact on the physiology and behaviour of animals as well as humans. As a clinically relevant issue it has been the topic of many clinical and basic research projects in the last few decades. Additionally, the influence stress has on brain and body functions, is in the focus of research into both gastrointestinal and neuropsychiatric disorders. Evidence rises for the negative impact of stress on the progression of several different diseases like gastrointestinal inflammation (1-3), primary psychiatric disorders (4,5), skin diseases (6) or wound healing (7).

In this present work I will focus on the effects of water avoidance stress (WAS), a psychological stressor and acute dextran sulfate sodium (DSS)-induced colitis, a physiological stressor on neuronal cell activation in several brain regions of interest (ROI) in mice. We chose DSS to induce colitis because this method that has been used for almost 20 years as a rodent model for acute and chronic colitis (8). Neuronal activation in the brain was measured by the quantification of the number of c-Fos expressing neuronal cells in the amygdala (basolateral amygdala (BLA), central amygdala (CeA) and medial amygdala (MeA)), the paraventricular hypothalamic nucleus (PVH), the medial prefrontal cortex (PFC; cingulate cortex (CC), infralimbic cortex (ILC)) and in subareas of the hippocampal formation (granular cell layer of the dentate gyrus (DG), CA1 and CA3 area). These brain regions are key elements of the central stress circuitries and important modulators of the stress response (1,9).

The underlying presumption of the study is the bi-directionality of gut-brain-interactions in health and disease. Hence, the possible effects the stressed or unstressed brain has on body functions such as inflammation of the gastrointestinal tract (GIT) are considered along the effects body functions and irritated body functions like inflammation have on the brain and neuronal cell activity.

To illustrate the rationale and the background of this project, I will first explain internal and external stress and its neuronal circuitries as well as its influence on

the GIT. A general overview on the topic of the gut-brain axis (GBA) will be given and the description of gastrointestinal inflammation will conclude the introduction.
The main part of the thesis consists of my research on 32 mice, subjected to WAS and DSS-induced acute colitis, including methodological approaches used and the results of my experiment.
In the résumé the specific ROI functions will be discussed to query the study´s outcome and to phrase further questions upon this subject.

1.2 Internal and external stressors

A lot of research has been done regarding the different processing of diverse stressors. Generally, a division can be made between internal (physiological) and external (psychological) stressors. In 1997, Herman and Cullinan proposed the terms “systemic” for physiological, and “processive” for so called psychological threats (10). This is supposed by the fact that restraint stress increased adrenocorticotrophic hormone (ACTH) and corticosterone in the PFC while no effects were induced by ether. Also effects on the hippocampus (HC), caused by corticosterone and ACTH, could be seen after restraint but not after hypoxia. This Herman and Cullinan (10) referred to as “limbic-sensitive” and “limbic-insensitive” kind of stressors, given that psychologically perceived threats need comparison/memory with previous experiences through higher neuronal systems. Notable is also that limbic stress pathways include multiple centres, including PFC, amygdaloid nuclei and hippocampal formation and multiple sensory modalities to respond. In contrast, physiological stressors like ether and hypoxia are direct threats to survival and don´t include the limbic system in the defence mechanism. Both kinds of stressors, however, activate the PVH. The “systemic” stimulus, like hypoxia, activates it directly through afferents via the nucleus tractus solitaries (NTS) and other direct pathways. The “processive” stress, like exposure to a new environment, activates the PVH through interlinked centres which process and interpret the stimulus before it reaches the PVH (10).

1.3 Stress and its pathways through body and brain

According to a definition given by H. Selye, pioneer in stress research from the middle of the 20th century, stress is defined as an acute threat to the homeostasis

of the body (2). The stressor can be both perceived (psychologically) and/or real (physiologically). The stress response describes the reaction of the whole being "to defend the stability of the internal environment and to ensure the survival of the organism" (1) also known as "allostasis" (11). Stress has gained a negative connotation when referred to by the general public. But it is already known that intermediate mild stress (psychological and physiological) has positive effects on stress resilience, the ability to cope with stress (12). Chronic stress can cause imbalance and damage in structure and function of body and brain dependent on e.g. high cortisol levels and to a certain amount on the individual, a situation called "allostatic overload". As proposed formerly, stressors may be broad in variety, but the known bodily responses were supposed to be rather monotonic and mostly independent of the stressor, according to Selye. Not all research could prove this unvaried response theory. In the meantime there is a lot of evidence for a variety of different stress responses, depending on duration, intensity and type of stress (13-15). Stress has an impact on the central nervous system (CNS) and leads to neuroendocrine effects on the body. It always includes a certain share of the hypothalamic-pituitary-adrenal axis (HPA), the autonomic nervous system (ANS) and descending monoaminergic pathways. The outflow system of stress-induced responses is termed as the emotional motor system. It is composed by autonomic motor, neuroendocrine and pain modulatory pathways (16). Crucial places for stress processing are the hypothalamus for the hypothalamus-pituitary-adrenal (HPA) axis and the brain stem with the locus coeruleus (LC) for the ANS. Amongst the higher centres connected to the HPA axis and ANS are the mesocortical/mesolimbic systems, influencing emotions and anticipatory phenomena. Amygdala and hippocampal formation act in context with initiation, propagation and termination of stress system activity, while the arcuate nucleus is involved in the setting of pain sensations (9). In the CNS both corticotropin releasing factor (CRF) and noradrenaline (NA) stimulate arousal, attention, the mesocortical dopaminergic system and the hypothalamic endorphin system, which decreases pain sensations. Corticosterone, as well as CRF, acts as the main effector components of the HPA axis, while NA is the main transmitter of the sympathetic branch of the ANS (9). HPA axis and ANS are reciprocally influencing each other (15). The stress response systems have effects on the whole body and further interlinked systems, e.g. the immune system and the enteric nervous

system (ENS). This relationship was already proved by Selye in 1936 experimentally. His findings showed adrenal hypertrophy, involution of the lymphatic nodes and gastric lesions conjoined in rats submitted to various noxae (17). Selye also claimed three stages of the stress response, depending on the time of ongoing stress. An initial “alarm reaction”, analogous to Cannon’s “fight or flight” response, a stage of adaptation, with resistance to the stressor, and eventually a stage of exhaustion and death of the organism” (15). My study could be assigned in between the initial alarm and the adaptation stage because of the relatively short exposure to the stressors.

Psychological and physiological stress is perceived through cortical and brainstem structures, which define an external situation as stress, and visceral and somatic afferents, which inform the brain about homeostatic disturbances of the body (16). The participants in the stress response system are linked to each other in a very complex manner and act in healthy populations simultaneously. In contrast patients with ulcerative colitis (UC) and Crohn´s disease (CD) show uncoupled reactions of HPA axis and ANS (18).

In the following chapters I will analyse the main players and their influence in the brain-gut axis (BGA).

1.3.1 The HPA axis

The hypothalamus influences the pituitary gland via CRF, in a circadian manner in healthy subjects, and it massively stimulates the pituitary gland in stressed individuals. Precisely, CRF induces the release of ACTH in the anterior pituitary gland. ACTH, after travelling through the systemic circulation and binding to adrenal ACTH receptors, leads to steroid outflow from the adrenal medulla into the blood stream. Glucocorticoids have a variety of roles. One of them is the negative feedback on the HPA axis itself and the ANS, and activation of fear centres like the amygdala (9). Specifically, the central negative feedback mechanism of corticosteroids on the HPA axis works through glucocorticoid receptors (GR) mainly in the pituitary and the PVH. Also, in other areas involved in the stress response, like the PFC, amygdala, and the HC, the expression of GRs is increased during stress. However, the role of glucocorticoids is not the same in all brain regions. Glucocorticoids have a mainly inhibitory role on the HPA axis, while

in the amygdala and HC they have both inhibitory and excitatory effects, which is also modulated through the ILC (19).

Likewise, the effector components of the HPA axis, the glucocorticoids, cause multiple changes in the body's peripheral homeostasis to enable the body to defend against the threatening stress. The main effects of glucocorticoids are on the metabolism, the immune system, the cardiopulmonary circuit and the electrolyte balance. Specifically, glucocorticoids increase gluconeogenesis in the liver and enhance the outflow of proteins and fatty acids from the skeletal muscles, bones, the lymphoid system and fatty tissue, to build enzymes needed to defend against the stressor and to provide the body with necessary substrates to build defending products like catecholamines, one of the links to the sympatho-adrenomedullar (SAM) pathway (20). Glucocorticoids also suppress the immune system. In fact, the corticosteroid influence on the immune system is not totally understood, as it seems to depend on the duration of stress and its blood levels (20). However, two pathways how glucocorticoids can influence the immune system have been described. On the one hand, hydrocorticosterone binds to intracellular NF-κB (nuclear factor 'kappa-light-chain-enhancer' of activated B-cells) in lymphocytes, which leads to a decreased outflow of proinflammatory proteins and blocks a big part of the lymphatic immune response. Another way of blocking the immune system by hydrocortisone is through induction of the protein lipocortin which in turn blocks the conversion of phospholipids to arachidonic acid, necessary for the synthesis of prostaglandins and leucotrienes (20). The cardiopulmonary effects of glucocorticoids are poorly understood. Verified effects include an increased blood volume through the impact of mineralocorticoids and an augmentation of catecholaminergic receptors on endothelial cells of blood vessels (20). Effects on electrolyte homeostasis only become relevant after higher doses of cortisol and may lead to an increase of sodium retention with concurrent water influx and a subsequent increase of blood pressure (20).

The other equally important chemical component of the HPA axis is CRF, mainly present in neurons of the PVH, the amygdala and the LC (21). CRF has not only effects on the CNS, but also affects non-neural tissues. CRF1 and CRF2 receptors and ligands are widely spread in the periphery, suppressing reproductive, thyroid, and growth functions (9), and are also present in the GIT (3). CRF causes an increase of colonic permeability in rats and colonic hyperalgesia (22). This

presumption is solidified by the observation that antagonism of CRF1 receptors decreases stress-induced stimulation of colonic motor function (17). CRF also induces mast cell degranulation, and their products lead to increased jejunal water secretion and propulsive gut motility contributing to pain and diarrhoea, especially in irritable bowel syndrome (IBS) (1). However, the reports on the pertinent CRF effects are inconsistent, which may be attributed to different types of inflammation used and the CRF receptor examined. Nevertheless evidence suggests that an imbalance in the CRF system may favour inflammation (3).

1.3.2 The autonomic nervous system

The ANS consists of the sympathetic nervous system (SNS), the parasympathetic nervous system (PNS) and the ENS. It is able to initiate body responses quickly as it works through mostly direct nervous innervations in contrast to the slower endocrine HPA system. The ANS serves on the one hand as a mediator between CNS and peripheral organs and on the other hand has a direct influence on the CNS itself. Central integrative structures involved in ANS processing are amongst others the NTS, the periaquaeductal grey, amygdaloid nuclei, thalamus and hypothalamus (23). The NTS gains information about current blood compositions through the area postrema and has an influence on gastrointestinal motility. The periaquaeductal grey takes part in nociception and autonomous responses to pain (23). The amygdala gathers information from associative cortical areas as well as from the HC and sensory thalamic nuclei. It processes both behavioural and autonomic reactions (23). The thalamus, as a hub between cortex and limbic structures is involved in processing body sensations and combines those with cortical information to initiate reactions of body and brain.

Acetylcholine serves as neurotransmitter in the preganglionic neurons of the SNS and PNS, while postganglionic transmitters are acetylcholine for the PNS and NA for the SNS (23). Other neuronal subpopulations, expressing a variety of neuropeptides, nitric oxide or lipid mediators, are also present in the ANS (9). Outputs of the ANS can be triggered both by ascending pathways through interoceptive gut signals and descending pathways, as mentioned above, from brain centres involved in cognitive and emotional function (16).

In most of our daily regular life PNS and SNS control the function of the effector organs in balance. However, in challenging situations and in periods of rest one of the branches predominates. If internal or external stimuli overcharge the system, imbalance can result and pathological changes can occur.

1.3.2.1 The sympathetic nervous system

As stress increases SNS activity while decreasing PNS activity, SNS is the favoured branch of the ANS in acute challenging situations (3). The central part of the ANS is the LC. It releases NA and has an activating impact on the HPA axis and the amygdala. NA exerts a negative feedback on LC activation itself and a reciprocal impact on CRF neurons (9). The SNS has direct, nervous, and indirect, humoral, influences on the body. Directly, sympathetic nerve fibres innervate organs, smooth muscles and glands in and at the head, the thorax, the abdomen and the extremities. Indirectly, it influences allostasis through stimulation of the adrenal medulla. There, adrenaline (80%) and NA (20%) are released as the humoral components of the SNS pathway. SNS afferents from the gut primarily transmit information about visceral pain (23). The effect the SNS has on the body follows the theme of “fight or flight” and its ultimate goal is to provide energy for the body in challenging or threatening situations. The SNS is hence the ANS trail of the stress response. Via α- and β-adrenoceptors the SNS has an inotropic and chronotropic effect on the heart, leads to vasoconstriction in smooth muscles and vasodilatation in skeletal muscles, depending on the amount of adrenoceptors expressed in the respective effector organ. Furthermore SNS activation leads to increased glycogenolysis as well as lipolysis to provide substrates for energy generation (23).

In the gut, sympathetic nerve fibres decrease motility and secretion and increase sphincter tone (23). Like CRF, the SNS has a mainly proinflammatory role (3). Inflammation is initiated and aggravated through stimulation of bacterial growth via catecholamines (24).

1.3.2.2 The parasympathetic nervous system

The PNS pathway is mostly built by the vagus nerves and sacral nerves. It innervates the organs directly via postganglionic efferents. PNS effects on the

body ensue the motto of “rest and digest” and manifest themselves in a decrease of inotropy and chronotropy of the heart, an increase of intestinal motility and sphincter relaxation. The PNS represents therefore the ANS part responsible for recovery and anabolism. The PNS afferents from the gut to the brain carry information concerning luminal osmolarity, carbohydrate levels and mechanical distortion of the mucosa as well as about the presence of chemical or biological noxae (23). Moreover, the intestinal PNS interacts strongly with the ENS as well as with the immune system, the intestinal microbiome and endocrine systems (16).

1.3.2.3 The enteric nervous system

The ENS, as the intestinal part of the ANS, consists of 80 – 100 million nerve cells (approximately the same number of neurons as in the spinal cord) (23). It is composed of two plexuses, the submucosal or Meissner plexus, which regulates secretion and absorption, and the myenteric or Auerbach plexus, which controls peristalsis and intestinal motility. The ENS operates mainly independently but it is also modulated by the ANS and is linked through it to the CNS. The ENS has intrinsic primary afferent neurons (PANs) to gather information about mechanical distension, chemical and nociceptive stimuli as well as intraluminal electrolyte balance and transmits it the intramural circuits of the ENS that subserve intestinointestinal communication and digestive control (23). Moreover it interacts with the intestinal microbiome which will be discussed below.

1.4 Gut - brain interactions

The brain-gut communication is comparatively well understood, as research in this field started about some 20 years ago (2,24-27). It is a very complex research area, and many aspects of its implications are still unknown or only rudimentarily understood. Especially the impact of the gut on brain function and further on behaviour, memory, mood and other neuronal changes calls for further exploration and has gathered increasing attention in the last few years. Already Hippocrates and ancient medical systems like Traditional Chinese Medicine or the Indian Ayurvedic Medicine have included body and mind into one system without any separation in between them (28,29). In the last two centuries the idea of body-brain integration was readopted in Europe from first reports by William James and

Carl Lange in the 1880´s. The theory proposed that peripheral changes like increased heart rate or respiration or painful sensations in the gut cause emotional feelings (16). Walter Cannon pleaded on the contrary in the late 1920´s that the bodily sensations are “by–products” from brain changes and that emotional feelings are generated only by subcortical brain regions. Current theories move past the directionality of brain–viscera communication and are talking about “body–loops” and emotional images of the body´s homeostasis (16).

Hints at brain-body parallelism are for example the benefits of antidepressants on IBD symptoms, like decreased relapse rates, corticosteroid use and endoscopies per year after the start of antidepressive medication (30). Also non-pharmacolgical treatments like hypnosis, meditation and yoga have been proven to have a positive impact on stress circuits and inflammatory processes even though few data are available for IBD (31-33). Higher prevalence rates of anxiety, panic, obsessive-compulsive and depressive disorders in IBD patients than in the control population combined with worsening of bowel and mood symptoms in stressful times (3) indicate the simultaneous processing of stimuli by body and brain. It is also provable that individuals suffering from IBD have differential outcomes in questionnaires assessing the quality of life, depending on current disease activity (34). Still it remains unclear whether the impact of IBD on the mood or vice versa is stronger, as there is evidence of higher depression rates before IBD diagnosis than in controls (3). In this context, the different effects of stressful early life events on the physiology and pathophysiology in human and animal studies are also important. Amongst others, HPA axis-dependent increases of intestinal mast cells, modifications of proinflammatory cytokines and Toll-like receptors (TLR) are consequences of early life stress like deficient maternal care, maternal separation or neonatal inflammation (3). In fact it is not possible to talk only about gut-brain interferences because other organs are always involved in the processing of internal and external stimuli. Concerning stress, heart rate variability has to be mentioned as it reliably indicates imbalances in the ANS, even though it must be said that heart rate variability changes have been shown to differ depending on the type of intestinal disease (35).

1.4.1 How the gut communicates

As reviewed by Mayer et al. (16) three main cell types serve as morphological signalling factors from gut to brain: PANs, immune cells and enteroendocrine cells (EE). The intestine builds our body's largest surface (100 times larger than the skin surface) and the human ENS is built by 200 to 600 million neurons, with different classes of afferents, interneurons and effector neurons. Moreover, more than two-thirds of the body´s immune cells reside in the gut-associated lymphoid tissue, a large population of commensal microorganisms with 100 times the number of genes present in the human genome hosts in the gut lumen and more than 20 different hormones released by the EE have been found (16).

1.4.1.1 Neuronal signalling

Neuronal communication between the gut and brain takes place through extrinsic (spinal and vagal afferents) whereas intrinsic afferents serve the communication within the ENS (36). Being polymodal, PANs are sensitive to physiological and noxious mechanical and chemical stimuli and signal through intermediate cells in the spinal cord. They receive chemical information from enterochromaffin cells, immune cells and the gut microbiota and build networks amongst each other (16). Interestingly, some afferents do not react under physiologic conditions but become sensitive in inflammation (37).

The visceral influence on the CNS is mainly mediated through vagal afferents activated directly or through immune and endocrine cell substrates. The main relay station for this information is the NTS and the LC. The two nuclei send cholinergic and noradrenergic projections, respectively, to higher structures, including the PFC, the amygdaloid nuclei and the hippocampal formation, which are again reciprocally connected (38). The actual effect of intestinal changes on emotional and cognitive functions has been under investigation, but is not fully understood. Obvious is the pleasant effect of food intake, which in rats may even decrease pain behaviour (39), and the reduced neural activation in presence of sad emotions after intragastric fatty acid infusion in humans (40). In contrast, inflammation or intoxication causes negative emotions like nausea, pain, fatigue and sickness behaviour through mucosal immune cell activation and their products (16). These basic gut-related positive and negative feelings develop in early

childhood as hunger represents the earliest impression of pain and satiety the one for pleasure. As Mayer et al. phrase it “...the gut...provid(es) the child with the first value-based map of the world”, which is linked to the concept of interoceptive memories (16). Accordingly, these early built gut-based responses to appetitive and aversive stimuli could also “...form the basis for emotional states that are relevant for complex social emotions, (...) and decision making”, as further drafted by Mayer et al.

1.4.1.2 Immunological signalling

Immune cells play a very important role in homeostasis and host defence against pathological bacteria in the body. However, to distinguish between commensal and pathological microorganisms and other noxious stimuli is a large task for the intestinal immune system. The barrier between lumen and tissue is built by a single layer of epithelial cells. To detect pathological stimuli epithelial cells and dendritic cells express pattern recognition receptors, such as TLR. Vagal afferent terminals, in close proximity to mucosal immune cells, receive signals from molecules (proteases, histamine, serotonin, CRF, cytokines) released by these and other cells. EEs are also influenced by immune cell products (16). Activation of the intestinal immune system results in hyperalgesia and sickness behaviour produced through “... cytokine-mediated vagal activation of the hypothalamus and related limbic brain regions” (16). The impact of the immune system is especially relevant under inflammatory conditions as it is known that some psychiatric diseases like depression and anxiety disorders, as well as stress is associated with increased levels of inflammatory biomarkers like Interleukin 6, tumour necrosis factor and C-reactive protein (41). Therapeutically administered inflammatory cytokines, used e.g. in the treatment of chronic hepatitis, cause an increase of depressive symptoms in the patients who previously positively responded to antidepressants. It has even been suggested that antidepressants could work partly through molecules like IL-10 as studies showed that antidepressive treatment increased IL-10 levels (42), which acts immune- as well as pain-suppressive (41). However, although cytokine receptors have been found in the CNS, mainly in circumventricular organs, it is still unknown how the influence on mood and cognition by cytokines is processed precisely (41).

1.4.1.3 Endocrine signalling

Endocrine and paracrine signals are transmitted via more than 20 different hormones, released by EEs, which constitute 1% of the gut's epithelial cells. Gut hormone signalling to the brain takes place through vagal afferents or the bloodstream. EEs are activated by depolarization of mechanosensitive cation channels, by fatty acids and taste receptors (27). Also TLRs, present on the EEs are activated by intraluminal pathogens. EE activation results in intracellular calcium increase, peptide release, and activation of NF-κB, an important proinflammatory transcriptional factor, which leads to further cytokine release, causing in turn an increased immune response (16). Well studied are 5-HT-containing enterochromaffin cells as one subtype of EE cells (43). Noxious stimulation of the intestine leads to an increased release of 5-HT and subsequently to accelerated motor activity and secretory reflexes as well as to nausea via an interaction between EEs and vagal afferent pathways to dispose the offending stimulus. Whether the intestinal 5-HT has modulatory effects on affective central brain circuits is still unknown (16).

1.4.1.4 Influence of the microbiota

Another important player in the BGA or GBA, respectively, is the microbiota of the gut. Stress alters the composition of the microbiota (41). For instance, the SNS increases the virulence of Escherichia coli and Campylobacter jejuni, and the HPA axis alters the adherence of bacteria to the mucosa through glucocorticoids (24,44). Exaggerated activity of the HPA axis, as it occurs in stress, alters the microbiota as well. Conversely, specific microbes are able to reduce HPA axis activity. Bifidobacterium infantis, an infant gut bacterium used for probiotic treatment in IBS patients, seems to be able to reduce proinflammatory cytokines, like interleukin 12, tumour necrosis factor α and interferon γ and, on the other hand, to increase anti-inflammatory cytokines, like interleukin 10 (IL-10) (45). The microbiota itself also has a direct influence on GIT motility, the type of influence depending on the predominant bacteria (27). Furthermore, elevated adherence of the microbiota to the mucosa combined with a stress-induced increase of mucosal permeability leads to increased inflammatory reactions. This has relevance for IBD

patients and could hint at new therapeutic strategies, like probiotic treatment options (27,46,47). Research on the influence of the GIT on the CNS and disorders like psychiatric diseases is still in its beginnings (41,48). Findings in rats by Diaz Heijtz et al. (2011) imply an early impact of microbiota on postnatal neuronal development (49), which may relate to the above-mentioned gut-based map of the world the child receives and builds at a very early stage. There is also emerging evidence for a possible influence of the microbiota on pain sensitivity, emotionality and cognition. Lactobacillus species can upregulate IL-10, known to have anti-inflammatory and possibly analgesic effects (50) as mentioned above. Moreover, Lactobacillus probiotics are able to reduce pain perception (51), and the Lactobacillus-evoked expression of μ-opioid and cannabinoid receptors in intestinal epithelial cells (52) may also reflect an analgesic impact. In contrast, oral administration of Campylobacter jejuni and Citrobacter rodentium induces anxiety-like behaviour in mice (53,54), while specific dietary changes influence memory positively and reduce anxiety-like behaviour concurrently with a higher microbe diversity (55). Lactobacillus is also able to produce γ-aminobutyric acid (GABA) and other neuroactive molecules (41,56), and converts nitrate to nitrogen monoxide, which is a strong vasodilatator (20). Among the metabolites produced by the microbiota to influence behaviour are short chain fatty acids which as endproducts of anaerobic microbes have a positive influence on major depressive and bipolar disorders (41). Furthermore, an alteration in the microbiota composition in obese patients is observed, emphasizing an impact of microbes on the host's metabolism and energy harvest (57).

1.5 Colitis

Acute colitis is defined as an inflammation of the large intestine with a rapid onset and a short, but potentially severe course (58). Inflammation implies increased blood circulation, activation of different intestinal immune cells and possibly oedema, erosions and ulcerations, depending on the inflammatory initiator. Clinical symptoms are, dependent on the aetiology, nausea, different types of diarrhoea as well as constipation and abdominal pain amongst others (2,59). Several types of colitis including idiopathic colitis, UC, CD and IBS are known to be aggravated by stress (1,2,16,21). The incidence of chronic gastrointestinal diseases (GID) is 3 to

33/100.000 per year. IBS concerns almost half the patients suffering from any gastrointestinal disorder (58).

2 Material and Methods

2.1 Experimental animals

The experiments were carried out with 32 adult male C57BL/6N mice obtained from Charles River (Sulzfeld, Germany). As previously described (60) the mice were housed 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 6:00 h, lights off at 18:00 h). All experiments were approved by an ethical committee at the Federal Ministry of Science and Research of the Republic of Austria (BMWF-66.010/0119-II/3b/2011) and conducted according to the Directive of the European Communities Council of 24 November 1986 (86/609/EEC). The experiments were designed in such a way that both the number of animals used and their suffering was minimized.

2.2 Experimental protocol

The aim of my diploma thesis was to investigate the effect of psychological stress and colitis on the expression of the immediate early gene product c-Fos, a marker of neuronal activation. Therefore mice were allocated to 4 treatment groups. As shown in Figure 1, after habituation to the local animal facility, mice were either treated with vehicle (plain drinking water) or DSS (2 %), added to the drinking water) for 7 days. One day after this treatment period half of the DSS- and vehicle-treated mice were submitted to a 30-minute session of WAS, while the other half remained unstressed controls. Following a 90-minute stress-free interval in their home cage, WAS-treated animals were euthanized with an overdose of pentobarbital (150 mg/kg body weight injected intraperitoneally) and brains were collected. Unstressed mice were taken directly from their home cage and brains were excised after euthanasia.

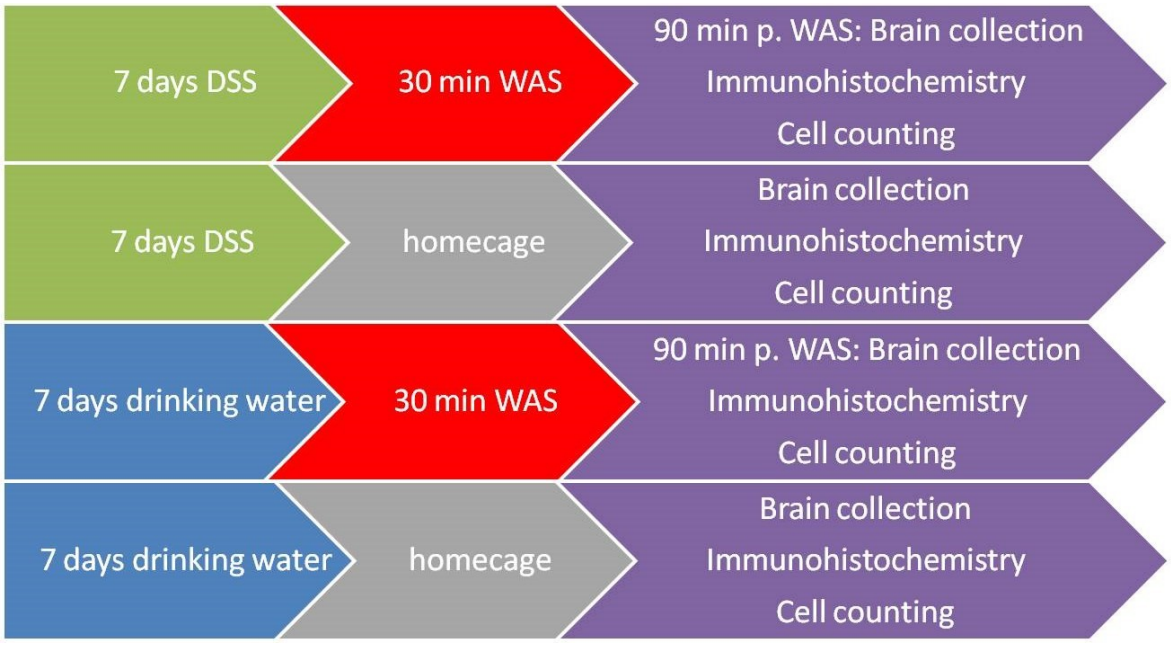

Figure 8. Treatment protocol. 32 C57BL/6N-mice were divided into four groups. Each group consisted of 8 animals. Mice received dextran sulfate sodium (DSS, 2 %, added to the drinking water) or tap water for 7 days. Half of the DSS- and water-treated animals were submitted to 30 minutes of water avoidance stress (WAS), the other half remained in their home cages. Ninety minutes post-WAS all mice were euthanized (pentobarbital 150 mg/kg body weight injected intraperitoneally). Brains were collected, frozen in 2-methyl-butan, stored at -70 °C and processed for immunohistochemistry. Cell counting was done with a computerized image analysis system (MCID Basic, version 7.0, Imaging Research Inc., Brock University, St. Catherines, Ontario, Canada). Abbreviations: DSS = dextran sulfate sodium; WAS = water avoidance stress; p. WAS = post WAS.

2.3 Administration of dextran sulfate sodium

As described previously (60) a mild colitis was induced in mice by adding DSS (molecular weight 36,000–50,000; MP Biomedicals, Illkirch, France) at a concentration of 2 % (w/v) to the drinking water for 7 days. The control animals received normal tap water. The DSS-containing drinking water was made up fresh every day to avoid bacterial contamination.

2.4 Water Avoidance Stress

The WAS procedure was used as a psychological stress model as mice are known to be water aversive. As described by Reichmann et al. (60) mice were placed on a small platform (6 × 3 × 6 cm, length × width × height) in the centre of a water-filled tank (61 × 40 × 22 cm, length × width × height), the level of the water (25 °C)

in the tank being 0.5 to 1 cm below the platform. The stress procedure was carried out in a brightly lit room (230 – 250 lux) for 30 minutes. Occasionally mice tried to escape from the WAS procedure. If they were able to jump onto the wall of the water filled tank, they were put back onto the platform. Following exposure to WAS the animals were returned to their home cage for 90 minutes and then euthanized.

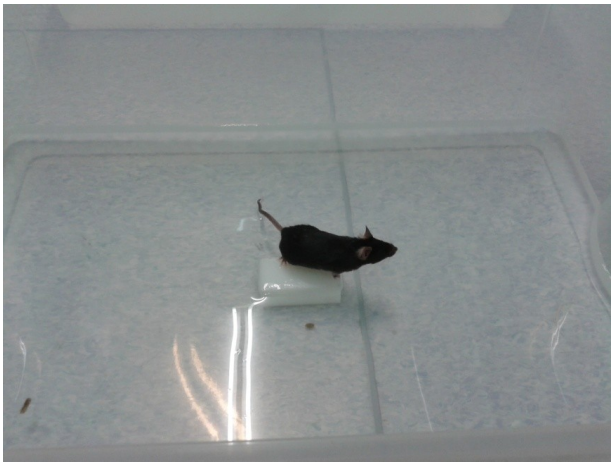

Figure 9. Water avoidance stress procedure. The image depicts the typical behaviour of a mouse during the 30 minutes water avoidance stress (WAS). The mouse sits on the usually dry platform and is surrounded by water (25 °C). This is a commonly used model of mild psychological stress in mice.

2.5 c-Fos Immunohistochemistry: Preparation and Process

As previously described (60) the activation of neurons in ROI was visualized by c-Fos immunohistochemistry (IHC). Thus WAS-treated and non-stressed animals (Control) were euthanized as mentioned above. Brains of WAS-treated animals were collected 2 hours after beginning of WAS since the maximal c-Fos protein expression takes place 1 to 3 hours after an acute stimulus (61). The brains were removed from the skull with scissors and forceps. After removing the dorsal skull, all nerve fibres connecting the brain with the spinal cord, the olfactory bulb and the eyes were cut with scissors. After removal, the brain was placed in ice-cold phosphate-buffered saline (PBS) to clean the tissue. Forebrain and cerebellum were cut apart using a razor blade. The forebrain was frozen placed on the razor blade in 2-methyl-butan. Afterwards brains were wrapped in aluminum foil and stored in labelled 50 ml falcon tubes in cardboard boxes at -70 °C. To obtain brain slices, coronal sections of 20 µm thickness were cut from the forebrain with a cryostat. Sections were mounted on Superfrost Plus slides (Menzel,

Braunschweig, Germany) and stored at -20 °C. Every sixth section was used for c-Fos IHC, while parallel sections were Nissl-stained for neuroanatomical orientation.

IHC was performed according to the protocol of Reichmann et al. (2013) (60). First the sections were surrounded with a hydrophobic barrier pen (ImmEdge Pen, Vector Laboratories, Burlingham, California, USA) and then incubated for 10 minutes in 4 % paraformaldehyde (Sigma-Aldrich, Vienna, Austria) in 0.1 M PBS of pH 7.4 for fixation. Then the slides were washed three times for 5 minutes in washing buffer (WB: 0.1 M PBS with 0.05 % Tween 20), to enable the following procedures to penetrate the tissue, and incubated in 0.3 % H_2O_2 for 15 minutes, a compound which blocks the activity of the peroxidase enzyme present in the tissue. This is necessary to prevent unwanted background staining derived from the activity of this endogenous enzyme. Then again the sections were washed three times in WB before they were incubated with 10 % normal (non-immune) goat serum in antibody diluent (AD: 0.1 M PBS containing 0.05 % Tween 20 and 1 % bovine serum albumin) for 5 minutes. Tween 20 is a detergent which facilitates the antibody penetration into the tissue, while goat serum blocks unspecific binding sites for the c-Fos antibody, reducing unwanted background staining.

Afterwards the slides were incubated overnight at 4 °C with the primary antibody (rabbit polyclonal anti-c-Fos SC-52, 1:2000, Santa Cruz Biotech, Santa Cruz, California, USA) in AD to bind to c-Fos protein. On the following day the sections were washed again three times in WB and incubated for 30 minutes in AD containing the biotinylated secondary antibody (goat-rabbit IgG 1:200, Vectastain Elite ABC Kit, Vector Laboratories) at room temperature. The secondary antibody is biotinylated so that the avidin-biotin-complex (ABC) binds with a free biotin site of the 4 biotin binding sites of avidin to the biotin of the secondary antibody. On the ABC attached is the peroxidase enzyme which enables staining of the primary/secondary antibody complex. Further three washes in WB and 30 minutes incubation in ABC (Vectastain Elite ABC Kit, Vector Laboratories) followed. Thereafter the slides were flushed with WB and developed for an equal amount of time with 3.3-diaminobenzidine substrate (DAB substrate kit for peroxidase, Vector Laboratories). The ABC-attached peroxidase uses DAB as substrate and chromogen (62). The end product of this reaction is the DAB´s polymerized insoluble brown precipitate which can be seen by light microscopy and quantified

with a computerized image analysis system (CAS) for cell counting. The duration of incubation in DAB is accountable for the colour intensity. To stop the enzyme activity and therefore the development of further colour precipitates, the sections were washed three times in distilled water for 5 minutes, air-dried overnight, cleared in 100 % xylol and coverslipped with Entellan (Merck, Darmstadt, Germany).

2.6 Cell counting and quantification

As previously described (60) a CAS (MCID Basic, version 7.0, Imaging Research Inc., Brock University, St. Catherines, Ontario, Canada) was used to quantify the immunohistochemically stained brain sections. The analysis system consisted of a light microscope (Axiphot, Zeiss, Oberkochen, Germany) connected to the CAS. To count only the c-Fos positive cells (brown/black reaction product of sufficient intensity within the nucleus) with the CAS, we defined an intensity-based background threshold for each ROI. The background threshold was set at an intensity which allowed quantification of c-Fos expressing cells without inclusion of any background staining. Images could be edited manually, which was necessary to eliminate CAS errors, e.g. artefacts or multiple cells being counted as only one big cell. The investigator was blinded by coding the slides.

The ROIs were identified with the help of the mouse brain stereotactic atlas of Paxinos and Franklin (63) and parallel Nissl-stained sections. To quantitate the expression of c-Fos, the c-Fos positive cells in each ROI were counted bilaterally in consecutive sections. Three consecutive sections were enumerated in the CC (Bregma + 1.10 to + 0.86), the BLA, CeA and MeA (Bregma - 1.06 to - 1.34) as well as in the CA1 field, the CA3 field and the granular cell layer of the DG of the HC (Bregma - 1.34 to - 1.58). Two consecutive sections were counted to assess the expression of c-Fos in the ILC (Bregma + 1.54 to + 1.42) and the PVH (Bregma - 0.58 to - 0.70). To ensure comparability, cell counting in a given ROI was always performed at the same Bregma level. Most of the ROIs where quantified within a square of 200 x 200 µm. Exceptions were the granular cell layer of the DG and the PVH where all c-Fos positive cells within this structures were quantified. The numbers of c-Fos positive cells counted in the different sections of each ROI in a given animal were used to calculate the mean number of c-Fos

positive cells within the distinct brain region of that animal. These mean values were used for statistical analysis.

2.7 Statistics

For the statistical evaluation of the results SPSS 20 (SPSS Inc., Chicago, IL, USA) was used. The data consisted of one variable (number of c-Fos expressing cells) and two factors (stress and colitis) and were thus analysed by two-way analysis of variance (ANOVA). The homogeneity of variances was estimated with the Levene test. Student´s t-tests were performed in case of a significant interaction between the two factors. Bonferroni correction was used to adjust for multiple testing. Probability values of ≤ 0.05 were regarded as statistically significant. P values < 0.001 were regarded as highly significant. All data are presented as means $\pm$ SEM, n referring to the number of mice in each group.

3 Results

3.1 c-Fos expression in the paraventricular hypothalamic nucleus

In the PVH, WAS caused a highly significant increase in the number of c-Fos expressing cells independently of DSS treatment ($F_{(1.22)}$ = 31.211; p < 0.001). In contrast, DSS treatment did not alter c-Fos expression and there was also no interaction between stress exposure and treatment condition (Figure 3).

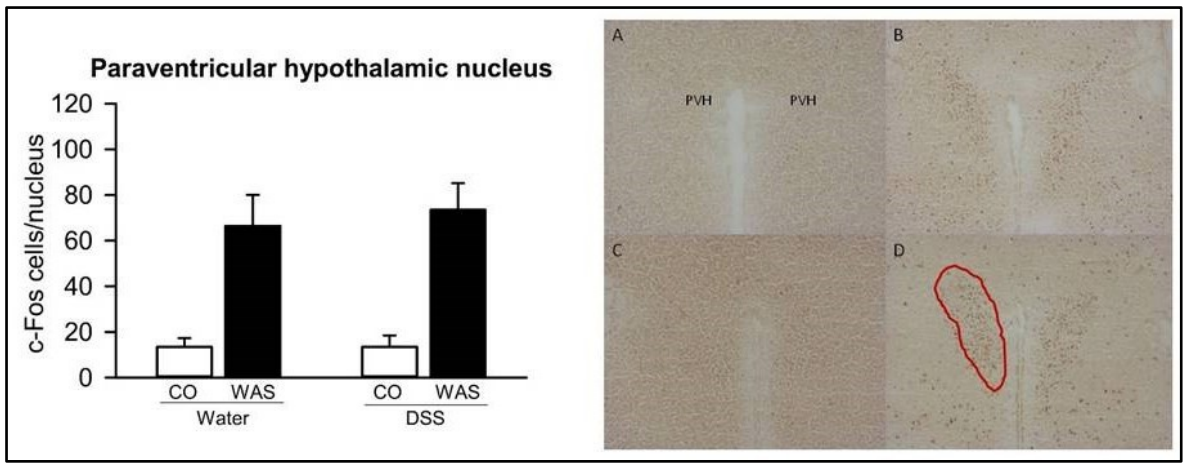

Figure 10. Water avoidance stress (WAS) increased the number of c-Fos expressing cells in the paraventricular nucleus of the hypothalamus (PVH) independently of dextran sulfate sodium (DSS)-

induced colitis. The strong impact of WAS, relative to control (CO) conditions, on the number of c-Fos expressing cells in the PVH is shown in the graph on the left. The images on the right show immunohistochemical micrographs of the PVH representative of the different treatment groups: Water/CO (A), Water/WAS (B), DSS/CO (C), DSS/WAS (D). In panel D an exemplary version of the PVH counting area is shown. High numbers of c-Fos expressing cells following exposure to WAS can be seen in panel B and D, while unstressed animals (CO) have low numbers of c-Fos expressing cells (panel A and C). DSS (2 %) was added to the drinking water for 7 days, while untreated mice drank plain water (Water). Expression of c-Fos was visualized under basal conditions (CO) or 2 h after the beginning of a 30-min exposure to WAS at the end of the treatment period (WAS). Unstressed mice remained in their home cages. Data are presented as means + SEM, n = 6 - 8/group.

3.2 c-Fos expression in the amygdaloid nuclei

The rise in the number of c-Fos expressing cells in the BLA (Figure 5) was highly significant in both DSS-treated mice and in mice drinking plain water ($F_{(1.24)}$ = 26.121; $p < 0.001$). In contrast, there was no effect of DSS treatment and no interaction between the two factors.

A similar result was obtained in the CeA (Figure 5) in which WAS caused a highly significant increase in the number of c-Fos expressing cells ($F_{(1.25)}$ = 19.823; $p < 0.001$) independently of DSS treatment and without interaction between the two factors.

In the MeA (Figure 6) stress, but not colitis, increased the number of c-Fos expressing cells ($F_{(1.24)}$ = 51.569; $p < 0.001$), with a significant interaction between the two factors ($F_{(1.24)}$ = 9.204; $p < 0.01$). Specifically, there was a large increase in the number of c-Fos expressing cells in the DSS/WAS group compared to the DSS/control group ($t_{(12)}$ = -7.002; $p < 0.001$) but only a small yet significant increase in the number of c-Fos expressing cells in the Water/WAS group compared to the Water/Control group ($t_{(12)}$ = -3.034; $p < 0.05$). In contrast, no significant difference between the two WAS groups (DSS/WAS vs. Water/WAS) and the two non-stressed groups (DSS/Control vs. Water/Control) was observed.

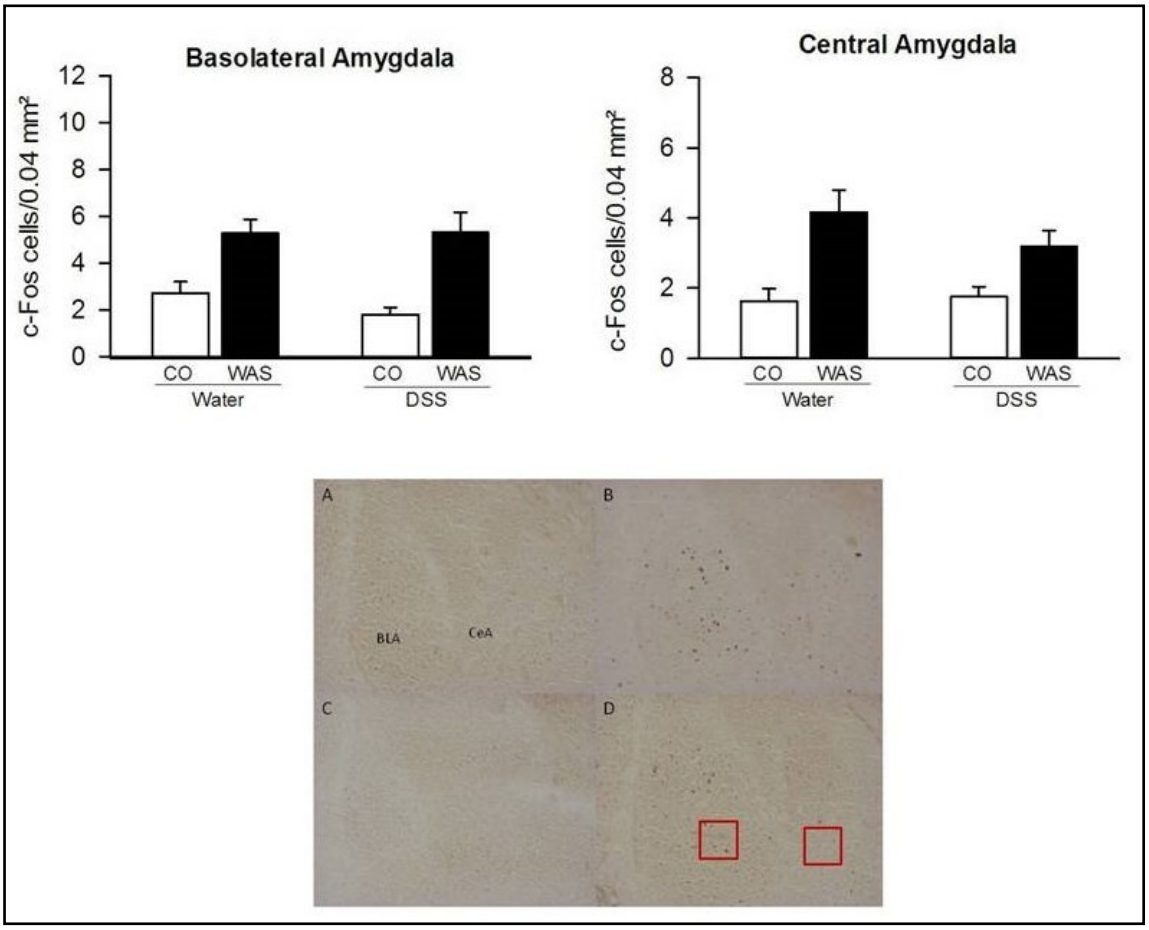

Figure 11. Water avoidance stress (WAS) increased the number of c-Fos expressing cells in the basolateral (BLA) and central amygdala (CeA) independently of dextran sulfate sodium (DSS)-induced colitis. The graphs show the significantly increased number of c-Fos expressing cells in BLA and CeA after submission to water avoidance stress (WAS), relative to control (CO) conditions. The images below show immunohistochemical micrographs of the BLA and CeA representative of the different treatment groups: Water/CO (A), Water/WAS (B), DSS/CO (C), DSS/WAS (D). Examples of the counting areas are shown in D. The stress-induced increase in the number of c-Fos expressing cells is illustrated in B and D, whereas no staining in A and C (control conditions) is seen. DSS (2 %) was added to the drinking water for 7 days, while untreated mice drank plain water (Water). Expression of c-Fos was visualized under basal conditions (CO) or 2 h after the beginning of a 30-min exposure to WAS at the end of the treatment period (WAS). Unstressed mice remained in their home cages until euthanasia. Data are presented as means + SEM, n = 7-8/group.

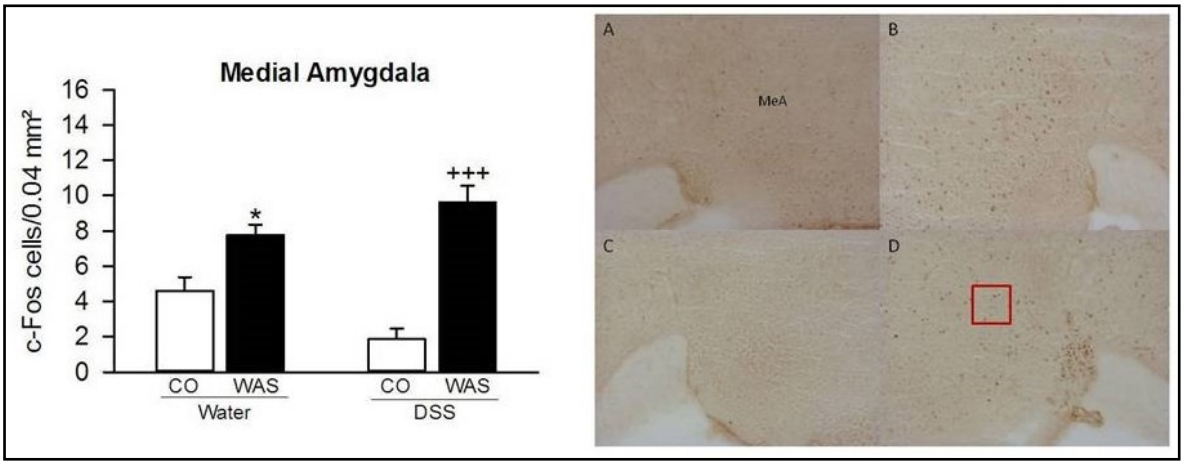

Figure 12. In the medial amygdala (MeA) water avoidance stress (WAS) increased the number of c-Fos expressing cells to a higher extent in DSS-treated than in plain water-drinking animals. In the graph on the left the impact of WAS on the number of c-Fos expressing cells is shown relative to control (CO) conditions. The increase in the number of c-Fos expressing cells in the DSS/WAS group (+++ $p < 0.001$ vs. DSS/CO) was larger than in the Water/WAS group (* $p < 0.05$ vs. Water/CO). On the right immunohistochemical micrographs of the MeA representative of the different treatment groups are shown: Water/CO (A), Water/WAS (B), DSS/CO (C), DSS/WAS (D). In panels B and D the high number of c-Fos expressing cells following WAS exposure is displayed, while panels A and C show only a small number of c-Fos positive cells under basal conditions. DSS (2 %) was added to the drinking water for 7 days, while untreated mice drank plain water (Water). Expression of c-Fos was visualized under basal conditions (CO) or 2 h after the beginning of a 30-min exposure to water avoidance stress (WAS) at the end of the treatment period. Unstressed mice remained in their home cages until euthanasia. Data are presented as means + SEM, n = 7-8/group.

3.3 c-Fos expression in the medial prefrontal cortex

Stress increased the number of c-Fos expressing cells in the ILC ($F_{(1.25)} = 29.304$; $p < 0.001$) in a highly significant manner, independently of, and without a significant interaction with, DSS treatment (Figure 4).

In the CC a highly significant elevation of cells expressing c-Fos could likewise be seen in stressed animals ($F_{(1.25)} = 18.901$; $p < 0.001$) independently of DSS-induced colitis. DSS itself had no significant effect and there was no interaction between WAS and DSS (Figure 4).

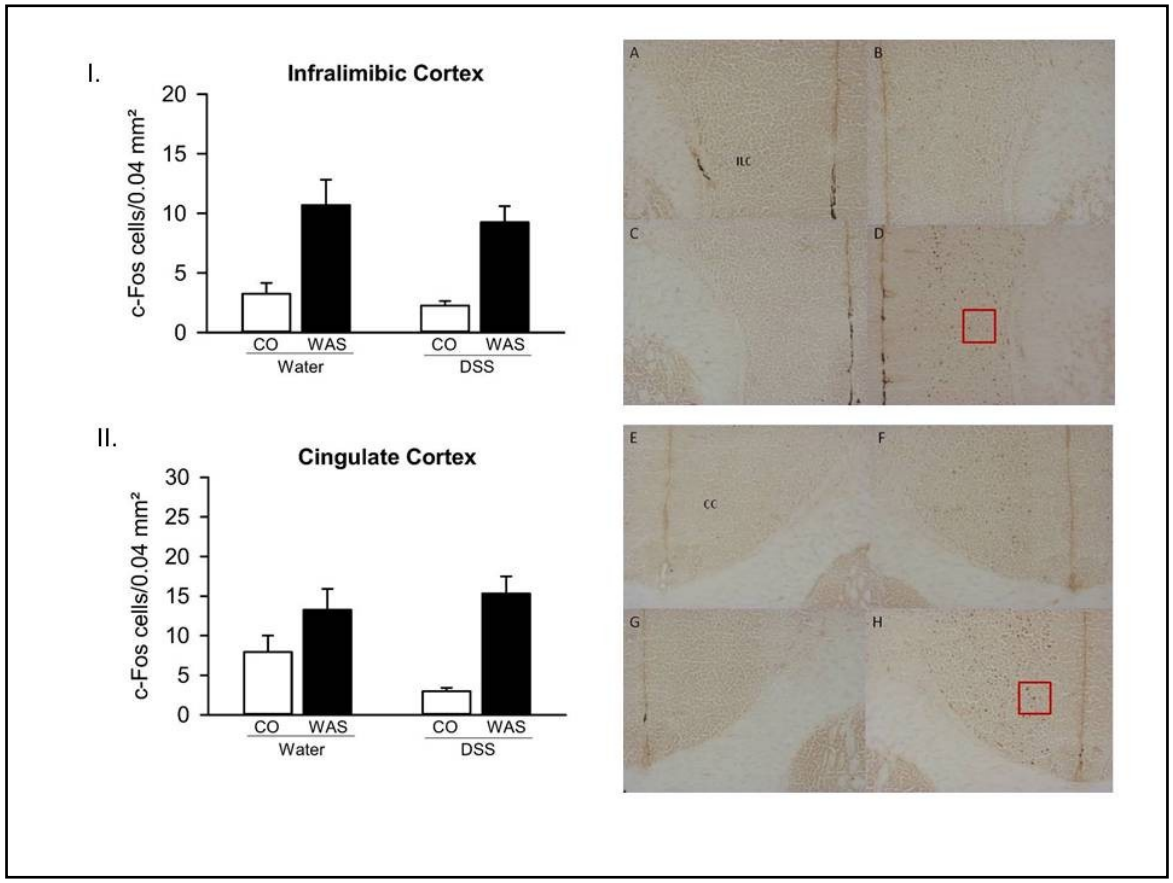

Figure 13. The number of c-Fos expressing cells in the medial prefrontal cortex increased after exposure to water avoidance (WAS), independently of dextran sulfate sodium (DSS)-induced colitis. Panels I. and II. show highly significant increases in the number of c-Fos expressing cells following exposure to WAS, relative to control (CO) conditions, in the infralimbic cortex (ILC) and cingulate cortex (CC), respectively. The images on the right show immunohistochemical micrographs of the ILC and CC representative of the different treatment groups: Water/CO (A/E), Water/WAS (B/F), DSS/CO (C/G), DSS/WAS (D/H). In D and H an exemplary version of the counting area in the ILC and CC, respectively, is shown. In panels B, D, F and H the WAS-induced increase of c-Fos expressing cells is shown relative to the expression of c-Fos under control conditions (A, C, E, G). DSS (2 %) was added to the drinking water for 7 days, while untreated mice drank plain water (Water). Expression of c-Fos was visualized under basal conditions (CO) or 2 h after the beginning of a 30-min exposure to WAS at the end of the treatment period (WAS). Unstressed mice remained in their home cages. Data are presented as means + SEM, n = 7-8/group.

3.4 c-Fos expression in the hippocampal area

In the DG (Figure 7) neither WAS nor DSS had an effect on the number of c-Fos expressing cells, but the two factors interacted with each other ($F(1.25) = 6.431$; $p < 0.05$). However, after adjusting for multiple testing, no significant differences were detected by the post-hoc test.

As in the DG, WAS and DSS did not affect c-Fos expression in the CA1 (Figure 7). There was also no interaction between the two factors in this brain region.

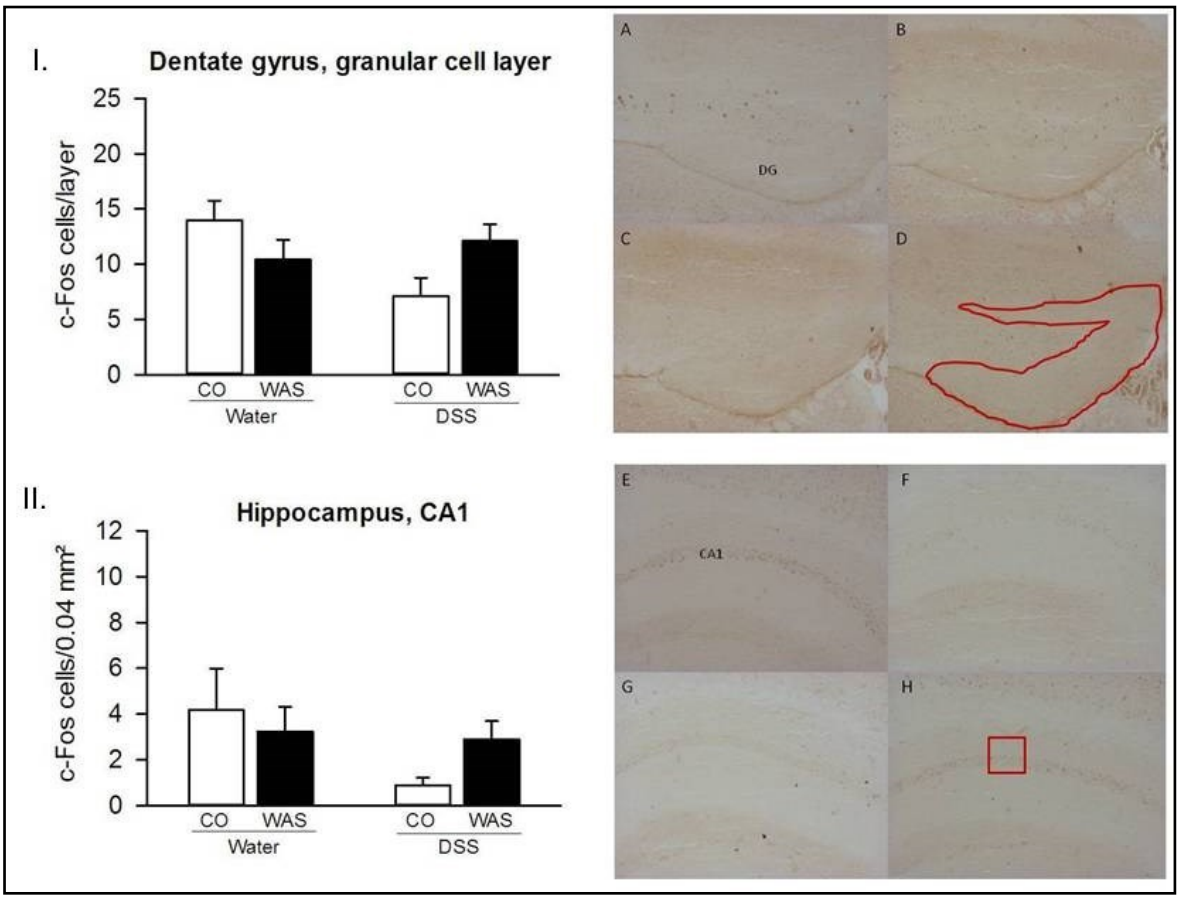

Figure 14. In the dentate gyrus (DG) and the CA1 area of the hippocampus neither water avoidance stress (WAS) nor DSS-induced colitis altered the number of c-Fos expressing cells. The graphs on the left demonstrate that WAS, relative to control (CO) conditions, and DSS treatment, relative to plain water, failed to significantly alter the number of c-Fos expressing cells. The panels on the right show immunohistochemical micrographs of the DG and CA1 representative of the different treatment groups: Water/CO (A/E), Water/WAS (B/F), DSS/CO (C/G), DSS/WAS (D/H). Panels D and H display exemplary counting areas. DSS (2 %) was added to the drinking water for 7 days, while untreated mice drank plain water (Water). Expression of c-Fos was visualized under basal conditions (CO) and 2 h after the beginning of a 30-min exposure to water avoidance stress (WAS) at the end of the treatment period. Unstressed mice remained in their home cages until euthanasia. Data are presented as means + SEM, n = 7-8/group.

The only area in the hippocampal formation showing stress-induced elevations in the number of c-Fos expressing cells was the CA3 region ($F_{(1.24)}$ = 4.996; $p < 0.05$). Similar to the other brain ROI, DSS treatment had no effect, and there was also no interaction between stress and treatment (Figure 8).

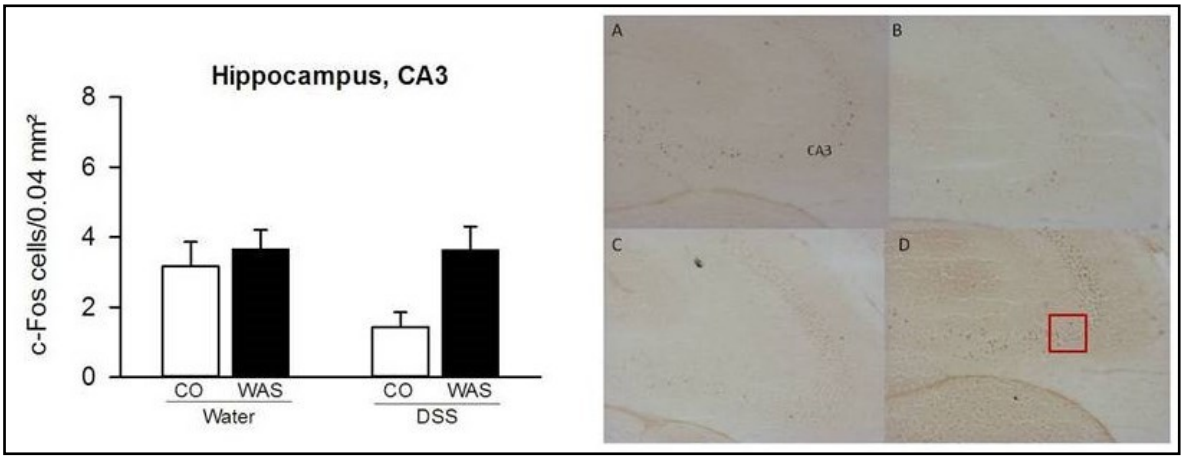

Figure 15. Water avoidance stress exposure (WAS) increased the number of c-Fos expressing cells in the CA3 area independently of DSS-induced colitis. The graph on the left shows the effect of water avoidance stress (WAS), relative to control (CO) conditions, and DSS treatment, relative to plain plain water, induced on the number of c-Fos expressing cells. The right panel presents immunohistochemical micrographs of the CA3 representative of the different treatment groups: Water/CO (A), Water/WAS (B), DSS/CO (C), DSS/WAS (D). In panel D an exemplary counting area is tagged. An increased number of c-Fos positive cells can be seen in panels B and D, relative to panels A and C. DSS (2 %) was added to the drinking water for 7 days, while untreated mice drank plain water (Water). Expression of c-Fos was visualized under basal conditions (CO) and 2 h after the beginning of a 30-min exposure to water avoidance stress (WAS) at the end of the treatment period. Unstressed mice remained in their home cages until euthanasia. Data are presented as means + SEM, n = 7-8/group.

4 Discussion

4.1 Rationale of the study

The purpose of the present study was to examine if and, if so, in which way DSS-induced colitis alters the WAS-induced neuronal c-Fos expression in limbic and paralimbic central brain structures in mice. This question was based on the accumulating evidence about gut-brain connections and peripheral impacts of sensory neurons as well as humoral factors on brain activity and further on emotions and behaviour. The results showed that WAS increased the number of c-Fos expressing cells in most of the ROIs examined, while DSS-induced colitis had only modest effects on this parameter. However, DSS administration caused remarkable alterations of c-Fos expression in the MeA. The increase in the number of c-Fos expressing cells in the MeA of WAS-exposed animals was highly significant ($p < 0.001$) in DSS-treated animals while it was less pronounced ($p < 0.05$) in water-drinking controls. In contrast, under non-stressed conditions, the

number of c-Fos expressing cells in the MeA was lower in the DSS-treated animals compared to unstressed controls. Noteworthy, DSS treatment also seemed to decrease the number of c-Fos expressing cells of unstressed mice in the hippocampal formation and the CC, although this difference failed to reach statistical significance.

In the following chapters I will discuss the impact of the current findings on GBA research and associate these data with previous findings related to colitis, anxiety, stress, depression and neuronal c-Fos expression in rodents and humans in the ROIs examined here. Special focus will be given to the MeA as it was the only brain area where DSS caused significant alterations. Moreover, CRF and corticosterone will be discussed more precisely as they play decisive roles in stress processing. However, since not only neuronal and humoral factors but also immunological and microbial factors have an impact on brain activity, they will also be discussed.

4.2 Influence of external and internal stress on neuronal activation patterns

Clinical and animal studies have shown that stress has an important influence on the course of GID. Vice versa inflammatory diseases like IBS are associated with altered behaviour and with depressive and anxiety disorders (3,16,60,64,65). In view of this bidirectional relationship, the present experiment used a chemical colitis model and a psychological stressor to investigate the influence of these factors on gut-brain-gut communication. Although DSS colitis does not represent IBS explicitly, it models inflammatory gut-brain signalling, which is also present in IBS patients. Furthermore, IBS development is often seen after gastrointestinal infections. This post-infectious IBS phenotype results in visceral hypersensitivity (66), which can also be seen in the DSS colitis model supporting the translational validity of the experimental approach used. As mentioned in the Introduction, IBS patients have a higher prevalence of mood disorders (3) with some ambiguity concerning the chronological order of their appearance. Also, a higher prevalence of traumatic childhood experiences was found in IBS (65) and increased visceral sensitivity (67). Recent findings by Felice et al. (68) demonstrate an important

aspect of the brain-gut-brain connectivity in IBS. For this purpose, they investigated the influence of maternal separation on open field behaviour and the impact of maternal separation, open field stress and colorectal distension (CRD) on neuronal c-Fos expression in rats. The study revealed that maternal separation altered the behaviour in the open field test by increasing anxiety-like behaviour. CRD alone led to increased c-Fos expression in the ILC and CC only in maternally separated animals. Interestingly open field stress had no impact on the number of c-Fos expressing cells in those brain areas, emphasising that it might be a less disturbing stressor compared to WAS, even though the behaviour of separated animals was altered (68). In the CeA, CRD increased c-Fos expression in both separated and not separated rats (68), supporting the idea that the CeA is activated by painful stimuli independently of early life experiences. However, open field stress also increased the number of c-Fos expressing cells in the CeA, emphasising an additional role of this nucleus in fear processing. These findings show the importance of the influence of early life experiences on the GBA, which was not investigated in my experiment. Nevertheless emphasizes the predominant influence of psychological factors on neuronal activation, which corresponds to my findings.

As mentioned in the Introduction, certain bacteria, like Campylobacter jejunii and Citrobacter rodentium, have the ability to induce anxiety-like behaviour in rodents (54). In this study the behavioural effects were accompanied by altered c-Fos expression in brain regions involved in emotional and autonomic processing. Precisely, a single Campylobacter jejunii administration several hours before behavioural testing enhanced the number of c-Fos expressing cells in the medial PFC, the PVH and the CeA compared to a saline control group. Similarly, the effect of behavioural testing in a holeboard, which also served as a stressor, led to strong increases in the number of c-Fos positive cells in the medial PFC, the PVH, CeA and BLA. The combination of Campylobacter jejunii and holeboard exposure increased the number of c-Fos positive cells in the PFC, BLA and the PVH compared to controls and Campylobacter jejuni infection alone. Only in the CeA the increase in the number of c-Fos expressing cells was not much further enhanced by the combination of infection and stress compared to Campylobacter jejuni infection alone. Moreover, only in the CeA and the BLA a mild increase in the number of c-Fos expressing cells in the holeboard exposed animals was

detected after Campylobacter jejuni infection. Furthermore the increase in the number of c-Fos expressing cells in these ROIs corresponded negatively with the exploratory behaviour (54). The comparison of the present work with the findings of this study suggest a different processing of bacterial and chemical colitis as well as different processing of WAS and holeboard exposure stress, especially in the CeA. In contrast to Campylobacter infection, DSS treatment did not affect the number of c-Fos expressing cells, and in none of the ROI, except in the MeA, was WAS and DSS able to cause an additive increase of c-Fos expression. The different effects of the two colitis models on c-Fos expression may be related to different modes of colitis induction and a different effect on the colonic microbiota, taking into account that in the case of DSS-induced colitis a microbial shift takes place (69).

Alterations in the number of c-Fos expressing cells were also seen in different models of chemical colitis. Trinitrobenzenesulfonic acid (TNBS)-induced colitis used by Porcher et al. was associated with an increased c-Fos expression in PVH and CeA, which peaked 2 h after colitis induction and returned to baseline after 6-12 h (70). Noticeable, TNBS was only administered once and represents a mixed psychological and physiological acute stressor because the rectal application itself can be considered stressful for the animal. It has also been shown that c-Fos expression is induced by intraperitoneally administered acetic acid and prevented by opioid receptor agonists in the PVH and NTS (71), suggesting cell activation after pain perception or the inclusion of stress circuits in pain responses as the PVH is a main relay station for stress processing. In contrast, in the present findings only the psychological stressor but not the internal stress of DSS-induced colitis activated cells in the PVH. The inability of DSS colitis to influence c-Fos expression may be related to the different method of colitis induction in DSS-treated animals. While TNBS and acetic acid are administered once in an invasive manner, DSS is applied via the drinking water for a longer time period. The contradictory findings underline the assumed influence of systemic as well as processive stressors on the activation of the PVH, the impact being in addition dependent on the aggressiveness of the stressor. Attention need also be given to possibly different pathways depending on the type of stressor (processive or systemic) and on the type of colitis, which may activate different brain regions. This was also seen in clinical studies. Mayer et al. (72) found increased cerebral

blood flow following CRD in limbic/paralimbic (e.g. amygdala, CC, hypothalamus) structures via PET imaging in IBS but not in UC patients or controls. Furthermore, Mayer et al. (16) showed that brain regions of IBS patients involved in the processing of afferent visceral information and emotional excitation are more activated in response to rectal distension than in healthy controls; changes of the cortical thickness in these brain areas were also suggested.

4.3 Functions and interconnections in the examined brain areas

4.3.1 The paraventricular hypothalamic nucleus

The PVH, the major source of brain CRF (70), has been shown to be activated after systemic and processive stress. PVH, but also CeA and BLA are established as regions which integrate internal challenges with autonomic and emotional responses to “processive” and “systemic” stressors (73). For instance, Bonaz et al. (74) examined WAS-induced c-Fos expression combined with CRF antagonists in rats. They found a strong increase in the number of c-Fos expressing cells mainly in the PVH. CRF antagonism before WAS exposure significantly reduced the number of c-Fos expressing cells in the PVH, indicating that PVH cells are activated by CRF following WAS. However only two third of all c-Fos expressing cells were also CRF-positive (74), suggesting that one third of stress-activated PVH cells express neuronal factors other than CRF. For example, oxytocin may be involved as Dayas et al. (75) found that restraint stress caused an increase in the number of c-Fos expressing cells in the PVH of rats; after double staining they were highly positive for CRF and oxytocin. It is also interesting to note that CRF receptor expression in the PVH has been shown to increase after acute stress, like immobilisation, but to decrease under chronic conditions (64). Furthermore, CRF receptor mRNA is increased in the PVH after TNBS-induced colitis, an effect absent in the CC, BLA, MeA and CA3. In these brain regions, CRF levels were already as high as in the activated PVH under basal conditions (70). This might indicate that WAS-induced c-Fos expression in the PVH depends on CRF receptors while in the CC, BLA and CA3, three brain regions also activated by WAS in my study, the neuronal activation depends on other factors. The impact of WAS combined with TNBS colitis on PVH CRF expression and corticosterone release was examined by Kresse et al. (76). They found significant differences

within the PVH, more precisely between the parvocellular and the magnocellular part of the PVH. TNBS increased CRF mRNA only in the magnocellular part. In contrast, the WAS-induced rise of plasma corticosterone decreased with TNBS treatment. TNBS is considered a chronic colitis model, which may explain this outcome (76). The PVH has several reciprocal connections to higher brain structures, the brainstem and the limbic system. For instance, deafferentation of ascending pathways from the brainstem decreases c-Fos activation in the PVH after inflammation but this deafferentation has no impact on c-Fos expression after foot-shock, implying a different pathway for this stimulus (10). A further transmitter active in the PVH is glutamate which activates neurosecretory neurons and GABA-ergic projections which seem to operate as negative feedback mechanisms in the HPA axis (10).

4.3.2 Amygdalar nuclei

The function of the amygdala in an overall view comprises emotions, like fear, reward, motivation, aggression, as well as sexual, maternal and ingestive behaviour and furthermore memory tasks associated with emotional significance (77). The amygdala obtains monoaminergic innervations (dopamine, NA, serotonin) as well as glutamatergic and cholinergic synapses (19). In addition, receptors for glucocorticoids and estrogen, as well as for opioids, oxytocin, vasopressin, CRF, melanocortin and neuropeptide Y are present in the amygdala, in order to highlight the complexity of amygdaloid chemical systems (77,78). Inputs are mostly excitatory, through glutamate, but interconnections are strongly GABAergic, “to keep spontaneous cellular activity low” (77). The complicated inhibitory circuits within the amygdala are feedback mechanisms, which inhibit projection neurons and may result in dampened output responses after repetition of stimuli (77). Challenged through stress, the extracellular concentrations of NA, serotonin and dopamine in the amygdala are increased (19). This affects the amygdalar neurotransmission as serotonin together with corticosterone excites GABAergic cells which, in turn, inhibit projection neurons (77). To achieve explicit memory, which is believed to be dependent on emotional significance, the amygdala has to be connected to the HC and these connections derive mainly from the BLA which is activated by glucocorticoids (77). Parts of the amygdala are

major portals through which sensory information enter the limbic system. The amygdala has connections to the HC as mentioned above, the thalamus, hypothalamus, cortex, brainstem, the olfactory system, striatum, PFC and the VAGUS NERVE as well as numerous connections within itself. It consists of several subnuclei with different functions which are more or less well understood. From clinical studies it is known that there are associations between psychiatric disorders and changes in the amygdala (77). This is endorsed by findings that direct electric stimulation of the human amygdala results in an increase of sadness, fear and anxiety (79).

The present study analyzed stress-induced activation of the CeA, BLA and MeA. The CeA receives inputs from the viscero-sensory cortex, the PFC, the sensory brainstem and the other amygdalar subnuclei. It is supposed to act as the main output region of the amygdala, initiating the expression of emotional and associated physiological responses like freezing, arousal and hypothalamic and parasympathetic systemic effects (77). The BLA also receives inputs from the HC and is bidirectionally connected to the PFC and associative cortices. Furthermore it is believed to have output connections to the striatum through which it is involved in “controlling actions”, like seeking for shelter (77). The increased number of c-Fos expressing cells after WAS independently of colitis in the BLA and CeA in my experiment underlines the influence of psychological stress on these parts of the amygdala. It also suggests that DSS-induced colitis may not induce appreciable pain, as no alterations in the number of c-Fos expressing cells were observed in the CeA following DSS-colitis. As the amygdala is strongly involved in fear circuits, an increased number of c-Fos expressing cells after WAS indicates a possible increase of fear in these animals. Additionally, a hypersensitivity to stress following intestinal inflammation is suggested as DSS-treated WAS-exposed animals had the highest c-Fos levels in the MeA. In contrast to my findings in the CeA, Bonaz et al. (74) found no alteration in the number of c-Fos expressing cells after WAS in the CeA, even though previous studies showed that immobilisation stress increased the number of c-Fos positive glucocorticoid containing cells in the CeA (80). This finding emphasizes again that the activity in the CeA depends on the type of stressor and, as immobilisation stress represents a stronger threatening stimulus than WAS, the increased activation of the CeA is explainable, as it is supposed to be the nociceptive part of amygdaloid regions (68). Interestingly the

location and type of pain seems to play an important role in amygdalar activation. Nakagawa et al. (81) found that intraplantar applied formalin did not alter c-Fos expression in the CeA but changed c-Fos levels in the BLA. In contrast, intraperitoneally applied acetic acid significantly increased the number of c-Fos expressing cells only in the CeA. This might indicate an important role of the CeA in visceral pain. Furthermore, in context with the present findings, where WAS significantly increased the number of c-Fos expressing cells in the CeA, I hypothesise a different activation pattern and reaction to WAS in mice compared to rats (or other underlying differences associated with the protocol or the experimental conditions). For instance, Bonaz et al. (74) exposed the animals 1 h instead of 30 min to WAS, which may have led to delayed c-Fos staining, some habituation or even activation changes of the CeA. Previous findings by Dayas et al. (75) showed an increase in the number of c-Fos positive cells mainly in the MeA and PVH and less activation of the CeA after restraint stress in rats. Lesions of the MeA and CeA in this study interestingly did not alter the behavioural response, like struggling, to restraint stress, pointing to a rather responsive than autonomous function of these amygdaloid nuclei. Nevertheless, in contrast to the CeA, lesions of the MeA caused a significant reduction in the number of c-Fos expressing, CRF and oxytocin positive cells in the PVH (75). Furthermore, lesions of the MeA and CeA decreased the ACTH and corticosterone release after restraint, but not after respiratory stress (10). This might indicate an underestimated strong influence of the MeA on the HPA axis via MeA-PVH connections. These connections were previously examined in retrograde tracing studies (75) and their importance is supported by findings that MeA stimulation leads to increased plasma corticosterone levels, anxiety-like behaviour and anorexia (78), the latter possibly implying connections to the gut. In previous studies, c-Fos increases in the MeA were especially observed after exposure to different processive stressors while the CeA responded stronger to systemic stressors (75,82). Concerning the function of the MeA, recent findings by Wang et al. (83) in male mice revealed its role in aggressive behaviour. The study showed a decrease of oxytocin and vasopressin containing neurons of the PVH after MeA lesion, accompanied by a reduction in aggressive behaviour (83). The MeA is highly responsive to processive stressors like forced swim stress, social defeat, immobilization and restraint (78). Furthermore it has been shown by Liu et al. (78)

that the MeA contains melanocortinergic neurons which are activated by restraint. Stimulation of the melanocortin-receptors in the MeA resulted in elevated corticosterone levels, anxiety-like behaviour and anorexia. Melanocortin plays an important role in energy balance, and this aspect also points out the importance of the physiological aspect of stress processing in the emotional centre of the amygdala (78). As WAS is partly comparable with restraint, the observed c-Fos increase is likely to result from activation of similar pathways. Still, it remains unclear why DSS led to a higher number of c-Fos expressing cells in stressed animals. In contrast to the present observation, Reichmann et al. (60) found a decrease in the number of WAS-induced c-Fos expressing cells after DSS treatment in the BLA, CeA and MeA. However, the different age of the mice in this study and in my experiment as well as differential housing conditions may account for the observed differences.

4.3.3 The medial prefrontal cortex

The PFC consists of several subdivisions which subserve various tasks (84). In the present study the ILC was examined, but it would have also been interesting to study the prelimbic PFC as it seems to be partly the ILC´s counterpart in fear processing (85). The ILC, an important mood-related part of the medial PFC, responds to stress as shown by an increased release of of dopamine, acetylcholine, NA and serotonin. Findings on the release of glutamate and GABA in the ILC under stressed conditions are controversial (19). Also, the ILC is involved in anxiety and depression-like behaviour (68) although findings are contradictory here as well (86). A higher prevalence of depressive symptoms in patients mainly with left-sided PFC damage has been observed (87). The involvement of the PFC in emotional processes is supposed to depend on inhibitory connections to the amygdala and vice versa (85). Intercalated cells and direct contralateral connections from the PFC to the BLA are supposed to play major roles in this task (88). The intercalated cells are GABAergic neurons, responsible for CeA inhibition and consequently for fear extinction (85,89). Interestingly, Reichmann et al. (60) found that after 9 weeks of enriched housing the number of WAS-induced c-Fos expressing cells decreased in the ILC and the CeA compared to standard housed animals, supporting the inhibitory role of the

ILC on the CeA. The PFC is connected to several cortical and limbic structures. Also afferent and efferent connections to visceral and somatic structures exist (84). It is also connected to the HC, since atrophy of the PFC leads to decreased hippocampal-dependent long-term memory (90). Posttraumatic stress disorder and panic disorder patients show decreased activation, reduced gray matter volume as well as reduced receptor binding for benzodiazepines in the PFC (91). An increase in the number of c-Fos expressing cells in the ILC of Wistar-Kyoto rats hypersensitive to CRD has been shown (92). In the present work, the ILC was activated only by stress, while the number of c-Fos expressing cells under basal conditions was very small. This supports the involvement of the ILC in mood and emotional processing, but is controversial to the findings by Gibney et al. mentioned above. This might indicate an additional pathway in the used rat strain or different processing of visceral stress in rats and mice.

The CC, or parts of it, is known to play important roles in pain perception, pain processing, mood, attention and anxiety (91-93). Importantly one has to keep in mind that there may be species differences in the function of the CC (83). Nevertheless, TNBS as well as anxiogenic drugs lead to an increase in the number of c-Fos expressing cells in the CC of rats (70,94). Gibney et al. (92) also found increased numbers of c-Fos expressing cells in the CC after CRD in viscerally hypersensitive Wistar-Kyoto as well as normosensitive Sprague-Dawley rats. In contrast, chronic WAS, as observed by Wang et al. (83), decreased the number of c-Fos expressing cells in the CC in rats, which may represent a habituation effect after multiple sessions of WAS. In the present study one session of WAS led to an increase in the number of c-Fos expressing cells independently of DSS-induced colitis. Compared to the findings of Reichmann et al. (60) where DSS treatment reduced the number of c-Fos expressing cells in the CC, my experiment study did not confirm this difference. However, I found an insignificant reduction in the number of c-Fos expressing cells in unstressed DSS-administered animals.

4.3.4 The hippocampal formation

In the hippocampal formation several studies revealed an increased release of glutamate, NA, serotonin, acetylcholine, dopamine and GABA under different

stressful conditions (19), emphasizing the complexity of the functionality of this area. Increased c-Fos expression after chronic stress (e.g. food deprivation, hot/cold stress) in rats was observed, too (95). In my present work involving acute stress, I found increased numbers of c-Fos expressing cells only in the CA3 area, but not in the CA1 or DG. Hippocampal volumes have been extensively examined in humans, e.g. concerning memory function and in association with depression, panic and posttraumatic stress disorder as well as with dissociative symptoms. Results of these studies are contradictory, but the majority showed a decrease of volume and activation in patients compared to healthy controls (91). One of the stress-related functions of the HC is to terminate the HPA axis response. Indeed if the HC is atrophied or destroyed the HPA axis response is prolonged (10). The HC has one of the highest levels of glucocorticoid receptors, which allows the HC to sense HPA axis activity and fulfil its inhibiting function (10). Furthermore the HC is well known to be strongly connected to the amygdala in memory tasks which are linked to emotions (96). Relating to the present findings, one could argue, based on the low impact of either stress or colitis on c-Fos expression in the HC that the memory function has not been activated or that memory has already been acquired at the time of stress exposure. In long-term studies like in the study of Reichmann et al. (60) an increase in the number of WAS-induced c-Fos expressing cells has been observed in the DG after 9 weeks of enriched housing. Furthermore, a DSS dependent decrease in the number of c-Fos expressing cells in WAS exposed animals in the DG and CA1 region of the HC has been seen (60). The former observation suggests that the improved function of the HC following enriched housing enhances the sensitivity to acute stress, whereas under non-enriched conditions the HC does not sufficiently respond to a relatively mild acute stress stimulus. The latter finding of a DSS dependent decrease of stress-induced c-Fos expression was not seen in the present study. Actually, especially in the DG and the CA1, no alterations, either by WAS or DSS, could be detected. However, as described above, the differences in study design may account for these differences in c-Fos expression.

4.4 Conclusion

The significance of the present results lies in the contribution of a small piece in the complex puzzle of the GBA. Thus, a clear influence of the acute stressor WAS on most of the limbic and paralimbic structures examined has been detected, which supports and extends previous findings using acute stressors and c-Fos IHC (92,97,98). The establishment of mild colitis over a course of 7 days resulted in an increase of the number of c-Fos expressing cells only in the MeA. The expression of the transcriptional factor c-Fos indicates activation of neuronal cells and is frequently used in functional neuroanatomy. The MeA is strongly involved in processing emotional aspects of stress (75) and its stronger activation in the course of colitis may indicate a higher level of fear and anxiety, which probably comes along with increased sickness. In confirmation of this argument it has previously been shown that mild DSS-induced colitis increases anxiety in male mice (99). The impact of colitis on the MeA is also consistent with findings on the influence of the gut on emotional processes in humans (16).

In contrast to WAS, the present results showed that colitis had no impact on neuronal activation in the other limbic and paralimbic structures examined. This suggests that there was no change in the activity of these areas probably because mild colitis was insufficient to effectively impact on gut-brain-communication. Importantly, DSS-induced colitis as studied in the present work cannot be considered either definitely acute or chronic, while c-Fos is an immediate early gene that is transcribed by acute cell activation, this transcription factor reaching its peak levels 1 – 3 hours after stimulus exposure (61). In contrast DSS was administered continuously for seven days. Therefore it cannot be regarded as an acute stimulus, which may explain to a certain extent its poor impact on the number of c-Fos expressing cells. Further research should focus on differences in c-Fos expression dependent on the duration and time course of DSS exposure and the dose of DSS to analyse the impact of this colitis model on brain activity in full detail. In some areas DSS-induced colitis even decreased the number of c-Fos expressing cells, suggesting either inhibition of particular brain pathways or a habituation of the c-Fos response, which is in line with other studies using chronic stressors (64,83,100). The findings of a decrease in the number of c-Fos expressing cells are contradictory and do may not only depend only on the duration of DSS exposure but also on the type of stressor. Thus, Matsuda et al.

(101) found that in socially challenged mice the number of c-Fos expressing cells persisted in increased number, in contrast to control animals.

Behavioural studies on the influence of colitis and stress on emotional-affective behaviour and social performance provide important information on gut-brain interactions (16,54,97). Therefore, performing behavioural studies with the same experimental protocol may advance the understanding of the clinical impact of colitis and stress and their interaction on emotionality and mood. It is also important to mention that c-Fos IHC only examines the quantity of activated cells in a given ROI, regardless of their function and their connections between each other. To overcome this limitation, double staining for a vast amount of neurotransmitters and their corresponding receptors would be needed. However, one has to bear in mind that various protocols as well as several colitis and stress models along with the use of other species and of both sexes are likely to result in different outcomes and to impede the comparability of studies.

Unfortunately, the internal and external challenges, especially for human beings, are rising these times and therefore increased research on the causes, mechanisms and therapeutic approaches for systemic and processive stressors is badly needed. In these efforts, an integrative approach comprising body and mind such as the GBA needs to be pursued. The growing number of studies in the field of the GBA is attesting to these efforts, and the outcome of these investigations will ultimately support the striving of humans for well-being in concert with physiological organ function.

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