Dissertation

Behavioral characterization and signaling mechanisms of visceral pain in a mouse model of psychological stress and experimental colitis

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

Abbreviations and definitions

AITC	Allyl isothiocyanate
Amy	Amygdala
ANOVA	Analysis of variance
BSA	Bovine serum albumin
CD	Crohn's disease
CGRP	Calcitonin gene related peptide
CNS	Central nervous system
Control	Con
CRD	Colorectal distension
CRF	Corticotropin-releasing factor
DAS	Disease activity score
DMSO	Dimethyl sulfoxide
DRG	Dorsal root ganglion
DSS	Dextran sulfate sodium
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
GC	Glucocorticoids
GI	Gastrointestinal
HBSS	Hank's balanced salt solution
HC	HC-030031

HRQOL	Health-related quality of life
Hth	Hypothalamus
i.p.	Intraperitoneal
IBD	Irritable bowel disease
IBS	Irritable bowel syndrome
JNK	C-jun n-terminal kinase
MAPKs	Mitogen-activated protein kinases
MPO	Myeloperoxidase
MRI	Magnetic resonance imaging
Pfc	Prefrontal cortex
PMSF	Phenylmethylsulphonyl fluoride
Rpm	Rounds per minute
RT-PCR	Reverse transcription polymerase chain reaction
s.c.	Subcutaneous
SC	Spinal cord
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TG	Trigeminal ganglion
Th	Thalamus
TRP	Transient receptor potential
TRPA1	Transient receptor potential ankyrin 1
TRPV1	Transient receptor potential vanilloid 1

TRPV4	Transient receptor potential vanilloid 4
UC	Ulcerative colitis
Vehicle	Veh
WAS	Water avoidance stress

Abstract in German

Einleitung: Funktionelle gastrointestinale Erkrankungen mit viszeralem Schmerz gehen mit zentraler Sensibilisierung und psychiatrischen Störungen einher und werden oft durch Stress ausgelöst oder verstärkt. Der Ionenkanal „Transient Receptor Potential Ankyrin 1“ (TRPA1) ist ein wichtiger Transducer schmerzhafter Faktoren und trägt zu Überempfindlichkeitsreaktionen bei. Die vorliegende Dissertation setzte sich zum Ziel, die multidimensionalen Aspekte viszeralen Schmerzes in einem Tiermodell mit Colitis und Stress zu untersuchen.

Methodik: Die Experimente wurden mit 4 Versuchsgruppen von männlichen C57BL/6N-Mäusen durchgeführt: Kontrollmäuse, Dextran Sulphate Sodium (DSS)-behandelte Mäuse, Water Avoidance Stress (WAS)-exponierte Mäuse und DSS+WAS-behandelte Mäuse. Nach diesen Behandlungen wurden verschiedene Verhaltensparameter gemessen und die Schmerzempfindlichkeit auf mechanische und thermische Reize getestet. Neurochemische Änderungen im Zentralnervensystem (ZNS; Rückenmark, Thalamus, Hypothalamus, Amygdala und präfrontaler Cortex) wurden anhand der Expression der Mitogen-Activated Protein Kinases (MAPK) pp38 und pp42/44 sowie c-Fos quantifiziert. In einem weiteren Versuch wurden Verhaltensänderungen (Lokomotion, Exploration und Freezing) und die Expression von c-Fos, pp38 und pp42/44 nach Gabe von Allylisothiocyanat (AITC) in das Kolon unter Kontrollbedingungen und nach Vorbehandlung mit dem Opioidanalgetikum Morphin bestimmt. Mit dem TRPA1-Antagonisten HC-030031 und genetischer TRPA1-Ausschaltung wurde die mögliche Implikation von TRPA1 bei DSS-induzierter Colitis geprüft. Außerdem wurde die Expression verschiedener TRP-Kanäle (mRNA) in den Dorsalwurzelganglien bei DSS-induzierter Colitis gemessen. Weiters untersuchte ich, ob die durch Colitis ausgelösten Schmerzempfindlichkeitsänderungen mit einer funktionellen Änderung von TRPA1-Kanälen in den Dorsalwurzelganglien und der Freisetzung von Calcitonin-Gene-Related-Peptide im Rückenmark einhergehen.

Ergebnisse und Diskussion: Die Induktion einer Colitis mit DSS ging mit einer Abnahme der Kolonlänge und einer Zunahme des Kolongewichts, des Disease

Activity Score und des Myeloperoxidase-Gehalts im Kolon einher, während WAS keinen Einfluss auf diese Parameter hatte. WAS- und DSS-behandelte Mäuse waren überempfindlich auf mechanische und thermische Reize (Abdomen und Hinterpfote), was mit einer erhöhten Expression von c-Fos im Rückenmark und Gehirn einherging. DSS-Behandlung verstärkte die Expression von pp42/44 im Rückenmark, während WAS die Expression von pp38 und pp42/44 in allen untersuchten Gehirnregionen steigerte. Die kombinierte DSS- und WAS-Behandlung blieb ohne Auswirkung auf die mechanische und thermische Schmerzempfindlichkeit und c-Fos-Expression im Gehirn. Die intrarektale Applikation von AITC induzierte ein Freezing-Verhalten, verminderte Lokomotion und Exploration und erhöhte die Expression von c-Fos, pp38 und pp42/44 im Gehirn, was die diskriminative, emotionale und autonome Dimensionen der viszeralen Schmerzverarbeitung widerspiegelt. Morphin unterdrückte spezifische Aspekte der verhaltensmäßigen und neurochemischen Schmerzreaktionen auf intrarektale AITC-Applikation, was für eine Beteiligung opioidsensitiver neuronaler Schmerzmechanismen spricht. TRPA1-Antagonismus und genetische TRPA1-Deletion reduzierte die mechanische Schmerzüberempfindlichkeit (Abdomen, Gesicht) DSS-behandelter Mäuse. DSS-Behandlung steigerte überdies die TRPA1 mRNA-Expression in den Dorsalwurzelganglien und erhöhte die neuronale Empfindlichkeit auf den TRPA1-Agonisten AITC. Diese Ergebnisse zeigen, dass die multidimensionalen Aspekte viszeralen Schmerzes in der Maus durch verschiedene verhaltensmäßige und neurochemische Parameter erfasst werden können. Außerdem konnte ich nachweisen, dass der TRPA1-Ionenkanal an der colitis-induzierten Schmerzüberempfindlichkeit im somatischen Bereich beteiligt ist.

Abstract in English

Introduction: Functional gastrointestinal disorders with abdominal pain are associated with central sensitization and psychological disorders that often are triggered or exacerbated by stress. The transient receptor potential ankyrin 1 (TRPA1) channel is a major transducer of nociceptive signals and is involved in hypersensitivity conditions. The current dissertation project set out to study the multi-dimensional aspects of visceral pain in an animal model involving colitis and stress.

Materials and methods: Four groups of mice (C57BL/6N) were studied: control mice, mice treated with dextran sulphate sodium (DSS), mice exposed to water avoidance stress (WAS) 1 hour daily, and mice treated with DSS+WAS for the duration of one week. Following these treatments, various dimensions of behavior were assessed, and the pain sensitivity to mechanical stimuli and thermal stimuli evaluated. Neurochemical alterations in the central nervous system (CNS; spinal cord, thalamus, hypothalamus, amygdala and prefrontal cortex) were measured by the expression pp38 and pp42/44 mitogen activated protein kinases (MAPKs). Behavioral changes (freezing, locomotion, rearing) and the expression of c-Fos and MAPKs in the CNS were also determined in response to intracolonic allyl isothiocyanate (AITC) following pre-treatment with an opioid analgesic (morphine). Pharmacological blockade of TRPA1 by the selective antagonist HC-030031 and genetic deletion of TRPA1 were used to investigate the role of TRPA1 in DSS-induced colitis. The expression of mRNA of different TRP channels was quantified in isolated dorsal root ganglion (DRG) neurons of control and DSS-treated mice. I further investigated whether the nociceptive alterations in colitis are reflected by potentiation of TRPA1 channels in DRG neurons and calcitonin gene related peptide release in the spinal cord.

Results and discussion: Induction of colitis was confirmed by a decrease in colon length but increased colonic weight, disease activity score and colonic myeloperoxidase levels, these changes remaining unaffected by WAS. Exposure to WAS led to increased locomotion and rearing as well as self-care and motivational behaviour. WAS-exposed and DSS-treated mice were hypersensitive

to mechanical and thermal pain stimuli (abdominal and plantar skin), changes that were paralleled by increased c-Fos expression in the spinal cord and brain. DSS treatment caused an increase in pp42/44 MAPK expression in the spinal cord, while WAS exposure caused an increase in pp38 and pp42/44 MAPK expression in all brain regions. Combined treatment with WAS and DSS did not change pain sensitivity (mechanical and thermal) and c-Fos expression in the CNS. Intrarectal AITC administration enhanced freezing behaviour, led to expression of c-Fos, pp38, and pp42/44 in the CNS and diminished locomotion and rearing, which reflects the discriminative, emotional and autonomic processing of a visceral pain stimulus. Morphine reduced specific aspects of the behavioral and neurochemical pain response to intrarectal AITC administration, which affirms that these aspects involved opioid-sensitive pain pathways in the CNS. In addition, DSS treatment increased the mechanical and thermal sensitivity (abdominal, facial and plantar skin). The TRPA1 antagonist and genetic deletion of TRPA1 reduced mechanical sensitivity of both the abdominal and facial region. DSS treatment caused an increase in TRPA1 mRNA expression in the DRG neurons. DSS-induced colitis increased the sensitivity of primary sensory neurons to the TRPA1 agonist, AITC. Taken together, the current findings indicate that the multidimensional aspects of visceral pain can be modeled in mice by the assessment of various behavioral parameters and neurochemical alterations in the CNS. Finally, I conclude that the TRPA1 channel participates in colitis-associated pain hypersensitivity at the somatic level.

1. Introduction

1.1. Visceral pain

According to a definition given by the International Association for the Study of Pain, pain is defined as 'an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage' (Merskey, 1994). Among the different types of pain, visceral pain is frequently reported in the clinic, as it is a common symptom of diseases (Cervero and Laird, 1999). Visceral pain is the pain of internal/visceral organs which are mainly located in the thoracic and abdominal cavities of the human body. Although the origin of visceral pain is in the internal organs, it can often be felt at distal parts of the body, and this form of pain is termed "referred pain". The causes of visceral pain vary from cellular (inflammation, injury, neoplasm) to neuronal (neurotransmitter, central sensitization) disturbances (Cervero and Laird, 1999; Molony et al., 2015). Abdominal visceral pain is the symptom of several diseases such as irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), and functional dyspepsia. Although these maladies are not considered life-threatening diseases, the patient's quality of life is often found to be severely affected by the associated visceral pain (Schirbel et al., 2010; Halpin and Ford, 2012). At the chronic stage, abdominal visceral pain can become a disease in itself, which is idiopathic and without effective treatment (Bielefeldt et al., 2009; Greenwood-Van Meerveld et al., 2015).

Studies showing an association of psychiatric disturbances with GI disorders were reported already in the 1970s (Baumann et al., 1977; Fukudo et al., 1992). However, mechanism-based studies on abdominal visceral pain with an emphasis on 'gut-brain interaction' have been done only in the last two decades (Mayer and Tillisch, 2011; Mayer, 2013). Among several factors, psychological stress is considered one of the major factors playing an important role (causal and functional) in the pathophysiology of functional GI disorders (Levy et al., 2006; Konturek et al., 2011).

1.2. Visceral pain pathways

The vagus, splanchnic and pelvic nerves innervate upper and lower abdominal visceral organs. Apart from containing efferent nerve fibers belonging to the autonomic nervous system, all of these nerves contain a considerable part of primary afferent neurons. The cell bodies of the vagal primary sensory nerve fibers reside in the nodose ganglia (vagal afferent neurons) whereas the cell bodies of the splanchnic and pelvic primary sensory neurons reside in the thoracolumbar and lumbosacral dorsal root ganglia (DRG). Primary sensory neurons innervate all abdominal organs, and their nerve endings in the abdominal viscera are primarily A-delta and C-fibers (Oudenhove et al., 2007; Sharma et al., 2009; Greenwood-Van Meerveld et al., 2015; Malony et al., 2015).

Upon activation of primary afferent neurons by the release of certain biochemicals, elicited by noxious stimuli or inflammatory mediators during inflammation, the pain signal propagates along vagal afferent neurons towards the the nucleus of the solitary tract in the medullary brainstem and along spinal afferent neurons towards the dorsal horn (laminae I,II, V, VI, VII and X) of the spinal cord. Secondary neurons in the spinal cord then carry these signals to higher centers of the brain, mainly *via* two pathways, named spinoreticular and spinothalamic sensory pathways. In the spinoreticular sensory pathway, the pain signals from the spinal cord travel to the reticular formation in the brainstem and reach the thalamus, and then are further transmitted to the somatosensory cortex, thus ascertaining the perception of location of the pain in the body. In the spinothalamic pathway, the pain signals from the spinal cord travel to the thalamus and hypothalamus, and then are further transmitted to the cerebral cortex (mainly prefrontal cortex and anterior cingulate cortex), thus ascertaining the motivational and emotional regulation of pain. Within the brain, these pain processing regions communicate with other subthalamic, hypothalamic and cortical regions, which form the pain matrix. Within the pain matrix, the amygdala is an important region for depicting the motivational and emotional regulation of visceral pain processing (Oudenhove et al., 2007; Sharma et al., 2009; Greenwood-Van Meerveld et al., 2015; Malony et al., 2015).

The activation of the pain matrix by colorectal distension (CRD) and in pathological conditions such as IBS and IBD can be visualized by Magnetic Resonance Imaging (MRI) or functional MRI and thus confirm emotional regulation of visceral pain processing. There are also pain inhibiting circuits in the central nervous system (CNS) which involve the anterior cingulate cortex, amygdala and periaqueductal gray before reaching the dorsal horn of the spinal cord via descending pathways. The convergence of different visceral and somatic afferents in the dorsal horn of the spinal cord is thought to be the main reason for referred pain in GI disorders (Oudenhove et al., 2007; Sharma et al., 2009; Greenwood-Van Meerveld et al., 2015; Malony et al., 2015).

1.3. Visceral pain in IBD

Inflammatory bowel diseases (IBD) are defined by the presence of inflammation in the GI tract. Among IBD-like conditions, ulcerative colitis (UC) is characterized by inflammation of the colon and disruption of the colonic mucosa, while discontinuous inflammation of the whole GI tract and disruption of deeper colonic structures are mostly seen in a condition called Crohn's disease (CD). Despite their dissimilarities in localization of the inflammation and different risk factors, UC and CD share several common pathophysiological features (disturbances of immune system, mucosal barrier function and gut microbiota) and clinical features (abdominal pain, presence of blood in stool, diarrhea and weight loss) (Baumgart and Sandborn, 2012; Ordas et al., 2012). In Western countries, the prevalence of IBD has increased more than 10 times in the last 50 years (Cosnes et al., 2011). Emerging evidence indicates that the prevalence of IBD has increased in Asia in recent years as well (Ng, 2014).

As with other GI disorders, visceral pain is a hallmark of IBS and IBD that affects patients' health-related quality of life (HRQOL) in terms of physical disease severity, behavioral dysfunction, physical activity, abdominal pain, and emotional well-being (Pace et al., 2003; Amouretti et al., 2006; Seres et al., 2008; Schirbel et al., 2010; Gray et al., 2011; Engelmann et al., 2015). Functional GI disorders with abdominal hypersensitivity are classically associated with central sensitization (Moshiree et al., 2006) and psychiatric disorders that are often triggered or

exacerbated by stress (Levy et al., 2006). In a recent study, a positive correlation was found between GI symptoms and psychological distress in both IBS and IBD, a correlation that was interestingly stronger in IBD compared to IBS. In the same study, psychological distress was found to be the main cause of poor HRQOL in IBD patients who were affected by severe GI symptoms (Naliboff et al., 2012). Abdominal pain in IBD also found to affect patients' HRQOL, and for clinicians the management of abdominal pain is one of the major challenges among other symptoms of IBD (Spiegel et al., 2011; Srinath et al., 2012)

The association of visceral pain with psychological distress mandates that visceral pain processing needs to be studied at the cerebral level, if efficacious therapeutics are to be developed (Mayer et al., 2008; Holschneider et al., 2011). However, the interaction between external stressors (e.g., psychological stress) and internal stressors (e.g. GI inflammation and nociception) in the sensory and emotional dimensions of visceral pain is insufficiently understood. With a clear need for improvement in chronic abdominal pain management, it is important to understand how colitis can disturb emotional well-being, along with changes in visceral and somatic pain sensitivity, and how psychological stress affects these disturbances (Jain et al., 2015).

1.4. Pain management in IBD

To manage visceral pain in IBD is a challenge for physicians, owing to its multifactorial origin and associated psychiatric comorbidities (Filipovic and Filipovic, 2014). Non-steroidal anti-inflammatory agents and selective cyclo-oxygenase-2 inhibitors are effective in the management of various inflammatory conditions. However, the use of non-steroidal anti-inflammatory agents is not recommended in IBD as it has been found to be associated with exacerbation and remission of the disease while the benefits of long-term treatment with cyclo-oxygenase-2 inhibitors has not been systemically studied in IBD (Kefalakes et al., 2009). GI motility changes have been linked to abdominal pain in IBD, and therefore antispasmodic agents are frequently used to control pain in IBD. The use of opiates is limited in IBD patients suffering from chronic or severe pain because

of their side effects, such as abuse, dependence, and GI as well as psychological disturbances (Srinath et al., 2012).

Psychotropic agents, such as tricyclic antidepressants, selective serotonin reuptake inhibitors, and serotonin norepinephrine reuptake inhibitors, are recommended to control the psychological comorbidities associated with IBD. These agents have been found to be effective in reducing abdominal pain associated with IBD (Larauche et al., 2012; Srinath et al., 2012). Disturbance of the physiological gut microbiota can potentiate on-going inflammation and abdominal pain in IBD, while the restoration of disturbed gut microbiota is a new approach towards an improved management of IBD symptoms. Other emerging therapeutic targets are mast cell mediators, endocannabinoids, gut hormones, neuropeptides (neuropeptide Y and peptide YY), corticotropin-releasing factor (CRF), and transient receptor potential channels, which may add to the management of IBD- and IBS-like conditions (Holzer et al., 2015).

Apart from neuropharmacological approaches, studies with psychological (cognitive behavioral therapy, hypnotherapy), psychosocial (coping skill modification, stress management), as well as procedural (acupuncture, nerve block, transcutaneous electrical nerve stimulation) approaches have also been reported for symptomatic management in IBD (Srinath et al., 2012). Among these, psychotherapy and stress management in IBD patients appeared to reduce abdominal pain to a larger extent than the usual treatment (McCombie et al., 2013). Several drugs used to treat IBD, such as 5-aminosalicylate, sulfasalazine, glucocorticoids (GC), azathioprine, 6-mercaptopurine, 6-thioguanine, methotrexate, cyclosporine and tacrolimus, are commonly associated with GI and liver adverse effects, and can even worsen the abdominal pain in IBD (Rogler, 2010).

1.5. Stress and visceral pain

The term ‘stress’ was first defined by Hans Selye, according to whom stress is an acute threat to the homeostasis of an organism (Selye, 1976). Stressful events have been found to induce either hyperalgesia or analgesia, the type of

response depending on genetic background as well as type, intensity, frequency and duration of the stressor (Ford and Finn, 2008; Jenning et al., 2014; Miguez et al., 2014). External stressors (psychological) are perceived mainly by the brain, leading to the activation of the CNS and neuroendocrine system in order to cope with the stressor and bring the body back to homeostasis. When the stressor is severe and chronic, the changes in the neural and endocrine mechanisms of the body may no longer be reversed to re-gain homeostasis and thus may result in diseases and disorders. Numerous studies suggest that visceral pain perception is highly affected by psychological stress. In addition, neuroimmune mediators, such as cytokines, noradrenaline, dopamine, substance P, CGRP, endocannobinoids, and mediators of the hypothalamic-pituitary-adrenal (HPA) axis such as CRF and GC (cortisol or corticosterone), are thought to play a pivotal role in stress-driven nociception. IBS and IBD with abdominal hypersensitivity are frequently associated with psychiatric disorders that are often triggered or exacerbated by stress. In addition, stress can enhance the permeability of the GI mucosa and increase intestinal vulnerability to injury (Larauche et al., 2011; Larauche et al., 2012; Greenwood-Van Meerveld et al., 2015; Malony et al., 2015).

Experimental stress was found to be associated with increased cytokine levels, gut motility, and decreased rectal pain threshold in healthy subjects (Rao et al., 1998; Gonlachanvit et al., 2005; Miller et al., 2009). In IBD patients, stress has been found to be associated with an exacerbation of symptom severity (Mawdsley et al., 2006; Kuroki et al., 2007), on the one hand, and activation of the HPA axis, on the other hand (Straub et al., 1998; Straub et al., 2002).

1.6. Dextran sulfate sodium (DSS)-induced colitis as a model of visceral hypersensitivity

Among various models of colitis, chemically induced GI inflammation models are widely used and adopted to study visceral hypersensitivity and pain. The advantages of a chemically induced model over other models of colitis are non-invasiveness, easy development, and reproducibility. Besides 2,4,6-trinitrobenzene sulfonic acid (TNBS), oxazolone and acetic acid, DSS is widely

used to induce experimental inflammation of the colon and to study IBD-like conditions (Randhawa et al., 2014). This model of GI inflammation (Dothel et al., 2013) involves a noninvasive route of DSS administration via the drinking water, which avoids additional stress. It reproduces several pathophysiological features of IBD, particularly of UC, such as increased expression of interleukin-6, interleukin-10, and tumor necrosis factor alpha, elevated colonic MPO levels, inflammatory histopathology, as well as symptomatic features of IBD such as bloody diarrhea and a decrease in food and water intake (Sainathan et al., 2008; Perse and Cerar, 2012). Induction of mild or severe colitis in rodents can be easily achieved by adjusting the concentration and time of exposure to DSS. Treatment with 2 % DSS in the drinking water induces mild colitis in mice (Mitrovic et al., 2010). Exposure of the colonic epithelium to DSS causes structural disruption and, therefore, increases the permeability of the mucosa. As a result, profiles of both T helper 1 and T helper 2 cytokines are affected, and most proinflammatory cytokine levels are increased at the site of inflammations well as in the circulation (Randhawa et al., 2014).

The presence of colonic visceral hypersensitivity in the post-inflammatory stage of DSS-induced colitis has been confirmed in rodents by using several methods. The most widely used method is to record the visceromotor response of the abdominal striated muscle (electromyographic recording) to colorectal distension. The drawback of this method is that the visceromotor recordings can be influenced by stress induced by surgical procedure, post-surgical single housing, and treatment with antibiotics. The activation of several brain regions involved in the processing of visceral pain by CRD in rodents (control and colitis) is comparable to observations made in healthy subjects and IBS patients. The possibility of interference by anesthetics with the animal's CNS response to CRD is another limitation of this approach (Larauche et al., 2012). Recording of spontaneous behavior and measurement of biochemical changes in the CNS after irritation of the colonic mucosa by an irritant stimulus such as allyl isothiocyanate (AITC) and/or capsaicin, is another commonly used approach to study visceral pain processing in experimental colitis. Intestinal hyperalgesia is associated with mechanical hypersensitivity of the abdominal skin, and this phenomenon of referred pain has frequently been used as a measure of intestinal pain (Laird et al.,

2001; Eijkelkamp et al., 2007; Jain et al., 2015). Referred pain on the surface of the abdomen in rodents can be measured by the conventional von Frey hair test, which is also used to measure mechanical allodynia in humans (Tena et al., 2012).

1.7. Water avoidance stress (WAS) as a model of visceral hypersensitivity

Psychological stress can affect GI function, induce visceral hypersensitivity, interfere with the pathophysiological features of IBD, and is considered as one of the major factors responsible for IBD associated psychological comorbidities in humans. Interestingly, the features of visceral pain in IBS and IBD, such as colonic hypersensitivity and activation of brain regions involved in visceral pain processing, can be observed in rodents in response to several psychological stressors. Although the nature of stressors is different in rodents from humans, the validity of the stressor response in rodents is comparable to humans, particularly in terms of physiological changes, such as an increase in HPA axis activity as reflected by increased levels of CRF and corticosterone. Exposure to stressors during the perinatal period (maternal prenatal stress) or early life period (maternal separation stress) is associated with altered visceral sensitivity in the adult life period, probably through epigenetic mechanisms (Mawdsley et al., 2006; Larauche et al., 2011; Larauche et al., 2012; Greenwood-Van Meerveld et al., 2015; Malony et al., 2015).

WAS has been widely used as a model of psychological stress in which mice or rats are placed on a small platform in the middle of a water-filled tank, a situation that cannot be avoided and thus is stressful for rodents, also due to their fear of water. The resulting activation of the HPA axis as well as changes in visceral sensitivity confirm the validity of this experimental paradigm as a psychological stress model in rodents, with considerable face and construct validity relative to psychosocial stress in humans. Both acute and chronic WAS have been found to affect GI permeability, motility and pain sensitivity in rodents (Bradesi et al., 2005; Schwetz et al., 2005; Larauche et al., 2010; Konturec et al., 2011). The continuous (daily) but intermittent WAS protocol is considered to be

comparable to human conditions in which subjects are exposed to psychological stress in their daily life, as regards the time of exposure to stressors. The visceromotor response to colorectal distension was found to be increased by WAS in rodents. Similarly, WAS exposure was also reported to increase the pain-related behavior induced by intracolonic administration of capsaicin. In rodents, the development of visceral hypersensitivity by WAS (acute and chronic) was linked to the involvement of GC-C receptors, CRF, 5-hydroxytryptamine-3 receptors, cannabinoid CB1 receptors, release of different peripheral mediators (histamine, prostaglandin E2, nerve growth factor) and disturbance of the physiologic gut microbiota. The development of colonic visceral hypersensitivity is thought to be regulated by the peripheral sensitization of sensory afferent neurons by TRP channels, on the one hand, and by central sensitization at the spinal cord and stress-responsive limbic areas of the brain, on the other hand (Greenwood-Van Meerveld et al., 2015; Reichmann et al., 2015).

1.8. Interaction between stress and colitis

Based on above information, the multifactorial origin of visceral pain under conditions of inflammation and stress can be summarised in Figure 1. Within the gut-brain axis, the communication from the gut to the brain is maintained by sensory afferent neurons and other factors (gut hormones, immune mediators and microbial metabolites), while the brain to gut communication is maintained by efferent neurons and neuroendocrine mediators. Under physiologic conditions, gut function is regulated by mediators of the nervous system (enteric, afferent, central and autonomic), endocrine system (gut hormones, CRF, HPA axis and adrenaline) and gut microbiota. These mediators can be disturbed by internal (GI inflammation, irritants) and external (psychological stress) stressors. As the connections between these mediators are interdependent, disturbance of one factor disturbs also others.

Both colitis and psychological stress share several common pathophysiologic mechanisms (peripheral and central) to induce colonic visceral hypersensitivity. The involvement of peripheral and central mediators responsible for visceral hypersensitivity are not completely understood, and this could be one

potential reason why the pharmacologic treatment of visceral pain is utterly unsatisfactory. The development of efficacious therapeutics is hampered by a lack of appropriate preclinical models to study the cerebral manifestations of abdominal nociception. Arguably, this goal can only be achieved if the interaction of internal stressors (e.g., GI inflammation and nociception) with external stressors (e.g., psychological stress) in the cerebral circuits underlying the sensory-discriminative, affective-motivational and cognitive-evaluative dimension of pain (Melzack, 1999; Fukudo, 2013) is better understood (Jain et al., 2015).

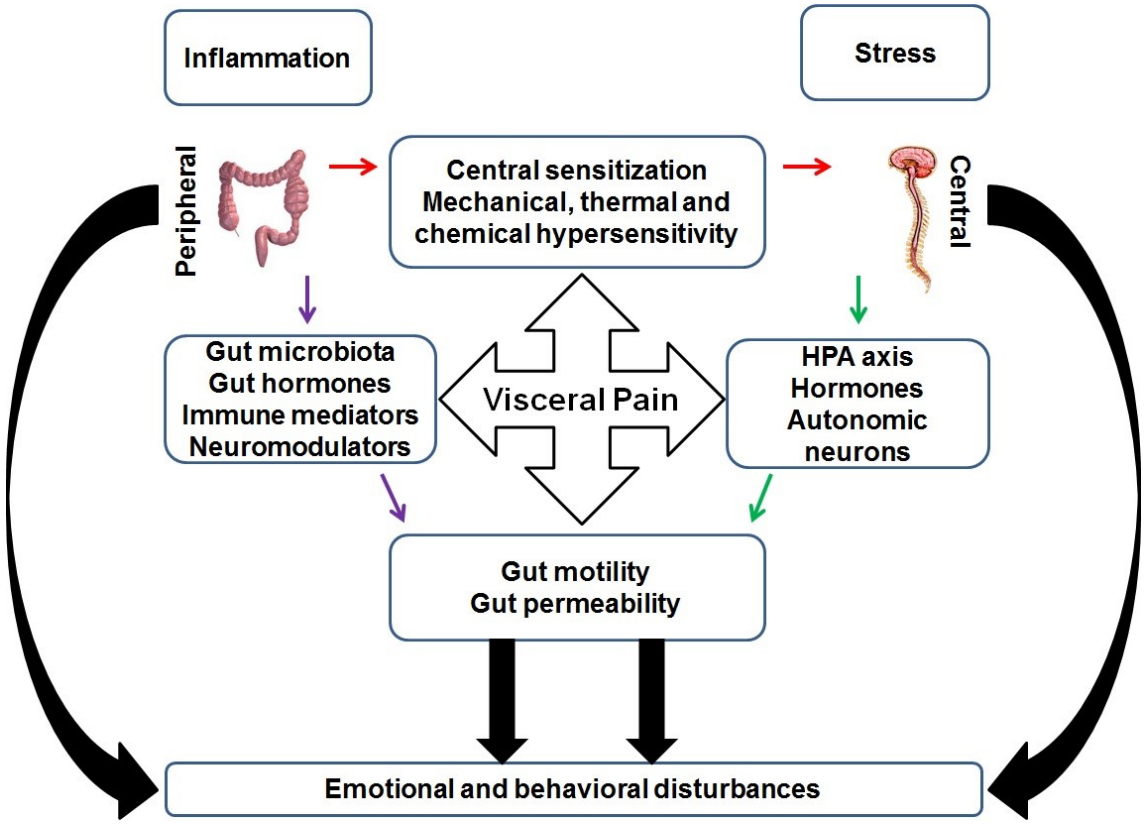

Figure 1. Multifactorial origin of visceral pain under conditions of inflammation and stress and interaction of peripheral and central mediators and factors. Inflammation in the colon disturbs GI microbiota and the release of immune mediators, gut hormones and neuromodulators (substance P, calcitonin gene-related peptide), which changes gut motility, increases gut permeability and elicits abdominal pain. Stress is perceived by the brain followed by activation of the hypothalamic-pituitary-adrenal (HPA) axis, autonomic neurons and neurotransmitters (norepinephrine) and release of neuroendocrine mediators

(glucocorticoids, adrenaline), which disturb gut motility and permeability. Visceral pain associated with colitis and stress is associated with central sensitization (mechanical, thermal and chemical hypersensitivity). The visceral hypersensitivity as well as disturbed gut motility and permeability induced by colitis and stress are associated with several behavioral disturbances.

1.9. MAPK and c-Fos in pain

The study of biochemical changes in the CNS is one promising approach to understanding the molecular mechanisms of somatic and visceral hypersensitivity. p38, p42/44 MAPKs and c-Jun N-terminal kinase (JNK), are serine/threonine-specific protein kinases that can be activated or deactivated in response to stress, inflammation, heat shock, and the like. Their activation leads to changes in different cellular functions and, with its cellular actions, the MAPK pathway plays an important role in the development of neuronal plasticity and initiation of pain hypersensitivity under conditions of inflammation, nerve injury and stress (Obata and Noguchi, 2004; Obata et al., 2004; Cruz and Cruz, 2007; Ji et al., 2009; Cargnello and Roux, 2011).

Western blot analysis of inflamed colonic tissues obtained from IBD patients showed activation of MAPKs (p42/44, p38-alpha and JNK); in addition, p38-alpha was highly expressed in macrophages and neutrophils as seen by immunohistochemistry. Only in a few studies MAPK inhibitors were tested in IBD patients and found to be effective to control IBD progression, mainly by interrupting the activity of proinflammatory cytokines. As reported in preclinical and clinical studies, GC and infliximab (tumor necrosis factor alpha inhibitor) play an important role in IBD remission through MAPK signaling, although the effect on visceral hypersensitivity has not been explored in these studies (Waetzig et al., 2002; Broom et al., 2009; Coskun et al., 2011).

Tissue injury and inflammation cause the release of several inflammatory mediators (for instance, cytokines) from non-neuronal cells and neurochemicals (brain-derived neurotrophic factor, substance P, glutamate, adenosine monophosphate) in the spinal cord dorsal horn. These mediators act on their

respective receptors in postsynaptic neurons and lead to the activation of MAPK kinases, which activate the p38 and p42/44 MAPKs by a separate cascade. Activated MAPKs can then induce central sensitization via post-translation changes, or maintain the central sensitization via posttranscriptional changes. The series of events which is responsible for central sensitization through p38 is still not completely understood (Ji et al., 2009).

c-Fos is an early transcriptional factor belonging to the Fos family. In animal studies, c-Fos is used as a marker to study neuronal excitation linked to nociception, and its expression in the CNS can be changed in conditions such as stress and colitis. GI pain induces expression of c-Fos in the spinal cord and brain, a process that is thought to portray neuronal excitation linked to nociception (Michl et al., 2001; Mitrovic et al., 2001; Eijkelkamp et al., 2007) and stress (Matsuda et al., 1996; Quintero et al., 2003). c-Fos, which is downstream of the p42/44 MAPK pathway, can be activated by tissue inflammation and injury in the spinal cord dorsal horn neurons. It is mainly involved in transcriptional changes that accompany central sensitization. Although the direct role of c-Fos in nociception is not clear, evidence indicates that it changes the signal transduction pathways and is responsible for intracellular changes, thus modifying the function of spinal nociceptive circuits. The role of c-Fos in nociception can be examined by the changes in its expression pattern by different stimuli and pharmacologic inhibitors (Gao and Ji, 2009).

Most of the studies involving c-Fos and pp42/44 as a marker of neuronal activation in response to colonic stimulation are limited to the dorsal horn neurons or the lumbosacral spinal cord (Gao and Ji, 2009; Larauche et al., 2012). However, the effect of colonic pain on the activation of MAPKs and induction of c-Fos in the brain under conditions of colitis and psychological stress has not yet been systematically explored.

1.10. TRPA1 in visceral pain

TRPA1 belongs to a subfamily of TRP channels activated by chemical, mechanical and thermal stimuli, subserving important roles in the regulation of

homeostasis and in tissue injury and inflammation. TRPA1 activation/sensitization has been shown to affect physiologic and pathophysiologic processes in the gut. Specifically, TRPA1 channels are involved in gut hypersensitivity and inflammation which are associated with the pathophysiology of various GI diseases. In humans and rodents, TRPA1 is mainly present in primary sensory neurons and highly expressed in DRG and TG neurons (Baraldi et al., 2010; Holzer, 2011a; Holzer, 2011b; Geppetti et al., 2014; Holzer and Izzo, 2014). It has already been demonstrated that TRPA1 contributes to inflammatory hypersensitivity to colonic distension in chemically induced colitis (Yang et al., 2008). CGRP release from peripheral fibers of DRG neurons leads to neurogenic inflammation and leukocyte migration into the tissue. CGRP release can also be activated by TRPA1 activation, whereas pharmacological inhibition of TRPA1 and TRPV1 on nociceptive sensory neurons attenuates experimental colitis (Eysselein et al., 1992).

TRPA1 is considered to be an important factor in nociceptive processing because activation of TRPA1 by various chemicals induces pain-related behavior, whereas pain responses are reduced in TRPA1 deficient mice or by pharmacological inhibition of TRPA1, for instance by HC-030031, a selective TRPA1 antagonist (McNamara et al., 2007; Fujita F., 2008). Taken many findings together, there is abundant evidence that TRPA1 is involved in the control of pain sensitivity in several somatic and visceral tissues.

1.11. AITC and its use in experimental studies

AITC is a chemical present in several plants such as onions, garlic, mustard and horseradish in which it is responsible for their pungent taste. AITC stimulates TRPA1 and TRPV1 receptors on nociceptive nerve fibers (Everaerts et al. 2011; Holzer et al. 2011a).

AITC produces chemonociception mainly via activating TRPA1 channels and is used to study visceral pain behavior when administered intracolonically in rodents (Laird et al., 2001). In a previous study Mitrovic and colleagues have shown that visceral hyperalgesia induced by DSS amplifies AITC-evoked c-Fos

expression in the sacral region of the spinal cord (laminae I-IIo of S1) (Mitrovic et al., 2010). Activation of c-fos in spinal cord correlates with the induction of spontaneous pain behavior by different stimuli, an effect that is reduced by the intrathecal administration of c-Fos antisense oligodeoxynucleotides (Gao and Ji., 2009).

Intracolonic administration of AITC is likewise associated with activation of p42/44 MAPK in the lumbosacral spinal cord (Galan et al. 2003), and inhibition of the MAPK pathway has been reported to attenuate visceral nociception (Lai et al., 2011). Activation of TRPA1 by AITC has been shown to trigger a nociceptive response and to evoke a calcium-dependent release of CGRP in the trigeminovascular system, effects that are prevented in TRPA1 deficient mice (Nassini et al., 2012). Although the role of CGRP in IBS is not fully understood, TRPA1-evoked release of CGRP from primary afferent neurons and p42/44 activation in these neurons has been linked to development of gut hyperalgesia (Kondo et al., 2010).

1.12. Aims and objectives of the study

The overall goal of the current study was to investigate the interaction of experimental colitis and psychological stress in their impact on nociception-related behavior and neurochemical dynamics in the mouse brain. In the present study, WAS was used as a psychological stressor. To study the interaction of WAS with an internal GI stressor, colitis induced by DSS added to the drinking water, was used.

The first aim was to study the interaction of DSS-induced colitis and repeated WAS exposure in mice with respect to inflammation, disease severity and behavior (locomotion, exploration, ingestion, motivation and self-care).

The second aim was to examine whether DSS-induced colitis, repeated WAS, and combined WAS+DSS treatment is associated with a change in the mechanical and thermal pain sensitivity of the abdominal and plantar skin. I was further interested to explore whether the nociceptive and/or behavioral alterations

evoked by colitis and/or stress are reflected by activation of MAPKs and expression of the immediate early gene c-fos in the CNS.

The third aim was to examine whether the behavioral and molecular CNS responses to acute colonic pain are affected by DSS-induced colitis and/or repeated WAS exposure. To elicit colonic pain I used AITC. In particular, I examined whether intracolonic AITC activates cerebral pain pathways as reflected by behavioral changes (freezing, locomotion, exploration) and parallel activation of both MAPKs and expression of c-Fos in the spinal cord, cortex (perceptional dimension of pain) and limbic system (emotional dimension of pain).

The fourth aim was to address the question whether the AITC-evoked behavioral and molecular CNS responses can be alleviated by an analgesic drug such as morphine, thus confirming the nociceptive nature of these effects (Mitrovic et al., 2010; Schwartz et al., 2013; Jain et al., 2015). In addition, the effect of morphine was studied in DSS-, WAS- and WAS+DSS-treated mice to shed light on the dynamics of cerebral pain processing under conditions of colitis and stress.

The fifth aim was to address the question whether TRPA1 play a causal role in behavioral and neurochemical manifestations of visceral pain in DSS-induced colitis. Specifically I investigated the mechanical pain sensitivity of abdominal and facial skin and thermal pain sensitivity of plantar skin in control and DSS-treated mice in the presence of a TRPA1 antagonist and following genetic deletion of TRPA1. I was further interested in examining whether the nociceptive alterations evoked by colitis are reflected by potentiation of TRPA1 channels in DRG neurons and CGRP release from central nerve endings in the spinal cord.

2. Materials and Methods

The description of Materials and Methods follows in part the text published in Jain et al. (2015).

2.1. Experimental animals

Studies 1-6 were performed at the Institute of Experimental and Clinical Pharmacology of the Medical University of Graz, Austria. Male C57BL/6N mice (8 weeks old, 20-24 g) were obtained from Charles River (Sulzfeld, Germany) and habituated to the laboratory environment for at least 2 weeks. The animals were housed either one (study 2, see below) or two (study 1, 3, 4, 5 and 6, see below) per cage under controlled conditions of temperature (set point 21°C), air humidity (set point 50%) and a 12 h light/dark cycle (lights on at 6:00 a.m., lights off at 6:00 p.m.). Standard laboratory chow (altromin 1324 FORTI, Altromin, Lage, Germany) was provided ad libitum throughout the studies. All experiments were approved by an ethical committee at the Federal Ministry of Science and Research of the Republic of Austria (BMWF-66.010/0118-II/3b/2011 and BMWFW-66.010/0054-WF/II/3b/2014) and conducted according to the Directives 86/609/EEC and 2010/63/EU of the European Communities Council.

Studies 7 and 8 were conducted at the Department of Health Science, Section of Clinical Pharmacology and Oncology, University of Florence, Italy. Male C57BL/6N mice (8 weeks old, 20-24 g) were obtained from Harlan Laboratories (Milan, Italy). Male or female wild type ($TRPA1^{+/+}$) or TRPA1 deficient ($TRPA1^{-/-}$) mice of C57BL/6 background (B6;129P-Trpa1tm1Kykw/J; Jackson Laboratories, Italy) were generated in the laboratory in Florence, Italy. The animals were housed two per cage under controlled conditions of temperature (set point 21°C), air humidity (set point 50%) and a 12 h light/dark cycle (lights on at 6:00 a.m., lights off at 6:00 p.m.). Animal experiments were carried out in conformity to the European Communities Council (ECC) guidelines for animal care procedures and the Italian legislation (DL 116/92) application of the ECC directive 86/609/EEC. Studies were conducted under the University of Florence research permit number 204/2012-B. The experiments were designed in such a way that both the number of animals used and their suffering was minimized.

2.2. Study design

Eight studies (study 1-8, Table 1) were carried out. In each study except studies 7 and 8, mice were randomly allocated to four treatment groups: group I (control; no treatment), group II (WAS, subjected to intermittent WAS for 7 days), group III (DSS, treated with DSS for 7 days) and group IV (WAS+DSS, subjected to intermittent WAS and treated with DSS for 7 days). Group II animals were challenged with intermittent WAS by placing them one hour/day (7 days) on a small platform (6 x 3 x 3 cm; length x width x height) in the center of a water-filled tank (50 x 32 x 30 cm; length x width x height) (Melgar et al., 2008). The water level in the tank was kept at 0.5 to 1 cm below the platform. Group III animals were treated with 2% (w/v) DSS (molecular weight 36,000-50,000; MP Biomedicals, Illkirch, France) in the drinking water for 7 days. Group IV animals underwent both the WAS challenge and DSS treatment for 7 days. The body weight of the animals was measured on day 1 before the start of any treatment and on day 8 (Jain et al., 2015).

After completion of the 7-day treatment period, the animals were randomly assigned to one of the following studies (Table 1).

In study 1, the animals were euthanized by intraperitoneal (i.p.) injection of pentobarbital (150 mg/kg) on day 8; then spinal cords and brains were isolated, homogenized and subjected to Western blot analysis. The expression of phosphorylated p38 and p42/44 MAPK as well as c-Fos was evaluated in the lumbosacral spinal cord and brain.

Study 2 and 3 were carried out to examine behavioral changes in response to the treatment regimens on day 8. In study 2, short-term activity (locomotion, exploration and ingestion) for a period of 60 min was measured with the LabMaster system (TSE Systems, Bad Homburg, Germany). Then study 2 was continued and the behavior of the mice was evaluated again on day 12 after the beginning of the 7-day treatment. In study 3, the motivational and self-care behavior of animals was estimated with the splash test.

Study 4 was designed to assess somatic pain sensitivity of the abdominal and plantar region. On day 8, the von Frey hair test for mechanical pain sensitivity and on day 9 the plantar test for thermal pain sensitivity were performed.

Study 5 was carried out to examine the effect of intrarectal administration of AITC (Sigma-Aldrich, Vienna, Austria) on visceral pain behavior and protein expression in the CNS of all four treatment groups (group I-IV). The animals received an intrarectal instillation (0.05 ml) of either AITC (2% v/v) in peanut oil (Sigma-Aldrich) or peanut oil (vehicle control for AITC). Immediately after AITC instillation, visceral pain behavior was recorded for 15 min. One hour post-AITC instillation, the mice were sacrificed and tissues collected.

Study 6 was carried out to examine the effect of intrarectal administration of AITC in combination with morphine (Gerot-Lannach, Lannach, Austria) premedication on visceral pain behavior and protein expression. These experiments were performed with all treatment groups. Group I received an intrarectal instillation (0.05 ml) of either AITC (2% v/v) in peanut oil (Sigma-Aldrich) or peanut oil (vehicle control for AITC), while groups II-IV received AITC only. One hour prior to AITC treatment, group I received a subcutaneous injection (2 ml/kg) of either saline (0.9% NaCl in water, w/v) or morphine (10 mg/kg in saline), while groups II-IV received morphine only. Immediately after AITC instillation, visceral pain behavior was recorded for 15 min. One hour post-AITC instillation, the mice were sacrificed and tissues collected.

In study 7, the effects of the TRPA1 antagonist HC-030031 (100 mg/kg, i.p.), relative to vehicle, as well as the effects of genetic deletion of TRPA1 on the severity of DSS-induced colitis and on somatic pain sensitivity in the abdominal and facial region were examined. These experiments comprised two different sets.

In set 1, Male C57BL/6N mice were randomly allocated to four treatment groups: group I (control, no treatment, vehicle-injected), group II (control, no treatment, HC-030031-injected) group III (DSS, treated with DSS for seven days, vehicle-injected) and group IV (DSS, treated with DSS for 7 days, HC-030031-injected). On day 8, the von Frey hair test for mechanical pain sensitivity and the hot plate test for thermal pain sensitivity were performed. One hour prior to the tests, the mice were treated with an i.p. injection (10 ml/kg) of either HC-030031

(100 mg/kg, i.p.) in 4% dimethyl sulfoxide (DMSO) and 4% Tween 20 or its vehicle (4% DMSO and 4% Tween 20 in isotonic NaCl solution).

In set 2, male and female *TRPA1*$^{+/+}$ and *TRPA1*$^{-/-}$ mice were randomly allocated to two treatment groups: group I (control, no treatment), group II (DSS, treated with DSS for seven days). On day 8, the von Frey hair test for mechanical pain sensitivity and the hot plate test for thermal pain sensitivity were performed.

In study 8, male C57BL/6N mice were used to study the effects of DSS treatment (added to the drinking water for 7 days) and, in some experiments, of HC-030031 treatment on various molecular and functional parameters in the DRG or spinal cord. These experiments comprised three different sets. After completion of the 7-day treatment with DSS (or no treatment in control mice), the animals were randomly assigned to one of the following experimental sets on day 8.

In set 1, the effects of DSS and the TRPA1 antagonist HC-030031 (100 mg/kg, i.p.), relative to vehicle, on mRNA expression in sensory neurons were examined. The animals were randomly allocated to four treatment groups: group I (control, no treatment, vehicle-injected), group II (control, no treatment, HC-030031-injected) group III (DSS, treated with DSS for seven days, vehicle-injected) and group IV (DSS, treated with DSS for 7 days, HC-030031-injected). One hour after injection of vehicle or HC-030031 the animals were euthanized followed by isolation of lumbosacral DRGs. The isolated DRGs were subjected to reverse transcription polymerase chain reaction (RT-PCR) analysis to study the expression of TRPA1, TRPV1 and TRPV4 mRNA.

In set 2, the effects of DSS and the TRPA1 agonist AITC on the release of calcitonin gene-related peptide (CGRP) in the spinal cord were examined. Following treatment with DSS for 7 days (or without treatment) the animals were euthanized followed by isolation of the thoracosacral spinal cords. The isolated spinal cord tissues were used for studying the release of CGRP evoked by AITC.

In set 3, the effects of DSS, the TRPA1 agonist AITC and the TRPV1 agonist capsaicin on functional parameters in sensory neurons were investigated. Following treatment with DSS for 7 days (or without treatment) the animals were

euthanized, after which the thoracosacral DRGs were isolated and cultured for 24 hours and then subjected to electrophysiological studies.

At the end of each study, the animals were euthanized by pentobarbital (150 mg/kg i.p., studies 1-6) or inhalation of CO_2 plus 10-50% O_2 (studies 7-8), after which the severity of colitis was assessed by recording of a disease activity score (DAS). In addition, colon length (without stretching), weight (after cleaning) and colonic MPO mass (studies 1-6) or MPO activity (studies 7-8) were estimated. At the end of study 1, 5 and 6, the spinal cords and brains were isolated, homogenized, and subjected to Western blot analysis, whereas DRGs and spinal cords were harvested in study 8.

Table 1: Experimental groups and study plan.

Studies	Days 1-7	Day 8	Day 9
Study 1 (40 mice)	Control, WAS, DSS, WAS+DSS	Western blot analysis	
Study 2 (41 mice)	Control, WAS, DSS, WAS+DSS	Recording of locomotion, exploration and ingestion	
Study 3 (32 mice)	Control, WAS, DSS, WAS+DSS	Splash test	
Study 4 (46 mice)	Control, WAS, DSS, WAS+DSS	von Frey test	Plantar test
Study 5 (80)	Control, WAS, DSS, WAS+DSS	Intrarectal AITC instillation followed by recording of visceral pain behavior and Western blot analysis	
Study 6 (55)	Control, WAS, DSS, WAS+DSS	Intrarectal AITC instillation in the absence or presence of morphine followed by recording of visceral pain behavior and Western blot analysis	

Study 7 **Set 1 (32)** **Set 2 (24)**	Control, DSS	i.p. HC-030031 administration followed by von Frey test and hot-plate test (set 1) von Frey test and hot-plate test in $TRPA1^{+/+}$ and $TRPA1^{-/-}$ mice (set 2)	
Study 8 **Set 1 (20)** **Set 2 (12)** **Set 3 (6)**	Control, DSS	Isolation of DRGs followed by RT-PCR analysis (set 1) Isolation of spinal cord followed by CGRP release measurement (set 2) Isolation and culture of DRGs (set 3)	Electro-physiological studies

2.3. Experimental protocols

2.3.1. Disease activity score

Following sacrifice, a DAS based on fur appearance and stool consistency was recorded. Normal and abnormal fur appearance scored 0 and 1, respectively. Normal, soft but formed and loose stool scored 0, 1 and 2, respectively, and the absence of bleeding or the presence of blood traces or gross bleeding in the perianal region scored 0, 1 and 2, respectively. The absence or presence of blood in the stool was evaluated with the HemdetectR test (DIPROmed, Weigelsdorf, Austria) and scored as 0 or 1. The sum of all scores in each category yielded scores of 0 to 6 (Reichmann et al., 2013; Jain et al., 2015).

2.3.2. Colonic MPO content

MPO mass in the distal colon was quantitated with a commercially available ELISA kit (Hycult Biotechnology, Uden, The Netherlands). Tissue samples were prepared according to the manufacturer's recommendations. Briefly, tissues were

weighed and homogenized in lysis buffer (200 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 10 mM trishydroxy methylaminomethane, 10% (v/v) glycerine, 1 mM phenylmethylsulphonyl fluoride (PMSF, Sigma-Aldrich-P7626), 1 mg/ml leupeptide, and 28 mg/ml aprotinin, pH 7.4) at a tissue:lysis buffer ratio of 1 mg:0.02 ml. Tissue debris was pelleted by centrifugation (two runs at 6,000 x g and 4°C for 15 min). Supernatants were used for measurement of MPO mass as marker for neutrophil infiltration. All standard and sample values were subtracted with blank control values, and MPO concentrations were quantitated using a standard curve. The sensitivity of the assay was 1 ng/ml at an intra- and inter-assay variation of around 10% (Reichmann et al., 2013; Jain et al., 2015).

2.3.3. Colonic MPO activity

Tissues were weighed and homogenized in lysis buffer (80 mM sodium acetate buffer, pH 5.4 plus 0.5% hexadecyltrimethylammonium bromide, pH 7.4) at a tissue:lysis buffer ratio of 1 mg:0.02 ml. Tissue debris was pelleted by centrifugation (two runs at 11,200 x g and 4°C for 20 min). Supernatants were used for measurement of MPO activity as marker for neutrophil infiltration. For assay, 10 µl of supernatant and 220 µl of 80 mM sodium acetate buffer (pH 5.4) were added in triplicate to a 96-well plate. The reaction was initiated by the addition of 20 µl of 18.4 mM tetramethylbenzidine. The mixture was incubated for 3 min at 37 °C and then immersed in an ice bath. The reaction was stopped by the addition of 30 µl of acetic acid and the absorbance was monitored at a wavelength of 630 nm. All standard and sample values were subtracted with blank control values, and MPO activity was quantitated using a standard curve. The MPO activity was expressed as MPO per g of tissue (Hoffmeister et al., 2011; Trevisan et al., 2013a).

2.3.4. Locomotor and ingestive activity

The mice were habituated to the water bottles used in the LabMaster system for 7 days before starting the 7-day treatment with WAS, DSS or

WAS+DSS. On day 8, the activity (locomotion, exploration and ingestion) of the animals was recorded with the LabMaster system. For this purpose, the animals were placed in test cages (42 x 26.5 x 15 cm, length x width x height) which were connected to the LabMaster system for the recording of locomotion, exploration (rearing), feeding and drinking on day 8 for 60 min without interruption (study 2). Subsequently from day 8 to 12, the mice were kept in the test cages without any interruption. The LabMaster system was composed of six recording units consisting of six moveable cages surrounded by two horizontal infra-red frames and a cage lid with two weight transducers (Painsipp et al., 2013). Two frames positioned above each other allowed to record ambulatory (locomotion) and vertical (rearing, exploration) movements. The two weight transducers were used to record food and water intake. The recordings of ingestion were normalized to body weight (Jain et al., 2015).

2.3.5. Splash test

The splash test was used to assess grooming behavior (Taksande et al., 2013). On day 8, a 10% (w/v) sucrose solution (0.1 to 0.2 ml) in water was sprayed on the dorsal surface of the mice. Subsequently, the mice were placed in small plexiglass cages with tiny bedding (study 3). The cages were connected to the LabMaster system to record locomotion and rearing. Simultaneously, the behavior of the animals during the first 5 min was recorded with a video camera to evaluate grooming behavior. The video recordings were analyzed by a trained blinded observer with the VideoMot2 (TSE Systems) event monitoring module. The time taken to start grooming (either nose/face or head/body grooming) was measured and considered as an index of self-care while grooming frequency was used to assess motivational behavior (Taksande et al., 2013). A decrease in grooming frequency and an increase in grooming latency is indicative of low motivational and reduced self-care behavior (Taksande et al., 2013), respectively (Jain et al., 2015).

2.3.6. von Frey hair test (abdominal and plantar region)

On day 8, the mechanical pain sensitivity of the abdominal (study 4 and study 7) and plantar region (study 4) was evaluated with von Frey filaments (Bioseb, Vitrolles, France) by a modification of the method of Laird et al. (2001). The mice were habituated in small plexiglas compartments on a perforated grid (Dynamic Plantar Aesthesiometer, Ugo Basile, Comerio, Italy) for one hour. Subsequently von Frey filaments were applied to the abdomen (between diaphragm and genitals) and the plantar side of the right and the left hind paw. The test was performed by a trained blinded observer. The individual filaments were tested in an ascending order covering 0.008, 0.02, 0.04, 0.07, 0.16, 0.4 and 0.6 g forces. Each force was applied 10 times to the abdominal surface, and 5 times to the right and the left hind paw. The maximal duration of each force application was 2 s, and the inter-stimulus interval was 2-3 min. Following each challenge, the withdrawal response was quantified either as 1 (withdrawal of abdominal wall or paw, licking or retraction of animal) or 0 (no response). All counts in response to an individual filament were averaged. Withdrawal responses to low forces reflect high mechanical pain sensitivity (Jain et al., 2015).

2.3.7. von Frey hair test (facial region)

On day 8 (study 7), the mechanical pain sensitivity of the facial region was evaluated with von Frey filaments. The mice were habituated in small plexiglass compartments on a perforated grid for one hour. Subsequently von Frey filaments were applied to the periorbital region. The test was performed by a trained blinded observer. The individual filaments were tested in an ascending or descending order covering 0.008, 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1, 1.4 g forces. The 0.16 g force was applied first to the periorbital region, and then the next filament was used based on the animal response. Following each challenge, the withdrawal response was quantified either as 1 (sharp withdrawal of face) or 0 (no response). In case of sharp withdrawal of face the next filament of lower intensity was used while the next filament of higher intensity was used in case of no response. Subsequently, 5 more responses were recorded from each mouse in the same manner. The maximal duration of each force application was 2 s, and the inter-

stimulus interval was 2-3 min. The withdrawal threshold was calculated according to the Dixon up-down method. Low withdrawal thresholds reflect high mechanical pain sensitivity (Dixon, 1965; Yan et al., 2011; Materazzi et al., 2013).

2.3.8. Plantar test

On day 9 (study 4), the thermal pain sensitivity of the plantar region was evaluated with the Hargreaves (plantar) test using the Ugo Basile Thermal Paw Stimulator (Allen and Yaksh, 2004). Before the plantar test, the mice were habituated in the small compartments of the Ugo Basile Dynamic Plantar Aesthesiometer for one hour. Subsequently, the plantar side of the right and the left hind paw was exposed to an infrared source through a glass plate (cut-off time: 15 s). The test was performed by a trained blinded observer. The withdrawal response was assessed at three infrared intensities (1, 2 and 3; low to high intensity). The time taken by the mice to move their paw from the infrared light beam (withdrawal latency) was recorded. Each hind paw was subjected to 3 trials at an inter-stimulus interval of 5 min, and the average latency was calculated. A short latency of the withdrawal response reflects high thermal pain sensitivity (Jain et al., 2015).

2.3.9. Hot-plate test

On day 8 (study 7), the thermal pain sensitivity of the plantar region was evaluated with the hot plate test using the Hot Plate Analgesia Meter (Ugo Basile, Varese, Italy) maintained at a constant temperature of 55 ± 0.3 ºC. Before the hot-plate test, the mice were habituated in the same room of the test for one hour. Subsequently, the mice were placed on the hot plate (cut-off time: 30 s). The test was performed by a trained blinded observer. The time taken by the mice to move their hind paw from the hot plate was recorded and taken to calculate the average latency in each treatment group. A short latency of the withdrawal response reflects high thermal pain sensitivity (Trevisan et al., 2013b).

2.3.10. Visceral pain behavior

Two dimensions of visceral pain behavior were simultaneously recorded after intrarectal administration of peanut oil or AITC (study 5 and study 6). For this purpose, mice were placed in small plexiglass cages with tiny bedding. Dimension 1 was assessed by a modification of the method of Laird et al. (Laird et al., 2001). In this test, the freezing behavior (typically associated with the AITC instillation) of the animals was recorded for 15 min with a video camera, and evaluated with the VideoMot2 (TSE Systems) event monitoring module by a blinded observer. Dimension 2 of the visceral pain behavior was a reduction of locomotion and rearing (Schwartz et al., 2013), which was recorded with the LabMaster system (TSE Systems) for 5 min. A decrease in locomotion and rearing is thought to reflect nociception-related behavior (Schwartz et al., 2013). Since the mice were not habituated to the test cages it cannot be ruled out that a component of novelty-induced behavior contributed to the recordings (Jain et al., 2015).

2.3.11. Tissue extraction and microdissection

After euthanization of the animals, the vertebral column was dissected from head to tail and cleaned. A 5 ml syringe filled with phosphate-buffered saline (PBS, pH 7.4) was used to flush the spinal cord from the caudal end of the vertebral column. The whole spinal cord was excised and the lumbosacral region was identified (Galan et al., 2003). Following dissection, the spinal cord was collected in homogenization tubes (Peqlab micro packaging vials, 2 ml, Peqlab Biotechnologie, Erlangen, Germany) containing 1.4 mm zirconium oxide beads (Peqlab Precellys beads), frozen in liquid nitrogen and stored at -70°C (Jain et al., 2015).

Brains were excised, placed on dry ice and stored at -70°C. Before microdissection, the brains were placed in a Microm HM 560 cryostat (Microm, Walldorf, Germany) at -20°C for 30 min. For microdissection, the brains were placed on a cold plate at -20°C (Weinkauf Medizintechnik, Forchheim, Germany), and the forebrains were cut into 8-10 slices. Subsequently the prefrontal cortex, hypothalamus, thalamus and amygdala were microdissected under a

stereomicroscope and collected in homogenization tubes, placed on dry ice and stored at -70°C (Brunner et al., 2014; Jain et al., 2015).

2.3.12. Western blot analysis

The spinal cord and microdissected brain tissues were homogenized in lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% (v/v) Triton X-100, 0.5% (v/v) sodium deoxycholate and 10 mM PMSF) and a Peqlab Precellys 24 homogenizer (two runs at 6,500 rpm for 20 s with a 5 min refractory period). The tissue homogenates were centrifuged (10,000 rpm, 4°C, 10 min) to pellet debris. The protein content was measured with the BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Total protein lysates (50 µg) were mixed with 8 µl of 4xNuPAGE LDS sample buffer and 2 µl of NuPAGE sample reducing agent and heated for 10 min at 70°C. Protein samples were subjected to electrophoresis on 4-12% gradient SDS-PAGE gels. After transfer to nitrocellulose membranes (45 µm; Invitrogen, Lofer, Austria), proteins were visualized with Ponceau's solution (Sigma-Aldrich). Then the membranes were blocked with non-fat milk (5% (w/v) Tris-buffered saline containing Tween 20 (25°C, 2 h) (Rauh et al., 2008) and incubated overnight at 4°C with the following primary antibodies diluted in bovine serum albumin (BSA) (5% (w/v) in Tris-buffered saline containing Tween 20: (i) anti-pp42/44 MAPK (1:1000, Cell Signaling-9106) and (ii) anti-c-Fos (1:1000, Cell Signaling-2250). After being washed, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:200,000, Biomol-6293, Hamburg, Germany) or goat anti-mouse IgG (1:100,000, Biomol-8101102). Immunoreactive bands were visualized with Super Signal West Pico Chemiluminescent substrate (Thermo Scientific, Vienna, Austria) and developed with Bio-Rad ChemiDoc MP Imaging System (Bio-Rad, Vienna, Austria) (Kitz et al., 2011). For normalization, membranes were stripped (58.4 g/l NaCl, 7.5 g/l glycine, pH 2.15) and incubated with anti-p42/44 MAPK (1:2,000, Santa Cruz-SC-94, Heidelberg, Germany) or anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies (1:1000, Santa Cruz-SC-25778). Densitometric evaluations of immunoreactive bands were performed with the Image Lab software volume module (Jain et al., 2015).

2.3.13. CGRP-like immunoreactivity assay

Slices (~ 400 µm) of isolated spinal cords were superfused with aerated (95% O2 and 5% CO2) Krebs solution (119 mM NaCl, 25 mM NaHCO3, 1.2 mM KH2PO4, 1.5 mM MgSO4, 2.5 Mm CaCl2, 4.7 Mm KCl, 11 mM D-glucose) which was maintained at 37 °C. BSA (0.1%), the angiotensin converting enzyme inhibitor captopril (1 mM), and the neutral endopeptidase inhibitor phosphoramidon (1 mM) were added to the Krebs solution to reduce peptide degradation. After a stabilization period (120 min), tissues were stimulated with AITC (10 µM and 100 µM, 10 min) dissolved in Krebs solution. Some tissues were challenged with capsaicin (0.3 µM, 10 min). Tissue superfusates (4 ml) were collected at 10 min intervals as follows: 2 fractions before, 1 fraction during and 2 fractions after stimulation. Subsequently, the fractions were freeze-dried and reconstituted with EIA buffer. CGRP like immunoreactivity was quantitated with a commercially available ELISA kit (Bertin Pharma, Montigny le Bretonneux, France). The sensitivity of the assay was 5 pg/ml at an intra- and inter-assay variation of around 10%. Results are expressed as femtomoles of peptide per g of tissue (Fusi et al., 2014).

2.3.14. RNA extraction and RT-PCR

RNA was extracted from the isolated DRGs using Trizol (Sigma Aldrich, Dorset, UK) according to the manufacturer's protocol. The RNA concentration and purity was assessed spectrophotometrically by measuring their absorbance at 260 nm and 280 nm. Aliquots of 100 ng RNA were reverse-transcribed with the iScript™ cDNA Synthesis kit (Bio-Rad, Hercules, USA) according to the manufacturer's protocol.

For relative quantification of mRNA, real time PCR was performed on Rotor Gene® Q (Qiagen, Hilden, Germany). The specific primers used for amplification and quantitation of mRNA are listed in Table 2. 18S was used as reference gene. The SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, USA) was used for amplification, and the cycling conditions were as follows: samples

were heated to 95°C for 1 min followed by 40 cycles of 95°C for 10 s and 40 cycles of 58°C for 20 s. The PCR reaction was carried out in triplicate.

The results were evaluated using the Rotor Gene® software and the threshold cycle (Ct) values were exported to Microsoft Excel. The 2–ΔΔCt method described by Livak and Schmittgen (2001) was used to analyse the results. The Ct values for each set of three reactions were averaged for all subsequent calculations (Livak and Schmittgen, 2001)

Table 2: TRP-specific primers used for amplification of TRP cDNA during PCR

Gene	Sequence forward primer	Sequence reverse primer	Reference
TRPA1	CAGGATGCTACGGTT TTTTCATTACT	GCATGTGTCAATG TTTGGTACTTCT	NCBI Ref Seq: NR_003278.3
TRPV1	TTCCTGCAGAAGAGC AAGAAGC	CCCATTGTGCAGA TTGAGCAT	NCBI Ref Seq: NM_001001445 .2
TRPV4	ATGCTTATCGCCCTCA TGGGTG	CAGGGAAGGAACG CTCGATGTC	NCBI Ref Seq: NM_022017.3
18S	CGCGGTTCTATTTTGT TGGT	AGTCGGCATCGTT TATGGTC	NCBI Ref Seq: NR_003278.3

2.3.15. Isolation and culture of primary sensory neurons

After euthanization of the animals, the vertebral column was dissected from head to tail and cleaned with Hank's Balanced Salt Solution (HBSS) containing penicillin and streptomycin (100 U/ml). The DRGs were identified and excised under the microscope. The ganglia were collected in HBSS and cleaned. Subsequently, ganglia were digested into HBSS containing 2 mg/ml of

collagenase type 1A and 1 mg/ml papain for 25 min at 37 °C. After being washed with HBSS, ganglia were pelleted and resuspended in Ham's-F12 containing 10% heat inactivated horse serum, 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml of penicillin and streptomycin, and 2 mM L-glutamine. Mechanical digestion of ganglia was performed by passing them through a 23G syringe (10x) followed by a 25G syringe (10x). The homogenates were centrifuged (11,000 rpm, 20-25°C, 5 min) to pellet neurons. The neurons were then suspended in Ham's-F12 containing 10% heat-inactivated horse serum, 10% heat-inactivated FBS, 100 U/ml of penicillin and streptomycin, 2 mM L-glutamine, and 100 ng/ml mouse nerve growth factor. Subsequently, the neurons were plated on glass coverslips coated with poly-L-lysine (8.3 µM) and laminin (5 µM) (Fusi et al., 2014).

Glass cover slips with a diameter 13 mm were used to plate the neurons for electrophysiology studies. Neurons were cultured for 24 hours before being used in this experiment (Fusi et al., 2014).

2.3.16. Electrophysiology

Patch-clamp recordings were performed on isolated DRG neurons. For recording, a glass cover slip with neurons was transferred to a compartment on the stage of a microscope (Olympus CKX41, Milan, Italy). Neurons were superfused with a standard extracellular solution (10 mM HEPES, 10 mM D-glucose, 147 mM NaCl, 4 mM KCl, 1 mM MgCl2 and 5 mM CaCl2) which was maintained at a flow rate of 2 ml/min and pH 7.4 (adjusted with NaOH). Sutter Instruments puller (model P-87) was used to pull and create borosilicate glass electrodes (Harvard Apparatus, Holliston, MA, USA) with a final tip resistance of 4-7 milliohm. The pipette solution which was used for recording contained 120 mM CsCl, 3 mM Mg_2ATP, 10 mM 1,2-bis(o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-Na adjusted to pH 7.4 (adjusted with CsOH). The temperature was maintained at 20-22 °C throughout the experiment. Recordings were made by using an Axopatch 200B amplifier (Axon Instruments, Union city, CA, USA) and analysed and stored with pClamp 9.2 software (Axon Instruments). The neurons were voltage-clamped at a holding potential of -60 mV, and signals were sampled at 1 kHz and low-pass

filtered at 10 kH. TRPA1 currents were detected as inward currents activated by AITC (100 μM). Capsaicin (1 μM) was used to identify capsaicin-sensitive nociceptive neurons. Consequently, the peak currents were recorded and normalized to membrane capacitance. The peak currents were averaged and expressed as a mean of the current density (pA/pF) (Fusi et al., 2014).

2.4. Statistics

The IBM SPSS 20 and SigmaPlot 12.5 packages were used to analyze and plot the results. Before applying any statistics, the data were checked for normal distribution with the Shapiro-Wilk test. The results of all experiments in studies 1-6 were analyzed by considering DSS treatment as one factor and WAS as the other factor. In experiments involving more than two treatment groups, two-way ANOVA or repeated measures ANOVA were used for statistical analysis, as appropriate. A DSS main factor effect was referred to by the term “DSS effect” when combined data from the DSS and WAS+DSS groups were compared with combined data from the control and WAS groups. Analogously, a WAS main factor effect was denoted by “WAS effect” when combined data from the WAS and WAS+DSS groups were compared with combined data from the control and DSS groups. In study 7 (set 1), DSS and treatment with HC were considered as the two main factors. In study 7 (set 2), DSS and genetic deletion of TRPA1 were considered as the two main factors. Interactions between the two factors (two-way ANOVA) or three factors (repeated measures ANOVA) were considered significant if $p \leq 0.05$. The post-hoc Bonferroni test was employed to compare differences among the treatment groups. When repeated measures ANOVA was applied, sphericity assumptions were checked by Mauchly's test and, in case of violation of sphericity, the Greenhouse-Geisser correction was used. After repeated measures ANOVA, the independent samples t-test was used to compare differences between two factors or post-hoc two-way ANOVA was used to test for differences and interactions between two factors (WAS and DSS). Comparisons between two groups were made with the independent samples t-test. In case of nonparametric distributions, the Kruskal–Wallis test was employed, and in post-hoc testing the

Mann–Whitney U-test. The Bonferroni correction was used for pairwise comparisons (Jain et al., 2015).

3. Results

The description of Results follows in part the text published in Jain et al. (2015).

3.1. DSS-induced colitis remained unaffected by WAS

In order to study the effect of WAS on colitis induced by DSS, I examined the severity of colitis in all treatment groups (I – IV). Treatment of mice with DSS (study 1) induced colitis as seen in treatment groups III (DSS) and IV (WAS+DSS). Analysis of colitis-associated parameters did not reveal any significant interaction between WAS and DSS. Treatment with DSS (DSS effect, $p<0.001$) led to body weight loss (Fig. 2A), increased DAS (Fig. 2B), reduced colon length (Fig. 2C), increased colon weight (Fig. 2D) as well as increased colonic MPO mass (Fig. 2E). WAS exposure alone did not alter any of these parameters (Fig. 1A-E). The changes of colitis-associated parameters shown in Fig. 2 (study 1) were representative of those seen in the other studies (studies 2-6). Thus, WAS did not alter colitis induced by DSS (Jain et al., 2015). The details of the statistical analysis are shown in Tables 3-8.

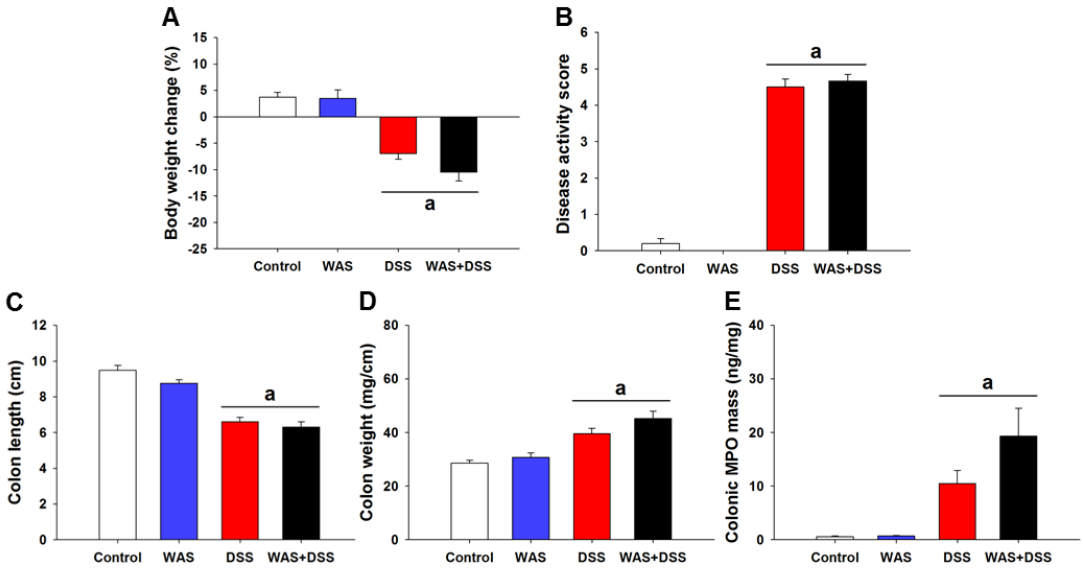

Figure 2. Effect of a 7-day treatment of mice with DSS, WAS and WAS+DSS on colitis-associated parameters measured on day 8 (study 1): (A) percentage of body weight change relative to the weight measured before the treatment started,

(B) disease activity score, (C) colon length, (D) colon weight, and (E) colonic MPO mass. Values are expressed as means + SEM or - SEM (n=8-12). Two-way ANOVA did not show any WAS x DSS interaction and any WAS effect but revealed a DSS effect (^{a} $p<0.05$) (Jain et al., 2015).

3.2. WAS exposure and WAS+DSS treatment modified locomotion and rearing in a differential manner

In order to examine whether DSS-induced colitis, WAS and combined WAS+DSS treatment affect spontaneous behavior in mice, I recorded short-term activity (locomotion, exploration and ingestion) for a period of 60 min. Analysis of activity-related parameters (study 2) disclosed a significant interaction between WAS and DSS in their influence on locomotion and rearing ($p<0.01$, Fig. 3A,B). Bonferroni's multiple comparison revealed that WAS exposure significantly increased locomotion ($p<0.05$ vs. control) and rearing ($p<0.01$ vs. control), whereas DSS treatment alone had no effect. In contrast, WAS+DSS treatment led to a significant decrease in locomotion and rearing ($p<0.001$ and $p<0.001$ vs. WAS, $p<0.05$ and $p=0.061$ vs. DSS). Analysis of food and water intake did not show any significant interaction between WAS and DSS (Fig. 3C,D). Taken together, WAS increased, whereas WAS+DSS treatment decreased, spontaneous activity (locomotion and rearing) in mice (Jain et al., 2015). The details of the statistical analysis are shown in Table 9.

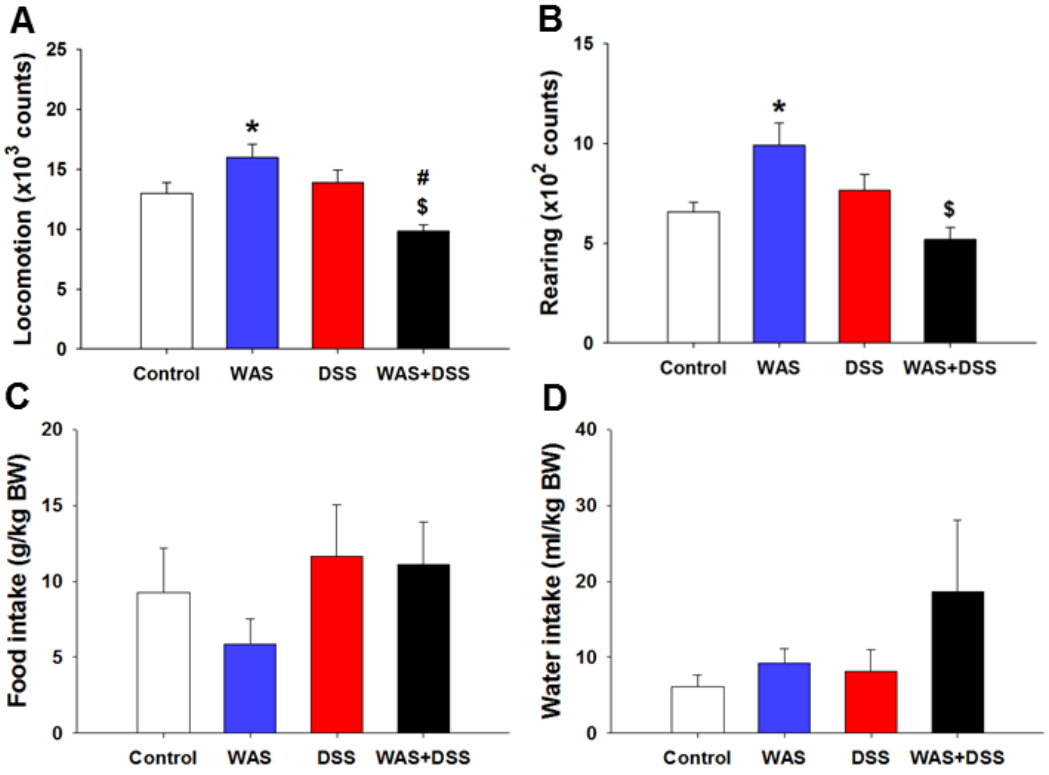

Figure 3. Effect of a 7-day treatment of mice with DSS, WAS and WAS+DSS on short-term activity (60 min) measured on day 8 (study 2): (A) locomotion, (B) rearing, (C) food intake, and (D) water intake. Water and food intake are expressed relative to the body weight (BW) of the animals. Values are expressed as means + SEM (n=6-8). Two-way ANOVA revealed a WAS x DSS interaction ($p<0.01$), and post-hoc analysis (Bonferroni's multiple comparison) demonstrated significant differences: * $p<0.05$ vs. Control; $^{\$}$ $p<0.05$ vs. WAS; $^{\#}$ $p<0.05$ vs. DSS (Jain et al., 2015).

In order to examine whether DSS-induced colitis, WAS and combined WAS+DSS treatment have an effect on long term behavior in mice, study 2 was continued for a period of 5 days during which I recorded long-term activity (locomotion, exploration and ingestion). Of the animals in the DSS and WAS+DSS groups only 50 % survived the long-term experiment (Fig. 4). Therefore, the recordings of the long term-activity experiments were not further analyzed.

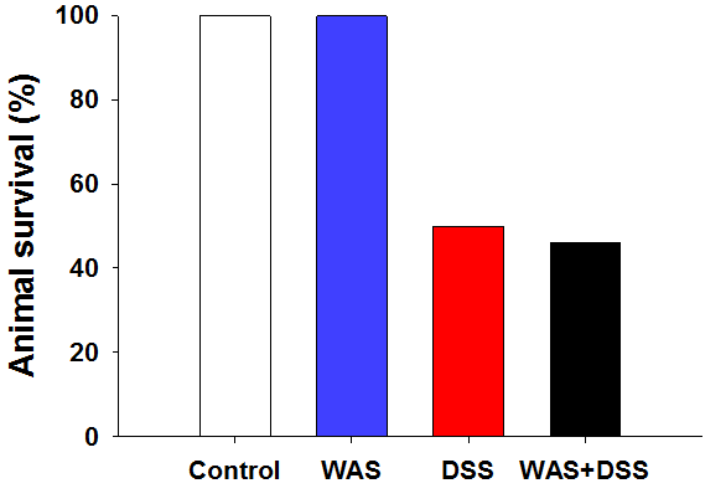

Figure 4. Effect of a 7-day treatment of mice with DSS, WAS and WAS+DSS on percentage of animal survival on day 12 relative to the total number of animals used before the treatment started.

3.3 WAS exposure improved self-care and motivational behavior

In order to investigate whether DSS-induced colitis, WAS and combined WAS+DSS treatment affect self-care and motivational behavior, I recorded grooming frequency and latency during the first 5 min after spraying a 10% (w/v) sucrose solution on the dorsal surface of mice. DSS treatment and WAS exposure (study 3) affected the behavior in the splash test in a disparate fashion (Fig. 5A-D), although statistical analysis of the data did not show any significant interaction between WAS and DSS in their influence on locomotion, rearing, total (face + body) grooming frequency and face grooming latency. However, locomotion and rearing of DSS-treated mice in response to spraying of a sucrose solution on the dorsal surface was significantly decreased (DSS effect, $p<0.001$, Fig. 5A,B). The total grooming frequency was increased in WAS-exposed mice while the face grooming latency was shortened (WAS effect, $p<0.05$, Fig. 5C,D). Face grooming typically preceded body grooming. Thus, WAS increased both motivational and self-care behavior of mice in the splash test (Jain et al., 2015). The details of the statistical analysis are shown in Table 10.

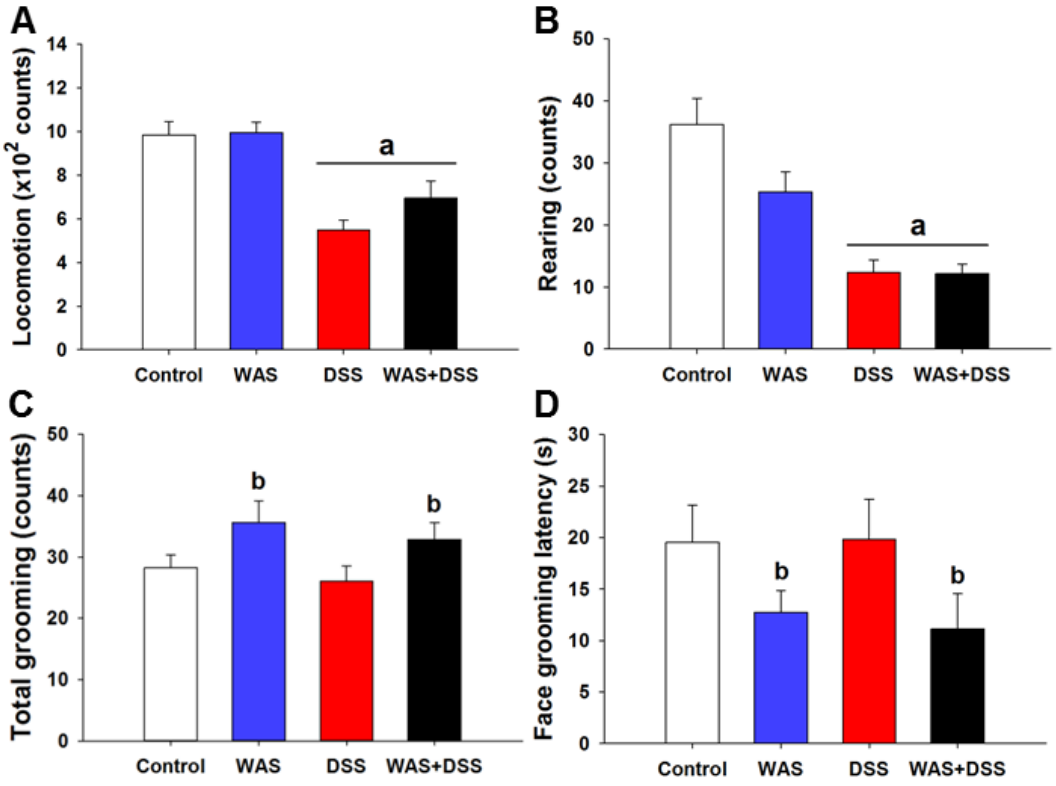

Figure 5. Effect of a 7-day treatment of mice with DSS, WAS and WAS+DSS on (A) locomotion, (B) rearing, (C) total (face + body) grooming frequency and (D) face grooming latency recorded in the splash test performed on day 8 (study 3). All parameters were recorded for 5 min after spraying the mice with sucrose solution. Values are expressed as means + SEM (n=8-12). Two-way ANOVA did not show any WAS x DSS interaction but revealed a DSS effect (^{a} $p<0.001$) and a WAS effect (^{b} $p<0.05$) (Jain et al., 2015).

3.4. WAS exposure and DSS treatment increased the somatic sensitivity to mechanical and thermal stimuli

In order to study the effect of DSS-induced colitis, WAS and combined WAS+DSS treatment on somatic nociception in mice, I assessed the sensitivity of the abdominal skin to mechanical stimuli and of the plantar skin to mechanical and thermal stimuli. Assessment of the mechanical sensitivity in the abdominal region

with the von Frey test did not reveal any significant interaction between WAS/DSS and the test forces in their influence on the withdrawal response (study 4). There was, however, a significant interaction between WAS exposure and test forces as well as between DSS treatment and test forces in their impact on the withdrawal response ($p<0.05$, Fig. 6A). To examine these effects further, post-hoc analysis with the independent samples t-test revealed only a DSS effect, as DSS treatment led to a significant increase in the withdrawal response to the 0.04 g force (DSS effect, $p<0.05$, Fig. 6A). The details of the statistical analysis are shown in Table 11.

Evaluation of the mechanical sensitivity of the plantar region with the von Frey test disclosed a significant interaction ($p<0.05$, Fig. 6B) between WAS/DSS and the test forces in their effect on the withdrawal response. Two-way ANOVA showed a significant interaction between WAS and DSS at three test forces; 0.02 g ($p<0.01$), 0.04 g ($p<0.001$), and 0.07 g ($p<0.05$). Bonferroni's multiple comparison analysis revealed that the withdrawal response to forces of 0.02 g ($p<0.01$ vs. control) and 0.04 g ($p<0.001$ vs. control) was significantly increased in WAS-exposed mice. In DSS-treated animals the withdrawal response to forces of 0.02 g ($p<0.001$ vs. control) and 0.04 g ($p<0.01$ vs. control) was also significantly enhanced. WAS+DSS treatment led to a significant decrease in the withdrawal response to forces of 0.04 g and 0.07 g ($p<0.01$ and $p=0.052$ vs. WAS) and to forces of 0.02 g and 0.04 g ($p=0.056$ and $p=0.054$ vs. DSS) (Fig. 6B). The details of the statistical analysis are shown in Table 12.

Assessment of the thermal sensitivity on the plantar side of the hindpaw with the plantar test showed that there was a significant interaction ($p<0.05$) between the two main factors (WAS and DSS) and the intensity of the infrared light stimulus (1, 2 and 3) in their impact on withdrawal latency (Fig. 6C). While, as disclosed by post-hoc analysis, there was no effect of intensity 1 (data not shown) on withdrawal latency, WAS exposure caused a significant decrease in withdrawal latency (WAS effect, $p<0.05$) at intensity 2. A significant interaction ($p<0.05$) between WAS and DSS was found at intensity 3. Bonferroni's multiple comparison revealed that the withdrawal latency was significantly decreased in both WAS-exposed and DSS-treated mice ($p<0.001$ and $p<0.05$ vs. control) (Fig. 6C). The details of the statistical analysis are shown in Table 13.

These data show that DSS causes mechanical hypersensitivity of the abdominal skin while both DSS and WAS induce mechanical and thermal hypersensitivity of the plantar skin. These effects were absent in animals treated with WAS+DSS (Jain et al., 2015).

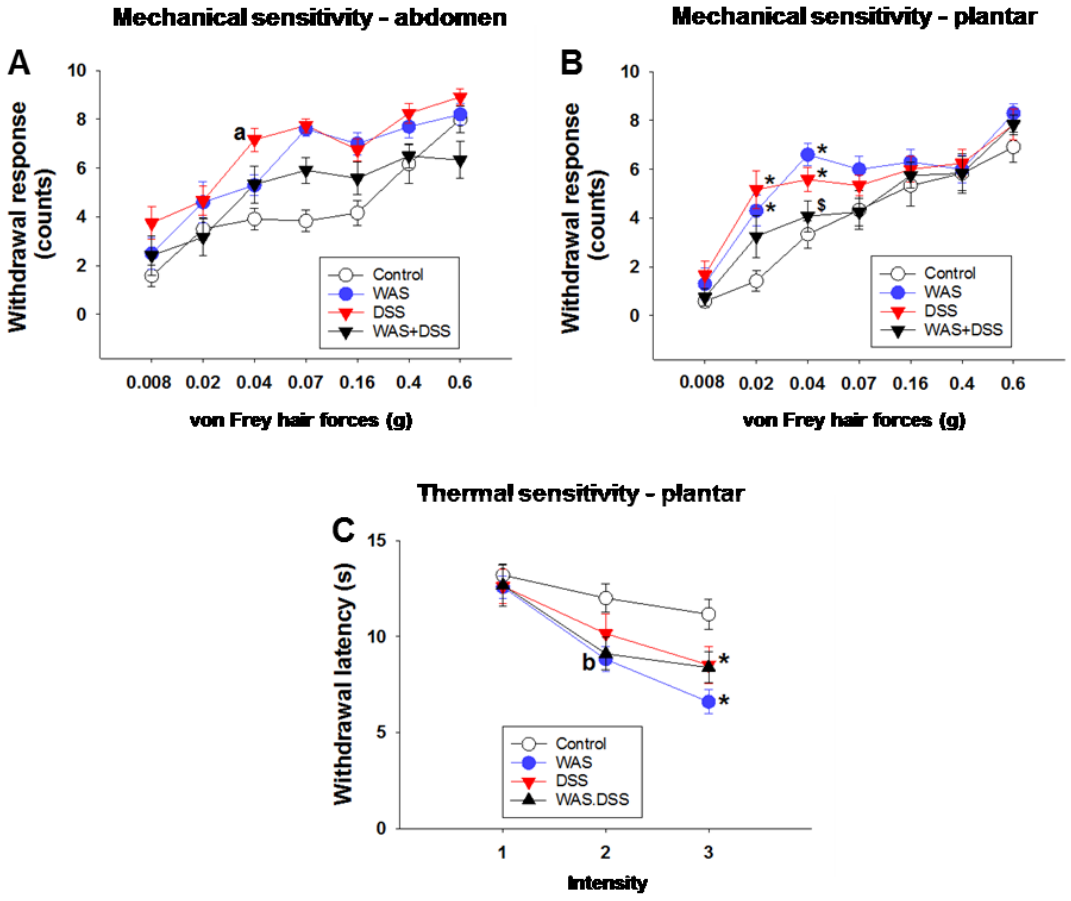

Figure 6. Effect of a 7-day treatment of mice with DSS, WAS and WAS+DSS on mechanical pain sensitivity of the abdomen (A) and plantar hind paw region (B) and on thermal pain sensitivity of the plantar hind paw region (C) recorded with the von Frey test on day 8 and the plantar test on day 9, respectively (study 4). Values are expressed as means ± SEM or + SEM (n=10-12). Repeated measures ANOVA (A) did not show any WAS x DSS x forces interaction in the withdrawal response from abdominal stimulation but revealed a WAS x forces and a DSS x forces interaction ($p<0.05$). Post-hoc analysis (independent samples t-test) demonstrated a DSS effect (^{a} $p<0.05$) as indicated in panel A. Repeated measures ANOVA disclosed a WAS x DSS x forces interaction ($p<0.05$) in the withdrawal

response to mechanical plantar stimulation (B) and a WAS x DSS x intensity interaction ($p<0.05$) in the withdrawal response to thermal plantar stimulation (C). Post-hoc (two-way ANOVA) analysis demonstrated a WAS x DSS interaction at three test forces; 0.02 g ($p<0.01$), 0.04 g ($p<0.001$), and 0.07 g ($p<0.05$) (B), a WAS effect (^{b} $p<0.05$) at infrared intensity 2 and a WAS x DSS interaction g ($p<0.05$) at intensity 3 (C). Post-hoc (Bonferroni's multiple comparison) analysis demonstrated significant differences: * $p<0.05$ vs. Control; $^{\$}$ $p<0.05$ vs. WAS (Jain et al., 2015).

3.5. WAS exposure and DSS treatment induced phosphorylation of p42/44 MAPK and c-Fos expression in the spinal cord

On the molecular level, I examined the effect of DSS-induced colitis, WAS and combined WAS+DSS treatment on p42/44 MAPK phosphorylation and c-Fos expression in the spinal cord. WAS exposure and DSS treatment (study 1) induced phosphorylation of p42/44 MAPK and c-Fos expression in the spinal cord (Fig. 7C,E). Densitometric evaluation of the immunoreactive pp42/44 MAPK and c-Fos bands showed a significant interaction between the WAS and DSS treatments ($p<0.05$, Fig. 7D,F). Bonferroni's multiple comparison revealed that DSS treatment increased p42/44 MAPK phosphorylation ($p<0.01$ vs. control) and c-Fos expression ($p<0.05$ vs. control), while WAS exposure enhanced only c-Fos expression ($p<0.001$ vs. control). WAS+DSS treatment led to a decrease in pp42/44 MAPK ($p<0.05$ vs. DSS) and c-Fos ($p<0.001$ vs. WAS) expression. No phosphorylation of p38 MAPK was detected in the spinal cord (data not shown). Thus, DSS, but not WAS and WAS+DSS, caused MAPK phosphorylation in the spinal cord while c-Fos expression was stimulated by WAS and DSS, but not WAS+DSS (Jain et al., 2015). The details of the statistical analysis are shown in Tables 14-15.

3.6. WAS exposure and DSS treatment induced phosphorylation of p38 and p42/44 MAPKs and c-Fos expression in the brain

I further explored the effect of DSS-induced colitis, WAS and combined WAS+DSS treatment on p38, p42/44 MAPK phosphorylation and c-Fos expression in the brain. WAS exposure and DSS treatment induced phosphorylation of p38 and p42/44 MAPKs and c-Fos expression (study 1) in distinct brain areas (Fig. 7A,C,E). Densitometric evaluation of the immunoreactive pp38 (thalamus, hypothalamus, and amygdala) and pp42/44 MAPK bands (thalamus, hypothalamus, amygdala, and prefrontal cortex) failed to show any significant interaction between the WAS and DSS treatments. WAS exposure induced p38 and p42/44 MAPK phosphorylation (Fig. 7B,D) in the thalamus ($p<0.05$, p=0,057), hypothalamus ($p<0.01$, $p<0.01$) and amygdala ($p<0.001$, $p<0.01$), respectively (WAS effect). In the prefrontal cortex ($p<0.01$), a WAS-evoked upregulation of pp42/44 MAPK (WAS effect) was observed. In addition, there was a significant interaction ($p<0.001$) between WAS and DSS in the phosphorylation of p38 MAPK in the prefrontal cortex. Bonferroni's multiple comparison revealed that WAS+DSS treatment reduced pp38 MAPK expression in the prefrontal cortex (P=0.050 vs. WAS). The details of the statistical analysis are shown in Tables 14 and 16.

WAS exposure and DSS treatment also induced c-Fos expression in specific brain areas. Densitometric evaluation of the immunoreactive c-Fos bands showed a significant interaction ($p<0.001$) between WAS and DSS in the thalamus, hypothalamus, amygdala, and prefrontal cortex (Fig. 7F). Bonferroni's multiple comparison revealed that WAS exposure and DSS treatment increased c-Fos expression in the thalamus, hypothalamus, amygdala ($p<0.001$ vs. control) and prefrontal cortex ($p<0.001$, $p<0.01$ vs. control). Conversely, WAS+DSS treatment decreased c-Fos expression ($p<0.001$ vs. WAS, $p<0.001$ vs. DSS) in the thalamus, hypothalamus, amygdala and prefrontal cortex. The details of the statistical analysis are shown in Table 15.

Thus, WAS and WAS+DSS, but not DSS, caused MAPK phosphorylation in the brain while c-Fos expression was stimulated by WAS and DSS, but not WAS+DSS (Jain et al., 2015).

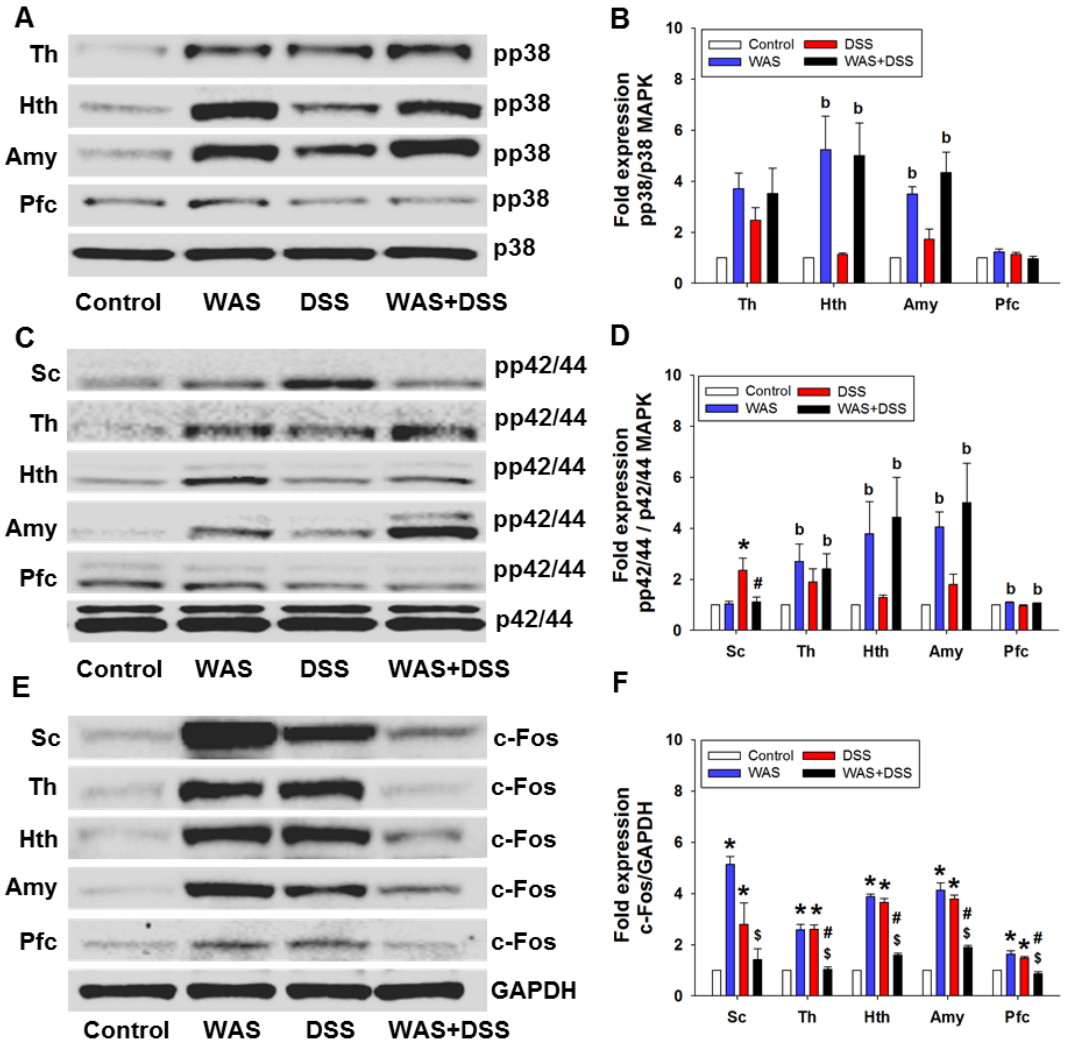

Figure 7. Effect of a 7-day treatment of mice with DSS, WAS and WAS+DSS on protein expression (A/B: pp38/p38 MAPK, C/D: pp42/44 / p42/44 MAPK, E/F: c-Fos/GAPDH) on day 8 in the spinal cord (Sc), thalamus (Th), hypothalamus (Hth), amygdala (Amy) and prefrontal cortex (Pfc) (study 1). Panels A, C and E show representative Western blots and panels B, D and F show quantitative data obtained by densitometric evaluation of the Western blots, expressed as x fold over control. Values shown in panels B, D and F are expressed as means + SEM (n=3-4). Two-way ANOVA showed a WAS x DSS interaction ($p<0.001$) in pp38 expression in the prefrontal cortex (B), a WAS x DSS interaction ($p<0.05$) in pp42/44 (D) and c-Fos (F) expression in the spinal cord, and a WAS x DSS interaction ($p<0.05$) in c-Fos expression in the thalamus, hypothalamus, amygdala, and prefrontal cortex (F). In addition, a WAS effect (^{b} $p<0.05$) was revealed as

depicted in panels B and D. Post-hoc (Bonferroni's multiple comparison) analysis demonstrated significant differences: * $p<0.05$ vs. Control; $^{\$}$ $p<0.05$ vs. WAS; $^{\#}$ $p<0.05$ vs. DSS (Jain et al., 2015).

3.7. Intrarectal AITC challenge induced freezing and reduced locomotion and rearing in control mice

The behavioral manifestations of acute visceral pain were studied following instillation of AITC into the mouse colon. Analysis of the visceral pain behavior data (dimension 1 and 2, study 5) demonstrated that intrarectal administration of AITC to control mice prolonged their freezing behavior ($p<0.01$ vs. vehicle, Fig. 8A) while locomotion ($p<0.01$ vs. vehicle, Fig. 8B) and rearing ($p<0.05$ vs. vehicle, Fig. 8C) were significantly shortened (Jain et al., 2015). The details of the statistical analysis are shown in Table 17.

3.8. Intrarectal AITC challenge reduced freezing in DSS-treated mice and induced locomotion and rearing in WAS-exposed mice

In order to examine whether DSS-induced colitis, WAS and combined WAS+DSS treatment affect visceral nociception in mice, pain-related behavior was recorded during the first 5 min after intrarectal AITC administration. The visceral pain behavior evoked by AITC (study 5) was modified by WAS exposure and DSS treatment in a differential manner, although there was no significant interaction between WAS and DSS in their influence on AITC-evoked changes of freezing, locomotion and rearing in the treatment groups. The freezing counts were significantly decreased in DSS-treated mice (DSS effect, $p<0.01$, Fig. 8D) while locomotion (WAS effect, $p=0.05$, Fig. 8E) and rearing (WAS effect, $p=0.06$, Fig. 8F) were prolonged in WAS-exposed mice. Thus, DSS reduced freezing and WAS increased locomotion in response to intrarectal AITC administration in mice (Jain et al., 2015). The details of the statistical analysis are shown in Table 18.

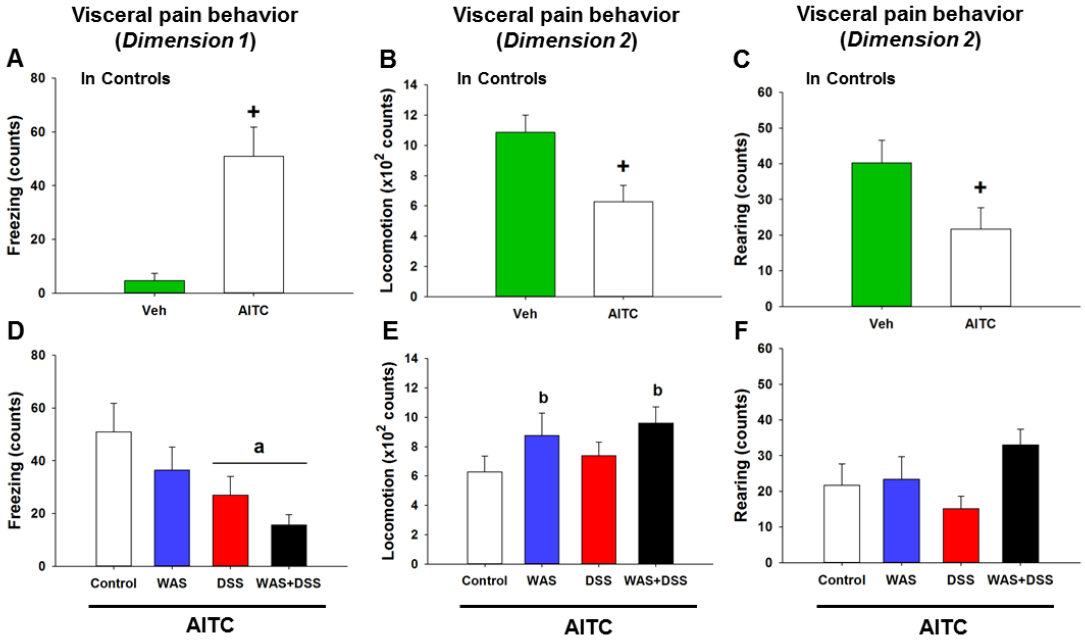

Figure 8. Effect of intrarectal instillation of peanut oil (Veh) and AITC 2% (v/v) to elicit visceral pain behavior in control mice (A,B,C) and in mice treated with WAS, DSS and WAS+DSS for 7 days, relative to control mice (D,E,F). The visceral pain behavior (prolonged freezing and reduced locomotion and rearing) was measured on day 8 (study 5). In dimension 1, freezing (A,D) was recorded for 15 min, and in dimension 2 locomotion (B,E) and rearing (C,F) were recorded for 5 min after intrarectal instillation. Note that the dimension of the y axis scale in panels B and E is x100. Values are expressed as means + SEM (n=8-12). The independent samples t-test disclosed a significant difference between Veh and AITC in panels A, B ($^{+}$ $p<0.01$) and C ($^{+}$ $p<0.05$). Two-way ANOVA did not show any WAS x DSS interaction but revealed a DSS effect (^{a} $p<0.01$) and a WAS effect (^{b} $p=0.05$) with regard to changes in locomotion and rearing as indicated in panels D, E and F (Jain et al., 2015).

3.9. WAS exposure and DSS treatment modified AITC-evoked phosphorylation of MAPKs and c-Fos expression in the spinal cord and brain

In a subsequent experiment I investigated the effect of DSS-induced colitis, WAS and combined WAS+DSS treatment on the AITC-induced p38 and p42/44 MAPK phosphorylation as well as c-Fos expression in the spinal cord and brain. The effect of intrarectal AITC challenge (study 5) to induce phosphorylation of p38, p42/44 MAPK and expression of c-Fos in the spinal cord and brain, as seen in control mice, was altered by DSS, WAS and WAS+DSS treatment in a region-related manner (Fig. 9A,C,E). Intrarectal AITC challenge altered MAPK phosphorylation and c-Fos expression in the spinal cord and brain of all four treatment groups (Fig. 9A,C,E). Densitometric evaluation of the immunoreactive bands revealed a significant interaction between WAS and DSS with respect to AITC-evoked levels of pp38 MAPK in the thalamus ($p<0.05$), hypothalamus ($p<0.01$) and amygdala ($p<0.01$) as well as pp42/44 MAPK in the thalamus ($p<0.05$), hypothalamus ($p<0.05$) and prefrontal cortex ($p<0.05$) (Fig. 9B,D). Bonferroni's multiple comparison revealed that the effect of AITC to enhance p38 MAPK phosphorylation in the thalamus ($p<0.01$), hypothalamus and amygdala ($p<0.001$) and p42/44 MAPK phosphorylation in the thalamus ($p<0.01$), hypothalamus ($p=0.05$) and prefrontal cortex ($p<0.01$) was mitigated in DSS-treated mice (vs. control mice). This inhibitory effect of DSS treatment on the AITC response in the brain was reversed by concomitant WAS exposure (WAS+DSS). When compared to DSS-treated mice, phosphorylation of p38 MAPK was increased in the thalamus ($p<0.05$) hypothalamus ($p<0.001$) and amygdala ($p<0.001$) and that of p42/44 MAPK in the thalamus ($p=0.06$), hypothalamus ($p<0.05$) and prefrontal cortex ($p<0.01$) of WAS+DSS-treated mice (Fig. 9B,D). There was no significant interaction between WAS and DSS with regard to the AITC-evoked pp38 MAPK expression in the prefrontal cortex and pp42/44 expression in the spinal cord. However, DSS treatment decreased AITC-evoked pp38 MAPK levels in the prefrontal cortex ($p<0.01$) and pp42/44 MAPK levels in the spinal cord ($p<0.05$) (DSS effect). Thus, DSS reduced AITC-evoked MAPK phosphorylation in the thalamus, hypothalamus and prefrontal cortex, an effect

that was absent in animals treated with WAS+DSS (Jain et al., 2015). The details of the statistical analysis are shown in Tables 19-20.

Densitometric evaluation of the immunoreactive bands revealed a significant interaction between WAS and DSS with regard to the AITC-evoked c-Fos expression in the spinal cord ($p<0.01$), thalamus ($p<0.05$), hypothalamus ($p<0.001$) and amygdala ($p<0.05$) (Fig. 9F). Bonferroni's multiple comparison showed that the effect of AITC to stimulate c-Fos expression was decreased in the thalamus ($p<0.001$), but increased in the hypothalamus ($p<0.05$) and amygdala ($p<0.001$) of WAS-exposed animals (vs. control animals). The effect of AITC to induce c-Fos expression in the thalamus was diminished ($p<0.01$) in DSS-treated animals, but enhanced ($p<0.05$) in the spinal cord (vs. control mice, Fig. 9F). The effects of WAS exposure and DSS treatment on the c-Fos response to AITC were reversed in particular regions of the spinal cord and brain when the two treatments were combined (Fig. 9F). Thus, in mice treated with WAS+DSS, the AITC-evoked expression of c-Fos in the spinal cord, thalamus, hypothalamus ($p<0.001$) and amygdala ($p<0.01$) was decreased relative to animals treated with DSS. Moreover, c-Fos expression was diminished in the spinal cord ($p<0.01$), hypothalamus ($p<0.01$) and amygdala ($p<0.001$) of mice treated with WAS+DSS (vs. WAS). Taken together, DSS, WAS and WAS+DSS treatment altered AITC-evoked expression of c-Fos in the spinal cord and brain in a treatment- and region-dependent manner (Jain et al., 2015). The details of the statistical analysis are shown in Table 21.

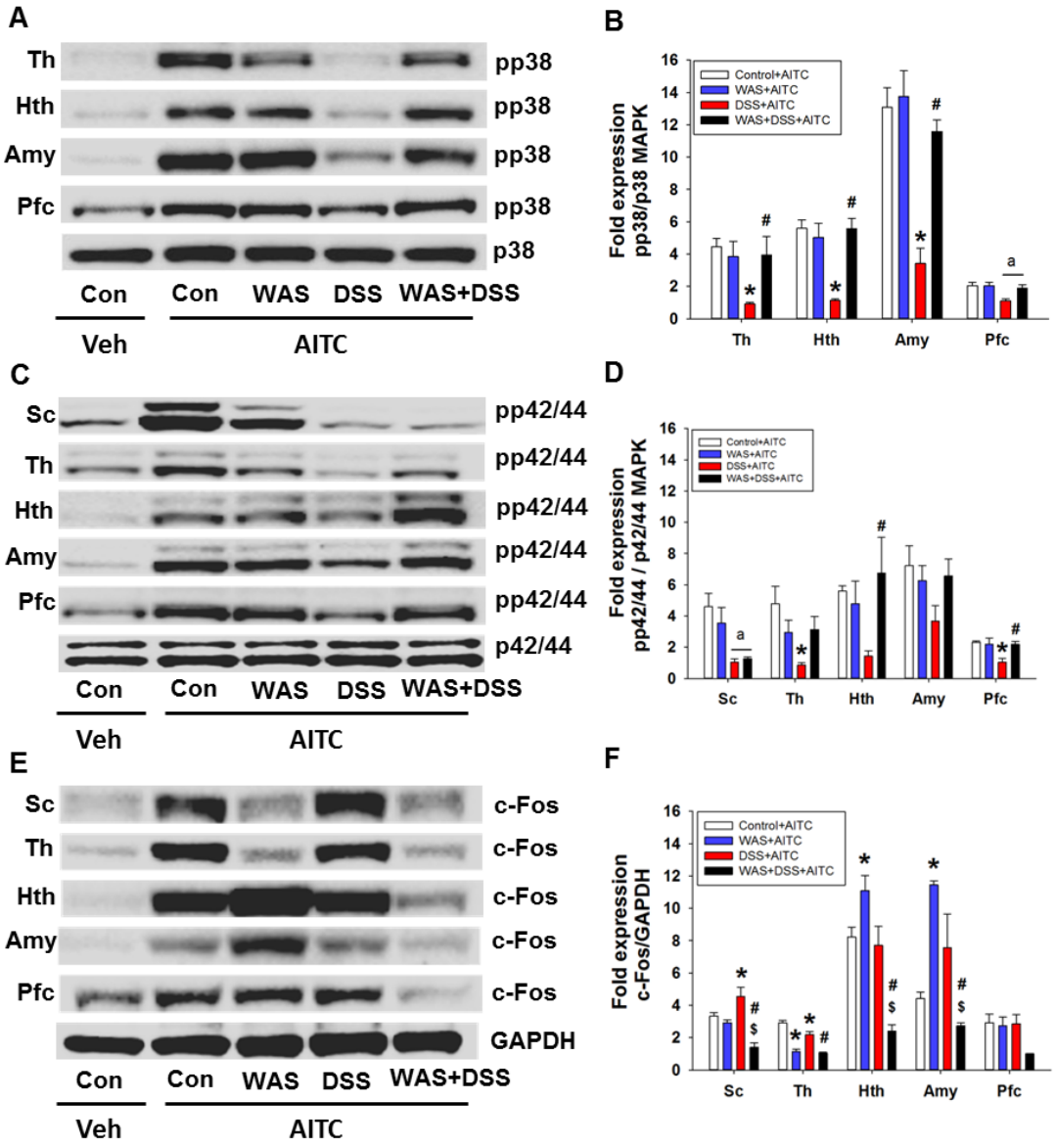

Figure 9. Effect of intrarectal instillation of peanut oil (Veh) and AITC 2% (v/v) on protein expression (A/B: pp38/p38 MAPK, C/D: pp42/44 / p42/44 MAPK, E/F: c-Fos/GAPDH) in the spinal cord (Sc), thalamus (Th), hypothalamus (Hth), amygdala (Amy) and prefrontal cortex (Pfc) of control (Con) mice and mice treated with WAS, DSS and WAS+DSS for 7 days (study 5). Protein expression was evaluated on day 8. Panels A, C and E show representative Western blots and panels B, D and F show quantitative data obtained by densitometric evaluation of the Western blots, expressed as x fold over control. Values shown in panels B, D and F are expressed as means + SEM (n=3-4). Two-way ANOVA demonstrated a WAS x DSS interaction in pp38 expression (B) in the thalamus ($p<0.05$),

hypothalamus ($p<0.01$) and amygdala ($p<0.01$), in pp42/44 expression (D) in the thalamus ($p<0.05$), hypothalamus ($p<0.05$) and prefrontal cortex ($p<0.05$) and in c-Fos expression (F) in the spinal cord ($p<0.01$), thalamus ($p<0.05$), hypothalamus ($p<0.001$) and amygdala ($p<0.05$). Post-hoc (Bonferroni's multiple comparison) analysis demonstrated significant differences: * $p<0.05$ vs. Control+AITC; $^{\$}$ $p<0.05$ vs. WAS+AITC; $^{\#}$ $p<0.05$ vs. DSS+AITC. Two-way ANOVA failed to show a significant WAS x DSS interaction in the expression of pp38 in the prefrontal cortex and pp42/44 in the spinal cord but disclosed a DSS effect (^{a} $p<0.05$) (Jain et al., 2015).

3.10. Morphine modified the effects of AITC on freezing, locomotion and rearing

In order to address the question whether the behavioral and molecular CNS responses to intrarectal AITC administration are related to pain, control mice were injected with morphine prior to AITC instillation. Morphine had distinct effects on the behavior of mice (study 6). The freezing counts recorded in morphine-treated control mice were not normally distributed, and analysis of the data with a non-parametric test revealed significant differences in freezing behavior ($p<0.001$, Fig. 10A) among the treatment combinations (saline + peanut oil, saline + AITC, morphine + peanut oil, morphine + AITC) in control animals. Post-hoc analysis with the Mann-Whitney U test confirmed that AITC instillation in saline-injected control mice prolonged freezing ($p<0.05$ vs. saline + peanut oil). Morphine caused an approximately 50% (although non-significant) reduction of freezing counts in AITC-treated mice (Fig. 10A). Morphine also interfered with the AITC-evoked freezing in the other treatment groups, although there was no significant interaction between WAS and DSS in the presence of morphine. In contrast, AITC-evoked freezing behavior was suppressed in morphine-injected mice of the WAS, DSS and WAS+DSS treatment groups ($p<0.05$, WAS and DSS effect) (Fig. 10B). The details of the statistical analysis are shown in Tables 22-23.

Locomotion and rearing were likewise affected by morphine. In morphine-injected control mice a 2-fold increase in locomotion and approximately 10-fold

increase in rearing was observed when compared to saline-injected control mice (Fig. 10C,D). The extensive increase in rearing was most likely a recording artifact caused by the morphine-evoked Straub phenomenon (upright position of the tail) (Faenzi, 1961). The behavioral parameters recorded after peanut oil instillation to saline-injected mice did not significantly differ from those recorded without prior saline injection (compare Fig. 8A,B,C with Fig. 10A,C,D). Taken all findings together, the effect of morphine on the AITC-evoked pain behavior proved difficult to evaluate because morphine per se increased the activity of the mice (Jain et al., 2015).

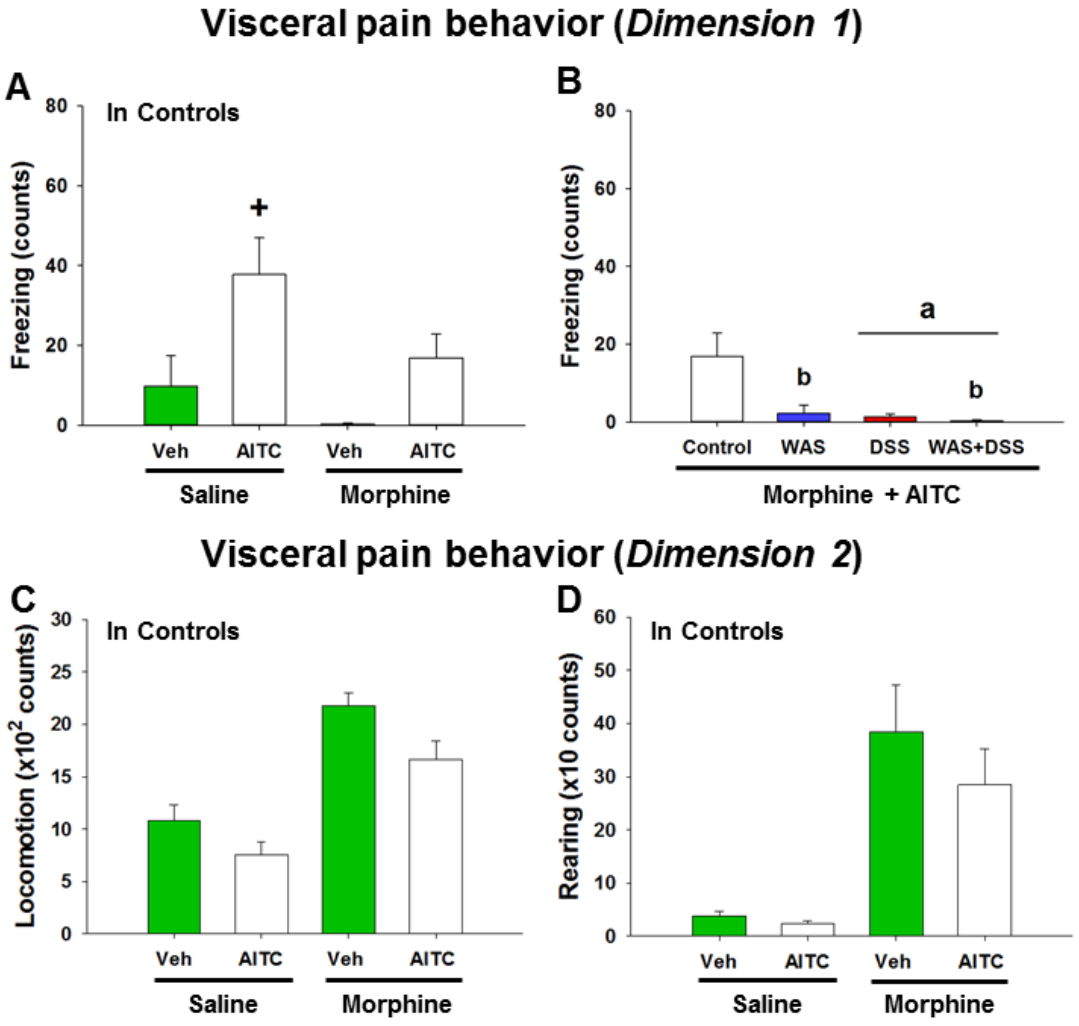

Figure 10. Visceral pain behavior (freezing, locomotion and rearing) in control mice (A,C,D) as well as in mice treated with WAS, DSS and WAS+DSS for 7 days (B) following intrarectal instillation of peanut oil (Veh) or AITC 2% (v/v) in the absence or presence of morphine. Saline or morphine (10 mg/kg, s.c.) was injected one hour prior to intrarectal instillation. The visceral pain behavior was measured on day 8 (study 6). In dimension 1, freezing (A, B) was recorded for 15 min, and in dimension 2 locomotion (C) and rearing (D) were recorded for 5 min after intrarectal instillation. Please note that the dimension of the y axis scale in panels C and D is x100 and x10, respectively. Values are expressed as means + SEM (n=7-8). Data in panel A were evaluated with the Kruskal-Wallis one-way ANOVA test ($p<0.001$), and the Mann-Whitney U test (post-hoc test) demonstrated a significant difference between Veh and AITC in saline-injected control mice ($^{+}$ $p<0.05$). Two-way ANOVA did not show any WAS x DSS interaction but revealed a DSS effect (^{a} $p<0.05$) and a WAS effect (^{b} $p<0.05$) (Jain et al., 2015).

3.11 Morphine blunted the AITC-evoked phosphorylation of p38 and p42/44 MAPKs and c-Fos expression in the spinal cord and brain of control mice

Intrarectal AITC challenge of control mice (study 5) induced phosphorylation of p38 and p42/44 MAPKs and expression of c-Fos in the thalamus, hypothalamus, amygdala, prefrontal cortex and, with the exception of p38, spinal cord (Fig. 9A,C,E). Densitometric evaluation of the immunoreactive bands confirmed the effect of AITC to increase ($p<0.05$ vs. vehicle) the phosphorylation of p38 (except spinal cord) and p42/44 MAPK and the expression of c-Fos (Fig. 11A,B,C) in the spinal cord and all brain regions examined. The AITC-induced levels of both MAPK and c-Fos were markedly diminished in morphine-injected control animals (study 6) (Fig. 12A,C,E). Densitometric evaluation of the immunoreactive bands confirmed that morphine blunted the AITC-evoked rise of c-Fos and MAPKs levels in the brain and spinal cord in a region-dependent manner (Fig. 11A,B,C). The expression of c-Fos and MAPKs in the CNS measured after peanut oil instillation to saline-injected mice did not significantly differ from that measured without prior saline injection (compare Fig. 9 with Fig. 12). Taken all data together, morphine

reduced the effect of intrarectal AITC to evoke freezing, MAPK phosphorylation and c-Fos expression in the CNS of control mice (Jain et al., 2015). The details of the statistical analysis are shown in Tables 24-25.

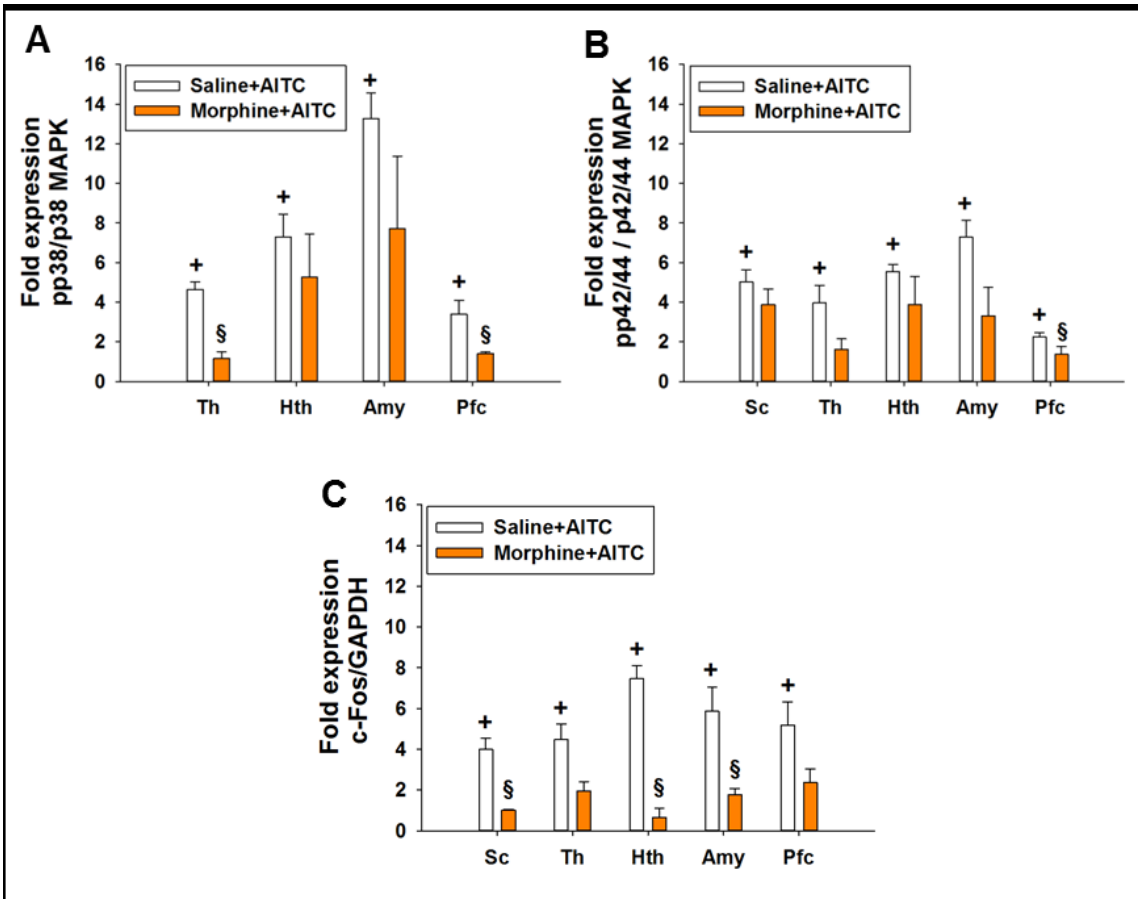

Figure 11. Protein expression (A: pp38/p38 MAPK, B: pp42/44 / p42/44 MAPK, C: c-Fos/GAPDH) in the spinal cord (Sc), thalamus (Th), hypothalamus (Hth), amygdala (Amy) and prefrontal cortex (Pfc) of control mice following intrarectal instillation of peanut oil (Veh) or AITC (2% v/v) in the absence or presence of morphine (study 5 and study 6). Saline or morphine (10 mg/kg, s.c.) was injected one hour prior to intrarectal instillation. The graph compares the AITC-induced protein expression levels in control animals (Veh + Con in Fig. 9 pooled with Saline + Veh + Con in Fig. 12) with those in morphine-treated animals (Morphine + Veh + Con in Fig. 12). Values are given as means + SEM (n=3-6). Analysis with the independent samples t-test disclosed significant differences: $^{+}$ $p<0.05$ vs. Veh; § $p<0.05$ vs. Morphine+AITC (Jain et al., 2015).

3.12. Morphine blunted the AITC-evoked phosphorylation of p38, p42/44 MAPKs and c-Fos expression in the spinal cord and brain of WAS-exposed and DSS-treated mice

Morphine caused region-specific changes in the AITC-evoked p38 and p42/44 MAPK phosphorylation in the brain of all treatment groups (Fig. 12A,C). As shown by densitometric evaluation of the immunoreactive bands, there was no significant interaction between WAS and DSS with regard to AITC-evoked phosphorylation of p38 (Fig. 12B, in the different brain regions) and p42/44 MAPKs (Fig. 12D, in the spinal cord and brain regions) in animals injected with morphine. Nevertheless, both DSS and WAS main factor effects were disclosed under the influence of morphine. Thus, the AITC-evoked expression of pp38 MAPK in the prefrontal cortex ($p<0.05$) and pp42/44 MAPK in the spinal cord ($p<0.01$) was blunted in DSS-treated mice (DSS effect). The AITC-evoked expression of pp42/44 MAPK in the hypothalamus ($p<0.05$) was also mitigated in WAS-exposed mice injected with morphine (WAS effect). The details of the statistical analysis are shown in Tables 26-27.

Morphine reversed the effect of AITC on c-Fos expression in the spinal cord and brain of all treatment groups (Fig. 12E), a result confirmed by densitometric evaluation of the immunoreactive bands (Fig. 12F). A significant interaction ($p<0.05$) between WAS and DSS in the AITC-evoked c-Fos induction under the influence of morphine was only found in the spinal cord (Fig. 12F). Bonferroni's multiple comparison demonstrated that morphine blunted AITC-evoked c-Fos expression in the spinal cord in both WAS-exposed and DSS-treated mice ($p<0.001$, vs. controls). The AITC-evoked expression of c-Fos in the amygdala ($p<0.05$) was alleviated in WAS-exposed and DSS-treated mice that had been injected with morphine (WAS and DSS effect). The details of the statistical analysis are shown in Table 28.

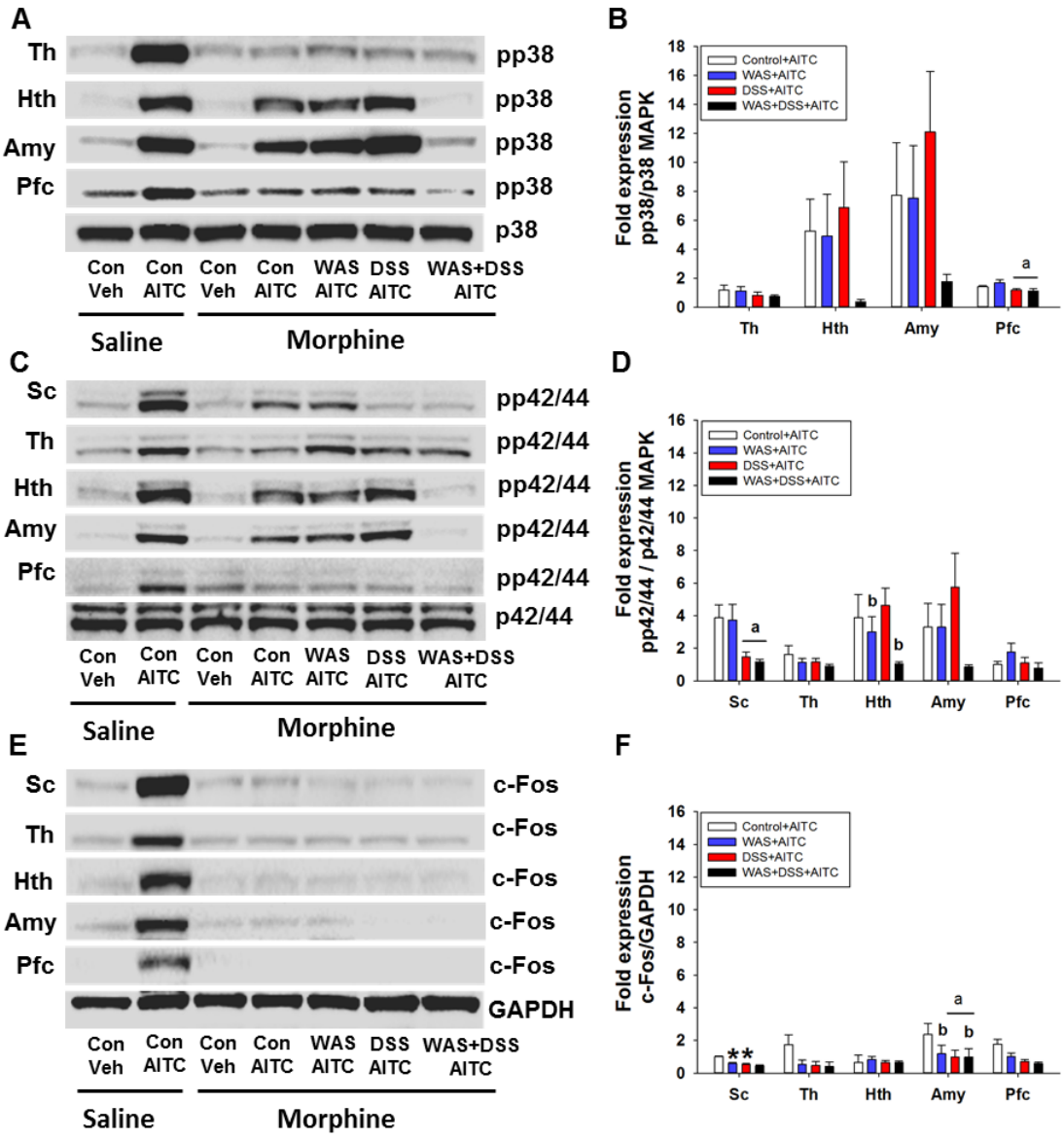

Figure 12. Protein expression (A/B: pp38/p38 MAPK, C/D: pp42/44 / p42/44 MAPK, E/F: c-Fos/GAPDH) in the spinal cord (Sc), thalamus (Th), hypothalamus (Hth), amygdala (Amy) and prefrontal cortex (Pfc) of control (Con) mice and mice treated with WAS, DSS and WAS+DSS following intrarectal instillation of peanut oil (Veh) or AITC 2% (v/v) in the absence or presence of morphine (study 6). The mice were treated with WAS, DSS and WAS+DSS for 7 days, and protein expression was evaluated on day 8. Saline or morphine (10 mg/kg, s.c.) was injected one hour prior to intrarectal instillation. Panels A, C and E show representative Western blots, and panels B, D and F show quantitative data obtained by densitometric evaluation of the Western blots, expressed as x fold

over control. Values shown in panels B, D and F are expressed as means + SEM (n=3-4). Two-way ANOVA revealed a WAS x DSS interaction in c-Fos expression (F) in the spinal cord ($p<0.05$) but not in the brain. There was no significant WAS x DSS interaction in pp38 (B) and pp42/44 (D) expression in any tissue examined. Two-way ANOVA showed a DSS effect (^{a} $p<0.05$) and a WAS effect (^{b} $p<0.05$). Post-hoc (Bonferroni's multiple comparison) analysis disclosed significant differences: *$p<0.05$ vs. Morphine+Control+AITC.

3.13. DSS-induced colitis remained unaffected by a TRPA1 antagonist

In order to explore the contribution of TRPA1 to DSS-induced colitis I tested the effect of the selective TRPA1 antagonist HC-030031 by examining the severity of colitis. Treatment of mice with DSS (study 7, set 1) induced colitis as seen in treatment groups DSS+Veh and DSS+HC. Analysis of colitis-associated parameters did not reveal any significant interaction between DSS and HC treatment. Treatment with DSS (DSS effect, $p<0.001$) led to body weight loss (Fig. 13A), increased disease activity score (Fig. 13B), reduced colon length (Fig. 13C) and increased colon weight (Fig. 13D) as well as enhanced colonic MPO activity (DSS effect, $p<0.05$) (Fig. 13E). HC-030031 treatment of control mice did not alter any of these parameters (Fig. 13A-E). Thus, the TRPA1 antagonist HC-030031 did not alter colitis induced by DSS. The details of the statistical analysis are shown in Table 29.

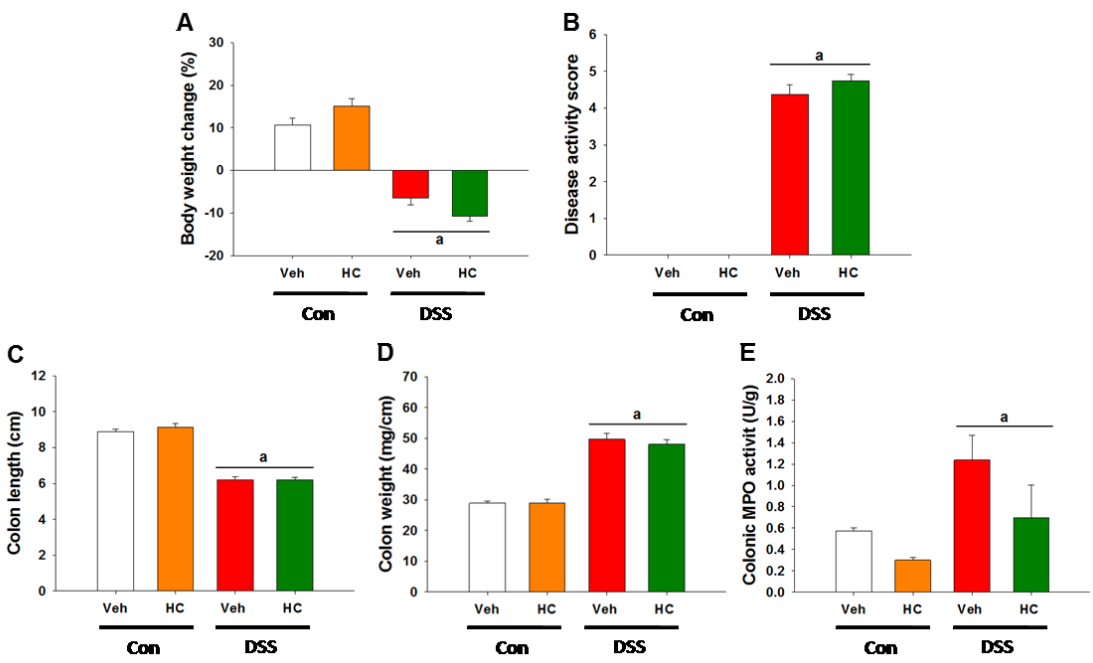

Figure 13. Effect of the selective TRPA1 antagonist HC-030031 (HC) or its vehicle (Veh) in control (Con) mice and mice treated with DSS for 7 days on colitis-associated parameters (A-E) measured on day 8 (study 7, set 1). HC-030031 (100 mg/kg, i.p.) or its vehicle was injected one hour prior to sacrifice of mice. (A) Percentage of body weight change relative to the weight measured before the treatment started, (B) disease activity score, (C) colon length, (D) colon weight, and (E) colonic MPO activity. Values are expressed as means + SEM or - SEM (n=4-8). Two-way ANOVA did not show any DSS x HC interaction and any HC effect but revealed a DSS effect (^{a} $p<0.05$).

3.14. DSS-induced colitis remained unaffected by genetic deletion of TRPA1

In order to further explore the contribution of TRPA1 to DSS-induced colitis I tested the effect of genetic deletion of TRPA1 (genetic deletion) by examining the severity of colitis in wild type ($TRPA1^{+/+}$) and TRPA1 deficient ($TRPA1^{-/-}$) mice. Treatment of mice with DSS (study 7, set 2) induced colitis as seen in the treatment groups DSS+$TRPA1^{+/+}$ and DSS+$TRPA1^{-/-}$. Analysis of colitis-associated parameters did not reveal any significant interaction between DSS and

genetic deletion. Treatment with DSS led to body weight loss (DSS effect, $p<0.01$) (Fig. 14A), increased disease activity score (DSS effect, $p<0.001$) (Fig. 14B), reduced colon length (DSS effect, $p<0.01$) (Fig. 14C) and increased colon weight (DSS effect, $p<0.001$) (Fig. 14D) as well as colonic MPO activity (DSS effect, $p<0.05$) (Fig. 14E). Genetic deletion of TRPA1 did not alter any of these parameters (Fig. 14A-E). Thus, the genetic deletion of TRPA1 did not alter colitis induced by DSS. The details of the statistical analysis are shown in Table 30.

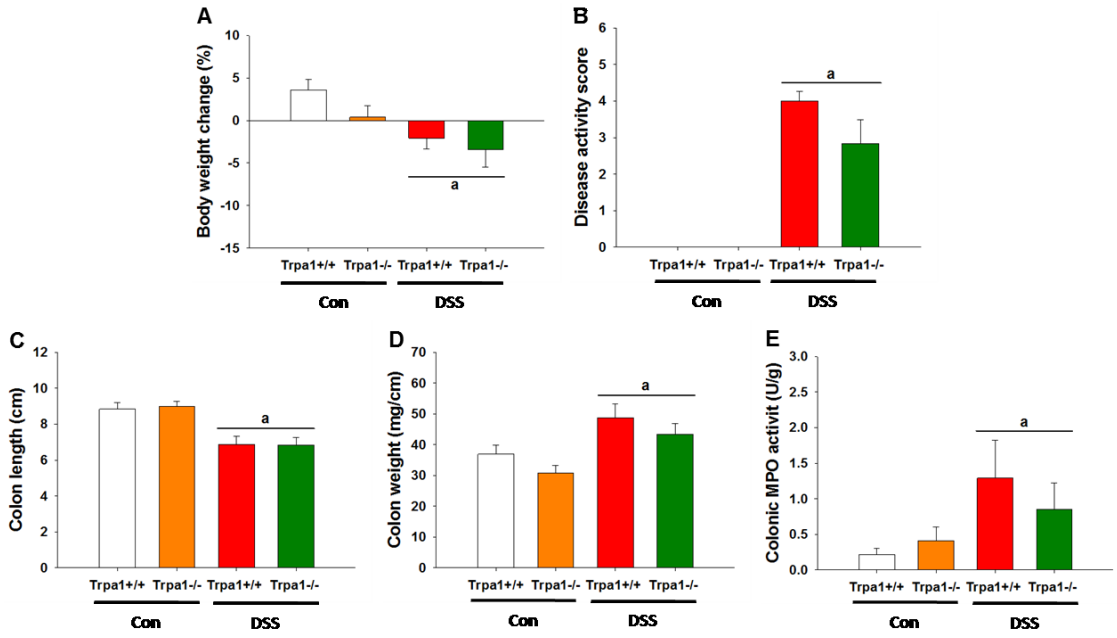

Figure 14. Effect of genetic deletion of TRPA1 in control (Con) mice and mice treated with DSS for 7 days on colitis-associated parameters (A-E) measured on day 8 (study 7, set 2). (A) Percentage of body weight change relative to the weight measured before the treatment started, (B) disease activity score, (C) colon length, (D) colon weight, and (E) colonic MPO activity. Values are expressed as means + SEM or - SEM (n=6). Two-way ANOVA did not show any DSS x genetic deletion interaction and any genetic deletion effect but revealed a DSS effect ([a] $p<0.05$).

3.15. The TRPA1 antagonist HC-030031 reduced colitis-evoked somatic sensitivity to mechanical and thermal stimuli

In order to further explore the contribution of TRPA1 to colitis-induced somatic nociception in mice, I assessed the effect of HC-030031, relative to its vehicle, on the sensitivity of the abdominal and facial skin to mechanical stimuli and of the plantar skin to thermal stimuli in control and DSS-treated mice.

The mechanical sensitivity in the abdominal and facial region was assessed with the von Frey test and the thermal sensitivity of the plantar region with the plantar test under three experimental condiditions (study 7, set 1): basal – prior to any treatment (Fig. 15A), pre-vehicle/HC-030031 – following control or DSS treatment but prior to injection of HC-030031 or its vehicle (Fig. 15B), and post-vehicle/HC-030031 – following control or DSS treatment and injection of HC-030031 or its vehicle (Fig. 15C). There was no difference among the animals in terms of the withdrawal response in the von Frey test under basal conditions (Fig. 15A). Under pre-vehicle/HC-030031 conditions there was no significant interaction between DSS/HC-030031 treatments and the test forces in their influence on the withdrawal response. There was, however, a DSS effect ($p<0.05$, Fig. 15B) at all test forces in their impact on the withdrawal response, DSS enhancing the withdrawal response. Assessment of the mechanical sensitivity in the abdominal region with the von Frey test under post-vehicle/HC-030031 conditions likewise disclosed a significant interaction ($p<0.01$, Fig. 15C) between DSS/HC treatments and the test forces in their effect on the withdrawal response. Two-way ANOVA showed a significant interaction between DSS and HC-030031 at three test forces: 0.008 g ($p<0.01$), 0.02 g ($p<0.01$), and 0.04 g ($p<0.001$). Bonferroni's multiple comparison analysis revealed that the withdrawal response to forces of 0.008 g ($p<0.001$ vs. Con+Veh), 0.02 g ($p<0.001$ vs. Con+Veh) and 0.04g ($p<0.001$ vs. Con+Veh) was significantly increased in DSS-treated mice. DSS+HC-030031 treatment led to a significant decrease in the withdrawal response to forces of 0.008 g ($p<0.01$ vs. DSS+Veh), 0.02 g ($p<0.001$ vs. DSS+Veh) and 0.04g ($p<0.001$ vs. DSS+Veh) (Fig. 15C). The details of the statistical analysis are shown in Tables 31-33.

Assessment of the mechanical sensitivity in the facial region with the von Frey test disclosed no difference in the withdrawal threshold among the animals under basal conditions (Fig. 15D). Under pre-vehicle/HC-030031 conditions there was no significant interaction between DSS and HC-030031 treatments in their influence on the withdrawal threshold but there was a significant DSS effect ($p<0.001$, Fig. 15D) in lowering the withdrawal threshold. Assessment of the mechanical sensitivity in the facial region with the von Frey test under post-vehicle/HC-030031 conditions disclosed a significant interaction ($p<0.01$, Fig. 15D) between the DSS and HC-030031 treatments in their effect on the withdrawal threshold (Fig. 15D). Bonferroni's multiple comparison analysis revealed that the withdrawal threshold was significantly decreased in DSS-treated mice ($p<0.001$ vs. Con+Veh) while the treatment with HC-030031 in DSS-treated mice (DSS+HC) led to a significant increase in the withdrawal threshold ($p<0.001$ vs. DSS+Veh) (Fig. 15D). The details of the statistical analysis are shown in Tables 34-36.

Assessment of the thermal sensitivity in the plantar region with the hot plate test disclosed no difference in withdrawal latency among the animals under basal conditions (Fig. 15E). Analysis of the data obtained under pre-vehicle/HC-030031 conditions did not reveal any significant interaction between DSS and HC-030031 treatments in their influence on the withdrawal latency. There was, however, a DSS effect ($p<0.001$, Fig. 15E) in shortening the withdrawal latency. There was likewise no significant interaction between DSS and HC-030031 in their influence on the withdrawal latency under post-vehicle/HC-030031 conditions. There was, however, a DSS effect ($p<0.01$, Fig. 15E) in its impact on the withdrawal latency, while HC-030031 failed to reverse the effect of DSS to shorten the withdrawal latency. The details of the statistical analysis are shown in Tables 37-39.

Taken together, these data show that DSS-induced colitis enhanced the mechanical and thermal sensitivity of the abdominal and facial region, and the plantar skin, respectively. Treatment with HC-030031 reversed the DSS-induced mechanical hypersensitivity in the abdominal and facial skin but did not affect the DSS-evoked thermal hypersensitivity in the plantar region.

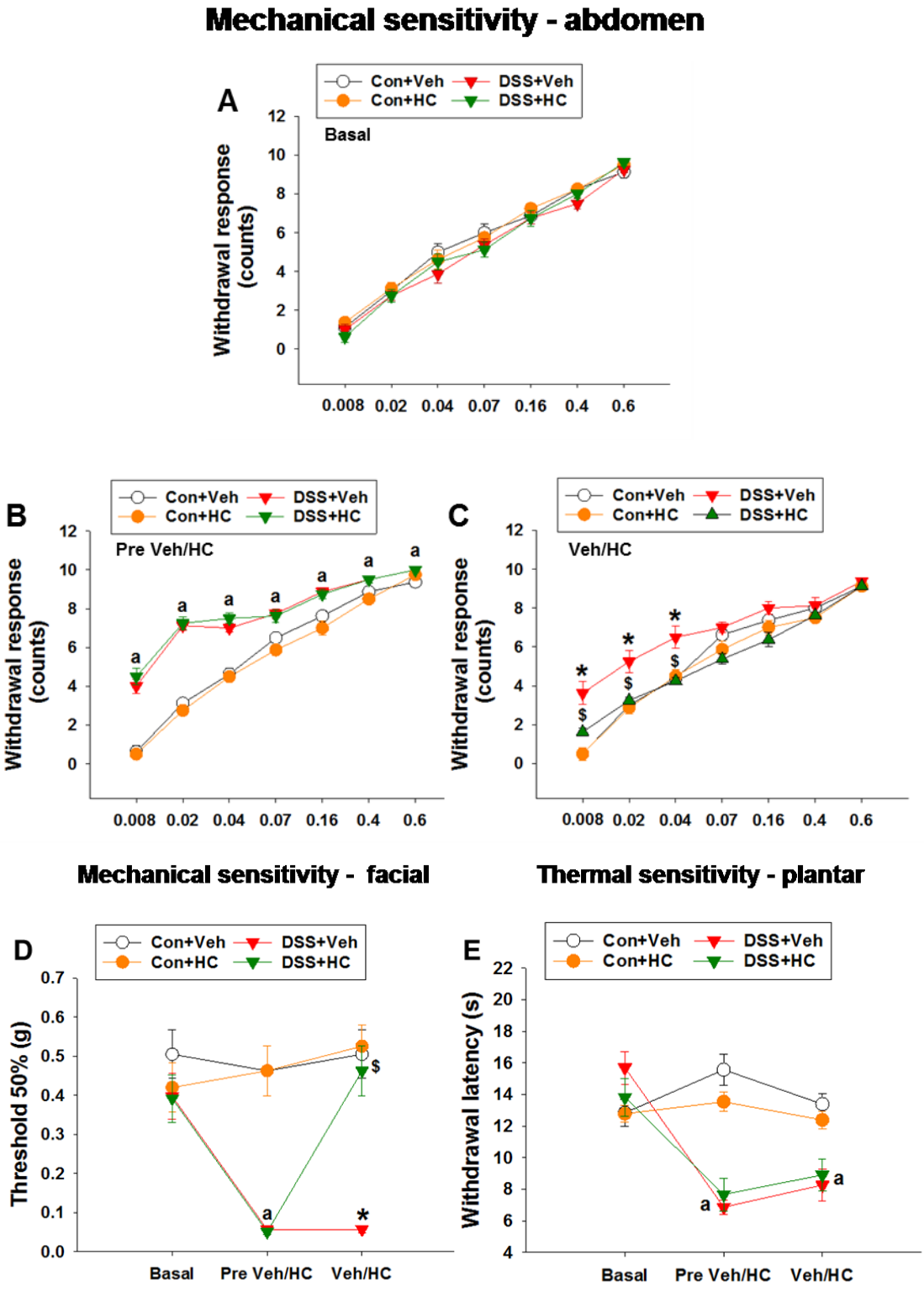

Figure 15. Effect of HC-030031 (HC) or its vehicle (Veh) in control (Con) mice and in mice treated with DSS for 7 days on mechanical pain sensitivity of the abdomen (A-C) and facial region (D) and on thermal pain sensitivity of the plantar region (E)

measured on day 8 (study 7, set 1). HC-030031 (100 mg/kg, i.p.) or its vehicle was injected one hour prior to the test. Values are expressed as means ± SEM or + SEM (n=7-8). Repeated measures ANOVA (A) did not show any DSS x HC x forces interaction in the withdrawal response from abdominal stimulation under basal conditions. Repeated measures ANOVA (B) did not show any DSS x HC x forces interaction in the withdrawal response from abdominal stimulation following control or DSS treatment but prior to injection of HC-030031 or its vehicle (Pre Veh/HC) but revealed a DSS x forces interaction ($p<0.001$). Post-hoc analysis (independent samples t-test) demonstrated a DSS effect (^{a} $p<0.05$) as indicated in panel B. Repeated measures ANOVA (C) disclosed a DSS x HC x forces interaction ($p<0.01$) in the withdrawal response from abdominal stimulation following control or DSS treatment and injection of HC-030031 or its vehicle (Veh/HC). Post-hoc (two-way ANOVA) analysis demonstrated a DSS x HC interaction at the 0.008, 0.02 ($p<0.01$) and 0.04 g forces ($p<0.001$). Two-way ANOVA (D, Basal) did not show any DSS x HC interaction and any DSS or HC effect in the withdrawal threshold from facial stimulation under basal conditions. Two-way ANOVA (D) did not show any DSS x HC interaction and any HC effect in the withdrawal threshold from facial stimulation under “Pre Veh/HC” conditions but revealed a DSS effect (^{a} $p<0.001$). Two-way ANOVA (D) disclosed a DSS x HC interaction ($p<0.01$) in the withdrawal threshold from facial stimulation under “Veh/HC” conditions. Two-way ANOVA (E, Basal) did not show any DSS x HC interaction and any DSS or HC effect in the withdrawal latency from plantar stimulation under basal conditions. Two-way ANOVA (E) did not show any DSS x HC interaction and any HC effect in the withdrawal latency from plantar stimulation under “Pre Veh/HC” conditions but revealed a DSS effect (^{a} $p<0.001$). Two-way ANOVA (E, Veh/HC) did not show any DSS x HC interaction and any HC effect in the withdrawal latency from plantar stimulation under “Veh/HC” conditions but revealed a DSS effect (^{a} $p<0.01$). Post-hoc Bonferroni’s multiple comparison analysis demonstrated significant differences: *$p<0.05$ vs. Con+Veh; $^{\$}$ $p<0.05$ vs. DSS+Veh.

3.16. Genetic deletion of TRPA1 reduced colitis-evoked somatic sensitivity to mechanical stimuli

In order to study the contribution of TRPA1 to colitis-induced somatic nociception in mice, I assessed the sensitivity of the abdominal and facial skin to mechanical stimuli and of the plantar skin to thermal stimuli in $TRPA1^{+/+}$ - and $TRPA1^{-/-}$ - control and DSS-treated mice.

The mechanical sensitivity in the abdominal and facial region was assessed with the von Frey test and the thermal sensitivity of the plantar region with the plantar test under two experimental conditions (study 7, set 2): basal – prior to any treatment (Fig.16A) and post-DSS – following control or DSS treatment (Fig. 16B). There was no difference among the animals in terms of the withdrawal response in the von Frey test under basal conditions (Fig. 16A). Assessment of the mechanical sensitivity in the abdominal region with the von Frey test under post-DSS conditions disclosed a significant interaction ($p<0.01$, Fig. 16B) between DSS/genetic deletion and the test forces in their effect on the withdrawal response. Two-way ANOVA showed a significant interaction between DSS and genetic deletion at five test forces: 0.008 g ($p<0.001$), 0.02 g ($p<0.01$), 0.04 g ($p<0.05$) 0.07 ($p<0.001$) and 0.16 g ($p<0.001$). Bonferroni's multiple comparison analysis revealed that the withdrawal response to forces of 0.008 g, 0.02 g 0.04 g, 0.07 g ($p<0.001$ vs. Con-$TRPA1^{+/+}$) and 0.16 g ($p<0.05$ vs. Con-$TRPA1^{+/+}$) was significantly increased in DSS-treated $TRPA1^{+/+}$ (DSS-$TRPA1^{+/+}$) mice. DSS treatment in $TRPA1^{-/-}$ (DSS-$TRPA1^{-/-}$) mice led to a significant decrease in the withdrawal response to forces of 0.008 g, 0.02 g, 0.04 g ($p<0.001$ vs. DSS-$TRPA1^{+/+}$), 0.07 g and 0.16 g ($p<0.01$ vs. DSS-$TRPA1^{+/+}$) (Fig. 16B). The details of the statistical analysis are shown in Tables 40-41.

Assessment of the mechanical sensitivity in the facial region with the von Frey test disclosed no difference in the withdrawal threshold among the animals under basal conditions (Fig. 16C). However, under post-DSS conditions a significant interaction ($p<0.01$, Fig. 16C) between DSS and genetic deletion in their effect on the withdrawal threshold was disclosed (Fig. 16C). Bonferroni's multiple comparison analysis revealed that the withdrawal threshold was significantly decreased in DSS-treated $TRPA1^{+/+}$ mice ($p<0.01$ vs. Con-$TRPA1^{+/+}$). This effect

of while DSS treatment was absent in TRPA1$^{-/-}$ (DSS-TRPA1$^{-/-}$) mice ($p<0.01$ vs. DSS-TRPA1$^{+/+}$) in which there was no decrease in the withdrawal threshold relative to Con-TRPA1$^{+/+}$ mice (Fig. 16C). The details of the statistical analysis are shown in Tables 42-43.

Assessment of the thermal sensitivity in the plantar region with the hot plate test disclosed no difference in withdrawal latency among the animals under basal and post-DSS conditions (Fig. 15D). Taken together, these data show that DSS-induced colitis enhanced the mechanical sensitivity of the abdominal and facial region in TRPA1$^{+/+}$ mice. Genetic deletion of TRPA1 in mice reversed the DSS-induced mechanical hypersensitivity in the abdominal and facial skin.

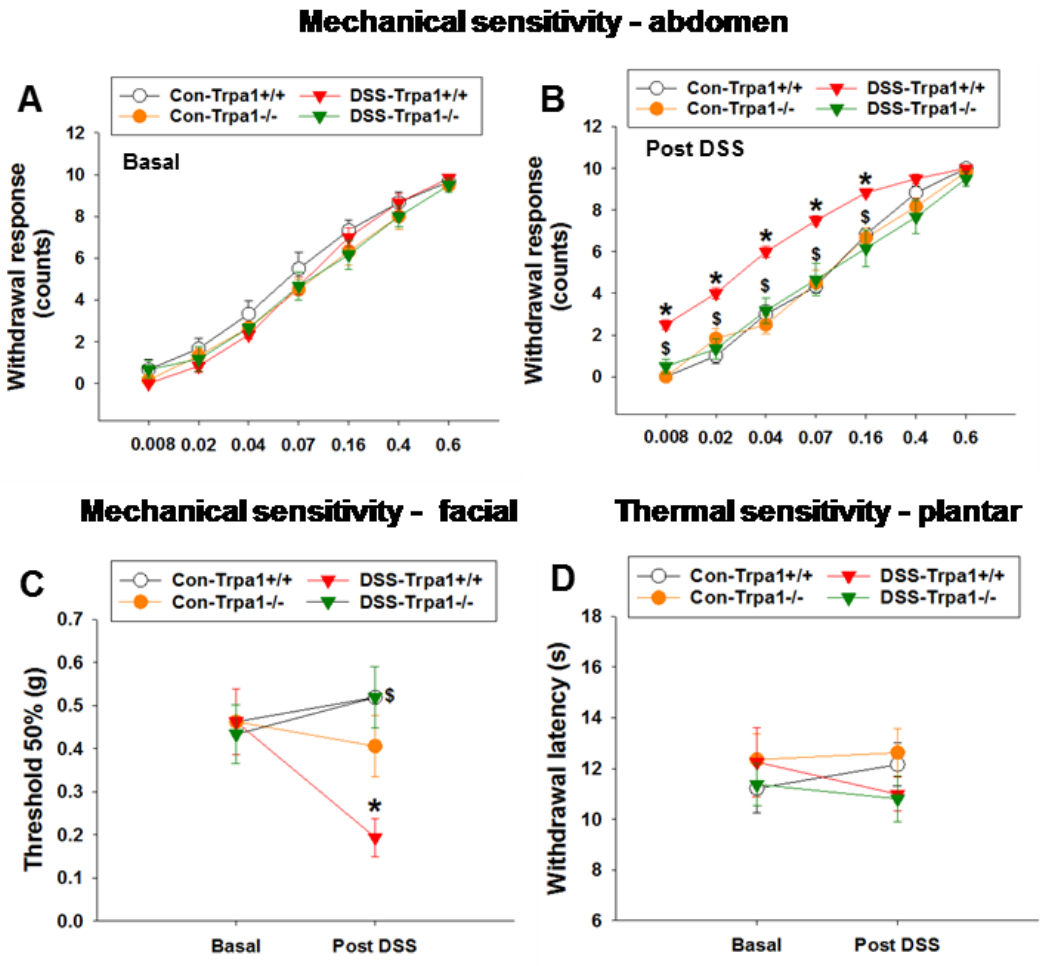

Figure 16. Effect of genetic deletion of TRPA1 in control (Con) mice and in mice treated with DSS for 7 days on mechanical pain sensitivity of the abdomen (A-B) and facial region (C) and on thermal pain sensitivity of the plantar region (D)

measured on day 8 (study 7, set 2). Values are expressed as means ± SEM or + SEM (n=6). Repeated measures ANOVA (A) did not show any DSS x genetic deletion x forces interaction in the withdrawal response from abdominal stimulation under basal conditions. Repeated measures ANOVA (B) showed a DSS x genetic deletion x forces interaction in the withdrawal response from abdominal stimulation following control or DSS treatment ($p<0.01$). Post-hoc (two-way ANOVA) analysis demonstrated a DSS x genetic deletion interaction at the 0.008 g ($p<0.001$), 0.02 g ($p<0.01$), 0.04 g ($p<0.05$), 0.07 g and 0.16 g forces ($p<0.001$). Two-way ANOVA (C, Basal) did not show any DSS x genetic deletion interaction and any DSS or genetic deletion effect in the withdrawal threshold from facial stimulation under basal conditions. Two-way ANOVA (C) disclosed a DSS x genetic deletion interaction ($p<0.01$) in the withdrawal threshold from facial stimulation under "post DSS" conditions. Two-way ANOVA did not show any DSS x genetic deletion interaction and any DSS or genetic deletion effect in the withdrawal latency from plantar stimulation under basal and post-DSS conditions (E). Post-hoc Bonferroni's multiple comparison analysis demonstrated significant differences: * $p<0.05$ vs. Con+TRPA1$^{+/+}$; $^{\$}$ $p<0.05$ vs. DSS+TRPA1$^{+/+}$.

3.17. DSS treatment increased the relative mRNA expression of TRPA1 in DRGs

On the molecular level, I examined the effect of DSS-induced colitis and HC-030031 treatment on the relative expression of TRPA1, TRPV1 and TRPV4 mRNA in lumbosacral DRGs (study 8, set 1). Statistical analysis of the data showed that there was no significant interaction between the DSS and HC-030031 treatments. However, DSS treatment increased expression of TRPA1 mRNA in the DRG neurons (DSS effect, $p<0.01$, Fig. 17A). The details of the statistical analysis are shown in Table 44.

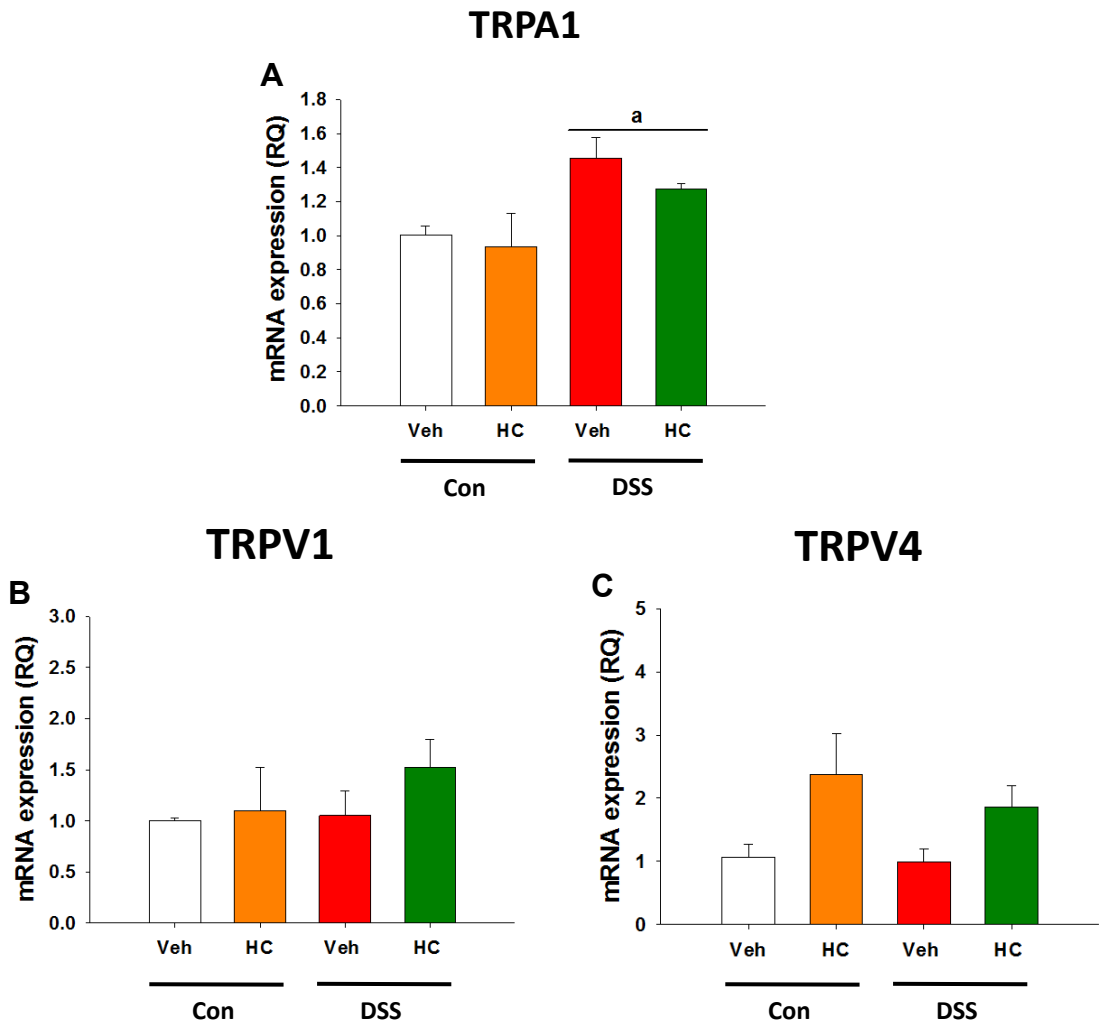

Figure 17. Effect of HC-030031 (HC) or its vehicle (Veh) on expression of TRPA1 (A) TRPV1 (B) and TRPV4 (C) mRNA in lumbosacral DRG neurons isolated from control (Con) mice or mice treated with DSS for 7 days (study 8, set 1). HC-030031 (100 mg/kg, i.p.) or its vehicle was injected one hour prior to sacrifice of mice. Values are expressed as means + SEM (n=4-5). Two-way ANOVA did not show any DSS x HC interaction and any HC effect but revealed a DSS effect in expression of TRPA1 mRNA (^{a} $p<0.05$). Two-way ANOVA did not show any DSS x HC interaction and any DSS or HC effect in expression of TRPV1 and TRPV4 mRNA.

3.18. DSS treatment did not alter the release of calcitonin gene-related peptide (CGRP) evoked by TRPA1 stimulation

In set 2 of study 8 I examined the effect of DSS-induced colitis on CGRP release from central endings of sensory neurons in the thoracosacral spinal cord, evoked by AITC or capsaicin. The data demonstrate that the AITC-evoked release of CGRP-LI from spinal cord preparations was not affected by DSS treatment. Statistical analysis of the data showed there was no significant difference in the release rates of CGRP measured after stimulation with AITC (10 µM and 100 µM) and capsaicin (CPS) (10 µM) between the control (Con) and DSS-treated groups (Fig. 18).

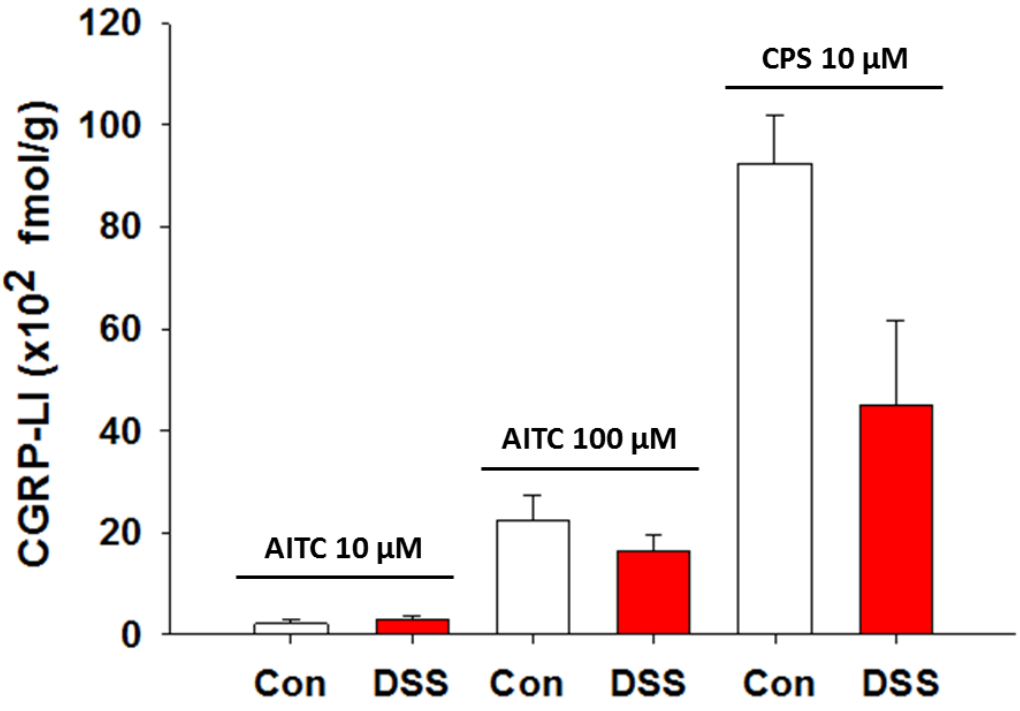

Figure 18. Effect of AITC or capsaicin (CPS) on CGRP-LI release from thoracosacral spinal cord preparations isolated from control (Con) mice or mice treated with DSS for 7 days (study 8, set 2). Values are expressed as means + SEM (n=3-4). Independent samples t-test did not show any significant difference among control (Con) and DSS groups.

3.19. DSS treatment led to sensitization of nociceptive sensory neurons

In study 8, set 3, I examined whether hypersensitivity caused by DSS-induced colitis in mice can be analyzed in primary cultures of nociceptive sensory neurons. Cultured thoracosacral DRG neurons were challenged with AITC or capsaicin in electrophysiological studies in which peak inward currents were recorded, normalized to membrane capacitance and expressed as current density (pA/pF). As shown in Fig. 19, both AITC and in particular capsaicin evoked inward currents at a voltage clamp of -60 mV. Inward currents induced by capsaicin were not affected by DSS treatment. In contrast, the response to AITC in capsaicin-sensitive neurons isolated from DRGs of DSS-treated mice was amplified when compared with that in control mice. As the data were not normally distributed, analysis with a non-parametric test revealed significant differences in inward current ($p<0.001$, Fig. 19) between control (Con) and DSS-treated groups. The details of the statistical analysis are shown in Table 45.

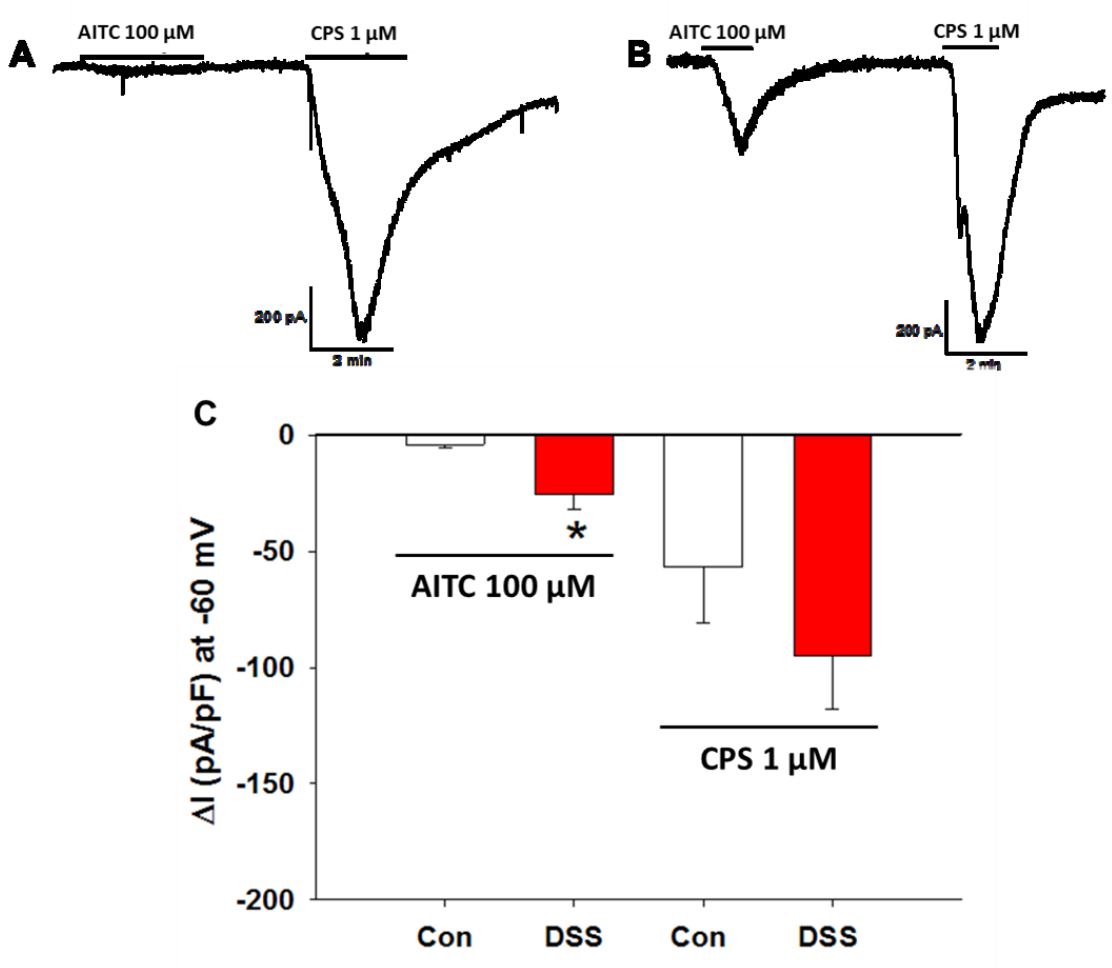

Figure 19. Inward currents evoked by AITC and capsaicin (CPS) DRG neurons (thoracosacral) isolated from control (Con) mice or mice treated with DSS for 7 days (study 8, set 3).The ΔI is the difference between current amplitude measured (at -60 millivolts) before and after the application of AITC/CPS to neurons. The inward currents are shown as current density pA/pF. Values are expressed as means - SEM (n=16 neurons from 3 mice/group). Mann-Whitney U test demonstrated a significant difference between control (Con) and DSS groups: *p<0.05 vs. Con.

3.20. Details of statistical analysis

Table 3: Details of statistical analysis performed in study 1

Two-way ANOVA			
Parameters (study 1)	DSS effect	WAS effect	WAS x DSS interaction
Body weight change (%)	F(1,36) = 79.2; p < 0.001	NS	NS
Disease activity score	F(1,36) = 658.3; p < 0.001	NS	NS
Colon length (cm)	F(1,36) = 92.8; p < 0.001	NS	NS
Colon weight (mg/cm)	F(1,36) = 37.8; p < 0.001	NS	NS
Colonic MPO mass (ng/mg)	F(1,33) = 15.2; p < 0.001	NS	NS

NS = not significant

Table 4: Details of statistical analysis performed in study 2

Two-way ANOVA			
Parameters (study 2)	DSS effect	WAS effect	WAS x DSS interaction
Body weight change (%)	F(1,24) = 131.73; p < 0.001	NS	NS
Disease activity score	F(1,24) = 389.61; p < 0.001	NS	NS
Colon length (cm)	F(1,24) = 105.55; p < 0.001	NS	NS
Colon weight (mg/cm)	F(1,24) = 92.54; p < 0.001	NS	NS
Colonic MPO mass (ng/mg)	F(1,13) = 16.48; p < 0.01	NS	NS

NS = not significant

Table 5: Details of statistical analysis performed in study 3

Two-way ANOVA			
Parameters (study 3)	DSS effect	WAS effect	WAS x DSS interaction
Body weight change (%)	$F(1,28) = 241.50$; $p < 0.001$	NS	NS
Disease activity score	$F(1,28) = 469.00$; $p < 0.001$	NS	NS
Colon length (cm)	$F(1,28) = 254.69$; $p < 0.001$	NS	NS
Colon weight (mg/cm)	$F(1,28) = 190.27$; $p < 0.001$	NS	NS
Colonic MPO mass (ng/mg)	$F(1,13) = 16.48$; $p < 0.01$	NS	NS

NS = not significant

Table 6: Details of statistical analysis performed in study 4

Two-way ANOVA			
Parameters (study 4)	DSS effect	WAS effect	WAS x DSS interaction
Body weight change (%)	$F(1,40) = 59.06$; $p < 0.001$	NS	NS
Disease activity score	$F(1,40) = 942.61$; $p < 0.001$	NS	NS
Colon length (cm)	$F(1,40) = 89.71$; $p < 0.001$	NS	NS
Colon weight (mg/cm)	$F(1,40) = 36.14$; $p < 0.001$	NS	NS
Colonic MPO mass (ng/mg)	$F(1,13) = 15.02$; $p < 0.01$	NS	NS

NS = not significant

Table 7: Details of statistical analysis performed in study 5

Two-way ANOVA			
Parameters (study 5)	DSS effect	WAS effect	WAS x DSS interaction
Body weight change (%)	$F(1,71) = 119.95$; $p < 0.001$	NS	NS
Disease activity score	$F(1,39) = 837.33$; $p < 0.001$	NS	NS
Colon length (cm)	$F(1,71) = 261.66$; $p < 0.001$	NS	NS
Colon weight (mg/cm)	$F(1,71) = 105.14$; $p < 0.001$	NS	NS
Colonic MPO mass (ng/mg)	$F(1,26) = 11.37$; $p < 0.01$	NS	NS

NS = not significant

Table 8: Details of statistical analysis performed in study 6

Two-way ANOVA			
Parameters (study 6)	DSS effect	WAS effect	WAS x DSS interaction
Body weight change (%)	$F(1,27) = 42.18$; $p < 0.001$	NS	NS
Disease activity score	$F(1,27) = 939.07$; $p < 0.001$	NS	NS
Colon length (cm)	$F(1,27) = 58.74$; $p < 0.001$	NS	NS
Colon weight (mg/cm)	$F(1,27) = 41.22$; $p < 0.001$	NS	NS
Colonic MPO mass (ng/mg)	$F(1,15) = 10.54$; $p < 0.01$	NS	NS

NS = not significant

Table 9: Details of statistical analysis performed in study 2

Two-way ANOVA				
Parameters (study 2)	DSS effect	WAS effect	WAS x DSS interaction	
Locomotion ($x10^3$ counts)	NA	NA	$F(1,24) = 13.62$; $p < 0.01$	
Rearing ($x10^2$ counts)	NA	NA	$F(1,24) = 12.27$; $p < 0.01$	
Post-hoc (Bonferroni's multiple comparison)				
Parameters	DSS vs. Control	WAS vs. Control	WAS+DSS vs. DSS	WAS+DSS vs. WAS
Locomotion ($x10^3$ counts)	$p = 0.505$	$p < 0.05$	$p < 0.05$	$p < 0.001$
Rearing ($x10^2$ counts)	$p = 0.369$	$p < 0.01$	$p = 0.061$	$p < 0.001$

NA = not applicable

Table 10: Details of statistical analysis performed in study 3

Two-way ANOVA			
Parameters (study 3)	DSS effect	WAS effect	WAS x DSS interaction
Locomotion ($x10^2$ counts)	$F(1,28) = 38.21$; $p < 0.001$	NS	NS
Rearing (counts)	$F(1,28) = 40.28$; $p < 0.001$	NS	NS
Total grooming (counts)	NS	$F(1,28) = 6.61$; $p < 0.05$	NS
Face grooming latency (s)	NS	$F(1,28) = 5.42$; $p < 0.05$	NS

NS = not significant

Table 11: Details of statistical analysis performed in study 4

Repeated measure ANOVA			
Parameters (study 4)	DSS x forces	WAS x forces	WAS x DSS x forces interaction
Withdrawal response (counts), abdomen	F(4.5,189.16) = 2.61; p < 0.05	F(4.5,189.16) = 2.67; p < 0.05	NS
Post-hoc (independent samples t-test)			
Force intensity (g)	DSS effect	WAS effect	
0.04	Bonferroni's correction p = 0.006 x 7 (p = 0.042)	NS	

NS = not significant, Bonferroni's correction: p value multiplied by 7 (total number of forces used of different intensities)

Table 12: Details of statistical analysis performed in study 4

Repeated measure ANOVA				
Parameters (study 4)	DSS x forces	WAS x forces	WAS x DSS x forces interaction	
Withdrawal response (counts), plantar	NA	NA	F(4.25,178.57) = 2.59; p < 0.05	
Post-hoc (two-way ANOVA)				
Force intensity (g)	DSS effect	WAS effect	WAS x DSS interaction	
Force 0.02	NA	NA	F(1,42) = 11.57; p < 0.01	
Force 0.04	NA	NA	F(1,42) = 18.81; p < 0.001	
Force 0.07	NA	NA	F(1,42) = 5.16; p < 0.05	
Post-hoc (Bonferroni's multiple comparison)				
Force intensity (g)	DSS vs. Control	WAS vs. Control	WAS+DSS vs. DSS	WAS+DSS vs. WAS
Force 0.02	p < 0.001	p < 0.01	p = 0.056	p = 0.310
Force 0.04	p < 0.01	p < 0.001	p = 0.054	p < 0.01
Force 0.07	p = 0.238	p = 0.064	p = 0.201	p = 0.052

NA = not applicable

Table 13: Details of statistical analysis performed in study 4

Repeated measure ANOVA			
Parameters (study 4)	DSS x intensity	WAS x intensity	WAS x DSS x intensity interaction
Withdrawal latency (s)	NA	NA	F(2,80) = 3.67; p < 0.05
Post-hoc (two-way ANOVA)			
Infrared intensity	DSS effect	WAS effect	WAS x DSS interaction
1	NS	NS	NS
2	NS	F(1,40) = 6.14; p < 0.05	NS
3	NA	NA	F(1,40) = 7.32; p < 0.05

Post-hoc (Bonferroni's multiple comparison)				
Infrared intensity	DSS vs. Control	WAS vs. Control	WAS+DSS vs. DSS	WAS+DSS vs. WAS
3	p < 0.05	p < 0.001	p = 0.914	p = 0.147

NA = not applicable, NS = not significant

Table 14: Details of statistical analysis performed in study 1

Fold expression, pp42/p44 / p42/p44 (study 1) Two-way ANOVA			
Region	DSS effect	WAS effect	WAS x DSS interaction
Spinal cord	NS	NS	F(1,8) = 6.16; p < 0.05
Thalamus	NS	F(1,12) = 4.44; p = 0.057	NS
Hypothalamus	NS	F(1,12) = 19.88; p < 0.01	NS
Amygdala	NS	F(1,12) = 13.67; p < 0.01	NS
Prefrontal cortex	NS	F(1,8) = 25.25; p < 0.01	NS

Post-hoc (Bonferroni's multiple comparison)				
Region	DSS vs. Control	WAS vs. Control	WAS+DSS vs. DSS	WAS+DSS vs. WAS
Spinal cord	p < 0.01	p = 0.899	p < 0.05	p = 0.856

NS = not t significant

Table 15: Details of statistical analysis performed in study 1

Fold expression, c-fos/GAPDH (study 1) Two-way ANOVA			
Region	DSS effect	WAS effect	WAS x DSS interaction
Spinal cord	NA	NA	$F(1,8) = 10.05$; $p < 0.05$
Thalamus	NA	NA	$F(1,12) = 120.0$; $p < 0.001$
Hypothalamus	NA	NA	$F(1,12) = 686.81$; $p < 0.001$
Amygdala	NA	NA	$F(1,12) = 226.00$; $p < 0.001$
Prefrontal cortex	NA	NA	$F(1,12) = 56.43$; $p < 0.001$

Post-hoc (Bonferroni's multiple comparison)				
Region	DSS vs. Control	WAS vs. Control	WAS+DSS vs. DSS	WAS+DSS vs. WAS
Spinal cord	$p < 0.05$	$p < 0.001$	$p = 0.073$	$p < 0.001$
Thalamus	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
Hypothalamus	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
Amygdala	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
Prefrontal cortex	$p < 0.01$	$p < 0.001$	$p < 0.001$	$p < 0.001$

NA = not applicable

Table 16: Details of statistical analysis performed in study 1

Fold expression, pp38/p38 (study 1) Two-way ANOVA			
Region	DSS effect	WAS effect	WAS x DSS interaction
Thalamus	NS	$F(1,12) = 8.70$; $p < 0.05$	NS
Hypothalamus	NS	$F(1,12) = 19.88$; $p < 0.01$	NS
Amygdala	NS	$F(1,12) = 30.35$; $p < 0.001$	NS
Prefrontal cortex	NA	NA	$F(1,12) = 5.36$; $p < 0.001$

Post-hoc (Bonferroni's multiple comparison)				
Region	DSS vs. Control	WAS vs. Control	WAS+DSS vs. DSS	WAS+DSS vs. WAS
Prefrontal cortex	$p = 0.294$	$p = 0.083$	$p = 0.192$	$p = 0.050$

NA = not applicable, NS = not significant

Table 17: Details of statistical analysis performed in study 5

Independent samples t-test	
Parameters (study 5)	Veh vs. AITC
Freezing (counts)	t(8.97)=-4.17; p<0.01
Locomotion (x10^2 counts)	t(19)=-2.92; p<0.01
Rearing (x10^2 counts)	t(19)=-2.11; p<0.05

Table 18: Details of statistical analysis performed in study 5

Two-way ANOVA			
Parameters (Study 5)	DSS effect	WAS effect	WAS x DSS interaction
Freezing (counts)	F(1,38) = 14.21; p < 0.01	NS	NS
Locomotion (x10^3 counts)	NS	F(1,34) = 4.13; p = 0.05	NS
Rearing (x10^2 counts)	NS	F(1,34) = 3.65; p = 0.06	NS

NS = not significant

Table 19: Details of statistical analysis performed in study 5

Fold expression, pp38/p38 (study 5) Two-way ANOVA			
Region	DSS effect	WAS effect	WAS x DSS interaction
Thalamus	NA	NA	F(1,12) = 5.37; p < 0.05
Hypothalamus	NA	NA	F(1,12) = 17.30; p < 0.01
Amygdala	NA	NA	F(1,12) = 10.42; p < 0.01
Prefrontal cortex	NS	F(1,12) = 4.22; p < 0.01	NS

Post-hoc (Bonferroni's multiple comparison)				
Region	DSS vs. Control	WAS vs. Control	WAS+DSS vs. DSS	WAS+DSS vs. WAS
Thalamus	p < 0.01	p = 0.597	p < 0.05	p = 0.867
Hypothalamus	p < 0.001	p = 0.522	p < 0.001	p = 0.538
Amygdala	p < 0.001	p = 0.699	p < 0.001	p = 0.216

NA = not applicable, NS = not significant

Table 20: Details of statistical analysis performed in study 5

Fold expression, pp42/p44 / p42/p44 (study 5) Two-way ANOVA				
Region	DSS effect	WAS effect	WAS x DSS interaction	
Spinal cord	F(1,12) = 19,43; $p < 0.05$	NA	NS	
Thalamus	NA	NA	F(1,12) = 6.61; $p < 0.05$	
Hypothalamus	NA	NA	F(1,12) = 4.93; $p < 0.05$	
Amygdala	NS	NS	NS	
Prefrontal cortex	NA	NA	F(1,12) = 6.20; $p < 0.05$	
Post-hoc (Bonferroni's multiple comparison)				
Region	DSS vs. Control	WAS vs. Control	WAS+DSS vs. DSS	WAS+DSS vs. WAS
Thalamus	$p < 0.01$	$p = 0.129$	$p = 0.068$	$p = 0.867$
Hypothalamus	$p = 0.054$	$p = 0.685$	$p < 0.05$	$p = 0.335$
Prefrontal cortex	$p < 0.01$	$p = 0.729$	$p < 0.01$	$p = 0.990$

NA = not applicable, NS = not significant

Table 21: Details of statistical analysis performed in study 5

Fold expression, c-fos/GAPDH (study 5) Two-way ANOVA				
Region	DSS effect	WAS effect	WAS x DSS interaction	
Spinal cord	NA	NA	F(1,12) = 15.50; $p < 0.01$	
Thalamus	NA	NA	F(1,12) = 5.17; $p < 0.05$	
Hypothalamus	NA	NA	F(1,12) = 24.05; $p < 0.001$	
Amygdala	NA	NA	F(1,12) = 30.74; $p < 0.05$	
Prefrontal cortex	NA	NA	NS	
Post-hoc (Bonferroni's multiple comparison)				
Region	DSS vs. Control	WAS vs. Control	WAS+DSS vs. DSS	WAS+DSS vs. WAS
Spinal cord	$p < 0.05$	$p = 0.407$	$p < 0.001$	$p < 0.01$
Thalamus	$p < 0.01$	$p < 0.001$	$p < 0.001$	$p = 0.704$
Hypothalamus	$p = 0.664$	$p < 0.05$	$p < 0.001$	$p < 0.01$
Amygdala	$p = 0.060$	$p < 0.001$	$p < 0.01$	$p < 0.001$

NA = not applicable

Table 22: Details of statistical analysis performed in study 6

Non parametric test: one-way ANOVA			
Parameter	Difference among the groups (control, WAS, DSS, WAS+DSS)		
Freezing (counts)	F(3,26) = 8.83; p < 0.001		
Post-hoc (Mann-Whitney U test)			
	Saline + AITC vs. Saline + Veh	Saline + AITC vs. Morphine + AITC	Morphine + AITC vs. Morphine + Veh
Freezing (counts)	p < 0.05	NS	NS

NS = not significant

Table 23: Details of statistical analysis performed in study 6

Two-way ANOVA			
Parameters	DSS effect	WAS effect	WAS x DSS interaction
Freezing (counts)	F(1,24) = 4.73; p < 0.05	F(1,24) = 5.86; p < 0.05	NS

NS = not significant

Table 24: Details of statistical analysis performed in study 5

Independent samples t-test			
Study 5/study 6	Fold expression, pp38/p38	Fold expression, pp42/p44 / p42/p44	Fold expression, c-fos/GAPDH
Region	AITC vs. Veh	AITC vs. Veh	AITC vs. Veh
Spinal cord	NA	t(6)=-4.28; p<0.05	t(6)=-11.21; p<0.01
Thalamus	t(6)=-6.52; p<0.01	t(6)=-3.38; p<0.05	t(6)=-14.06; p<0.01
Hypothalamus	t(6)=-8.71; p<0.01	t(3)=-13.77; p<0.01	t(6)=-11.98; p<0.01
Amygdala	t(6)=-10.02; p<0.01	t(3)=-4.95; p<0.001	t(6)=-8.59; p<0.01
Prefrontal cortex	t(6)=-5.04; p<0.05	t(6)=-15.86; p<0.01	t(6)=-3.50; p<0.05

NA = not applicable

Table 25: Details of statistical analysis performed in study 6

Independent samples t-test			
Study 6	Fold expression, pp38/p38	Fold expression, pp42/p44 / p42/p44	Fold expression, c-fos/GAPDH
Region	Saline + AITC vs. Saline + Veh	Saline + AITC vs. Morphine + AITC	Morphine + AITC vs. Morphine + Veh
Spinal cord	NA	NS	$t(8) = 4.34$; $p < 0.01$
Thalamus	$t(8) = 6.58$; $p < 0.001$	NS	NS
Hypothalamus	NS	NS	$t(8) = 6.56$; $p < 0.001$
Amygdala	NS	NS	$t(8) = 2.70$; $p < 0.05$
Prefrontal cortex	$t(6.16) = 2.83$; $p < 0.05$	$t(8) = 4.14$; $p < 0.01$	NS

NA = not applicable, NS = not significant

Table 26: Details of statistical analysis performed in study 6

Fold expression, pp38/p38 (study 6) Two-way ANOVA			
Region	DSS effect	WAS effect	WAS x DSS Interaction
Thalamus	NS	NS	NS
Hypothalamus	NS	NS	NS
Amygdala	NS	NS	NS
Prefrontal cortex	$F(1,8) = 7.42$; $p < 0.05$	NS	NS

NS = not significant

Table 27: Details of statistical analysis performed in study 6

Fold expression, pp42/p44 / p42/p44 (study 6) Two-way ANOVA			
Region	DSS effect	WAS effect	WAS x DSS interaction
Spinal cord	$F(1,8) = 15.41$; $p < 0.01$	NS	NS
Thalamus	NS	NS	NS
Hypothalamus	NS	$F(1,12) = 4.96$; $p < 0.05$	NS
Amygdala	NS	NS	NS
Prefrontal cortex	NS	NS	NS

NS = not significant

Table 28: Details of statistical analysis performed in study 6

Fold expression, c-fos/GAPDH (study 6) Two-way ANOVA			
Region	DSS effect	WAS effect	WAS x DSS interaction
Spinal cord	NA	NA	F(1,12) = 7.78; p < 0.05
Thalamus	NS	NS	NS
Hypothalamus	NS	NS	NS
Amygdala	F(1,12) = 14.54; p < 0.05	F(1,12) = 5.16; p < 0.05	NS
Prefrontal cortex	NS	NS	NS

Post-hoc (Bonferroni's multiple comparison)				
Region	DSS vs. Control	WAS vs. Control	WAS+DSS vs. DSS	WAS+DSS vs. WAS
Spinal cord	p < 0.001	p < 0.001	p = 0.387	p = 0.111

NA = not applicable, NS = not significant

Table 29: Details of statistical analysis performed in study 7, set 1

Two-way ANOVA			
Parameters (study 7)	DSS effect	HC effect	DSS x HC interaction
Body weight change (%)	F(1,28) = 170.53; p < 0.001	NS	NS
Disease activity score	F(1,28) = 867.51; p < 0.001	NS	NS
Colon length (cm)	F(1,28) = 302.89; p < 0.001	NS	NS
Colon weight (mg/cm)	F(1,28) = 214.27; p < 0.001	NS	NS
Colonic MPO activity (U/g)	F(1,13) = 6.60; p < 0.05	NS	NS

NS = not significant

Table 30: Details of statistical analysis performed in study 7, set 2

Two-way ANOVA			
Parameters	DSS effect	Genetic deletion effect	DSS x Genetic deletion interaction
Body weight change (%)	F(1,20) = 10.04; p < 0.01	NS	NS
Disease activity score	F(1,20) = 28.82; p < 0.001	NS	NS
Colon length (cm)	F(1,20) = 12.89; p < 0.01	NS	NS
Colon weight (mg/cm)	F(1,20) = 94.43;	NS	NS

	p < 0.001		
Colonic MPO activity (U/g)	F(1,15) = 5.77; p < 0.05	NS	NS

NS = not significant

Table 31: Details of statistical analysis performed in study 7, set 1

Repeated measures ANOVA (Basal)			
Parameters (study 7)	DSS x forces	HC x forces	DSS x HC x forces interaction
Withdrawal response (counts), abdomen	NS	NS	NS

NS = not significant

Table 32: Details of statistical analysis performed in study 7, set 1

Repeated measures ANOVA (Pre Veh/HC)			
Parameters (study 7)	DSS x forces	HC x forces	DSS x HC x forces interaction
Withdrawal response (counts), abdomen	F(3.83,107.32) = 39.12; p < 0.001	NS	NS
Post-hoc (independent samples t-test)			
Force intensity (g)	DSS effect	HC effect	
0.008	Bonferroni's correction p = 0.000 x 7 p = 0.000)	NS	
0.02	Bonferroni's correction p = 0.000 x 7 (p = 0.000)	NS	
0.04	Bonferroni's correction p = 0.000 x 7 (p = 0.000)	NS	
0.07	Bonferroni's correction p = 0,000 x 7 (p = 0.000)	NS	
0.16	Bonferroni's correction p = 0.000 x 7 (p = 0.000)	NS	
0.4	Bonferroni's correction p = 0.000 x 7 (p = 0.000)	NS	
0.6	Bonferroni's correction p = 0.004 x 7 (p = 0.028)	NS	

NS = not significant, Bonferroni's correction: p values multiplied by 7 (total number of forces used of different intensities)

Table 33: Details of statistical analysis performed in study 7, set 1

Repeated measures ANOVA (Post Veh/HC)			
Parameters (study 7)	DSS x forces	HC x forces	DSS x HC x forces interaction
Withdrawal response (counts), abdomen	NA	NA	F(4.39,123.00) = 4.40; p < 0.01
Post-hoc (two-way ANOVA)			
Force intensity (g)	DSS effect	HC effect	DSS x HC interaction
Force 0.008	NA	NA	F(1,28) = 13.23; p < 0.01
Force 0.02	NA	NA	F(1,28) = 12.41; p < 0.01
Force 0.04	NA	NA	F(1,28) = 15.51; p < 0.001

Post-hoc (Bonferroni's multiple comparison)				
Force intensity (g)	Con+HC vs. Con+Veh	DSS+HC vs. DSS+Veh	DSS+Veh vs. Con+Veh	Con+HC vs. DSS+HC
Force 0.008	p = 0.684	p < 0.01	p < 0.001	p = 0.16
Force 0.02	p = 0.811	p < 0.001	p < 0.001	p = 0.811
Force 0.04	p = 0.495	p < 0.001	p < 0.001	p = 0.364

NA = not applicable

Table 34: Details of statistical analysis performed in study 7, set 1

Two-way ANOVA (Basal)			
Parameters	DSS effect	HC effect	DSS x HC interaction
Withdrawal response (counts), facial	NS	NS	NS

NS = not significant

Table 35: Details of statistical analysis performed in study 7, set 1

Two-way ANOVA (Pre Veh/HC)			
Parameters	DSS effect	HC effect	DSSx HC interaction
Withdrawal threshold (g), facial	F(1,28) = 80.52; p < 0.001	NS	NS

NS = not significant

Table 36: Details of statistical analysis performed in study 7, set 1

Two-way ANOVA (Post Veh/HC)				
Parameters	DSS effect	HC effect		DSS x HC interaction
Withdrawal threshold (g), facial	NA	NA		F(1,28) = 13.39; p < 0.01
Post-hoc (Bonferroni's multiple comparison)				
	Con+HC vs. Con+Veh	DSS+HC vs. DSS+Veh	DSS+Veh vs. Con+Veh	Con+HC vs. DSS+HC
Withdrawal threshold	p = 0.783	p < 0.001	p < 0.001	p = 0.404

NA = not applicable

Table 37: Details of statistical analysis performed in study 7, set 1

Two-way ANOVA (Basal)			
Parameters	DSS effect	HC effect	DSS x HC interaction
Withdrawal latency (s)	NS	NS	NS

NS = not significant

Table 38: Details of statistical analysis performed in study 7, set 1

Two-way ANOVA (Pre Veh/HC)			
Parameters	DSS effect	HC effect	DSS x HC interaction
Withdrawal latency (s)	F(1,28) = 82.46; p < 0.001	NS	NS

NS = not significant

Table 39: Details of statistical analysis performed in study 7, set 1

Two-way ANOVA (Post Veh/HC)			
Parameters	DSS effect	HC effect	DSS x HC interaction
Withdrawal latency (s)	F(1,28) = 27.52; p < 0.01	NS	NS

NS = not significant

Table 40: Details of statistical analysis performed in study 7, set 2

Repeated measures ANOVA (Basal)			
Parameters (study 7)	DSS x forces	Genetic deletion x forces	DSS x Genetic deletion x forces interaction
Withdrawal response (counts), abdomen	NS	NS	NS

NS = not significant

Table 41: Details of statistical analysis performed in study 7, set 2

Repeated measures ANOVA (Post DSS)			
Parameters (study 7)	DSS x forces	Genetic deletion x forces	DSS x Genetic deletion x forces interaction
Withdrawal response (counts), abdomen	NA	NA	$F(6,120) = 3.04$; $p < 0.01$
Post-hoc (two-way ANOVA)			
Force intensity (g)	DSS effect	Genetic deletion effect	DSS x Genetic deletion X Forces interaction
Force 0.008	NA	NA	$F(1,20) = 24.00$; $p < 0.001$
Force 0.02	NA	NA	$F(1,20) = 15.97$; $p < 0.01$
Force 0.04	NA	NA	$F(1,20) = 6.71$; $p < 0.05$
Force 0.07	NA	NA	$F(1,20) = 8.02$; $p < 0.001$
Force 0.16	NA	NA	$F(1,20) = 5.54$; $p < 0.001$

Post-hoc (Bonferroni's multiple comparison)				
Force intensity (g)	Con-Trpa1$^{+/+}$ vs. Con-Trpa1$^{-/-}$	DSS-Trpa1$^{-/-}$ vs. DSS-Trpa1$^{+/+}$	DSS-Trpa1$^{+/+}$ vs. Con-Trpa1$^{+/+}$	Con-Trpa1$^{-/-}$ vs. DSS-Trpa1$^{-/-}$
Force 0.008	$p = 1$	$p < 0.001$	$p < 0.001$	$p = 0.099$
Force 0.02	$p = 0.4383$	$p < 0.001$	$p < 0.001$	$p = 0.769$
Force 0.04	$p = 0.442$	$p < 0.001$	$p < 0.001$	$p = 0.308$
Force 0.07	$p = 0.826$	$p < 0.01$	$p < 0.001$	$p = 0.826$
Force 0.16	$p = 0.827$	$p < 0.01$	$p < 0.05$	$p = 0.513$

NA = not applicable, NS = not significant

Table 42: Details of statistical analysis performed in study 7, set 2

Two-way ANOVA (Basal)			
Parameters	DSS effect	Genetic deletion effect	DSS x genetic deletion interaction
Withdrawal response (counts), facial	NS	NS	NS

NS = not significant

Table 43: Details of statistical analysis performed in study 7, set 2

Two-way ANOVA (Post DSS)				
Parameters	DSS effect		Genetic deletion effect	DSS x Genetic deletion interaction
Withdrawal threshold (g), facial	NA		NA	F(1,20) = 11.09; p < 0.01
Post-hoc (Bonferroni's multiple comparison)				
	Con-Trpa1$^{+/+}$ vs. Con-Trpa1$^{-/-}$	DSS-Trpa1$^{-/-}$ vs. DSS-Trpa1$^{+/+}$	DSS-Trpa1$^{+/+}$ vs. Con-Trpa1$^{+/+}$	Con-Trpa1$^{-/-}$ vs. DSS-Trpa1$^{-/-}$
Withdrawal threshold (g)	p = 0.238	p < 0.01	p < 0.01	p = 0.238

NA = not applicable, NS = not significant

Table 44: Details of statistical analysis performed in study 8, set 1

Two-way ANOVA			
Parameters	DSS effect	HC effect	WAS x HC interaction
TRPA1 mRNA, DRG	F(1,12) = 10.77; p < 0.01	NS	NS

NS = not significant

Table 45: Detail of statistical analysis performed in study 8, set 3

Mann-Whitney U test		
	Inward current, 100 M	**Inward current, 1 M**
	Con+AITC vs.DSS+AITC	Con+CPS vs. DSS+CPS
DRG	p < 0.001	NS
DRG	Non parametric test	NS

NS = not significant

4. Discussion

The description of Discussion is partly reused from Jain et al. (2015) with modifications.

Clinical evidence indicates that chronic visceral pain in IBD and IBS is exaggerated by psychosocial stress, but the mechanisms behind this relationship are little understood. In a translational perspective I therefore examined, in mice, in which way DSS-induced mild colitis and repeated intermittent WAS, a psychological stressor, interact in shaping visceral pain-related behavior and altering molecular signaling in distinct pain relays of the CNS. I also explored whether the change in pain sensitivity caused by colitis and WAS was limited to the gut or extended to somatic tissues. I went on to shed light on specific molecular mechanisms behind the changes in visceral pain processing caused by colitis. Specifically, I investigated whether TRPA1 plays a causal role in some of the behavioral and neurochemical manifestations of visceral pain and colitis-evoked hypersensitivity.

4.1. The major findings of my experimental study in mice can be summarized as follows

(i) WAS had no major effect on DSS-induced colitis but increased locomotion, rearing (exploration), motivation and self-care behavior and enhanced MAPK phosphorylation in the thalamus, hypothalamus and amygdala.

(ii) DSS did not change locomotion and exploration but in combination with WAS decreased these parameters.

(iii) DSS increased the mechanical pain sensitivity of the abdominal skin while both WAS and DSS enhanced the mechanical and thermal pain sensitivity of the plantar skin (Jain et al., 2015). These states of hyperalgesia were paralleled by augmented c-Fos expression in the lumbosacral spinal cord, thalamus, hypothalamus, amygdala and prefrontal cortex. While WAS stimulated the MAPK

pathway, DSS activated another signaling pathway, both of which converged on c-Fos but inhibited each other when activated in parallel by WAS+DSS. The induction of c-Fos in the CNS is of relevance to central sensitization processes because the DSS- and WAS-induced hyperalgesia in the abdominal and plantar skin and the DSS- and WAS-induced c-Fos expression in the CNS disappeared when the mice were subjected to WAS+DSS treatment.

(iv) Intrarectal AITC instillation evoked aversive behavior (freezing, reduction of locomotion and rearing) in association with MAPK phosphorylation and c-Fos expression in the spinal cord and all brain regions examined. These effects were prevented by morphine, which attests to their relationship with nociception.

(v) DSS increased the effect of intrarectal AITC to induce c-Fos expression in the spinal cord but mitigated the effect of AITC to evoke freezing and p42/44 MAPK phosphorylation in the spinal cord.

(vi) Locomotion and c-Fos expression in the hypothalamus and amygdala recorded after intrarectal AITC instillation were enhanced in WAS-exposed mice whereas c-Fos expression in the thalamus was diminished.

(vii) MAPK phosphorylation in all brain regions examined and c-Fos expression in the thalamus recorded after intrarectal AITC instillation were attenuated in DSS-treated mice. The inhibitory effect of DSS on MAPK phosphorylation was canceled by concomitant WAS.

(viii) Morphine premedication inhibited the effect of intrarectal AITC to evoke freezing, MAPK phosphorylation and c-Fos expression in the CNS in all treatment (WAS, DSS, WAS+DSS) groups.

(ix) A TRPA1 antagonist and genetic deletion of TRPA1 reduced mechanical pain hypersensitivity evoked by DSS treatment in both the abdominal and facial skin but failed to alter thermal hypersensitivity in the plantar skin.

(X) DSS treatment caused an increase in TRPA1 mRNA expression in DRG neurons.

(Xi) The AITC-evoked inward current was increased in cultured DRG neurons taken from DSS-treated mice.

4.2. WAS fails to modify the severity of colitis

In the interpretation of the current observations it is important to state that WAS failed to significantly alter the severity of DSS-induced colitis as judged by body weight, colon length, colon weight, colonic MPO content and DAS (Fig. 2A-E). Although repeated WAS has been found to disturb GI function (Soderholm et al., 2002; Velin et al., 2004), my findings confirm previous reports that WAS fails to modify experimentally induced colitis in rodents (Deiteren et al., 2014; Hassan et al., 2014). I therefore conclude that the interactions between WAS and DSS with regard to emotional and pain-related behavior, pain sensitivity and signaling processes in the CNS do not reflect WAS-induced alterations of colitis. To the contrary, I propose that these interactions take place primarily within the brain (Jain et al., 2015).

4.3. Colitis and WAS are linked to alterations in spontaneous behavior and CNS signaling

Internal (inflammation) and external (psychological) stressors are known to affect several aspects of behavior related to emotionality, pain (Cattaruzza et al., 2013; Banozic et al., 2014) and sickness (Farzi et al., 2015; Zhang et al., 2014). Increased activity in rats exposed to unpredictable chronic mild stress resembles the psychomotor agitation seen in patients with psychiatric disorders (Gronli et al., 2005). In the current study I found that WAS increases spontaneous activity (locomotion and rearing) in mice whereas a reduction of spontaneous activity was observed in stressed mice with colitis (WAS+DSS) (Fig. 3A,B). It should be noted that these alterations of spontaneous behavior included a component of novelty-induced behavior since the animals were not habituated to the test cages. These behavioral changes occurred independently of changes in ingestion (Fig. 3C,D)

and thus do not seem to reflect sickness but are rather of an emotional-affective nature (Jain et al., 2015).

In the splash test (Fig. 5A-D), locomotion and rearing recorded after the spraying intervention was reduced in DSS-treated, but not WAS-exposed, animals. This differential change in intervention-related activity may have a bearing on grooming behavior which, however, was enhanced only in WAS-exposed mice but left unaltered in DSS-treated animals. Other studies have shown that unpredictable chronic mild stress is associated with a decrease in motivational and self-care behavior, which is thought to reflect a depression-like state because it can be reversed by antidepressants (Taksande et al., 2013). Unlike unpredictable chronic mild stress, repeated WAS has been argued to represent a model of predictable chronic stress (Hassan et al., 2014). This may explain why I found WAS to increase both motivational (increase in grooming frequency) and self-care (decrease in grooming latency) behavior. The beneficial influence of WAS is consistent with its ability to counteract the effects of DSS-induced colitis to reduce social interaction and elevate anxiety, plasma corticosterone and hypothalamic neuropeptide Y mRNA expression (Hassan et al., 2014; Jain et al., 2015).

WAS and DSS were also found to cause alterations in c-Fos expression and MAPK phosphorylation in the CNS independently of any acute experimental intervention (Fig. 7A-F). I argue that these changes in CNS signaling may be related to the alterations of spontaneous behavior seen in WAS-exposed and DSS-treated mice. The induction of immediate early genes such as c-Fos occurs downstream of the stimulation of MAPKs but can also be regulated by other signaling pathways (Kovacs, 1998; O'Donnell et al., 2012; Reul, 2014), which is likely to explain the dissimilar MAPK phosphorylation and c-Fos expression patterns observed in the different treatment groups. The expression of c-Fos in the CNS in response to inflammation, pain and stress has been investigated in a number of studies (Matsuda et al., 1996; Sun and Luo, 2004; Welch et al., 2010; Nishi et al., 2014). In the current work I found that both DSS and WAS are associated with an induction of c-Fos in the lumbosacral spinal cord, thalamus, hypothalamus, amygdala and prefrontal cortex. This stimulation of CNS signaling may have a bearing on the change in spontaneous behavior associated with colitis

and on the change in the cerebral processing of pain stimuli, as will be further discussed below (Jain et al., 2015).

Unlike c-Fos expression, cerebral MAPK phosphorylation was not significantly affected by DSS but enhanced by WAS, most prominently in the thalamus, hypothalamus and amygdala. It is tempting to propose that activation of the p38 and p42/44 MAPK pathways is of relevance to the emotional and motivational alterations seen in WAS-exposed mice. This argument is supported by the finding that increased expression of MAPK phosphatase-1, a negative regulator of MAPKs, causes depression-like behavior in rodents exposed to stress (Shen et al., 2004; Akbarian and Davis, 2010; Duric et al., 2010). Similarly, MAPK activation in the rat amygdala during forced swim stress has been suggested to play a role in the manifestation of depression-like behavior (Huang and Lin, 2006). I surmise that p42/44 MAPK activation is of particular relevance to the emotional and motivational alterations seen in WAS-exposed mice (Jain et al., 2015).

It is worth mentioning that combined WAS+DSS treatment induced less c-Fos in the CNS than WAS or DSS separately (Fig. 7E,F). This result may be linked to the finding that WAS+DSS treatment increases circulating corticosterone to a larger extent than WAS or DSS alone (Hassan et al., 2014), given that dexamethasone prevents c-Fos induction by stress and inflammatory pain (Buritova et al., 1996; De Medeiros et al., 2005). Such a mechanism may also apply to the effect of combined WAS+DSS treatment to rduce locomotion whereas WAS enhances this parameter and DSS has no effect (Fig. 3A). Since, in addition, WAS prevents the anxiogenic effect of DSS (Hassan et al., 2014), I assume that WAS-evoked locomotion reflects psychomotor agitation which is reflected by MAPK activation and c-Fos expression in the CNS (Jain et al., 2015).

4.4. Central sensitization induced by WAS and colitis is mirrored by alterations in CNS signaling

Visceral hypersensitivity to distention of the colon has repeatedly been described to occur after DSS or WAS exposure (Larauche et al., 2010; Larauche et al., 2011; Chen et al., 2013; Deiteren et al., 2014). The present findings (Fig.

6A-C) demonstrate that DSS and WAS also lead to mechanical and thermal hypersensitivity of the plantar skin. While the enhanced withdrawal response to mechanical and thermal pain stimuli is indicative of somatic hyperalgesia, the DSS-induced mechanical hyperalgesia of the abdominal skin may reflect referred pain associated with DSS-induced colitis. This inference receives support from the finding that WAS failed to significantly alter the mechanical pain sensitivity of the abdomen. Referred pain on the abdominal surface has previously been observed after induction of experimental colitis and used as an indirect measure of intestinal pain (Laird et al., 2001; Eijkelkamp et al., 2007). The presence of somatic hyperalgesia in mice with DSS-induced colitis is consistent with the presence of somatic hyperalgesia in IBS patients (Stabell et al., 2013). Since both colitis and WAS induce visceral and/or somatic pain hypersensitivity, I conclude that pain sensitization takes place at the CNS level (Jain et al., 2015).

The results from different studies regarding the effect of WAS on visceral pain are inconsistent. Several reports attest to WAS-induced mechanical hypersensitivity in male Wistar, Long-Evans and Fischer-344 rats (Bradesi et al., 2005; Schwetz et al., 2005; Johnson et al., 2015), while other reports hold that repeated exposure to WAS does not alter mechanical sensitivity in female Wistar rats (Deiteren et al., 2014) or even causes immediate post-stress hypoalgesia in female Wistar rats (Larauche et al., 2012) and male Long-Evans rats (Schwetz et al., 2005). This and another study in male and female mice (Larsson et al., 2009), respectively, failed to observe any repeated WAS-induced mechanical hypersensitivity, which could relate to the absence of changes in CRF and GC receptor mRNA expression in the mouse amygdala (Hassan et al., 2014). In male Fischer-344 rats, however, both CRF and GC signaling in this region contribute to visceral hypersensitivity (Myers and Greenwood-Van Meerveld, 2012; Johnson et al., 2015). Species, strain and sex differences need therefore to be considered as factors that affect the ability of WAS to affect visceral sensitivity. In female Wistar rats, the failure of repeated WAS to cause visceral hyperalgesia is associated with a failure to activate the HPA axis (Deiteren et al., 2014). In male Long-Evans rats, acute exposure to WAS induces visceral hypersensitivity only in maternally separated animals, this effect seems to involve central, but not peripheral, CRF receptors (Schwetz et al., 2005; van den Wijngaard et al., 2012). The potential

effect of sex was studied in Wistar rats in which Larauche et al. (2012) observed visceral analgesia immediately after acute WAS exposure, an effect that depended on opioid signaling in female, but not male, animals. Apart from species, strain and sex, factors such as the method used to record visceral sensitivity, housing condition and animal handling may in addition affect WAS-associated changes in visceral sensitivity (Jain et al., 2015).

The mechanical and thermal hyperalgesia recorded from the abdominal and plantar skin of DSS-treated or WAS-exposed mice was absent when the animals were treated with WAS+DSS. While the precise mechanisms behind this observation remain unexplained, it can be surmised that the sensitization processes induced by DSS and WAS involve different CNS signaling pathways that interact with each other in a negative way. This contention is supported by the observation that WAS and WAS+DSS, but not DSS, caused MAPK phosphorylation in the thalamus, hypothalamus, amygdala and prefrontal cortex. In contrast, c-Fos expression in all CNS regions examined was stimulated by both WAS and DSS, but not WAS+DSS. These results show that DSS and WAS stimulate different signal transduction pathways that both converge on c-Fos but inhibit each other in inducing c-Fos when activated in parallel (Jain et al., 2015).

The observation of MAPK phosphorylation by WAS and of c-Fos induction by both WAS and DSS is indicative of major alterations in CNS signaling (Fig. 7A-F) which are most probably of relevance to the central pain sensitization observed. This conclusion is strongly backed by the finding that the DSS- and WAS-induced hyperalgesia in the abdominal and plantar skin and the DSS- and WAS-induced c-Fos expression in the CNS disappeared when the animals were treated with WAS+DSS. Activation of MAPKs (Edelmayer et al., 2014) and induction of c-Fos (Harris, 1998; Coggeshall, 2005; Sandkuhler, 2009) in nociceptive afferent pathways have been extensively used to investigate the molecular mechanisms of pain signaling induced by acute noxious stimuli. It needs to be emphasized that alterations of MAPK phosphorylation and c-Fos induction were found here in the absence of any acute intervention and thus are likely to have a bearing on the alterations in spontaneous behavior seen in WAS-exposed and DSS-treated mice (Jain et al., 2015).

4.5. Colitis and WAS modify cerebral processing of acute visceral pain in a differential manner

Apart from intervention-independent alterations in emotional behavior and CNS signaling, I was also able to show that an acute visceral pain stimulus, intrarectal AITC, evokes aversive behaviour (Fig. 8A-F), activates MAPKs and leads to c-Fos expression in the brain (Fig. 9A-F). Being an agonist at TRPA1 and TRPV1 (Holzer, 2011), intrarectal AITC has previously been found to cause visceral pain and to induce pp42/44 MAPK and c-Fos in the lumbosacral spinal cord, the molecular responses reaching a peak about 1 h after administration (Galan et al., 2003; Mitrovic et al., 2010). In the present study I extended these observations by demonstrating that intrarectal AITC instillation in control mice evokes MAPK phosphorylation and c-Fos induction in all brain regions examined. This cerebral signaling response to AITC is associated with behavioral alterations that involve the brain: prolonged freezing (Laird et al., 2001) and decreased activity, a response that has been proposed to be a readout of visceral pain in mice (Schwartz et al., 2013). It is hence tempting to postulate that MAPK phosphorylation and c-Fos expression in the spinal cord, thalamus, hypothalamus, amygdala and prefrontal cortex mirror the sensory-discriminative, emotional and autonomic processing of a visceral pain stimulus. This contention is affirmed by the ability of morphine to attenuate AITC-evoked aversive behavior (freezing) in parallel with MAPK and c-Fos activation in the CNS. The susceptibility to morphine has previously been used as an argument that central c-Fos expression elicited by a visceral pain stimulus is a correlate of visceral nociception (Mitrovic et al., 2010; Schwartz et al., 2013; Jain et al., 2015).

IBD and IBS are associated with changes in visceral pain sensitivity and stress coping (Bielefeldt et al., 2009; Elsenbruch, 2011; Fukudo, 2013). The present study involving DSS and WAS enabled me to compare the central processing of an acute visceral pain stimulus (intrarectal AITC) under conditions of hyperalgesia with and without colonic inflammation. The pertinent experiments revealed an interaction between DSS and WAS in modifying molecular brain signaling, but not pain-related freezing. WAS and DSS likewise failed to modify the visceromotor pain response to colorectal distention in mice (Larsson et al., 2009),

and in the present study was likewise found to be devoid of an effect on the freezing response to intrarectal AITC. This observation indicates that the effect of WAS to upregulate TRPA1 and TRPV1 expression in colonic afferent neurons (Yu et al., 2010) does not result in an exaggeration of the freezing reaction to AITC, an agonist at TRPA1 and TRPV1 (Everaerts et al. 2011; Holzer, 2011a). While AITC-evoked freezing remained unaltered in WAS-exposed mice, locomotion and c-Fos expression in the hypothalamus and amygdala were augmented in WAS-exposed mice whereas c-Fos expression in the thalamus was diminished. A similar region-specific effect of WAS on the cerebral processing of visceral pain emerges from functional brain mapping studies. Thus, colorectal distention activates the hypothalamus and amygdala more strongly than the prefrontal cortex of rats exposed to repeated WAS (Wang et al., 2013). Functional imaging studies in IBS patients likewise indicate that chronic stress enhances activation of limbic and paralimbic brain regions by colorectal distention, which points to exaggerated emotional regulation of pain (Tillisch et al., 2011). These studies (Tillisch et al., 2011; Wang et al., 2013) point to exaggerated emotional regulation of distention-evoked colorectal pain (Jain et al., 2015).

The effects of DSS on AITC-evoked pain behavior and spinocerebral MAPK phosphorylation and c-Fos expression differed in many respects from those of WAS. For instance, DSS mitigated the ability of intrarectal AITC to induce p42/44 MAPK phosphorylation but enhanced its ability to evoke c-Fos expression in the spinal cord. In the brain areas examined, DSS blunted the AITC-evoked phosphorylation of p38 and p42/44 MAPKs in the absence of any major effect on c-Fos expression. The DSS-initiated inhibition of cerebral MAPK activation was paralleled by an inhibition of the freezing reaction to AITC. Since the cerebral c-Fos expression in response to AITC was, by and large, not blunted, I hypothesize that DSS-induced colitis leads to inhibition of MAPK pathways in the CNS that are unrelated to c-Fos expression but linked to pain-related behaviors such as freezing. This contention is in keeping with the ability of MAPK inhibitors to prevent visceral pain behavior (Matsuoka and Yang, 2012; Galan-Arriero et al., 2014) and suggests that MAPK activation rather than c-Fos expression is relevant to nociception-related behavior. The current observations in mice are reminiscent of functional imaging studies in humans in which the visceral afferent input to

limbic/paralimbic circuits of the brain in IBS patients with colitis has been found to be smaller than in patients without inflammation (Mayer et al., 2005; Jain et al., 2015).

In this context it is worth mentioning that experimentally induced colitis fails to alter the visceromotor pain response to colorectal distention in mice (Larsson et al., 2009) but augments it in rats (Elsenbruch, 2011; Perse and Cerar, 2012; Edelmayer et al., 2014). The present findings in mice show that combined WAS+DSS treatment blunts the freezing reaction to intrarectal AITC (a DSS effect) and prolongs the locomotor reaction (a WAS effect). These behavioral alterations are associated with distinct changes in molecular CNS signaling, which further attest to a differential engagement of MAPK and c-Fos signaling pathways. While the inhibitory effect of DSS on MAPK phosphorylation in the thalamus, hypothalamus, amygdala and prefrontal cortex was neutralized by additional exposure to WAS, combined WAS+DSS treatment inhibited AITC-evoked c-Fos expression in all CNS regions examined. These observations add to the contention that experimental colitis and repeated WAS impact on different signaling pathways in brain areas relevant to the central processing of pain. It is hence important to study various signaling pathways if one wants to understand the interaction between colonic inflammation and chronic stress on brain activity. The present results also emphasize that stress can, to a certain extent, counteract the molecular manifestations of DSS-induced colitis in the brain, an observation that may explain why repeated WAS prevents the effect of DSS to enhance anxiety and reduce social interaction in mice (Hassan et al., 2014; Jain et al., 2015).

4.6. The effect of morphine indicates that AITC-evoked changes in MAPK and c-Fos activation are related to nociception

The ability of morphine to reduce the effect of intrarectal AITC to evoke freezing (Fig. 10A), MAPK phosphorylation and c-Fos expression in the CNS in all treatment (WAS, DSS, WAS+DSS) groups (Fig. 11A-C) (Fig. 12A-F) indicates that these responses involve opioid-sensitive neural pathways and reflect

manifestations of visceral nociception. The effect of AITC to inhibit MAPK phosphorylation in the hypothalamus and amygdala of control, WAS-exposed and DSS-treated mice was relatively resistant to inhibition by morphine. It is unlikely that this finding is related to the action of morphine to stimulate locomotion and induce the Straub phenomenon because morphine blunted the AITC-evoked c-Fos expression in all CNS areas of all treatment groups. These observations reaffirm the contention that MAPK and c-Fos signaling make differential contributions to visceral pain processing in the CNS. The site of action of morphine is thought to be within the CNS because the colitis parameters did not differ between the different treatment groups (Jain et al., 2015). A similar conclusion has been reached in a previous study (Mitrovic et al., 2010).

4.7. A TRPA1 antagonist and genetic deletion of TRPA1 fails to modify the severity of colitis

Neuronal and non-neuronal cells express TRPA1 in the GI tract. Non-neuronal cells (such as epithelial cells) are activated by colonic inflammation and in turn release inflammatory mediators. Consequently, primary sensory afferent nerve endings are excited by these inflammatory mediators and release inflammatory neuropeptides such as substance P and CGRP. These neuropeptides are responsible for several intracellular changes and thus determine whether progression of, or protection from, further cell injury as well as hypersensitivity to mechanical, thermal and chemical stimuli ensue. TRPA1 is able to modulate directly or indirectly the function of inflammatory mediators as well as inflammatory neuropeptides, an activity profile that has been used to propose TRPA1 as a gatekeeper of GI inflammation (Basbaum et al., 2009; Bautista et al., 2013; Brierley and Linden, 2014).

DSS treatment in rodents and IBD (UC and CD) in humans cause an increase in TRPA1 mRNA expression in the colon. DSS treatment in TRPA1 knockout mice has been found to be associated with increased DAS and an elevation of proinflammatory cytokine and inflammatory neuropeptide concentrations. TRPA1 activation has been found to be cause protection from

colitis by reducing several proinflammatory cytokines, neuropeptides and chemokines (Kun et al., 2014). In the interpretation of the current observations it is important to state that the TRPA1 antagonist HC-030031 as well as genetic deletion of TRPA1 failed to significantly alter the severity of DSS-induced colitis as judged by body weight, colon length, colon weight, colonic MPO activity and DAS (Fig. 13A-E) (Fig. 14A-E). These findings confirm a previous report that acute treatment with HC-030031 fails to modify experimentally induced colitis in mice (Mitrovic et al., 2010). To the contrary, other reports indicate that genetic deletion of TRPA1 can protect from colitis, while other evidence shows that TRPA1 is not involved in the pathogenesis of colitis (Cattaruzza et al., 2010; Engel et al., 2011). Collectively, these results permit me to contend that the pharmacological blockade of TRPA1 with HC-030031 and genetic deletion of TRPA1 has no effect on DSS-induces colitis.

4.8. The TRPA1 channel contributes to somatic pain hypersensitivity in experimental colitis

TRPA1 is well characterized for its role in the physiologic mechanosensation process (Bautista et al., 2006). Genetic deletion and pharmacological inhibition of TRPA1 by HC-030031 is associated with reduced responses to CRD in mice and reduced inflammatory and neuropathic pain in rats (Petrus et al., 2007; Eid et al., 2008; Brierley et al., 2009). Oral administration of HC-030031 (75 mg/kg) one hour prior to colonic AITC administration has been reported to reduce the mechanical sensitivity of the abdominal skin (Pereira et al., 2013). The presence of somatic hyperalgesia to mechanical stimuli in the facial skin in mice with DSS-induced colitis is consistent with the results of mechanical hypersensitivity in the abdominal and plantar skin (Jain et al., 2015) and shows that DSS treatment leads to widespread mechanical hypersensitivity in somatic tissues. Somatic hypersensitivity is not confined to the mechanical modality but also includes thermal hypersensitivity as shown in the plantar skin of DSS-treated mice. These widespread increases in pain sensitivity indicate that colitis not only evokes referred hyperalgesia in dermatomes of overlapping visceral and somatic

primary afferent neurons but also gives rise to widespread central sensitization to mechanical and thermal stimuli (Fig. 15A-D).

The present observations made with HC-030031 (Fig. 15A-D) and TRPA1 deficient mice (Fig. 16A-C) add a significant aspect to the mechanisms of colitis-induced central sensitization to somatic pain stimuli. While this TRPA1 antagonist was without effect on mechanical and thermal pain sensitivity in the abdominal, facial and plantar skin of untreated control mice, it reversed the colitis-induced mechanical hypersensitivity in the abdominal and facial skin. The thermal hypersensitivity in the plantar skin associated with colitis, however, was not ameliorated by HC-030031 (Fig. 15E) or genetic deletion of TRPA1 (Fig. 16D) . These observations attest to a modality-specific involvement of TRPA1 in central sensitization processes caused by visceral inflammation. This outcome is consistent with abundant evidence that TRPA1 is involved in the transduction of mechanical but not heat stimuli in mice (Laursen et al., 2014). However, the precise sites and mechanisms whereby TRPA1 channels mediate somatic mechanical hypersensitivity in DSS-induced colitis remain to be explored.

4.9. TRPA1 links colitis to increased sensitivity of primary sensory neurons

Diabetic neuropathy in rats is associated with an increase in TRPA1 mRNA expression in DRGs, which contributes to the cold hypersensitivity observed under these conditions (Ji et al., 2008). Treatment of diabetic rats with peripheral neuropathies with HC-030031 reduces the associated mechanical hypersensitivity (Wei et al., 2009). TRPA1 in nociceptors is known to be activated by oxidative stress byproducts, and this pathway is responsible for nociceptor sensitization in a neuropathic pain model (Nassini et al., 2012). However, the role of TRPA1 in visceral pain and its association with somatic hyperalgesia has not been systemically explored at the neuronal level. In rodents, colonic irritation by AITC or acetic acid (Winston et al., 2007; Christianson et al., 2010), colitis induced by TNBS (De Schepper et al., 2008; Yang et al., 2008; Malin et al., 2011) and treatment with DSS for 10 days (Chen et al., 2014) cause an increase in the

expression of TRPA1 and TRPV1 mRNA in DRGs (L6-S1), which has been linked to visceral hypersensitivity.

In my study, treatment with DSS for 7 days increased the expression of TRPA1 mRNA but not of TRPV1 and TRPV4 mRNA in lumbosacral DRGs (Fig. 17A-C). This result suggests that the increased transcription of TRPA1 in DRGs is related and/or contributes to the increased sensitivity to intrarectal administration of AITC and potentially to the mechanical hypersensitivity of tissues that are innervated by sensory neurons of the DRGs under investigation. This contention is supported by other findings using a different approach. In TNBS-induced colitis in mice, the increased visceromotor response to CRD has been found to be exclusively TRPA1-dependent and related to an increase of TRPA1 mRNA expression in primary sensory neurons (Cattaruzza et al., 2010). In keeping with this observation, the increased visceromotor response to CRD in rats with TNBS-induced colitis has been found to be reduced after i.p. or intrathecal injection of both TRPA1 or TRPV1 antagonists (Vermeulen et al., 2013).

Changes in electrophysiological properties such as a decrease in the resting membrane potential, a lowered rheobase, and sensitization of DRG neurons isolated from DSS-treated mice have been reported previously (Chen et al., 2015). Here I demonstrate that DSS treatment in mice increases the inward membrane current of DRG neurons in response to the TRPA1 agonist AITC but not to capsaicin, a TRPV1 agonist (Fig. 19A-C). This finding is consistent with the hypothesis that TRPA1 plays an important role in the colitis-evoked hypersensitivity (behavioural and molecular) to intrarectal administration of AITC. It should also be noted that TRPA1 activation is associated with a reduction in contractility of the isolated colon as well as a decrease in colonic motility in mice (Poole et al., 2011). I therefore cannot rule out a possible involvement of other TRPA1-expressing cells in the GI tract (epithelial cells, enteric neurons) in controlling visceral sensory processes.

CGRP and substance P are important mediators of primary sensory neurons. Within the gut, CGRP is released mainly from the extrinsic sensory nerve endings while substance P originates mainly from intrinsic enteric neurons (Suzuki et al., 1997, Green and Dockray, 1998., Reinshagen et al., 1998; Holzer and

Holzer-Petsche, 2001). In almost all types of experimental colitis CGRP has a protective role, while substance P is rather involved in the pathogenesis of inflammation (Reinshagen et al., 1998; Clifton et al., 2007; Engel et al., 2008; Thompson et al., 2008; Engel et al., 2011). In the present study, DSS-induced colitis did not change the release of CGRP from isolated DRG neurons evoked by either AITC or capsaicin (Fig. 18). This finding indicates that the colitis-induced upregulation of TRPA1 in DRG neurons does not translate into a change of CGRP release from these neurons.

4.10. Conclusions

The current study represents an attempt to study visceral pain in the brain as it pertains to behavioral perturbations and alterations in molecular signaling pathways. In addition, the current results provide new perspectives for the understanding of the interaction between colitis and psychological stress in the molecular and behavioral manifestations of visceral nociception. While repeated WAS did not modify the severity of DSS-induced colitis, it altered behavior (locomotion, exploration, motivation and self-care) and MAPK phosphorylation in the thalamus, hypothalamus and amygdala. Exposure to WAS and induction of colitis caused somatic and visceral hyperalgesia, and the underlying central sensitization was linked to c-Fos expression in distinct brain regions. Further analysis revealed that DSS and WAS stimulate different signal transduction pathways that converge on c-Fos but inhibit each other in inducing c-Fos when activated in parallel. Colitis and WAS also modified the behavioral and molecular responses to an acute visceral pain stimulus in a differential manner, which further affirms that the MAPK and c-Fos transduction mechanisms subserve different aspects of nociception in the brain. These results emphasize the need for studying multiple signaling pathways in parallel in order to advance the understanding of the central processing of pain. The ability of morphine to inhibit the molecular responses to intrarectal AITC indicates that the changes in MAPK and c-Fos activation are related to nociception. Collectively, the current results demonstrate that the study of spontaneous and intervention-evoked behavior as well as MAPK phosphorylation and c-Fos expression in the CNS are important approaches to

address the sensory-discriminative and emotional processing of a visceral pain stimulus. Further studies are required to identify the precise relationship between cerebral MAPK and c-Fos signaling and distinct behavioral manifestations of visceral pain. In addition, the current findings indicate that the TRPA1 channel participates in colitis-associated pain hypersensitivity at the somatic level.

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