Behavioral Characterization of Experimental Colitis in Mice: Role of Psychological Stress, Peptide YY, and Glucagon-like Peptide-1

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 organizations 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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Abbreviations

ACTH	Adrenocorticotropic hormone
AITC	Allyl isothiocyanate
AUs	Facial expression action units
BCG	Bacille Calmette-Guerin
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CD	Crohn's disease
CGRP	Calcitonin gene related peptide
CNS	Central nervous system
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CRF	Corticotropin-releasing factor
DPP-IV	Dipeptidyl peptidase-IV
DSS	Dextran sulfate sodium
EIA	Enzyme immunoassay
ENS	Enteric nervous system
FST	Forced swim test
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GIP	Glucose-dependent insulinotropic peptide
GIT	Gastrointestinal tract
GLP-1	Glucagon-like peptide-1
GR	Glucocorticoid receptors
HPA	Hypothalamic-pituitary-adrenal
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IL-6	Interleukin 6
IL-10	Interleukin 10

IL-12	Interleukin 12
IL-18	Interleukin 18
IP	Intraperitoneal
LPS	Lipopolysaccharide
MGS	Mouse grimace scale
ΔMGS	Difference in mouse grimace scale
MPO	Myeloperoxidase
MPT	Mechanical pain threshold
ΔMPT	Difference in mechanical pain threshold
MR	Mineralocorticoid receptors
NPY	Neuropeptide Y
NS	Not significant
NSAIDs	Nonsteroidal anti-inflammatory drugs
NTS	Nucleus tractus solitarii
OF	Open field test
PGE2	Prostaglandin E_2
Pgk	Phosphoglycerate kinase 1
PYY	Peptide YY
$PYY^{(-/-)}$	Peptide YY knockout mice
SC	Subcutaneous
SEM	Standard error of the mean
SI	Social interaction test
SUDO	Simplified up-down method for measuring mechanical pain threshold
TNBS	Trinitrobenzene sulfonic acid
TNF-α	Tumor necrosis factor alpha
TST	Tail suspension test
UC	Ulcerative colitis
WT	Wild-type mice

Abstract

Background: Inflammatory bowel disease (IBD) is associated with an increased risk of anxiety and depression disorders, and psychological stress can lead to exacerbation of IBD. This work aimed at exploring endocrine signaling pathways between the gut and brain in order to advance the understanding of gut-brain communication in IBD. The work involved three sets of experiments performed in male mice. In the first set of experiments, the behavioral consequences of experimental colitis, psychological stress, and their combination were investigated. In the second set of experiments, the status and the role of peptide YY (PYY) and glucagon like peptide 1 (GLP-1) in experimental colitis were assessed. Visceral pain is one of the chief complaints in IBD, and the third set of experiments explored the role of PYY in visceral pain.

Methods: Seven-day treatment with dextran sulfate sodium (DSS; 2% in drinking water) was used to induce colitis, and 1-hour sessions of water avoidance stress (WAS) for 7 days were used as a model of psychological stress. The behavioral battery included the open field (OF) test, the social interaction (SI) test, the tail suspension test (TST) and the forced swim test (FST). To assess the role of PYY and GLP-1 in colitis, colitis severity, food and water intake as well as OF and SI behavior were assessed in mice treated with the Y2 receptor antagonist BII0246 combined with the GLP-1 receptor antagonist exendin (9-39). The effect of PYY knockout ($PYY^{(-/-)}$), PYY (3-36) and BII0246 on visceral pain sensitivity were evaluated by measuring pain-related behaviors after rectal administration of 2% allyl isothiocyanate (AITC). In $PYY^{(-/-)}$ mice, visceral pain sensitivity was additionally evaluated by referred hyperalgesia and the mouse grimace scale.

Key findings: (1) DSS treatment decreased locomotion and enhanced anxiety-like behavior in the OF and reduced SI. **(2)** DSS treatment also increased circulating neuropeptide Y (NPY) and hypothalamic expression of cyclooxygenase-2 (COX 2) mRNA and decreased hippocampal expression of NPY mRNA, brain-derived neurotrophic factor (BDNF) mRNA and mineralocorticoid receptor mRNA. **(3)** Repeated WAS for 7 days had little influence on behavior but prevented the DSS-induced behavioral disturbances in the OF and SI tests. In contrast, repeated WAS did not modify colon length, colonic myeloperoxidase (MPO) content and circulating

proinflammatory cytokines, parameters used to assess colitis severity. **(4)** Repeated WAS significantly decreased the expression of corticotropin-releasing factor (CRF) mRNA in the hippocampus. The effect of repeated WAS to blunt the DSS-evoked behavioral disturbances was associated with a rise of circulating corticosterone and an increase in the expression of hypothalamic NPY mRNA. **(5)** DSS treatment increased colonic PYY and preproglucagon mRNA and circulating PYY and active GLP-1. **(6)** BII0246 + exendin (9-39) increased the colonic MPO content independently of DSS treatment. Additionally, the treatment of DSS-treated mice with BII0246 + exendin (9-39) reduced food intake and led to behavioral impairment in the OF. **(7)** $PYY^{(-/-)}$ mice showed enhanced pain-related behaviors and increased referred hyperalgesia in response to intrarectal treatment. **(8)** BII0246 increased pain-related behaviors in response to intrarectal treatment with AITC while PYY (3-36) was without effect.

Conclusions: Reduced hippocampal NPY and BDNF and increased hypothalamic COX-2 are likely to contribute to colitis-induced behavioral impairment. Repeated predictable stress causes resilience against behavioral impairment induced by colitis. The resilience induced by repeated predictable stress is related to increased hypothalamic NPY and increased circulating corticosterone. PYY and GLP-1 have a protective role against colitis and colitis-induced behavioral impairment. Endogenous PYY has a hypoalgesic effect which seems to be mediated by peripheral Y2 receptors.

Zusammenfassung

Einleitung: Chronisch-entzündliche Darmerkrankungen (CED) gehen oft mit Angsterkrankungen und Depression einher, und chronischer Stress kann zu einer Exazerbation von CED führen. Diese Dissertation hatte zum Ziel, endokrine Signalwege zwischen Gastrointestinaltrakt und Gehirn und ihre Rolle in der Darm-Hirn-Kommunikation bei Entzündung zu erforschen. Hierfür wurden drei unterschiedliche experimentelle Ansätze in männlichen Mäusen gewählt. Im Ansatz I wurden Verhaltensänderungen bei experimenteller Colitis, nach psychologischem Stress und einer Kombination beider Interventionen analysiert. Im Ansatz II wurde die Beteiligung der Darmhormone Peptid YY (PYY) und Glucagon-like peptide-1 (GLP-1) bei experimenteller Colitis untersucht. Ansatz III befasste sich mit der Bedeutung von PYY bei viszeralem Schmerz.

Methodik: Eine Colitis wurde durch Zugabe von Dextran Sulfate Sodium (DSS, 2%, 7 Tage) zum Trinkwasser induziert, psychologischer Stress durch Exposition auf Water-Avoidance-Stress (WAS, 1 Stunde täglich, 7 Tage) ausgelöst. Das Verhalten wurde mittels Open-Field-Test (OF), Sozialinteraktionstest (SI), Tail-Suspension-Test (TST) und Forced-Swim-Test (FST) analysiert. Entzündungsgrad, Futter- und Wasserkonsum sowie Verhalten im OF und SI wurden auch nach kombinierter Gabe des Y2-Rezeptorantagonisten BII0246 und GLP-1-Rezeptorantagonisten Exendin-(9-39) gemessen. Die Auswirkungen eines PYY-Knockout und einer Behandlung mit dem Y2-Rezeptoragonisten PYY-(3-36) und Y2-Rezeptorantagonisten BII0246 auf schmerzassoziierte Verhaltensweisen, lokomotorische Aktivität, übertragene Schmerzüberempfindlichkeit (referred hyperalgesia) und Mausgesichtsausdruck (mouse grimace scale) wurden nach intrarektaler Gabe von Allylisothiocyanat (1 oder 2%) untersucht.

Ergebnisse: **(1)** Die DSS-induzierte Colitis führte zu einer Abnahme der Lokomotion und des Sozialverhaltens und einer Zunahme der Ängstlichkeit. **(2)** Die Colitis ging mit einem Anstieg der Plasmaspiegel von Neuropeptid Y (NPY) und der Expression von Cyclooxygenase-2 (COX 2) mRNA im Hypothalamus sowie mit einer Abnahme der Expression von NPY, Brain-Derived Neurotrophic Factor (BDNF) und Mineralkortikoidrezeptor mRNA im Hippocampus einher. **(3)** Wiederholte Exposition auf WAS hatte kaum Einfluss auf das Verhalten, verhinderte

jedoch die durch DSS hervorgerufenen Verhaltensänderungen im OF und SI. WAS hatte keinen Einfluss auf den Colitis-Schweregrad, da Colon-Länge, Colon-Gehalt an Myeloperoxidase (MPO) und Zytokin-Plasmaspiegel unverändert blieben. **(4)** Nach WAS kam es zu einer Abnahme der Expression von Corticotropin-Releasing Factor (CRF) mRNA im Hippocampus. Der protektive Effekt von WAS gegenüber DSS-induzierten Verhaltensänderungen war mit einem Anstieg der Corticosteronspiegel und der Expression von NPY mRNA im Hypothalamus assoziiert. **(5)** Die DSS-induzierte Colitis steigerte die PYY- und Preproglucagon-Expression im Colon und den PYY- und GLP-1-Spiegel im Plasma. **(6)** BIl0246 plus Exendin-(9-39) erhöhte die MPO-Konzentration im Colon unabhängig von der DSS-Behandlung und bewirkte bei DSS-behandelten Tieren eine Abnahme des Futterkonsums sowie Verhaltensänderungen im OF. **(7)** Genetische PYY-Deletion verstärkte schmerzassoziierte Verhaltensreaktionen und die übertragene Schmerzempfindlichkeit auf intrarektale Verabreichung. **(8)** BIl0246 erhöhte ebenfalls die schmerzassoziierten Verhaltensreaktionen auf Allylisothiocyanat, während PYY-(3-36) wirkungslos war.

Schlussfolgerung: Die Abnahme der NPY- und BDNF-Expression im Hippocampus und der Anstieg der COX-2-Expression im Hypothalamus tragen wahrscheinlich zu den Verhaltensänderungen bei experimenteller Colitis bei. Die WAS-induzierte Resilienz gegenüber Colitis-induzierten Verhaltensänderungen hängt mit dem Anstieg der hypothalamischen NPY-Expression und Corticosteronspiegel zusammen. PYY und GLP-1 vermindern colitis-induzierte Verhaltenseinschränkungen. Außerdem wirkt PYY über periphere Y2-Rezeptoren hypoalgetisch.

1 INTRODUCTION

1.1 Inflammatory bowel disease (IBD)

Inflammatory bowel disease (IBD) includes mainly two disorders: ulcerative colitis (UC) and Crohn's disease (CD). Histologically, both diseases lead to architectural distortion of the intestine and to the accumulation of inflammatory cells in the intestinal mucosa (Hanauer 2006). There are several pathological and clinical distinctions that can usually but not always differentiate between CD and UC. CD is characterized by transmural inflammation and can affect any part of the gastrointestinal tract (GIT). CD commonly leads to complications such as abscesses, fistulas and strictures. In contrast, UC is characterized by mucosal inflammation and limited to the colon (Abraham and Cho 2009). IBD is more common in Western countries where the prevalence is more than 200 per 100,000 inhabitants, but the incidence and prevalence are increasing in different areas all over the world (Cosnes et al. 2011, Molodecky et al. 2012).

IBD patients suffer from several symptoms which affect their quality of life. In UC, patients usually suffer from the presence of blood and mucus mixed with stool, accompanied by lower abdominal cramping. In CD, the symptoms vary depending on the site and severity of lesion; for example, those patients with ileocolonic involvement suffer from abdominal pain which is usually postprandial and may be referred to the periumbilical area. In contrast, gastroduodenal CD leads to early satiety, nausea, emesis, epigastric pain, and/or dysphagia. Due to postprandial pain and delay in gastric emptying, patients with gastroduodenal CD often limit their caloric intake to diminish their discomfort. Extensive small bowel disease causes diffuse abdominal pain, anorexia, diarrhea, and weight loss and may result in lactose malabsorption. Extraintestinal manifestations are also common in IBD and they include fever, weight loss, arthritis and arthralgia, ocular complications, hepatic complications, and renal complications (Hendrickson et al. 2002).

The aetiology of IBD remains unclear, but there is accumulating evidence that IBD results from an inappropriate inflammatory response to intestinal microbes in genetically susceptible subjects (Abraham and Cho 2009). It is likely that the pathogenesis of IBD involves a complex interaction between genetic,

environmental, and microbial factors and the immune response (Zhang and Li 2014).

Several categories of medications are used in the treatment of IBD. Aminosalicylates (mesalazine) are indicated for controlling mild-to-moderate episodes of UC, preventing relapses and maintaining remission. Corticosteroids are recommended only for short-term use in order to achieve remission, while thiopurines (azathioprine) and mercaptopurine are used for maintaining clinical remission in steroid-dependent IBD. Methotrexate can be employed in patients with CD resistant or intolerant to thiopurines or mercaptopurines. Calcineurin inhibitors (cyclosporine and tacrolimus) can be effective in the management of steroid-refractory UC. Monoclonal antibodies targeting tumor necrosis factor alpha (TNF-α) (infliximab and adalimumab) are effective therapies in IBD and can be used in severe cases (Quetglas et al. 2015, Mowat et al. 2011). A new treatment modality for both UC and CD is represented by vedolizumab which is a monoclonal antibody that interferes with lymphocyte migration to the intestinal mucosa by specifically binding to integrin α4β7 (Lau & Tsai 2016)

Several mental disorders including major depression, panic and generalized anxiety disorders are more common in IBD patients than in community controls (Walker et al. 2008, Graff et al. 2009). The comorbidity with psychiatric disorders in addition to the potential role of psychological stress in the development or exacerbation of IBD directed the attention to gut-brain communication as a relevant player in IBD (Mackner et al. 2011, Bonaz and Bernstein 2013)

1.2 Gut-brain communication in IBD

For two decades, irritable bowel syndrome (IBS) has been considered as a model of disordered gut-brain communication. In the light of recent epidemiological and experimental data evidence for a disordered gut-brain communication is also emerging in several mental and gastrointestinal disorders including IBD. The footprint of the bidirectional communication between gut and brain can increasingly be seen in the relationship between IBD and mental disturbances in which both directions of gut-brain and brain-gut signaling are affected, and ‘top-down’ effects of

the brain on the gut and ‘bottom-up’ effects of the gut on the brain need be considered (Mayer 2011, Bonaz and Bernstein 2013).

One of the early observations that attracted the attention towards gut-brain axis communication in IBD was provided by Salem and Shubair in 1976 who reported spontaneous UC in Arab Bedouins who were forced to leave their familiar environment in the desert and live in houses provided by the government. Salem and Shubair suggested that psychological and environmental disturbances predispose these patients to IBD (Salem and Shubair 1967, Reber 2012). This early finding was supported by more recent studies which investigated the role of gut-brain signaling in modulating IBD. For example, a Canadian prospective study which recruited 704 IBD patients evaluated disease flares every 3 months in relation to potential triggers which included nonsteroidal anti-inflammatory drugs (NSAIDs), antibiotics, infections, and stress (Bernstein et al. 2010). Among the afore-mentioned potential triggers, only stress perceived as being of high level was associated with an increased risk of flare. A Swiss study which recruited 486 adults with CD revealed a significant association between perceived stress and disease exacerbation (Camara et al. 2011). The study which aimed at identifying the exact stress component which is responsible for the exacerbation found that the association between perceived stress and exacerbation of CD was fully attributable to mood components, specifically anxiety and depression. The investigators of the study suggested management of anxiety and depression as a strategy for the potential prevention of CD exacerbations (Camara et al. 2011). Moreover, psychiatric disturbances negatively affect prognosis and response to treatment. In a study with 100 patients suffering from CD, infliximab was less efficacious in patients suffering from major depression (Persoons et al. 2005).

In addition to studies which confirmed the effect of stress on IBD, several well designed studies in the past decade revealed a higher prevalence of some psychiatric disorders among IBD patients (Graff et al. 2009). In a Canadian study which recruited 351 IBD patients and 779 matched controls, IBD patients had higher rates of panic, generalized anxiety, and obsessive-compulsive disorders and major depression within a follow-up period of 12 months (Walker et al. 2008). The results are in line with a retrospective American study which evaluated 2,144 young CD

patients and their 10,720 matched controls and found a greater risk of developing anxiety disorders and depression in CD patients (Loftus et al. 2011).

The manifold co-morbidities of IBD and mental disorders are not surprising if we consider the several signaling pathways which connect the gut to the brain. Therefore, gastrointestinal disorders are commonly associated with neuropsychiatric disorders and vice versa (Mayer 2011).

1.3 Gut-brain signaling pathways

The gut and the brain are connected via several neuronal, endocrine and immune signaling pathways in an afferent and efferent direction. The neuronal connections include vagal and spinal afferent neurons as well as sympathetic and parasympathetic efferent signaling pathways while the endocrine pathways include gut hormones in addition to the hypothalamic-pituitary-adrenal (HPA) axis. Since IBD is an inflammatory disease, inflammatory mediators also can participate in signaling to the brain in IBD patients (Bonaz and Bernstein 2013). The gut-brain axis communication pathways will be briefly discussed in the following pages

1.3.1 Nervous connections between the gut and the brain

The extrinsic innervation of the gut is provided by the vagus nerve and the spinal nerves. Compared to the innervation of somatic tissues, the situation in the gut is much more complicated due to the presence of four extrinsic neuronal systems (vagal afferent and efferent, spinal afferent and efferent) in addition to the intrinsic enteric nervous system (ENS; afferent and efferent). The spinal afferent neurons comprise several classes of mechano- and chemosensitive nerve fibers (Knowles and Aziz 2009).

In addition to spinal nerves, the vagus nerve which is one of the most widespread sensory pathways in the body contributes to visceral pain sensation. The vagus nerve innervates the entire gut, except the transverse and distal portion of the colon, with up to 80% of the vagal fibers being afferents. Evidence from animal studies supports the involvement of the vagus in mechano- and chemosensitive nociception. In addition, vagal afferents respond to many gut hormones and

transmit of information on the luminal contents in the GIT. The vagal afferents reach the central nervous system (CNS) through projection to the nucleus tractus solitarii (NTS), located in the medulla oblongata, while spinal gut afferents reach the CNS through projection to the dorsal horn in thoracolumbar and sacral spinal cord, respectively. After reaching the CNS, visceral information is carried on to several brain regions involved in the autonomic, endocrine, motor, and behavioral responses to visceral sensations (Holzer 2009, Knowles and Aziz 2009, Bonaz and Bernstein 2013, Vermeulen et al. 2014).

IBD is frequently associated with visceral hyperalgesia; this hyperalgesia can be explained in part by sensitization of primary sensory afferents in response to inflammatory mediators. However, peripheral sensitization alone is not sufficient to explain all pain symptoms in IBD patients, since some patients still suffer from pain in remission, and pain is poorly correlated with inflammatory markers. It was therefore suggested that secondary changes in higher-order neuronal circuits in the spinal cord or brain contribute in the pain symptoms in IBD patients (Bielefeldt et al. 2009).

1.3.2 Gut hormones

The gut is a major endocrine organ which secretes more than 20 hormones. Gut hormones are formed in specialized endocrine cells of the gastrointestinal mucosa and are involved in the coordination of digestion, the signaling of hunger and satiety, and the regulation of energy homeostasis (Murphy and Bloom 2006, Holzer et al. 2012). Evidence from knockout mice hints at the involvement of gut hormones in emotional-affective behavior as well (Holzer et al. 2012). In this thesis, the role of peptide YY (PYY) and glucagon like peptide-1 (GLP-1) in experimental colitis was investigated, so these two hormones are discussed in some detail.

Peptide YY (PYY)

PYY was first isolated in 1980 from pig intestinal mucosa. Due to the presence of a tyrosine residue at each terminus of the 36 amino acid polypeptide chain it was named peptide YY since Y is the abbreviation used for the amino acid tyrosine. The peptide is secreted by L-cells in the GIT which release it in the form of PYY (1-36).

The amino acids tyrosine and proline are cleaved from the N-terminus of the polypeptide by the di-peptidyl peptidase IV (DDP-IV) enzyme to produce PYY (3-36) which constitutes approximately 50% of the PYY immunoreactivity in human blood. Circulating PYY is increased in response to food intake, reaching a peak level at 1-2 hours after food intake, and remains elevated several hours thereafter. The dynamics of PYY blood levels depend on the composition of the meal (Neary and Batterham 2009). In addition to regulation by food composition, PYY release is regulated by the parasympathetic nervous system, inflammatory mediators, and other gut hormones including cholecystokinin, vasoactive intestinal polypeptide, gastrin, and GLP-1 (Ballantyne 2006, Cox 2007, Coskun et al. 2013).

In addition to its expression in the intestine, the presence of PYY in the CNS of humans and rodents has also been reported, but the evidence for the expression of PYY in the brain remains relatively sparse (Morimoto et al. 2008, Holzer et al. 2012).

The cleavage of PYY (1-36) into PYY (3-36) changes the pharmacological properties of the peptide. While PYY (1-36) binds to all Y receptor subtypes, PYY (3-36) has higher affinity to the Y2 subtype (Neary and Batterham 2009). The two peptides have paracrine actions in the GIT where they act as brakes to inhibit gastric emptying, intestinal motility, and mouth to anus transit time, which provides more time to digest high fat content meals. PYY also inhibits electrolyte secretion in the intestine as well as exocrine and endocrine (insulin) pancreatic secretion (Ballantyne 2006, Cox 2007).

PYY is one of the appetite-regulating gut hormones. Peripheral administration of PYY (3-36) inhibits food intake in rodents and human subjects, and this effect remains intact in obese subjects. The satiety or anorexic effect of PYY (3-36) is mediated by Y2 receptors as it can be blocked in rodents by genetic deletion of Y2 receptors or by Y2 receptor-specific antagonists (Neary and Batterham 2009). However, the reports on the anorexic effect of PYY (3-36) are inconsistent, as several groups failed to reproduce them (Tschop et al. 2004). This inconsistency in the literature may in part be due to the variable stress levels that the experimental animals had been exposed to, as the anorectic effect of PYY (3-36) is deactivated

under stress conditions (Batterham et al. 2004). In contrast to PYY (3-36), central administration of PYY (1-36) in rats increases food intake and this effect appears to be mediated by Y1 receptors (Ballantyne 2006).

It is not clear whether the physiological effect of PYY (3-36) on appetite is mediated via central or peripheral Y2 receptor activation. On the one hand, the peptide crosses the blood-brain barrier by a non-saturable mechanism (Nonaka et al. 2003), and injection of the selective Y2 receptor antagonist BIIE0246 into the arcuate nucleus blocks the effect of endogenous as well as exogenously (peripherally) injected PYY (3-36) on food intake (Abbott et al. 2005). On the other hand, peripherally injected BIIE0246 blocks the anorexic effect of peripherally injected PYY (3-36) (Scott et al. 2005, Talsania et al. 2005). BIIE0246 has poor brain penetration due to its high molecular weight (molecular weight of 896) and large polar surface area (219 Å2) (Brothers et al. 2010). Peripheral PYY (3-36) may affect the brain via Y2 receptor–mediated activation of the afferent vagus nerve. In rats, vagotomy blocks PYY (3-36)-induced anorexia and hypothalamic c-Fos expression (Koda et al. 2005).

In addition to their role in the regulation of feeding behavior, Y receptors have several roles in the regulation of emotional-affective behavior and pain (Naveilhan et al. 2001, Brumovsky et al. 2007, Holzer et al. 2012). These diverging roles of Y receptors can explain the pronounced behavioral changes observed in PYY knockout ($PYY^{(-/-)}$) mice which show depression-like behavior (Painsipp et al. 2011a) and exhibit ehnhanced sickness behavior when they receive Bacille Calmette-Guerin (BCG) as an immune challenge (Painsipp et al. 2013).

While the role of neuropeptide Y (NPY) and Y receptors in nociception has been thoroughly investigated (Brumovsky et al. 2007, Holzer et al. 2012), the role of PYY in somatic and gastrointestinal pain regulation remains unclear.

Glucagon-like peptide-1 (GLP-1)

GLP-1 is produced by posttranslational processing of pro-glucagon in the GIT and brain. In the GIT, GLP-1 is secreted from L cells in which it is nearly always co-

localized with either glucose-dependent insulinotropic peptide (GIP) or PYY. Upon secretion, the majority of GLP-1 is rapidly cleaved by DPP-IV to inactive metabolites leaving only less than 25% of active GLP-1 reaching the liver and less than 10% of GLP-1 reaching the systemic circulation (Holst 2007, Punjabi et al. 2011).

GLP-1 has an important incretin effect, which means that it increases insulin release. Together with PYY, GLP-1 reduces GIT motility and secretion and acts as satiety signal (Holst 2007). Both peripheral and central GLP-1 reduces appetite (Williams et al. 2009). Vagotomy and peripherally administered GLP-1 receptor blockers inhibit the effect of intraperitoneal (IP) GLP-1, which suggests a contribution of the vagus nerve to the transmission of peripheral GLP-1 signaling to the brain (Williams et al. 2009, Punjabi et al. 2011). On the other hand, vagotomy does not block the effect of GLP-1 administered directly to the inferior vena cava, so central signaling of peripheral GLP-1 cannot be excluded (Punjabi et al. 2011).

Apart from its effect on appetite, relatively little is known about any behavioral effects of GLP-1. In one study which evaluated the effect of peripherally administered GLP-1 analogues on locomotion and anxiety-like behavior in mice, locomotion was reduced whereas anxiety-like behavior was not affected (Krass et al. 2012).

The effects of GLP-1 on pain were investigated in both humans and rodents (Hellstrom et al. 2009; Gong et al. 2014, Fan et al. 2015). ROSE-010 is a GLP-1 analogue which showed promising results in relieving visceral pain in patients suffering from IBS (Hellstrom et al. 2009). The primary rationale for targeting GLP-1 receptors in the treatment of IBS was derived from its known inhibitory effect on intestinal motility and the hypothesis that blocking disordered GIT motility is beneficial for IBS patients (Hellstrom et al. 2009). However, it seems that GLP-1 also blocks pain by a central mechanism. In a recent study, intrathecal GLP-1 agonists potently alleviated peripheral nerve injury-, bone cancer-, and diabetes-induced pain in rats and formalin-induced pain in rats and mice without affecting acute nociceptive responses (Gong et al. 2014). The same study revealed that the expression of GLP-1 receptors in the dorsal spinal horn is specifically confined to microglia. Furthermore, GLP-1 receptor agonists evoked β-endorphin release from both the spinal cord and cultured microglia, and the antiallodynic effect of GLP-1

agonists could be blocked completely by microglial inhibitors and opioid receptor antagonists (Gong et al. 2014, Fan et al. 2015). The existence of related mechanisms in IBS patients and animal models of visceral hypersensitivity awaits to be investigated.

1.3.3 Inflammatory mediators

IBD is characterized by increased colonic production of proinflammatory cytokines, eicosanoids and other immune mediators (Zhang and Li 2014). Several proinflammatory cytokines and eicosanoids possess well known psychogenic activities. Behavioral effects can be evoked in experimental settings either directly through injection of the cytokines themselves or indirectly by injection of lipopolysaccharide (LPS) or BCG which are known to increase circulating cytokine levels. Both interventions consistently cause behavioral impairment in rodents and human subjects. The behavioral consequences of cytokine administration include sickness behavior, anxiety-like behavior, depression-like behavior, social isolation and anorexia (Dantzer et al. 2008). Major depression is one of the psychiatric disorders which has been linked to inflammation, and several investigators consider the available connection between inflammation and major depression as sufficient evidence to consider inflammation as a causal factor involved in major depression pathogenesis (Miller et al. 2009).

1.3.4 Hypothalamic-pituitary-adrenal (HPA) axis

The HPA axis is one of the pathways through which psychological stress can affect the severity of intestinal inflammation. Psychological stress leads to activation of the HPA axis and thereby to increased glucocorticoids levels. Circulating glucocorticoids can either suppress colonic inflammation via their anti-inflammatory properties or promote colitis via their immunosuppressive action, which may allow the colonic microbiota to proliferate and translocate the mucosal barrier (Reber et al. 2011, Reber 2012).

On the other hand, the HPA axis might contribute to IBD effects on the brain through different mechanisms. First, chronic colitis can suppress corticotropin-releasing factor (CRF) gene activation in the hypothalamus in response to stress, with a

consequent reduction of plasma corticosterone. Glucocorticoids are necessary for coping with stress, and a blunted HPA axis response has been linked to several psychiatric disorders (Bonaz and Bernstein 2013). Moreover, IBD is occasionally accompanied by glucocorticoid resistance which can be due to different mechanisms (Farrell and Kelleher 2003). Glucocorticoid resistance has also been observed in patients with major depression, and a lack of brain sensitivity to glucocorticoids is likely to be involved in major depression pathophysiology (Pace et al. 2007).

1.3.5 Neuropeptides

In addition to their well-established role in regulating CNS functions, neuropeptides control also ENS functions and immune responses in the gut (Genton and Kudsk 2003, Souza-Moreira et al. 2011). These properties elect neuropeptides to be among the key connections between the gut and the brain. In this work, I investigated the gene expression of 2 neuropeptides in the brain in response to experimental colitis: NPY and CRF.

Neuropeptide (NPY)

NPY is a 36-amino acid peptide which was isolated for the first time from brain extracts. We now know that NPY is one of the most abundant neuropeptides within the brain. In the CNS, NPY regulates many physiological functions such as food intake, energy homeostasis, circadian rhythm, and cognition, in addition to being a key component in regulating the stress response. NPY and its two close relatives, PYY and pancreatic polypeptide (PP) act on Y receptors which comprise 5 subtypes in mammals (Y1, Y2, Y4, Y5, and Y6). NPY shows a strong affinity to Y1, Y2, and Y5 receptors (Reichmann and Holzer 2016). NPY and Y receptors regulate pain sensitivity but most studies evaluated the relevance of the NPY system in somatic pain, while relatively few studies addressed its role in visceral pain (Holzer et al. 2012).

In the gut, the major source of NPY is the ENS. NPY is also found in sympathetic neurons supplying the vascular system but is largely absent from sympathetic neurons innervating the gastrointestinal mucosa. Primary afferent neurons originating in the dorsal root ganglia compose an additional minor source of NPY in

the gut. NPY is generally inhibitory on gut motility and secretory function, while evidence from animal studies suggests a proinflammatory role of NPY in animal models of IBD (Holzer et al. 2012).

Experimental colitis in rodents leads to an increase in the number of enteric neurons containing NPY and to hyperplasia of NPY nerve fibers. A similar observation was made in the ileum of CD patients (El-Salhy and Hausken 2016). NPY is likely to be causally involved in the pathogenesis of colitis since NPY gene knockout attenuates experimental colitis (Chandrasekharan et al. 2008).

Corticotropin-releasing factor (CRF)

CRF is a 41-amino acid polypeptide that stimulates the synthesis and release of adrenocorticotropic hormone (ACTH) and β-endorphin from the anterior pituitary. It was isolated by Vale and colleagues from the hypothalamus in 1981. More recently, three other mammalian CRF-related peptides have been characterized: urocortin 1 (Ucn 1), urocortin 2 (Ucn 2), and urocortin 3 (Ucn 3). There are 2 main types of CRF receptors in humans and mammals: CRF1 and CRF2. The CRF signaling system is widely expressed in the brain, and animal experiments confirm a role of central CRF signaling in controlling the response to psychological stress and in the regulation of gastrointestinal motility and pain sensitivity (Stengel and Taché 2010).

Similarly to several neuropeptides, the CRF system also exists in the gut. In line with its role in the brain, human and animal studies showed that the peripheral CRF system mediates the gut motor response to stress. In spite of the converging evidence from preclinical studies, CRF1 receptor antagonists failed to improve GIT symptoms of IBS in clinical trials (Stengel and Taché 2010).

Peripheral and central CRF can affect gastrointestinal inflammation by several mechanisms. Peripherally, CRF is considered generally proinflammatory. During inflammation, CRF and its related peptides can stimulate mast cells and other immune cells to secrete inflammatory cytokines and to increase IBD severity. Central CRF can also modulate IBD severity via activation of the HPA axis and increasing circulating corticosterone, or via reducing vagal tone, and consequently

reducing the cholinergic anti-inflammatory action of the efferent vagus (Kiank et al. 2010).

1.3.6 Brain derived neurotrophic factor (BDNF)

BDNF is a member of the family of neurotrophic factors which include nerve growth factor (NGF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). In 1982 BDNF was isolated from the pig brain and found to support the survival of neurons. BDNF is necessary for survival and growth of a variety of neurons, including dorsal root ganglia, hippocampus and frontal cortex neurons. BDNF and its receptors are widely distributed in the CNS and involved in several physiological functions including learning, memory and neurogenesis. BDNF dysregulation has been linked to several diseases including epilepsy, Alzheimer's disease, bipolar disorder, anxiety and depression (Binder and Scharfman 2004, Duman and Monteggia 2006).

BDNF is upregulated in the dorsal root ganglia of rodents with experimental colitis. This upregulation is likely to contribute to visceral pain hypersensitivity observed in models of experimental colitis as BDNF heterozygous mice exhibit less colitis-induced pain hypersensitivity than wild-type (WT) mice (Yang et al. 2010).

Brain BDNF gene expression is also affected by gut conditions. For example, infection of mice with the intestinal parasite Trichuris muris leads to a reduction of hippocampal BDNF gene expression, an effect that can be abolished by the probiotic Bifidobacterium longum NCC3001 (Bercik et al. 2010).

1.3.7 Intestinal microbiota

Preclinical studies involving germ-free mice, antibiotic-treated mice and probiotic-treated mice reveal that the intestinal microbiota influences rodent behavior and brain neurochemical signaling. In humans relatively little evidence for these implications is available due to limitations in the experimental approaches. The relatively few studies which assessed behavioral responses to probiotics in humans yielded some contradictory results, and larger well-designed clinical trials are required to obtain solid evidence for an effect of intestinal microbiota manipulation on human brain function and behavior (Mayer et al. 2014).

IBD in humans and experimental colitis in rodents are associated with intestinal dysbiosis (Sartor and Mazmanian 2012, Gkouskou et al. 2014). Changes in the intestinal microbiota (dysbiosis) might have a role in gut signaling to the brain, given that probiotics can improve anxiety-like behavior in the DSS-induced colitis model of IBD (Bercik et al. 2011).

1.4 Animal models of IBD: do they involve gut-brain axis communication?

As indicated in the previous sections, there is a complex system for gut-brain communication in IBD. To understand this complex pathways, animal models of IBD which provide evidence of gut-brain axis communication would be of a great value.

Several animal models of IBD which include genetic models of experimental colitis, infection-induced colitis, and chemically induced colitis possess adequate translational validity (Hoffmann et al. 2002, Jurjus et al. 2004, Dothel et al. 2013). With regard to gut-brain communication, chemically induced colitis has been most extensively investigated, particularly trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats and DSS-induced colitis in mice (Reber 2012), a model which was adopted in this dissertation.

Dextran is a complex polymer of glucose which includes straight and branched chains of variable molecular weights starting from 5,000 to up to 1.4 million. DSS is a polyanionic derivative of dextran, produced by esterification with chlorosulfonic acid. In 1985, DSS was first reported to induce acute colitis in hamsters, and since then the model was adapted to be used in mice, rats, and guinea-pigs. DSS is usually added to the drinking water, after which gastrointestinal inflammation does develop within days. The duration, severity and location of inflammation induced by DSS can vary depending on several factors including species, concentration of DSS, and molecular weight of DSS. Though its exact mechanism is still not clear, the gastrointestinal inflammation which develops in response to DSS has many clinical and pathological similarities to human IBD and responds to several standard IBD therapeutics such as steroids, cyclosporine, and anti-TNF antibody (Solomon et al. 2010, Perse and Cerar 2012)

There is ample evidence that gut-brain communication takes place in the DSS-induced model of experimental colitis. For example, the effect of psychological stress has repeatedly been tested in the DSS model of IBD and in most studies found to aggravate the severity of inflammation (Milde and Murison 2002, Reber et al. 2006, Reber et al. 2008). Moreover, male mice with DSS-induced colitis exhibit anxiety-like behavior, while female mice with DSS-induced colitis exhibit depression-like behavior (Bercik et al. 2011, Painsipp et al. 2011a). Except for the involvement of the HPA axis which was previously investigated in the DSS- and TNBS-induced colitis model (Reber 2012), relatively little is known about the molecular and hormonal signaling mechanisms that can explain the influence of experimental colitis on emotional-affective behavior.

1.5 The water avoidance stress (WAS) paradigm as a tool to study gut-brain axis communication in rodents

Psychological stress is a risk factor for the development of several psychiatric and gastrointestinal disorders including anxiety disorders, major depression, and IBD exacerbations (Mayer et al. 2001, Turner and Lloyd 2004, Bonaz and Bernstein 2013,). For this reason, animal models of psychological stress have frequently been used to analyze the influence of psychological stress on gut-brain axis signaling and on GIT physiology (Stengel and Taché 2009).

Water avoidance stress (WAS) is a model of psychological stress in rodents, in which animals are placed on a platform surrounded by water which prevents them from escaping. WAS efficiently simulates unescapable stressful situations in humans. WAS induces anxiety-like behavior and visceral hyperalgesia in rats. The rat model is particularly popular among research groups in the field of neurogastroenterology, and the effects of single and repeated sessions of WAS on visceral pain sensitivity and experimental colitis have been characterized to a considerable extent (Bonaz and Taché 1994, Bradesi et al. 2005, Deiteren et al. 2014).

Apart from its effect on murine visceral pain sensitivity which has been studied previously (Larauche et al. 2010), relatively little is known about the behavioral

effects of WAS in mice. However, measurements of stress hormones and central c-Fos activation in response to WAS indicate activation of stress-sensitive brain circuits in response to single and repeated sessions of WAS (Reichmann et al. 2013, Ait-Belgnaoui et al. 2014, Reichmann et al. 2015).

1.6 Aim of the work

As outlined before, gut-brain interactions operate in IBD patients in whom an increased incidence of several psychiatric disorders is observed and in whom IBD relapses might be enhanced by psychological stress (Bonaz and Bernstein 2013). Gut-brain communication is mediated by complex pathways which include neuronal, endocrine, immune, and microbial factors (Mayer 2011, Holzer et al. 2012). The DSS-induced colitis model of IBD exhibits some behavioral changes parallel to some of the mental disorders seen in IBD (Painsipp et al. 2011a). Therefore, the DSS-induced colitis model of IBD appears to be a valid model to study gut-brain signaling mechanisms in IBD. This work addressed four objectives concerning the DSS-induced colitis animal model of IBD and its impact on the gut-brain axis.

The first objective was to characterize the effect of DSS-induced colitis on mouse behavior, brain neuropeptides, and HPA axis activity. Unlike the effects of psychological stress on colitis severity which has been frequently investigated [(1)], relatively little is known about the effect of this interaction on the brain. **The second objective** of this work was therefore to investigate the in which way DSS-induced colitis and repeated sessions of WAS affect colitis-induced behavioral and neuropeptide changes.

As the gut hormones PYY and GLP-1 are strong candidates to participate in gut-brain communication in IBD, **the third objective** of this work was to evaluate the roles of PYY and GLP-1 in DSS-induced colitis. Since visceral pain is a cardinal symptom in IBD (Bielefeldt et al. 2009) and no information is available in the literature regarding the effect of PYY on abdominal nociception, the **fourth objective** of this work was to assess the role of PYY in visceral pain.

In addressing these objectives, I asked the following specific research questions:

[(1)] For a list of these studies see (Reber 2012)

1. Does experimental colitis induced by DSS lead to anxiety-like behavior, social isolation, depression-like behavior, and anorexia in male mice?
2. Does combining psychological stress with colitis influence experimental colitis severity and colitis-induced behavioral changes?
3. In which way do experimental colitis, psychological stress, and their combination affect HPA axis activity?
4. Will experimental colitis, psychological stress, and their combination affect gene expression of the neuropeptides CRF, NPY, and BDNF?
5. Does experimental colitis affect secretion of the gut hormones PYY and GLP-1?
6. Will blocking the effect of GLP-1 and PYY (3-36) affect experimental colitis severity and colitis-induced anorexia, anxiety and social isolation?
7. Are $PYY^{(-/-)}$mice more sensitive to colonic pain?
8. Will exogenous PYY (3-36) or the Y2 receptor antagonist, BII0246, affect colonic pain sensitivity?

To answer these questions, I performed 8 experiments: The first three experiments used DSS-induced colitis as an experimental model of IBD and WAS as a model of psychological stress. This set of experiments was designed to answer the first 4 research questions.

The fourth and fifth experiment also took use of the DSS-induced colitis model and were designed to answer the 5^{th} and the 6^{th} research question.

The sixth, the seventh, and the eighth experiment were designed to answer the 7^{th} and the 8^{th} research question. For this purpose, rectal administration of allyl isothiocyanate (AITC) was employed as a model of chemically induced colonic pain (Laird et al. 2001).

2 MATERIAL AND METHODS

2.1 Experimental animals

All experiments except the sixth and eighth experiment were carried out with male C57BL/6N mice obtained from Charles River (Sulzfeld, Germany). C57BL/6N mice were received at 8 weeks of age and housed 2 per cage in the first three experiments, 5 per cage in the fourth experiment, 2 per cage in the fifth experiment, and 2-3 per cage in the seventh experiment. These differences in housing conditions were due to space limitations in the animal facility. It should be noted, however, that animals were always housed 2 per cage in those experiments in which the emotional-affective or social behavior was assessed. Animals were housed at a temperature of 21°C under a 12 h light/dark cycle (lights on at 6:00 h, lights off at 18:00 h). Standard laboratory chow was provided ad libitum throughout the studies. In all experiments, the mice were habituated in the animal facility for at least two weeks before any intervention. All experiments were approved by an ethical committee at the Federal Ministry of Science, Research, and Economy 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 Directive of the European Communities Council of 24 November 1986 (86/609/EEC) and the Directive of the European Parliament and of the Council of 22 September 2010 (2010/63/EU).

The sixth and eighth experiment were carried out with male $PYY^{(-/-)}$ mice. The genetic background of the knockout as well as WT mice was a 1:1 mixture C57Bl/6 and129/SvJ. These mice were bred in the animal facility of the Institute of Experimental Clinical Pharmacology, Medical University of Graz (Graz, Austria) but were originally obtained from the Neurobiology Research Program of the Garvan Institute of Medical Research (Sydney, Australia) via the Institute of Pharmacology of the Medical University of Innsbruck (Innsbruck, Austria). $PYY^{(-/-)}$ mice and their WT were housed 2-5 per cage. The $PYY^{(-/-)}$ mice and the age-matched WT were 2-4 months old when used in the sixth experiment and 3-9 months old in the eighth experiment.

2.2 Experimental protocols

The overall aim of this work was (1) to assess behavioral changes caused by experimental colitis induced by DSS, (2) to evaluate the effect of psychological stress on these behavioral changes, (3) to evaluate the role of the gut hormones PYY and GLP-1 in these behavioral changes, and (4) to assess the role of PYY in visceral pain. To achieve the study objectives, 8 experiments were designed.

2.2.1 First experiment

Objective: To assess behavioral changes caused by DSS-induced experimental colitis and the effect of WAS on these behavioral changes.

Experimental design: As described in the original publication (Hassan et al. 2014), 68 mice were allocated to 4 experimental groups:

(1) Control group (n = 16), handled once daily from day 1 to day 7

(2) Water avoidance stress (WAS) group (n = 16), exposed to intermittent WAS once daily for 1 h from day 1 to day 7

(3) DSS colitis group (n = 18), receiving DSS (2 %) in the drinking water and handled once daily from day 1 to day 7

(4) WAS+DSS group (n = 18), subjected to both WAS and DSS treatment from day 1 to day 7

Body weight was measured on day 1 and day 8. Anxiety-like behavior and locomotor activity were assessed with the OF test on day 8. On day 9, social activity was evaluated with the SI test. On day 10, depression-like behavior was evaluated with the TST. On day 11, the mice were sacrificed by decapitation after they had been deeply anaesthetized with pentobarbital (150 mg/kg IP) to collect the colon for myeloperoxidase (MPO) determination. The behavioral tests were carried out between 08:00 and 13:00 h.

2.2.2 Second experiment

Objective: to assess hormonal and brain molecular changes induced by DSS, WAS and DSS plus WAS without interference from behavioral tests.

Experimental design. As described in the original publication (Hassan et al. 2014), the effects of a 7-day treatment with WAS, DSS, or WAS+DSS on molecular factors in the colon, blood, and brain were assessed in the absence of any behavioral tests. In this study the daily food and daily water intake were assessed by weighing the food pellets and the water bottles of the housing cages at the beginning of the WAS session. On day 8, the animals were sacrificed as described in the first experiment, and heart blood, colon and brain collected. The treatment groups were similar to those used in the first experiment. The total number of mice used in the second experiment was 32 (n = 8 per group). Plasma and tissue collection was carried out between 8:00 and 13:00 h.

2.2.3 Third experiment

Objective: to evaluate the effect of DSS, WAS, and DSS + WAS in combination on depression-like behavior assessed by the FST and on HPA axis activation assessed by the plasma corticosterone level.

Experimental design: 38 mice were allocated to 5 groups. One group, the group of naïve mice, was not subjected to the FST, while the other groups underwent the FST at the end of the study.

(1) Naïve mice (n = 6), handled once daily from day 1 to day 7.

(2) Control group (n = 8), handled once daily from day 1 to day 7

(3) Water avoidance stress (WAS) group (n = 8), exposed to intermittent WAS once daily for 1 h from day 1 to day 7

(4) DSS colitis group (n = 8), receiving DSS (2 %) in the drinking water and handled once daily from day 1 to day 7

(5) WAS+DSS group (n = 8), subjected to both WAS and DSS treatment from day 1 to day 7

On day 8 the mice were subjected to FST between 08:00 and 13:00 h, then sacrificed immediately after the test by cervical dislocation. Trunk blood and colon were collected for analysis of plasma corticosterone and colonic MPO, respectively.

Apart from the parameters of colitis severity determined in the previous 2 experiments, occult blood in the fecal pellets collected from the colon was evaluated.

2.2.4 Fourth experiment

Objective: to assess the effect of DSS-induced colitis on the plasma levels of the gut hormones PYY and active GLP-1. To measure active GLP-1 accurately, a DPP-IV inhibitor was added to the blood collected in this experiment

Experimental design: 20 mice were allocated to two groups:

(1) Control group (n = 10), handled once daily from day 1 to day 7
(2) DSS colitis group (n = 10), receiving DSS (2 %) in the drinking water and handled once daily from day 1 to day 7

On day 8, the animals were sacrificed as described in the first experiment, and heart blood, colon and colonic fecal pellets were collected.

2.2.5 Fifth experiment

Objective: to assess the effect of blocking Y2 receptors and GLP-1 receptors on DSS-induced colitis and on colitis-induced behavioral changes.

Experimental design: 42 mice were allocated to 4 groups:

(1) Control+ vehicle group (n = 10), receiving normal drinking water and vehicle injections
(2) Control + BII0264 + exendin (9-39) group (n = 8), receiving normal drinking water and IP injections of BII0246 and exendin (9-39)
(3) DSS + vehicle group (n = 12), receiving 2% DSS in drinking water and vehicle injections.
(4) DSS + BII0246 + exendin (9-39) group (n = 12), receiving 2% DSS in drinking water and IP injections of BII0264 and exendin (9-39).

Peripherally administered exendin (9-39) increased food intake in rats when delivered in the mid-light phase or one hour after the beginning of dark phase but not when delivered immediately before the dark phase (Williams et al. 2009). For

this reason, the effect of BII0246 + exendin (9-39) was assessed at two time points, mid-light cycle and beginning of dark cycle.

The experiment was performed in the LabMaster setup which allows 24-hour monitoring of locomotor activity, feeding, and drinking. Mice housed two per cage in the home cages were first habituated to the LabMaster drinking bottles for 3 days. Then they were transferred to the LabMaster cages where they received 2% DSS or normal tap water. Mice were left in the LabMaster cages uninterrupted during days 1 and 2, while during days 3-5 they received IP saline injections for habituation

On day 6, food pellets were removed at 9:00-10:00 h. Four hours later (at 13:00-14:00) the mice received BII0246 + exendin (9-39) or their vehicles, after which food was reintroduced to record the effect of medications on feeding, drinking and locomotion in the light cycle.

On day 7, food pellets were removed at 13:00-14:00 h. Four hours later (at 17:00-18:00) the mice received BII0246 + exendin (9-39) or their vehicles, after which food was reintroduced to record the effect of medications on feeding, drinking and locomotion in the dark cycle.

On day 8, mice received BII0246 + exendin (9-39) or their vehicles 15 minutes before the OF test.

On day 9, mice received BII0246 + exendin (9-39) or their vehicles 15 minutes before the SI test. After the SI test the mice were sacrificed 30-35 minutes after receiving the IP injections **(Figure 1)**.

The OF and SI tests were performed between 11:00 and 14:00 h.

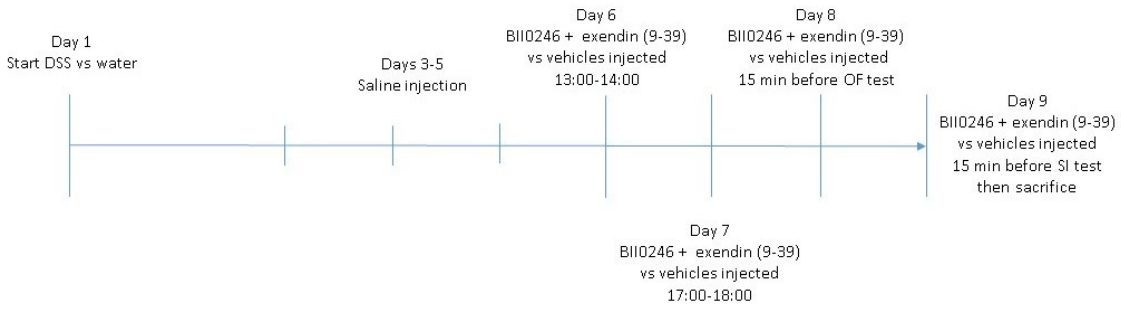


Figure 1: Study design of the fifth experiment

2.2.6 Sixth experiment

Objective: to evaluate the effect of knocking out PYY on chemically induced visceral nociception.

Experimental design: $PYY^{(-/-)}$ and WT mice were allocated to receive a rectal instillation of either 2% AITC or its vehicle, peanut oil (PO). The four study groups were:

(1) WT PO group (n=7)
(2) WT 2% AITC group (n=8)
(3) $PYY^{(-/-)}$ PO group (n=6)
(4) $PYY^{(-/-)}$ 2% AITC group (n=7)

After the rectal treatment, the behavior of the mice was video-recorded for 15 minutes. At the same time, horizontal and vertical locomotor activity was recorded by the LabMaster system. Mice were sacrificed after monitoring of the behavior as described in the first experiment. Pain recording was performed in the light cycle between 09:00 and 13:00 h.

2.2.7 Seventh experiment

Since I found increased pain-related behaviors in $PYY^{(-/-)}$ mice, I went on to investigate the effect of PYY (3-36), the circulatory form of PYY, and its targeted receptors, Y2 receptors on chemical nociception in the colorectal region.

Experimental design: C57Bl/6N mice were allocated to 7 groups. Animals received a subcutaneous (SC) injection of BIIE0246 or its vehicle, 5 minutes later IP PYY (3-36) or its vehicle, and after a further 5 minutes PO or 2% AITC intrarectally.

Mice were allocated to seven groups:

(1) IP vehicle, SC vehicle, PO intrarectally (PO + vehicle group) (n = 6)
(2) IP vehicle, SC vehicle, 2% AITC oil intrarectally (2% AITC + vehicle group) (n = 12)
(3) IP PYY (3-36), SC vehicle, PO intrarectally (PO + PYY (3-36) group) (n = 6)

(4) IP PYY (3-36), SC vehicle, 2% AITC intrarectally (2% AITC + PYY (3-36) group) (n = 10)

(5) IP vehicle, SC BIIE0246, PO intrarectally (PO + BIIE0246 group) (n = 6)

(6) IP vehicle, SC BIIE0246, 2% AITC intrarectally (2% AITC + BIIE0246 group) (n = 10)

(7) IP PYY (3-36), SC BIIE0246, 2% AITC intrarectally (2% AITC + PYY (3-36) + BIIE0246 group) (n = 9)

Pain-related behavior and locomotor activity were assessed as in the sixth experiment.

2.2.8 Eighth experiment

Neither pain-related behavior nor locomotion was affected in response to 2% AITC in WT mice in the sixth experiment. To further explore this observation I assessed mechanical pain sensitivity and referred hyperalgesia, in addition to the mouse grimace scale in response to 1% AITC injected intrarectally in 0.05 ml (one quarter of the AITC amount used in the 6th experiment).

Animals were allocated to four groups:

(1) WT PO group (n=7)

(2) WT 1% AITC group (n=7)

(3) PYY$^{(-/-)}$ PO group (n=6)

(4) PYY$^{(-/-)}$ 1% AITC group (n=7)

Pain recording was performed in the light cycle between 10:00 and 17:00 h. One hour after the intrarectal treatment, mice were sacrificed as described in the first experiment and spinal cords were collected for PCR.

2.3 Drug treatment

Pentobarbital, human PYY (3-36) lyophilized powder, polyethylene glycol 200, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Vienna, Austria).

Exendin (9-39) acetate salt was purchased from Bachem (Bubendorf, Switzerland), and BIIE0246 from Tocris Bioscience (Bristol, UK).

The two peptides, PYY (3-36) and exendin (9-39), were dissolved in 1% BSA in saline. Aliquots were stored at -70°C in polypropylene tubes and thawed on the day of experiment. BSA (1%) and polypropylene tubes were used as they improve the recovery of several peptides including PYY (Goebel-Stengel et al. 2011). BIIE0246 was dissolved in 30% polyethylene glycol 200 in distilled water, and was prepared freshly on the day of the experiment.

In the fifth experiment, BIIE0246 (2 mg/kg) and exendin (9-39) (0.2 mg/kg) were injected IP. These doses successfully block the effects of the exogenous agonists, PYY (3-36) and exendin-4 on food intake (Talsania et al. 2005).

In the seventh experiment, BII0246 was injected SC at the dose of 0.03 mmol/kg which is equivalent to 27 mg/kg. This dose was used previously to evaluate the role of Y2 receptors in vagal afferent signaling (Wultsch et al. 2005), while PYY (3-36) was injected IP at the dose of 0.2 mg/kg (Stadlbauer et al. 2013).

2.4 Induction of colitis

As described in the original publication (Hassan et al. 2014), colitis was induced by adding DSS (molecular weight 36,000 - 50,000; MP Biomedicals, Illkirch, France) at a concentration of 2 % (w/v) to the drinking water for 7 days (Mitrovic et al. 2010). Control animals received normal tap water.

2.5 Water avoidance stress (WAS)

As described in the original publication (Hassan et al. 2014), mice were placed on a small platform (6 × 3 × 3 cm, length × width × height) in the center of a water-filled tank (50 × 32 × 30 cm, length × width × height), the level of the water in the tank being 0.5 to 1 cm below the platform. After a 60 min stay on the platform, the animals were returned to their home cages (Melgar et al. 2008). The mice were not pre-trained to avoid jumping into the water. During the WAS session the mice were surveyed by an investigator. If mice jumped into the water, the investigator put them

back immediately on the platform. It was observed that mice tried to escape more frequently in the first 2 days of the 7-day treatment period, whereas in the remaining 5 days the trials to escape were uncommon. The WAS procedure was carried out between 10:00 and 13:00 hours. Each individual mouse was exposed to WAS every day at the same time of the day.

2.6 Open field test (OF)

The test which was used in the first and fourth experiments was performed as described in the original publication (Hassan et al. 2014). The OF consisted of an opaque grey plastic box (50 × 50 × 50 cm, length × width × height). The ground area of the box was divided into a 36 × 36 cm central area and the surrounding border zone. The mice were placed individually in a corner of the OF, and their behavior during a 5 min test period was tracked by a video camera positioned above the center of the OF and recorded with the software VideoMot2 (TSE Systems, Bad Homburg, Germany). The software was used to evaluate the time spent in the central area, the number of entries into the central area, the total distance travelled in the OF, and the distance travelled in the central area. A reduction of the central area time, of the distance travelled in the central area, and/or of the central area entries was interpreted as an increase in anxiety-like behavior (Bailey and Crawley 2009). The OF box was cleaned with water after each mouse had been tested.

2.7 Social interaction test (SI)

The test which was used in the first and the fifth experiments was performed as described in the original publication (Hassan et al. 2014). The SI test was performed in the OF box as described previously (de Theije et al. 2014, Tabuchi et al. 2007). An empty cylindrical meshwork container (7 × 10 cm, diameter × height) was placed adjacent to the middle of one wall of the OF. The test mouse was placed adjacent to the middle of the opposite wall of the OF and allowed to explore the field for 3 min, after which the mouse was returned to its home cage. A novel mouse (target mouse) was placed in the cylindrical container, and then the test mouse was allowed to explore the OF for another 3 min. The time spent in the interaction zone which was within 8 cm of the cylindrical container was calculated with the VideoMot2 software in both sessions. Social activity was expressed as SI percent which was

defined as the percent ratio between the time spent in the interaction zone in the presence of the target mouse divided by the time spent in the interaction zone in the absence of the target mouse. The OF box was cleaned with 70 % ethanol after each mouse had been tested (de Theije et al. 2014).

2.8 Tail suspension test (TST)

The test which was used in the first experiment was done as described in the original publication (Hassan et al. 2014), the mice were suspended by their tail with a 1.9 cm wide strapping tape (Leukotape classic, BSN Medical S.A.S., Le Mans, France) for 6 min, and their behavior was recorded by a video camera. A trained blinded observer analyzed the video recordings with the VideoMot2 software event monitoring module for 3 types of behavior: swinging, curling and immobility. The mouse was considered swinging when it continuously moved its paws while keeping the body straight and/or moving the body from side to side. The mouse was considered curling when the mouse twisted its trunk (Berrocoso et al. 2013). The time spent swinging, curling and being immobile was calculated. Increased immobility was described as increased depression-like behavior, and mice which climbed over their tails were excluded as they had learned that escape is possible (Cryan et al, 2005).

2.9 Forced swim test (FST)

The test which was used in the third experiment was performed as described previously (Porsolt et al. 1977, Painsipp et al. 2011a). The mice were placed individually in a glass cylinder (diameter:16 cm, height: 23 cm) containing tap water of 25°C at a depth of 16 cm, which prevented the mice from touching the bottom with their paws or the tail. The mice were tested for 6 min, and their behavioral activity was scored by a trained blinded observer. The time each mouse spent climbing, swimming and floating (immobile) was calculated. Mice were considered immobile when they floated passively in the water, performing only movements that enabled them to keep their heads above the water level. An increase in the immobility time was interpreted as an increase in depression-like behavior.

2.10 LabMaster system

The LabMaster system (TSE Systems) was used in the fifth, sixth, and seventh experiment. The system allowed continuous recording of the animals without intervention by any investigator. As described previously (Painsipp et al. 2013, Farzi et al. 2015), the LabMaster system consisted of test cages (type III, 42.0 × 26.5 × 15.0 cm, length × width × height), surrounded by two external infrared frames and a cage lid equipped with three weight transducers. For recording locomotion and exploration, the two external infrared frames were positioned in a horizontal manner above one another at a distance of 4.3 cm, with the lower frame being fixed 2.0 cm above the bedding floor. The bottom frame was used to record horizontal activity of the mice, whereas the top frame detected vertical movements (rearing, exploration). The measures of activity (locomotion, exploration) were derived from the light beam interruptions (counts) of the corresponding infrared frames.

In the fifth experiment, two lid transducers were used to record the consumption of standard rodent chow and of for the drinking water with or without 2% DSS, respectively. Food intake, water intake, horizontal and vertical activities were analyzed 1 hour, 2 hours, and 12 hours after injection of drugs or vehicles.

In the sixth and seventh experiments, only locomotor activity was measured for 15 minutes and no food and water were available to the mice during this period. In these experiments, the cages were covered with transparent plates to allow video recording.

2.11 Colonic chemonociception

Chemically induced nociception in the colon was induced by intrarectal AITC administrated through a Teflon feeding cannula (length 38.1 mm, gauge 20) (Scanbur, Karlslunde, Denmark). Vaseline (Rösch and Handel, Vienna, Austria) was applied to the perianal region and to the tip of the cannula before the intrarectal administration to reduce the pain induced by the procedure of application itself. Then 2% AITC (or 1% in the eighth experiment) or its vehicle, PO, were injected

intrarectally through the feeding cannula (Laird et al. 2001, Mitrovic et al. 2010, Jain et al. 2015).

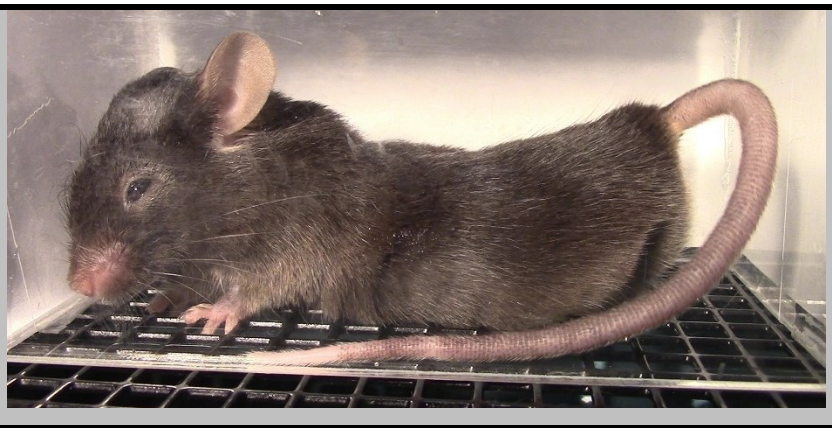
2.12 Assessment of pain-related behaviors

After intrarectal administration of AITC or vehicle the behavior of mice was video-recorded in the LabMaster system for 15 minutes. A blinded trained investigator used the Videomot 2 event marker module to evaluate the video-recordings with regard to the following pain-related behaviors (Laird et al. 2001) **(Figure 2)**:

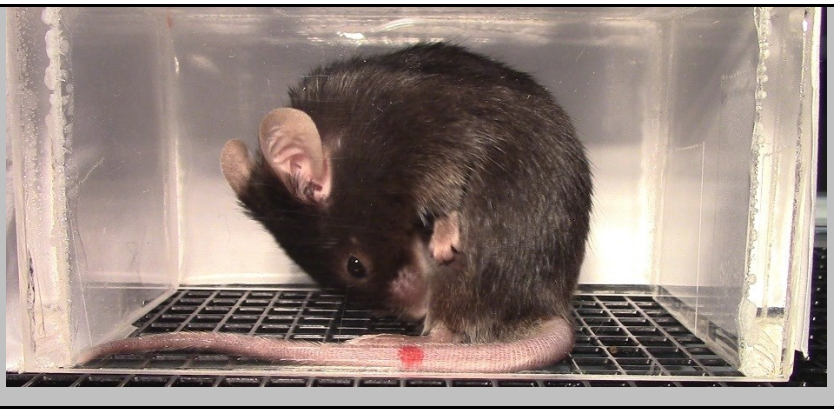
a. Stretching of the trunk and squashing (pressing the abdomen towards the floor of the cage and stretching of body)
b. Licking of the lower part of the abdomen
c. Arching of the trunk

The time spent in grooming and freezing was also calculated from the video recordings. Horizontal and vertical locomotor activity were measured simultaneously with the LabMaster system (Jain et al. 2015).

Squashing



Abdominal licking



Arching

Figure 2: Pain-related behaviors used to assess visceral pain

2.13 The mouse grimace scale (MGS)

The scale was evaluated as described previously (Langford et al. 2010) with a slight modification. The modifications were introduced to allow simultaneous assessment of referred hyperalgesia in the same settings. Mice were kept on a wire mesh (Ugo Basile, Varese, Italy) in a homemade plexiglass box (9 cm × 5 cm × 5 cm; length × height × width). All sides of the box except one (9 cm × 5 cm) were covered with white paper. Mice were left for 30 minutes to habituate in the box, then a 20-minute video recording under basal conditions ("no pain") was taken with a Canon Legira HF R406 video camera. After taking the video, the mechanical pain threshold (MPT) was measured as described in section 2.14. Afterwards the mice received 1% AITC or the vehicle (PO) intrarectally as described in section 2.11, then another 20 minutes video recordings for the assessment of the MGS were taken followed by a second measurement of MPT.

For MGS analysis frames that showed clear facial expressions were captured from the video. The analyzer took the earliest frame showing a clear facial expression in the video as JPEG file, then started searching for another clear frame after 1.5 min. Since the mice were not always in a favorable position to take the next clear frame, the usual framing interval was 1.5-3 min.

The resultant JPEG files were cropped such that body position was no longer visible. Then a blinded analyzer evaluated the following specific facial expression action units (AUs):

1. Orbital tightening which is narrowing of the orbital area, with a tightly closed eyelid or an eye squeeze (denoted by a wrinkle around the eye).
2. Nose bulge which is a rounded extension of the skin visible on the bridge of the nose.
3. Cheek bulge which refers to a convex appearance of the cheek muscle (between eye and whiskers) relative to its baseline position, or shortening of the distance from eye to whisker pad.
4. Position of the ears which can be rotated outward or back (away from the face) during pain.

5. Position of the whiskers which can be pulled backward or forward, or be clumped together.

The score for each AU was 0 (not present), 1 (moderately visible) or 2 (clearly visible) **(Figure 3)**. Since the whiskers were not clear in many images, they were not included in the analysis, as MGS assessment without scoring of whisker position was reported previously (Leach et al. 2012). After unblinding, all photos of each mouse under “no pain” and “pain” conditions were compiled together, and the average “no pain” MGS and “pain” MGS were calculated for each mouse. The ΔMGS value was used as an indicator of visceral pain and was calculated by subtracting the “no pain” MGS from “pain” MGS.

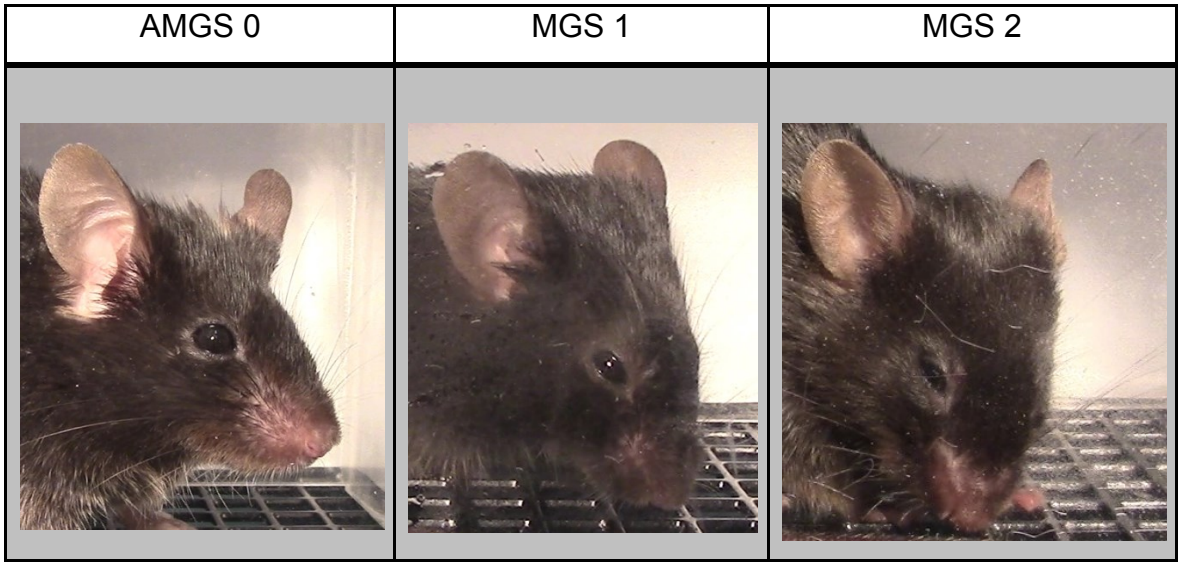


Figure 3: Photographs showing scores 0, 1, and 2 of the mouse grimace scale (MGS)

2.14 Assessment of mechanical pain threshold (MPT) and referred hyperalgesia

Referred hyperalgesia was described as a method to measure colonic pain sensitivity. The method depends on the phenomenon that visceral pain leads to increased pain sensitivity of the corresponding skin dermatome (Laird et al. 2001). Referred hyperalgesia was evaluated in parallel to MGS assessment and determined twice by assessment of the MPT over the hind paw and the abdomen. The first measurement was taken after the “no pain video” was recorded but before

the intrarectal administration of PO or 1% AITC was performed, while the second measurement was taken after the “pain video” had been recorded (i.e. 20 min after intrarectal administration of PO or 1% AITC). The MPT was evaluated with von Frey filaments (Bioseb, Vitrolles, France) by using the simplified up-down method (SUDO method) (Bonin et al. 2014). The forces applied with the test filaments were 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1.0, and 1.4 grams. Testing began with a filament of 0.16 g force for the paw and 0.07 g force for the abdomen. the testing sequence progressed with applications of 5 different force filaments following an up-down sequence such that a positive response to a filament indicated the next lower force filament to be used, while a negative response indicated the next higher force filament to be used. Finally, the MPT was calculated with the following formula: **MPT = force of 5th hair applied + adjustment factor**. The adjustment value equals + 0.5 of the last stimulus interval if the last hair produced a negative response. Alternatively, the adjustment factor equals - 0.5 of the last stimulus interval if the last hair produced a positive response. MPT was measured on both hind paws while two consecutive measurements were taken over the belly. The hairs were applied for 1–2 s, with an inter-stimulus interval of at least 10 seconds. Over the belly, stimuli were applied on the lower to mid abdomen, avoiding the external genitalia, and care was taken not to stimulate the same point twice in succession. Sharp retraction of abdomen, immediate licking or scratching of the site of hair application, and trials of escaping or jumping were considered as withdrawal responses (Laird et al. 2001). The average MPT of both paws and of the two abdominal measurements were used in the statistics. ΔMPT was used as an indicator of visceral pain and was calculated by subtracting the “no pain” MPT from “pain” MPT. Some mice showed prolonged squashing under pain conditions and did not respond to the hairs; these mice were excluded from the analysis.

2.15 Collection of blood and tissues

As described in the original publication (Hassan et al. 2014), blood was collected by cardiac puncture with 3.8 % citrate as anticoagulant during sacrifice within 3.5 min after injection of pentobarbital. In the third experiment, in which mice were sacrificed by cervical dislocation, trunk blood was collected in EDTA coated tubes. In the 4th experiment, a DPP-IV inhibitor (DDP-4, Millipore, St Charles, MO, USA, 10 µL DPP-

IV inhibitor per milliliter of blood) was added to the samples to prevent GLP-1 deactivation. After centrifugation at 1,600 × g for 15 min at 4 °C, the plasma was frozen immediately on dry ice and stored at -70 °C until assay.

In the experiments involving colitis, colon length and colon weight were used to assess colitis severity (Vowinkel et al. 2004). After decapitation of the mice, the colon extending from the proximal end at the caecum to the anal end was rapidly removed and its length measured. Subsequently the colon was opened longitudinally, washed under running water, dried with tissue paper, and its weight (mg) determined. Then the distal part of the colon was shock-frozen in liquid nitrogen and stored at -70 °C until MPO assay.

In the third, fourth, and fifth experiment, stool was collected from the colon and tested for occult blood using the Hemdetect ® kit as described by the manufacturer (DIPRO med, Weigelsdorf, Austria). This measurement was used as an additional readout for colitis.

The brains were removed, frozen on dry ice, wrapped in aluminum foil, and stored at -70 °C until microdissection while the spinal cords where shock frozen in liquid nitrogen stored at -70 °C until RNA extraction.

2.16 Colonic myeloperoxidase measurement

MPO is an enzyme found mainly in neutrophils, monocytes and macrophages and has frequently been used to quantify experimental colitis severity (Krawisz et al. 1984). As described in the original publication (Hassan et al. 2014), the MPO content of the colon was measured with an enzyme-linked immunosorbent assay kit specific for the rat and mouse protein (Hycult Biotechnology, Uden, The Netherlands). The tissue samples were prepared according to the manufacturer's instructions. After weighing, the frozen tissues were placed in MPO lysis buffer (pH 7.4) at a ratio of 1 mg: 0.02 ml. The composition of the lysis buffer was: 200 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 10 mM trishydroxy methylaminomethane, 10 % glycerine, 1 mM phenylmethylsulphonyl fluoride, 1 mg/ml leupeptide, and 28 mg/ml aprotinin.

The samples were homogenized on ice with an Ultraturrax (IKA, Staufen, Germany) and then subjected to two centrifugation steps at 6,000 × g at 4 °C for 15 min. The MPO content of the supernatant was measured with the kit. The sensitivity of the assay was 1 ng/ml at an intra- and inter-assay variation of around 10 %.

2.17 Multiplex measurement of plasma cytokines

As described in the original publication (Hassan et al. 2014), the plasma levels of interleukins 6, 10, 12, and 18 (IL-6, IL-10, IL-12, and IL-18) were measured with a multiplex immunoassay (ProcartaPlex Multiplex Immunoassays, eBioscience, Vienna, Austria). The assay was performed according to the manufacturer's instructions. The fluorescent signal was measured with the Bio-Plex 200 multiplex suspension array system in combination with the Bio-Plex 5.0 Software (Bio-Rad, Hercules, CA, USA). Standard curves were generated with a five-parameter logistic curve-fitting method. Cytokines that were below detection limit were assigned a value of zero. The sensitivities of the assay were 0.9, 0.35, 0.35, and 8.09 pg/ml for IL-6, IL-10, IL-12, and IL-18, respectively.

2.18 Plasma NPY, corticosterone, PYY, and active GLP-1 measurements

The plasma levels of corticosterone, NPY, PYY, active GLP-1 and corticosterone were determined with enzyme immunoassay (EIA) kits according to the manufacturer's instructions (**Table 1**).

Table 1: EIA kits used in the work

EIA kit	Manufacturer	Quantification limit
Corticosterone	Assay Designs, Ann Arbor, MI, USA	0.027 ng/ml
NPY	Phoenix Pharmaceuticals, Burlingame, CA, USA	0.09 ng/ml
PYY	ALPCO, Salem, NH, USA	0.15 ng/ml
Active GLP-1	Millipore, St Charles, MO, USA	0.46 pg/ml

The specificity of the NPY and PYY kits were checked against plasma samples from NPY and PYY knockout mice. The NPY kit showed satisfying specificity with very

low plasma levels of NPY-like immunoreactivity in knockout mice, while the PYY-like immunoreactivity in plasma of $PYY^{(-/-)}$ mice was slightly lower than in WT mice, indicating a poor specificity of the PYY EIA kit.

2.19 Brain microdissection

As described in the original article (Hassan et al. 2014), the frozen brains were transferred to a cryostat at -20 °C and cut manually into approximately 1 mm thick slices. These brain slices were placed on a cold plate (Weinkauf Medizintechnik, Forchheim, Germany) set at -20 °C, on which hypothalamus, amygdala and hippocampus were microdissected under a stereomicroscope. Hypothalamic tissue was collected from the preoptic area (Bregma: +0.26) to the end of the mammillary bodies (Bregma: -2.92), amygdalar tissue from the anterior edge of the optical tract (Bregma: -0.58) to the posterior part of the basolateral and basomedial amygdala (Bregma: -2.54), and hippocampal tissue from the limit of the hippocampal formation (Bregma: -0.94) to the caudal end of the dentate gyrus (Bregma: -4.04). The microdissected brain areas were kept in homogenization tubes on dry ice and subsequently stored at -70 °C until further processing (Reichmann et al. 2015).

2.20 RNA extraction and real time PCR

As described in the original article (Hassan et al. 2014), RNA from the microdissected brain regions and spinal cord was extracted with the RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) while RNA from the colon was extracted with the RNeasy Tissue Mini Kit (Qiagen, Hilden, Germany). Aliquots of 1 µg RNA were reverse-transcribed with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA).

For relative quantification of mRNA, real time PCR was performed with the CFX Connect™ Real-Time PCR detection system in combination with the CFX Manager™ software 3.1 (Bio-Rad). The specific primers used for amplification and quantitation of mRNA are listed in **Table 2**. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Mm_Gapdh_3_SG QuantiTect Primer Assay, Qiagen) was used as reference gene in the brain, while phosphoglycerate kinase 1 (pgk) was used as reference gene in the colon. Both GAPDH and pgk were used as

reference genes in the spinal cord. The stability of GAPDH and pgk as reference genes was confirmed with the M value (Hellemans et al. 2007, Vandesompele et al. 2002) as assessed by the CFX Manager™ 3.1 software. The PCR SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) was used for amplification, and the cycling conditions were as follows: samples were heated to 95 °C for 30 s followed by 39 cycles of 95 °C for 3 s, and 60 °C for 30 s. Except for the GAPDH primers which have been validated by the manufacturer, the products of all other primers were sequenced to confirm specificity. The sequencing was performed by VBC Biotech (Vienna, Austria). Quantitative values of mRNA relative to control were calculated with the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008).

Table 2: Primers used in the study

Gene	Primer Sequence (5' -->3')	Reference
BDNF forward	GTGACAGTATTAGCGAGTGG	Designed by Primer-Blast (Ye et al. 2012)
BDNF reverse	TTCTCTAGGACTGTGACCGT	
COX-1 forward	ATGAGTCGAAGGAGTCTCTCG	Harvard Primer Bank (Spandidos et al. 2010) ID: 6679537a1
COX-1 reverse	GCACGGATAGTAACAACAGGGA	
COX-2 forward	TTCAACACACTCTATCACTGGC	Harvard Primer Bank ID: 31127110a1
COX-2 reverse	AGAAGCGTTTGCGGTACTCAT	
CRF forward	GAATTTCTTGCAGCCGGAGC	Designed by Primer-Blast
CRF reverse	CAGCGGGACTTCTGTTGAGA	
GR forward	GACTCCAAAGAATCCTTAGCTCC	Harvard Primer Bank ID: 121247452c1
GR reverse	CTCCACCCCTCAGGGTTTTAT	
MR forward	GAAGAGCCCCTCTGTTTGCAG	Harvard Primer Bank ID: 17384011a1
MR reverse	TCCTTGAGTGATGGGACTGTG	
NPY forward	CAGATACTACTCCGCTCTGCGACA CTACAT	(Ferenczi et al. 2010)

NPY reverse	TTCCTTCATTAAGAGGTCTGAAATC AGTGTCT	
Pgk forward	ATGTCGCTTTCCAACAAGCTG	Harvard PrimerBank ID: 6679291a1
Pgk reverse	GCTCCATTGTCCAAGCAGAAT	
Preproglucagon forward	TTACTTTGTGGCTGGATTGCTT	Harvard PrimerBank ID: 33468853a1
Preproglucagon reverse	AGTGGCGTTTGTCTTCATTCA	
PYY forward	ACGGTCGCAATGCTGCTAAT	Harvard PrimerBank ID: 21703868a1
PYY reverse	GACATCTCTTTTTCCATACCGCT	
Y1 receptor forward	TGATCTCCACCTGCGTCAAC	(Reichmann et al. 2015)
Y1 receptor reverse	ATGGCTATGGTCTCGTAGTCAT	
Y2 receptor forward	TCCGGGAATACTCCCTGATTG	(Reichmann et al. 2015)
Y2 receptor reverse	GCAAAACGTACAGGATGAGCAG	

Abbreviations: BDNF (brain-derived neurotrophic factor), COX-1 (cyclooxygenase-1), COX-2 (cyclooxygenase-2), CRF (corticotropin-releasing factor, GR (glucocorticoid receptors), MR (mineralocorticoid receptors), NPY (neuropeptide Y), Pgk (phosphoglycerate kinase 1), PYY (peptide YY).

2.21 Statistics

SPSS (21 and 22) and SigmaPlot (12.1 and 13) were used for statistical analysis and graphic presentation of the results. The data were analyzed with t test, one way ANOVA, two way ANOVA or chi square test, as appropriate. Log transformation was considered whenever needed to meet one-way and two-way ANOVA assumptions. If log transformation was not sufficient to meet two-way ANOVA assumptions, rank transformation was applied. The Bonferroni post-hoc test was carried out in the presence of statistically significant interactions between the two factors in two way

ANOVA. If log transformation was not sufficient to meet the equal variability assumption of one way ANOVA as tested by Levene's test, the Welch correction and post hoc Games Howell test were used. The Kruskal-Wallis test and post-hoc Mann-Witney U test with Bonferroni correction were used in the case of non-parametric distribution of the readouts. Daily food and water intake were analyzed with repeated measures ANOVA. Sphericity assumptions were checked by Mauchly's test and, in case of violation of sphericity, the Greenhouse-Geisser correction was used.

3 RESULTS

3.1 First and second experiments

The results of the first and second experiments were published in the original paper (Hassan et al. 2014).

3.1.1 Dextran sulfate sodium (DSS) treatment induced experimental colitis while WAS had a minor effect

Treatment with 2 % DSS induced colitis as deduced from body weight loss, reduced colon length, increased colon weight, and enhanced levels of colonic MPO **(Table 3 and 4 and Figure 4)**. In the first experiment two-way ANOVA revealed a significant main factor effect of DSS treatment on body weight change ($F_{(1,64)} = 55.7$; $p < 0.001$), colon weight ($F_{(1,64)} = 148.2$; $p < 0.001$), and colon length ($F_{(1,64)} = 182.3$; $p < 0.001$) **(Table 3** and **Figure 4A)**. Similarly, DSS treatment led to a significant increase of the colonic MPO content ($F_{(1,62)} = 187$; $p < 0.001$) **(Figure 4A)**.

No significant effect of WAS and no significant interaction between WAS and DSS could be detected in any of the parameters used to assess colitis severity in the first experiment **(Table 3, Figure 4A)**.

In the second experiment, in which mice were not subjected to behavioral tests, DSS treatment had a similar effect as in the first experiment (compare **Tables 3** and **4**). Two-way ANOVA revealed a significant main factor effect of DSS on body weight change ($F_{(1, 28)} = 106.8$; $p < 0.001$), colon length ($F_{(1, 28)} = 54.7$; $p < 0.001$), and colonic MPO content ($F_{(1, 28)} = 86.7$; $p < 0.001$) (**Table 4, Figure 4B)**. Unlike in the first experiment, a significant main factor effect of WAS was observed with regard to body weight loss ($F_{(1, 28)} = 9.2$; $p = 0.005$), and a significant interaction between the factors WAS and DSS were found to exist for colon weight ($F_{(1, 28)} = 5.7$; $p = 0.024$). Post-hoc analysis revealed a significant WAS-induced increase in colon weight only in the presence of DSS, while DSS increased colon weight both in the absence and presence of WAS **(Table 4)**.

Table 3: Effect of DSS and WAS, alone and in combination, on body weight change, colon weight, and colon length in mice that underwent behavioral tests. The mice were sacrificed 4 days after the end of treatment with DSS and WAS

	Control n=16	WAS n=16	DSS n=18	WAS + DSS n=18	WAS main factor effect	DSS main factor effect	Interaction
Body weight change (g) on day 8	+ 0.31 (0.18)	- 0.06 (0.28)	- 1.39 (0.22)	- 1.83 (0.28)	NS	p <0.001	NS
Colon weight (mg/cm) on day 11	29.9 (0.86)	31.7 (0.97)	49.6 (1.99)	47.7 (1.54)	NS	p <0.001	NS
Colon length (cm) on day 11	7.80 (0.19)	7.63 (0.13)	5.81 (0.15)	5.51 (0.14)	NS	p <0.001	NS

Animals were subjected to a 7-day treatment (days 1-7) with DSS (2 % in the drinking water) and WAS (1 hour daily), alone or in combination, followed by behavioral testing during days 8-10. The mice were sacrificed on day 11, i.e. 4 days after the end of treatment with DSS and WAS. The data are presented as means, with SEM given in brackets. NS, not significant. This table was published in the original publication (Hassan et al. 2014).

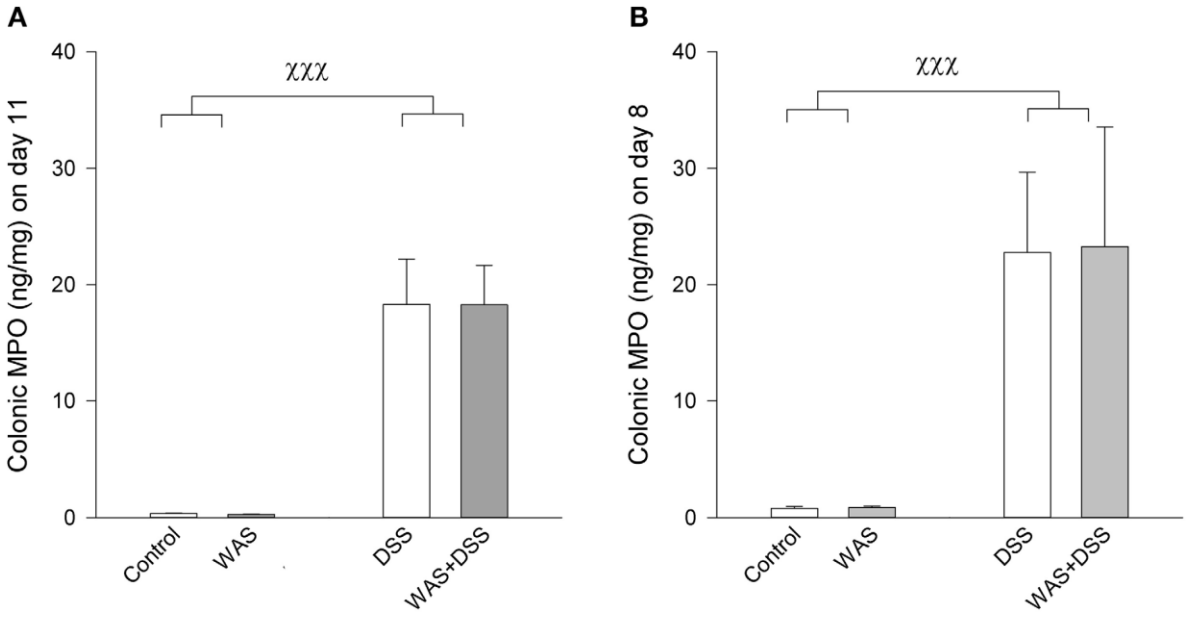


Figure 4. Colonic MPO content in mice subjected to a 7-day treatment with WAS, DSS or WAS+DSS (n=15-18 per group) followed by behavioral testing during days 8-10 as carried out in the first experiment (A) or sacrificed on day 8 (n=8 per group) without behavioral testing as examined in the second experiment (B). In both experiments, two-way ANOVA disclosed a significant DSS effect ($p < 0.001$) but no WAS effect and no significant interaction. The data shown are means + SEM, χχχ p <0.001 for DSS main factor effect. This figure was published in the original publication (Hassan et al. 2014).

Table 4: Effect of DSS and WAS, alone and in combination, on body weight change, colon weight, and colon length in mice used for the assay of biochemical factors in the colon, blood and brain in the absence of any behavioral testing. The mice were sacrificed 1 day after the end of treatment with DSS and WAS

	Control n=8	WAS n=8	DSS n=8	WAS+DSS n=8	WAS main factor effect	DSS main factor effect	Interaction
Body weight change (g) on day 8	+ 0.64(0.17)	+ 0.02(0.17)	- 2.47(0.29)	- 3.90(0.57)	p <0.01	p <0.001	NS
Colon weight (mg/cm) on day 8	30.9(1.49)	28.6(0.76)	41.7(1.71) ***	46.7(1.87) §###	NS	p <0.001	p <0.05
Colon length (cm) on day 8	8.65 (0.35)	8.75 (0.27)	6.51 (0.40)	5.95 (0.30)	NS	p <0.001	NS

Animals were subjected to a 7-day treatment (days 1-7) with DSS (2 % in the drinking water) and WAS (1 hour daily), alone or in combination, and sacrificed for the assay of biochemical factors in the colon, blood and brain on day 8, i.e. 1 day after the end of treatment with DSS and WAS. The data are presented as means, with SEM given in brackets. *** $p < 0.001$ versus control (colon weight), § $p < 0.05$ versus DSS (colon weight) , ### $p < 0.001$ versus WAS (colon weight). NS, not significant. This table was published in the original publication (Hassan et al. 2014).

3.1.2 DSS treatment reduced the daily food intake

Although no significant differences could be detected in the cumulative water intake over the treatment period **(Figure 5A)**, repeated measures ANOVA **(Figure 5C)** showed significant changes in daily water intake during the treatment period ($F_{(6,13)}$ = 13.1; $p < 0.001$), with a significant interaction with DSS ($F_{(6,13)}$ = 11.1; $p < 0.001$) but not WAS. The DSS-treated mice had a tendency to drink more during days 1-4 but this difference was statistically significant only on day 3 **(Figure 5C)**. Subsequently, starting from day 5, daily water intake dropped below the daily water

intake of control mice. This reduction of water intake in response to DSS was statistically significant on day 6 **(Figure 5C)**.

The effect of DSS treatment on food intake was more prominent **(Figure 5B, D)** than on water intake. Cumulative food intake over the treatment period was significantly reduced in response to DSS ($F_{(1,12)}$ = 68.7; p < 0.001) but not WAS **(Figure 5B)**, with no significant interaction between the two factors in the two-way ANOVA assessment. In the same line, repeated measures ANOVA showed a significant decrease of the daily food intake **(Figure 5D)** during the treatment period ($F_{(2.6,31.2)}$ = 26.8; p < 0.001), with a significant interaction with DSS ($F_{(2.6,31.2)}$ = 20.3; p < 0.001), but not WAS. A statistically significant reduction of food intake in response to DSS treatment could be detected by post-hoc testing during days 5-7 **(Figure 5D)**.

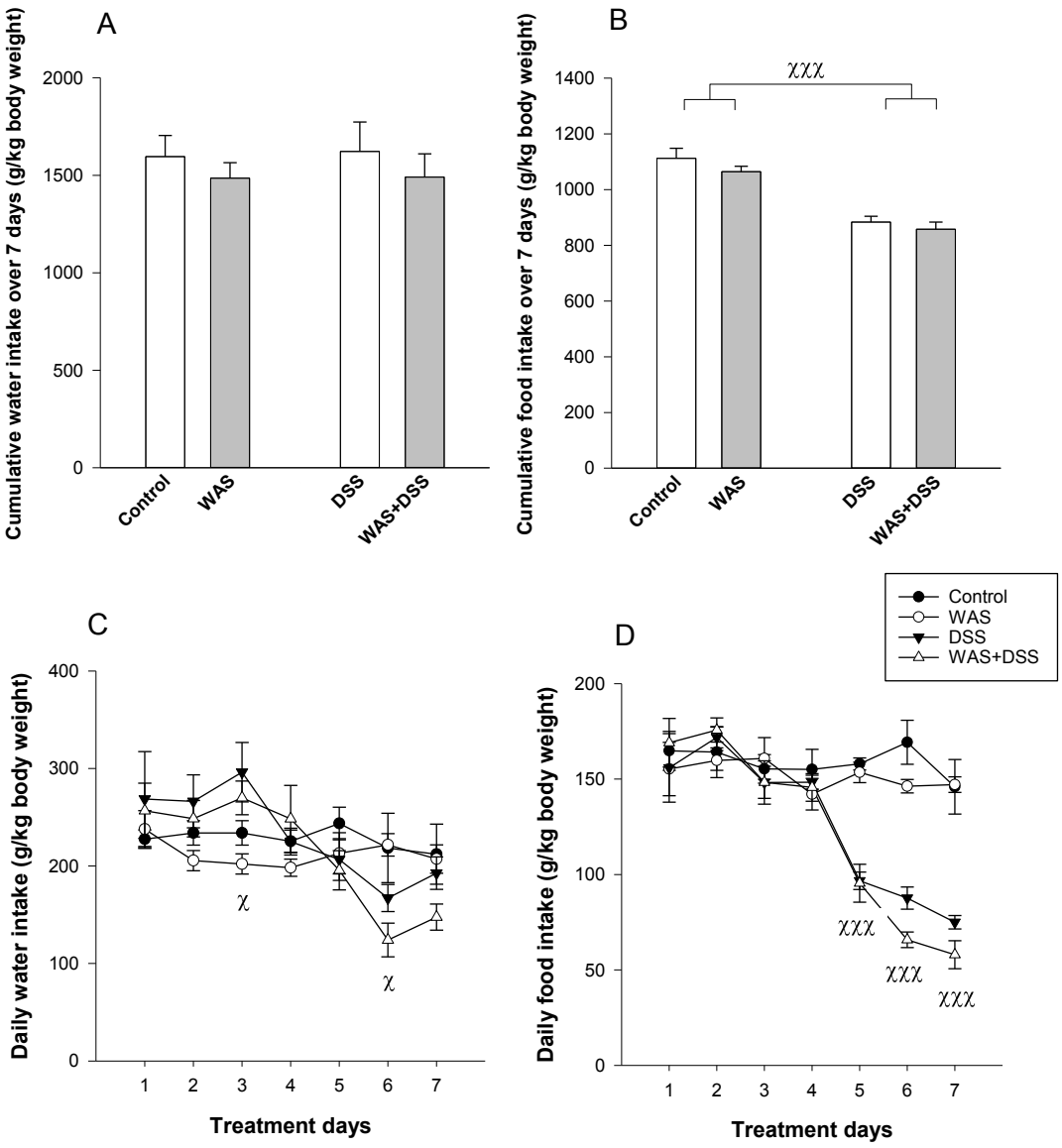


Figure 5: Effect of a 7-day treatment with DSS and WAS, alone and in combination, on water and food intake as examined in the second experiment. (A) Cumulative water intake over the 7-day treatment period. There were no significant differences. (B) Cumulative food intake over the 7-day treatment period. There was a significant main factor effect of DSS on total food intake ($p < 0.001$). (C) Daily water consumption during the 7-day treatment period. Repeated measures ANOVA disclosed that DSS significantly modified the daily water intake during the 7-day observation period. Post-hoc testing showed a significant main factor effect of DSS on days 3 and 6 ($p < 0.05$). (D) Daily food consumption during the 7-day treatment period. Repeated measures ANOVA revealed that DSS significantly suppressed the daily water intake. Post-hoc testing showed a significant main factor effect of DSS during days 5-7 ($p < 0.001$). The data shown are means + SEM (A,B) and means ± SEM (C,D), n = 4 cages per group each including two mice, χ $p < 0.05$ DSS main factor effect, χχχ $p < 0.001$ DSS main factor effect. This figure was published in the original publication (Hassan et al. 2014).

3.1.3 WAS caused resilience against DSS-induced behavioral changes in the OF and SI tests but not in the TST

DSS treatment significantly influenced behavior in the OF test, which can be interpreted as anxiogenic effect or sickness-like behavior, while concomitant WAS treatment protected from this effect **(Figure 6)**. Locomotor activity as indicated by the total traveling distance in the OF test was significantly shortened by DSS treatment (main factor effect: $F_{(1,62)} = 19.3$; $p < 0.001$) and significantly prolonged by WAS (main factor effect: $F_{(1,62)} = 28$; $p < 0.001$), with no significant interaction between the two factors. DSS and WAS significantly interacted in their effect on anxiety-related parameters in the OF test which included number of central area visits, percentage traveling distance in the central area, and percentage time spent in the central area. The interaction statistics for these parameters were, respectively: $F_{(1,62)} = 6.1$, $p = 0.013$; $F_{(1,62)} = 7.0$, $p = 0.01$; and $F_{(1,62)} = 6.3$, $p = 0.015$. Post-hoc analysis revealed a significant increase in anxiety-like behavior by DSS only in the absence of WAS **(Figure 6).** In contrast, in the presence of WAS, DSS did not alter anxiety-related behavioral parameters in the OF **(Figure 6)**.

In the SI test, there was a significant interaction between WAS and DSS ($F_{(1,61)} = 5.2$; $p = 0.027$). Post-hoc analysis disclosed a significant reduction of the social activity of mice in response to DSS treatment, an effect that was only seen in the absence of WAS, whereas in the presence of WAS, DSS failed to affect social activity **(Figure 7)**.

Unlike in the OF and SI tests, no significant interaction between the factors WAS and DSS could be identified in the TST **(Figure 8)**. Additionally, neither DSS nor WAS had a significant effect on immobility time as revealed by two-way ANOVA. On the other hand, DSS had a significant main factor effect on swinging time ($F_{(1,61)} = 9.7$; $p = 0.003$) and curling time ($F_{(1,61)} = 8.6$; $p = 0.005$), with no significant WAS effect and no significant interaction between the two factors **(Figure 8)**.

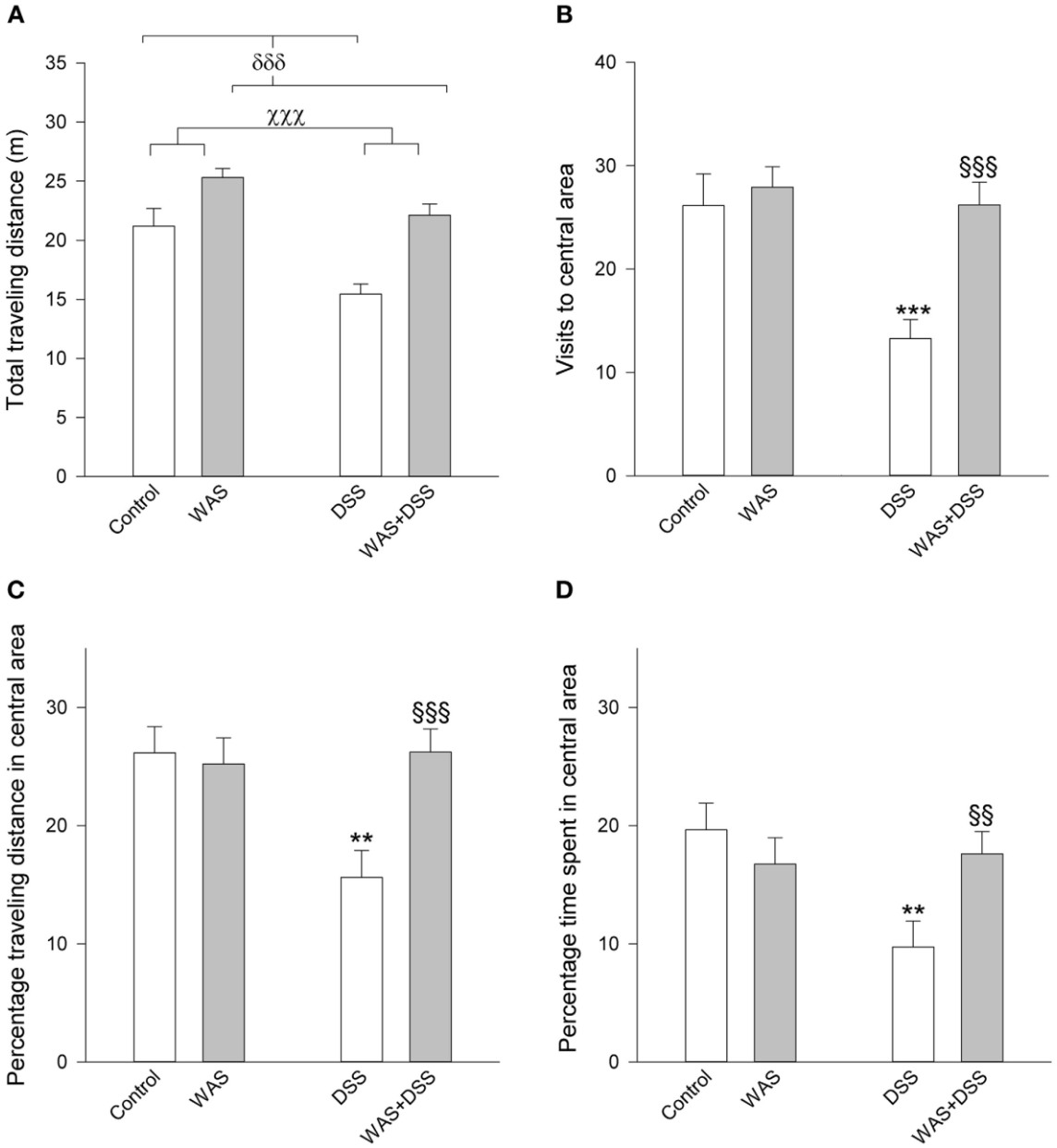


Figure 6: Effect of a 7-day treatment with DSS and WAS, alone and in combination, on behavioral parameters of the OF test as carried out 1 day after the end of the treatment period. (A) Total traveling distance. Both WAS and DSS had a main factor effect ($p < 0.001$), but there was no significant interaction between the two factors. (B) Visits to central area. There was a significant interaction between WAS and DSS in this parameter ($p < 0.05$). (C) Percentage traveling distance in central area, calculated as a percentage of the total traveling distance. There was a significant interaction between WAS and DSS in modifying this parameter ($p < 0.05$). (D) Percentage time spent in central area, calculated as a percentage of the total time spent in the OF. There was a significant interaction between WAS and DSS in modifying this parameter ($p < 0.05$). The data shown are means + SEM, n=14-18 per group; χχχ $p < 0.001$ DSS main factor effect, δδδ $p < 0.001$ WAS main factor effect, ** $p < 0.01$, *** $p < 0.001$ versus control, §§ $p < 0.01$, §§§ $p < 0.001$ versus DSS. This figure was published in the original publication (Hassan et al. 2014).

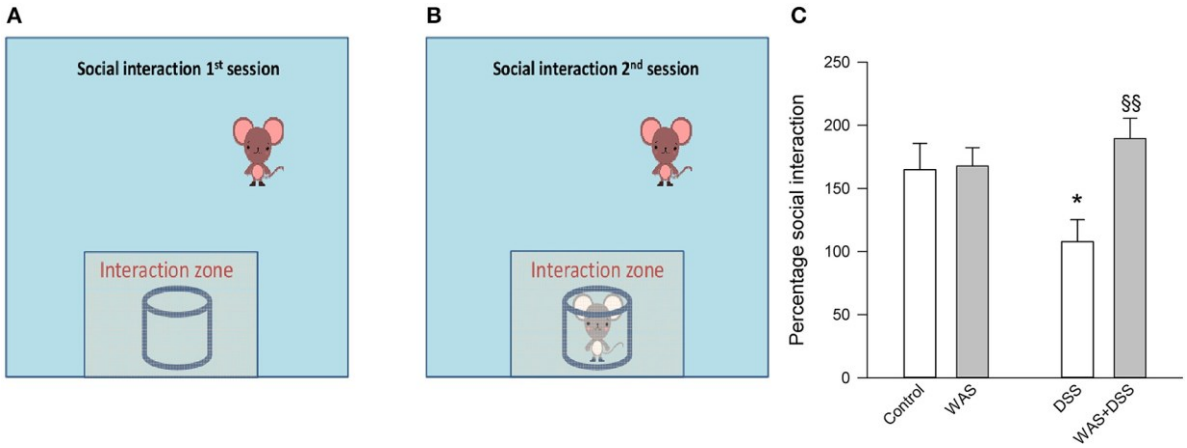


Figure 7: Effect of a 7-day treatment with DSS and WAS, alone and in combination, on behavior in the SI test as carried out 2 days after the end of the treatment period. The left (A) and middle (B) panel explain the procedure of the SI test that consisted of two sessions, the first session being conducted without a target mouse in the cylindrical container. Panel (C) presents the results of the SI test as percentage social interaction, calculated as the percent ratio of the time spent in the interaction zone in the presence of the target mouse divided by the time spent in the interaction zone in the absence of the target mouse. There was a significant interaction between WAS and DSS in modifying social interaction percent (p <0. 05). The data shown are means + SEM, n= 16-17 per group; * p <0. 05 versus control, §§ p < 0.01 versus DSS. This figure was published in the original publication (Hassan et al. 2014).

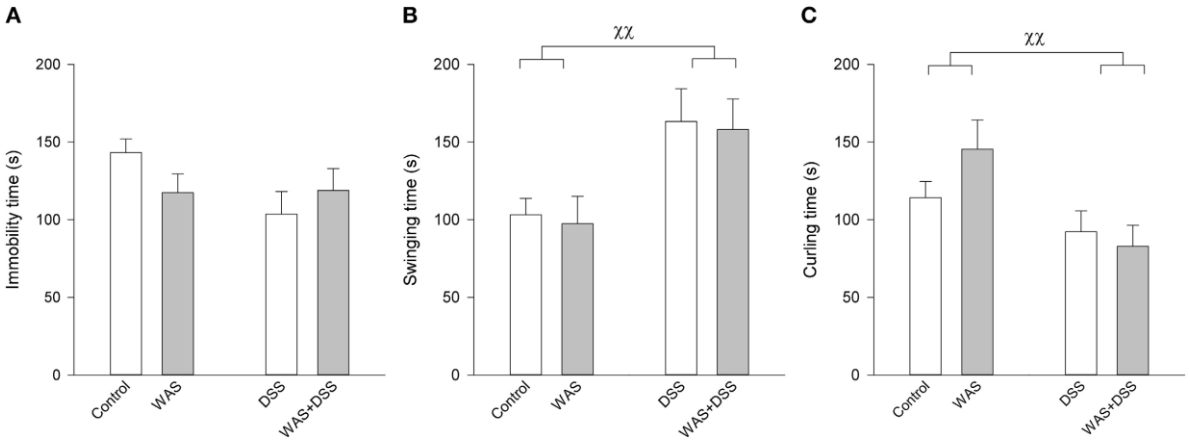


Figure 8: Effect of a 7-day treatment with DSS and WAS, alone and in combination, on behavior in the TST as carried out 3 days after the end of the treatment period. (A) Immobility time. Neither WAS nor DSS had a main factor effect, nor was there any interaction. (B) Swinging time. There was a main factor effect of DSS (p < 0.01) but not WAS. (C) Curling time. There was likewise a main factor effect of DSS (p < 0.01) but not WAS. The data shown are means + SEM, n=16-17 per group, χχ p < 0.01 DSS main factor effect. This figure was published in the original publication (Hassan et al. 2014).

3.1.4 DSS increased plasma levels of interleukin 6 (IL-6) and interleukin 18 (IL-18)

Plasma levels of IL-6 were below detection limit in the control and WAS groups. For statistical analysis the Kruskal-Wallis test was applied as the data violated normality and homogeneity assumptions of ANOVA. The Kruskal-Wallis test revealed significant differences among the treatment groups with regard to IL-6 ($H = 23.9$; $p < 0.001$) and IL-18 ($H = 19.5$; $p < 0.001$) **(Figure 9)**. Post-hoc analysis disclosed that the plasma levels of IL-6 and IL-18 were significantly higher in the DSS and WAS+DSS groups compared to the control group. No significant differences were observed between the control and WAS groups and between the DSS and WAS+DSS groups **(Figure 9)**. The plasma levels of IL-10 and IL-12 were below the detection limit in all groups.

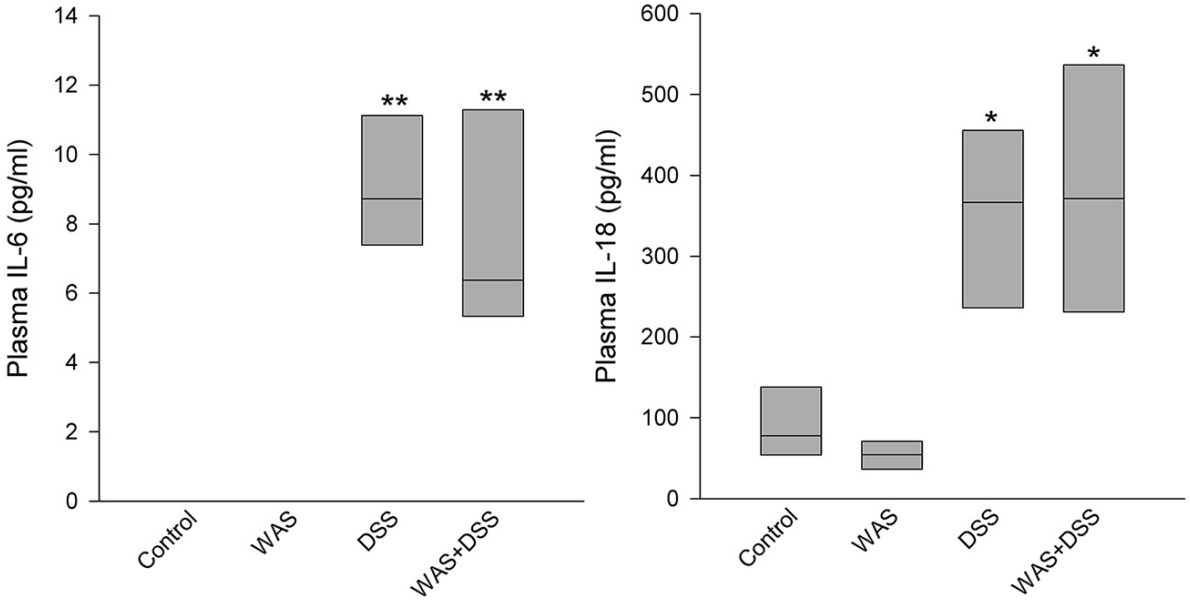


Figure 9: Effect of a 7-day treatment with DSS and WAS, alone and in combination, on plasma levels of IL-6 and IL-18 as measured 1 day after the end of the treatment period. The IL-6 plasma levels in the control and WAS groups were below detection limit. The data shown are medians ± quartiles, n = 7-8 per group; * $p < 0.05$, ** $p < 0.01$ versus control. This figure was published in the original publication (Hassan et al. 2014).

3.1.5 DSS increased plasma NPY independently of WAS

Two-way ANOVA revealed elevated plasma levels of NPY in response to DSS treatment ($F_{(1,27)}$ = 65.5; p < 0.001), with no significant effect of WAS and no significant interaction between the two factors **(Figure 10A)**.

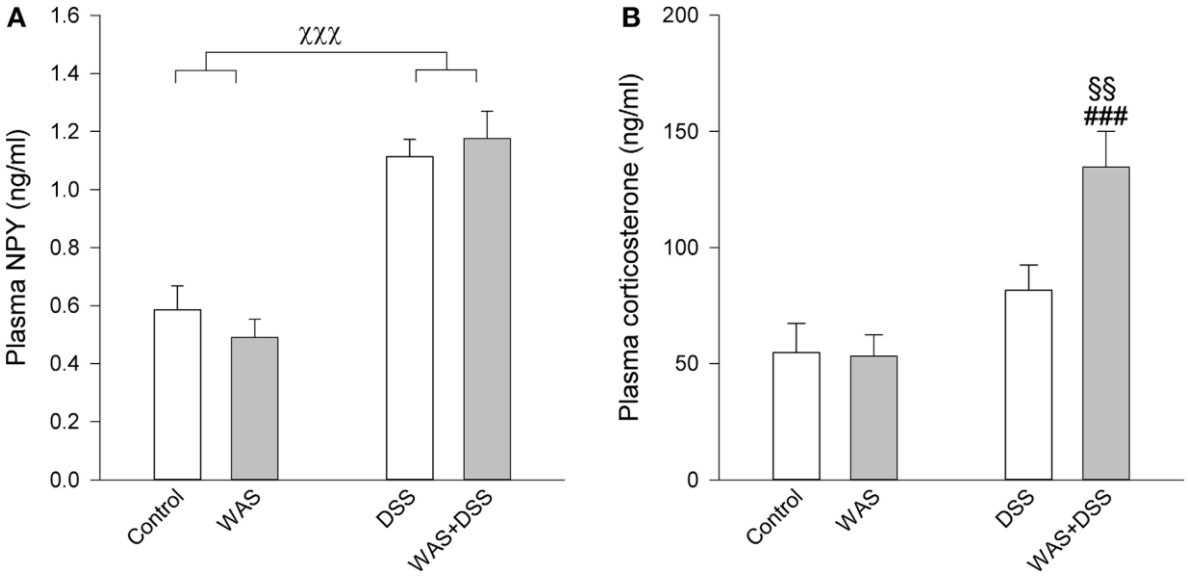


Figure 10: Effect of a 7-day treatment with DSS and WAS, alone and in combination, on plasma levels of NPY (A) and corticosterone (B) as measured 1 day after the end of the treatment period. With regard to plasma NPY, DSS but not WAS had a significant main factor effect (p < 0.001), with no significant interaction. As regards plasma corticosterone, there was a significant interaction between DSS and WAS (p < 0.05). The data shown are means + SEM, n=7-8 per group; χχχ p < 0.001 DSS main factor effect, §§ p < 0.01 versus DSS, ### p < 0.001 versus WAS. This figure was published in the original publication (Hassan et al. 2014).

3.1.6 DSS increased plasma corticosterone in the presence of WAS

DSS treatment and WAS significantly interacted with each other in modifying circulating corticosterone levels ($F_{(1,27)}$ = 5.1; p = 0.031). Post-hoc analysis revealed a significant increase in the plasma corticosterone levels in response to combined WAS and DSS treatment, while neither WAS nor DSS affected plasma corticosterone in the absence of the other factor **(Figure 10B)**.

3.1.7 DSS treatment suppressed expression of BDNF, NPY, and mineralocorticoid receptors (MR) mRNA while WAS suppressed expression of CRF mRNA in the hippocampus.

In the hippocampus, DSS treatment suppressed the relative expression of BDNF, NPY, and MR mRNA, with no significant WAS main factor effect and no significant interaction between the two factors **(Table 5)**. The DSS main factor effect statistics were: $F_{(1,25)} = 15.4$, $p < 0.001$; $F_{(1,25)} = 13.8$, $p = 0.001$; and $F_{(1,25)} = 15.4$, $p < 0.001$ for BDNF, NPY, and MR respectively. In contrast, the relative expression of CRF mRNA was reduced in response to WAS ($F_{(1,25)} = 4.8$; $p = 0.038$) but was not affected by DSS, with no significant interaction between the two factors **(Table 5)**. No significant changes could be detected with respect to the relative expression of COX-1, COX-2, and GR mRNA **(Table 5)**.

Table 5: Effect of DSS and WAS, alone and in combination, on the relative gene expression of various molecular factors in the hippocampus

mRNA	Control (n=8)	WAS (n=7)	DSS (n=8)	WAS+DSS (n=6)	WAS main factor effect	DSS main factor effect	Interaction
COX-1	1.0 (0.03)	1.0 (0.04)	0.9 (0.09)	0.9 (0.06)	NS	NS	NS
COX-2	1.0 (0.07)	1.0 (0.05)	0.9 (0.10)	1.0 (0.05)	NS	NS	NS
GR	1.0 (0.04)	1.0 (0.03)	1.0 (0.05)	0.9 (0.07)	NS	NS	NS
MR	1.0 (0.04)	1.0 (0.06)	0.8 (0.06)	0.7 (0.05)	NS	$p < 0.001$	NS
BDNF	1.0 (0.06)	1.0 (0.04)	0.8 (0.06)	0.8 (0.06)	NS	$p < 0.001$	NS
CRF	1.0 (0.10)	0.7 (0.08)	0.8 (0.10)	0.7 (0.09)	$p < 0.05$	NS	NS
NPY	1.0 (0.06)	0.9 (0.06)	0.7 (0.05)	0.8 (0.06)	NS	$p < 0.01$	NS

Animals were subjected to a 7-day treatment (days 1-7) with DSS (2 % in the drinking water) and WAS (1 hour daily), alone or in combination, and sacrificed for the assay of molecular factors in the microdissected brain on day 8. The data are presented as means with SEM given in brackets. NS, not significant. This table was published in the original publication (Hassan et al. 2014).

3.1.8 DSS treatment increased expression of cyclooxygenase-2 (COX-2) mRNA, while expression of NPY mRNA increased in response to combined WAS+DSS in the hypothalamus

In the hypothalamus, DSS treatment increased the relative expression of COX-2 mRNA ($F_{(1,24)}$ = 18; p < 0.001), with no significant WAS effect and no significant interaction between the two factors **(Table 6)**. Combined DSS and WAS treatment exerted a significant interaction on the relative expression of NPY mRNA ($F_{(1,24)}$ = 8.3; p = 0.008). Post-hoc testing revealed a significantly enhanced expression of NPY mRNA in response to the combined treatment with WAS+DSS while WAS alone and DSS alone had no significant influence **(Table 6)**. No significant changes could be detected with regard to the relative expression of COX-1, GR, BDNF, and CRF mRNA **(Table 6)**.

Table 6: Effect of DSS and WAS, alone and in combination, on the relative gene expression of various molecular factors in the hypothalamus

mRNA	Control (n=7)	WAS (n=7)	DSS (n=8)	WAS+ DSS (n=6)	WAS main factor effect	DSS main factor effect	Interaction
COX-1	1.0 (0.07)	1.3 (0.28)	0.9 (0.07)	1.1 (0.13)	NS	NS	NS
COX-2	1.0 (0.18)	0.8 (0.12)	1.6 (0.13)	1.6 (0.23)	NS	p < 0.001	NS
GR	1.0 (0.03)	1.0 (0.03)	1.0 (0.04)	1.0 (0.09)	NS	NS	NS
MR	1.0 (0.04)	1.3 (0.20)	0.9 (0.06)	1.0 (0.10)	NS	NS	NS
BDNF	1.0 (0.05)	0.9 (0.10)	0.9 (0.06)	0.9 (0.05)	NS	NS	NS
CRF	1.0 (0.12)	1.5 (0.17)	1.2 (0.10)	1.1 (0.24)	NS	NS	NS
NPY	1.0 (0.11)	1.0 (0.15)	1.6 (0.29)	2.9 (0.31) §§§ ###	p <0.05	p <0.001	p <0.01

Animals were subjected to a 7-day treatment (days 1-7) with DSS (2 % in the drinking water) and WAS (1 hour daily), alone or in combination, and sacrificed for the assay of molecular factors in the microdissected brain on day 8. The data are presented as means with SEM given in brackets. §§§ p < 0.001 versus DSS, ### p < 0.001 versus WAS. NS, not significant. This table was published in the original publication (Hassan et al. 2014).

3.1.9 DSS treatment and WAS interacted in modifying the relative expression of NPY mRNA in the amygdala

In the amygdala **(Table 7)**, DSS treatment and WAS exposure exhibited a significant interaction in modifying the relative expression of NPY mRNA ($F_{(1,25)}$ = 5.1; p = 0.033). Post-hoc analysis revealed a significantly reduction of the relative expression of NPY mRNA in response to WAS alone, an effect which was absent after combined treatment with both WAS and DSS **(Table 7)**. No significant changes could be detected in the amygdalar COX-1, COX-2, GR, MR, BDNF, and CRF mRNA expression.

Table 7. Effect of DSS and WAS, alone and in combination, on the relative gene expression of various molecular factors in the amygdala

mRNA	Control (n=8)	WAS (n=7)	DSS (n=8)	WAS+ DSS (n=6)	WAS main factor effect	DSS main factor effect	Interaction
COX-1	1.0 (0.07)	1.3 (0.28)	0.9 (0.07)	1.1 (0.13)	NS	NS	NS
COX-2	1.0 (0.13)	0.8 (0.08)	1.0 (0.09)	1.1 (0.08)	NS	NS	NS
GR	1.0 (0.03)	1.0 (0.05)	1.1 (0.03)	1.1 (0.08)	NS	NS	NS
MR	1.0 (0.04)	1.3 (0.20)	0.9 (0.06)	1.0 (0.1)	NS	NS	NS
BDNF	1.0 (0.05)	1.0 (0.08)	1.0 (0.05)	1.0 (0.05)	NS	NS	NS
CRF	1.0 (0.09)	0.9 (0.08)	1.3 (0.15)	1.1 (0.12)	NS	NS	NS
NPY	1.0 (0.06)	0.8 (0.04) *	1.0 (0.09)	1.1 (0.06) #	NS	NS	p <0.05

Animals were subjected to a 7-day treatment (days 1-7) with DSS (2 % in the drinking water) and WAS (1 hour daily), alone or in combination, and sacrificed for the assay of molecular factors in the microdissected brain on day 8. The data are presented as means with SEM given in brackets. * p < 0.05 versus control, # p < 0.05 versus WAS. NS, not significant. This table was published in the original publication (Hassan et al. 2014).

3.2 Third experiment

This experiment included a naïve group of mice in addition to the four groups of animals that were also studied in the first two experiments; control animals and mice treated with WAS, DSS, or WAS+DSS. For reasons of consistency, parameters of the naïve group were compared with those of the control group by t test, while the other four groups (including control) were compared with two-way ANOVA, similarly to the analysis in the first two experiments.

3.2.1 DSS induced colitis which was not affected by WAS

Rank transformation of the MPO data was required to meet two-way ANOVA assumptions. As in the previous two experiments, DSS induced colitis which was confirmed by increased colonic MPO content **(Figure 11A),** reduced body weight, shortened colon length, and increased colon weight **(Table 8).** A significant WAS effect was observed only with respect to body weight change, with no significant interaction between the two factors **(Table 8)**. Similarly, DSS but not WAS treatment resulted in the appearance of occult blood in the stool (for DSS $\chi^2_1 = 12.3$; $p < 0.001$). No significant differences (t test) in colitis severity parameters were observed between naïve mice and animals of the control group (data not shown).

The colonic MPO content in naïve mice (0.25±0.05 ng/mg, mean ± SEM) did not differ significantly from that in mice of the control group (0.31.07±0.07 ng/mg, mean ± SEM). Two-way ANOVA showed a significant main factor effect of DSS but not WAS on colonic MPO content (main factor effect: $F_{(1, 28)} = 29.7$; $p < 0.001$), with no significant interaction between the two factors **(Figure 11A)**.

Table 8: Effect of DSS and WAS, alone and in combination, on body weight change, colon weight, and colon length in mice used for FST

	Control n = 8	WAS n = 8	DSS n = 8	WAS + DSS n = 8	WAS main factor effect	DSS main factor effect	Interaction
Body weight change (g) on day 8	+ 0.46 (0.24)	- 0.14 (0.30)	- 2.10 (0.34)	-3.76 (0.55)	p <0.01	p <0.001	NS
Colon weight (mg/cm) on day 8	33.3 (1.33)	32.8(0.94)	50.4 (2.15)	48.1 (2.29)	NS	p <0.05	NS
Colon length (cm) on day 8	8.62 (0.18)	8.18 (0.24)	6.15 (0.16)	6.17 (0.13)	NS	p <0.001	NS

Animals were subjected to a 7-day treatment (days 1-7) with DSS (2 % in the drinking water) and WAS (1 hour daily), alone or in combination, followed by FST. The mice were sacrificed on day 8 immediately after FST. The data are presented as means with SEM given in brackets. NS, not significant.

3.2.2 Effect of FST on plasma corticosterone levels

Compared with naïve mice, animals of the control group had significantly elevated levels of plasma corticosterone ($t_{(11)}$ = 11.8; p < 0.001).

Two-way ANOVA showed a significant interaction between WAS and DSS in their effect on plasma corticosterone after FST ($F_{(1,20)}$ = 7.0; p = 0.015). Post-hoc testing revealed higher plasma corticosterone levels in the WAS+DSS group compared to DSS alone and WAS alone. The corticosterone concentrations measured in animals treated with DSS alone and WAS alone were not significantly different from those in the control group **(Figure 11B)**.

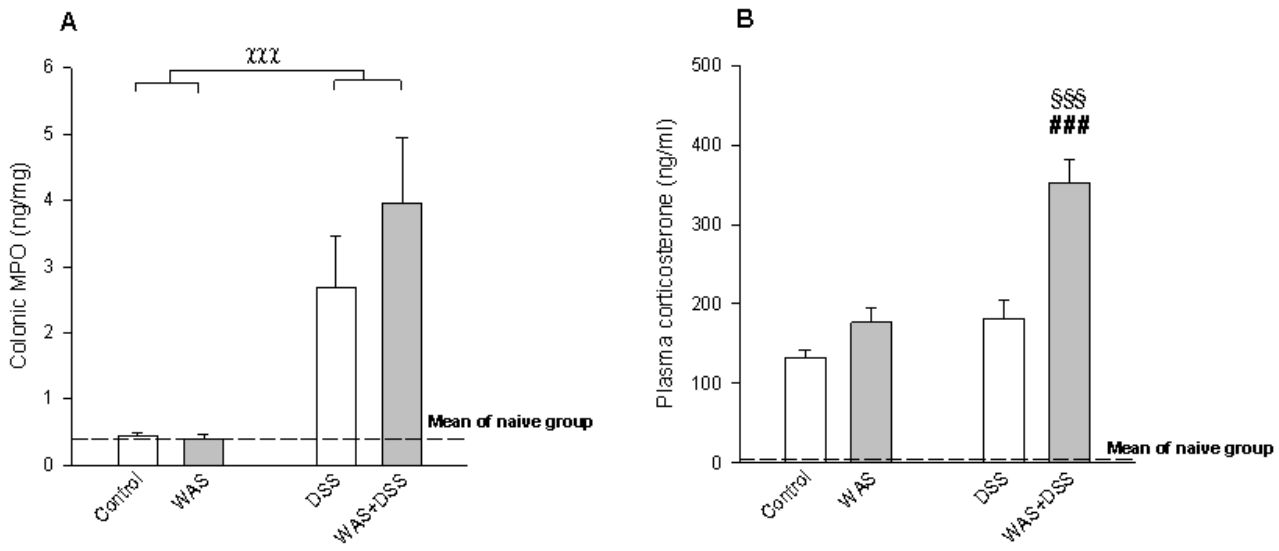


Figure 11: Effect of a 7-day treatment with DSS and WAS, alone and in combination, on colonic MPO (A) and plasma corticosterone (B) as measured immediately after FST performed on day 8. Statistical analysis was performed by two-way ANOVA. With regard to colonic MPO, DSS but not WAS had a significant main factor effect (*p* <0.001), with no significant interaction. As regards plasma corticosterone, there was a significant interaction between DSS and WAS (*p* <0.05). The data shown are means + SEM. For the naïve group, n=6 in MPO assay and 4 in corticosterone assay, while for the other groups, n=8 per group in colonic MPO assay and 5-7 in corticosterone assay; χχχ *p* < 0.001 DSS main factor effect, §§§ *p* < 0.001 versus DSS, ### *p* < 0.001 versus WAS.

3.2.3 DSS-induced colitis and WAS independently reduced immobility time and increased swimming time in the FST

To meet homogeneity assumptions, two-way ANOVA was performed on ranks of immobility time and log transformation of swimming time. No transformation was needed in the analysis of the climbing time. Two-way ANOVA disclosed a significant DSS main factor effect on immobility time ($F_{(1,28)}$ = 14.5; p < 0.001) and swimming time ($F_{(1,28)}$ = 11.1; p = 0.002), but not on climbing time. Similarly, a significant WAS effect was observed on immobility time ($F_{(1,28)}$ = 14.1; p < 0.001) and swimming time ($F_{(1,28)}$ = 13.2; p = 0.001) but not on climbing time. No significant interaction between the two factors on any of the FST parameters was identified **(Figure 12)**.

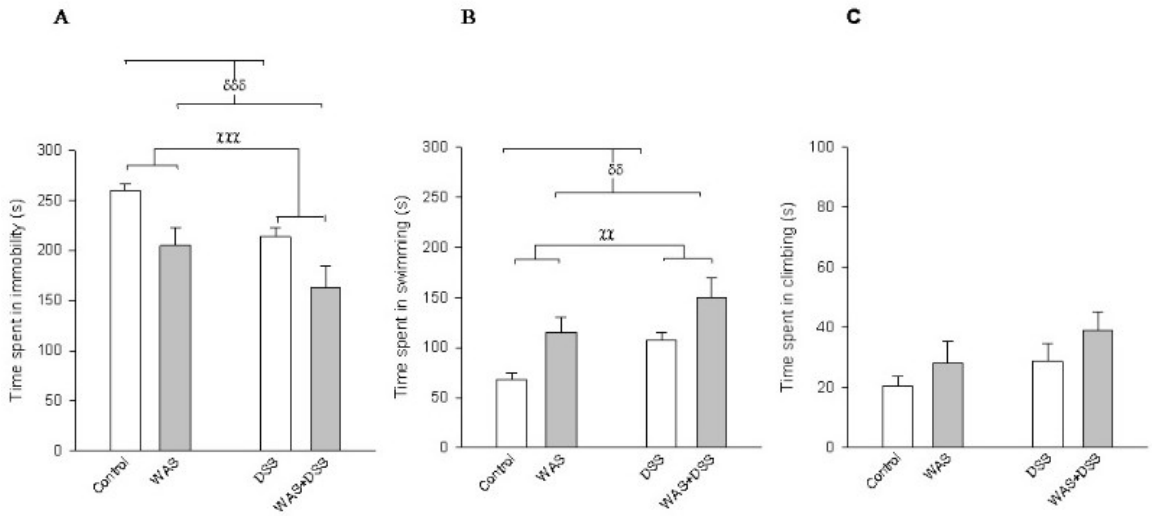


Figure 12: Effect of a 7-day treatment with DSS and WAS, alone and in combination, on behavior in the FST which was performed on the day 8 after starting the treatment. Two-way ANOVA showed main factor effects of both WAS and DSS on immobility time (A) and on swimming time (B), with no significant interaction between the two factors. No statistically significant differences could be identified with regard to climbing time (C). The data shown are means + SEM, n=8 per group; χχ $p < 0.01$, χχχ $p < 0.001$ DSS main factor effect, δδ $p < 0.01$, δδδ $p < 0.001$ WAS main factor effect.

3.3 Fourth experiment

In this experiment, the effects of DSS-induced colitis on colonic PYY and preproglucagon mRNA and on plasma PYY and active GLP-1 were evaluated.

3.3.1 DSS induced colitis

DSS treatment induced colitis as indicated by its effects on body weight change (t_{18} = 9; $p < 0.001$), colon length (t_{18} = 7.9; $p < 0.001$), and colon weight (t_{18} = -6.5; $p < 0.001$) **(Table 9)**. Occult blood in the stool was positive in all DSS-exposed mice but only in 2 control mice (χ^2_1 = 12.3; $p < 0.001$).

Table 9: Effect of DSS on body weight change, colon weight, and colon length in mice used for PYY and GLP-1 assays.

	Control n = 10	DSS n = 10	p value
Body weight change (g) on day 8	+ 0.59 (0.13)	- 3.16 (0.39)	p <0.001
Colon weight (mg/cm) on day 8	29.2 (1.34)	40.7 (1.16)	p <0.001
Colon length (cm) on day 8	8.87 (0.23)	6.17 (0.26)	p <0.001

Animals were subjected to a 7-day treatment (days 1-7) with DSS (2 % in the drinking water) relative to control animals which drank water without DSS. The mice were sacrificed on day 8. The data are presented as means with SEM given in brackets.

3.3.2 DSS-induced colitis increased colonic PYY and preproglucagon mRNA

The colons of mice that suffered from DSS-induced colitis contained significantly higher PYY mRNA ($t_{6.3}$= -2.4; p = 0.049) and higher preproglucagon mRNA ($t_{6.6}$= -4.0; p = 0.006) levels **(Figure 13)**.

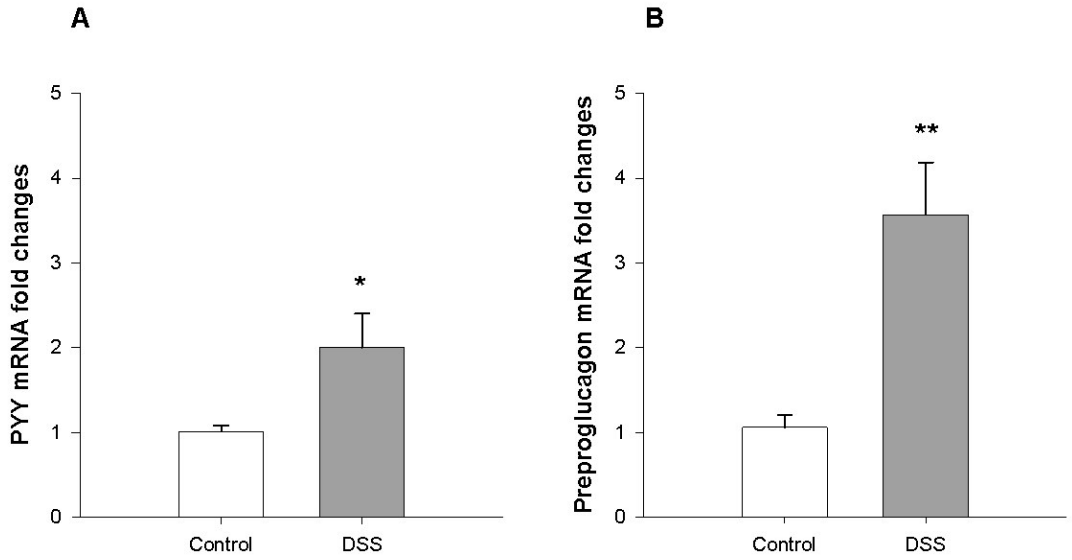


Figure 13: Effect of a 7-day treatment with DSS (2% in drinking water) on colonic PYY mRNA (A) and colonic preproglucagon mRNA (B) measured on day 8. The data shown are means + SEM, n=7 per group; * $p < 0.05$; ** $p < 0.01$.

3.3.3 DSS-induced colitis increased plasma PYY and GLP-1

DSS-induced colitis significantly increased both plasma PYY ($t_{9.8} = -5.7$; $p < 0.001$) and plasma active GLP-1($t_{15} = -3$; $p = 0.01$) levels **(Figure 14)**.

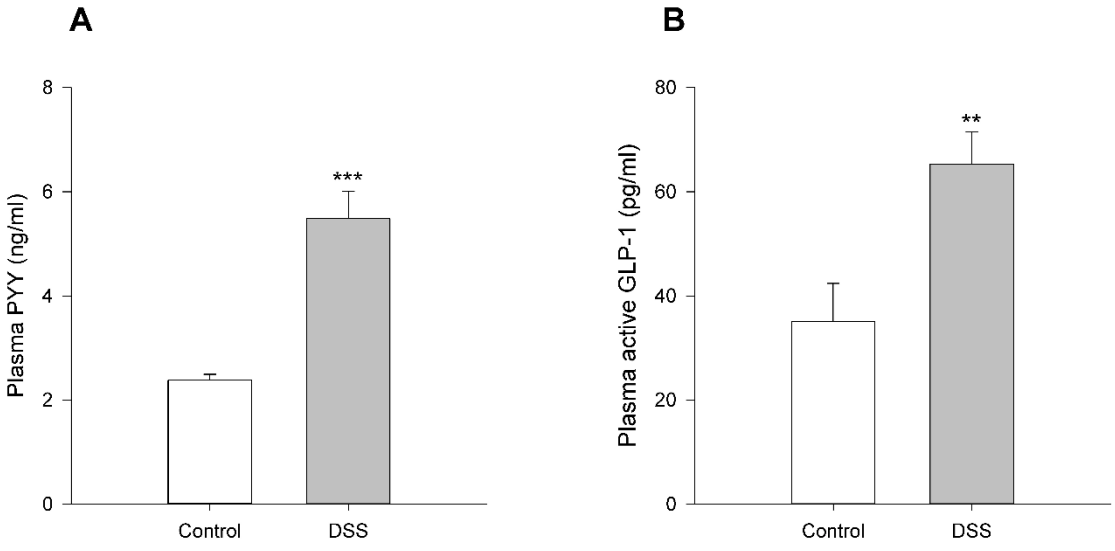


Figure 14: Effect of a 7 day treatment with DSS (2% in drinking water) on plasma PYY (n=10 per group) (A) and on plasma GLP-1 (n=10 for control and n=7 for DSS) (B). The data shown are means + SEM; ** $p < 0.01$, *** $p < 0.001$.

3.4 Fifth experiment

In this experiment, the effects of blocking Y2 receptors and GLP-1 receptors on DSS-induced colitis and on colitis-induced behavioral changes were evaluated. Mice received 2% DSS in drinking water for 7 days. On day 6, mice received BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) or their vehicles at 13:00-14:00, then LabMaster parameters were analyzed for 12 hours. On day 7, mice received BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) or their vehicles at 17:00-18:00, then LabMaster parameters were analyzed for 12 hours. On day 8 and 9, mice received BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) or their vehicles 15 minutes before the OF test and the SI test, respectively. Mice were sacrificed after the SI test, and colons and plasma were collected for MPO and corticosterone assays.

Results revealed that BII0246 + exendin (9-39) increased the colonic MPO content independently of DSS treatment. Additionally, the treatment of DSS-treated mice with BII0246 + exendin (9-39) reduced food intake and locomotor activity and led to behavioral impairment in the OF.

3.4.1 BII0246 + exendin (9-39) treatment increased colitis severity

Rank transformation of the weight change data and log transformation of the colonic MPO data was required to meet two-way ANOVA assumptions. The induction of colitis by DSS was confirmed by two-way ANOVA which revealed a significant DSS main factor effect on body weight change ($F_{(1,31)} = 70.3$; $p < 0.001$), colon weight ($F_{(1,31)} = 6.1$; $p = 0.02$), colon length ($F_{(1,31)} = 56.9$; $p < 0.001$) **(Table 10)**, and colonic MPO ($F_{(1,31)} = 73.64$; $p < 0.001$) **(Figure 15A)**. Combined treatment with BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) evoked a significant main factor effect on body weight ($F_{(1,31)} = 6.7$; $p = 0.014$) and colonic MPO ($F_{(1,31)} = 5.4$; $p = 0.027$) but not on colon length and colon weight **(Table 10 and Figure 15A)**. There was no significant interaction between the two factors in affecting the colitis severity variables. Similarly, occult blood in the stool was significantly more frequent in DSS treated mice (14 of 19 DSS-treated mice had occult blood in the stool compared to just 3 of 16 control mice) ($\chi^2_1 = 11.8$; $p < 0.001$). BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) treatment had no significant effect on occult blood in the stool.

Two mice from the DSS + vehicle group and two mice of the DSS + BII0246 + exendin (9-39) group died before the end of experiment and were not included in the analysis. Two mice (one cage) from control + vehicle group received a wrong injection on day 7, so they were not included in the analyses performed after this wrong injection. One mouse of the DSS + vehicle group was excluded from the analysis of colitis severity as it was sacrificed at a wrong time point after performing the SI test.

Table 10: Effect of DSS and BII0246 + exendin-(9-39) on body weight change, colon weight, and colon length

	Control+ vehicle n = 8	Control+ BII0246+ exendin (9-39) n = 8	DSS+ vehicle n = 9	DSS+ BII0246+ exendin (9-39) n = 10	DSS main factor effect	BII0246+ exendin (9-39) main factor effect	Interaction
Body weight change (g) on day 9	-1.03(0.28)	-1.52(0.28)	-3.48(0.36)	-5.59(0.65)	p <0.001	p <0.05	NS
Colon weight (mg/cm) on day 9	40.5(3.35)	42.3(1.51)	48.6 (5.19)	54.2(4.45)	p <0.05	NS	NS
Colon length (cm) on day 9	9.05(0.30)	8.63(0.23)	6.38(0.39)	6.42(0.31)	p <0.001	NS	NS

Animals were subjected to a 7-day treatment (days 1-7) with DSS (2 % in the drinking water) relative to control animals which drank water without DSS. BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) were injected IP on days 6, 7, 8 and 9. Mice were sacrificed on day 9 after performing the SI test. The data are presented as means with SEM given in brackets. NS, not significant.

3.4.2 DSS-induced colitis increased plasma corticosterone after IP injection of BII0246 + exendin (9-39) and the SI test

Following the IP injections of BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) and performing the SI test, the plasma corticosterone concentration was increased in DSS-treated mice ($F_{(1,31)}$ = 9.9; p = 0.004), with no significant effect of BII0246 +

exendin (9-39) treatment and no significant interaction between the two factors **(Figure 15B)**.

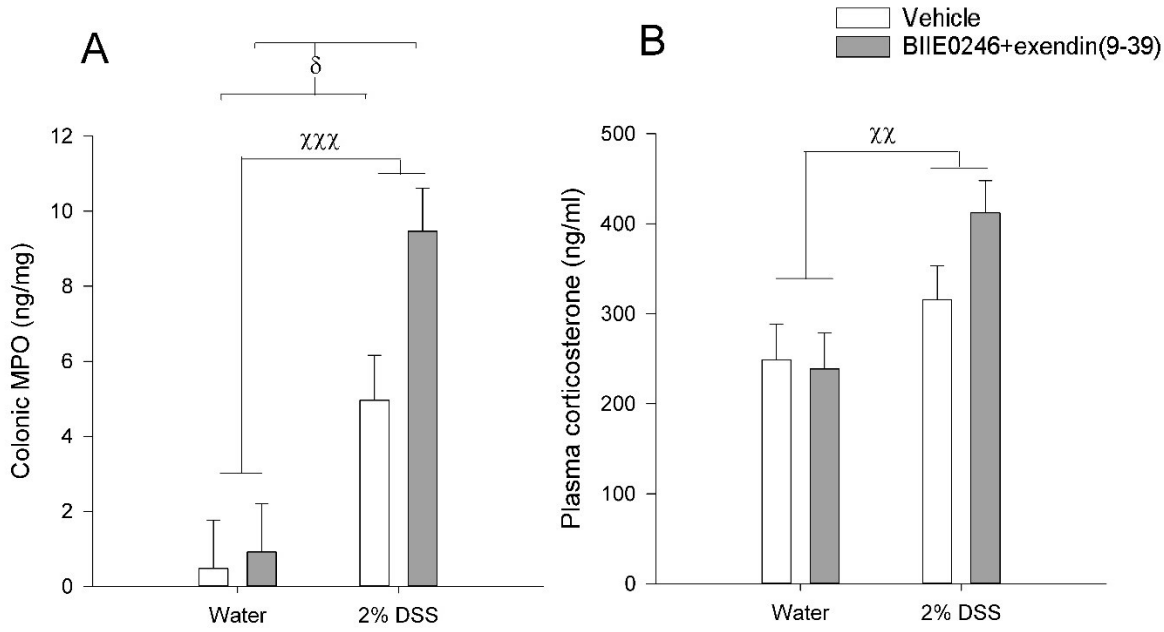


Figure 15: Effect of DSS and BII0246 + exendin (9-39) on colonic MPO (A) and plasma corticosterone (B). Mice received DSS (2% in drinking water) for 7 days (days 1-7). BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) were injected IP on days 6, 7, 8 and 9. Mice were sacrificed on day 9 after performing the SI test. DSS exerted a significant main factor effect on colonic MPO (p <0.001) and plasma corticosterone (p < 0.01). BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) had a significant main factor effect on colonic MPO (p < 0.05) but not on plasma corticosterone. The data shown are means + SEM, n = 8-10 per group; χχ p < 0.01, χχχ p < 0.001 DSS main factor effect, δ p < 0.05 WAS main factor effect.

3.4.3 Effect of BII0246 + exendin (9-39) on feeding, drinking, and locomotion in the light cycle

Food intake, fluid intake, locomotor activity and rearing as measured by the LabMaster system were assessed on day 5 as baseline variables before the treatment with BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) started. In the statistical analysis the two factors were DSS treatment and allocation to the BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) group. Rank transformation was applied on the water intake readouts to meet two-way ANOVA assumptions. However, no

significant differences between the groups could be identified with respect to food intake and locomotor activity, while a non-significant trend for a diminished water intake and rearing was observed in the DSS + BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) group **(Table 11)**.

Table 11: Food intake, water intake, horizontal activity, and vertical activity before administration of BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg)

	Control+ vehicle n =5 cages	Control + BII0246 + exendin (9-39) n = 4 cages	DSS + vehicle n = 5 cages	DSS + BII0246 + exendin (9-39) n = 5 cages	DSS main factor effect	BII0246 + exendin (9-39) main factor effect	Interaction
Food intake (g/kg body weight)	142.7 (7.1)	142.8(8.1)	141.4(6.0)	130.2(22.9)	NS	NS	NS
Water intake(g/kg body weight)	136.9(9.2)	169.7(5.6)	156.0(14.8)	128.1(18.4)	NS	NS	$p = 0.051$
Horizontal activity (counts x 10^3)	98.5(17.9)	131.2(12.1)	100.4(9.2)	92.0(9.9)	NS	NS	NS
Rearing (counts x 10^2)	63.7(16.6)	116.4(29.1)	72.2(20.5)	55.3(10.4)	NS	NS	$p = 0.091$

Animals were subjected to a 7-day treatment with DSS (2 % in the drinking water) relative to control animals which drank water without DSS. The parameters were recorded on day 5, i.e. before subsequently BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) were administered. The data are presented as means with SEM given in brackets. NS, not significant.

On day 6 mice received IP injections of BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) between hour 13:00 and 14:00 to check the effects of the blockers on the LabMaster parameters in the light cycle. In order to meet two-way ANOVA assumptions, log transformation was applied to the water intake data recorded 2 hours after the IP injection of BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) and rank transformation was applied to the vertical movement recordings 1 hour after the injection. The effects of interventions on food intake, water intake, and locomotor activity are shown in **Table 12**. A reduction of food and water intake as well as

locomotor activity was evident 12 hours after the injection of BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) in DSS-treated mice. Two-way ANOVA revealed a significant interaction between BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) and DSS treatment on food intake 1 hour ($F_{(1,15)} = 6.1$; $p = 0.026$) and 12 hours after IP injection of BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) ($F_{(1,15)} = 6.6$; $p = 0.021$). Post-hoc testing showed that mice treated with DSS ate significantly more in the first hour after the injection of vehicle. This effect was lost in mice treated with BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg). BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) significantly reduced food intake 1 hour and 12 hours after their administration to DSS-treated mice but not to control mice.

A significant interaction between the factors DSS and BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) was also observed in horizontal activity at 1 hour ($F_{(1,15)} = 7.1$; $p = 0.018$) and on vertical activity at 1 hour ($F_{(1,15)} = 4.6$; $p = 0.049$) post-injection. Post-hoc testing revealed significantly higher horizontal activity in DSS + vehicle-treated mice compared to control + vehicle-treated mice. In contrast, administration of BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) to DSS-treated mice caused a significant decrease of locomotor activity. Post-hoc testing failed to find significant differences in vertical activity among the groups 1 hour after the injection of BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg). However, a significant DSS main factor effect on horizontal activity ($F_{(1,15)} = 11.5$; $p = 0.004$) and vertical activity ($F_{(1,15)} = 6.0$; $p = 0.027$) could be identified 12 hours after the injection, with no significant effect of BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) and no significant interaction between the two factors.

Table 12: Food and water intake and locomotor activity measured 1, 2, and 12 hours after injection of BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) in the light cycle

	Control+ vehicle n = 5 cages	Control+ BII0246+ exendin (9-39) n = 4 cages	DSS+ vehicle n = 5 cages	DSS+ BII0246+ exendin (9-39) n = 5 cages	DSS main factor effect	BII0246+ exendin (9-39) main factor effect	Interaction
Food intake (g/kg body weight)							
1 hour	1.5(1.0)	2.4(0.9)	4.1(0.7)*	0.8(0.6)§	NS	NS	$p < 0.05$
2 hours	4.9(1.6)	4.5(1.6)	8.3(1.0)	5.5(1.4)	$p = 0.06$	NS	NS
12 hours	118.4(7.8)	125.0(5.5)	102.6(7.0)	71.6(7.8) ###§§	$p < 0.001$	NS	$p < 0.05$
Water intake (g/kg body weight)							
1 hour	2.1(1.2)	8.8(3.0)	8.9(3.0)	9.1(3.2)	NS	NS	NS
2 hours	3.9(1.5)	11.9(4.7)	9.4(2.8)	14.7(4.7)	NS	$p = 0.063$	NS
12 hours	120.4(7.3)	141.3(9.1)	105.1(8.0)	91.5(8.9)	$p < 0.001$	NS	$p = 0.06$
Horizontal activity (counts x 10^3)							
1 hour	0.9(0.2)	1.4(0.4)	1.7(0.2)*	0.9(0.2)#	NS	NS	$p < 0.05$
2 hours	2.2(0.4)	2.5(0.7)	3.1(0.3)	2.5(0.3)	NS	NS	NS
12 hours	70.7(12.7)	96.6(11.9)	56.1(7.4)	42.2(8.1)	$p < 0.01$	NS	$p = 0.07$
Vertical activity (counts x 10^2)							
1 hour	0.3(0.1)	0.5(0.1)	0.7(0.1)	0.3(0.2)	NS	NS	$p < 0.05^+$
2 hours	1.0(0.4)	0.8(0.2)	1.2(0.3)	1.2(0.3)	NS	NS	NS
12 hours	43.4(11.1)	104.7(40.9)	36.6(7.7)	22.9(4.8)	$p < 0.05$	NS	$p = 0.06$

Animals were subjected to a 7-day treatment with DSS (2 % in the drinking water) relative to control animals which drank water without DSS. On day 6, BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) were injected IP between 13:00 and 14:00 hours. The variables were subsequently recorded 1, 2 and 12 h post-injection. The data are presented as means with SEM given in brackets. $^+$ Significant interaction with two-way ANOVA while post-hoc testing failed to identify significant differences among the groups; * $p < 0.05$ versus control + vehicle, § $p < 0.05$ versus DSS + vehicle, §§ $p < 0.01$ versus DSS + vehicle; ### $p < 0.001$ versus control + BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg); NS, not significant.

3.4.4 Effect of BII0246 + exendin (9-39) on feeding, drinking, and locomotion when injected immediately before the beginning of dark cycle

On day 7 mice received IP injections of BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) between hour 17:00 and 18:00 (immediately before the beginning of the dark cycle) to check the effects of the blockers on the LabMaster parameters in the dark cycle. Rank transformation of vertical activity recordings was required to meet two-way ANOVA assumptions. The effects of interventions on food intake, water intake, and locomotor activity are shown in **Table 13.**

There was a statistically significant reduction of food intake in DSS-treated mice 2 hours ($F_{(1,14)} = 5.1$; $p = 0.039$) and 12 hours post-injection ($F_{(1,14)} = 12.1$; $p = 0.004$) and a statistically significant reduction of water intake 12 hours post-injection ($F_{(1,14)} = 24.8$; $p < 0.001$). No significant interaction, and no significant main factor effect of BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) on food intake was observed at any time point, while a significant interaction was observed with regard to water intake at 1 hour ($F_{(1,14)} = 4.6$; $p = 0.05$) post-injection although post-hoc testing could not reveal statistically significant differences among the groups.

BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) and DSS showed a significant interaction with respect to horizontal activity 1 hour ($F_{(1,14)} = 22.7$; $p = 0.02$), 2 hours ($F_{(1,14)} = 4.9$; $p = 0.04$), and 12 hours post-injection ($F_{(1,14)} = 13.3$; $p = 0.003$), as well as vertical activity 1 hour ($F_{(1,14)} = 9.6$; $p = 0.008$), 2 hours ($F_{(1,14)} = 5.7$; $p = 0.031$), and 12 hours post-injection ($F_{(1,14)} = 8.9$; $p = 0.01$). Post-hoc testing revealed a significant increase in horizontal and vertical activity in response to BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) administration to control animals. This increase in activity in response to BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) was not observed in DSS-treated mice in which BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) treatment actually decreased vertical and horizontal activity.

Table 13: Food and water intake and locomotor activity measured 1, 2, and 12 hours after injection of BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) immediately before the beginning of dark cycle

	Control+ vehicle n = 4 cages	Control+ BII0246+ exendin (9-39) n = 4 cages	DSS+ vehicle n = 5 cages	DSS+ BII0246+ exendin (9-39) n= 5 cages	DSS main factor effect	BII0246+ exendin (9-39) main factor effect	Interaction
Food intake (g/kg body weight)							
1 hour	8.2(3.3)	11.3(3.4)	9.2(1.5)	5.4 (1.5)	NS	NS	NS
2 hours	18.2(6.5)	26.2(47)	14.5(2.1)	11.7(2.3)	$p < 0.05$	NS	NS
12 hours	105.3(21.2)	139.1(17.2)	86.7(13.8)	67.5(6.8)	$p < 0.01$	NS	$p = 0.06$
Water intake (g/kg body weight)							
1 hour	10.5(3.5)	18.8(2.3)	16.8(2.3)	12.6(3.2)	NS	NS	$p < 0.05^{+}$
2 hours	24.6(5.1)	31.9(4.9)	23.0(4.6)	20.9(4.9)	NS	NS	NS
12 hours	122.8(13.9)	157.6(7.4)	92.4(8.8)	96.2(6.3)	$p < 0.001$	$p = 0.07$	NS
Horizontal activity (counts x 10^3)							
1 hour	3.0(0.9)	6.0(1.5)*	3.7(0.7)	2.2(0.5)##	NS	NS	$p < 0.05$
2 hours	8.6(2.5)	16.2(4.0)*	6.6(1.2)	4.3(0.7)##	$p < 0.01$	NS	$p < 0.05$
12 hours	54.3(10.9)	113.5(13.8) **	49.0(10.8)	30.7(7.0) ###	$p < 0.001$	$p = 0.08$	$p < 0.01$
Vertical activity (counts x 10^2)							
1 hour	1.1(0.4)	5.5(2.9)*	3.4(0.9)	1.6(0.9)#	NS	NS	$p < 0.01$
2 hours	4.4(1.4)	17.5(7.1)	6.1(1.7)	3.1(1.4)#	NS	NS	$p < 0.05$
12 hours	32.6(9.7)	117.7(35.3) *	42.5(12.2)	19.8(3.4) ##	$p < 0.05$	NS	$p < 0.001$

Animals were subjected to a 7-day treatment with DSS (2 % in the drinking water) relative to control animals which drank water without DSS. On day 7, BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) were injected IP immediately before the dark cycle. The variables were subsequently recorded 1, 2 and 12 h post-injection. The data are presented as means with SEM given in brackets. + Significant interaction with two-way ANOVA while post-hoc testing failed to identify significant differences among the groups; * $p < 0.05$ versus control + vehicle, ** $p < 0.01$ versus control + vehicle, ## $p < 0.001$ versus control + BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg), ### $p < 0.001$ versus control + BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg); NS, not significant.

3.4.5 BII0246 + exendin (9-39) treatment reduced exploration and induced anxiety-like behavior in mice with experimental colitis but not in control mice

On day 8, mice were injected with BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) or vehicles 15 min before the OF test to evaluate the effect of the blockers on anxiety-like behavior. Rank transformation of the total traveling distance readout was required to meet two-way ANOVA assumptions. Two-way ANOVA revealed a significant interaction between DSS and BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) treatment on all OF test parameters which included total traveling distance ($F_{(1,33)} = 8.4$; $p = 0.007$), central area visits ($F_{(1,33)} = 17.4$; $p < 0.001$), percent traveling distance in central area ($F_{(1,33)} = 10.8$; $p = 0.003$), and percent time spent in central area ($F_{(1,33)} = 14.6$; $p < 0.001$) **(Figure 16)**. Post-hoc testing revealed a significant decrease of all parameters in DSS-treated mice injected with BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) as compared mice treated with BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) alone or DSS alone **(Figure 16)**. No change in anxiety-like behavior was observed in the DSS + vehicle group compared to the control + vehicle group. In contrast, mice treated with DSS + vehicle spent more time in the central zone compared to mice of the control + vehicle group **(Figure 16)**.

3.4.6 BII0246 + exendin (9-39) had no effect on social interaction

On day 9, mice were injected with BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) or vehicles 15 min before the SI test to evaluate the effect of the blockers on social behavior. Two-way ANOVA failed to disclose any significant differences in the social interaction among the experimental groups in which the effect and interaction of DSS and BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) was studied **(Figure 17)**. Mice which had zero interaction in the first session were excluded from the analysis.

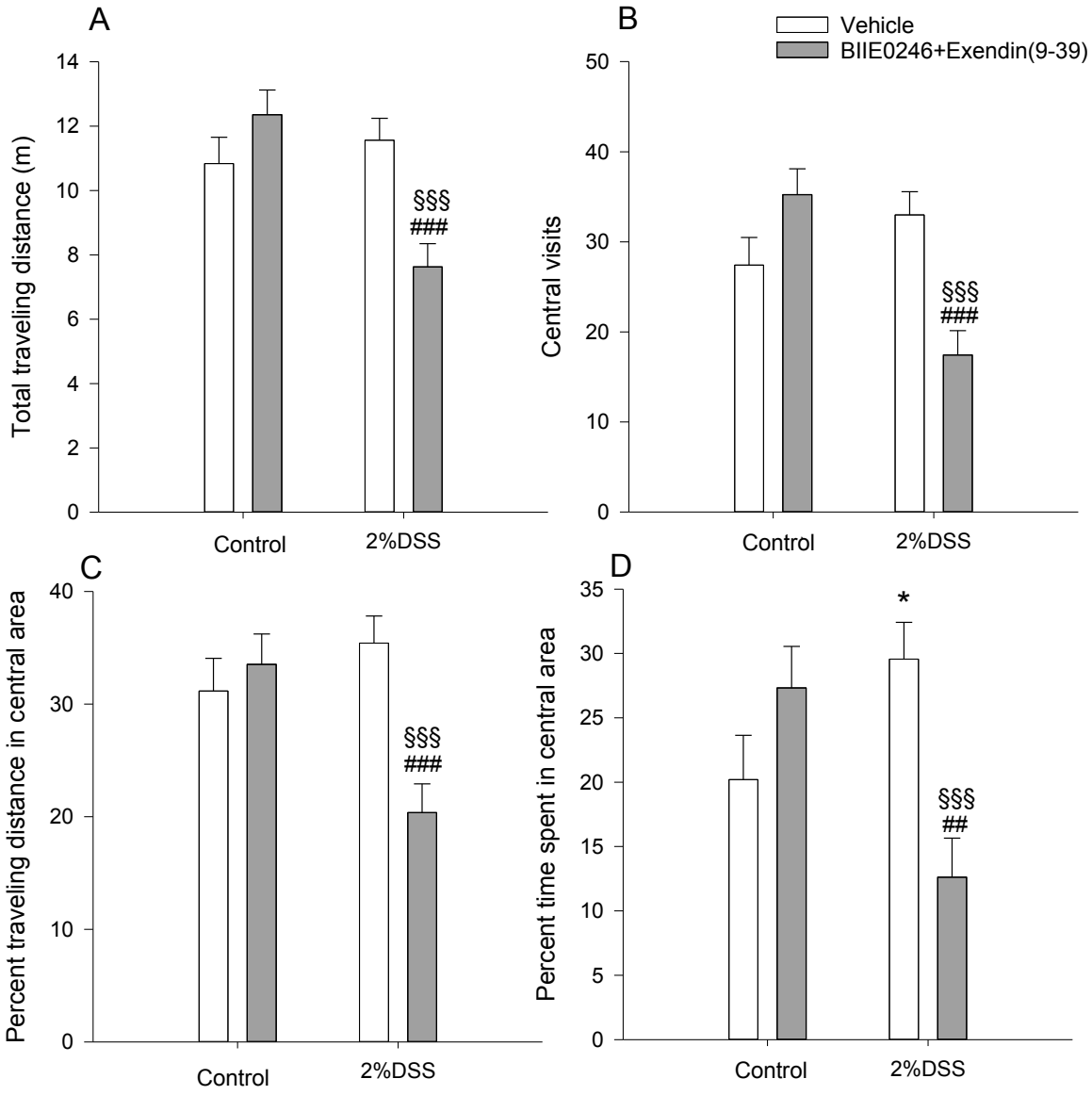


Figure 16: Effect of DSS and BII0246 + exendin-(9-39), alone and in combination, on behavioral parameters in the OF test. Mice received DSS (2% in drinking water) for 7 days (days 1-7). BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) were injected IP on days 6, 7, 8 and 9. Mice were tested in the OF on day 8. There was a significant interaction between DSS and BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) in all OF test parameters including: total traveling distance (A), central area visits (B), percentage traveling distance in central area, calculated as a percentage of the total traveling distance (C), and percentage time spent in central area, calculated as a percentage of the total time spent in the OF (D). The data shown are means + SEM, n=7-10 per group; *$p < 0.05$ versus control + vehicle, §§§ $p < 0.001$ versus DSS + vehicle, ## $p < 0.01$, ### $p < 0.001$ versus control+ BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg).

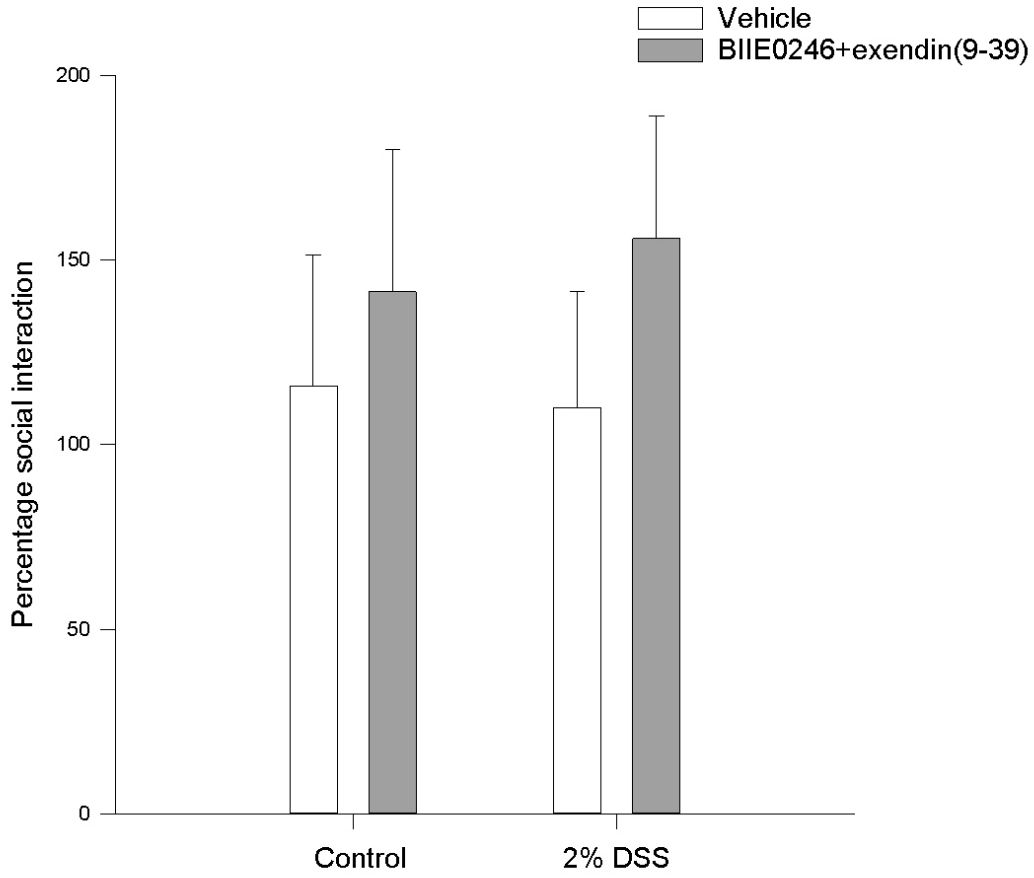


Figure 17: Effect of DSS and BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg), alone and in combination, on behavioral parameters in the SI test. Mice received DSS (2% in drinking water) for 7 days (days 1-7). BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) were injected IP on days 6, 7, 8 and 9. Mice were studied in the SI test on day 9. Two-way ANOVA failed to show any significant differences among the groups. The data shown are means + SEM, n=7-9 per group.

3.5 Sixth experiment

The results of the sixth experiment revealed that PYY$^{(-/-)}$ mice have a higher pain sensitivity. Compared to WT mice, PYY$^{(-/-)}$ mice showed enhanced pain-related behavior in response to intrarectal treatment; this effect was independent of the presence of the chemical irritant (AITC).

3.5.1 PYY$^{(-/-)}$ mice showed exaggerated pain-related behavior independent of the presence of AITC

Log transformation of total pain-related behavior, time spent freezing, latency to exhibit pain-related behavior, and time spent grooming was required to meet two-way ANOVA assumptions. Two-way ANOVA revealed a significant increase in pain-related behavior in PYY$^{(-/-)}$ mice independently of the intrarectal treatment ($F_{(1, 24)}$ = 8.8; p = 0.007) **(Figure 18A)**. The response to intrarectal 2% AITC was highly variable, and there was just a trend towards an increase in pain-related behavior in response to intrarectal 2% AITC ($F_{(1, 24)}$ = 3.2; p = 0.085). No significant interaction between genotype and intrarectal treatment with PO or 2% AITC was observed **(Figure 18A)**. The time spent freezing measured after intrarectal PO treatment tended to increase in PYY$^{(-/-)}$ mice ($F_{(1, 24)}$ = 3.1; p = 0.088), while rectal 2% AITC significantly increased the time spent freezing ($F_{(1, 24)}$ = 14.6; p < 0.001) with no significant interaction between the two factors **(Figure 18B)**. No significant effects or interactions were observed with regard to the latency to show first pain-related behavior, while the time spent grooming tended to decrease in response to intrarectal 2% AITC ($F_{(1, 24)}$ = 3.6; p = 0.068) **(Figure 18C, D)**.

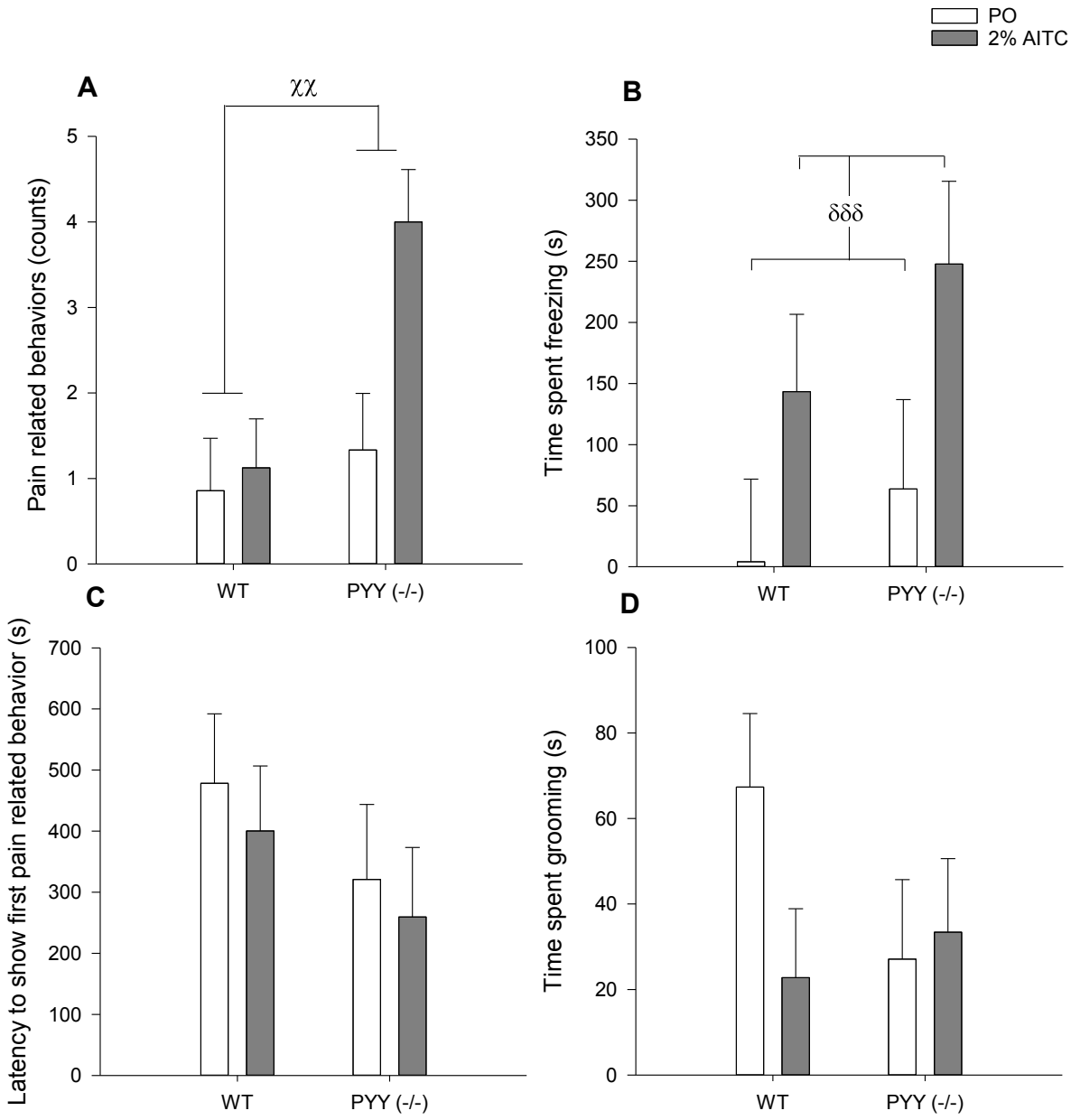


Figure 18: Effect of intrarectally administered AITC (2%, 0.1 ml) and PYY knockout (PYY$^{(-/-)}$) on pain-related behavior (A), time spent freezing (B), latency to show first pain-related behavior (C), and time spent grooming (D). Behavior was evaluated blindly from 15 min videos recorded immediately after intrarectal treatment. Two-way ANOVA revealed a significant main factor effect of genotype on pain-related behavior ($p < 0.01$) and a significant main factor effect of AITC on the time spent freezing ($p < 0.001$). The data shown are means + SEM, n=6-8 per group, χχ $p < 0.01$ genotype main factor effect, $\delta\delta\delta$ $p < 0.001$ AITC main factor effect.

3.5.2 Lack of effect of PYY knockout and intrarectal irritation with AITC on locomotor activity

No significant differences in horizontal activity, vertical activity, and traveling distance (measured for 5 min) were encountered after the rectal injection of PO and 2% AITC **(Figure 19)**. The same result was obtained when the recording period was extended to 15 minutes (data not shown).

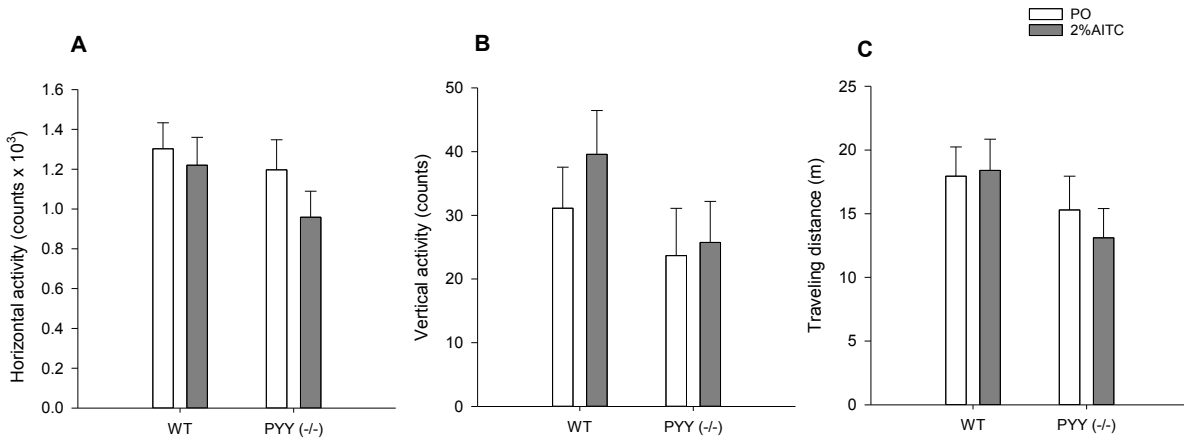


Figure 19: Effect of intrarectally administered AITC (2%, 0.1 ml) and PYY knockout ($PYY^{(-/-)}$) on horizontal locomotor activity (A), vertical locomotor activity (B), and traveling distance (C). Behavior was recorded with the LabMaster system for a 5-min period immediately after intrarectal treatment. Two-way ANOVA revealed no significant differences among the groups. The data shown are means + SEM, n=6-8 per group.

3.6 Seventh experiment

In this experiment the effects of Y2 receptor agonist PYY (3-36) (0.2 mg/kg) and the Y2 receptor antagonist BII0246 (0.03 mmol/kg) on visceral pain sensitivity were evaluated in conjunction with intrarectal administration of AITC (2%). The results revealed that the Y2 antagonist, BII0246 (0.03 mmol/kg), increased pain-related behaviors induced by intrarectal AITC while the Y2 agonist, PYY (3-36) (0.2 mg/kg) had no effect.

3.6.1 BII0246 increased pain-related behaviors induced by intrarectal AITC

The Levene's test of homogeneity revealed significantly inhomogeneous groups. As this inhomogeneity was not correctable by log transformation, one-way ANOVA with Welch's correction was applied and Games Howell post-hoc testing was used for pairwise comparisons. ANOVA with Welch's correction showed statistically significant differences among the groups with regard to pain-related behavior (Welch's $F_{(6, 21.5)}$=9.4; $p < 0.001$), time spent freezing (Welch's $F_{(6, 19.1)}$=26.1; $p < 0.001$), latency to show first pain-related behavior (Welch's $F_{(6, 18.2)}$=15.6; $p < 0.001$), and time spent grooming (Welch's $F_{(6, 18.7)}$=10.9; $p < 0.001$),.

Post-hoc test failed to reveal any significant differences in pain-related behavior counts between the PO + vehicle group and 2% AITC + vehicle group. Significantly higher pain-related behavior counts were recorded in the AITC + BIIE0246 and AITC + PYY (3-36) + BIIE0246 groups compared to the PO + vehicle and PO + BIIE0246 groups. The AITC + BIIE0246 group also exhibited significantly higher pain-related behavior counts compared to the AITC + vehicle group **(Figure 20A)**. The time spent freezing was significantly higher in all groups receiving AITC compared to PO, but no significant differences were observed within the groups receiving PO or within the groups receiving AITC **(Figure 20B)**. Similarly, the time spent grooming and the latency to show first pain-related behavior were shorter in all groups receiving AITC than in the PO group, while no significant differences were observed within groups receiving PO or within groups receiving AITC **(Figure 20C,D)**.

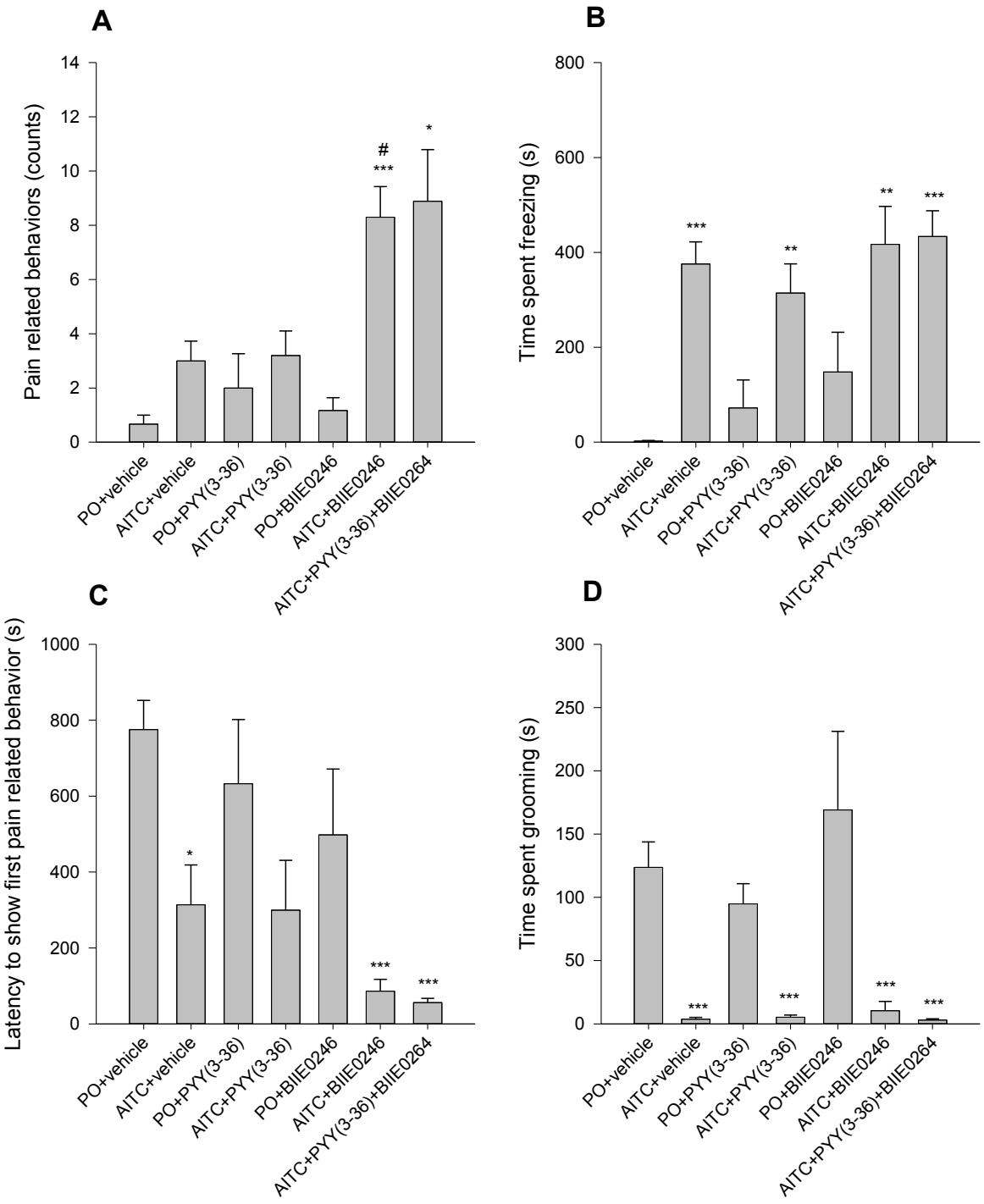


Figure 20: Effect of intrarectally administered AITC (2%, 0.1 ml), SC injected BIIE0246 (0.03 mmol/kg), and IP injected PYY (3-36) (0.2 MG/KG) on pain-related behavior (A), time spent freezing (B), latency to show first pain-related behavior (C), and time spent grooming (D). Behavior was evaluated blindly from 15-min videos recorded immediately after intrarectal treatment. One-way ANOVA revealed significant differences among the groups. The data shown are means + SEM, n=6 for PO groups and 9-12 for AITC groups; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to PO+vehicle, # $p < 0.05$ compared to AITC+vehicle.

3.6.2 Lack of effect of BII0246, PYY (3-36) and AITC on locomotor activity

Horizontal activity, vertical activity, and traveling distance measured for 5 min after intrarectal injection of PO or AITC were not significantly different among the experimental groups **(Figure 21)**. Similarly, no significant differences were observed when these parameters were recorded for 15 min (data not shown).

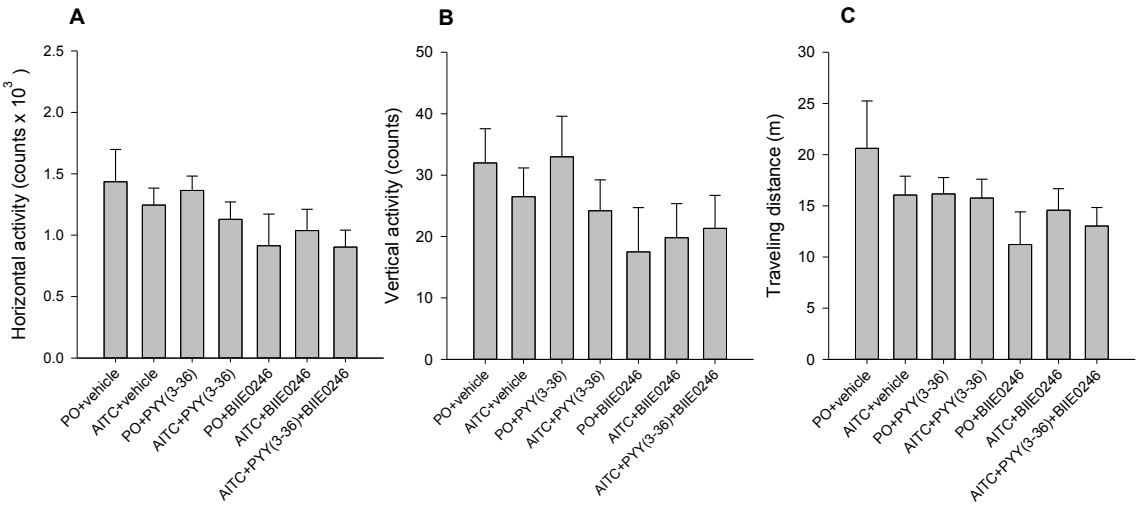


Figure 21: Effect of intrarectally administered AITC (2%, 0.1 ml), SC injected BIIE0246 (0.03 mmol/kg), and IP injected PYY (3-36) (0.2 MG/KG) on horizontal locomotor activity (A), vertical locomotor activity (B), and traveling distance (C). Behavior was evaluated with the LabMaster system for 5 min immediately after intrarectal treatment. One-way ANOVA revealed no significant differences among the groups. The data shown are means + SEM, n=5-6 for PO groups and 9-12 for AITC groups.

3.7 Eighth experiment

The results of the eighth experiment revealed that PYY$^{(-/-)}$ mice showed by trend a more pronounced MGS which approached significance level. Moreover PYY$^{(-/-)}$ mice showed more referred hyperalgesia compared to WT mice. Similar to sixth experiment, the PYY$^{(-/-)}$ genotype effects were independent on the presence of the chemical irritant AITC.

3.7.1 MGS significantly increased in response to intrarectal AITC treatment

In this experiment the concentration of AITC administered intrarectally was 1%. Two-way ANOVA revealed a significant main factor effect of intrarectal AITC on ΔMGS ($F_{(1, 23)}$ = 152.8; p < 0.001). PYY$^{(-/-)}$ mice exhibited a tendency towards a higher ΔMGS which approached statistical significance ($F_{(1, 23)}$=4.1; p = 0.054) with no significant interaction between the two factors **(Figure 22)**.

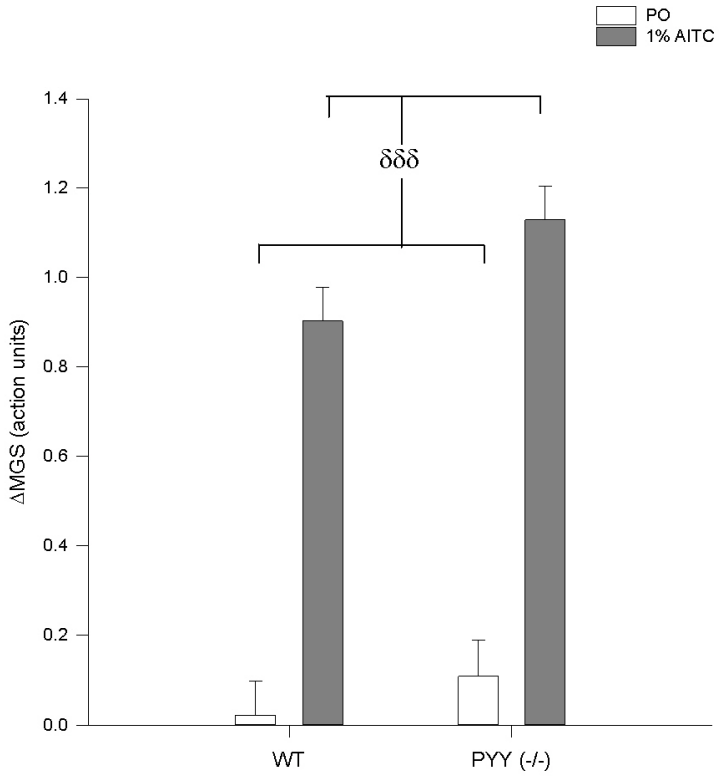


Figure 22: Effect of intrarectally administered AITC (1%, 0.05 ml) and PYY knockout (PYY$^{(-/-)}$) on ΔMGS expressed as action units. Two-way ANOVA revealed a significant main factor effect of intrarectal AITC on ΔMGS. The data shown are means + SEM, n=6-7 per group. $\delta\delta\delta$ p < 0.001 AITC main factor effect.

3.7.2 AITC and knockout of PYY significantly increased referred hyperalgesia of the paws

The mechanical pain threshold (MPT) on the hindpaws was measured in WT and PYY$^{(-/-)}$ mice before and after intrarectal treatment with PO or AITC (1%). Two-way ANOVA failed to disclose any statistically significant differences among the groups under “no pain” conditions **(Figure 23A)**. Under “pain conditions”, two-way ANOVA revealed a trend towards a lower MPT in AITC-treated mice ($F_{(1, 20)} = 3.4$; $p = 0.08$) and in PYY $^{(-/-)}$ mice ($F_{(1, 20)} = 3.5$; $p = 0.074$), with no significant interaction between the two factors **(Figure 23B)**. ∆MPT, however, was significantly changed in response to both AITC treatment ($F_{(1, 20)} = 5.5$; $p = 0.029$) and PYY knockout ($F_{(1, 20)} = 4.84$; $p = 0.04$) with no significant interaction between the two factors **(Figure 23C)**.

The quantitative MPT and ∆MPT values over the abdomen showed a similar pattern but with higher variability, and no significant differences could be detected among the groups **(Figure 24)**.

3.7.3 AITC increased spinal NPY and Y1 receptor mRNA expression

In this experiment the effect of intrarectal administration of PO and AITC (1%, 0.05 ml) on the expression of NPY as well as Y1 and Y2 receptor mRNA in the spinal cord was examined in WT and PYY$^{(-/-)}$ mice. As shown in **(Table 14)**, two-way ANOVA revealed a significant increase of NPY mRNA ($F_{(1, 18)} = 5.6$; $p = 0.029$) and Y1 receptor mRNA ($F_{(1, 18)} = 7.6$; $p = 0.013$) in response to intrarectal AITC treatment while the effect on Y2 receptor mRNA expressions was not statistically significant ($F_{(1, 18)} = 3.6$; $p = 0.075$). In PYY$^{(-/-)}$ mice a trend towards enhanced spinal NPY mRNA was seen, which was statistically not significant ($F_{(1, 18)} = 4.2$; $p = 0.055$). No effect of genotype could be detected on Y1 and Y2 receptor mRNA levels. In addition, no significant interaction between intrarectal treatment and genotype in any of the three measured mRNA was observed.

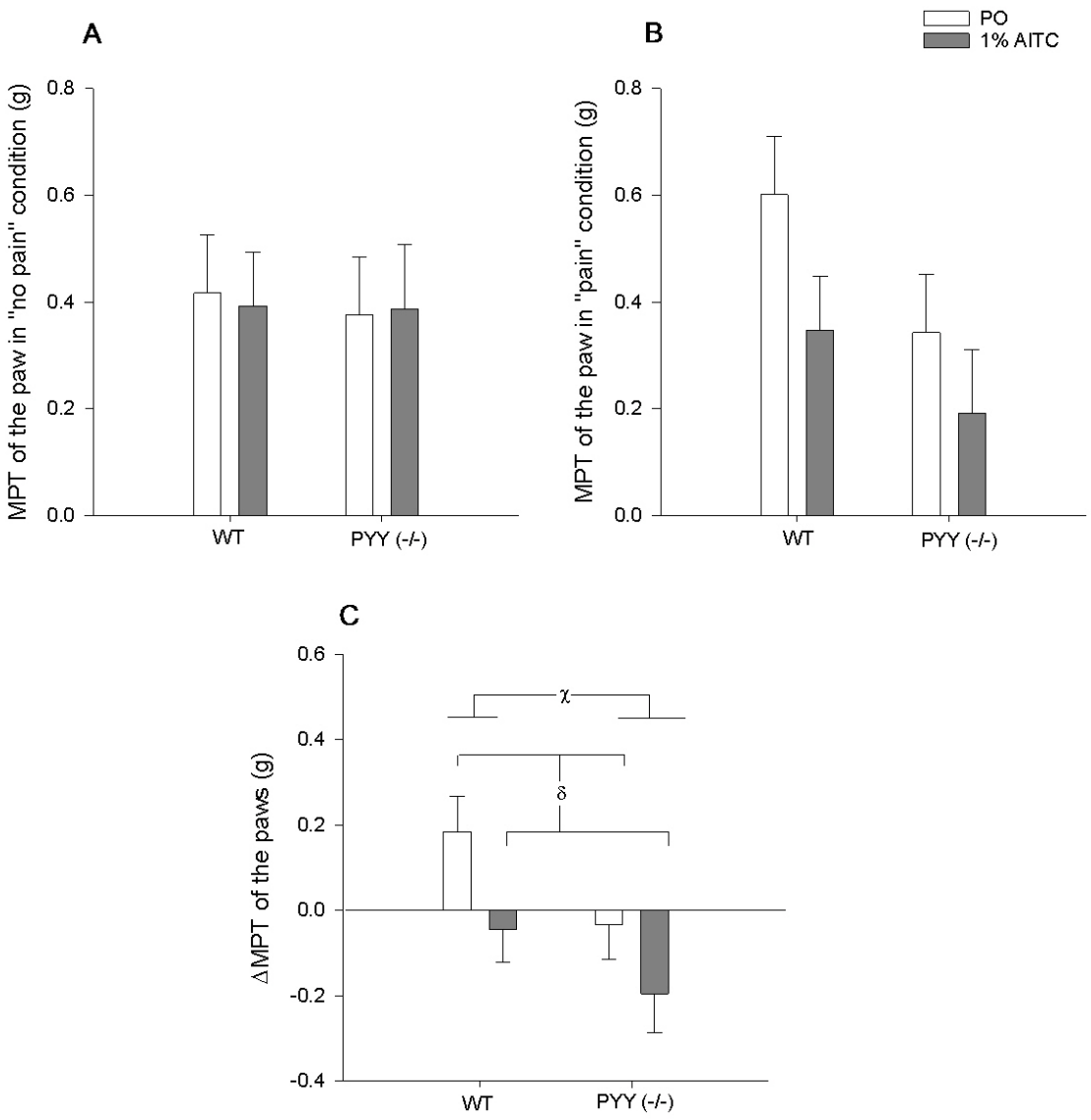


Figure 23: MPT of the paws in WT and PYY$^{(-/-)}$ mice before (A) and after (B) intrarectal administration of PO or AITC (1%; 0.05 ml) and the difference between the two measurements (ΔMPT) (C). MPT was assessed by von Frey hairs, and the values represent the average of both hind paws. With regard to ΔMPT, two-way ANOVA revealed a significant main factor effect of genotype as well as a significant main factor effect of intrarectal treatment (p <0.05). The data shown are means + SEM, n=5-7 per group; χ p < 0.05 genotype main factor effect, δ p < 0.05 AITC main factor effect.

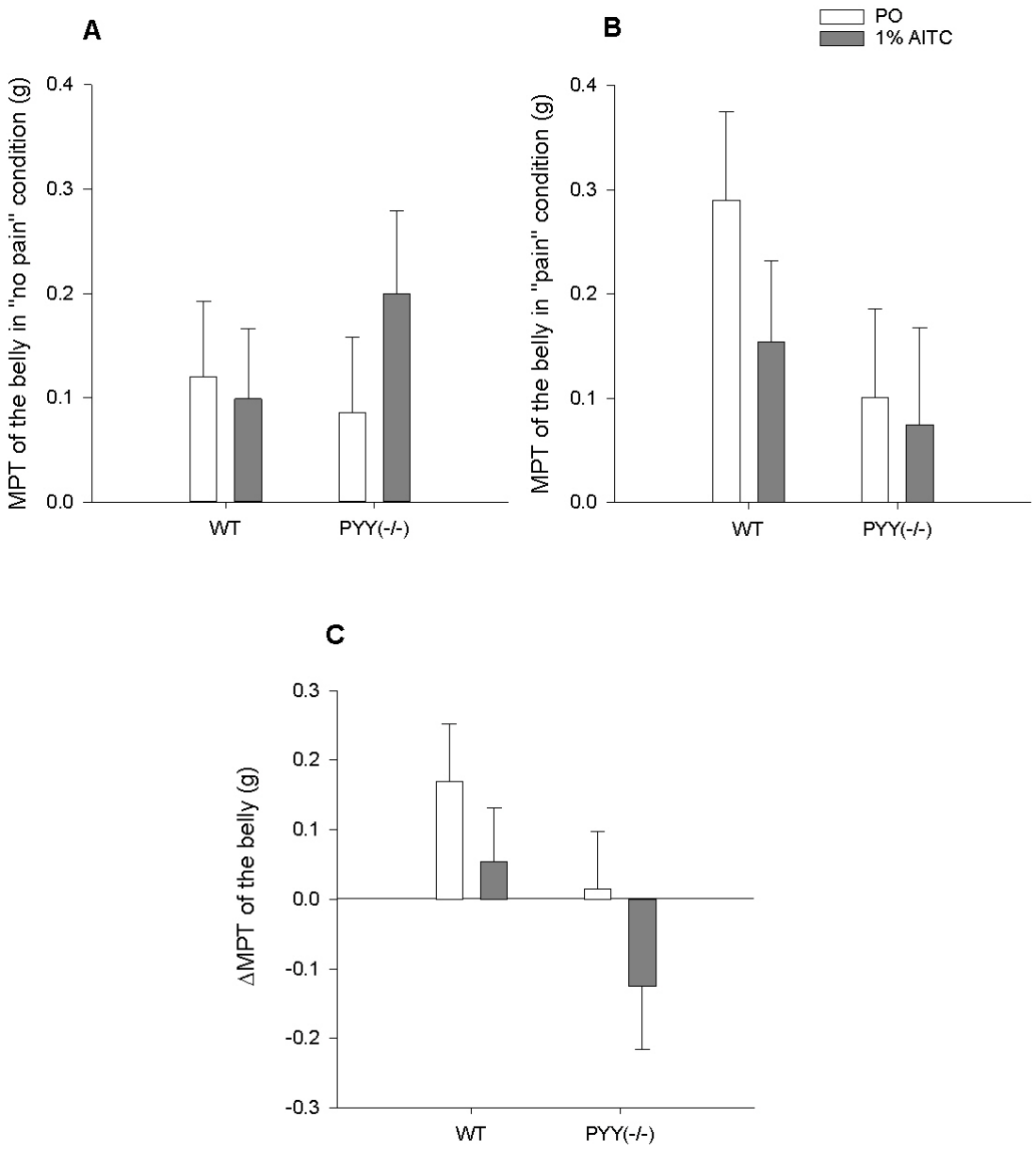


Figure 24: MPT of the belly in WT and PYY$^{(-/-)}$ mice before (A) and after (B) intrarectal administration of PO or AITC (1%; 0.05 ml) and the difference between the two measurements (C). MPT was assessed by von Frey hairs, and the values represent the average of two measurements. Two-way ANOVA failed to disclose any significant differences among the groups. The data shown are means + SEM, n=5-7 per group.

Table 14: Effect of intrarectal AITC and PYY knockout on the relative expression of NPY, Y1, and Y2 receptor mRNA in the lumbosacral spinal cord

mRNA	WT PO (n=5)	WT 1% AITC (n=6)	PYY$^{(-/-)}$ PO (n=5)	PYY$^{(-/-)}$ 1% AITC (n=6)	AITC effect	Genotype effect	Interaction
NPY	1.0(0.05)	1.2(0.09)	1.2(0.03)	1.4(0.17)	$p < 0.05$	$p = 0.055$	NS
Y1 receptor	1.0(0.04)	1.1(0.08)	1.0(0.08)	1.3(0.08)	$p < 0.05$	NS	NS
Y2 receptor	1.0(0.06)	1.2(0.08)	1.1(0.11)	1.3(0.15)	$p = 0.075$	NS	NS

WT and PYY$^{(-/-)}$ mice were intrarectally treated with PO or AITC (1%, 0.05 ml). One hour later the spinal cords were collected for measurement of mRNA expression. The data are presented as means with SEM given in brackets. NS, not significant.

4 DISCUSSION

In this work I examined gut-brain axis communication in three animal models, DSS-induced colitis, WAS, and AITC-induced colonic nociception. The work can be aligned into three studies with particular aims.

I. In the first study which included the first 3 experiments, I assessed behavioral, hormonal, and neuropeptide changes induced by experimental colitis and analyzed the effect of repeated predictable stress on these changes. The majority of these results has already been published (Hassan et al. 2014).

II. In the second study which included the fourth and the fifth experiment, I assessed the effect of experimental colitis on the gut hormones PYY and GLP-1. Furthermore I evaluated the effect of blocking Y2 receptors and GLP-1 receptors on colitis severity and colitis-induced behavioral changes.

III. In the third study which included the sixth, seventh and eighth experiment, I assessed the role of PYY in colonic pain. In the sixth experiment, I examined colonic pain in $PYY^{(-/-)}$ mice, and in the seventh experiment, I confirmed the results observed in $PYY^{(-/-)}$ mice by using pharmacological tools: the Y2 receptor agonist PYY (3-36) and the Y2 receptor antagonist BII0246. In the eighth experiment I used additional behavioral and molecular readouts to assess colonic pain in WT and $PYY^{(-/-)}$ mice.

The discussion of the related findings has been divided into three parts; each part discusses one of the three studies. The majority of the first part of discussion has been published in the original publication (Hassan et al. 2014).

4.1 Behavioral and neurochemical effects of DSS-induced colitis, repeated WAS and their combination in male C57BL/6N mice

The aim of the first three experiments was to investigate the interaction between repeated WAS, a model of mild psychological stress, and DSS-induced colitis as a model of IBD on the severity of colitis and their impact on emotional-affective behavior as well as on potential endocrine and brain molecular correlates. The main

findings of the first three experiments can be summarized in the following statements.

(i) DSS-induced colitis induced several behavioral changes such as reduced locomotion, enhanced anxiety-like behavior in the OF, reduced sociability in the SI test, altered mobility pattern in the TST, and reduced immobility in the FST.

(ii) Repeated WAS alone failed to induce colitis but enhanced locomotion in the OF, and reduced immobility time in the FST.

(iii) The combination of WAS and DSS treatment counteracted the colitis-induced anxiety-like behavior and colitis-induced hypolocomotion and reduced sociability with minimal influence on colitis severity parameters.

(iv) DSS-induced colitis led to an increase in circulating IL-6, IL-18, and NPY concentrations.

(v) DSS-induced colitis increased the expression of COX-2 mRNA in the hypothalamus, and reduced the expression of BDNF, NPY, and MR mRNA in the hippocampus.

(vi) The beneficial effects of WAS on DSS-evoked behavioral impairments was associated with a significant increase in circulating corticosterone and in the expression of hypothalamic NPY mRNA.

4.1.1 DSS-induced colitis leads to distinct behavioral alterations

In a previous work from this lab, Painsipp et al. (2011a) reported behavioral changes in response to experimental colitis in a gender-dependent manner. Although Painsipp et al. (2011a) performed their study on a different mouse strain, they made observations similar to those made in the current study as they described anxiety-like behavior but not depression-like behavior in male mice suffering from DSS-induced colitis. These consistent observations confirm the robustness of an anxiogenic phenotype in the model of DSS-induced colitis. The current work extended the observation of Painsipp et al. (2011a) by including additional behavioral tests, such as the SI test and the TST, and by assessing the impact of

WAS on colitis severity and behavioral profile. In the current work, DSS-induced colitis led to a reduction of locomotion in the OF test. In the presence of reduced locomotion it is difficult to distinguish whether the reduced time spent in, and the reduced number of visits to, the central zone of the OF as observed in DSS-treated mice reflect anxiety-like behavior or generalized sickness behavior. However, (Painsipp et al. 2011a) reported also anxiety-like behavior in the elevated plus maze test in response to DSS-induced colitis without a reduction of locomotion (Painsipp et al. 2011a), which indicates that the anxiety-like behavior in the DSS-induced colitis model is not necessarily a result of hypolocomotion or reflects generalized sickness behavior.

Social interaction was impaired in mice with colitis, a finding which is in line with social dysfunction observed in IBD patients (Casati and Toner 2000, Bernklev et al. 2005).The social isolation could be a consequence of the gastrointestinal inflammation, provided that immune challenges in rodents induce social withdrawal (Marvel et al. 2004, Dantzer et al. 2008). As with the anxiety-like behavior observed in the OF, the influence of reduced locomotion on the SI cannot be excluded.

DSS-induced colitis led to changes in the pattern of escape behavior in the TST. The TST evaluates behavioral despair in an inescapable situation as an indication of depression-like behavior (Cryan et al. 2005). While the immobility time, which is the classical and direct index of despair/depression-like behavior, was not changed in response to DSS-induced colitis, the time spent in swinging was prolonged and the time spent in curling was shortened in response to experimental colitis. Unlike the conventional analysis of TST which is restricted to the quantitation of mobility vs immobility, Berrocoso et al. (2013) described a differentiation of mobility time into curling and swinging and disclosed the pharmacological significance of this differentiation. In their work, opioid receptor agonists were found to affect mainly the time spent curling while noradrenaline and/or serotonin reuptake inhibitors affect mainly the time spent swinging (Berrocoso et al. 2013). In the current work I showed for the first time that, in the absence of a significant change in the immobility time, an intervention can lead to changes in the pattern of escape behavior. My findings require follow-up studies including appropriate neurochemical analyses to understand the ethological significance of swinging and curling.

In the FST, DSS-induced colitis led to a reduction of the immobility time and a prolongation of swimming time. A shortening of the immobility time is usually interpreted as a reduction of despair behavior and improved coping with stress, and factors and substances which reduce the immobility time in the FST are generally considered antidepressants (Cryan and Mombereau 2004). The divergent findings I saw with DSS- treated mice in the TST and FST are at variance with the mainstream of reports in the literature in which animal models of peripheral inflammation (including animals injected with LPS) usually cause a prolongation of immobility in the FST. Following injection of LPS, immobility appears at first as part of sickness behavior in which depression-like behavior is obscured by reduced locomotor activity. At later time points, locomotor impairment resolves while depression-like behavior remains (Dantzer et al. 2008). Although in a recent study TNBS-induced colitis was found to increase immobility in mice subjected to the FST (Heydarpour et al. 2016), a more comprehensive review of the literature reveals that peripheral inflammation is occasionally associated with reduced immobility in the FST. For example, Painsipp et al. (2011b) reported reduced immobility in response to LPS in group-housed C57BL/6N mice on the second day after LPS injection, while in the same work immobility was increased in group-housed CD1 mice, indicating a significant strain effect on the FST response to LPS injection (Painsipp et al. 2011b). Moreover, in an interesting finding, LPS injection could reverse immobility and disrupted microglial dysfunction induced by chronic unpredictable stress (Kreisel et al. 2014). Furthermore, the prolonged swimming time in the FST in the third experiment correlates with the increased swinging time in the TST in the first experiment, given that both behavioral traits seem to involve enhanced serotonergic signaling (Berrocoso et al. 2013, Cryan and Mombereau 2004).

I think that the converging findings of reduced immobility in the FST in response to peripheral inflammation reflect an occasional response to peripheral inflammation in rodents, indicating that the FST response to peripheral inflammation is the product of interaction of several opposing mechanisms rather than a simple one-way mechanism.

By putting all findings together, I conclude that DSS-induced colitis lead to behavioral alterations in mice which include: anxiety-like behavior, reduced locomotor activity, reduced sociability, and anorexia. DSS-induced colitis also

altered the response to an unescapable stressful situation as seen in the TST and FST, but did not cause a clear depression-like behavior.

4.1.2 Repeated WAS fails to induce colitis and to aggravate colitis severity

Psychological stress can activate the HPA axis and subsequently increase glucocorticoid secretion. Glucocorticoids can have either a colitis-suppressing effect via their anti-inflammatory properties, or colitis-promoting effects due to local immune suppression in the colon, which probably allows luminal and translocated bacteria to proliferate without constraint. Besides glucocorticoids, other factors may be involved in stress-induced effects on colitis severity, for instance, catecholamines and a leaky epithelial barrier (Reber et al. 2011, Reber 2012). Therefore, psychological stress may increase or decrease the severity of chemically induced colitis, and occasionally be devoid of any effect (Reber 2012, Reichmann et al. 2015).

In the current work, the severity of colitis was assessed via measuring colonic MPO content, colon length, colon weight, body weight and plasma IL-6 and IL-18 levels. As indicated by these parameters, DSS treatment induced colitis, while WAS failed to affect colitis severity or induce colitis on its own. It is worth noting that colon weight increased 1 day after the end of treatment with WAS+DSS (in the second experiment), an effect that could not be detected 4 days after the end of the treatment (in the first experiment). This divergence could be related either to the difference in the time points of analysis or to effects of the behavioral tests in the first experiment. Apart from its effect on colon weight, WAS treatment did not affect any of the colitis severity parameters on its own and failed to influence colitis induced by DSS.

4.1.3 Repeated predictable psychological stressors can induce stress resilience

Repeated sessions of one-hour WAS enhanced anxiety-like behavior in Wistar rats (Bradesi et al. 2005), and a single session of WAS led to activation of stress circuits in the brain as indicated by c-Fos expression (Reichmann et al. 2013). From these findings I anticipated that the WAS protocol used here would induce anxiety-like

behavior and colitis-induced behavioral changes would be exaggerated by WAS. However, the experimental findings of the first experiment rejected my hypothesis as WAS counteracted the behavioral impairments induced by experimental colitis in the OF and SI tests.

Beneficial effects of repeated predictable mild stress have been reported previously. For example, 5-min restraint stress sessions over a period of 28 days improved mood, hippocampal neurogenesis, and memory functions in rats (Parihar et al. 2011). Moreover, if applied in adolescence, the same stress protocol induced resilience against chronic unpredictable stress in adulthood (Suo et al. 2013). Stress experiences are not necessarily harmful, and exposure to a dose of stress that does not overcome stress-coping mechanisms can induce future resilience to stress and consequently prevent the development of stress-dependent psychiatric disorders (Russo et al. 2012). It has been surmised that the relationship between stress exposure and its effect on stress-coping mechanisms can be described by an inverted U shape curve; in this curve, both low and high doses of psychological stress reduce stress coping, while intermediate doses of psychological stress promote optimum stress coping mechanisms (Yuen et al. 2009, McEwen and Gianaros 2011, Russo et al. 2012).

The WAS protocol used in this study could be considered as a model of repeated predictable mild stress, given that the exposure to daily WAS followed a strict scheme in terms of duration and time of WAS. To the best of my knowledge, this is the first report that psychological stress induces resilience towards behavioral manifestations induced by experimental colitis. It is worth highlighting that the beneficial effects of WAS did not extend to all behavioral alterations induced by colitis. Thus, the DSS-evoked anorexia and the DSS-induced changes of behavioral pattern in the TST were not affected by repeated WAS. In a related observation, rats which were subjected to chronic stress developed resilience to stress-induced cognitive dysfunction but they were still sensitive to stress-induced weight loss and metabolic changes (Sweis et al. 2013). Therefore, the authors suggested that stress resilience is a domain-specific rather than a global characteristic (Sweis et al. 2013). My data support this contention.

It is worth noting that repeated WAS had some positive effects which were independent of colitis. Specifically, WAS increased locomotor activity in the OF, reduced immobility, and increased swimming in the FST, indicating that the beneficial effects of the repeated WAS sessions are not confined to behavioral changes induced by experimental colitis.

4.1.4 DSS-induced colitis leads to distinct changes in circulating pro-inflammatory cytokine levels and expression of NPY, BDNF and COX-2 in the brain

To identify potential signaling mechanisms which mediate the behavioral alterations in experimental colitis, circulating factors and cerebral factors were investigated. Since IBD is associated with an increased risk for anxiety and depression disorders (Walker et al. 2008, Graff et al. 2009), I focused on particular messenger molecules that are known for their role in the regulation of anxiety and mood, and at the same time are known for their role in gut-brain axis communication, namely, pro-inflammatory cytokines, corticosterone, NPY, BDNF, and CRF (Holmes et al. 2003, Martinowich et al. 2007, Pace et al. 2007, Bercik et al. 2010, Stengel and Taché 2010, Holzer et al. 2012).

DSS treatment for 7 days increased the circulating levels of IL-6 and IL-18, which indicates that these cytokines may play role in gut brain signaling in colitis. In contrast, the plasma levels of corticosterone were not enhanced in DSS-treated mice. The effect of DSS-induced colitis on plasma corticosterone is quite inconsistent across the literature. While some publications report unaltered corticosterone concentrations in response to colitis (Reber et al. 2006, Mitrovic et al. 2010), others report an increase of plasma corticosterone in DSS-treated mice (Reichmann et al. 2015). It is not clear whether this variability in plasma corticosterone across the literature is related to variability in colitis severity or variability in other experimental conditions, including baseline stress which is likely to vary across different animal facilities.

Plasma NPY was significantly increased in DSS-treated mice. This observation is in line with previous observations of increased NPY in the plasma of mice with TNBS-induced colitis and in enteric ganglia of DSS-treated mice (Chandrasekharan et al. 2008, Baticic et al. 2011). The observation provides additional evidence for the

engagement of the NPY system in the pathophysiology of IBD, given that the deletion of the NPY gene attenuates the severity of DSS-induced colitis in mice (Chandrasekharan et al. 2008). In contrast, repeated WAS did not alter circulating NPY, which is consistent with a previous study (Kuo et al. 2007).

In the brain, NPY is one of the most abundant neuropeptides, playing a role in the regulation of several brain functions such as food intake, coping with psychological and immunological stress, pain perception, and cognition (Heilig 2004, Morales-Medina et al. 2010, Holzer et al. 2012, Goebel-Stengel et al. 2014). Hippocampal NPY has a protective role against stress disorders; therefore, local administration of NPY into the hippocampus induces resilience against experimental models of chronic mild stress-induced depression and posttraumatic stress disorder (Luo et al. 2008, Cohen et al. 2012,). In the current work, I found that experimental colitis reduced the expression of NPY in the hippocampus, which may in part be responsible for the behavioral impairments observed in mice suffering from colitis. It is worth mentioning that the expression of NPY in the hypothalamus tended to increase in response to DSS-induced colitis, which is similar to the increase in hypothalamic NPY found in mice and rats with TNBS-induced colitis (Ballinger et al. 2001, Baticic et al. 2011). NPY is an orexigenic peptide, and the rise of its expression in the hypothalamus may reflect a counter-regulatory response to the colitis-evoked anorexia (Ballinger et al. 2001). However, in spite of the higher NPY expression in mice with colitis, anorexia still persisted, which indicates that the upregulation of NPY was not able to normalize food intake under colitis conditions.

Neuroinflammation is involved in the pathophysiology of several mental disorders including major depression (Miller et al. 2009). Extension of peripheral inflammation to the brain is one of the possible mechanisms which can lead to neuroinflammation and consequently to mental disorders (Berk et al. 2013). The current work provided an example of such an extension of GIT inflammation to the brain as indicated by the enhanced expression of hypothalamic COX-2 in DSS-treated mice. COX-2 has been involved in LPS- and IL1β-induced anorexia (Asarian and Langhans 2010). PGE2 which is produced by COX-2 is likely involved in the anorexia induced by DSS-induced colitis, provided that LPS- and IL-1beta-induced anorexia could be blocked by COX-2 but not COX-1 inhibitors (Lugarini et al. 2002, Asarian and Langhans 2010). Anorexia is also a symptom of IBD and was consistently reported

to be present in animal models of IBD, including DSS-induced colitis in mice (DeBoer et al. 1985, Ballinger et al. 2000). This anorexic effect may in part be explained by the increase in hypothalamic COX-2 gene expression and by the subsequently increased prostaglandin production in the brain under colitis conditions. Interestingly, WAS which did not affect hypothalamic COX-2 mRNA, did not alter food intake.

The effects of corticosteroids in the brain are mediated by two types of receptors; MR and GR. GR have a 10 fold lower affinity for corticosterone than MR; thus, GR are only activated when levels of corticosterone are markedly increased either in response to stress or during the peak of its circadian rhythm. The two receptors have two distinctive functions: while MR promote the initial stress coping behavior, the GR activation pathway acts as a break for the initial MR reaction, causing suppression of the acute stress response towards baseline conditions and initiating long-term stress coping behaviors. According to the MR:GR balance hypothesis, MR and GR work in a complementary manner; any imbalance between them leads to HPA axis dysfunction and behavioral impairment (de Kloet 2013). This MR:GR imbalance and HPA axis dysfunction have been shown in chronic stress-induced depression and maternally deprived infant rats (Vázquez et al. 1996, Lopez et al. 1998). Since DSS treatment affected hippocampal MR but not hippocampal GR expression, it is likely that DSS treatment disrupted the MR:GR balance in the hippocampus.

DSS-induced colitis reduced hippocampal BDNF mRNA, which may contribute to the behavioral phenotype in DSS-treated mice. Anxiety-like behavior has been reported in mouse models of impaired BDNF signaling (Martinowich et al. 2007). In humans, a systematic review of the studies concluded that circulating BDNF is lowered in individuals with anxiety disorders and attributed the inconsistency of the results to different sampling procedures used and different anxiety subtypes studied (Suliman et al. 2013). The data of the current work support previous findings of reduced hippocampal BDNF levels being associated with anxiety-like behavior induced by GIT inflammation (Bercik et al. 2010) and emphasize the involvement of hippocampal BDNF in gut brain axis signaling.

4.1.5 Repeated WAS-induced resilience towards colitis-related behavioral disturbances may involve the HPA axis and hypothalamic NPY

Since repeated WAS protected from some of the behavioral impairments which were induced be experimental colitis, it was of particular interest to explore which signaling pathways were specifically altered in the WAS+DSS group and thus could be involved in the relative resilience observed in this group. Among the targets under study, two systems were significantly amplified in WAS+DSS-treated mice compared to the other treatment groups: plasma corticosterone and hypothalamic NPY. Both systems are known for their protective effects towards inflammation-induced behavioral impairments. Combined WAS+DSS treatment for 7 days significantly increased plasma basal corticosterone, while WAS alone or DSS alone failed to modify basal plasma corticosterone levels. This observation was robust even after FST which caused a several-fold rise of plasma corticosterone, but the relative relationship in circulating corticosterone levels between the 4 groups was still intact. In a similar finding, Reber et al. (2006) reported that the diurnal circulating corticosterone was not affected by DSS-induced colitis alone or chronic stress alone, while the combination of the two treatments elevated circulating corticosterone (Reber et al. 2006). The elevated plasma corticosterone in the presence of combined experimental colitis and chronic psychological stress could be explained by the synergistic effects of inflammation and psychological stress on the HPA axis, since pro-inflammatory cytokines can stimulate the HPA axis (Turnbull and Rivier 1995). In the current work, hypothalamic CRF was not affected by experimental colitis. However, the effect of cytokines on the HPA axis is not necessarily mediated via CRF, as there is evidence that pro-inflammatory cytokines could affect the adrenal gland themselves. For example, IL-6 can stimulate ACTH-induced release of corticosterone from rat primary adrenal gland cells (Salas et al. 1990), and the cortisol response to ACTH correlates with the blood IL-6 concentration in healthy humans (Zarkovic et al. 2008). On the other hand, a protective effect of endogenous corticosterone against the behavioral perturbations induced by inflammation has been repeatedly and consistently reported in several models of inflammation including injection of LPS, IL-1β and viral infection. In these models, the behavioral perturbations were enhanced by adrenalectomy or glucocorticoid antagonists (Goujon et al. 1995, Johnson et al. 1996, Pezeshki et al. 1996, Silverman et al. 2007, Wang et al. 2011). Therefore, the increase in circulating

corticosterone in response to the combined WAS+DSS treatment is likely to have a role in the relative resilience observed in this group.

The combined WAS+DSS-treatment enhanced the expression of NPY mRNA in the hypothalamus compared to either WAS alone or DSS alone. A role of NPY and NPY receptors in promoting resilience towards psychological stressors (Cohen et al. 2012, Russo et al. 2012, Sweis et al. 2013) and peripheral immune challenge (Painsipp et al. 2008, Painsipp et al. 2013) is well established. In analogy with other data (Thorsell et al. 2006, Holzer et al. 2012, Sweis et al. 2013), I postulate that NPY expression in the arcuate nucleus of the hypothalamus is of relevance to the relative resilience which was observed in the WAS+DSS group. Though there is no direct evidence indicating the implication of hypothalamic NPY in stress resilience, neurons from the arcuate nucleus project to several brain regions that are well known for their involvement in the stress resilience effects of NPY including lateral septum, amygdala, periaqueductal grey, and locus coeruleus (Kask et al. 2002). In addition, hypothalamic NPY interacts with the HPA axis and this interaction exists in both directions. On the one hand, circulating glucocorticoids can increase hypothalamic NPY and, on the other hand, hypothalamic NPY can stimulate the HPA axis and increase circulating corticosterone (Krysiak et al. 1999). As research on hypothalamic NPY was mainly concerned with its role in the regulation of food intake and to a lesser extent with HPA axis regulation, my data indicate a potential role in stress resilience, an implication which requires further confirmation.

In conclusion, the results of the first three experiments show that the DSS model of IBD has a good face and construct validity as regarding IBD-associated mental disturbances. The DSS-induced behavioral syndrome includes a reduction of locomotion and exploration, an increase in anxiety, a decrease in social activity, and anorexia. This behavioral phenotype is associated with enhanced expression of COX-2 in the hypothalamus and reduced expression of BDNF, MR, and NPY in the hippocampus. Repeated predictable WAS prevents the DSS-induced alterations of locomotion, anxiety and social interaction, but not DSS-induced anorexia. The resilience induced by repeated predictable stress involves stimulation of the HPA axis and upregulation of NPY in the hypothalamus.

4.2 Role of PYY and GLP-1 in DSS-induced colitis in C57BL/6N mice

Gut hormones play an important role in gut-brain signaling, but their role in IBD remains largely unclear. In study 2 of this thesis, involving the fourth and fifth experiment, I investigated the status and the role of two related gut hormones in experimental colitis in mice, PYY and GLP-1. I chose to screen the effect of both hormones together, as both hormones are secreted by L cells and both share several effects on gut and feeding behavior. Accordingly, there is good reason to investigate both hormones in combination in human and rodents studies since they have an additive effect (Neary et al. 2005, De Silva et al. 2012).

The results I obtained showed that PYY and active GLP-1 are increased in mice with experimental colitis. To better understand their pathophysiological roles I used pharmacological blockers of Y2 receptors and GLP-1 receptors. The pertinent experiments revealed a potential protective role of PYY and GLP-1 in experimental colitis, since mice treated with Y2 and GLP-1 blockers had enhanced colonic MPO, decreased body weight, increased anorexia, and impaired performance in the OF.

4.2.1 DSS-induced colitis increases plasma PYY and active GLP-1

In the fourth experiment, DSS-induced colitis caused a several-fold increase of colonic PYY and preproglucagon mRNA. Consistently with the colonic mRNA increase, the plasma levels of PYY increased by more than 2-fold and the plasma levels of active GLP-1 rose by more than 1.5 fold. To my best knowledge, this is the first report to show that circulating levels of PYY and GLP-1 are enhanced in experimental colitis in mice. In contrast, Hirotani et al, (2008) found a reduction of PYY in the large intestine of rats suffering from DSS-induced colitis. In the study by Hirotani et al. (2008), intestinal PYY levels were normalized relative to the wet weight of the intestine. The relevance of these wet weight-normalized PYY values may be questioned as colon weight increases in DSS-induced colitis. Thus it is not clear whether the colitis-evoked change in of PYY observed by Hirotani et al. (2008) reflected a true reduction of intestinal PYY. Unfortunately, neither colon weights nor plasma levels of PYY were reported in the paper by Hirotani et al. (2008). It cannot go unnoticed that the measurements of PYY conducted in the present study need to be considered with caution because of the lack of specificity of the PYY EIA kit in

use. Nevertheless, the parallel increase of intestinal PYY mRNA and the rise of plasma GLP-1 which is secreted from the same enteroendocrine cells supports the contention of a simultaneous release of PYY and GLP-1 into the circulation. Further support for this argument can be deduced from the findings that COX-2 and its product PGE_2 are increased in the intestine of IBD patients and rodents with DSS-induced colitis (Wang and Dubois 2010, Gravaghi et al. 2011), because PGE_2 increases the secretion of both PYY and GLP-1 from the L cells via the prostaglandin E_2 receptor 4 (Coskun et al. 2013).

Total plasma PYY but not plasma GLP-1 was found to be increased in patients suffering from active small bowel CD (Moran et al. 2013). In another study, Moran et al. (2012) reported an increase of GLP-1 mRNA in the terminal ileum of patients suffering from CD. Plasma GLP-1 has also been reported to be increased in patients suffering from UC (Keller et al. 2009). These reports of increased PYY and GLP-1 production in IBD patients indicate that my finding of increased PYY and active GLP-1 in mice suffering from DSS-induced colitis has translational significance. It is worth noting that contradictory reports of decreased intestinal PYY in subjects suffering from IBD also exist (Tari et al. 1988, El-Salhy et al. 2002).

4.2.2 BII0246 + exendin (9-39) treatment increases colonic MPO and induces weight loss

PYY (3-36) is a psychogenic compound in rodents (Stadlbauer et al. 2013), and the role of both PYY (3-36) and GLP-1 as satiety signals is well established in rodents and humans (De Silva and Bloom 2012). The fifth experiment was designed to assess the role of elevated PYY and GLP-1 levels in the behavioral changes that occurred in mice suffering from DSS-induced colitis, especially anorexia which might be related to increased PYY (3-36) and GLP-1 production. The effects of PYY (3-36) on food intake are stress-sensitive and might be blocked if mice are overstressed (Batterham et al. 2004). To reduce experimentation-related stress on mice while examining the effect of pharmacological blockers, I evaluated their effects on food intake, fluid intake, and locomotion while the mice were housed two per cage in their home cage. This was possible by the use of the LabMaster system. Additionally, the mice received three injections of saline over three days to habituate them to the IP injections.

BII0246 and exendin (9-39) were injected IP as there is sufficient evidence from the literature that peripherally injected BII0246 and exendin (9-39) can block peripherally injected PYY (3-36) and GLP-1, respectively. Circulating PYY (3-36) can cross the blood brain barrier and work directly on Y2 receptors in the brain (Nonaka et al. 2003). Although the Y2 blocker BII0246 does not cross the blood brain barrier (Brothers et al. 2010), peripherally administrated BII0246 can efficiently block the anorexic effect of peripherally administrated PYY (3-36) (Scott et al. 2005, Talsania et al. 2005). This can be explained by the involvement of vagal afferent Y2 receptors in the anorexic effect of PYY (3-36), which is supported by the loss of the anorexic effect of PYY (3-36) in rats after vagotomy (Koda et al. 2005). In mice the situation is more complicated. Although vagotomy fails to block the anorexic effect of PYY (3-36) in mice (Halatchev and Cone 2005), peripherally injected BII0246 can still block the anorexic effect of the hormone (Talsania et al. 2005). The GLP-1 peptide is found in gut, circulation and brain. The satiety effect of GLP-1 can be observed after both peripheral and central administration of the peptide or its analogues. Exendin (9-39) is an inverse agonist at GLP-1 receptors (Serre et al. 1998). In rats exendin (9-39) is effective when injected IP to block the effects of peripherally injected GLP-1 while centrally injected exendin (9-39) blocks the effect of centrally injected GLP-1 (Williams et al. 2009).

BII0246 and exendin (9-39) affected two important colitis severity parameters, body weight and colonic MPO. Weight loss in mice treated with the antagonists may be secondary to the colonic inflammation in these mice or could be related to blockade of Y2 receptors in the periphery. I base the latter argument on the finding that adult-onset conditional knockdown of Y2 receptors in peripheral tissues has been found to reduce weight gain and adiposity and increase energy expenditure in mice receiving a high-fat diet (Shi et al. 2011).

4.2.3 BII0246 + exendin (9-39) reduce food intake and locomotion in DSS-treated mice

To evaluate a role of PYY and GLP-1 in food and fluid intake and locomotor activity in colitis, DSS-treated mice and control mice were treated with BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) or vehicle on day 6 at 13:00 – 14:00 hours and on day 7 at 17:00 – 18:00 hours. Following this treatment, locomotion, food and water intake

were recorded with the LabMaster system. For quantitative assessment the readouts were calculated at 1 hour, 2 hours, and 12 hours post-injection. On both day 6 and 7, a statistically significant DSS effect was observed 12 hours after the injection; at this time point, DSS-treated mice ate less, drank less and moved less. Interestingly, mice treated with DSS + vehicle ate more than control mice in the first hour after the injection in the light cycle which may indicate stress-induced feeding in the DSS + vehicle group in the light cycle. This observation in DSS-treated mice was abolished by blockade of Y2 and GLP-1 receptors. A significant interaction between DSS and BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) was observed in locomotor activity 12 hours post-injection when mice received the injection at the beginning of dark cycle; under these conditions mice which received DSS + BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) had lower locomotion counts compared to the DSS alone or BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) alone groups, which is consistent with the results of the OF test.

In the OF, mice receiving DSS + BII0246 (2 mg/kg) + exendin (9-39) (0.2 mg/kg) exhibited impaired performance in all OF parameters compared to mice receiving DSS + vehicle. The behavior of mice with colitis receiving DSS+ BII0246 + exendin (9-39) in the OF can be interpreted either as sickness behavior or anxiety-like behavior. This type of behavior was absent in the DSS + vehicle group, indicating a protective role of endogenous Y2 and/or GLP-1 receptor ligands against development of this type of behavior in mice with colitis.

Mice treated with DSS + vehicle did not show the behavioral profile that was observed in the first experiment, which was to some extent consistent with what was observed by Painsipp et al. (2011a). The loss of the DSS-induced behavioral pattern (anxiety-like behavior and reduced sociability) in the DSS + vehicle group of the fifth experiment is probably due to the different experimental conditions, as in the fifth experiment mice received IP injections only 15 minutes before behavioral testing. Moreover, mice received several injections during the experiment, so the experimental setting of the fifth experiment is not comparable to that of the first experiment.

Social interaction was reduced in the first experiment in DSS-treated mice. Since PYY (3-36) has been found to reduce social interaction (Stadlbauer et al. 2013), I

wanted to investigate whether social isolation observed in DSS-treated mice is related to the enhanced plasma levels of PYY following DSS treatment. This was not possible, however, as the interventions in the fifth experiment abolished several of behavioral effects of DSS-induced colitis, including social isolation.

In conclusion, the findings of the fifth experiment reject the hypothesis of an involvement of PYY and GLP-1 in the anorexia and social isolation seen in mice with experimental colitis undisturbed by IP injections. To the contrary, PYY and GLP-1 whose formation was increased in mice suffering from colitis seem to have a protective effect against colitis itself and against colitis-induced behavioral changes. Concerning the effect of blocking Y2 receptors, the results are rather in line with previous observations of Painsipp et al. (2008, 2013), who reported on a protective role of Y2 receptors and PYY against behavioral disturbances evoked by LPS and BCG immune challenges. The behavioral effects of the Y2 and GLP-1 receptor blockers might be secondary to their effect on colitis severity, and follow-up studies are required to analyze the individual effects of PYY and GLP-1, and to confirm the protective effect of PYY and/or GLP-1 in colitis with different pharmacological and genetic approaches.

Although the role of Y receptors in the nociception is well established (Brumovsky et al. 2007), relatively little is known about the specific role of PYY in pain regulation. Given the increased colonic expression of PYY mRNA in mice suffering from colitis, I investigated a possible implication of PYY in visceral pain which is a key component of gut-brain axis communication. To this end I examined the effects of the $PYY^{(-/-)}$ genotype and of Y2 receptor ligands on visceral pain sensitivity. $PYY^{(-/-)}$ mice showed enhanced pain-related behavior in response to intrarectal treatment which was independent on the presence of the chemical irritant, AITC. In analogy with these results, the Y2 receptor antagonist BII0246 also increased pain-related behaviors in response to rectal AITC. With regard to mechanical pain sensitivity of the paws, $PYY^{(-/-)}$ mice exhibited a similar MPT as WT animals at baseline, but after intrarectal treatment had the paw MPT lowered to a greater extent than WT mice. In $PYY^{(-/-)}$ mice there was also a trend towards an enhanced grimace response to intrarectal treatment, which however was not statistically significant. These results are consistent with a previous observation of increased thermal pain sensitivity in $PYY^{(-/-)}$ mice (Jain et al. 2012) and attest to an involvement of PYY in the regulation of somatic and visceral pain.

4.3.1 $PYY^{(-/-)}$ mice show enhanced pain-related behavior in response to intrarectal treatment

Laird et al. (2001) described a model of pain-related behavior and visceral hyperalgesia for the assessment of visceral pain in rodents, which is relatively less invasive compared to other visceral pain models that may require surgery. This model was adapted by using LabMaster monitoring of locomotor activity as an indicator of visceral pain instead of referred hyperalgesia (Jain et al. 2015). In the sixth and seventh experiment, I used pain-related behaviors in addition to locomotor activity as recorded with the LabMaster system in response to intrarectal AITC treatment to evaluate visceral pain sensitivity. The main observation of the sixth experiment was increased pain-related behavior in $PYY^{(-/-)}$ mice compared to WT mice independently of the type of intrarectal treatment. The response of the WT mice to rectal nociception was not typical, as they showed very little pain-related

behavior following AITC treatment. The reason for this observation is not clear but may reflect a strain effect, given that the genetic background of the animals is different from that of C57BL/6 mice.

4.3.2 The Y2 receptor blocker BIIE0246 exaggerates visceral pain while the Y2 receptor agonist PYY (3-36) has no effect

PYY (3-36), the circulatory form of PYY, has a higher affinity towards Y2 receptors than PYY itself (Holzer et al 2012). In the seventh experiment I checked the effects of the Y2 receptor agonist PYY (3-36) (0.2 mg/kg) and the Y2 receptor antagonist BII0246 (0.03 mmol/kg) on visceral pain induced by 2% AITC. The results revealed a visceral hyperalgesic effect of BII0246 (0.03 mmol/kg), while PYY (3-36) (0.2 mg/kg) had no effect. The in vivo failure of PYY (3-36) to induce expected pharmacological effects is a common observation in the literature (Tschöp et al. 2004). The inconsistent efficacy of PYY-(3-36) might be due to handling of the animals, a poor pharmacokinetic profile in the rodents, or concomitant activation of Y1 and Y5 receptors, as PYY (3-36) is not totally receptor-selective. These limitations may be solved in future studies by using more selective peptides or by PEGylating PYY (3-36) to improve its pharmacokinetic profile (DeCarr et al. 2007).

4.3.3 Colonic 1% AITC induces a clear increase in MGS

To overcome the limitation that WT mice exhibited little pain-related behavior in response to intrarectal AITC treatment, I tried to further substantiate a role of PYY in nociception by studying the MGS and referred hyperalgesia. The MGS has been described by Langford et al. (2010) as a novel model for assessment of the nociceptive response to several noxious stimuli including IP acetic acid and magnesium sulfate. In the eighth experiment I evaluated the MGS in mice in response to 1% AITC, as an add-on readout for pain that can be combined with recording of pain-related behavior and referred hyperalgesia described by Laird et al. (2001). A combination of these methods may overcome strain-related differences in the nociceptive responses to noxious stimuli, provided that, e.g., the MGS recording does not interfere with the other recording methods. Though the WT mice did not show the typical pain-related behavior described by Laird et al. (2001) in response to intrarectal AITC instillation, they exhibited a very prominent MGS response: the ΔMGS was more than 20 fold larger in response to 1% AITC than

after administration of PO. Facial expressions reflect a component of the pain experience which is different from pain-related behaviors of the trunk as they represent the function of different CNS levels in the pain pathway. For that reason, chemical lesions of the rostral anterior insula in mice causes attenuation of the MGS response without affecting abdominal constriction behavior (Langford et al. 2010). Consequently, it is not surprising to find a clear effect of AITC on MGS in WT mice while trunk pain-related behaviors were very sparse. In the same context, the PYY genotype effect was not as clear with regard to the MGS reaction as it was with regard to trunk pain-related behaviors and referred hyperalgesia on the paw.

4.3.4 PYY$^{(-/-)}$ mice show enhanced referred hyperalgesia on the paw

Visceral pain leads to increased sensitivity on the corresponding dermatome, which can be used to evaluate visceral pain in humans and experimental animals as it is proportional to visceral pain severity (Laird et al. 2001, Drewes et al. 2003). In the original method described by Laird et al. (2001), referred hyperalgesia was assessed by applying 10 stimuli of 5 von Frey hairs over the paws, lower abdomen and tail before and after colonic administration of noxious stimuli. With this protocol, some 300 potentially painful stimuli need to be tested in total, which is exhausting for the animal and cumbersome for the examiner. The SUDO method is a well validated method for the assessment of mechanical pain threshold over the paw with just 5 applications of von Frey hairs (Bonin et al. 2014). I used the SUDO method to assess visceral hyperalgesia in response to intrarectal AITC and was able to detect statistically significant visceral hyperalgesia on the paw but not on the lower abdomen. Like the lower abdomen, the hind paw is a somatotopically appropriate location for referred pain from the colon, and referred hyperalgesia over the hind paw can be used as an indicator for visceral pain severity (Laird et al. 2001).

It is worth noting that there was no significant interaction between intrarectal AITC treatment and PYY genotype in all parameters including pain-related behavior, MGS, and referred hyperalgesia, indicating that the enhanced response of PYY$^{(-/-)}$ mice to intrarectal treatment is independent of AITC. Intracolonic administration of vehicle or AITC is likely to cause some distension and mechanical irritation of the colon. In AITC-treated mice, another pain component is added to the mechanical irritation of the colon: stimulation of transient receptor potential ankyrin 1 (TRPA1)

and transient receptor potential vanilloid 1 (TRPV1) ion channels which are activated by AITC and give rise to pain because they are expressed by nociceptive afferent nerve fibers (Mitrovic et al. 2010, Everaerts et al. 2011). In the absence of a statistically significant interaction between AITC response and PYY genotype, the hypersensitivity of $PYY^{(-/-)}$ mice could be interpreted to indicate increased sensitivity of $PYY^{(-/-)}$ mice towards the mechanical component of intrarectal treatment which takes place in both vehicle-treated and AITC-treated mice.

4.3.5 Potential mechanisms explaining the role of PYY in controlling pain sensitivity

PYY can activate all types of Y receptors through its two isoforms PYY (1-36) and PYY (3-36) (Neary and Batterham 2009). Y1 and Y2 receptors are widely expressed over the primary somatic and visceral pain pathways, including the skin and colon, the dorsal root ganglia, the inferior ganglion of the vagus nerve, and the dorsal horn of the spinal cord (Ghilardi et al. 1994, Zhang et al. 1997, Goumain et al. 1998, Brumovsky et al. 2005, Landry et al. 2000). In this study, intrarectal nociceptive stimulation by AITC increased the expression of NPY and Y1 receptors in the spinal cord, which is consistent with analogous findings in somatic pain (Ji et al. 1994). The NPY system with its different Y (Y1, Y2, Y4, Y5) receptors has a regulatory role on pain perception, and knockout of Y1 receptors is associated with thermal, chemical, and mechanical somatic hyperalgesia and exaggerated acetic acid- and MgSO4-induced visceral pain (Naveilhan et al. 2001, Holzer et al. 2012). Analgesic effects of NPY ligands have been reported repeatedly, and electrophysiological studies confirm the role of both Y1 and Y2 receptors in controlling postsynaptic currents in various neuronal circuits (Smith et al. 2007, Moran 2004). NPY acting through Y1 receptors controls the production of several neuropeptides including substance P and calcitonin gene related peptide (CGRP), which subserve pronociceptive functions in the CNS (Brumovsky et al. 2007). In addition to their effect on spinal pain pathways, Y receptor ligands affect visceral pain mediated by vagal afferent pathways. In a previous study from this lab, Y2 receptor knockout and Y4 receptor knockout mice had a higher c-Fos expression in the NTS following intragastric acid challenge (Wultsch et al. 2005). Furthermore, Y1 receptors are involved in the descending control of pain signaling; for example, NPY injection into

the arcuate nucleus reduces somatic pain in rats, an effect which could by blocked by Y1 but not Y2 receptor antagonists (Li et al. 2005).

Since BII0246 acts primarily by blocking peripheral Y2 receptors (Brothers et al. 2010), it is likely that peripheral Y2 receptors play a role in controlling visceral pain signaling. Y2 receptors are expressed by primary sensory neurons in in the skin and colon of mice (Goumain et al. 1998, Brumovsky et al. 2005; Brumovsky et al. 2007), which may be of relevance to the hyperalgesia observed in $PYY^{(-/-)}$ mice and in mice treated with the Y2 antagonist BII0246.

The observation of increased visceral pain sensitivity in $PYY^{(-/-)}$ mice and in BIIE0246-treated mice could have a translational value. The density of PYY-producing cells is reduced in patients suffering from IBS (El-Salhy et al. 2014). This finding is consistent enough to use the density of PYY-producing cells in the colon as a clinically useful marker for the three subtypes of IBS (El-Salhy et al. 2015). The results of this thesis suggests that the decreased PYY content of the lower GIT of IBS patients may contribute to enhanced visceral pain sensitivity, mostly due to reduced peripheral Y2 signaling. Future therapeutic strategies for IBS may target Y2 signaling in the colon with more advanced Y2 receptor agonists which have a better pharmacokinetic/pharmacodynamic profile compared to the conventional Y2 agonist PYY (3-36).

In conclusion, endogenous peptide YY has a hypoalgesic effect against visceral pain, which is likely mediated by peripheral Y2 receptors. Peripheral Y2 receptors could be a target in future therapeutic strategies for visceral pain.

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