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An investigation of the impact of experimental colitis
and inflammatory bowel disease on behaviour and on
systemic and central inflammation



Thesis submitted for the degree of Doctor of Philosophy

By

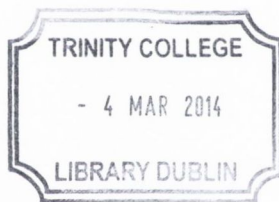
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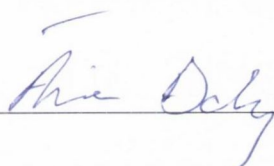


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Áine Abautret-Daly
January 2013

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Áine Abautret-Daly
January 2013

The absence of stress is death

—HANS SELYE

Summary

Inflammatory bowel diseases (IBD) are chronic conditions characterised by uncontrolled inflammation of the intestinal mucosa. Clinical symptoms include weight loss, diarrhoea, rectal bleeding and abdominal pain. In addition to physical symptoms, patients with IBD are at increased risk of depression and anxiety. Whether these psychological disturbances occur due to stress associated with the unpleasant symptoms of IBD, or as a result of biological mediators of inflammation is unknown. It has been suggested that pro-inflammatory cytokines themselves can induce mood changes, or that cytokines (particularly interferon γ (IFN γ)) can induce depression and anxiety by degrading tryptophan to kynurenine via induction of indolamine 2,3 dioxygenase (IDO). In addition, tryptophan can be metabolised to kynurenine via tryptophan 2,3 dioxygenase (TDO).

The main aim of this thesis was to investigate the association between colonic inflammation or IBD symptoms and mood/anxiety disturbances in patients, and to examine the implications of colonic inflammation on central markers of immune activation in animal models of colitis. 18 IBD patients and 19 patient controls were scored using the Hamilton-depression (HAM-D), HAM-anxiety (HAM-A), profile of mood states (POMS), and disease activity scores (Inflammatory bowel disease questionnaire, Mayo index, and Crohn's disease activity index). Intestinal biopsies were analysed by PCR for interleukin-6 (IL-6), IL-1 β , tumor necrosis factor TNF α , inducible nitric oxide synthase (iNOS), IFN γ , IDO, and matrix metalloproteases 9 (MMP9). Whole blood PAXgene analysis of IFN γ , IDO, MMP9, and iNOS was examined by PCR. Circulating concentrations of IL-6, IFN γ , and C-reactive protein (CRP) were analysed by ELISA, and kynurenine and tryptophan concentrations were examined by High performance liquid chromatography (HPLC). A significant increase in HAM-D scores was found, which was independent of the psychological impact of acute symptoms, but was associated with increased colonic and circulating cytokine expression and kynurenine:tryptophan ratio.

Two models of colitis were established in Wistar rats. Dextran sulphate sodium (DSS) induced symptoms of ulcerative colitis, ultrastructural damage to the intestinal wall, increase inflammatory gene expression in the distal colon, and behavioural disturbances in commonly used rodent behavioural tests (open field, light/dark box, elevated plus maze, marble burying). Colonic inflammation was associated with increased circulating kynurenine:tryptophan ratio, and increased hippocampal IL-6, IL-1 β , iNOS, tryptophan and

decreased serotonin. iNOS expression remained increased up to 7 days post DSS administration in the frontal cortex, hippocampus and hypothalamus. The trinitrobenzenesulphonic acid (TNBS) model was used for comparative purposes and confirmed the main findings from the DSS model: upregulation of central inflammatory mediator, decreased circulating tryptophan, increased central tryptophan, and behavioural disturbances. The influence of restraint stress on DSS-induced colonic inflammation was examined at various time points. The most significant stress-induced enhancement of colitis was with initial stress exposure immediately prior to commencing DSS. This resulted in increased disease activity index, enhanced weight loss, and increased MMP9 and IL-6 expression within the colon compared to non-stressed colitic rats.

Together these results suggest that inflammatory mediators may influence anxiety and/or mood state in IBD patients, however, the psychological implications of chronic illness will need further investigation. Irrespective of the cause, this indicates that IBD patients should be screened for depression or anxiety over the course of the disease.

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List of Abbreviations

3-HK	:	3-Hydroxykynurenine
3-HAA	:	3-Hydroxyanthranilic Acid
5-HT	:	Serotonin
5-HIAA	:	5-Hydroxyindolacetic Acid
AG	:	Aminoguanidine
ALE	:	Adverse Life Events
BBB	:	Blood Brain Barrier
BDI	:	Becks Depression Index
BAS	:	Beck Anxiety Scale
B5A	:	Bovine Serum Albumin
B5I	:	Big Five Inventory
CC	:	Colon Cancer
CDI	:	Children Depression Inventory
CD	:	Crohn's Disease
CRP	:	C-reactive Protein
CES-D	:	Centre for Epidemiological Studies-Depression Scale
CGAS	:	Children's Global Assessment Scale
CHQ	:	Child Health Questionnaire
CRS	:	Cold Restraint Stress
CSK	:	Clinical Score of Kozarek
CSTAS	:	Cognitive and Somatic Trait Anxiety Scale
CSF	:	Cerebrospinal Fluid
CNS	:	Central Nervous System
CUMS	:	Chronic Unpredictable Mild Stress
DAB	:	Di-Amino-Benzidine
DAI	:	Disease Activity Index
DAQ	:	Disease Activity Questionnaire

DNA	:	Deoxyribonucleic Acid
DSS	:	Dextran Sulfate Sodium
DSQ	:	Defense Style Questionnaire
DPX	:	Di-N-Butyl Phthalate in Xylene
ECM	:	Extracellular Matrix
ELISA	:	Enzyme Linked Immuno Sorbent Assay
EPM	:	Elevated Plus Maze
ESR	:	Erythrocyte Sedimentation Rate
FAD	:	Flavin-Adenine Dinucleotide
FMN	:	Flavin Mononucleotide
FRET	:	Fluorescence Resonance Energy Transfer
FST	:	Forced Swim Test
FQCI	:	Freiburg Questionnaire of Coping with Illness
FH-RDC	:	Family History Research Diagnostic Criteria
GLS	:	General Life Satisfaction
GI	:	Gastrointestinal
GIT	:	Gastrointestinal Tract
GERD	:	Gastro-esophageal Reflux Disease
GIBDI	:	German Inflammatory Bowel Disease Activity Index
HADS	:	Hospital Anxiety and Depression Scale
HAMD	:	Hamilton Rating Scale for Depression
HAM-D-21	:	Hamilton Depression Inventory-21
HAM-A-14	:	Hamilton's Anxiety Inventory-14
HP	:	Helicobacter pylori
HPLC	:	High Performance Liquid Chromatography
HRLS	:	Health Related Life Satisfaction
HRP	:	Horse Radish Peroxidase
IDO	:	Indoleamine 2,3-dioxygenase
IL	:	Interleukin
IFN	:	Interferon
IBD	:	Inflammatory Bowel Disease
IBDQ	:	Inflammatory Bowel Disease Questionnaire
IBQ	:	Illness Behaviour Questionnaire
IBS	:	Irritable Bowel Syndrome

IBS-SFQ	: IBS-Symptom Frequency Questionnaire
IBS-BQ	: IBS-Bothersomeness Questionnaire
IPAA	: Ileal Pouch-Anal Anastomosis
IRF	: Interferon Regulatory Factor
K-SADS	: Schedule for Affective Disorders and Schizophrenia for School-Age Children
KYNA	: Kynurenic Acid
KYN	: Kynurenine
LOI	: Leyton Obsessional Inventory
LSI	: Life Style Index
LPS	: Lipopolysaccharide
MLN	: Mesenteric Lymph Nodes
MAS	: Medication Adherence Scale
MMP	: Matrix Metalloproteinase
NMDA	: N-Methyl-D-Aspartic Acid
NO	: Nitric Oxide
NOS	: Nitric Oxide Synthase
PM	: Project Management
PSQ	: Perceived Stress Questionnaire
PGWB	: Psychological General Well-being
PHQ-9	: Patient Health Questionnaire-9-item
PCDAI	: Paediatric Crohn's Disease Activity Index
PCSC	: Perceived Control Scale for Children
PASCET-PI	: Primary and Secondary Control Enhancement Therapy-Physical Illness
PCR	: Polymerase Chain Reaction
PS61	: Paykel's Scale for Stressful Events-61 item
RCMAS	: Revised Children's Manifest Anxiety Scale
RA	: Rheumatoid Arthritis
RNA	: Ribonucleic Acid
SF-36	: Short-Form 36 Health Survey
SIBO	: Small Intestinal Bacterial Overgrowth
STAI	: Spielberger's Trait-Anxiety Inventory

STAIC	:	State Trait Anxiety Inventory for Children
SLEQ	:	Stressful Life Events Questionnaire
SCID	:	Structured Clinical Interview for Axis-1 DSM-IV
SCID-NP	:	SCID-Non-Patients
SDS-PAGE	:	Sodium Dodecyl Sulphate-Polyacrylamide
SSL-I	:	Social Support List-Interactions
SPAS	:	Spencer Children's Anxiety Scale
SMP	:	Stress Management Psychotherapy
SCL-90-R	:	Symptom Distress Checklist-90-R
TCI	:	Temperament and Character Inventory
TAS-20	:	Toronto Alexithymia Scale-20 item
TAU	:	Treatment As Usual
TDO	:	Tryptophan-2,3-dioxygenase
TNBS	:	Trinitrobenzene Sulphonic Acid
TNF	:	Tumour Necrosis Factor
TST	:	Tail Suspension Test
UC	:	Ulcerative Colitis
VPMI	:	Vanderbilt Pain Management Inventory
WHO	:	World Health Organisation
Zung-SDS	:	Zung self-rating Depression Scale

Part I

General Introduction

Chapter 1

Background: A review of the co-morbidity of depression and anxiety in inflammatory bowel disease patients

1.1 Abstract

Inflammatory bowel disease (IBD) is a chronic relapsing and remitting disorder of the gastrointestinal tract. Psychological disturbances are common symptoms of many diseases involving chronic inflammation. The same appears to be true for IBD patients. The aim of this review was to determine whether systematic analysis of the recent literature can further clarify the association and interaction of IBD with psychological welfare. This review includes articles published between 2002 to 2012. A literature search between July and August 2012 was carried out which included the following search terms: Inflammatory bowel disease OR Crohn's or ulcerative colitis AND (Depression OR anxiety OR psychological). Search filters were set to "Publication date: 10 years" and "Species: Human". Thirty seven relevant articles over the 10 years were obtained which related to the co-morbidity of depression and/or anxiety and IBD. Overall there is a general consensus that IBD leaves patients more vulnerable to mood disorders compared to healthy controls and this increased prevalence of mood disorders is especially true for patients during active disease, but is not specific to either Crohn's disease or ulcerative colitis. It is therefore, widely recommended that patients with IBD should be screened for psychological disorders.

1.2 Introduction

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are chronic relapsing and remitting disorders of the gastrointestinal tract (Bouma and Strober, 2003). In acute UC, common symptoms include frequent diarrhoea with blood and mucus, abdominal pain, fever and weight loss. Inflammation in UC usually remains in the rectum and colon. CD can affect any part of the gastrointestinal (GI) tract, however most commonly involves illeocecal inflammation. UC and CD have similar symptomology, however in CD, symptoms can depend on the site of inflammation. Common symptoms include pain, abdominal distension, vomiting, constipation and weight loss. As many as 1.4 million persons in the United States and 2.2 million persons in Europe suffer from IBD (Loftus, 2004).

Historically, there has been a long standing interest in the co-morbidity of psychological well-being, psychiatric illness and personality differences associated with IBD (Murray, 1930; Sullivan, 1935; Daniels, 1942; Straker, 1960). Psychological disturbances are a common symptom of many chronic diseases such as atherosclerosis, congestive heart failure, and rheumatoid arthritis, all of which involve inflammation (O'Connor et al., 2008). The same appears to be true for inflammatory bowel disease in patients of all age groups. In a small group of 53 children with IBD, Burke et al. (1989) determined that the lifetime prevalence of depression was significantly higher in both CD (29 %) and UC (21 %), using the Kiddie-Schedule for Affective Disorders and Schizophrenia. In older children and adolescents with IBD, Szigethy et al. (2004) also reported a significant increase in depressive symptoms. They also found, however, that this correlated with steroid treatment and with the dose administered, suggesting that the treatment itself may be involved in the appearance of psychological symptoms. Many studies in adults have come to the same conclusion as reached in children, that both UC and CD are related to higher incidence of psychological disorders (Addolorato et al., 1997; Guthrie et al., 2002; Farrokhyar et al., 2006). Of particular interest is the study by Addolorato et al. (1997), as only patients naive to steroid treatment and surgical intervention were included, and again both CD (41.9 %) and UC (50.0 %) had significantly higher prevalence of depressive symptoms than controls (11.1 %). They also found that there was a significant association between mood and disease activation.

In a review, Mikocka-Walus et al. (2007) published a systematic review of 17 articles directly related to the co-morbidity of psychological disorders with IBD, based on a PubMed search of available articles between 1980 and 2005. The main aim of the review was to highlight the following 5 controversies:

1. Do psychological disorders co-occur with IBD more often than expected by chance?
2. Do psychological disorders appear during relapse or during remission of the disease?
3. Are particular psychological disorders specific to CD or UC?
4. Is the frequency of psychological disorders in IBD similar to, or higher than in other groups of medically ill patients?
5. Do psychological disorders precede and/or follow onset of the disease?

They conclude that methodological weakness of the reviewed data make it impossible to resolve some of these issues. However, they are satisfied that the results indicate a high frequency of interaction between IBD and anxiety/depression (Mikocka-Walus et al., 2007).

The aim of the current review is therefore, to determine whether systematic analysis of the recent literature can further clarify the association and interaction of IBD and psychological disturbances with regard to the questiones outlined above.

1.3 Methods

This review includes articles from 2002 to 2012 to avoid repetition of the Mikocka-Walus et al. (2007) study, which included articles between 1980 and 2005. A PubMed search between July and August 2012 was carried out including the following keywords: Inflammatory bowel disease OR Crohn's or ulcerative colitis AND (Depression OR anxiety OR psychological). PubMed filters were set to "Publication date: 10 years" and "Species: Human". The search produced 589 hits, the titles and abstracts of these were then examined, and if they contained evidence of both an IBD condition and evidence of depression, anxiety or psychological self/interview based assessment, they were included in the review. A further Google scholar search was carried out to ensure no large body of the literature was excluded from the PubMed search. Exclusion criteria included all articles that only contained information on irritable bowel syndrome (IBS), personality evaluations rather than psychological evaluation, external stressors, review articles, and letters. Where only the abstracts were available to the authors they were included in the summary table so as not to eliminate possible international variation, however, the detail could not be discussed further, other than what was in the abstract.

1.4 Results

Thirty seven relevant articles over the previous 10 years were obtained, which related to the co-morbidity of depression and/or anxiety and Inflammatory bowel disease. Twenty-six of these papers involved either IBD alone (CD or UC), or included negative control subjects for comparison (Table 1.1). Twelve papers involved comparison of IBD versus other inflammatory or autoimmune conditions or other gastrointestinal disorders (Table 1.2). These comparison disorders included Chronic Liver disease (CLD), coeliac disease, gastro-esophageal reflux disease (GERD), multiple sclerosis (MS), irritable bowel syndrome (IBS), helicobacter pylori (HP) infection, small intestinal bacterial overgrowth (SIBO), sugar malabsorption, gastritis HP negative, food allergies, and rheumatoid arthritis (RA). Seven studies were conducted in Spain, five in the USA, four in Germany and Canada, three in the UK, two in Austria, and one in Serbia, Turkey, Hungary, Tunisia, Ireland, Greece, Belgium, Sweden, Brazil, Netherlands, Croatia and Italy.

1.5 Discussion

1.5.1 Do psychological disorders co-occur with IBD more often than expected by chance?

In order to determine whether psychological risks are greater in IBD versus the general population it is important to compare IBD patients to appropriate control groups. Ideally the control groups should be matched across a number of factors including age, sex and possibly social class. Of the 37 studies, only 13 include either healthy controls or comparisons based on norms from the relevant general population (Ben Thabet et al., 2012; Goodhand et al., 2012b; Häuser et al., 2011, 2010; Loftus et al., 2011; Graff et al., 2009; Walker et al., 2008; Kovács and Kovács, 2007; Calvet et al., 2006; Gómez-Gil et al., 2003; Solmaz et al., 2003; Guthrie et al., 2002; Simrén et al., 2002). Of 103 UC patients, 101 CD patients and 124 patient controls, hospital anxiety and depression scores (HADS) were increased in IBD patients compared to the controls, however the average scores themselves especially for HADS-depression (D) were quite low, 4.1 and 4.7 and 1.7 for UC, CD and controls respectively (Goodhand et al., 2012b). Rather than reporting the average score Ben Thabet et al. (2012) reported the percentage of patients scoring above 10 in the HADS scores, and reported overall increases in prevalence of depression and anxiety in the IBD patients versus age and sex matched controls. In a large cohort of 422 IBD patients versus 422 age and sex matched controls of the German general population, HADS scores were

also used to demonstrate a higher frequency of anxiety and depression with IBD (Häuser et al., 2010).

Various other interviews and questionnaires for psychiatric disorders were also used, including the Composite International Diagnostic Interview (CIDI), Beck Depression Inventory (BDI) and Hamilton (HAM) scores, which also confirmed an increased risk of psychological disturbances in IBD patients. However, differences exist in the reported percentage of patients affected by psychiatric changes. In a Spanish cohort of 70 IBD patients, 42.4 % scored above 11, which is indicative of a probable psychiatric disorder, on the depression or anxiety subscale of the HADS (Gómez-Gil et al., 2003) while only 25.9 % of 112 IBD patients in a UK study scored above 11 on the same scale (Guthrie et al., 2002). These differences suggest that other factors such as the level of disease activity may influence anxiety and depression scores.

Increased risk for psychiatric disorders is not unique to adult IBD, in a large paediatric patient study, Loftus et al. (2011) compared 2,144 patients with CD to 10,720 matched paediatric controls. As with the adult studies, young patients with CD were found to be at greater risk of developing persistent anxiety disorders and depression. Interestingly, they found steroids to be a risk factor for developing anxiety disorders, however, even after controlling for this, CD itself was found to be a risk factor for mood disorders. Their analysis of prescription drug types suggest that the psychiatric conditions observed in the CD patients are being managed to some degree as the usage of antidepressants, antipsychotics, anxiolytics, mood stabilizers and benzodiazepines was higher in CD patient group. Overall, these studies all report a decreased psychological well being amongst IBD patients', and suggest that psychiatric evaluation in IBD maybe a beneficial adjunct to treatment.

1.5.2 Do psychological disorders appear during relapse or during remission of the disease?

Increased depression and/or anxiety are consistently reported to be increased in the active phase of IBD, however, the persistence of these disorders during the remission phase remains controversial. In a recent study, Ben Thabet et al. (2012) observed that of 44 % of IBD patients with high anxiety scores in the HAD-A (score > 10), 73 % were in the active disease state. Similar results were observed for the depression scores, whereby of 52 % of IBD patients who scored above 10 in the HAM-D 73 % were in an active disease phase. Häuser et al. (2011) also reported significantly increased mental disorders in IBD patients, again the highest rates were observed in moderate/severe disease activity (49.3

% versus 27.7 % with slight disease activity and 10.4 % in the general population). In an analysis of CD patients, Mardini et al. (2004) found a significant association between the BDI score and the Crohn's disease activity index (CDAI) score. This association was also true for CDAI and all the other psychological distress indices. Aside from the direct disease activity evaluations, Fernández et al. (2010) compared depression scores using the BDI to the levels of extraintestinal symptoms. They found a significant association between the levels of depression and the systemic manifestations of IBD.

In a separate study that included healthy controls and CD patients with active disease, based on a score of >2 in the Harvey-Bradshaw index, investigators demonstrated increased anxiety (HAM-A scores of 6.5 versus 16.2 for healthy controls versus active CD respectively) and depression (HAM-D scores of 3.7 versus 10.9 respectively for healthy controls versus active CD respectively) in active IBD (Calvet et al., 2006). Interestingly, a third group of CD patients, being treated with Thiopurinic immunomodulator, was also included. In this group, Thiopurinic-induced remission, restored psychological well-being to normal range in CD patients (5.5 anxiety score and 3.3 depression score). When taken together, these studies suggest that the risk of psychological manifestations is clear during the active phase, however, the results for patients during remission are less certain. Iglesias et al. (2009) conclude that despite clinical remission, a significant number of CD patients present with anxiety or depressive symptoms, and that CD patients in remission would benefit from psychological support. This is in contrast to the conclusions of Simrén et al. (2002), where IBD patients in remission demonstrated a level of psychological well-being comparable to that of the general population.

1.5.3 Are particular psychological disorders specific to CD or UC?

In a Swedish study of IBD patients compared to normal values for the Swedish population, Simrén et al. (2002) observed higher rates of borderline depression score (between 8 and 10 in HADS-D) and reduced well-being in CD patients versus UC patients. However, the more recent papers contradict this finding and it is now widely reported that no psychological differences between UC and CD exist. Goodhand et al. (2012b) conducted a study of 204 IBD patients (103 with UC and 101 with CD) and found no differences in the prevalence of mild, moderate, and severe anxiety and depression in UC or CD. Ben Thabet et al. (2012) came to the same conclusion in a smaller Tunisian population of 50 IBD patients (38 UC, 12 CD) based on the HAD scores for depression and anxiety. The lack of difference between risk of depression or anxiety in UC and CD was confirmed in

other studies (Häuser et al., 2011; Vidal et al., 2008; Guthrie et al., 2002). Although Vidal et al. (2008) did not find statistically significant differences between anxiety and depression HAD scores in UC and CD, they do report slightly higher scores for both disorders in the CD cohort. In a paediatric study of UC and CD patients, Szigethy et al. (2004) also report a lack of differences between the two conditions in Children's Depression Inventory (CDI) scores.

Although the general consensus is that no differences exist between risk of psychological symptoms for UC and CD patients, some interesting differences within each disorder have been reported. Maunder et al. (2006) report differences in measures of psychological distress between UC patients positive and negative for perinuclear anti-neutrophil cytoplasmic antibodies (pANCA), whereby a correlation between disease activity and depression was seen in the pANCA negative patients but not in the pANCA positive patients. Addolorato et al. (2008) report differences in the type of anxiety observed between UC and CD patients. They observed a relationship between state anxiety and active UC, while CD remission was inversely related to trait anxiety. Janke et al. (2005) also report differences in risk of psychiatric factors on health related life satisfaction (HRLS), whereby in CD patients psychiatric factors predicted reduced HRLS, whereas in UC it did not.

1.5.4 Is the frequency of psychological disorders in IBD similar to or higher than in other groups of medically ill patients?

There are 12 papers that compared IBD to other inflammatory and chronic diseases (see Table 1.2). These papers suggest that IBD patients are not more at risk than all other diseases when compared for psychiatric disorders, as the risk is higher or lower depending on the comparison illness. The most common comparison to IBD is IBS (Tkalcic et al., 2010; Kovács and Kovács, 2007; Crane and Martin, 2004; Solmaz et al., 2003). Solmaz et al. (2003) reported an increase in depression and anxiety scores in IBS patients versus IBD patients based on a variety of structured questionnaires. This observation was confirmed by Kovács and Kovács (2007), and Tkalcic et al. (2010). Tkalcic et al. (2010) also report that despite the increase in anxiety in the IBS patients, IBD patients suffer from lower health related quality of life (HRQOL). Crane and Martin (2004) slightly contradict these findings, whereby anxiety scores are increased in IBS compared to IBD, however, unlike the other studies, the depression score is higher in the IBD patients. The reason for the difference is not clear, but it may be due to the low disease activity level of the illness, as the depression scores are very low (5.5 and 3.4 for IBD and IBS respectively as assessed by HADS). These results are important in terms of psychological well-being in IBD, as 33

% of UC patients and 57 % of CD patients had IBS-like symptoms (Simrén et al., 2002). They reported that the group with IBS-like symptoms (both UC and CD) had higher levels of anxiety and depression, and reduced well-being compared to those without. They also concluded that anxiety and reduced vitality were found to be independent predictors for IBS-like symptoms in these patients.

In another gastrointestinal disorder, Brandi et al. (2009) compared IBD patients and gastro-esophageal reflux disease (GERD) patients. They report a higher level of overall psychological distress and depressed mood in the IBD patient group. They also report that this increase in depressed mood is related to active disease and CDAI scores.

Filipovic et al. (2007) compared the psychological impact of newly diagnosed IBD to newly diagnosed colon cancer. They observe an increase in percentage of severely depressed IBD patients compared to colon cancer patients (22/32 IBD patients > 16 score on the HAMD versus 5/30 colon cancer patients scoring > 16). They therefore suggest, as per previous studies, that IBD patients should be screened for depression, and that this is very important even in the early stages of disease diagnosis.

Häuser et al. (2010, 2011) carried out two separate investigations comparing IBD patients to patients with coeliac disease and chronic liver disease (CLD). In the initial study, they found no difference in levels of anxiety or depression between IBD or coeliac patients, however, when they adjusted for social class index, they did observe an increased depression score for IBD versus coeliac disease. Interestingly, they did not observe any differences between depression in the IBD patients versus the general population initially (Häuser et al., 2010), whereas they do report increased depression in a more recent investigation (Häuser et al., 2011). The differences most likely lie in the lack of active disease in the earlier study, as they make a clear differentiation of moderate/severe and slight disease activity in the latest report. They also report a higher depression score in CLD compared to IBD, with no difference in anxiety scores. Miehsler et al. (2008) compared IBD patients to patients suffering from Rheumatoid arthritis (RA), and observed increased anxiety in IBD, however, depression was higher in RA. Despite increased prevalence of depression in RA, the IBD patients expressed a higher need for psychological interventions, which the authors suggest stems from the psychosocial restrictions the symptoms of IBD imposes.

1.5.5 Do psychological disorders precede and/or follow onset of the disease?

Although there has been a substantial amount of literature on the increased prevalence of depression and anxiety in IBD, less investigation into the affect this might have on the

course of the disease itself has been carried out. This may be due to the longitudinal and more protracted nature of this kind of study. In a slightly more dated study than the 10 year remit of the review, Kurina et al. (2001) carried out an extensive analysis of general hospital admissions in southern England between 1963 and 1999, to determine whether patients suffering from IBD had a greater rate of developing depression than would be expected by chance, and whether depression or anxiety preceded or came after diagnosis of IBD. Results showed that with both CD and UC there is a greater chance of suffering from depression, but that in UC the depression is usually diagnosed in the year before onset of the disease symptoms, while in patients with CD the depression followed the diagnosis of the disease. They therefore suggest that onset of depression in UC might be causally related to UC, a result of living with an undiagnosed bowel condition, or was not recorded appropriately in the medical documents. The results suggest that there might be psychoneuroimmunological components that predispose some people to the development of UC (Kurina et al., 2001). For CD they suggest that depression might be a result of the disease symptoms or treatment of the illness.

No recent studies have completed such an extensive analysis into the temporal nature of psychological disorders with regard to the diagnosis of IBD. However, Walker et al. (2008) did investigate the lifetime risk of depression in the Manitoba IBD patient cohort, and carried out a longterm analysis of these patients over 12 months. They report a higher lifetime risk of depression, and a possible higher lifetime risk of some anxiety disorders in IBD patients versus a general Canadian population group. In the majority of patients with lifetime anxiety or depression, the psychological disorders preceded the diagnosis of IBD. It is therefore suggested that further research into the possible risk of psychological disorders on IBD development is needed.

Some earlier and later investigations have attempted to analyse the influence of either anxiety or depression on the risk of relapse in IBD. In a study of 112 patients with inactive IBD, Vidal et al. (2008) reported that neither depression nor anxiety increased the risk of relapse in UC and CD patients. This is contradictory to two other studies where BDI depression scores were predictive of future changes in IBD activity. In a small study of 18 CD patients, depressive symptoms, as per BDI scores, were positively associated with future changes in the CDAI scores (Mardini et al., 2004). Mittermaier et al. (2004) also reported a predictive value to the depressive scores, whereby BDI scores correlated with the time until first relapse. Persoons et al. (2005) also reported decreased remission rates in patients with a major depressive disorder. These studies suggest that psychological assessment may help to identify patients at risk of disease exacerbation or decreased rates of remission.

1.5.6 Association between psychological disorders and health related quality of life

A final observation in the literature is the association between depressive and anxiety scores to health related quality of life (HRQOL) (Hyphantis et al., 2010; Kiebles et al., 2010; Walker et al., 2008; Vidal et al., 2008; Mittermaier et al., 2004; Guthrie et al., 2002). Hyphantis et al. (2010) analysed 185 IBD patients confirmed by radiological, endoscopic and histological evaluation. Quality of life questionnaires revealed an association between psychological distress and HRQOL. They also report that somatization mediated the relationships of anxiety and depression with HRQOL. The same association between patients with and without affective disorders revealed an association between HRQOL disease activity or psychological distress in Canadian and Spanish patients (Walker et al., 2008; Vidal et al., 2008). Guthrie et al. (2002) also report psychiatric disorders as an independent predictor of poor HRQOL in their study of 112 IBD patients. Mittermaier et al. (2004) suggest that factors such as depressed mood and impaired HRQOL could exert a negative influence on the course of the IBD itself. If this is the case, it suggests that treating the psychiatric disorder could be beneficial in terms of the overall course of IBD in patients.

1.6 Conclusion

Overall, there is a general consensus that IBD leaves patients more vulnerable to mood disorders compared to healthy controls, and increased prevalence of mood disorders is especially true for patients during active disease, but is not specific to either CD or UC. Treating the psychological disorder could also be beneficial in improving HRQOL in IBD patients. It is recommended that patients with IBD should be screened for psychological disorders and that psychiatrists should be involved in the overall treatment of IBD.

1.7 Future directions

Despite a substantial increase in the number of relevant articles compared to the Mikocka-Walus et al. (2007) review, which has led to a number of the questions being answered, there has been a distinct lack of investigation into the cause of increased psychological disturbances. Due to the nature of the symptoms of IBD it is possible that the psychological impact of these could be sufficient to induce depression or anxiety. However, it is also possible that inflammatory mediators themselves could be responsible for altered mood, and

therefore, it is suggested that further attention to specific circulating mediators should be investigated in relation to psychological changes. This would be beneficial as antidepressants may not be as efficient in minimising the depression like symptoms if inflammatory mediators are responsible for the psychological changes. It is also possible that a combination of immunomodulators and conventional antidepressant therapy, or complimentary psychological management techniques may be the most appropriate treatment approach.

Table 1.1: Reports of psychological disturbance in IBD

Study	Participants	Method	Results and Authors Conclusions
Ben Thabet et al. (2012) (Tunisia)	Adults: UC, CD, control patients	CDAI, HADS, TAS-20	22% more patients than controls had HAD-D score >10, 26% more patients had HAD-A score >10, 72.7% and 73% of high HAD-D and HAD-A scores respectively were within an active disease phase
Goodhand et al. (2012b) (UK)	Adults: UC, CD, healthy controls	HADS, disease activity score, endoscopy, fecal calprotectin, CRP	Higher HAD-A scores in UC and CD versus controls (HADS-A: 8.5 , 8.6 , 3.2, respectively), higher HADS-D scores in UC and CD versus controls (HAD-D: 4.1, 4.7, 1.7, respectively). There were no differences in the severity of anxiety or depression between UC and CD.
Iglesias-Rey et al. (2012) (Spain)	Adults: CD, UC	HADS, SF-36, IBDQ, TAS-26	
Häuser et al. (2011) (German)	Adults: CD, UC, CLD, GP controls	HADS	Increased mental disorders in IBD patients versus general controls. Increased disease activity leads to increased risk of mental disorders (percentage of patients: 49.3%, 27.7% and 10.4% for severe activity, slight activity and controls respectively.
Loftus et al. (2011) (USA)	Children: CD, pediatric controls	ICD-9-CM epidemiologic evidence, prescription medication, diagnosis of psychological disorders,.	74% Increased risk of developing anxiety disorders after CD diagnosis. Increased risk of developing persistent anxiety and depression in CD patients. Increased usage rates of psychotropic medication in CD patients
Kilroy et al. (2011) (Ireland)	Children: CD, UC	IMPACT III scale, SPAS,	39% of patients had increased anxiety symptoms. Anxiety levels were a predictor of QOL
Kiebles et al. (2010) (USA)	Adults: CD, UC	IBDQ, SF-12v2, IPQ-R, BSI, DDAQ, PSQ, Brief cope, PDS	PDS scores were negatively correlated with scores of bowel and systemic health in the IBDQ. PDS scores were positively correlated with pain severity, perceived stress and GI visits.
Vidal et al. (2009) (Spain)	Adults: inactive CD and UC	SCID-I, DSM IV, Harvey-Bradshaw Index, SCAI	31.1% of IBD patients had at least one psychiatric disorder. Anxiety and depression were the most prevalent (17.9% an 11.6% respectively)
Fernández et al. (2010) (Spain)	Adults: UC, CD	BDI	Increased BDI scores in IBD patients suffering from extraintestinal symptoms

Hyphantis et al. (2010) (Greece)	Adults: CD, UC	IBDQ, GHQ-28, SCL-90-R, DSQ LSI	HRQOL were associated with psychological distress. Somatization is independently correlated to disease specific HRQOL in IBD patients.
Graff et al. (2009) (Canada)	Adults: Manitoba IBD population, community controls		Decreased psychological wellbeing/health in IBD. Increased distress in IBD. Association between psychological factors and perceived health. During quiescent disease psychological health is comparable to normal levels.
Iglesias et al. (2009) (Spain)	Adults: CD	CDAI, CRP ELISA, HADS	39% of CD patients had anxiety symptoms. 24% of CD patients had depressive symptoms. Despite clinical remission, anxiety and depressive symptoms were still present in an important number of CD patients
Walker et al. (2008) (Canada)	Adults: CD, UC, community controls. Also comparison to New Zealand and USA cohorts.	CIDI	Social anxiety decreased in IBD versus controls. No significant differences in panic disorder. No significant difference in agoraphobia (no panic). Increased major depression in IBD versus controls. Anxiety or mood disorders lead to decreased QOL perception and earlier onset of IBD symptoms.
Maunder et al. (2006) (Canada)	Adults: UC	ELISA, immuno-fluorescence staining, St. Mark's index, CES-D, IBQ	In UC patients positive for pANCA no association between disease activity and psychological disorders. In UC patients negative for pANCA the relationship between anxiety and depression during disease activity was significant
Vidal et al. (2008) (Spain)	Adults: UC, CD	IBDQ, CDAI, HADS, TCI	Slight increases in depression and increased anxiety in CD versus UC, however, this was not significant. 44.6% scored >8 in HADS-D or HADS-A. 15.8% scored >11 on one or both scales. Psychological distress and disease activity were the strongest predictors of QOL impairment.
Calvet et al. (2006) (Spain)	Adults: CD, healthy controls	SF-36, HAM-A-14, HAM-D-17	Decreased SF-36 score in active disease versus healthy controls and Thiopurinic-induced remission. HAM-A and HAM-D scores were also significantly higher in patients with active disease versus healthy controls and Thiopurinic-induced remission (HAM-A: 16.2, 6.5, 5.5 respectively, HAM-D 10.9, 3.7, 3.3 respectively)
Maunder et al. (2005) (Canada)	Adults: UC		Increased correlation between time to disease activity and depression

Häuser et al. (2005) (Germany)	Adults: UC	HADS-D, PDAI, IBDQ	No difference in probable mental disorders in UC patients with IPAA versus UC patients without IPAA and general German population
Janke et al. (2005) (Germany)	Adults: CD, UC	GLS, HRLS, FLZ, HADS, GIBDI	Increased psychiatric illness, medical co-morbidity and disease activity were risk factors of reduced HRLS in CD. Increased disease activity was the only predictive factor in UC patients.
Persoons et al. (2005) (Belgium)	Adults: CD	CDAI, PHQ-9, HADS, TAS-20, SSL-I, infliximab, CRP measured	Major depression in CD is predictive of lower remission rates.
Szigethy et al. (2004) (USA)	Adolescents: UC, CD	PCDAI, CSK, K-SADS, CDI,	30.4% of IBD patients had subsyndromal depression (>9). 24.5% scored >12. Later age of diagnosis is a risk factor for development of depressive symptomology. No correlation between IBD severity measures and depression severity. There is a relationship between steroid use and depression
Mardini et al. (2004) (USA)	Adults: CD	BDI, BAI, BHS, RLC, CDAI	BDI scores were strongly associated with CD activity (based on CDAI). All other psychological distress indices with high scores were suggestive of higher CDAI. Increased depressive symptomology was positively associated with future changes in CDAI
Mittermaier et al. (2004) (Austria)	Adults: CD, UC	CDAI, CAI, CRP, orosomucoid, ESR, BDI, STAI, IBDQ, PSQ, RFIPC	At baseline 28% of patients had >13 on the BDI score. Depressed patients did not differ in terms of lab data, age, disease duration, severity of disease, smoking, steroid use, or NSAID use. BDI scores were correlated with the time to first relapse. Depression was associated with anxiety and HRQOL
Gómez-Gil et al. (2003) (Spain)	Adults: CD, UC (25 relatives as controls)	HADS	22% of patients had been visited by a psychiatrist during relapse. 42.4% of patients reached >11 on the HADS-D or HADS-A
Guthrie et al. (2002) (UK)	Adults: CD, UC, general population representatives	HADS, SF-36	25.9% of patients scored >11 or more on HAD-A or HAD-D. Psychiatric disorders were an independent predictor of poor HRQOL. No difference in risk of mood disorders in CD versus UC

Simrén et al. (2002) (Sweden)	Adults: UC, CD, (No controls, however PGWB, GSRS and HADS compared to normal values for Swedish population, and STAI compared to norms from working Americans)	PGWB, GSRS, HADS, STAI	IBD patients in remission have similar psychological well-being to the general population. CD patients reported reduced wellbeing, GI symptoms and psychosocial dysfunction than UC. IBS was associated with increased levels of anxiety and depression
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Abbreviations Table 1.1: Adverse Life Events (ALE), Anxiety Subscale of the Hospital Anxiety and Depression Scale (AS-HADS), Beck Depression Inventory (BDI), Beck Anxiety Scale (BAS), Big Five Inventory (B5I), C-reactive protein (CRP), Center for Epidemiologic Studies-Depression Scale (CES-D), Children's Depression Inventory (CDI), Children's Global Assessment Scale (CGAS), Child Health Questionnaire (CHQ), Clinical Score of Kozarek (CSK), Cognitive and Somatic Trait Anxiety Scale (CSTAS), Child Health Questionnaire parent (CHQ-50) and child (CHQ-87), Colon Cancer (CC), Disease Activity Questionnaire, an unpublished questionnaire developed by Dr Israeli and Dr La (DAQ), Defense Style Questionnaire (DSQ), Centre for Epidemiological Studies - Depression Scale (CES-D), Erythrocyte Sedimentation Rate (ESR), Freiburg Questionnaire of Coping with Illness (FQCI), Family History Research Diagnostic Criteria (FH-RDC), General life satisfaction (GLS), Gastrointestinal (GI), Gastro-esophageal reflux disease (GERD), gastrointestinal symptom rating scale (GSRS), General Health Questionnaire (GHQ-28), German version of the HADS (HADS-D), General symptoms (GS), German inflammatory bowel disease activity index (GIBDI), Hamilton's depression inventory consisting of 21 items (HAM-D-21), Hamilton's anxiety inventory consisting of 14 items (HAM-A-14), Hamilton Rating Scale for Depression (HAM-D), Helicobacter pylori (HP), Health-related life satisfaction (HRLS), Hospital Anxiety and Depression Scale (HADS), Inflammatory Bowel Disease Questionnaire (IBDQ), IBS Symptom Frequency Questionnaire (IBS-SFQ), IBS Symptom Bothersomeness Questionnaire (IBS-BQ), Ileal pouch-anal anastomosis (IPAA), Illness Behavior Questionnaire: (IBQ), Indeterminate colitis (IC), Irritable Bowel Syndrome (IBS), Leyton Obsessional Inventory (LOI), Life Style Index (LSI), Medication Adherence Scale (MAS), Project management (PM), Perceived Stress Questionnaire (PSQ), PSYCHOLOGICAL GENERAL WELL-BEING (PGWB), Patient Health Questionnaire 9-items (PHQ-9). Pediatric Crohn's Disease Activity Index (PCDAI), Perceived Control Scale for Children (PCSC), Pouch Disease Activity Index (PDAI), Primary and Secondary Control Enhancement Therapy-Physical Illness (PASCET-PI), Paykel's scale for stressful events-61 item (PS61). Psychischer und Sozial-kommunikativer Befund (PSKB), German questionnaire known as Quality of Life (QL), Questions of Life Satisfaction (FLZ), Revised Children's Manifest Anxiety Scale (RCMAS), Rheumatoid Arthritis (RA), Schedule for Affective Disorders and Schizophrenia for School-Age Children (K-SADS), Short-Form 36 Health Survey, SF-36, Small intestinal bacterial overgrowth (SIBO), Spielberger's Trait-Anxiety Inventory (STAI), State Trait Anxiety Inventory for Children (STAIC), Stressful Life Events Questionnaire (SLEQ), Structured Clinical Interview for Axis-I DSM-IV disorders (SCID), Structured Clinical Interview for DSM-Non-patients (SCID-NP), Social Support List - Interactions (SSL-I). Spencer Children's Anxiety Scale (SPAS), Stress Management Psychotherapy (SMP), Symptom Distress Checklist-90-R (SCL-90-R), Temperament and Character Inventory (TCI), Toronto Alexithymia Scale 20 items (TAS-20), Treatment as usual (TAU), Vanderbilt Pain Management Inventory (VPMI), Zung self-rating Depression Scale (Zung-SDS).

Table 1.2: Reports of psychological disturbances in IBD patients when compared with other inflammatory conditions

Study	Participants	Method	Results and Authors Conclusions
Häuser et al. (2011) (Germany)	Adults: CD, UC, CLD, GP controls.	HADS	Increased depression is related to disease activity severity. Increased depression in CLD compared to IBD
Häuser et al. (2010) (Germany)	Adults: coeliac Disease, IBD, GP controls	HADS	Increased anxiety in IBD versus the general population, however, there were no difference in depression. No difference between IBD patients and coeliac patients for depression or anxiety
Greenley et al. (2010) (USA)	Children: CD, UC, IC	Self reports of anxiety, RC-MAS, STAIC, LOI, CDI, KADS	Meta-analysis
Bol et al. (2010) (Netherlands)	Adults: MS, UC	HADS, Expanded Disability Status Scale and CAI	Physical and mental fatigue were significantly higher in MS patients, but the contribution of disease severity, depression and negative affectivity to both physical and mental fatigue was the same for MS and UC
Brandi et al. (2009) (Brazil)	Adults: CD, GERD	BDI, AS-HADS, CDAI	Depressed mood associated with disease activity and higher CDAI. Increased depressed mood in IBD than GERD. In CD depression and anxiety are highly concurrent conditions.
Tkalcic et al. (2010) (Croatia)	Adults: IBS, IBD	SF-36, BDI, STAI, Big Five Inventory, SLEQ	Increased anxiety and neuroticism in IBS than IBD. Lower HRQOL in IBD than IBS.
Addolorato et al. (2008) (Italy)	Adults: GI disorders (Hp infection, SIBO, Sugar malabsorption, IBS, coeliac disease, GERD, Gastritis Hp neg, UC, Food allergies, CD)	STAI, Zung-SDS	State anxiety is related to active UC. Trait anxiety is inversely related to CD in remission. UC in remission is inversely related to depression. Most patients who seek medical consultation for GI problems show associated affective disorders
Miehsler et al. (2008) (Austria)	Adults: IBD, RA	ADAPT, HADS, SF-36, SUZU-K-22, RFIPC	There is some evidence of higher risk of anxiety in IBD versus RA (HAD-A >8). Higher HAD-D in RA versus IBD. IBD patients express higher need for psychological intervention than RA
Kovács and Kovács (2007) (Hungary)	Adults: IBD, IBS, GP healthy controls	Self reported questionnaires	Both IBS and IBD patients had increased depression and anxiety scores versus healthy controls. IBS had increased depression scores versus IBD. GI patients status is associated with depression and anxiety

Filipovic et al. (2007) (Serbia)	Adults: IBD, CCa	HAM-D-21, HAM-A-14, PS-61, CDAI	Newly diagnosed IBD patients had increased depression and anxiety compared to newly diagnosed colon cancer. Initial IBD indices show no correlation with depression or anxiety
Crane and Martin (2004) (UK)	Adults: IBD, IBS	HADS, GS, IBS-BQ, IBS-SFQ, VPMI	IBD patients scored significantly higher than IBS patients in the HAD-D, however, means for both groups were within normal range. Higher anxiety in IBS than IBD however, this was not significant
Solmaz et al. (2003) (Turkey)	Adult: IBD, IBS, healthy hospital workers	SCID-NP, SCL-90-R, BDI, BAS, HAMD. FH-RDC	Increased psychiatric and mood disorders in the IBS group than the control groups. IBS patients scored higher on anxiety and depression scales than the other groups.

Abbreviations Table 1.2: Adverse Life Events (ALE), Anxiety Subscale of the Hospital Anxiety and Depression Scale (AS-HADS), Beck Depression Inventory (BDI), Beck Anxiety Scale (BAS), Big Five Inventory (B5I), C-reactive protein (CRP), Center for Epidemiologic Studies-Depression Scale (CES-D), Children's Depression Inventory (CDI), Children's Global Assessment Scale (CGAS), Child Health Questionnaire (CHQ), Clinical Score of Kozarek (CSK), Cognitive and Somatic Trait Anxiety Scale (CSTAS), Child Health Questionnaire parent (CHQ-50) and child (CHQ-87), Colon Cancer (CC), Disease Activity Questionnaire, an unpublished questionnaire developed by Dr Israeli and Dr La (DAQ), Defense Style Questionnaire (DSQ), Centre for Epidemiological Studies - Depression Scale (CES-D), Erythrocyte Sedimentation Rate (ESR), Freiburg Questionnaire of Coping with Illness (FQCI), Family History Research Diagnostic Criteria (FH-RDC), General life satisfaction (GLS), Gastrointestinal (GI), Gastro-esophageal reflux disease (GERD), gastrointestinal symptom rating scale (GSRS), General Health Questionnaire (GHQ-28), German version of the HADS (HADS-D), General symptoms (GS), German inflammatory bowel disease activity index (GIBDI), Hamilton's depression inventory consisting of 21 items (HAM-D-21), Hamilton's anxiety inventory consisting of 14 items (HAM-A-14), Hamilton Rating Scale for Depression (HAMD), *Helicobacter pylori* (HP), Health-related life satisfaction (HRLS), Hospital Anxiety and Depression Scale (HADS), Inflammatory Bowel Disease Questionnaire (IBDQ), IBS Symptom Frequency Questionnaire (IBS-SFQ), IBS Symptom Bothersomeness Questionnaire (IBS-BQ), Ileal pouch-anal anastomosis (IPAA), Illness Behavior Questionnaire: (IBQ), Indeterminate colitis (IC), Irritable Bowel Syndrome (IBS), Leyton Obsessional Inventory (LOI), Life Style Index (LSI), Medication Adherence Scale (MAS), Project management (PM), Perceived Stress Questionnaire (PSQ), PSYCHOLOGICAL GENERAL WELL-BEING (PGWB), Patient Health Questionnaire 9-items (PHQ-9). Pediatric Crohn's Disease Activity Index (PCDAI), Perceived Control Scale for Children (PCSC), Pouch Disease Activity Index (PDAI), Primary and Secondary Control Enhancement Therapy-Physical Illness (PASCET-PI), Paykel's scale for stressful events-61 item (PS61). Psychischer und Sozial-kommunikativer Befund (PSKB), German questionnaire known as Quality of Life (QL), Questions of Life Satisfaction (FLZ), Revised Children's Manifest Anxiety Scale (RCMAS), Rheumatoid Arthritis (RA), Schedule for Affective Disorders and Schizophrenia for School-Age Children (K-SADS), Short-Form 36 Health Survey, SF-36, Small intestinal bacterial overgrowth (SIBO), Spielberger's Trait-Anxiety Inventory (STAI), State Trait Anxiety Inventory for Children (STAIC), Stressful Life Events Questionnaire (SLEQ), Structured Clinical Interview for Axis-I DSM-IV disorders (SCID), Structured Clinical Interview for DSM-Non-patients (SCID-NP), Social Support List - Interactions (SSL-I). Spencer Children's Anxiety Scale (SPAS), Stress Management Psychotherapy (SMP), Symptom Distress Checklist-90-R (SCL-90-R), Temperament and Character Inventory (TCI), Toronto Alexithymia Scale 20 items (TAS-20), Treatment as usual (TAU), Vanderbilt Pain Management Inventory (VPMI), Zung self-rating Depression Scale (Zung-SDS).

Chapter 2

Introduction: potential links between immune activation in IBD and psychological symptoms

2.1 Introduction

This general introduction discusses IBD in terms of symptoms, potential causes, intestinal barrier breakdown, bacterial translocation and the evidence for increased psychological disturbances during the active phase of disease. Immune activation, including increased inflammatory cytokine expression, kynurenine pathway activation and increased inducible nitric oxide synthase expression have been reported to be involved in altered behaviour. These immune factors are discussed in the context of IBD and depression/anxiety. The review concludes with a summary of the potential immune factors that may communicate with the central nervous system, and influence patient mood during active inflammation in IBD.

2.2 Inflammatory Bowel Disease

Peak diagnosis of IBD occurs during early adulthood, between 15 and 40 years of age, however, there are also cases of paediatric IBD, and later diagnosis with a second smaller peak between ages 50 and 80 (Langan et al., 2007). Incidence and prevalence rates of ulcerative colitis (UC) and Crohn's disease (CD) are highest in developed countries. Molodecky et al. (2012) reviewed geographical incidence and prevalence rates, and reported highest rates in Northern Europe, Canada and North America. They also concluded that the

incidence rates of IBD have been increasing since the earlier studies in the 1960s. Paediatric incidence rates are also increasing, in a recent study between 2000 and 2010 in Irish children (<16), a substantial and sustained increase in incidence rates in paediatric IBD was reported (Hope et al., 2012)

2.2.1 Causes of IBD

The causes of IBD are still unclear, however, a poorly understood combination of increased genetic predisposition, combined with environmental triggers are thought to be necessary (Brown, 2007). As no single gene has been linked to either UC or CD, it is thought that multiple genes may be involved. A large number of environmental factors have been proposed to be involved in the pathogenesis of IBD, these include smoking, dietary factors, drugs, geographical, social, economical, educational and occupational status, stress, microbial factors, intestinal permeability and appendectomy (Danese et al., 2004).

Of the potential environmental factors listed above, Danese et al. (2004), suggest that smoking, and the change in gut microflora are the most likely environmental causes. There is significant evidence to suggest that differences exist in the microbial flora of healthy individuals compared to patients suffering from IBD. The GI tract of healthy individuals contains very high concentrations of microbial flora, which are necessary for the correct functioning of the GI tract. In order for the microbial flora to inhabit the GI tract, without causing an immune response, humans have built up a tolerance to them, known as oral tolerance. A breakdown of this tolerance is thought to be involved in the development of IBD (Brown, 2007).

In a review article, patients with IBD were described as having loss of normal anaerobic bacteria or increased number of pathogenic bacteria (Shih and Targan, 2008). Differences between patients with UC compared to patients with CD were also reported, whereby there is a decrease of anaerobic bacteria in UC and an increase in enterobacteria in CD (Wen and Fiocchi, 2004).

Other possible causes of IBD could be an excessive activity of effector T cells or a decreased activity of regulatory T cells, as these are involved in experimentally-induced inflammation. Evidence for increased effector T cell activity has come from animal models that have altered immune systems, such as the TNF^{ARE} mice that overproduce tumour necrosis factor (TNF) (Bouma and Strober, 2003). The most important difference between UC and CD is that there is a different helper T cell mediated response. Traditionally, CD is viewed as being a Th1 mediated inflammatory response, while there is a predominantly Th2 cell mediated response in UC. This difference in Th1/Th2 cell balance leads to a

difference in cytokine production between the two diseases, with the Th1 cell mediated response leading to an induction of pro-inflammatory cytokines, while the Th2 response leads to production of anti-inflammatory cytokines (McClane and Rombeau, 1999). Only antigen presenting cells known as dendritic cells can determine the differentiation of pre helper T cells into either Th1 or Th2 cells. Recent evidence has shown that there is an increased number of mature dendritic cells present in the inflamed regions of tissue biopsies from patients suffering from CD, which may be the cause of the increased Th1 mediated response. No difference was seen in the numbers of dendritic cells present in biopsies of patients suffering for UC (Middel et al., 2006). More recently, the Th17 related cytokines have also been found in both UC and CD (for review see Monteleone et al. (2012)).

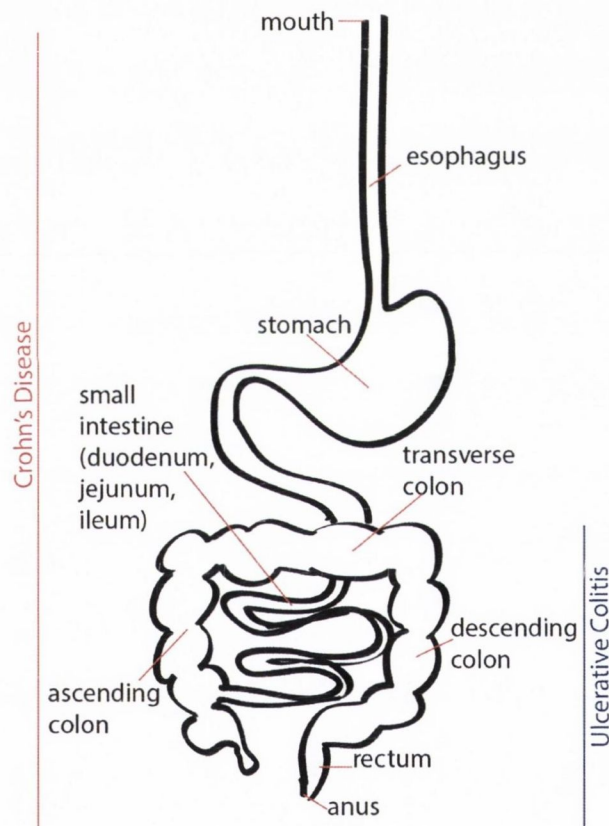


Figure 2.1: **Simplified diagram of the gastrointestinal tract:** Inflammation in UC usually remains in the rectum and colon. CD can affect any part of the gastrointestinal (GI) tract, however, most commonly involves illeocecal inflammation.

2.2.2 Gastrointestinal tract

The GIT can be broadly divided into two regions: the small intestine and the large intestine (see Figure 2.1). The small intestine is responsible for most of the digestion of lipids,

proteins and carbohydrates, and nutrient absorption, while the large and small intestine are responsible for water absorption and sodium regulation (Kohan et al., 2011; Pouokam et al., 2011). The wall of the GIT provides a physical barrier between the contents of the lumen and the circulation. The barrier is composed of an outer mucus layer, inner mucus layer and tightly connected epithelial cells (see Figure 2.2). The integrity of this barrier can become compromised during GI pathologies. For instance in IBD the mucosal layer can be damaged due to a dysregulated immune response, leaving the epithelial cells exposed to the luminal bacteria. This can leave patients vulnerable to enhanced immune activation and to the translocation of bacteria from the intestinal lumen into the systemic circulation causing the release of inflammatory cytokines (Laukoetter et al., 2008).

2.2.3 Bacterial translocation

As described above, the large intestine hosts a complex balance of bacteria which in the healthy bowel act in a symbiotic relationship with the host to break down molecules that the intestine is not capable of digesting. These bacteria also serve a number of other purposes including: immune development and regulation of gut physiology (for review see Sommer and Backhed (2013)). Under pathological conditions, where the intestinal wall becomes compromised, these bacteria can enter the mucosal layers and translocate to the lymphatic or systemic circulation in a process known as bacterial translocation. Bacterial translocation might be an important means of communication between the diseased bowel and the CNS, potentially responsible for the precipitation of mood and/or anxiety disorders during active IBD. Berg (1995) suggests that this translocation can occur in three discrete stages (see Figure 2.2):

1. In healthy bowels small, amounts of bacteria translocate however they are in very low numbers and are killed by the host immune defenses, including macrophages and dendritic cells
2. If bacterial overgrowth occurs, translocation can increase to the lymphatic organs, mainly the nearby mesenteric lymph nodes (MLN), but this does not enter systemic circulations and is normally clinically asymptomatic. In more severe cases, the bacteria spread beyond the MLN to various organs including the liver, spleen or kidney
3. Finally translocating bacteria can spread systemically to the peritoneal cavity and blood, which can lead to severe sepsis and death.

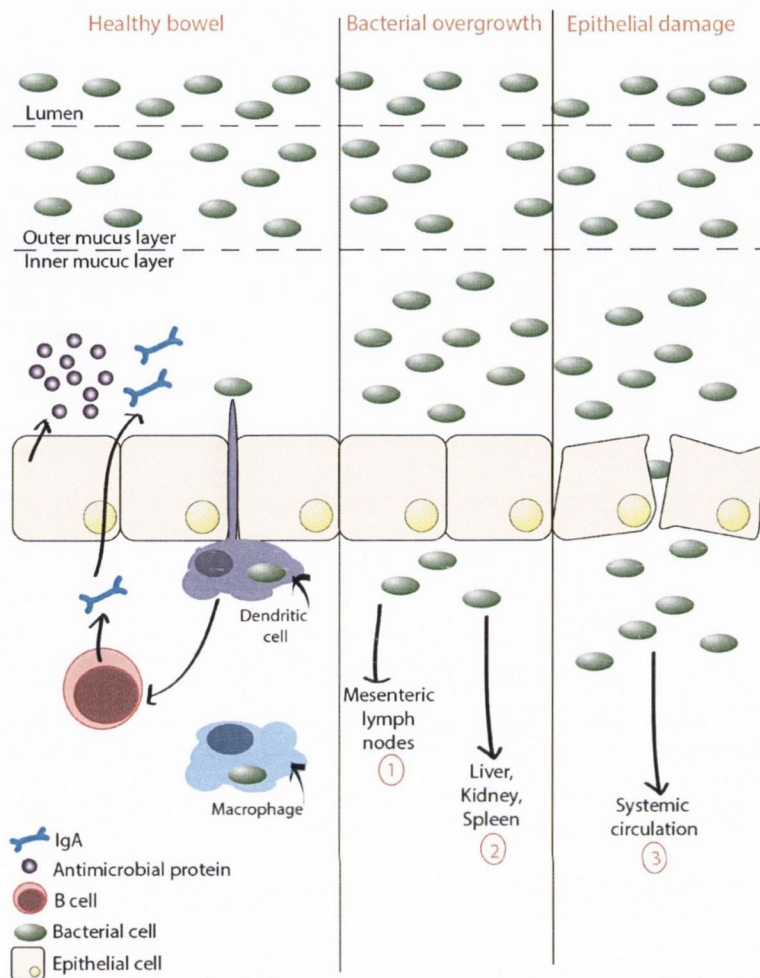


Figure 2.2: **Bacterial translocation from the gastrointestinal tract:** Three discrete stages of bacterial translocation. In **healthy bowels** small amounts of bacteria translocate however, they are in very low numbers and are killed by the host immune defenses, including macrophages and dendritic cells. If **bacterial overgrowth** occurs, translocation can increase to the lymphatic organs, mainly the nearby mesenteric lymph nodes (MLN) (1), but this does not enter systemic circulations and is normally clinically asymptomatic. In more severe cases the bacteria spread beyond the MLN to various organs including the liver, spleen or kidney (2). Finally with **epithelial damage**, translocating bacteria can spread systemically to the peritoneal cavity and blood (3) which can lead to severe sepsis and death. (based on Berg (1995))

2.3 Potential immune mediated induction of mood and anxiety disorders in IBD

Systematic review of the literature on co-morbidity of psychological disorders in IBD revealed an overall agreement that IBD leaves patients more vulnerable to increased mood

disorders compared to healthy controls, and this is especially true for patients during active disease (see chapter 1).

2.3.1 Psychological disorders: Depression and anxiety

Depression

Depression is the most common of the affective disorders, the world health organisation (WHO) estimates that approximately 121 million people are affected worldwide. With a lifetime prevalence of over 15%, it is currently the 4th leading contributor to the global burden of disease, and is expected to be the second leading contributor by 2020 (Elhwuegi, 2004; Irwin and Miller, 2007). In approximately 75% of patients with unipolar depression, symptoms are associated with stressful life-events such as death of loved ones (known as reactive depression). The remaining 25% suffer from endogenous depression, with no related external stresses (Rang et al., 2003). According to Rang et al. (2003), there are two components of depression: emotional symptoms and biological symptoms. Emotional symptoms can include apathy, pessimism, feelings of guilt, low self esteem, and loss of motivation. The biological symptoms of depression can include disturbances in sleep patterns, loss of appetite, and loss of libido.

Anxiety

Anxiety disorders involve exaggerated fear responses, which include autonomic reflexes, arousal and alertness, corticosteroid secretion, and negative emotions despite no external stimulus. As with depression they are highly prevalent disorders with a lifetime prevalence of over 15 % (Somers et al., 2006). Anxiety disorders can be categorised into various clinically distinct subgroups: generalised anxiety disorders, panic disorders, phobias and post traumatic stress disorders (Rang et al., 2003). Wang et al. (2000) report increased prevalence of anxiety among patients suffering with greater numbers of physical co-morbidities.

Monoamine theory of depression and anxiety

The monoamine theory of depression is the main biochemical hypothesis of depression. It was proposed in 1965 and implicates deficits in the monoamine neurotransmitters as a cause of depression (Schildkraut, 1965; Schildkraut and Kety, 1967). Noradrenaline was the main culprit in the original hypothesis, however, it later became apparent that serotonin (5-hydroxytryptamine (5-HT)) also played an important role in depression (Rang

et al., 2003). The greatest support for the monoamine hypothesis has been pharmacological, whereby drugs that increase availability of monoamines have anti-depressant effects, and drugs that inhibit monoamine availability have depressant effects (Rang et al., 2003). This theory is now widely accepted, as there is a vast literature supporting the role of monoamines in major depression (for review see Elhwuegi (2004)). However, due to the high level of treatment resistant depression, between 15 % and 33 % of patients will not respond to multiple drug interventions (Little, 2009), other possible biochemical mechanisms of depression are also being explored. Altered serotenergic systems are not unique to depressive behaviour and have also been implicated in anxiety (Graeff et al., 1996; Hale et al., 2012).

Following review of the psychoneuroimmunology literature I suggest two potential mechanisms which could alter psychological function during active intestinal inflammation.

1. Inflammatory mediators (Cytokine theory of depression)
2. Kynurenine pathway activation

2.3.2 Cytokines: implications for IBD and psychological disorders

Cytokines are soluble, regulatory proteins, such as the interleukins (IL), interferons (IFN) and tumour necrosis factors (TNF), which are released by cells of the immune system and act as intercellular mediators. Ultimately, their upregulation results in gene transcription through activation of signaling pathways. Cytokines can also interact with the central nervous system (CNS), thus providing a means of communication between the immune system and the brain. They are thought to enter or affect the brain via one or more of the following mechanisms which are proposed in two reviews on the role of inflammatory mediators in major depression (Licinio and Wong, 1999; Schiepers et al., 2005).

1. Disruption of the blood brain barrier (BBB), e.g. $\text{TNF}\alpha$ promotes BBB disruption
2. Penetration in to the brain through the circumventricular organs
3. De novo synthesis in the CNS (including the hypothalamus, hippocampus, cerebellum, forebrain regions, basal ganglia and brainstem nuclei)
4. Vagus nerve stimulation, which signals to the brain

5. Active transport at particular sites or induction of second messengers.

As well as influencing the CNS from the periphery, cytokines can also be produced within neurons and glial cells of the CNS. There is some evidence for basal cytokine expression in the CNS. Based on their review of the literature, Vitkovic et al. (2000) suggest that due to the constitutive expression and activity of cytokines in the brain, these may be important neuromodulators required for normal brain functions such as sleep. However, their detrimental effects in the CNS are also evident from the large body of literature reporting their over-expression in a number of neuropathologies (see Table 2.1)

Table 2.1: **Recent reviews implicating cytokines in neurological disorders**

Disorder	Authors
Epilepsy	Vezzani et al. (2008)
Alzheimer's	Heneka et al. (2010)
Multiple Sclerosis	Opdenakker and Van Damme (2011)
Dementia and Delerium	Cunningham (2011)
Schizophrenia	Mansur et al. (2012)
Neuropathic pain	Calvo et al. (2012)
Parkinson's	Panaro and Cianciulli (2012)
Depression	Leonard and Maes (2012)

Cytokine expression in IBD

In IBD, cytokines are thought to be involved in the initiation and amplification of the inflammatory response. They have the ability to modulate T cell recruitment as well as altering permeability and ion transport in the intestine (McClane and Rombeau, 1999). There are differences in cytokines expressed in patients suffering from IBD compared to healthy controls. A further difference exists between the cytokines that are up-regulated or down-regulated in CD versus UC. As mentioned above, there is a difference in helper T cell mediated response, which accounts for some of the cytokines differences between the two diseases; traditionally CD is viewed as Th1 mediated, while UC is viewed as Th2 mediated (McClane and Rombeau, 1999; Monteleone et al., 2011). In CD, the Th1 mediated response is manifested as increased concentrations of IL-1, IL-6, IL-8, IL-12 and IFN γ , and a decrease in IL-4 and IL-5 in colonic biopsies (McClane and Rombeau, 1999).

In inflamed colon samples, from UC patients, an increase in IL-12, IFN γ , TNF α , IL-1 β and IL-6 is also observed; however, there is also a significant increase in the Th2 mediated cytokine IL-4 and no increase in IL-2 (Moriconi et al., 2007). Bouma and Strober (2003) have also reported a Th2 type response in UC, characterised by an increase in both IL-5 and IL-13. Differences in cytokine expression are made more complex by differences in the concentrations of the various cytokines during different stages of the disease. More recently, with the identification of the Th17 subset of cells, which are regulated by IL-17 and IL-23, this inflammatory response has been reported in IBD patient biopsies and animal models of IBD (Liu et al., 2009; He et al., 2012; Alex et al., 2009; Monteleone et al., 2011).

Due to the nature of GIT breakdown in IBD, the over-production of cytokines is not confined to the bowel wall and increases in a number of pro-inflammatory cytokines have been reported in the systemic circulation in IBD patients (Szkaradkiewicz et al., 2009). In their investigation of 15 healthy controls, 20 UC patients and 12 CD patients, an increase in IL-6, IL-8, TNF α , and IFN γ in the circulation of both UC and CD patients versus controls was seen. They also report an increase in IL-10 in the serum of UC patients, which is not present in CD patients, supporting the Th2 mediated response reported in the colons of UC patients. *In-vivo* evidence of increased circulating cytokines in animal models of IBD has also been reported: using a novel approach of comprehensive multiplex cytokine profiling in mouse models of colitis, Alex et al. (2009) report a heightened circulating Th1-Th17 response in murine TNBS-induced colitis (increased IL-12 and IL-17), while in the DSS-induced colitis model there is a Th1-Th17 mediated acute inflammatory phase (increased TNF α , IL6, and IL-17), which changes to a mainly Th2 mediated inflammation as chronic colitis develops (increase in IL-4 and IL-10 and concomitant decrease in TNF α , IL6, and IL-17).

Cytokine expression in depression

There is increasing evidence, based on animal studies and case studies of patients suffering from inflammatory diseases, to suggest that cytokines are involved in depression (cytokine theory of depression)(for reviews see Leonard and Maes (2012); Dantzer et al. (2008); DellaGioia and Hannestad (2010); Irwin and Miller (2007)). Support for this hypothesis comes from three main findings. Firstly, activation of the inflammatory response system is linked to depression, and there is an increased innate immune response in patients suffering from depression (Maes et al., 1998). Maes et al. (1998) reviewed the evidence and report an increase in circulating IL-6, IL-8, IL-1 β , and IFN γ in patients suffering from

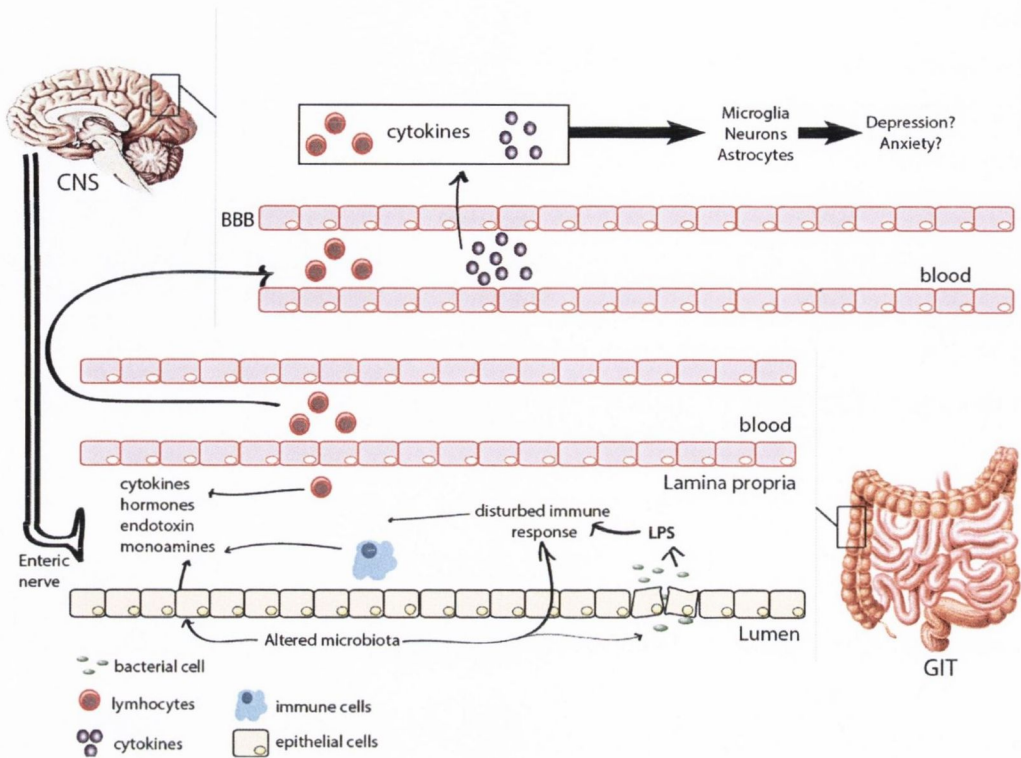


Figure 2.3: **Potential mechanism of immune activation-induced depression or anxiety in IBD:** Intestinal barrier disruption in IBD can lead to bacterial translocation and cytokine (IL-6, IFN γ , TNF α , IL-1 β) synthesis in the intestine. This can in turn induce systemic inflammation involving activation of circulating immune cells, increased circulating endotoxin from bacterial cells, and increase circulating cytokine concentrations. A combination of these factors penetrating the blood brain barrier (BBB) at leaky regions, or inducing *de novo* synthesis of immune mediators within the brain can result in modulation of normal CNS function resulting in behavioural disturbances. Immune activation within the intestine can also activate the vagus nerve which can signal to the CNS directly (based on de Theije et al. (2011))

depression. This cytokine profile provides evidence for a Th1 type immune response during major depression, which is very similar to the circulating cytokine profile reported in IBD patients. Secondly, it has been shown that patients treated with cytokine therapy, for cancer or hepatitis, develop both emotional and biological symptoms of depression, including depressed mood, dysphoria, anhedonia, helplessness, fatigue, anorexia and weight loss, hypersomnia, psychomotor retardation, decreased concentration and confusion, due to the treatment (Yirmiya, 2000; Capuron and Dantzer, 2003). In one study of 30 patients suffering from hepatitis C, who were treated with IFN α , 40.7% of the patients suffered from major depression after 3 months of treatment (Bonaccorso et al., 2002). Thirdly, in animals treated with substances that lead to an elevation in pro-inflammatory cytokines,

such as lipopolysaccharide (LPS), or administered the cytokines directly, there is an increase in sickness behaviour and depressive symptoms. Systemic administration of LPS leads to an increased release of pro-inflammatory cytokines such as $\text{TNF}\alpha$, $\text{IL-1}\beta$ and IL-6 in the brain (Harden et al., 2006). Two studies provide considerable evidence to suggest that IL-6 is an important mediator of sickness behaviour in rats (Harden et al., 2006, 2008). Using antisera against a variety of pro-inflammatory cytokines post LPS stimulation, Harden et al. (2006) demonstrated that inhibition of IL-6 significantly decreased fever and also decreased the reduction in voluntary wheel running and food consumption. However, although the results were significant, they confirmed that peripherally mediated IL-6 only partially mediates the suppression of voluntary movement induced by LPS. Further research, in 2008, involving injection of IL-6 and $\text{IL-1}\beta$ directly into the brains of conscious rats demonstrated a dose dependent effect of both cytokines on sickness behaviour, and also showed that when co-administered can have synergistic effects (Harden et al., 2008)

Based on this information, there is considerable evidence to suggest that cytokine production becomes dysregulated in IBD and these cytokines may be leading to the depressive symptoms experienced (see Figure 2.3). This finding is not surprising, as cytokines provide communication between the immune system and the CNS, therefore, they inform the brain of systemic sickness which could cause the behavioural changes. On the other hand, there is the possibility that the cytokines are having effects on other pathways, for example through activation of the kynurenine pathway, which in turn could lead to the depressive and or anxiety related symptoms. The kynurenine pathway is a major pathway involved in the degradation of the amino acid tryptophan (see section 2.4)

2.3.3 Matrix metalloproteinases

Matrix Metalloproteinases (MMPs) are a family of endopeptidases that can cleave most of the constituents of the extracellular matrix (ECM). They are mainly involved in the degradation of the interstitial connective tissue and basement membranes (Birkedal-Hansen et al., 1993). Transcription of MMPs can be induced by growth factors, cytokines and hormones leading to their distribution in many cell types including skin fibroblasts, keratinocytes, endothelial cells and monocytes. MMP9 is a member of the gelatinase subgroup of MMPs, it is not specifically a marker of inflammation however it does provide significant evidence of damage to colonic epithelial layers. Numerous studies have previously demonstrated the importance of colonic MMP9 induction in the DSS animal model and in human IBD (Santana et al., 2006; Medina et al., 2001; Medina and Radomski, 2006; Medina et al., 2003). The damage to the ECM associated with increases in MMP9 may

be involved in altered intestinal permeability and increased bacterial migration through the intestinal wall in IBD (Medina and Radomski, 2006).

The implication of MMP's extend beyond the bowel wall. MMPs are responsible for a number of functions within the CNS involved in synaptic plasticity and long term potentiation (LTP). MMP9 specifically is reported to be involved in regulation of hippocampal plasticity in *in vitro* studies (discussed in Domenici et al. (2010)). In their study, increase in MMP9 levels, by Multi Analyte Profiling (MAP), in depressed patients versus controls was reported.

2.3.4 Inducible Nitric Oxide Synthase: implications for IBD and psychological disorders

Nitric Oxide (NO), molecule of the year 1992, is a free radical gas discovered relatively recently as being a biological mediator produced by mammalian cells. This unexpected discovery, earned R. Furchgott, L. Ignarro, and F. Murad a Nobel Prize in physiology or medicine in 1998, and stimulated a large amount of interest in the physiological and pathological roles of NO. The enzymes responsible for the production of NO, nitric oxide synthase's (NOS), were first identified and described in 1989 (Alderton et al., 2001), leading to a scientific race to purify and clone NOS. Within the next four years, three genetically different isoforms of NOS had been characterised, including neuronal NOS (nNOS) (Bredt et al., 1991), inducible NOS (iNOS) (Xie et al., 1992; Lyons et al., 1992; Lowenstein et al., 1992; Wood et al., 1993; Geller et al., 1993) and endothelial NOS (eNOS) (Lamas et al., 1992; Marsden et al., 1992; Sessa et al., 1992; Janssens et al., 1992) (See review by Wang and Marsden (1995)). The nomenclature is based on the cell types they were originally purified from: neurons (nNOS), cytokine induced macrophages (iNOS), and vascular endothelium (eNOS). Although they are now known to be expressed in a variety of cell types (see Table 2.3), with some cell types capable of expressing more than one isoform the original names are still widely used. This section is intended to summarise the current knowledge on structure, induction and transcription of iNOS, as well as the potential impact of iNOS in IBD and the CNS.

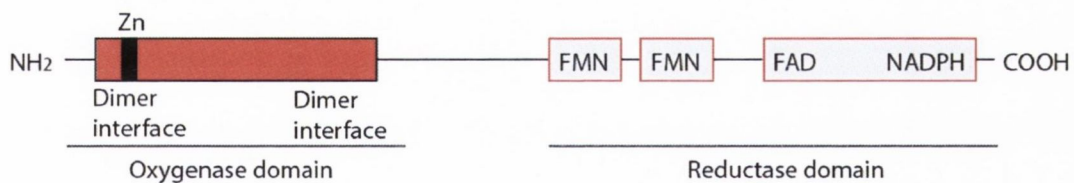
Structure, Induction and Transcription of iNOS

The NOSs are best characterized as cytochrome P-450 like haemoproteins (Bredt et al., 1991). Aside from iNOS being calcium independent (Table 2.2), the three NOS isoforms are structurally related. They can each be broadly divided into 2 distinct structural domains: the reductase domain at the COOH terminus and the oxidative domain at the

Table 2.2: Gene isoforms of human Nitric oxide synthase (Based on Wang and Marsden (1995); Alderton et al. (2001); Heneka and Feinstein (2001))

NOS isoform	Cell types	Molecular mass (Human)	Human gene structure, size and location	Co-Factors
nNOS, Type I, NOS-I, NOS-1	Neuronal tissue, Skeletal muscle, Cardiac muscle, smooth muscle	161 kDa	29 exons, 28 introns, >200kbp, Chromosome 12	Calmodulin, NADPH, FAD, FMN, Ca ²⁺ dependant
iNOS, Type II, NOS-II, NOS-2	Astrocytes, microglial cells, endothelial cells, neurons, motor neurons, macrophages, lymphocytes, hepatocytes, tumor cells, pancreatic islets, vascular smooth muscle cells, platelets, mesangial cells, renal tubular epithelial cells	131 kDa	26 exons, 25 introns, 37 kbp, Chromosome 17	Calmodulin, NADPH, FAD, FMN, Ca ²⁺ independant
eNOS, Type III, NOS-III, NOS-3	Endothelial cells, cardiac myocytes, platelets, certain brain neurons, renal tubular epithelial cells	133 kDa	26 exons, 25 introns, 21-22 kbp, Chromosome 7	Calmodulin, NADPH, FAD, FMN, Ca ²⁺ dependant

NH₂ terminus (see Figure 2.4). The reductase domain contains binding sites for NADPH, flavin-adenine dinucleotide (FAD), and flavin mononucleotide (FMN), and the calmodulin binding site lies between the oxidative and reductase domains (Alderton et al., 2001).

Figure 2.4: **Domain structure of iNOS:** Oxygenase and reductase domains are denoted by solid boxes (based on Alderton et al. (2001)).

Unlike constitutively expressed nNOS and eNOS, the expression of iNOS requires stimulation. This process is dependent on *de novo* mRNA and protein synthesis, with low basal rates of transcription being increased following exposure of cells to cytokines or LPS. Central iNOS induction has been demonstrated in astrocytes, neurons, and microglia in a wide variety of brain regions (Table 2.3). Once iNOS has been induced via transcriptional regulation it produces large amounts of NO, which can have neurotoxic effects. At the DNA level there is an NF κ B binding site in the proximal region of the promoter, which has been shown to be responsible for the induction of the iNOS gene by LPS (Xie et al., 1993; Lowenstein et al., 1993). A binding site for the IFN γ -induced interferon regulatory factor-1 (IRF-1), further down from the NF κ B binding domain, also results in increased

Table 2.3: Selected examples of studies reporting iNOS induction in various CNS cell types and brain regions

Paper	Cell type	Brain region	Method	iNOS induction method
Knerlich et al., 1999	Microglia (CD11 β), anti-granulocytes	Lesion penumbra	mRNA and immunostaining, RT-PCR	Cryogenic cortical lesion (rats)
Suarez et al., 2005	Bergmann glial cells, Purkinje cells	Cerebellum	Immunoblotting, Immunostaining	PCA (1m and 6m) (rats)
Carpuzano et al., 2008	Neurons (NeuN), Neutrophils (MPO), Microglia (TL), Astrocytes (GFAP)	Striatum	Immunofluorescence	NMDA lesion (aged and adult rats)
Ono et al., 2010	microglia (MAC-1)	around the striatal injury	PCR, Greiss assay, immunohistochemistry	Brain injury due to ethanol injection (mice)
Amrouni et al., 2010	Neurons (NeuN), Astrocytes (GFAP), microglia (Integrins)	Thalamus and hypothalamus	Immunohistochemistry, iNOS activity, immunofluorescence	T.b brucei infection (rats)
Goryaheva et al., 2010	-	Hippocampus, cortex	Nitrite and nitrate conc, western blotting	A β injections (rats)
Liu et al., 2010	-	Hippocampus, cortex	Western blotting	Chronic unpredicted mild stress (rats)

iNOS transcription (see Figure 2.5).

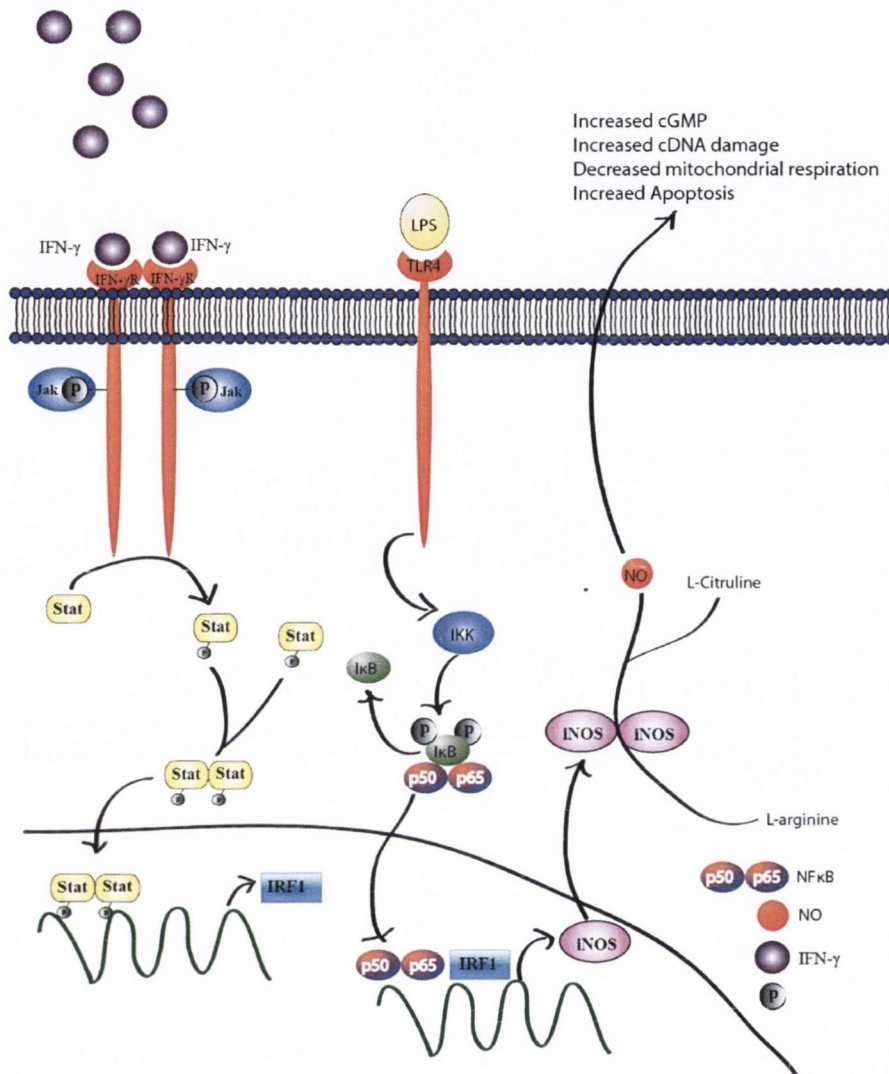


Figure 2.5: **Induction of iNOS mRNA transcription via IFN γ and LPS:** iNOS is transcribed and synthesised following cellular exposure to certain cytokines and endotoxins, such as IFN γ and LPS. IFN binds to the IFN γ R and signals through the JAK-STAT pathway. Receptor occupation and dimerisation induces the phosphorylation of associated STATs, these activated STATs dimerise and translocate to the nucleus where they increase expression of the transcription factor, IRF-1, that, in turn, binds to specific DNA elements in the iNOS gene promoter region to increase iNOS gene expression. LPS binds to TLR4 leading to the activation of an alternative pathway leading to the dissociation of I κ B from NF κ B, allowing NF κ B to translocate to the nucleus where it binds to specific DNA elements in the iNOS gene promoter region to increase iNOS gene transcription. NO is generated following, the dimerisation of iNOS in the cytosol, from L-arginine. Nitric oxide (NO) can signal within the cell in which it is produced or by penetrating cell membranes to affect adjacent cells.

Involvement of iNOS in IBD

In IBD, iNOS is of particular interest as it can be synthesized following induction by pro-inflammatory cytokines, endotoxin or hypoxia, and it has been widely reported in the intestine of IBD patients (Leonard et al., 1998; Kolios et al., 2004). Leonard et al. (1998) report increased iNOS immunohistochemical staining in the colons of both UC and CD patients. Increased iNOS mRNA expression has also been reported in the bowels of animal models of IBD (McCafferty et al., 1999; Beck et al., 2004). Induction of iNOS extends beyond the bowel: Oudkerk Pool et al. (1995) examined serum from patients with active and inactive CD or UC and reported a significant increase in circulating nitrate in active IBD which they suggest may be implicated in the pathogenesis of the disease. Palatka et al. (2006) have also demonstrated that exposing human umbilical vein endothelial cells to sera from IBD patient resulted in a significant increase in iNOS expression compared to control sera, and Villaran et al. (2010) also report increased iNOS mRNA expression in the substantia nigra in an animal model of colitis.

Involvement of iNOS in pathologies of the CNS

Physiological concentrations of NO play a crucial role in synaptic plasticity, neuromodulation and has other physiological functions in the brain. However, over production of NO due to iNOS may induce oxidative damage and has been demonstrated to induce neurotoxicity. Increased iNOS has been implicated in many pathologies of the CNS including Alzheimer's disease patients and models (Lüth et al., 2001; Haas et al., 2002; Nathan et al., 2005; Medeiros et al., 2007), Huntington's disease patients and models (Thomas et al., 2004; Ryu et al., 2004; Aguilera et al., 2007), neurodegeneration in Parkinson's disease (Hunot et al., 1996; Knott et al., 2000; Hunot and Hirsch, 2003), and possible roles of iNOS induced NO mediating sickness behaviour, depression or stress (Khovryakov et al., 2010; Yamaguchi et al., 2010; Montezuma et al., 2012; Chung et al., 2010).

2.4 The kynurenine Pathway: implications for IBD and psychological disorders

The kynurenine pathway has gained interest from neuroscientists as it provides a link between the more recent theory of immune mediated depressive symptoms and the traditional monoamine theory of depression. The kynurenine pathway is involved in the degradation of tryptophan to kynurenine either by tryptophan-2,3-dioxygenase (TDO) in

the liver or by indoleamine-2,3-dioxygenase (IDO), which is present in a range of immune cells, including macrophages and dendritic cells (Schrocksadel et al., 2006). TDO and IDO are induced via different mechanisms: TDO is induced by cortisol or tryptophan itself, while IDO is induced by inflammatory cytokines such as $\text{IFN}\gamma$, and to a lesser extent by $\text{TNF}\alpha$, and $\text{IL-1}\beta$ (Maes et al., 2011; Carlin et al., 1989; Yeung et al., 2012; Zunszain et al., 2012). Following the degradation of tryptophan to kynurenine there is a sequence of enzymatic events that lead to the generation of a variety of metabolites known as kynurenines. These include quinolinic acid, kynurenic acid (KYNA), and picolinic acid (Guillemin et al., 2007) (see Figure 2.6).

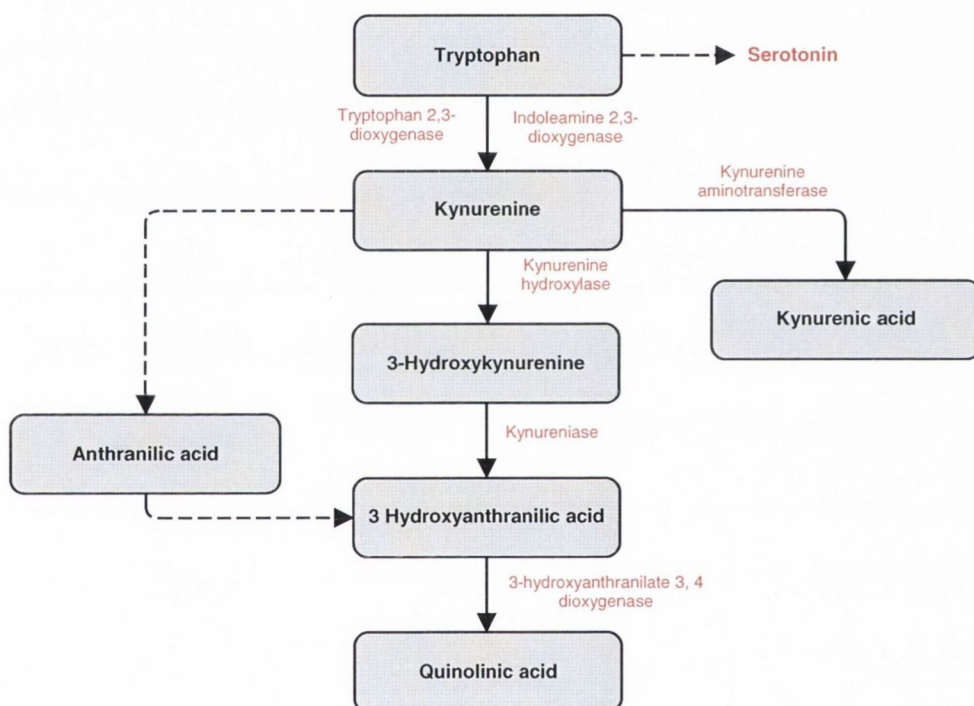


Figure 2.6: **Simplified diagram of the kynurenine pathway.** Following the degradation of tryptophan to kynurenine, there is a sequence of enzymatic events that lead to the generation of a variety of metabolites known as kynurenines. Metabolites are represented in solid boxes and enzymes are in red text.

2.4.1 Kynurenine pathway dysregulation in IBD

The physiological role of tryptophan and its metabolites in the GIT appear to be in immunomodulation, as several of the metabolites including kynurenine, suppress the proliferation of activated T cells, thereby promoting tolerance to non-harmful antigens (Terness et al., 2002). In IBD, there is increased IDO expression in epithelial cells flanking ulcers

or bordering crypts. This increased IDO expression results in decreased tryptophan concentrations, which is thought to have an anti-inflammatory role as it decreases tryptophan availability for tryptophan-dependent microorganisms, thereby decreasing their growth (Ferdinande et al., 2008). Wolf et al. (2004) report increased IDO in colonic explant cultures of CD patients. This IDO was associated with an increase in kynurenine and the kynurenine:tryptophan ratio in the supernatant. In a recent investigation, Gupta et al. (2012) found that increased intestinal IDO expression in CD patients was associated with decreased circulating tryptophan concentrations and an increased kynurenine:tryptophan ratio. Disruption to circulating tryptophan and the kynurenine:tryptophan ratio has been reported in two further investigations: Torres et al. (2007) found significant decreases in serum tryptophan and increased kynurenine:tryptophan ratio in CD patients versus controls, Hisamatsu et al. (2012) confirmed the tryptophan changes in CD patients but report no differences between UC patients and controls. The disruption to circulating tryptophan and kynurenine concentrations provides a mechanism by which the inflammation in the bowel could communicate with the CNS, potentially leading to mood disturbances.

2.4.2 Kynurenine pathway dysregulation in depression

The biochemical link between kynurenine pathway activation and psychological disturbances is still controversial, however, two potential factors are discussed in the literature: altered kynurenine metabolite concentrations and decreased tryptophan may influence behaviour.

Tryptophan depletion and depression

Tryptophan is the precursor to both kynurenine and 5-HT biosynthesis, and in humans altered serotonergic transmission is involved in the development of depression. There is also evidence to suggest that peripheral markers such as lower plasma tryptophan levels, and reduced 5-Hydroxyindolacetic acid (5-HIAA) levels in the cerebrospinal fluid (CSF) can be used to differentiate depressed patients relative to controls (Neumeister, 2003). For this reason it was suggested that experimentally depleting tryptophan using a specific amino acid diet should lead to reduced 5-HT in the CNS, and therefore, increase symptoms of depression. As 5-HT can not be measured directly in humans brains, support for this hypothesis has come from examination of CSF, where dietary tryptophan depletion leads to decreased tryptophan and 5-HIAA in the CSF (Williams et al., 1999). In humans, when tryptophan was depleted in healthy subjects, who were not at genetic risk of depression, some mood changes were noted, but it did not result in clinical depression. In contrast,

in patients who had a family history of depression, depletion of tryptophan resulted in increased risk of suffering depressive symptoms, and patients who had suffered from major depression in the past had a temporary return of depression (Neumeister, 2003).

A study by Capuron et al. (2002) revealed that cancer patients treated with cytokines had an increased level of depression that correlated with a decrease in serum tryptophan concentration. They hypothesise that it is the decreased availability of tryptophan for 5-HT synthesis that leads to the development of depression (Capuron et al., 2002). However, in a study by O'Connor et al. (2008), where they demonstrated altered kynurenine/tryptophan ratio, they found no difference in 5-HT levels of control and LPS treated mice. Although, in their study 5-HT was examined on whole brain homogenates, there is still the possibility that 5-HT may be decreased in specific brain regions when there is reduced availability of tryptophan, and this may influence mood (O'Connor et al., 2008). In a more recent investigation involving Poly I:C-induced immune activation, Gibney et al. (2012) also report altered kynurenine and tryptophan levels without changes to hippocampal or cortical 5-HT. In another study involving a starved rat group with a restricted diet, where plasma tryptophan levels were significantly decreased, the depletion in tryptophan corresponded with a significant decrease in 5-HT levels and an increase in 5-HIAA in the hypothalamus of both male and female rats. In male rats tryptophan depletion also corresponded with decreased 5-HT in the rest of the brain; by contrast the same decrease was not seen in the rest of the brain in female rats (Haider and Haleem, 2000).

Kynurenines in depression

Recent evidence supports a role for the excitatory amino acid glutamate in the development of depression. This hypothesis suggests depressed mood is associated with an increased activity of the glutamatergic system and activation of the glutamate receptor subtype, the NMDA receptor (Muller and Schwarz, 2007). Further evidence in favour of this hypothesis has been the discovery that NMDA antagonists, such as ketamine, lead to rapid antidepressant effects in patients with treatment-resistant major depression (Maeng and Zarate Jr., 2007). The kynurenines themselves can affect the NMDA receptors and therefore may be directly involved in depressed mood (for review see Ruddick et al. (2006).

Kynurenine

Kynurenine is the central compound of the kynurenine pathway, it is not thought to be neuroactive, but it can readily cross the blood brain barrier via the large neutral amine-acid transporter or get formed in the brain (Fukui et al., 1991; Nemeth et al., 2007), and in do-

ing so can lead to the generation of kynurenine metabolites by perivascular macrophages, microglial cells and astrocytes within the CNS (O'Connor et al., 2008). Increased kynurenine also appears to be an important factor in the development of inflammation induced depressive symptoms in mice (O'Connor et al., 2008). Their results showed a link between the dose of kynurenine and increased immobility in behavioural tests of depression. However, the doses used in the experiments were very high and therefore results might not represent the effect of kynurenine in disease states.

Quinolinic acid

Quinolinic acid is an NMDA receptor agonist. Quinolinic acid activation of the NMDA receptors leads to the generation of reactive oxygen and reactive nitrogen species, which have neurotoxic effects (Guillemin et al., 2007; Nemeth et al., 2007). It has similar toxic effects as glutamate in the neocortex, striatum and hippocampus (Nemeth et al., 2007). In addition to this, quinolinic acid also accumulates in astrocytes and neurons in depression (Leonard, 2007).

3-Hydroxykynurenine and 3-Hydroxyanthranilic acid

3-Hydroxykynurenine, produced directly from kynurenine and its metabolite 3-hydroxyanthranilic acid are also neurotoxic, however, they are less toxic than quinolinic acid. Damage caused by these metabolites is thought to be due to generation of free radicals, oxidative stress and lipid peroxidation rather than through interaction with NMDA receptors (Ruddick et al., 2006; Nemeth et al., 2007).

Kynurenic acid

While the other kynurenine metabolites are known to cause neurotoxicity, kynurenic acid has antagonistic effects on the NMDA receptor and has neuroprotective effects (Guillemin et al., 2007).

2.5 Summary of suggested mechanisms which might link IBD with depression or anxiety

In conclusion, it is possible that the immune activation may be involved in the increased psychological disturbances experienced during active IBD. Further research is needed in this area as it would be therapeutically beneficial to determine the role of chronic inflammation on normal CNS function, with an aim to reduce depression in patients suffering from IBD or other chronic inflammatory diseases (see Figure 2.7).

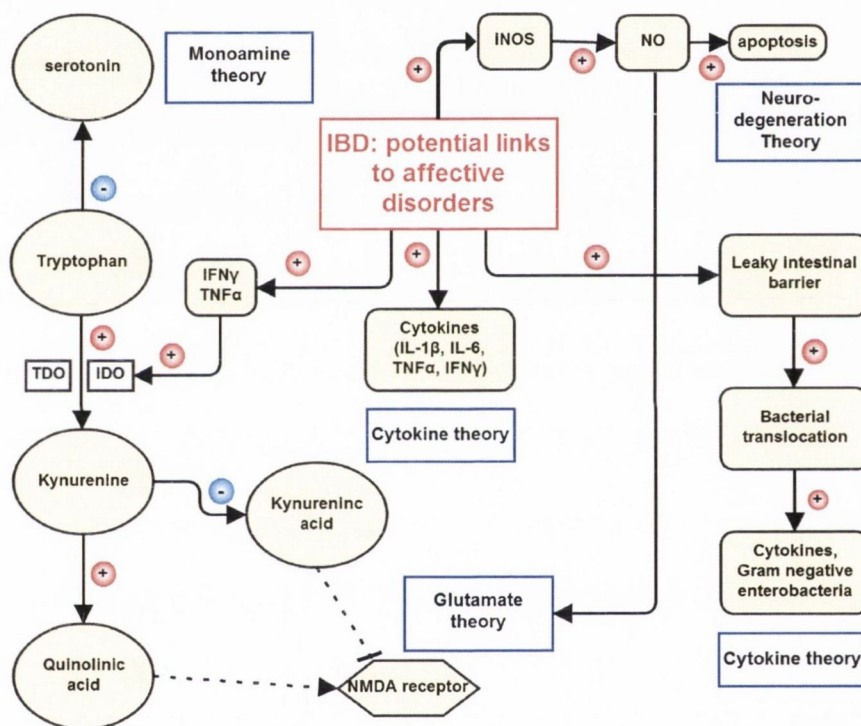


Figure 2.7: Summary of immune mediators which could induce depression or anxiety in IBD patients

Chapter 3

Bidirectional communication between the gut and central nervous system in animal models of inflammatory bowel disease

3.1 Introduction

IBD is a chronic relapsing and remitting disorder of the GI tract, which affects as many as 1.4 million people in the United States and 2.2 million people in Europe (Loftus, 2004). Common symptoms of IBD include diarrhoea with blood and mucus, constipation, vomiting, abdominal pain, fever and weight loss. IBD can be divided into two main disorders: ulcerative colitis (UC) and Crohn's disease (CD). Inflammation in UC usually remains in the rectum and colon, while CD can affect any part of the GI tract. Historically, there has been significant interest in the co-morbidity of psychological well-being and disorders of the gastrointestinal tract (Murray, 1930; Sullivan, 1935; Daniels, 1942; Straker, 1960). Recent research has confirmed that there is an increased risk of depression/anxiety in patients suffering from IBD, and these psychological symptoms are most often present during the active disease phase (see review of the literature and Ben Thabet et al. (2012); Häuser et al. (2011); Mardini et al. (2004); Calvet et al. (2006)). Despite this finding and the recent surge in psychoneuroimmunology research, there is a lack of investigation into the potential association between inflammatory mediators and psychological disturbances during the active phase.

As well as IBD affecting the CNS, there is evidence to suggest that a bi-directional

communication axis exists between the gut and brain, and that psychological stress and/or depression has negative implications for normal gut function. It is also hypothesised that psychological factors may be risk factors for relapse in patients suffering from IBD, although this remains controversial, with some studies reporting no effect of major stress on development of IBD or increased risk of relapse (Li et al., 2004; Vidal et al., 2008). Other studies found decreased remission rates in IBD patients with depression and that depression scores can predict future changes in IBD activity (Persoons et al., 2005; Mardini et al., 2004; Mittermaier et al., 2004).

Due to the lack of molecular evidence in humans and the questions surrounding the impact of psychological disturbances on IBD, the aim of this section was to review the animal model literature: firstly to determine whether colonic inflammation can induce molecular disturbances in the CNS, and secondly to establish the effect of psychological stress/depression on colonic damage in models of IBD.

3.2 Animal models

3.2.1 Animal models of IBD

As with most human diseases, there has been an attempt to create animal models of IBD to aid in characterising the disease process, and to test the efficacy of potential pharmaceutical therapies. According to Jurjus et al. (2004) this has led to the development of over 20 different animal models of IBD, some which represent the symptoms of UC better, and others that are more similar to CD. When creating these models, the aim is to generate animals that exhibit similar morphological changes, inflammation, symptoms, pathophysiology, and a course similar to human IBD (Jurjus et al., 2004). Various methods have been used in the development of these models: gene knockout, transgenics, spontaneous colitis, and chemically inducible colitis.

DSS and TNBS-induced colitis as animal models of IBD

Despite the development of a large number of IBD models, the main body of behavioural and brain research has been carried out in the trinitrobenzenesulphonic acid (TNBS) and dextran sulphate sodium (DSS) models of colitis. Okayasu et al. (1990) were the first to describe the DSS induced colitis model. This model involves oral administration of DSS in the drinking water of the animals, which leads to the development of acute and chronic colitis. Early clinical findings in the acute colitis include haemoccult positive stools, loose stools or diarrhoea, and weight loss. By day 7 of 5% DSS exposure symptoms are severe

and coincide with a 53% mortality rate in rats (Kullmann et al., 2001). Gaudio et al. (1999) assessed the structural, ultrastructural, immunohistochemical and clinical aspects of DSS colitis in Sprague Dawley rats in both acute and chronic DSS-induced colitis. They report slower weight gain and decreased colon length in colitic rats. They also demonstrate that the distal colon is predominantly involved. From a structural, ultrastructural and clinical point of view, they suggest that the DSS model is more representative of UC than CD. Although histologically the DSS model appears to be similar to that of human UC.

The TNBS model of colitis was first reported by Morris et al. (1989). This model of IBD involves a single enema of the toxin TNBS in an ethanol solution. The ethanol serves to break down the intestinal barrier allowing the TNBS access to the bowel wall where it induces an inflammatory response. Grisham et al. (1991) also report that intramural injection of TNBS in saline results in inflammation. TNBS-induced colitis results in a Th1 mediated inflammatory response, which is described as being Crohn's like in nature (Bouma and Strober, 2003). Unlike the DSS model, which predominantly involves the distal colon, TNBS can induce a more widespread colitis, involving macroscopic ulceration of the large intestine of varying severity.

Although these models are not ideal in mimicking IBD, as suggested by Te Velde et al. (2007) based on gene upregulation and downregulation assessment, they do result in immune activation in the gut, and are histologically representative of IBD, therefore, making them suitable models for examining the potential interaction between intestinal immune activation and the CNS.

3.2.2 Experimental stress induction

Various methods have been developed to test the effects of psychological stress in animal models including physical and psychological stressors: water avoidance stress, restraint stress, isolation housing or overcrowding, social defeat, white noise stress, electric shock, cold temperature exposure, and the communication box (Mawdsley and Rampton, 2005; Jaggi et al., 2011). These stressors can be administered once to investigate the effect of acute stress or are repeated and/or varied over a number of days to mimic the effects of chronic stress. Chronic stress protocols range from extremely severe, such as the chronic variable stress model first described by Katz et al. (1980), which involved 20 days of various stressors (including shock, food deprivation, cold swim, water deprivation (48 h), food deprivation (48 h), cage switching, cage mate switching, and isolation housing), to less severe models of chronic variable stress, which do not involve the intense foot shock, 48 h food/water deprivation and foot shock used by Katz et al. (1980), and mild models

involving only daily restraint stress or water avoidance stress (Willner, 1997; Israeli et al., 2008). Chronic stress paradigms have also been used to develop models of anxiety and depression, as they result in molecular and behavioural changes similar to anxiety or depression, and respond to some anxiolytic and antidepressant treatments (for reviews see Joëls et al. (2004); Treit et al. (2010))

The maternal stress (MS) model is based on the hypothesis that early life stress can result in long lasting psychological disturbances. The model involves daily separation of pups from as early as post natal day (PND) one for varying lengths of time (routinely 10-180 minutes) and for various numbers of days (14-22 days). MS adults are reported to have a depressive like phenotype: decreased sucrose preference and increased immobility in the forced swim test and tail suspension test, which is reversible with antidepressant treatment (Varghese et al., 2006; Oines et al., 2012). These adults are also reported to express an irritable bowel syndrome (IBS)-like phenotype supporting the idea of a CNS effect on normal bowel function (O'Mahony et al., 2011).

3.3 Gut-Brain communication: CNS disturbances in models of IBD

Evidence of disturbances to normal CNS functioning following induction of intestinal inflammation in models of IBD has been reported. These disturbances can be classified into five subgroups;

1. BBB permeability
2. Neuronal activation
3. HPA axis activation
4. Neuroinflammation
5. Behavioural changes

3.3.1 Increased BBB permeability

The blood brain barrier (BBB) provides a barrier between the potentially damaging substances within the circulation and the CNS. It achieves this physical barrier through tight junctions between the endothelial cell, which restricts the passage of substances into the

brain. Several areas of the brain are within 'leaky' areas of the BBB, including the circumventricular organs, which are therefore potentially more vulnerable to molecular changes within the circulation.

Based on the now controversial evidence of vascular lesions in the brains of IBD patients, Hathaway et al. (1999, 2000) investigated potential disruption to the BBB in rabbits exposed to TNBS. Barrier disruption was assessed following IV administration of low molecular weight fluorescein (mol. wt. 376 Da) or a higher molecular weight molecule FITC-dextran (mol. wt. 71000) 48 h post TNBS administration. Results demonstrated a significant increase in the permeability of the BBB to fluorescein, however, no difference in the permeability to the higher molecular weight FITC-dextran was found.

More recently, Natah et al. (2005) further analysed the BBB disruption in Sprague-Dawley rats exposed to TNBS to determine the anatomical sites of the BBB disruption using sodium fluorescein or IgG as a marker of increased permeability. As per Hathaway et al. (1999, 2000), they revealed an increased permeability to the low molecular weight sodium fluorescein, but not to the larger IgG molecules. The regions of higher permeability were located at the circumventricular organs: specifically the organum vasculosum of the lamina terminalis (OVLT), subfornical organ (SFO) and median eminence (ME) during days 1 and 2 following TNBS administration (see Figure 3.1).

Sans et al. (2001) measured expression of vascular cell adhesion molecule-1 (VCAM-1) and Intercellular Adhesion Molecule-1 (ICAM-1) using a dual radiolabelled antibody technique in 4 different models of colitis including the DSS and TNBS models. VCAM and ICAM are endothelial CAMs of the immunoglobulin super family, which are responsible for the adhesion of leukocytes in various inflammatory diseases. This study demonstrated that there is a significant increase in VCAM expression in the brain of all 4 models of colitis, which corresponded with colonic VCAM expression and colon weight. They also report that TNBS-induced colitis also induces ICAM expression, although this is not the case in DSS. These changes were not associated with increased leukocyte infiltration to the brain and are not representative of BBB disruption, however, they provide further evidence of molecular alterations at the BBB following colonic inflammation.

3.3.2 Neuronal activation

C-Fos is an immediate-early gene, which is used to indirectly measure neuronal activity as it is often expressed following an action potential (for reviews see Kovács (2008); Okuno (2011)). Central c-Fos expression has been well characterised in animal models of colitis (Sinniger et al., 2005; Cattaruzza et al., 2010; Porcher et al., 2004; Welch et al.,

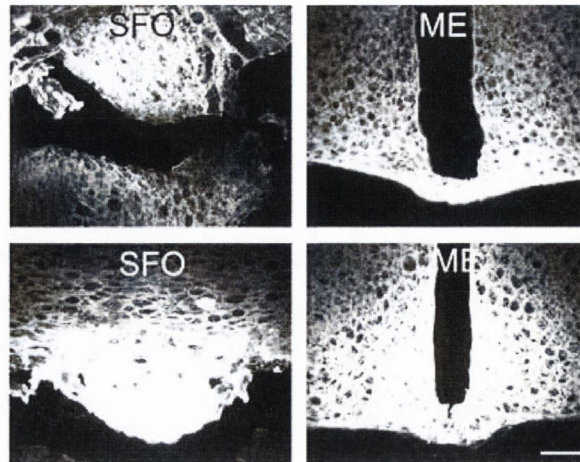


Figure 3.1: **Blood brain barrier disruption in the TNBS animal model of IBD.** Sodium fluorescein leakage in the circumventricular organs 2 days after saline (upper panels) or TNBS treatment (lower panels). Note that sodium fluorescein extravasation was significantly increased in the subfornical organ (SFO) and median eminence (ME) Scale bar: 200 μm . (images from Natah et al. (2005))

2005, 2010; Miampamba and Sharkey, 1999). Original evidence of *c-Fos* activation in the nervous system following induction of colitis was published by Miampamba and Sharkey (1999). Colitis was induced following a perendoscopic injection of formalin and rats were euthanised 2 h later. Immunohistochemical analysis demonstrated a significant increase in *c-Fos* in the lumbrosacral spinal cord (LSC), Nucleus of the solitary tract (NST) and Area postrema (AP). They also report that the α_2 adrenoceptor agonist xylazine dose dependently inhibits this increased *c-Fos* expression. A later study by Porcher et al. (2004) extensively analysed the expression of *c-fos* 2 h post TNBS administration throughout the brain. They report significant increases in *c-Fos* density across a number of brain regions, including the telencephalon, neocortex, thalamus, hypothalamus, as well as the pons and medulla, which include the AP and NST previously included in the Miampamba and Sharkey (1999) study. At 6 hours post TNBS administration *c-fos* mRNA expression in the PVN had completely returned to basal levels.

Welch et al. (2005, 2010) focused on TNBS-induced *c-Fos* activation in a number of brain regions; periventricular gray, hypothalamic/visceral thalamic stress axes and cortical domains, and septal /preoptic/amygdala brain areas. In the earlier study, on day 20 (13 days post final TNBS enema) rats were euthanised and brains were fixed for immunohistochemical analysis. Results support previous evidence of increased *c-Fos* induction following colitis, however, here the results suggest prolonged neuronal activation (Welch et al., 2005). In a later study they confirmed these findings, however, one group of rats

was also vagotomised to determine the implication of the vagus nerve on c-Fos induction in the paraventricular nucleus of the hypothalamus, basolateral amygdala, central amygdala, and periform cortex. Subdiaphragmatic vagotomy did not inhibit the observed increase in c-Fos induction, suggesting a different communication mechanism is involved.

3.3.3 HPA axis activation

The HPA axis represents a major axis of the neuroendocrine system that controls reactions to stress, and regulates many physiological processes including digestion, immune system, mood, and emotions. Dysregulation of the HPA axis has been linked to a number of mood disorders including depression, anxiety, bipolar disorders and ADHD. Greenwood-Van Meerveld et al. (2006) studied the long term effects of acute colitis on the expression of central corticotrophin releasing factor (CRF). They found a significant increase in CRF mRNA expression in the Para Ventricular Nucleus (PVN) of the hypothalamus 3 days post TNBS administration, which persisted up to 30 days post TNBS. The increased CRF expression was also present in the central nucleus of the amygdala (CeA) 3 days post TNBS administration, however, they had returned to basal levels in this region 30 days post TNBS. Kresse et al. (2001) also report increased CRF expression in the hypothalamus following induction of colitis. Porcher et al. (2004) reported increased expression of the CRF1 receptor mRNA expression in the PVN following TNBS induced colitis, however, unlike CRF, the CRF1 receptor mRNA levels had returned to baseline within 12 hours of TNBS administration.

Results by Kojima et al. (2002) conflict with the reported increase in CRF in the PVN following TNBS-induced colitis. They report the opposite effect: decreased CRF expression at 3 and 7 days post TNBS administration. They do however report increased circulating corticosterone on days 1, 3, 7 and 14 post TNBS. Both studies were carried out in male Sprague-Dawley rats, however, the doses of TNBS were much lower in the Kojima et al. (2002) study (0.2ml containing 20mg of TNBS versus 0.5ml containing 50mg of TNBS). The higher dose of TNBS may provoke a more severe colitis necessary for increased CRF expression in the brain.

3.3.4 Increased inflammatory mediator expression

Cytokines are soluble, regulatory proteins, released by immune cells, which act as intercellular mediators. Cytokines can interact with the CNS, thus providing a means of communication between the immune system and the brain. As well as influencing the brain following peripheral immune activation cytokines can be produced within neurons

and glial cells in the brain, and their involvement has been proposed in the pathophysiology of a number of psychiatric disorders including depression (Leonard and Maes, 2012). There is evidence of altered cytokine expression in the brains of rodents exposed to TNBS. Following reports of increased seizure susceptibility in patients with chronic inflammatory conditions, Riazi et al. (2008) investigated the influence of TNBS-induced colitis on hippocampal $\text{TNF}\alpha$ concentrations and microglial activation in male Sprague-Dawley rats. They found an increase in both hippocampal $\text{TNF}\alpha$ protein concentrations and microglial activation at 4 days post TNBS administration, both of which had returned to basal concentrations at day 10. In a later study, Medhi et al. (2009) confirmed that a single enema of TNBS induces increases in circulating $\text{TNF}\alpha$ concentrations, which are paralleled by increased brain $\text{TNF}\alpha$ protein concentrations. However, unlike the Riazi et al. (2008) study, the increase was still present at day 15 post TNBS administration, possibly due to differences in the strain of rat used.

Wang et al. (2010) investigated the effect of TNBS-induced colitis on IL-6 expression in the brains of female Wistar rats at 3, 7, 14, 21 and 28 days post enema. They report an increase in brain IL-6 mRNA expression and IL-6 protein concentration in the hypothalamus and cerebral cortex, which peaks at 7 days post enema. Concentrations of brain IL-6 are also increased in mice exposed to TNBS, however, peak IL-6 concentrations were at 2 days post enema and remained increased 7 and 15 days post TNBS administration (Baticic et al., 2011). In their study, they also report a decrease in the concentrations of the anti-inflammatory cytokine IL-10 2 and 7 days post TNBS. Finally, further confirmation of increased inflammatory cytokine expression following TNBS-induced colitis was also reported by Alhouayek et al. (2011). Three days post TNBS administration there was an increase in IL-1 β and $\text{TNF}\alpha$ mRNA expression in the brains of C57BL6 mice, which was associated with an increase in circulating endotoxin concentrations.

Although there is less research into the effect of DSS administration on central cytokine expression, one study did investigate the effect of acute inflammation on $\text{TNF}\alpha$, IL-6, IL-1 β , and iNOS mRNA expression in the substantia nigra (Villaran et al., 2010). They reported a significant increase in all the inflammatory cytokines investigated and iNOS in the substantia nigra of male Wistar rats during acute DSS-induced colonic inflammation.

3.3.5 Evidence of anxiety/depression-like behavioural changes in models of colitis

Painsipp et al. (2011) analysed female and male mouse behaviour in the elevated plus maze (EPM), openfield (OF) and forced swim test (FST) on days 8, 9 and 11 respectively of an

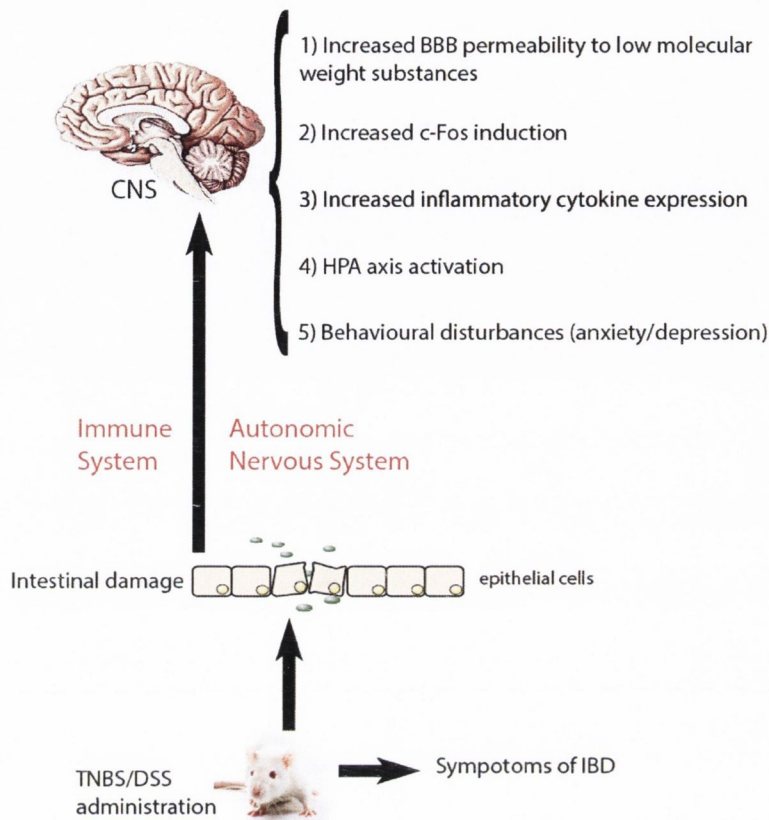


Figure 3.2: **Summary of molecular disturbances in the CNS of animals with colitis.** Results have shown that induction of inflammation in the bowel results in symptoms of IBD accompanied by altered blood brain barrier permeability, increased c-Fos expression, activation of the HPA axis, and increased inflammatory cytokines in the central nervous system of rats exposed to 2,4,6 trinitrobenzenesulphonic acid (TNBS) or dextran sulfate sodium (DSS). Animals with colitis have also been reported to behave differently to control animals.

11 day DSS exposure protocol (2% DSS). Colitis had some behaviour modulating effects, which were sex dependant: male mice spent significantly less time in the open arms of the EPM indicative of anxiety like behaviour, while female rats had increased immobility in the FST indicative of a depressive like phenotype. They conclude that colitis modified anxiety- and depression-related behaviour in a sex-, genotype- and test-related manner, and knockout experiments indicated that Peptide YY (PYY) and neuropeptide Y (NPY) were involved in some of these behavioural effects of colitis. In a different model of IBD, infection with *Citrobacter rodentium*, Lyte et al. (2006) examined anxiety like behaviour in a hole-board open field apparatus. Male mice were tested 7-8 h post infection and results provide evidence for an anxiety like phenotype: decreased exploration of the inner

zone of the openfield, decreased number of pokes into the holes as well as a preference for the first corner hole compared to control mice. The infected mice were also reported to exhibit an increased level risk assessment behaviours.

Anxiety like behaviour has also been reported in the DSS model of colitis (Bercik et al., 2011). In their investigation, mice received DSS (3%) in drinking water during three 1-week cycles. Behaviour was assessed by step-down test and results demonstrated increased anxiety in the colitic rats, which was vagally mediated. They also demonstrated that the probiotic *Bifidobacterium longum* NCC3001 is anxiolytic, potentially through activation of vagal pathways.

In an earlier study, Messaoudi et al. (1999) analysed lever pushing behaviour in an aversive light stimulus avoidance test in rats exposed to TNBS. They found that colitic rats had a lower number of total active lever pressings, and did not discriminate the active lever from the inactive one. This behavioural disturbance was attributed to TNBS induced pain, as morphine returned lever pressing to control levels. Despite not being suggestive of depression or anxiety, this highlights the importance of accounting for the potential influence of pain on behavioural disturbances in these animal models.

3.3.6 Summary of gut-brain communication during colitis

The mechanisms summarised in figure 3.2 illustrate how a significant central disturbance, as well as behavioural changes may occur following experimentally-induced colitis in rodents. Results to date suggest that the correlation between psychological disturbances and immune mediators should be investigated in patients, as targeting specific mediators could be beneficial in reducing psychological disturbances experienced.

Table 3.1: Colitis-induced CNS disturbances

Author	Colitis model	CNS molecular/behavioural changes
BBB permeability		
Hathaway et al. (1999)	TNBS (rabbit)	increased BBB permeability to low molecular weight fluorescein
Hathaway et al. (2000)	TNBS (rabbit)	free radical damage is not responsible for increased BBB permeability to low molecular weight fluorescein
Natah et al. (2005)	TNBS (rat)	increased BBB permeability occurs around the CVOs (OVLT, SFO, ME)
Sans et al. (2001)	DSS, TNBS (rat)	increased VCAM in both DSS and TNBS, and increased ICAM in TNBS
Neuronal activation		
Porcher et al. (2004)	TNBS (rats)	Increased c-Fos 2 hrs post TNBS: telencephalon, neocortex, thalamus, hypothalamus, pons, medulla. Basal c-Fos levels by 6 hrs post TNBS. Increased CRF1 receptor mRNA in the PVN, basal levels by 12 hrs post TNBS.
Cattaruzza et al. (2010)	TNBS (mice)	Increased spinal c-Fos expression (*check)
Welch et al. (2005)	TNBS (rat)	Prolonged neuronal activation, as per c-Fos expression, following TNBS-induced colitis
Welch et al. (2010)	TNBS (rat)	Prolonged neuronal activation following TNBS-induced colitis is not mediated through vagal communication
Miampamba and Sharkey (1999)	formalin (rat)	increased c-Fos in the LSC, NST, and AP 2 hrs post formalin injection. This was dose dependently inhibited by xylazine
HPA axis activation		
Greenwood-Van Meerveld et al. (2006)	TNBS (rat)	prolonged increase in CRF mRNA expression in the PVN (up to 30 days post TNBS)
Kresse et al. (2001)	TNBS (rat)	increased CRF mRNA expression in the PVN and supraoptic nucleus

Kojima et al. (2002)	TNBS (rat)	decreased CRF mRNA expression
Inflammatory markers		
Riazi et al. (2008)	TNBS (rat)	increased hippocampal TNF α and microglial activation at 3 days post TNBS
Medhi et al. (2009)	TNBS (rat)	increased central TNF α up to 15 days post TNBS
Wang et al. (2010)	TNBS (rat)	increased brain IL-6 mRNA and protein which peaks at 7 days post TNBS
Baticic et al. (2011)	TNBS (mice)	increased brain IL-6 which peaked at 2 days post TNBS, also decreased brain IL-10
Alhouayek et al. (2011)	TNBS (mice)	increased brain IL-1 β and TNF α which was associated with increased circulating endotoxin
Villaran et al. (2010)	DSS (rat)	increased TNF α , IL-6, IL-1 β and iNOS mRNA in the SN
Behavioural changes		
Painsipp et al. (2011)	DSS (mice)	sex dependent behavioural changes in the EPM (male) and FST (female)
Lyte et al. (2006)	CRI (mice)	anxiety like behaviour in the hole-board open field apparatus
Bercik et al. (2011)	DSS (mice)	vagally mediated anxiety-like behaviour following DSS
Messaoudi et al. (1999)	TNBS (rat)	pain-induced behavioural disturbance in lever pushing task

Abbreviations Table 3.1: Area postrema (AP), blood brain barrier (BBB), citrobacter rodentium infection (CRI), corticotrophin releasing factor (CRF), circumventricular organ (CVO), dextran sulphate sodium (DSS), elevated plus maze (EPM), forced swim test (FST), intercellular adhesion molecule (ICAM), interleukin (IL), inducible nitric oxide synthase (iNOS), lumbrosacral spinal cord (LSC), median eminence (ME), nucleus of the solitary tract (NST), organum vasculosum of the lamina terminalis (OVLT), paraventricular nucleus (PVN), subfornical organ (SFO), 2,4,6-trinitrobenzenesulphonic acid (TNBS), tumour necrosis factor (TNF), vascular cell adhesion molecule (VCAM)

3.4 Brain-gut communication

3.4.1 Influence of psychological stress on healthy gut function

Repeated investigation with various psychological stressors have been employed to confirm the negative effects of stress on GI function (see Figure 3.3). Review of the literature by Konturek et al. (2011) suggests a number of stress induced disturbances to normal GI physiology:

1. Altered GI motility
2. Increased visceral perception
3. Altered GI secretion
4. Increased intestinal permeability
5. Altered GI mucosa and mucosal blood flow
6. Altered intestinal microbiota

Previous investigations have shown that chronically stressed animals can develop spontaneous inflammation in the bowel (Wood et al., 2000; Reber et al., 2007). Reber et al. (2007) examined the effect of chronic psycho-social stress on histological changes in the murine colon. Their results demonstrate that exposure to chronic subordinate colony housing leads to colonic inflammation, resulting in macroscopic damage to the muscosal layers of the colon, and an increased secretion of pro and anti-inflammatory markers by the mesenteric lymph nodes. Johnson et al. (1996) also report spontaneous colitis in cotton-top tamarins due to environmental factors. The repeatedly reported influence of stress on healthy gut function has strengthened the hypothesis that stress could be a risk factor in the development of IBD, or could influence relapse rates in IBD patients.

3.4.2 Influence of psychological stress on models of colitis

The effect of stress on models of IBD has been relatively well studied, and numerous psychological stressors have been tested including restraint stress, cold restraint stress, water avoidance stress, chronic subordinate colony housing, and electric shock. These stress protocols have been carried out pre, during, or post induction of colitis (see Figure 3.4). As per the gut-brain research carried out in animal models, the majority of this research has been carried out in the DSS and TNBS models (see Table 3.2).

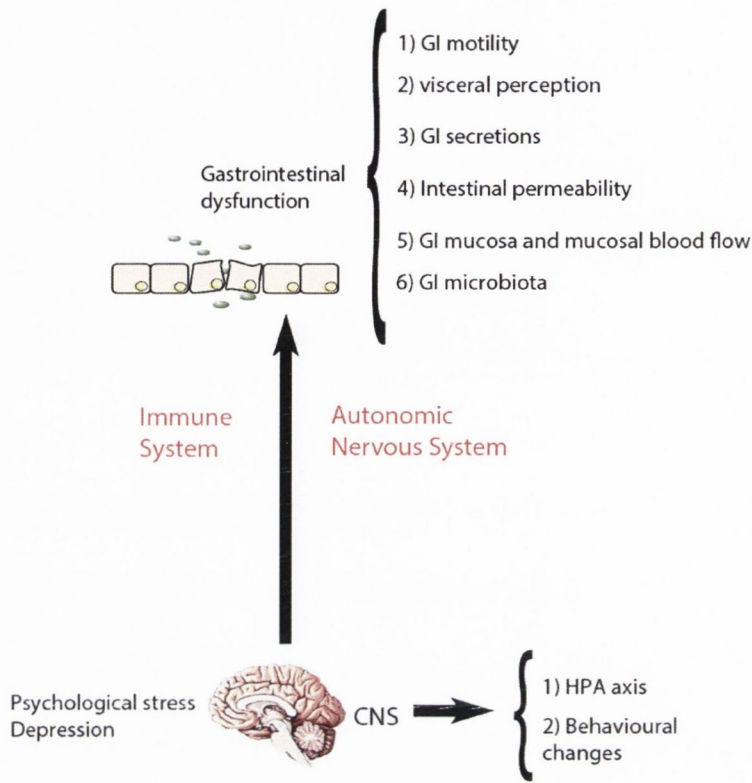


Figure 3.3: **Summary of known disturbances in healthy gut function following exposure to psychological stress.** Exposure to psychological stress can result in altered gastrointestinal (GI) motility, increased visceral perception, altered GI secretion, increased intestinal permeability, altered GI mucosa and mucosal blood flow, and altered intestinal microbiota.

Influence of psychological stress prior to colitis

The most severe impact of psychological stress on colitis has been reported by Reber et al. (2006b) in mice exposed to varying social defeat and overcrowding for 20 days. Following 20 days of stress, mice were allowed one day recovery before being exposed to low dose DSS (1%). The stress protocol itself was severe enough to induce decreased weight gain, decreased thymus weight and increased adrenal weight. It also rendered the adrenals hyporesponsive to stimulation with ACTH, resulting in lower corticosterone release. Stress also sensitised the mice to later DSS exposure: increased weight loss, increased histological score, decreased colon length, increased corticosterone, increased ACTH, and increased mesenteric lymph node (MLN) response to anti-CD3¹ exposure

¹Anti-CD3 monoclonal antibodies binds to CD3 on the surface of T cells

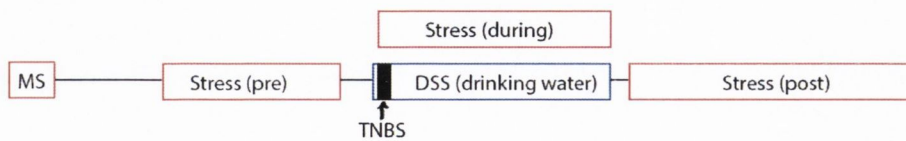


Figure 3.4: **Representation of the time of stress exposure in DSS and TNBS models of colitis.** Various stressors have been tested at each stage: pre stressors include social defeat, overcrowding, restraint stress, electric shock, water avoidance stress, chronic subordinate housing, post stressors include water avoidance stress, restraint stress, and the communication box, and stressors during acute colitis include restraint stress and water avoidance stress (see Table 3.2 for summary of outcomes and authors for each study). Maternal separation (MS)

in vitro. Recently Bartlang et al. (2012) exposed mice to a 20 day stress protocol only involving SD. They report a significant difference in the increased vulnerability to 1% DSS depending on the time of day mice were exposed to the stressor: SD during the dark hours was more effective than light phase SD at aggravating colitis. Further research has also shown that the effect of a 20 day SD protocol extends beyond the acute DSS activity, Peters et al. (2012) report increased risk of inflammation-related colorectal cancer (CRC) following 3 cycles of DSS in previously azoxymethane (AOM)² exposed mice that were also exposed to chronic subordinate colony housing.

A number of other studies have examined the effect of restraint stress on sensitivity to later chemically-induced colitis (Pfeiffer et al., 2001; Milde and Murison, 2002; Israeli et al., 2008; Gulpinar et al., 2004). In the earliest restraint stress investigation, Pfeiffer et al. (2001) examined the effect of restraint stress, at 4°C, on TNBS-induced colitis in rats. The restraint stress protocol alone altered the colonic mucosa resulting in decreased mucin glycoprotein, decreased goblet cells, and decreased mast cell numbers. After one day restraint stress there was also a significant increase in permeability of the colon, but this had returned to basal levels after 7 days restraint stress. They also report that prolonging the number of days of stress exposure increases the sensitivity to later TNBS colitis: increased colitis score and increased colonic myeloperoxidase (MPO). Increased MPO has been confirmed following restraint stress exposure, as well as increased substance P in the colonic mucosa (Israeli et al., 2008). Milde and Murison (2002) report decreased time to symptom expression in DSS rats previously exposed to restraint stress, in a separate study involving electric shock pre DSS exposure, they report a sensitising effect of stress on later vulnerability to intestinal permeability (Milde et al., 2005).

Water avoidance stress is not as effective at increasing the vulnerability to colitis as

²Azoxymethane carcinogen used to induce colon cancer in rats and mice

restraint stress or the chronic subordinate colony housing methods described above. Larsson et al. (2009) found no increased vulnerability to DSS-induced colitis following 1 h water avoidance stress exposure daily for 10 days, with two days recovery prior to initiation of DSS. No differences in body weight gain, colon length, or sensitivity to colorectal distension were observed post DSS. Cetinel et al. (2010) also exposed rats to repeated water avoidance stress, 30 min daily for five days with only one day recovery prior to TNBS administration. Some minor increases in macroscopic score and colonic malondialdehyde (MDA) levels were found, however, stress did not exaggerate increased colon weight, increased colonic MPO or decreased colonic glutathione (GSH) in colitic rats.

The majority of the evidence from chronic stress studies suggests a negative effect of previous stress on colitis outcome, however, Gulpinar et al. (2004) report an anti-inflammatory effect of acute stress prior to TNBS administration: decreased MPO activity and decreased macroscopic damage following electric shock. They suggest that this anti-inflammatory effect is potentially mediated through central corticotrophin releasing factor (CRF) or cholecystokinin (CCK) receptor, with the participation of the HPA axis and SNS.

Influence of psychological stress in combination with colitis

Colón et al. (2004) exposed rats to restraint stress five days before and five days after TNBS administration. Stress increased sensitivity to low dose TNBS (5 mg) but had no effect on the higher dose TNBS (30 mg). In the 5 mg group stress increased macroscopic damage, MPO activity, iNOS activity, cNOS activity, and colonic MDA. Xu et al. (2008) found enhanced sensitivity in mice exposed to DSS including increased disease activity index (DAI) and decreased weight gain, colonic IL-6 concentrations, and histological score. Strain differences in susceptibility to stress induced vulnerability to colitis have also been reported. Both Lewis and Fischer rats were equally affected by TNBS, however, following exposure to TNBS in combination with psychological stress Lewis rats had a more exaggerated response. These strain differences are reported to be related to differences in CRF expression (Million et al., 1999).

Influence of psychological stress post colitis

The influence of stress in animal models post colitis induction has aimed to answer two questions:

1. *Can stress prolong the time to recovery in models of colitis (Milde and Murison, 2002; Larsson et al., 2009; Matsunaga et al., 2011)?*

Early examination of the effect of stress immediately post DSS cessation report no significant effect of stress on colitis. Milde and Murison (2002) found no significant effects of exposure to restraint stress on recovery from DSS colitis. Larsson et al. (2009) report a similar lack of effect of water avoidance stress on DSS-induced recovery: colon weight was significantly increased, however, no difference in weight loss, fecal output, macroscopic damage score or sensitivity to later colorectal distension (CRD). Acute water avoidance stress 7 days post TNBS administration also failed to enhance MPO concentrations or macroscopic scores (Kresse et al., 2001). More recently, Matsunaga et al. (2011) report that mice exposed to communication box stress following exposure to 3% DSS had IL-18 dependant exacerbation of colitis.

2. *Can stress following an extended recovery phase reactivate quiescent colitis (Collins et al., 1996; Qiu et al., 1999; Saunders et al., 2006; Melgar et al., 2008)?*

Collins et al. (1996) allowed TNBS-induced colitic rats to recover for six weeks prior to exposure to mild restraint stress for three consecutive days. Although stress failed to induce clinically significant colitis it did induce some inflammatory differences including decreased IL-1 β and decreased noradrenaline release. The investigation was carried out in both Sprague-Dawley and Wistar-Kyoto rats as it was hypothesised that the Wistar-Kyoto rats might have a heightened vulnerability to stress-induced relapse. This did not occur, and the Wistar-Kyoto and Sprague-Dawley rats are reported as having a similar response in terms of GI alterations. In two follow up studies, dinitrobenzenesulphonic acid (DNB) and DSS-induced colitis was followed by restraint stress and water avoidance stress 6 weeks post colitis respectively (Saunders et al., 2006; Melgar et al., 2008). Saunders et al. (2006) report a significant reactivation of colitis only when stress is co-administered with further low dose DNB administration. This stress-induced reactivation of colitis was partially inhibited by hexamethazone ³ or a combination of atropine ⁴ and bretylium ⁵. Together, these results suggest a noradrenergic and cholinergic mediated stress-induced reactivation. Melgar et al. (2008) also report reactivation of colitis in rats exposed to stress six weeks after DSS administration in C57BL6 mice. Results have also shown that acute stress (restraint and sonic stress) can reactivate quiescent colitis in TNBS mouse models, possibly by reducing the protective barrier in the colon, and therefore, they suggest that stress management could be beneficial in conjunction

³a nicotinic acetylcholine receptor antagonist

⁴competitive antagonist for the muscarinic acetylcholine receptor

⁵inhibits noradrenaline release from nerve terminals

with drug therapy (Qiu et al., 1999).

3.4.3 Influence of Maternal separation on normal gut function

Review of maternal separation has also reported disturbances to gut function as a result of early life stress (O'Mahony et al., 2011). These maternally separated rodents can be used as a model of IBS due to their IBS-like functional symptoms, they are also reported to have altered neurotransmitter activity in the enteric nervous system, GI tract immune dysregulation, increased intestinal permeability, and disturbed intestinal microbiota. This further implicates stress in gut disorders, and in the possibility of a link between early stressful life events and later development of bowel disorders.

3.4.4 Influence of Maternal separation on models of experimental colitis

The influence of MS on later induction of colitis more closely models the effect of depression on GI inflammation. To our knowledge five papers have reported on the influence of neonatal MS on enhancement of colitis in adulthood (Barreau et al., 2004; Milde et al., 2004; Varghese et al., 2006; Veenema et al., 2008; Oines et al., 2012). In the earliest investigation, pups were maternally separated, and individually housed, for three consecutive hours from PND 2 to 14. Pups were then left to develop normally until 12 weeks of age when they were exposed to TNBS, and euthanised four days later. MS significantly affected total gut and colonic permeability, increased MPO concentrations, and increased mast cell numbers. MS rats were also found to have a significant inflammatory profile in the colon, spleen and liver, with significant increases in $\text{IFN}\gamma$, $\text{IL-1}\beta$, IL-4 and IL-10 ; splenic and colonic IL-2 were also significantly increased. These differences lead to an increased vulnerability to TNBS induced colitis in MS versus control rats, whereby increases in MPO, colonic damage score and permeability was found. Unfortunately, it is difficult to determine whether these effects are due to enhanced response to the DSS, or simply an additive effect of the already disrupted gut. More recently, Varghese et al. (2006) also report increased susceptibility to colitis in MS mice (C57BL6). Again MS alone resulted in increased GI permeability in adulthood. Following 6% DSS administration for 5 days from PND 60, MS mice had increased weight loss, increased clinical score, increased microscopic score, and increased colonic MPO, $\text{IL-1}\beta$, and serum amyloid P-component (SAP) compared to controls also exposed to DSS. Again, the colonic MPO activity in MS mice not exposed to DSS was not reported, making it difficult to determine whether this MS-DSS

effect is an interaction or an additive effect. One key finding in this paper is the benefit offered by pre-treatment, until PND 36, with the tricyclic antidepressant desipramine: a significant decrease in both microscopic scores and MPO activity in MS mice exposed to DSS. Antidepressant treatment did not offer any benefit to DSS mice that had not been exposed to MS. Attempts by Varghese et al. (2006) to replicate this DSS study with dinitrobenzenesulphonic acid (DNB) was not possible due to the high mortality rate in the combined MS and DNB group.

Three further studies examined the effect of MS on vulnerability to adult psychological stress enhancement of DSS-induced colitis (Milde et al., 2004; Veenema et al., 2008; Oines et al., 2012). Each of these investigations report an increased vulnerability to stress induced enhancement of colitis in the MS groups. Veenema et al. (2008) exposed MS mice to 19 days chronic subordinate colony housing immediately prior to 7 days 1% DSS. This low dose DSS-induced colitis was significantly enhanced by chronic subordinate colony housing as previously described (Reber et al., 2007). The chronic subordinate colony housing-induced changes in weight and colon length were further enhanced in the MS group. Results also demonstrate a significant increased histological score and mesenteric lymph node (MLN) IFN γ expression in the MS group. The two other investigations allowed a longer recovery between the adult stressor and DSS administration, which resulted in a less robust enhancement of the DSS-induced colitis (Milde et al., 2004; Oines et al., 2012). One interesting finding by Milde et al. (2004) is that a brief maternal separation appears to protect from DSS-induced colonic erosions, although this will need to be confirmed in future investigations.

3.5 Limitations of studying the implications of stress in these models

One major limitation when studying the effect of psychological stress or MS on DSS or TNBS-induced colitis is that both result in increased permeability of the intestinal tract. Therefore, any enhanced colitic effect may be due to increased permeability to the DSS or TNBS themselves, rather than due to altered immune activation, bacterial translocation or neuroendocrine function. Support for this possibility comes from the studies that required low dose administration of DSS in combination with psychological stress in order for the stressor to have any contributing effect to intestinal inflammation.

Table 3.2: Effect of psychological stress in models of colitis

Author	Colitis	Psychological intervention	Timing of intervention	Authors conclusions
Bartlang et al. (2012)	1% DSS (mice)	SDL or SDD (ED 1-20)	pre	SDD is more effective at inducing enhanced colitis
Reber et al. (2006b)	1% DSS (mice)	SD or OC (ED i-18)	pre	Pre exposure to psychosocial stress increases the severity of inflammation and impairs healing
Gulpinar et al. (2004)	TNBS (rats)	PRS ES (ED 0-4)	pre	Pre stress had anti-inflammatory effects on colonic macroscopic damage and MPO activity following TNBS. This is potentially mediated CRF and CCK receptor and the HPA axis and SNS system
Cetinel et al. (2010)	TNBS (rats)	WAS (ED 1-5)	pre	Increased TNBS-induced macroscopic damage and MDA levels in the WAS group
Israeli et al. (2008)	TNBS (rats)	RS (ED 1-4)	pre	Slight increases in MPO and increases in SP in pre-stressed colitis group
Milde et al. (2005)	4% DSS (rats)	ES (ED 21)	pre	Pre-shock and DSS results in increased permeability (interaction unclear)
Peters et al. (2012)	0.5-1% DSS (mice)	CSC (ED 1-20)	pre	CSC increases the risk of inflammation-related CRC following 3 cycles of DSS in AOM exposed mice
Pfeiffer et al. (2001)	TNBS (rats)	RS at 4°C (ED 1-7)	pre	Increasing number of prior stressors enhanced colitis score and MPO expression
Larsson et al. (2009)	3% DSS (mice)	WAS (ED 1-10 or ED 24-33)	pre and post	DSS colitis-induced sensitivity to colorectal distension, or fecal output was not effected by pre or post WAS stress
Milde and Murison (2002)	4% DSS (rats)	RS (ED 1-4)	pre and post	Some effects on latency to development of colitis following pre stress
Collins et al. (1996)	TNBS (rat)	RS (ED 42-44)	post	Previous TNBS-induced colitis sensitives the gut to later stress induced GI changes

Melgar et al. (2008)	3 then 1% DSS (mice)	WAS (ED 34-40)	post	WAS reactivates DSS-induced inflammation when low dose DSS is given in combination
Matsunaga et al. (2011)	3% DSS (mice)	CB (ED 6-10)	post	Stress induced exacerbation of DSS-induced colitis, this was IL-18 dependent
Saunders et al. (2006)	DNB (rat)	RS (ED 42-44)	post	Stress-induced reactivation of colitis is mediated by noradrenergic and cholinergic neural pathways
Colón et al. (2004)	TNBS (rat)	RS (ED 1-10)	pre and during	Increased sensitivity to low dose but not high dose TNBS-induced colitis following exposure to RS
Million et al. (1999)	TNBS (rat)	WAS and RS (ED 2-7)	during	Lewis rats are more sensitive to stress induced exacerbation of TNBS-induced colitis
Xu et al. (2008)	2.5% DSS (mice)	RS (ED 1-7)	during	IFN γ Independent exacerbation of DSS-induced colitis following RS
Maternal Separation				
Barreau et al. (2004)	TNBS (rat)	MS (PND 2-14)		MS leads to altered GI function in adulthood, associated with exaggerated response to TNBS
Milde et al. (2004)	4% DSS (rats)	MS (PND 1-14), Shock (PND 114)	pre	MS alone did not lead to exaggerated DSS response
Varghese et al. (2006)	6% DSS (mice)	MS (PND 1-21)		MS increases vulnerability to intestinal inflammation. Reversed by TCA DMI.
Veenema et al. (2008)	1% DSS (mice)	MS (PND 1-21), CSC (wk 8-11)	pre	Increased histological damage and MLN IFN γ and TNF α secretion in MS or CSC alone
Oines et al. (2012)	4% DSS (rats)	MS (PND 2-14), CSI (PND 72-75)	pre	CSI was needed to observe exaggerated colitis in MS rats exposed to DSS

Abbreviations Table 3.2: Azoxymethane (AOM), communication box (CB), cholecystokinin (CCK), colorectal cancer (CRC), corticotrophin-releasing factor (CRF), chronic subordinate colony housing (CSC), chronic social instability (CSI), desipramine (DMI), dinitrobenzenesulphonic acid (DNB), dextran sulphate sodium (DSS), experimental day (ED), electric shock (ES), gastrointestinal (GI), hypothalamic-pituitary-adrenocortical axis (HPA), interferon (IFN), malondialdehyde (MDA), mesenteric lymph

nodes (MLN), myeloperoxidase (MPO), maternal separation (MS), overcrowding (OC), postnatal day (PND), partial restraint stress (PRS), restraint stress (RS), social defeat (SD), social defeat during dark (SDD), social defeat during light (SDL), sympathetic nervous system (SNS), substance P (SP), tricyclic antidepressants (TCA), tumour necrosis factor (TNF), 2,4,4-trinitrobenzenesulphonic acid (TNBS), water avoidance stress (WAS)

3.6 Aims and objectives of the thesis

Systematic review of the literature revealed an increased risk of mood and anxiety disorders in IBD patients versus healthy controls and that increased prevalence of psychological disturbances was most significant for patients during the active disease phase (Mardini et al., 2004; Calvet et al., 2006; Häuser et al., 2011; Ben Thabet et al., 2012). Despite this, there have been no investigations into the potential role of inflammatory mediators in inducing these mood changes. Therefore, the aim of this thesis was to examine colonic and circulating inflammatory mediators in IBD patients, and determine whether any associations between inflammation and psychological scores exist. Secondly, the impact of colonic inflammation on anxiety and depression related behaviours, inflammatory mediators and neurotransmitters within the CNS was assessed in animal models of IBD. As bidirectional communication exists between the bowel and the CNS, and due to the numerous reports of stress impacting on intestinal function and possibly affecting the course of IBD (Levenstein et al., 2000; Hart and Kamm, 2002; Mawdsley et al., 2006; Sajadinejad et al., 2012), the final aim of the thesis was to examine the effects of mild restraint stress exposure on colonic and extraintestinal inflammatory markers. The specific objectives of the thesis were:

1. To examine IBD patients compared to a patient control group to determine if any associations exist between inflammatory mediators in IBD and psychological scores for depression, anxiety and general well-being: Hamilton-depression score, Hamilton-anxiety score, inflammatory bowel disease questionnaire, and profile of mood states (POMS).
2. To establish two animal models of IBD (dextran sulfate sodium (DSS) and trinitrobenzenesulphonic acid (TNBS)) to investigate the implication of colonic inflammation on behaviour and determine whether circulating or central molecular changes occur which may account for behavioural changes.
3. To investigate the effects of stress on DSS-induced colitis in terms of symptom expression, colonic MMP9 expression and activity, colonic inflammatory gene expression (IL-1 β , IL-6, TNF α and iNOS), and upregulation of extra-intestinal markers of inflammation in the liver and CNS.

Part II

Methods, Results and Discussion

Chapter 4

Materials and Methods

Materials

Animals

Male Wistar Rats	: Bioresources, TCD
Ketamine (Vetalar)	: Bioresources, TCD
Xylazine (Chanazine)	: Bioresources, TCD
Rat Diet	: Red Mills
Dextran Sulphate Sodium (DSS)	: TdB Consultancy AB
Trinitrobenzene Sulphonic Acid (TNBS)	: Sigma Aldrich
Saccharin	: Sigma Aldrich

General Laboratory Chemicals

β -mercaptoethanol	: Sigma Aldrich
2-Propanol	: Sigma Aldrich
Acrylamide	: Sigma Aldrich
Ammonium Persulfate (APS)	: Sigma Aldrich
Bis Acrylamide	: Sigma Aldrich
Bovine Serum Albumin 96% (BSA)	: Sigma Aldrich
Bromophenol Blue	: Sigma Aldrich
Diethyl Pyrocarbonate (DEPC)	: Sigma Aldrich
Di-Sodium Hydrogen Orthophosphate (Na_2HPO_4)	: BDH Chemicals
Glycerol	: Sigma Aldrich
Glycine	: Sigma Aldrich
Hydrochloric Acid (HCl)	: BDH Chemicals

Isopentane	: Sigma Aldrich
Magnesium Chloride (MgCl ₂)	: Sigma Aldrich
Methanol	: BDH Chemicals
NP-40	: Amersham
Paraformaldehyde	: Sigma Aldrich
Phosphatase Inhibitor Cocktail I and II	: Sigma Aldrich
Potassium Chloride (KCl)	: Sigma Aldrich
Protease Inhibitor Cocktail	: Sigma Aldrich
Sodium Bicarbonate (NaHCO ₃)	: BDH Chemicals
Sodium Carbonate (Na ₂ CO ₃)	: BDH Chemicals
Sodium Chloride (NaCl)	: BDH Chemicals
Sodium Dodecyl Sulphate 99% (SDS)	: Sigma Aldrich
Sodium Hydroxide (NaOH)	: Sigma Aldrich
Sodium Phosphate Dibasic (NaH ₂ PO ₄)	: Sigma Aldrich
Sucrose	: Sigma Aldrich
Sulphuric Acid 98% H ₂ SO ₄	: BDH Chemicals
Tetamethylethylene-Diamine (TEMED)	: Sigma Aldrich
Triton-X	: Sigma Aldrich
Tris-HCl	: Sigma Aldrich
Trizma Base	: Sigma Aldrich
Tween-20	: Sigma Aldrich

General Laboratory Plastics and Hardware

0.45 μ m nylon filter	: Cronus
50 ml Yellow Lid Containers	: Sarstedt
96 Well Plates	: Sarstedt
Blood Collection Tubes (12 ml)	: Sarstedt
Cover Slips	: Fischer Scientific
Glass Inserts	: Fischer Scientific
Glass screw top vials	: Labquip
Pasteur Pipettes	: Sarstedt
Pipette tips	: Sarstedt

Plastic Syringe (1 ml)	: Becton Dickenson
Microtubes (1.5ml)	: Sarstedt
Microscope Slides	: Fischer Scientific
Parafilm Laboratory Rolls	: Sarstedt

HPLC: Kynurenine

3-Hydroxyanthranilate	: Sigma Aldrich
3-Hydroxykynurenine	: Sigma Aldrich
Acetonitrile	: Sigma Aldrich
Glacial Acetic Acid	: Fisher Chemical
HPLC Grade Water	: Fisher Chemical
Kynurenic Acid	: Sigma Aldrich
L-Kynurenine	: Sigma Aldrich
L-Tryptophan	: Sigma Aldrich
N-methy-5-HT	: Sigma Aldrich
Perchloric Acid	: BDH Chemical
Quinolinic Acid	: Sigma Aldrich
Zinc Acetate	: Sigma Aldrich

HPLC: Monoamine

5-Hydroxyindoleacetic Acid (5-HIAA)	: Sigma Aldrich
Citric Acid	: Sigma Aldrich
Ethylenediaminetetra-acetic Acid (EDTA)	: BDH Chemicals
HPLC Grade Water	: Fisher Chemical
Methanol, 100 %	: Lab-Scan
N-methy-5-HT	: Sigma Aldrich
Octane-1-Sulphonic Acid	: Sigma Aldrich
Serotonin (5-HT)	: Sigma Aldrich
Sodium Phosphate monobasic monohydrate (NaH ₂ PO ₄)	: Sigma Aldrich

PCR

96 well PCR plates	: Applied Biosystems
Filter Pipette Tips	: Sarstedt

High Capacity cDNA archive Kit	: Applied Biosystems
Molecular Grade Water	: Sigma Aldrich
Optical Adhesive Covers	: Applied Biosystems
RNAlater	: Ambion
RNase Away	: Invitrogen
RNase Zap wipes	: Ambion
RNA extraction Kit	: Macherney-Nagel
TaqMan Gene Expression assays	: Applied Biosystems
TaqMan Universal PCR Master Mix	: Applied Biosystems

Immunostaining

3,3-Diaminobenzidine (DAB)	: BioRad
Ethanol	: Sigma Aldriche
Gelatin	: Fluka
iNOS Antibody	: Santa Cruz
Normal Goat Serum	: Sigma Aldriche
Tissue-tek O.C.T. Compound	: Sakura
Vectasheild	: Vector Laboratories
Vectastain Elite ABC kit	: Vector Laboratories
Xylene	: BDH Chemicals

Western Immunoblotting

Anode Buffer 1	: BioRad
Filter Paper	: Whatman Ltd.
Broad Range Molecular Weight Marker (Precision Plus)	BioRad

Methods

4.1 Animal husbandry

Male Wistar rats (175-210 grams) were obtained from the Bioresources Unit in Trinity College Dublin, and housed in hard-bottomed polypropylene cages with wood shavings as bedding. Animals were housed 4 per cage on arrival, under standard laboratory conditions, with an ambient temperature of 20-24° Celsius (C) and a 12 h light: 12 h dark cycle (lights on 08.00, lights off: 20.00). Animals had free access to food and water and were fed a standard laboratory diet (Red Mills, Ireland). A record of body weight was maintained as a general indication of the health and well-being of each animal throughout each study. All in-vivo work was approved by the Animal Ethics Committee Trinity College Dublin.

4.2 Animal models

4.2.1 Dextran sulphate sodium-induced colitis

Rats were randomly divided into control and colitis groups. Control groups received normal tap water for 7 days while colitis groups received 5 % dextran sulphate sodium (DSS, MW 36,000-44,000) in their drinking water (tap water) for seven consecutive days. Animals were observed daily for fluid intake, weight changes, and for major symptoms of colitis such as loose stools, diarrhoea, and rectal bleeding.

For recovery studies DSS was administered as above, once the seven days were complete animals were returned to normal tap water and allowed to recover for 2, 7 or 21 days. Animals were observed daily for fluid intake, weight changes, and for major symptoms of colitis such as loose stools, diarrhoea, and rectal bleeding over the course of the study.

4.2.2 Trinitrobenzene sulphonic acid-induced colitis

1. **TNBS: Dose response:** rats were singly housed and then divided into four groups (n=4-6). All groups were fasted for 24 h prior to trinitrobenzene sulphonic acid (TNBS) administration, during this period they received a 20% sugar solution containing a laxative. Following the 24 h fast, rats were anaesthetised (1.5ml ketamine and 1.5ml xylazine). Group one received a 1ml vehicle (30% ethanol) rectal enema, Group 2, 3 and 4 each received a 1ml enema containing 15, 30, or 60 mg/ml TNBS respectively. Rats were returned to clean cages and remained singly housed for the

remainder of the experiment. All groups were euthanised 8 days post enema. Rats were observed daily for fluid intake, weight changes, and for major symptoms of colitis such as loose stools, diarrhoea, and abdominal swelling.

2. **TNBS: Timecourse:** rats were housed and anaesthetised as above. Group one received a 1ml vehicle (30% ethanol) rectal enema, Group 2, 3 and 4 each received a 1ml enema containing 60 mg/ml TNBS. Rats were returned to clean cages and remained singly housed for the remainder of the experiment. Control group was euthanised 8 days post vehicle enema, group 2, 3 and 4 were euthanised 3, 8 and 21 days respectively post TNBS administration. Rats were observed daily for fluid intake, weight changes, and for major symptoms of colitis such as loose stools, diarrhoea, and abdominal swelling.

4.2.3 Chronic restraint stress

Male Wistar rats were exposed to 2 h restraint stress for 7 consecutive days. Rats were restrained in well ventilated, adjustable, plexiglass restrainers (35 cm length x 7 cm internal diameter), normally used for collecting tail blood (Harvard Apparatus, UK). The restrainer was then put back in the rats home cage under standard laboratory conditions, brightly lit with an ambient temperature of 20-24°, for 2 h.

4.2.4 Disease Activity Index

A Disease Activity Index (DAI) was constructed to examine DSS-induced colitis severity using the criteria of weight loss, stool consistency and rectal bleeding. Weight loss was scored on a scale of 0-4; 0 for no weight loss, 1 for 1-5%, 2 for 6-10%, 3 for 11-15% and 4 for >20%. Stool consistency was scored 0-4; 0 for normal stools, 1-2 for loose stools and 3-4 for diarrhoea. Rectal bleeding was scored 0-4; 0 for none, 1 for light bleeding, 2 for moderate bleeding, 3 for heavy bleeding and 4 for very heavy bleeding. Scores for each of the 3 criteria were averaged to give the Disease Activity Index score (Cooper et al., 1993).

4.2.5 Behavioural tests

Saccharin Preference test

Saccharin preference testing was carried out over the full course of the TNBS timecourse experiment. Two water bottles, one containing tap water, the other containing a 0.01%

Saccharin solution were placed with each cage. Fluid intake from each bottle was monitored every 24 h and daily percentage saccharin preference was calculated. Decreased saccharin preference was used as an indicator of anhedonia.

Open field testing

The open field consisted of a cylindrical plastic arena (1 m diameter). The base of the open field was black, and the walls were mirrored. A video camera was mounted on the ceiling directly above the centre of the open field. Room lights were dimmed during the testing phase. Rats were placed in the open field for 10 minutes each. Automated video recording of the rats movement between zones, speed, path length, time still and thigmotaxis were recorded using the HVS image software (HVS image Ltd.) Time spent grooming was also observed and recorded. Rats were considered to be grooming when they start by licking the paws, then rubs them over the head, this is often followed by licking and rubbing the side of the body, the anogenital region, and the tail.

Novel object Open Field test

In order to eliminate the effect of learning if rats were re-exposed to the open field test slight adaptations were made. The open field itself was identical to previously described however a small black rectangle (6 cm x 6 cm x 12 cm) was placed in the centre of quadrant SW and room lights were left on. Rats were left to explore for 10 min and recorded using the HVS image software. An observer also monitored the number of approaches the rat made to the object.

Elevated Plus maze

The Elevated plus maze (EPM) consisted of a grey plus maze elevated 50 cm off the ground. 50cm high black plexiglass surrounded two of the opposing open arms and the other two remained open. Each arm measured 50 cm x 10 cm (length by width respectively). The central junction measured 10 cm x 10 cm. Regular light-phase room lighting was maintained during the testing phase. Rats were placed in the centre of the EPM facing an open arm and tested for 5 min each. Automated video recording of the rats entries between arms, path length within open and closed arms, and time spent in each arm was recorded using the HVS image software (HVS image Ltd.) (the test was performed based on a combination of protocols described by Walf et al., 2007, and Carri et al., 2011)

Marble burying

The majority of marble burying studies are carried out with mice, therefore this protocol was based on the Schneider et al., (2007) adaptation of the protocol in female Wistar rats. Small hard-bottomed polypropylene cages (45 cm x 27 cm x 15 cm, length x width x height respectively) were filled 4.5-5 cm deep with regular wood shaving bedding. Two rows of marbles (2.3 cm diameter) were lined approximately 2 cm apart along the same short wall of the cage. Room lighting was dimmed and rats were placed in the cage and left alone for 10 min, following this rats were removed and marbles were counted. Marbles covered at least two thirds with bedding were considered buried.

Light Dark Box

The original light dark box is based on Crawley and Goodwin (1980), is now a standard test often combined with the EPM test. In this protocol, the light chamber consisted of a white walled, clear glass top with a bright light in the centre, measuring 30 x 30 cm, the dark chamber had black walls and a solid black top (30 x 30 cm). The two chambers were connected via a rectangular passage (10 cm). Rats were placed in the light chamber and observed for 10 min. Number of entries to each arm, time spent in each arm (s), and fecal boli count was recorded. Head poking was also recorded as an indication of the rats investigatory tendencies (Marino et al., 2005).

4.2.6 Tissue Collection

Rats were sacrificed by decapitation. Tissue samples of interest (Colon, liver, spleen and brain tissue) were retrieved, snap frozen on dry ice, and stored at -80°C until use, unless stated otherwise (HPLC tissue preparation). Trunk blood was collected immediately following decapitation in Z10 tubes and centrifuged at 2000 rpm for 15 min at 4°C. The pellets were discarded and supernatants (serum) were transferred to 1.5ml eppendorfs and stored at -80°C.

4.3 Histology

Distal colon samples (1-2 cm) were dissected and stored in Camoys Solution (60% alcohol; 30% chloroform; 10% glacial acetic acid) for 2-3 h, then transferred to 100% ethanol until embedding. Colon samples were removed from ethanol and halved in a cross-sectional manner and were then cut longitudinally. Care was taken only to cut through the muscle

layer of the colon sample; this allowed previously trapped luminal debris to be washed out from the exposed lumen while preventing damage to the mucosal, lamina propria and submucosal layers. Colon samples were replaced in fresh 100% ethanol until embedding. Both segments of colon samples were added to perforated embedding cartridges which were then put in a Leica PT Embedder for embedding. Embedding consisted of samples being pre-treated with 9 steps for 45 min each; 50% ethanol, 70% ethanol, 95% ethanol, absolute ethanol (x3), 50% absolute ethanol/50% chloroform, 100% chloroform (x2). Samples were then placed in wax twice for an hour each time.

After samples were embedded, molten liquid paraffin was poured into metal moulds, colon tissue samples were removed from their embedding cartridges and the samples were placed in metal moulds. More molten liquid paraffin was then poured into the metal moulds covering the samples completely; care was taken to ensure that samples were orientated optimally for sectioning. This involved keeping the longitudinal axis of the colon perpendicular to the base of the mould. For identification purposes the bottom of the embedding cartridge was attached to the liquid paraffin samples. When the paraffin had set, slices of embedded colon samples were sectioned with a microtome at a thickness of 10 μm (Leica Microtome). Excess paraffin was removed with a scalpel. Sections of embedded colon were added to a water bath where they stretched due to interactions between the embedding agent and water to eliminate any creases present. The expanded sections were removed from the water bath and placed on microscope slides. Microtomed slides were put in an oven at 60°C for 30 min to set before being placed in a Leica Autostainer. Microtomed slices were stained using hematoxylin and eosin stain which stain nuclei blue and eosinophilic structures red, pink and orange. Slides were mounted with cover slips using Di-N-Butyl Phthalate in Xylene (DPX) mounting medium. Slides were digitally recorded and analysed using an Olympus BX51 video-camera microscope for histological scoring.

Histological scoring used a modified version of a protocol to examine DSS damage in the mouse colon (Cooper et al., 1993); crypt damage and inflammation were scored in ten randomly chosen areas of colon sample slides. Crypt damage was rated on a scale of 0-4; 0 for no visible shortening or damage to the crypts, 1 for mild crypt destruction, 2 for moderate crypt destruction, 3 for complete to near complete destruction of crypts with the presence of surface epithelial cells and 4 for complete destruction of crypts together with an absence of surface epithelial cells. Inflammation was subjectively scored on a scale of 0-3; 0 for no visible enlargement of the lamina propria, 1 for mild enlargement of the lamina propria, 2 for moderate expansion of the lamina propria with increased infiltration of nuclei and 3 for substantial increases in lamina propria size in conjunction with heavy

infiltration of nuclei into the surrounding areas. Crypt damage and inflammation scores were recorded for the 10 randomly selected areas of each colon sample; scores were averaged to give approximate scores of colonic crypt damage and inflammation respectively.

4.4 High performance liquid chromatography (HPLC)

4.4.1 Detection of tryptophan and kynurenine pathway metabolites by HPLC coupled to UV/fluorescent detection

Instrumentation

HPLC analysis was conducted with an automated HPLC system (Shimadzu ADVP module). 20 μ l was injected into a reverse phase analytical column (Kinetex Core Shell Technology, Phenomenex UK) with specific area of 100mm x 4.6mm and particle size of 2.6 μ and fitted with a guard column (Lichrosorb RP18, specific surface area 30 x 4 mm, Phenomenex). Two detectors were connected in series; a PDA-UV detector (SPD-M10A VP, Shimadzu) and a fluorescent detector (RF-10A XL, Shimadzu). The PDA-UV detector was calibrated to analyze a UV spectrum from 240nm to 370nm while integrating at 250 nm. The fluorescence wavelengths were set to 254 nm excitation and 404nm emission. The flow rate was 1 ml/min (LC-10AT pump, Shimadzu) and the acquisition time was 18 min. CLASS-VP software (Shimadzu) was used for chromatographic control, data collection and processing. The signal to noise ratio was taken as 3 to 1. Peak heights compared with internal and external standards allowed quantification of concentrations of metabolites in samples.

Reagents

The mobile phase consisted of 50 nM glacial acetic acid, 100 mM zinc acetate (Sigma) and 3% acetonitrile dissolved in double-distilled NANOpure water HPLC grade H₂O (Sigma). The pH was adjusted to 4.9, using 5 M NaOH.

HPLC standards (N-methyl-5-hydroxy-tryptamine (Internal standard), L-kynurenine, L-Tryptophan and kynurenic acid) were obtained from Sigma Aldrich. Standard solutions of 10 mg/10ml were prepared by dissolving the standards in 10 ml of HPLC mobile phase and stored refrigerated in glass volumetric flasks. A 10 ml standard mix containing 200 ng/20 μ l of each standard was prepared as well as 10 ml solutions of each individual standard at 200 ng/20 μ l. Standard curves were generated using the these standards to determine the limits of detection (LOD) (see Table 4.1).

Table 4.1: Tryptophan, KYNA and kynurenine Limits of Detection

Metabolite	R ² value	LOD
Tryptophan	0.999	80 ng/ml
Kynurenine	0.998	20 ng/ml
KYNA	0.999	1 ng/ml

(a) Kynurenine HPLC Chromatographs

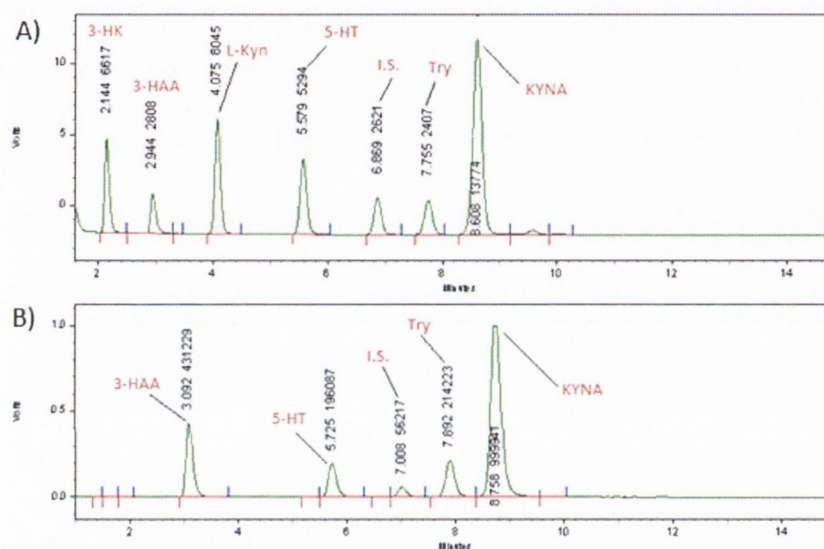


Figure 4.1: **Kynurenine HPLC Chromatograph** The standard mix was analyzed at 250nm by UV detection (A). Sharp peaks corresponding to 3-HK, 3-HAA, L-KYN, IS, L-TRP and KYNA are shown. This standard mix was also analysed by Fluorescence detection (254nm excitation (Ex) and 404nm emission (Em))(B).

Retention Times and resolvability

A 150 μ l aliquot of each 200 ng/20 μ l standard was pipetted into an individual 0.2 ml conical insert (Labquip, Dublin) and transferred into a 2 ml vial (Labquip, Dublin). Analysis was carried out by HPLC as described in section 4.4.1 in the mobile phase in order to determine the retention times of each standard (see Figure 4.1).

Tissue Preparation for Kynurenine HPLC Analysis

Colon, spleen, liver and brain samples were weighed (approximately 50 mg) and placed in ice cold homogenising buffer. Tissue was homogenised by sonication (MSE sonicator)

for approximately 30 s, in 500 μl of homogenising buffer (mobile phase containing IS (200 ng/20 μl N-methyl-5-hydroxytryptamine) and 6% perchloric acid). The samples were then centrifuged at 14,000 g for 20 min at 4°C. The supernatants were transferred to new 1.5 ml eppendorfs. Each supernatant was filtered using 4mm syringe filters (non-sterile 0.45 μm nylon, AF0-0420, Phenomenex) and kept frozen at -80°C until analysis.

Serum Preparation for HPLC Analysis

Serum was thawed and 300 μl was added to 300 μl of homogenising buffer (mobile phase containing 6% perchloric acid and 200 ng/20 μl of IS) and immediately vortexed for 30 seconds. The samples were then centrifuged at 14,000 g for 20 min at 4°C. The supernatants were transferred to new 1.5 ml eppendorfs. Each supernatant was filtered using 4 mm syringe filters (non-sterile 0.45 μm nylon, AF0-0420, Phenomenex) and analyzed immediately or kept frozen at -80°C until analysis.

HPLC Measurement of tryptophan and kynurenine pathway metabolites

The mobile phase was prepared as previously described and supernatants were thawed at room temperature and then analysed by injection into an automated HPLC system coupled with UV and fluorescent detection in an injection volume of 20 μl . The flow rate was 0.8 ml/min with a run time of 18 min. For each HPLC system, a mobile phase was injected intermittently and a standard mix was injected every six samples in order to recalibrate the system and curtail divergences in the retention times during sample runs. The auto-samplers were pre-programmed to self-rinse with methanol between each sample injection. Peak heights obtained from the chromatographs were used to calculate ng of metabolite per g wet weight of tissue, or ml of serum.

4.4.2 Measurement of Monoamines by HPLC coupled to electrochemical detection

Instrumentation

HPLC analysis was conducted with an automated HPLC system (Shimadzu ADVP module). 10 μl was injected into a reverse phase analytical column (Kinetex Core Shell Technology, Phenomenex UK) with specific area of 100 mm x 4.6 mm and particle size of 2.6 μm , maintained at 30°C, and fitted with a guard column (Lichrosorb RP18, specific surface area 30 x 4 mm, Phenomenex). An electrochemical detector (Digital Electrochemical Amperometric Detector, Antech ECD) was connected to the automated HPLC system.

The flow rate was 0.8 ml/min (LC-10AT pump, Shimadzu) and the acquisition time was 60 min. CLASS-VP software (Shimadzu) was used for chromatographic control, data collection and processing. The signal to noise ratio was 3 to 1. Peak heights compared with internal and external standards allowed quantification of concentrations of analytes in samples.

Reagents

The mobile phase consisted of 0.1 M citric acid monohydrate (Sigma), 0.1 M sodium dihydrogen phosphate monohydrate (Merck), 1.4 mM octane-1 sulphonic acid (BDH) and 0.1 M EDTA disodium salt dihydrate (Sigma) dissolved in double-distilled NANOpure HPLC grade water (Sigma). The pH was adjusted using a pH meter (Jenway) to 2.8 by the addition of 5 M NaOH. Monoamine standards (5-hydroxyindoleacetic acid, 5-hydroxytryptamine and N-methyl-5-hydroxytryptamine) were all obtained from Sigma Aldrich. Standard solutions of 10 mg/10 ml were prepared by dissolving standards in 10 ml of HPLC mobile phase and stored refrigerated in glass volumetric flasks. A 10 ml solution of each standard (5 ng/20 μ l) and a standard mix containing 5 ng/20 μ l of each standard was also prepared.

Retention Times and Resolvability

A 150 μ l aliquot of each 5 ng/20 μ l standard was pipetted into an individual 0.2 ml conical insert (Labquip ltd. Dublin) and transferred into a 2 ml vial (Labquip, Dublin). Analysis was carried out by HPLC in order to determine the retention times of each standard. Standard mixes were also injected with the settings as above in order to assess the columns ability to separate and resolve for each of the standards individually.

Tissue Preparation for Monoamine HPLC Analysis

Colon and cortex samples were weighed and immediately placed in ice cold homogenising buffer. Tissue was homogenised by sonication (MSE sonicator) for approximately 30 s, in 500 μ l of homogenising buffer (mobile phase consisting of 5 ng/20 μ l of IS (N-methyl-5-HT)). The homogenates were then centrifuged at 14,000 g for 20 min at 4°C. The supernatants were transferred to new 1.5 ml eppendorfs. Each supernatant was filtered using 4mm syringe filters (non-sterile 0.45 μ m nylon, AF0-0420, Phenomenex) and kept frozen at -80°C until analysis.

HPLC Measurement of Monoamines

The monoamine mobile phase was prepared as previously described and supernatants were thawed at room temperature and then injected on to an automated HPLC system coupled with electrochemical detection in an injection volume of 10 μ l. The flow rate was 0.8ml/min with a run time of 60 min.

For each HPLC system, a mobile phase was injected intermittently and a standard mix was injected every five samples in order to recalibrate the system and curtail divergences in the retention times during sampling. The auto-samplers were pre-programmed to self-rinse between each sample injection with methanol. Peak heights obtained from the chromatographs were used to calculate ng of metabolite per g wet weight of tissue.

4.5 Real Time Polymerase Chain Reaction

4.5.1 Total RNA extraction from tissue samples

Isolation of total RNA from all tissue samples was carried out using Nucleospin RNA II kits (Macherey-Nagel) as per manufacturers protocol. All instruments used were wiped with Rnase Away and rinsed with DEPC water before using. When ready for extraction ≤ 30 mg of tissue was homogenized in lysis buffer (350 μ l RA1 buffer and 3.5 μ l β -mercaptoethanol) using a Polytron for several minutes until all tissue was completely homogenized. Due to the solid consistency of the liver and colon samples, it was essential to chop the tissue into several pieces with a sterile scalpel prior to homogenisation. Following this, the lysate was filtered through Nucleospin Filter units for 1 min at 11,000 g. 350 μ l of 70% ethanol was applied to the lysate and pipetted 5 times until dissolution occurred. The total lysate was loaded to a nucleospin II column and centrifuged for 30 s at 11,000 g. The next step involved desalting the membrane of the column by applying 350 μ l membrane desalting buffer and centrifuging at 11,000 g for 1 min. Following this, any potential DNA contamination present was digested by adding 95 μ l Dnase reaction mixture directly onto the centre of the silica membrane of the column, and incubated at room temperature for 15 min. The additional steps involved washing. 200 μ l of RA2 buffer (provided with the kit to inactivate Dnase) was added and centrifuged for 30 s at 11,000 g, and column was placed into a new collecting tube. A second wash was carried out and involved adding 600 μ l buffer RA3 (wash buffer provided with the kit) to the column and centrifuging for 30 s at 11,000 g and the final wash was with 250 μ l RA3 and centrifuged for 2 min at 11,000 g to allow the membrane to dry completely. Finally, the

Table 4.2: cDNA master mix solution for one sample

Component L/Reaction	μl /Reaction
Reverse Transcription Buffer	4
Random primers	4
dNTPs	1.6
Nuclease-free water	8.4
MultiScribe TM Reverse Transcriptase	2

column was placed in a nuclease-free micro-centrifuge tube, RNA was eluted with 60 μl Rnase-free H₂O, and centrifuged for 1 min at 11,000 g. RNA was stored at -80°C.

4.5.2 RNA quantification and Equalization

RNA concentration and quality was performed using a NanoDrop©ND-1000 spectrophotometer (Thermo scientific). 1 μl of RNA was placed on the NanoDrop and sample RNA concentration and purity was determined based on an absorbance at 260 nm. It was essential to wipe the spectrophotometer between each sample to eliminate contamination between samples. The RNA present in each sample was then equalised to the lowest individual concentration detected, by addition of RNA free H₂O.

4.5.3 cDNA preparation

Following RNA equalisation, samples were assembled for cDNA production using the ABI High Capacity cDNA kit (Applied Biosystems) as it is very sensitive and synthesises a large volume of cDNA. A master-mix solution was prepared and stored on ice (see Table 4.2). Equal volumes of the master mix were added to an equivalent volume of diluted RNA, e.g. 20 μl of master mix was added to 20 μl RNA in PCR minitubes. The minitubes containing samples were placed in a thermocycler and program was set according to protocol as follows, step 1 was set for 10 min at 25°C and step 2 set for 120 min at 37°C. When the thermocycler was finished performing the reverse transcription stage, samples were removed and stored at -20°C or used immediately for real time PCR analysis.

4.5.4 Real-time PCR (Multi Plex PCR)

Real-time PCR was performed using Taqman Gene Expression Assay's (Applied Biosystems), which contain forward and reverse primers, and a FAM-labelled MGB Taqman probe to each gene of interest. The assay IDs for the genes examined are listed in Table 4.3. A 1 in 5 dilution of cDNA was prepared with sigma water. Samples were assayed using ABI's universal cycling conditions using a fast protocol on the StepOnePlus Real-time PCR system (Applied Biosystems, UK). A 10 μl volume was added to each well (4 μl of diluted cDNA, 0.5 μl of primer, 0.5 μl of endogenous control and 5 μl of Taqman®Fast advanced PCR Master Mix). Electronic pipettes (EDP3 20-200 μl , 2-20 μl and 10-100 μl) were used to ensure pipetting accuracy. Samples were assayed in a run (40-47 cycles), which was composed of 3 stages, 50°C for 2 min for uracil-N-glycosylation (UNG) incubation and 95°C for 20 s for polymerase activation (holding stage), 95°C for 1 s for each cycle (denaturation) and finally the annealing step at 60°C for 20 s. β -actin was used as an endogenous control, as its expression levels tend to remain stable, to normalise gene expression data. β -actin expression was measured using gene expression assays containing forward and reverse primers (primer limited) and a VIC-labelled MGB Taqman probe (Applied Biosystems). Gene expression was calculated relative to the endogenous control samples and to the control sample, giving an RQ value $2^{\Delta\Delta CT}$, where CT is the threshold cycle.

In relation to the various stages carried out during the PCR reaction, initially samples are heated to 95°C for 1 s, which allows the double stranded cDNA to denature. Hereafter, the temperature of the reaction decreases to allow annealing and extension of the cDNA, however, the target probe must anneal to the single-stranded cDNA since it has a higher melting temperature than that of the target primers (Applied Biosystems). This probe contains a FAM/VIC dye and a proprietary non-fluorescent quencher (NFQ) dye, this quencher blocks the dye from emitting a fluorescent signal by fluorescence resonance energy transfer (FRET) technology (Applied Biosystems). Once the reaction temperature reaches 60°C the primers anneal to the strand of cDNA and it is extended by 5' nuclease activity of the Taq polymerase. This induces the release of the FAM/VIC-labelled probe causing the FRET between the dye and quencher to be broken, and the generation of a fluorescent signal. Due to the specificity of the probe and primers for the cDNA sequence, one fluorescent signal is produced for each new cDNA copy, and measured during the annealing stage of the PCR cycle (60°C).

Table 4.3: List of rat genes used in RT-PCR studies with the gene expression assay and genbank ref sequence numbers.

Gene Symbol	Gene Name	Taqman Gene Assay ID
MMP9	Matrix metalloproteinase 9	Rn00579162_m1
IDO	Indoleamine 2,3-Dioxygenase	Rn00576778_m1
IFNγ	Interferon-gamma	Rn00594078_m1
TDO	tryptophan 2,3-dioxygenase	Rn00574499_m1
IL-1β	Interleukin-1 β	Rn00580432_m1
IL-6	Interleukin-6	Rn00561420_m1
TNFα	Tumour necrosis factor- α	Rn99999017_m1
β-actin	β -actin	4352340E
GFAP	Glial fibrillary acidic protein	Rn00566603_m1

4.5.5 Real-time PCR analysis

The $\Delta\Delta$ CT method (Applied Biosystems RQ software, Applied Biosystems, UK) was used to assess gene expression for all real-time PCR analysis. This method is used to assess relative gene expression by comparing gene expression of treated/experimental samples to a normal or untreated sample (control), rather than quantifying the exact copy number of the target gene. In this manner the fold-difference (increase or decrease) can be assessed between treated and control samples. The fold-difference is assessed using the cycle number (CT) difference between samples. Briefly, a threshold for fluorescence is set, against which CT is measured. To accurately assess differences between gene expression, the threshold is set when the PCR reaction is in the exponential phase, when the PCR reaction is optimal or 100% efficient. Thus, samples with low CT readings demonstrate high fluorescence, indicating greater amplification and hence, greater gene expression. When a PCR is 100% efficient a one-cycle difference between samples means a 2-fold difference in copy number (2^2), similarly a 5-fold difference is a 32-fold difference (2^5).

To measure this fold-difference relative to control, the CT of the endogenous control (β -actin) is subtracted from the CT of the target gene for each sample, thus accounting for any difference in cDNA quantity that may exist. This normalised CT value is called the (CT). The CT difference (Δ CT) of the control is subtracted from itself to give 0, and subtracted from all other samples, this is the $\Delta\Delta$ CT value. The $\Delta\Delta$ CT (cycle difference corrected for β -actin) is then converted into a fold-difference. As a one-cycle difference corresponds to a two-fold increase or decrease relative to control, 2 to the power of the

$\Delta\Delta CT$ (difference in control and sample CT corrected for actin) gives the fold-difference in gene expression between the control and treated samples. The control sample always has a $\Delta\Delta CT$ value of 0, thus 0^2 gives a $2^{\Delta\Delta CT}$ of 1, against which all other samples are referenced.

4.6 Zymography

4.6.1 Tissue preparation

15-20 mg of tissue or 20 μl of serum was collected and added to a 2 ml round-bottomed tube containing lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH8.0, 1% v/v NP-40, 50 $\mu\text{l}/10\text{ml}$ Phosphatase Inhibitor Cocktail I (Sigma-Aldrich), 50 $\mu\text{l}/10\text{ ml}$ Phosphatase Inhibitor Cocktail II (Sigma-Aldrich) and 100 $\mu\text{l}/10\text{ ml}$ Proteinase Inhibitor Cocktail (Sigma-Aldrich)). A Polytron was used to homogenize the samples; care was taken to wash the Polytron blade in lysis buffer between samples to avoid cross-contamination. Homogenised samples were then centrifuged at 14,000 rpm at 4°C for 15 min. Supernatants were transferred to clean Eppendorf tubes and stored at -80°C. Prior to electrophoresis protein concentrations of each sample were determined, as per section 4.6.2, and each sample was equalized: Cortex: 1000 $\mu\text{g}/\text{ml}$, Colon: 2000 $\mu\text{g}/\text{ml}$ and Serum: 4000 $\mu\text{g}/\text{ml}$.

4.6.2 Protein Quantification: Bradford Assay

The Bradford Assay was used as a method of protein detection and quantification. A 1000 $\mu\text{g}/\text{ml}$ solution of bovine serum albumin (BSA) was made up in a TBS-T (1x). Serial dilution was carried out to give 8 solutions with final protein concentrations of 1000, 500, 250, 125, 62.5, 31.25, 15.625 and 0 $\mu\text{g}/\text{ml}$. 10 μl of the protein standards/samples were pipetted in duplicate into the wells of a 96 well (Sarstedt plate). BioRad solution (Bradford reagent) was prepared and 200 μl was pipetted into each well containing sample/standard. The 96 well plate was allowed to incubate for 5 min before using an ELX 800 Universal Microplate Reader (Bio-Tek Instruments) to read absorbance at 595 nm. A standard curve was constructed by plotting the standards against the absorbance, and results obtained expressed as $\mu\text{g}/\text{ml}$ of protein.

4.6.3 Gel preparation and electrophoresis

Glass plates and 1 mm spacers (BioRad) were cleaned with 70% ethanol and assembled into the electrophoresis block. dH_2O was used to check for leaks between the glass spacers

and the position of the stacking gel was marked to just below the bottom of the well comb. Separating gels (30% acrylamide (8 ml), 1.5 M 4x Tris-Cl pH 8.8 (7.5 ml), 20 mg/ml porcine gelatin containing 1% sodium dodecyl sulphate (3 mL), ddH₂O (11.5ml), 10% ammonium persulphate (100 μ l), and TEMED (24 μ l)) were then made up and vortexed thoroughly before loading gently between the glass spacers and up to just below the bottom of the comb. A layer of ddH₂O was added to avoid bubbles forming in the gel. The gel was left for 45 min to polymerise. Once the separating gel solidified, water was drained, and the stacking gel was prepared (30% acrylamide (1.3 ml), 1.5 M 4x Tris-Cl pH 6.8 (2.5 ml), ddH₂O (6 ml), 10% APS (50 μ l) and TEMED (12 μ l)). The stacking gel was vortexed vigorously before loading onto the separating gel, and a comb was inserted to allow wells to be formed in the gel. The gel was allowed to solidify for 30 min and the comb was removed. The glass plates containing the gels were secured onto the electrophoresis stand and placed into an electrophoresis box. Ice cold tank buffer (For 10x: 30.3 g Tris base, 144 g glycine, 10 g SDS, to 1 L ddH₂O, pH 8.3) was poured into the electrophoresis box until half full and between the two glass plates to the very top to ensure current could pass through the gels. When thawed, 6 μ l aliquots of loading buffer were added to 18 μ l of each equalised sample. Positive control was also prepared by adding 6 μ l of loading buffer (0.5 M 4X Tris-Cl pH 6.8 (5 ml), glycerol (4 ml), SDS (0.8 g), ddH₂O (0.4 ml) and Bromophenol blue (0.2 mg) were dissolved in 10 ml ddH₂O) to 18 μ l HT standard (HT standard was kindly provided by Dr. Carlos Medina). 20 μ l of samples and standard were then loaded carefully into the wells. The electrophoresis boxes were then placed into a tank containing ice and the electrophoresis electrodes were connected to the power supply (BioRad). The voltage was set to 150 V, current was set to 300 mA and electrophoresis was run for approximately 2 h. When complete, the gel was carefully removed from the glass plates and the stacking gel layer was cut away. The gels were then washed for 20 min, 3 times with Triton buffer with mild agitation. Two further washes with zymography buffer (2 M Tris-Cl (50 ml), NaCl (18 g), CaCl₂ (1.47 g) and NaN₃ (1 g) dissolved in 2 L ddH₂O) for 20 min each time were then carried out. The gels were then incubated in fresh zymography buffer overnight in an incubator at 37°C. The next day, the gels were stained with staining solution (250 ml MeOH, 100 ml acetic acid, 650 ml ddH₂O, and 250 mg of Coomassie Brilliant Blue G-250) for 3 h and then destained using destaining solution (880 ml ddH₂O, 80 ml acetic acid, and 40 ml MeOH.). Gels were stored in destaining solution until analysis (see Figure 4.2 for example zymogram). Gelatinase activity of MMP9 and MMP2 was analysed by detection of intensity of band clearing at the same position as in the positive control. A calibrated densitometer (GS-800 Bio-Rad) and Quantity One analysis software (Version 4 Bio-Rad) was used to measure intensity x mm.

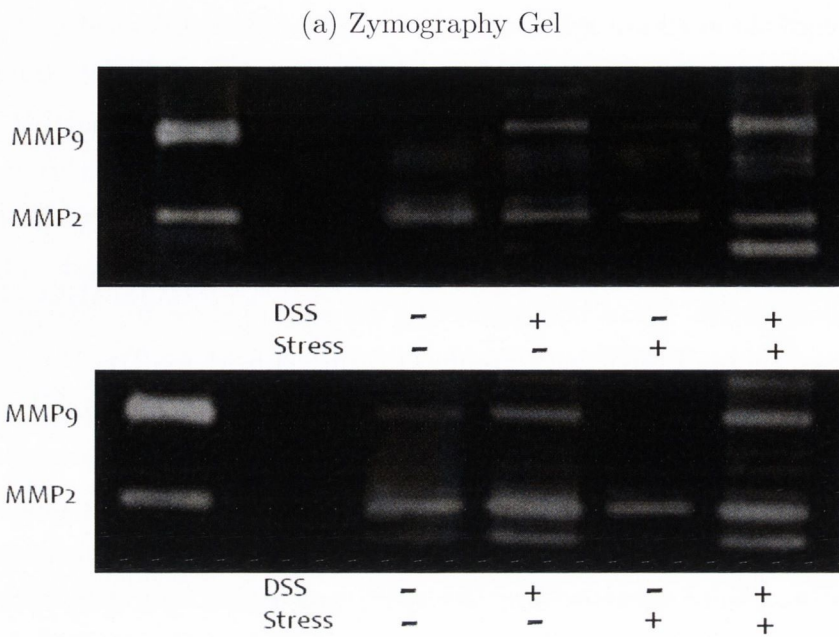


Figure 4.2: **Example zymogram** Zymogram, from acutely colitic rat colon samples, showing gelatinase activity.

4.7 Nuclear and cytoplasmic extraction

Nuclear fractions were isolated using the NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce Biotechnology) according to the manufacturer's instructions. Briefly, 20 mg of tissue was washed in 200 μ l PBS and centrifuged at 500 g for 5 min at 4°C. The PBS was removed and tissue was homogenised in CERI and CERII buffers to disrupt the cell membrane and release cell contents. The homogenate was centrifuged at 16,000 g for 5 min at 4°C. The supernatant (cytoplasmic extract) was transferred to a clean pre-chilled tube and stored at -80°C. The pellet fraction was resuspended in NER buffer and vortexed for 15 s every 10 min, for a total of 40 min. The tube was centrifuged at 16,000 g for 10 min at 4°C. The supernatant (nuclear extract) was transferred to a clean pre-chilled tube and stored at -80°C. Prior to performing the NF κ B p65 assay, the protein concentration of each sample was determined and equalised using NER buffer (supplied with extraction kit). After equalisation one final protein assay was performed and the values were taken into account when calculating NF κ B p65 activity.

4.8 NF κ B p65 detection

NF κ B p65 activation was measured using the Thermo Scientific Pierce NF κ B p65 Transcription Factor Kit. The kit contains a streptavidin-coated 96-well plate with the bound biotinylated-consensus sequence for NF κ B p65 which specifically binds only the active forms of NF κ B p65. This chemiluminescent ELISA-based assay provides greater sensitivity than traditional methods for measuring active NF κ B p65 including gel-shift or electrophoretic mobility shift assays (EMSA) and colorimetric ELISA-based assays.

The assay was performed according to manufacturer's instructions. Briefly, a working binding buffer (supplied with kit) was prepared according to the manufacturer's recommendations, and 50 μ l was added to each well along with 10 μ l of the nuclear extract which was prepared from the NE-PER nuclear and cytoplasmic extraction kit. The plate was incubated for 1 h at RT with mild agitation after which the well contents were discarded and the plate was tapped to remove all liquid. The plate was washed three times with 200 μ l of wash buffer. 100 μ l of the diluted primary antibody (1:1000) was added to each well and incubated for 1 h at RT without agitation. The plate was washed three times with 200 μ l of wash buffer. 100 μ l of diluted secondary antibody (1:10,000) was added to each well and incubated for 1 hour at RT without agitation. Finally, the plate was washed four times with 200 μ l of wash buffer and 100 μ l of the chemiluminescent solution was added to each well. Chemiluminescence was measured using a luminometer set to 200 ms and a sensitivity value of 135. Data was expressed as relative light units (RLU)/ μ g protein.

4.9 Immunohistochemistry

4.9.1 Brain Collection

Rats were anaesthetised by intraperitoneal urethane injection (12.5 g in 30 mls dH₂O). The rats forelimbs were pinned to the dissection apparatus and a scalpel was used to make a median incision from underneath the chin to below the sternum. The connective tissue connecting the skin to the ribcage was cut and an incision made in the diaphragm to gain entry to the thoracic cavity. The ribs were removed to expose the heart. A cannula, which was connected via tubing to a perfusion pump (Gilson Mini Pulse 3 perfusion pump), was inserted into the left ventricle, and the right atrium was pierced. 10 mM PBS (minimum of 5 min) followed by 4% paraformaldehyde (both pH 7.4) (minimum of 10 min) was peristaltically pumped through the heart into the vascular system. The rat was decapitated and the brain was collected and immersed in 4% paraformaldehyde at 4°C.

After 2 days fixation the brains were stored in a 30% sucrose solution for 3-4 days at 4°C to cryoprotect prior to snap freezing in isopentane (Sigma-Aldrich) on dry ice, and stored at -80°C until sectioning. A liver sample collected prior to perfusion was snap frozen on dry ice and stored at -80°C for use in RT-PCR.

4.9.2 iNOS immunostaining

Frozen brains were sectioned on a Leica CM1850 cryostat at -21°C to 30 μ m thickness. Six series were collected per brain. Each series of slices was placed in freezing storage solution (30% v/v ethylene glycol, 30% w/v sucrose in 10 mM PBS) and stored at -80°C until use.

For staining, brains were left to defrost at room temperature. A paint brush was used to transfer the sections into two netwells per brain, along with a third netwell for negative control slices. The netwells were put through three 5 min washes in 10 mM PBS (pH7. 4) before being incubated for 20 min in 0.75% H₂O₂ solution to block endogenous peroxidases. The sections then underwent three more washes in 10 mM PBS before being incubated in 10% normal goat serum (NGS) solution for 20 min. The sections were washed for 5 min in 10 mM PBS, and were then incubated overnight with agitation in a 1:300 dilution of the primary antibody (polyclonal rabbit iNOS - Santa Cruz) in antibody dilution buffer. The negative control slices were incubated in antibody dilution buffer only. Following incubation, sections were washed (3 x 5 min) in 10 mM PBS. A dilution of the secondary antibody was made up using Vectastain Elite ABC kit (polyclonal rabbit) (Vector Labs). The sections were incubated in the secondary antibody solution for 90 min at room temperature on a rock and roller. A/B solution was made up 30 min before use according to instructions supplied with the kit. Sections were washed (3 x 5 min) in 10 mM PBS and then incubated for 90 min with shaking in A/B solution. Sections were washed (3 x 5 min) in 10 mM PBS. A paintbrush was used to put sections into a small amount of 10 mM PBS in a petri dish. 10% Di-Amino-Benzidine (DAB) solution (DAB, 0.4% v/v H₂O₂ in 10 mM PBS) was prepared and added. Sections were left to incubate until the negative control slices began to turn brown (approximately 10 min). dH₂O was added to the petri dish to stop the reaction. Sections were washed twice in dH₂O and twice in 10 mM PBS. Sections were then mounted onto gelatin coated slides in a small amount of 10 mM PBS, and left to air dry at RT overnight.

4.9.3 Dehydrating and Mounting Slides

Slides were desalted by immersing in dH₂O for approximately 20 s. They were then placed in slide holders, and dehydrated by dipping in a series of alcohols (70% ethanol, 90% ethanol, 100% ethanol and xylene (Fisher Chemical)) in the fume hood. Slides were left to air dry. A few drops of DPX were used to carefully mount glass coverslips on the slides. The slides were left to dry in the fume hood.

4.9.4 iNOS expression analysis

Brain slices were visualised using Olympus BX-51 microscope linked to a computer, allowing them to be imaged and analysed using the Olympus Cell-D software program. Initial images were taken at 1.25 x magnification for identification according to the Paxinos and Watson (1998) rat brain atlas. Higher magnification (x10) images of regions of interest were used for quantification. iNOS positive cells were defined as dark brown stained cells and were counted by a researcher blind to the rat group.

4.10 Statistical Analysis of Results

All data was analysed using GB-STAT v.10 (Dynamic Microsystems Inc) and graphs were generated using GraphPad prism v 4.0 (GraphPad Software). Data are expressed as mean \pm standard error of the mean (SEM) and were analysed by Pooled variance Student's *t* test, one-way analysis of variance (ANOVA), or two-way ANOVA where appropriate. If any statistically significant differences were revealed by ANOVA, *post hoc* comparisons were performed using a Newman-Keuls test. Spearman's rank-order correlation was used for all correlations. In all cases a *p* value of < 0.05 was considered significant.

Chapter 5

Assessment of the association between psychological measures and inflammatory and disease related markers of Inflammatory Bowel Disease

5.1 Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory disease of the gastrointestinal (GI) tract, the two main types of IBD are ulcerative colitis (UC) and Crohn's disease (CD) (Bouma and Strober, 2003). Symptoms of IBD can include diarrhoea, pain, abdominal swelling, constipation, weight loss, nausea and rectal bleeding (mainly in UC). The cause of IBD is unknown, however, it is thought to arise from a combination of genetic predisposition and environmental triggers (Brown, 2007).

Beyond the physical symptoms, previous literature suggests that there is an increased risk of depression or anxiety in IBD. Historically associations between co-morbidity of psychological well-being, psychiatric illness and personality differences and IBD have been discussed (Murray, 1930; Sullivan, 1935; Daniels, 1942; Straker, 1960). Recent literature supports this increased risk of psychiatric disturbances in adult and pediatric IBD patients (Gómez-Gil et al., 2003; Loftus et al., 2011). The level of disease activity further increases the risk of psychological disturbances, whereby a number of investigations report increased psychological symptoms during active IBD (Mardini et al., 2004; Häuser et al., 2011; Ben

Thabet et al., 2012). It has also been reported that immunomodulation therapy induces remission of both disease activity and psychological symptoms (Calvet et al., 2006). Depression and anxiety in IBD patients are also correlated with decreased health related quality of life (HRQOL), and a link between poor drug compliance and psychological disorders has also been suggested (Walker et al., 2008; Vidal et al., 2008; Guthrie et al., 2002; Hyphantis et al., 2010; Kiebles et al., 2010; Selinger et al., 2011). Despite this evidence for increased risk of psychological disturbances during the active inflammatory phase and the recent surge in psychoneuroimmunology research, there are no investigations to date which have determined if mediators of inflammation, such as cytokines, are responsible for the psychological symptoms.

Although it has been suggested that inflammatory mediators could be involved in mediating psychological disturbances in IBD (Szigethy et al., 2004) only one study has attempted to correlate psychological scores to laboratory data (Mittermaier et al., 2004). In their study no correlation was found between circulating C-reactive protein (CRP) concentrations and psychological scores. Based on the recent psychoneuroimmunology literature two other potential molecular mechanisms are proposed here, which could influence normal psychological function: increased cytokine expression and kynurenine pathway activation.

5.1.1 Cytokine Expression

Increased intestinal permeability can lead to increased circulating cytokine concentrations. Cytokines are soluble mediators of inflammation produced by a variety of cell types including immune cells, which can interact with the central nervous system (CNS) (for review see Licinio and Wong (1999); Schiepers et al. (2005)). Cytokines can affect the brain via a number of mechanisms including penetration through the circumventricular organs, vagal nerve stimulation, or receptor activation, which in turn induces second messengers (for review see (Licinio and Wong, 1999; Schiepers et al., 2005)). Different cytokine profiles exist between UC and CD, however, some are common to both diseases: IL-1 β , TNF α , IL-6, IFN γ (McClane and Rombeau, 1999; Moriconi et al., 2007). As no differences in risk of psychological disturbances exist between UC and CD these are potential cytokine candidates for increased risk of psychological disturbances. These same cytokines have also previously been implicated in patients with depression and in animal models of sickness behaviour (Schiepers et al., 2005; Harden et al., 2006, 2008). In active IBD, there can be damage to the protective intestinal barrier, MMPs are capable of cleaving most constituents of the bowel, MMP9 in particular is increased in tissue biopsies from IBD patients and in intestinal samples from animal models of IBD (Santana et al., 2006; Medina et al.,

2001; Medina and Radomski, 2006; Medina et al., 2003). Measurement of MMP9 therefore, can provide a guide to the damage within the gut wall. Damage to the intestinal wall renders it leaky, thus leaving the body vulnerable to the toxic contents of the intestine, which can in turn induce a local inflammatory response or systemic immune activation.

5.1.2 Kynurenine pathway activation

The amino acid tryptophan is the precursor to both serotonin and kynurenine. During immune activation, through induction of indoleamine 2,3 dioxygenase (IDO), the metabolism of tryptophan can become unbalanced in favour of kynurenine synthesis. This is thought to affect normal CNS functioning in two ways: firstly increased metabolism in favour of kynurenine could decrease the availability of tryptophan for serotonin synthesis, and decreased serotonin availability is involved in both depression and anxiety (Rang et al., 2003; Graeff et al., 1996), secondly the products of kynurenine metabolism, kynurenines, are neuromodulators and could influence CNS function in their own right (Guillemin et al., 2007; Nemeth et al., 2007; Ruddick et al., 2006).

5.2 Aims and Objectives

As treatment of the psychological disturbances would be greatly beneficial to improve patients' quality of life, the mechanisms behind this increased risk are worth investigating. The overall aim of this investigation was to determine if symptoms or inflammatory mediators are associated with increasing psychological disturbances in IBD patients. Specifically the objectives were to:

1. Quantify the expression of inflammatory markers (IL-1 β , TNF α , IFN γ , IL-6, MMP9, iNOS and IDO) in intestinal biopsies from IBD patients during active and inactive inflammation
2. Quantify circulating concentrations of CRP, IFN γ , IL-6, tryptophan, kynurenine, and whole blood MMP9, iNOS and IDO mRNA expression as systemic biomarkers of active disease
3. Analyse psychometric scores including HAM-D, HAM-A, and POMS in IBD patients versus controls

5.3 Methods

5.3.1 Subjects

18 patients with IBD, in addition to 19 age balanced patient controls were included in the study. Inclusion criteria were patients of between 30 and 70 years with confirmed ulcerative colitis (10 patients) or Crohn's disease (11 patients). Patients with normal colonoscopy attending for screening were included as controls. Exclusion criteria included general exclusion criteria for colonoscopy, infective colitis, pregnancy, terminal illness and patients unfit to provide informed consent. A fasting sample of blood was obtained from each of the study participants. Serial biopsies were obtained from inflamed areas during colonoscopy. Ethical approval, patient recruitment, clinical interviews, and colonoscopy were carried out by the Department of Gastroenterology, Hospital Universitario Central de Asturias, Oviedo, Spain. Signed consent was obtained from each participant prior to assessment. All subsequent tissue analysis was carried out in Trinity College Dublin.

5.3.2 Well-being, mood and anxiety scores

POMS score

Profile of Mood States (POMS) was administered to all study participants by interview as an instrument to measure mood state and subjective well-being. The POMS rating scale consists of 65 adjectives that are rated by subjects on a 5-point scale (see Pollock et al., 1979). Seven factors have been derived from this, including, tension-anxiety, depression-dejection, anger-hostility, fatigue-inertia, vigor-activity and confusion-bewilderment. Each adjective is awarded the scores except relaxed (tension) and efficient (confusion) in which case the score was reversed. A number of dummy items including friendly, clear headed, considerate, sympathetic, helpful, good-natured and trusting were incorporated but not included in the overall score. Participants can choose from 0 (not at all) to 4 (extremely). Total mood disturbance is calculated by adding the raw scores from tension, depression, anger, fatigue and confusion and then subtracting the vigor score. This yields a value between -24 and 177, with lower scores indicative of people with stable mood profiles.

EuroQol-5D

Quality of life was assessed using the Spanish Version of the EuroQol-5D (EQ-5D) (EuroQol group, 1990). This is one of the most widely used health related quality of life (HRQOL) questionnaires. It assesses five dimensions, mobility, self-care, usual activities,

pain/discomfort, and anxiety/depression. Each dimension has 3 levels: no problems, some problems, and severe problems at the time of completion. For this study, the dimensions were divided into two groups: no problems versus problems.

IBDQ index

The inflammatory bowel disease questionnaire (IBDQ) 9, is a shortened version of the IBDQ-36 questionnaire, that is widely used for HRQOL assessment in patients with IBD (Alcala et al., 2004; Pallis et al., 2004). The questionnaire consists of 9 questions relating to stool frequency, fatigue, energy, cancelling social events, cramps, general discomfort, nausea, gas, and general life satisfaction. Patients are asked to respond to questions with respect to the previous two weeks. The questions are rated from 1 to 7, resulting in a total score ranging from 10 to 63, with higher scores representing higher quality of life. A modified version of the IBDQ9 (IBDQs), which only contains the five questions relevant to the bowel symptoms was also constructed for the purpose of this study (see appendix A). This results in a total score ranging from 7 to 35, with lower scores indicating higher symptom expression. A score of < 32 for the IBDQs was considered to be symptomatic.

Mayo index

The Mayo index is used to assess disease activity in UC patients. The score involves 4 separate dimensions 1) stool frequency, 2) rectal bleeding, 3) endoscopic score, 4) physicians global assessment. Each section is scored from 0 to 3 resulting in a total range from 0 to 12, with higher scores representing more severe disease activity. A score ≥ 4 indicates active disease.

CDAI

The Crohn's disease activity index (CDAI), first published by Best et al. (1976), is calculated based on an 8 item check list. Items 1 to 3 are completed daily by the patient over the course of a week for, 1) daily stool count, 2) abdominal pain, and 3) general well-being. Items 4 to 8 are based on clinical evaluation of 4) extraintestinal symptoms, 5) antidiarrheal use, 6) abdominal mass, 7) haematocrit values and 8) weight. Possible values range from 0 to 600, with scores above 450 relating to extremely severe disease and 150-450 relating to active disease.

Hamilton depression rating scale

Depressive symptomology was assessed in controls and IBD patients using the Hamilton depression rating scale (HAM-D) (Hamilton, 1960). The scale provides an indication of depression, and is one of the most widely used and accepted outcome measures for evaluating the severity of depression symptoms. 8 items are scored on a 5 point scale, ranging from 0 = not present to 4 = severe and 9 are scored from 0 to 2 as follows: depressed mood (0 to 4), feelings of guilt (0 to 4), suicide (0 to 4), insomnia early, middle or late (0 to 2), work and activities (0 to 4), psychomotor retardation (0 to 4), agitation (0 to 4), psychological anxiety (0 to 4), somatic anxiety (0 to 4), loss of appetite (0 to 2) general somatic symptoms (0 to 2), genital symptoms (0 to 2), hypochondriasis (0 to 4), loss of weight (0 to 3), insight or denial (0 to 2), diurnal variation (0 to 2) and severity of variation (0 to 2), depersonalization and derealisation (0 to 4), paranoid symptoms (0 to 3), obsessional compulsive symptoms (0 to 2). Although HAM-D lists 21 items, the scoring is based on the cumulative score of the first 17. The remainder provide additional clinical information. A score of 0-7 is within the normal range; 8-13, 14-18 and 19-22 fall within mild, moderate and severe categories, whereas a score of > 23 is considered a very severe condition.

Hamilton anxiety rating scale

Anxious symptomology was assessed in controls and IBD patients using the Hamilton anxiety rating scale (HAM-A) (Hamilton, 1959). The scale is the first scale developed to assess the severity of anxiety, and is one of the most widely used for evaluating the severity of anxious symptoms. 14 items are scored on a 5 point scale, ranging from 0 = not present to 4 = severe as follows: anxious mood, tension, fears, insomnia, intellectual, depressed mood, somatic (muscular), somatic (sensory), cardiovascular symptoms, respiratory symptoms, gastrointestinal symptoms, genitourinary symptoms, autonomic symptoms, behaviour at interview. A score of <17 indicates mild severity, 18-24 mild to moderate severity and 25-30 moderate to severe.

5.3.3 Processing of blood samples, preparation of plasma and sample preparation for HPLC

Whole blood was collected, by venous puncture, into commercially available EDTA-treated tubes. Plasma was obtained after centrifugation for 15 minutes at 2,000 x g. The resulting supernatant was immediately transferred into a clean polypropylene tube and the samples

were stored at -80°C until required for analysis. Plasma samples were defrosted on ice and added to an equal volume of 50nM glacial acetic acid, 100 mM zinc acetate, 3% acetonitrile dissolved in double-distilled HPLC grade H_2O , pH 4.9 with diluted NaOH. This buffer constituted for the mobile phase used in the HPLC assay. In order to extract the analytes of interest de-proteination was carried out by the addition of perchloric acid (final concentration 6% and 200 ng/20 μl of internal standard (N-methyl-serotonin) was added as a quality control measure for subsequent HPLC analysis. Samples were vigorously vortexed for approximately 10 s, and then centrifuged at 4°C and 14,000 rpm for 20 min. The supernatants containing the total fraction were subsequently filtered into eppendorf tubes by 0.45 μm filter tipped syringe (Phenomenex). In order to differentiate analytes bound to plasma proteins from those freely circulating, plasma samples were also processed by inclusion of an ultra-filtration step and treated with the following modifications: plasma samples were thawed and 300 μl was ultra-filtered at 14000 g for 30 min at 4°C using AmiconUltra-0.5 3K centrifugal filters (Millipore) to separate free from protein bound analytes. Internal standard was added to the ultra-filtrate, containing the free fraction, and injected onto the HPLC as described above. The bound fraction remaining on the filter was re-suspended in 300 μl of mobile phase containing perchloric acid and internal standard as described above. The samples were then sonicated for 10 seconds and vortexed vigorously prior to centrifugation and filtration as per total samples.

5.3.4 HPLC for determination of tryptophan and kynurenine

Tryptophan and kynurenine were analysed using the Shimadzu ADVP HPLC system coupled to PDA-UV (Shimadzu SPD-M10A VP set to integrate at 230 and 250 nm) and fluorescence (Shimadzu RF-20A XS prominence set at excitation 254 nm and emission 404 nm) detectors. A reverse phase analytical column (Kinetex Core Shell Technology, Phenomenex UK) with specific area of 100 mm x 4.6 mm and particle size of 2.6 μm and fitted with a guard column was used to separate the analytes in the sample. Standards (5 ng/20 μl) of tryptophan and kynurenine were prepared in mobile phase and standards and samples were injected in a volume of 20 μl with a continuous flow rate of 0.8 ml/min and acquisition time of 20 min. The signal to noise ratio was 3:1. Chromatograms were generated using Class VP software and peak heights and retention times of tryptophan, kynurenine and internal standard (N -methyl-5-HT) were used to calculate the concentrations in ng/ml plasma.

5.3.5 Plasma IL-6, IFN γ and C-reactive protein ELISA measurements

Plasma IL-6 and IFN γ concentrations were measured using ELISA MAXTM Deluxe kits (Biolegend, UK), and plasma C-reactive protein (CRP) concentrations were measured using a CRP ELISA DuoSet (R&D systems, UK). Immunoassays were performed according to manufacturer's instructions. Absorbance was read at 450 nm using a microplate reader (Sunrise Tecan, Reading, UK) and then re-calculated as concentration (pg/ml for IL-6 and IFN γ and mg/L for CRP). Limits of detection for the ELISAs were 4 pg/ml for IL-6 and IFN γ , and 5 pg/ml for CRP.

5.3.6 Zymography

Plasma MMP2 and MMP9 protein activity in the circulation was assessed by zymography as described previously in section 4.6. Briefly plasma samples were added to 2ml round-bottomed tubes containing lysis buffer (150mM NaCl, 50mM tris-HCl pH 8.0, 1% v/v NP-40, 50 μ l/10 ml Phosphatase Inhibitor Cocktail I (Sigma-Aldrich)). Samples were then vortexed vigorously and centrifuged at 14,000 rpm at 4°C for 15 min. Supernatants were transferred to clean Eppendorf tubes and stored at -80°C. Prior to analysis samples were equalised to 4000 μ g/ml. 18 μ l of sample and 6 μ l of loading buffer were added to each well, and electrophoresis (voltage was set to 150V, current was set to 300 mA) was run for 2 h and 30 min. Gels were washed and left to incubate for 2 nights in zymography buffer at 37°C. Following incubation gels were stained for 3 h, then destained in destaining solution. Gelatinase activity of MMP9 and MMP2 was analysed by detection of intensity of band clearing at the same position as in the positive control. A calibrated densitometer (GS-800 Bio-Rad) and Quantity One analysis software (Version 4 Bio-Rad) was used to measure intensity x mm.

5.3.7 Real-time PCR analysis of mRNA expression of target genes in colonic biopsies and whole blood samples

RNA was isolated from patient biopsies using a Nucleospin RNA II kit (Macherey-Nagel, Germany) according to the kit protocol. RNA isolation from patient blood samples was performed using a PAXgene blood RNA kit (Qiagen, UK) according to the kit protocol. Following RNA quantification and equalisation, cDNA was synthesised using a cDNA archive kit (High capacity cDNA reverse transcription kit, Applied Biosystems, UK). Gene expression analysis was conducted using real-time PCR employing Taqman[®]Gene

Expression Assays (Applied Biosystems, UK) as per (Hughes et al., 2012). To quantify expression of target genes of interest Taqman Gene Expression Assays containing FAM-labelled probes were used (Assay IDs: IDO1 Hs00984148_m1, IFN γ Hs00989291_m1, iNOS Hs01075529_m1, MMP9 Hs00234579_m1, TNF α Hs00174128_m1, IL-6 Hs00985639_m1 and IL-1 β Hs01555410_m1 Applied Biosystems, UK). PCR reactions were in a duplex format also containing a Taqman Gene Expression Assay (primer-limited) containing a VIC-labelled probe for the endogenous control gene GAPDH (Assay ID: 4326317E). Samples were assayed using ABI's universal cycling conditions using a fast protocol on the StepOnePlus Real-time PCR system (Applied Biosystems, UK). Fold change in gene expression from the control group was calculated using the $\Delta\Delta C_t$ method (see section 4.5), and GAPDH served as endogenous control in the amplification system. Data are expressed as fold change in gene expression relative to the control group.

5.3.8 Statistical Analysis

Data are presented as mean \pm standard error of the mean (SEM). As appropriate Student's *t* test, One-way ANOVA, and Two-way ANOVA were used to compare groups, followed by a Newman-Keuls *post hoc* test. All statistical analyses were considered to be significant when $p < 0.05$. Graphs and statistics were generated using GraphPad Prism Software Version 4.00 (GraphPad software, Inc) and GB-STAT v.10 (Dynamic Microsystems Inc) respectively.

5.4 Results

Eighteen IBD patients and 19 patient controls were recruited. For IBD patients clinical characteristics see Table 5.1. Of the 19 patient controls 1 (5.3%) has previously suffered from depression (female), while 3 (16.6%) of the IBD patients had a previous diagnosis of depression (two males and one female).

Table 5.1: IBD patient clinical characteristics

Variable	n
female	11
male	7
<i>IBD diagnosis</i>	
UC	10
CD	8
<i>Location</i>	
left-colitis	6
ileocolitis	6
pancolitis	3
proctitis	1
ileal	1
colon	1
<i>Treatment</i>	
5-aminosalicylic acid	7
azathiopine	5
none	3
methotrexate	2
infliximab	1

5.4.1 Increased intestinal and circulating inflammation related markers in IBD

Patients were divided into patient controls and IBD patients based on medical records and colonoscopy. Gene expression analysis by PCR supports increased inflammation in the IBD patients. Student's *t* test revealed significant increases in the expression of IDO mRNA [$t=-2.93$; $df\ 35$; $p = .0059$], IFN γ mRNA [$t=-2.29$; $df\ 35$; $p = .0281$], IL-6 mRNA [$t=-2.01$; $df\ 35$; $p = .05$], MMP9 mRNA [$t=-2.38$; $df\ 35$; $p = .02$], TNF α [$t=-3.56$; $df\ 35$; $p = .038$], and iNOS mRNA [$t=-3.56$; $df\ 35$; $p = .001$] in intestinal biopsy samples from IBD patients versus patient controls. IL-1 β [$t=-1.93$; $df\ 35$; $p = .06$] is approaching significance. Increases in circulating inflammatory mediators including IL-6 [$t=-2.07$; $df\ 35$; $p = .046$] and IFN γ [$t=-2.81$; $df\ 35$; $p = .0079$], and a trend towards increased CRP [$t=-1.97$; $df\ 35$; $p = .056$] were also revealed, as well as increases in the total kynurenine:tryptophan ratio [$t=-2.21$; $df\ 35$; $p = .034$]. No significant differences in the HAM-D scores, HAM-A scores, POMS score or IBDQ scores were revealed between the IBD patient and patient control groups (see Table 5.2).

Comparison of the UC and CD groups were also analysed by Student's *t* test (see Table 5.2). No differences in the inflammatory markers in the tissue biopsies or in the circulation were found. Psychological scores were not significantly different, however, there was a trend towards increased POMS scores, and decreased IBDQ index in the CD patients versus the UC patients.

Table 5.2: Evidence of intestinal and circulating immune mediators in IBD patients

	Patient Control	IBD	UC	CD
M:F	10:9	7:10	3:7	4:4
Age	54.3 ± 2.3	49 ± 2.69	49.7 ± 3.62	48.13 ± 4.26
n	19	18	10	8
<i>Biopsy</i> (Fold change)				
IDO	1.0 ± 0.1	24.0 ± 8.1 **	15.8 ± 5.5	34.2 ± 16.7
IFNγ	1.0 ± 0.3	12.1 ± 5.0 *	6.5 ± 2.7	19.2 ± 10.5
IL-6	1.0 ± 0.6	42.5 ± 21.2 *	19.4 ± 15.9	71.3 ± 42.7
IL-1β	1.0 ± 0.5	26.2 ± 13.4	13.8 ± 11.3	41.7 ± 26.7
TNFα	1.0 ± 0.2	2.4 ± 0.7 *	1.8 ± 0.4	30.7 ± 1.4
MMP9	1.0 ± 0.3	9.4 ± 3.7 *	9 ± 4.8	10.3 ± 6.2
iNOS	1.0 ± 0.2	7.6 ± 1.9 **	9.9 ± 2.7	4.9 ± 2.6
<i>Plasma</i>				
IL-6	5.9 ± 0.9	11 ± 2.3 *	9.8 ± 2.8	12.4 ± 4.1
IFNγ	2.6 ± 0.4	8.2 ± 2.2 **	9.3 ± 3.6	7.0 ± 2.5
CRP	2.4 ± 0.5	5.3 ± 1.4 (0.06)	4.5 ± 1.8	6.3 ± 2.3
kyn:try	4.4 ± 0.2	5.7 ± 0.6 *	5.2 ± 0.8	6.4 ± 0.8
<i>Scores</i>				
POMS	12.9 ± 4.3	11.2 ± 6.2	2.6 ± 5.5	21.9 ± 11.4 (0.1)
HAM-A	6.2 ± 1.0	8.5 ± 1.6	8.9 ± 2.8	8.0 ± 1.3
HAM-D	2.8 ± 0.8	5.1 ± 0.9	5.5 ± 1.5	4.6 ± 1
IBDQ	52.0 ± 1.4	53.0 ± 1.8	55.7 ± 1.5	49.6 ± 3.2 (0.08)
IBDQs	30.6 ± 1.1	30.3 ± 1.3	32.1 ± 1.2	28.0 ± 2.4 (0.1)

Data represent mRNA fold change for biopsy samples, circulating concentrations of IL-6 (pg/ml), IFN γ (pg/ml), and CRP (mg/L), the circulating total kynurenine:tryptophan ratio, and psychological scores. Data are expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$ vs patient controls (Pooled variance Student's t test). Numbers in brackets represent p values approaching significance. C-reactive protein (CRP), Crohn's disease (CD), female (F), Hamilton anxiety (HAM-A), Hamilton depression (HAM-D), indoleamine 2,3-dioxygenase (IDO), inducible nitric oxide synthase (iNOS), inflammatory bowel disease (IBD), inflammatory bowel disease questionnaire (IBDQ), interferon (IFN), interleukin (IL), male (M), matrix metalloproteinase (MMP), profile of mood states (POMS), tumour necrosis factor (TNF), ulcerative colitis (UC)

5.4.2 Significant increases in inflammatory mediators during moderate/severe intestinal inflammation

The aim of this section was to investigate whether there is a more pronounced psychological disturbance or circulating inflammatory response during moderate to severe intestinal inflammation in IBD patients compared to mild intestinal inflammation, inactive intestinal inflammation and patient controls (see Table 5.3). Intestinal inflammatory severity was evaluated by the physician during colonoscopy and IBD patients were assigned to one of three groups: inactive, mild, and moderate/severe inflammation. Biopsy PCR analysis of inflammatory markers confirmed the clinical diagnosis of severity, whereby the most severe increases in inflammatory markers were found in the moderate/severe IBD patient group. One-way ANOVA followed by Newman-Keuls *post hoc* analysis revealed significant differences in expression of IDO mRNA [$F(3,33) = 6.25, p = .0018$], IFN γ mRNA [$F(3,33) = 5.28, p = .0044$], IL-6 mRNA [$F(3,33) = 10.12, p < .0001$], IL-1 β mRNA [$F(3,33) = 8.35, p = .0003$], TNF α mRNA [$F(3,33) = 4.24, p = .012$], MMP9 mRNA [$F(3,33) = 9.03, p = .0002$], and iNOS mRNA [$F(3,33) = 10.56, p < .0001$]. Significant differences in the circulating markers of inflammation were also revealed during moderate/severe inflammation: IL-6 [$F(3,33) = 14.58, p < .0001$], IFN γ [$F(3,33) = 7.39, p = .0006$], CRP [$F(3,33) = 30.38, p < .0001$], and total kynurenine:tryptophan [$F(3,33) = 20.41, p < .0001$]. However no significant differences in POMS, HAM-D, HAM-A or IBDQ index were revealed when patients were divided based on the colonic inflammatory state.

Table 5.3: Moderate/severe IBD was associated with the Most significant increase in cytokines and kynurenine:tryptophan ratio

	Patient Control	Inactive inflammation	Mild inflammation	Moderate/severe inflammation
M:F	10:9	3:4	3:2	1:5
Age	54.3 ± 2.3	48.1 ± 4.2	46.4 ± 4.4	52.2 ± 5.7
n	19	7	5	6
<i>Biopsy</i> (Fold change)				
IDO	1.0 ± 0.1	7.4 ± 6.6	24.4 ± 11.9	42.9 ± 19.2 **
IFNγ	1.0 ± 0.3	1.5 ± 0.8	12.4 ± 7.6	24.3 ± 12.5 **
IL-6	1.0 ± 0.6	1 ± 0.6	4.7 ± 3.1	122.3 ± 51.4 **
IL-1β	1.0 ± 0.5	1.2 ± 0.6	4.2 ± 1.6	73.7 ± 33.9 **
TNFα	1.0 ± 0.2	1.0 ± 0.3	2.8 ± 0.9	3.7 ± 1.7 *
MMP9	1.0 ± 0.3	1.4 ± 0.4	5.5 ± 3.2	22.5 ± 9.1 **
iNOS	1.0 ± 0.2	2.4 ± 1.5	12.8 ± 3.8 **	9.4 ± 3.6 **
<i>Plasma</i>				
IL-6	5.9 ± 0.9	6.7 ± 1.7	4.4 ± 1.7	21.4 ± 4.1 **
IFNγ	2.6 ± 0.4	9.0 ± 4.9	1.1 ± 0.4	13.3 ± 1.5 **
CRP	2.4 ± 0.5	1.1 ± 0.3	2.5 ± 1.0	12.6 ± 1.8 **
kyn:try	4.4 ± 0.2	4.4 ± 0.4	4.2 ± 0.5	8.5 ± 0.8 **
<i>Scores</i>				
POMS	12.9 ± 4.3	12.0 ± 14.0	13.2 ± 9.5	8.5 ± 7.3
HAM-A	6.2 ± 1.0	7.4 ± 1.6	8.0 ± 3.9	10.2 ± 3.4
HAM-D	2.8 ± 0.8	4.4 ± 1.4	4.0 ± 1.6	6.8 ± 1.9
IBDQ	52.0 ± 1.4	54.0 ± 3.1	54.0 ± 1.9	51.0 ± 3.8
IBDQs	30.6 ± 1.1	32.0 ± 1.8	30.4 ± 1.9	28.2 ± 3.0

Data represent mRNA fold change for biopsy samples, circulating concentrations of IL-6 (pg/ml), IFN γ (pg/ml), and CRP (mg/L), the circulating total kynurenine:tryptophan ratio, and psychological scores. Data are expressed as mean ± SEM. * p < 0.05, ** p < 0.01 vs patient controls (One-way ANOVA followed by Newman-Keuls *post hoc* test). C-reactive protein (CRP), Crohn's disease (CD), female (F), Hamilton anxiety (HAM-A), Hamilton depression (HAM-D), indoleamine 2,3-dioxygenase (IDO), inducible nitric oxide synthase (iNOS), inflammatory bowel disease (IBD), inflammatory bowel disease questionnaire (IBDQ), interferon (IFN), interleukin (IL), male (M), matrix metalloproteinase (MMP), profile of mood states (POMS), tumour necrosis factor (TNF) ulcerative colitis (UC)

5.4.3 Significant increases in HAM-A and HAM-D scores in patients with higher disease activity indices

Previous studies have used disease activity index (DAI) scores to determine active versus inactive IBD. Here comparison of the UC and CD patients scoring higher, on the Mayo index (≥ 4), and CDAI (≥ 150) respectively, versus patient controls revealed a significant increase in the HAM-A and HAM-D tests as well as in the circulating concentrations of IL-6, CRP and the total kynurenine:tryptophan ratio (see Table 5.4). One-way ANOVA followed by Newman-Keuls *post hoc* analysis: HAM-D [F(2,34) = 4.57, p = 0.018], HAM-A [F(2,34) = 4.29, p = 0.022], circulating IL-6 [F(2,34) = 21.37, p \leq 0.0001], circulating IFN γ [F(2,34) = 4.66, p = 0.0166], circulating CRP [F(2,34) = 13.66, p \leq 0.0001], and circulating kynurenine:tryptophan ratio [F(2,34) = 14.14, p \leq 0.0001]. One-way ANOVA also revealed significant differences in mRNA expression from the intestinal biopsy samples for IDO [F(2,34) = 4.17, p = 0.024], IFN γ [F(2,34) = 4.56, p = 0.018], IL-6 [F(2,34) = 12.47, p \leq 0.0001], IL-1 β [F(2,34) = 12.13, p = 0.0001], TNF α [F(2,34) = 3.83, p = 0.032], MMP9 [F(2,34) = 11.03, p = 0.0002], and iNOS [F(2,34) = 7.17, p = 0.003].

Table 5.4: Increased cytokine expression, HAM-A, and HAM-D scores in IBD patients with a high disease activity index

	Patient Control	low DAI	high DAI
M:F	10:9	6:6	1:5
Age	54.3 ± 2.3	46.2 ± 3.2	54.7 ± 4.3
n	19	12	6
CD:UC		6:6	2:4
<i>Biopsy</i> (Fold change)			
IDO	1.0 ± 0.1	24.0 ± 11.2 *	23.8 ± 10.6
IFNγ	1.0 ± 0.3	7.7 ± 3.7	21.0 ± 12.9 *
IL-6	1.0 ± 0.6	5.3 ± 3.1	116.8 ± 53.5 ***
IL-1β	1.0 ± 0.5	3.0 ± 1.0	72.7 ± 34.3 ***
TNFα	1.0 ± 0.2	1.8 ± 0.5	3.5 ± 1.7 *
MMP9	1.0 ± 0.3	3.6 ± 1.5	21.4 ± 9.5 ***
iNOS	1.0 ± 0.2	6.5 ± 2.1 *	9.9 ± 4.0 **
<i>Plasma</i>			
IL-6	5.9 ± 0.9	5.8 ± 1.2	21.4 ± 4.1 ***
IFNγ	2.6 ± 0.4	7.0 ± 3.1	10.8 ± 2.5 *
CRP	2.4 ± 0.5	2.6 ± 1.0	10.7 ± 2.6 ***
kyn:try	4.4 ± 0.2	4.6 ± 0.3	7.9 ± 1.2 ***
<i>Scores</i>			
POMS	12.9 ± 4.3	11.3 ± 8.9	11.0 ± 6.5
HAM-A	6.2 ± 1.0	6.3 ± 1.2	13.0 ± 3.7 *
HAM-D	2.8 ± 0.8	3.8 ± 0.9	7.8 ± 1.7 *
IBDQ	52.0 ± 1.4	54.8 ± 1.9	49.5 ± 3.5
IBDQs	30.6 ± 1.1	29.3 ± 1.7	31.3 ± 2.0

Data represent mRNA fold change for biopsy samples, circulating concentrations of IL-6 (pg/ml), IFN γ (pg/ml), and CRP (mg/L), the circulating total kynurenine:tryptophan ratio, and psychological scores. A Mayo score ≥ 4 was considered high for UC, and a CDAI ≥ 150 was considered high for CD. Data are expressed as mean \pm SEM. * $p < 0.05$, *** $p < 0.001$ vs patient controls (One-way ANOVA followed by Newman-Keuls *post hoc* test). C-reactive protein (CRP), Crohn's disease (CD), disease activity index (DAI), female (F), Hamilton anxiety (HAM-A), Hamilton depression (HAM-D), indoleamine 2,3-dioxygenase (IDO), inducible nitric oxide synthase (iNOS), inflammatory bowel disease (IBD), inflammatory bowel disease questionnaire (IBDQ), interferon (IFN), interleukin (IL), male (M), matrix metalloproteinase (MMP), profile of mood states (POMS), tumour necrosis factor (TNF), ulcerative colitis (UC)

5.4.4 Increased HAM-D scores in IBD patients experiencing higher levels of symptoms

Depression was significantly associated with increased IBDQs symptom related score (see Table 5.5 and figures 5.1 and 5.2). Two-way ANOVA of HAM-D score revealed a significant interaction between symptom score (IBDQs) and IBD [$F(1,33) = 5.501$, $p = 0.025$]. Two-way ANOVA for HAM-A scores did not reveal an interaction between symptom expression and IBD [$F(1,33) = 3.3$, $p = 0.078$], however, symptoms do influence HAM-A score [$F(1,33) = 8.77$, $p = 0.006$]. Interaction between symptom expression and IBD was also revealed for IL-6 and IL-1 β mRNA expression in the intestinal biopsies, and circulating IL-6 concentrations: [$F(1,33) = 4.79$, $p = 0.036$], [$F(1,33) = 4.92$, $p = 0.034$] and [$F(1,33) = 11.278$, $p = 0.002$] respectively. For the other intestinal and circulating inflammatory markers significance was revealed for IBD alone: IDO mRNA [$F(1,33) = 7.73$, $p = 0.0089$], IFN γ mRNA [$F(1,33) = 5.31$, $p = 0.028$], iNOS mRNA [$F(1,33) = 11.56$, $p = 0.0018$], MMP9 mRNA [$F(1,33) = 7.14$, $p = 0.012$], and TNF α mRNA [$F(1,33) = 4.79$, $p = 0.036$].

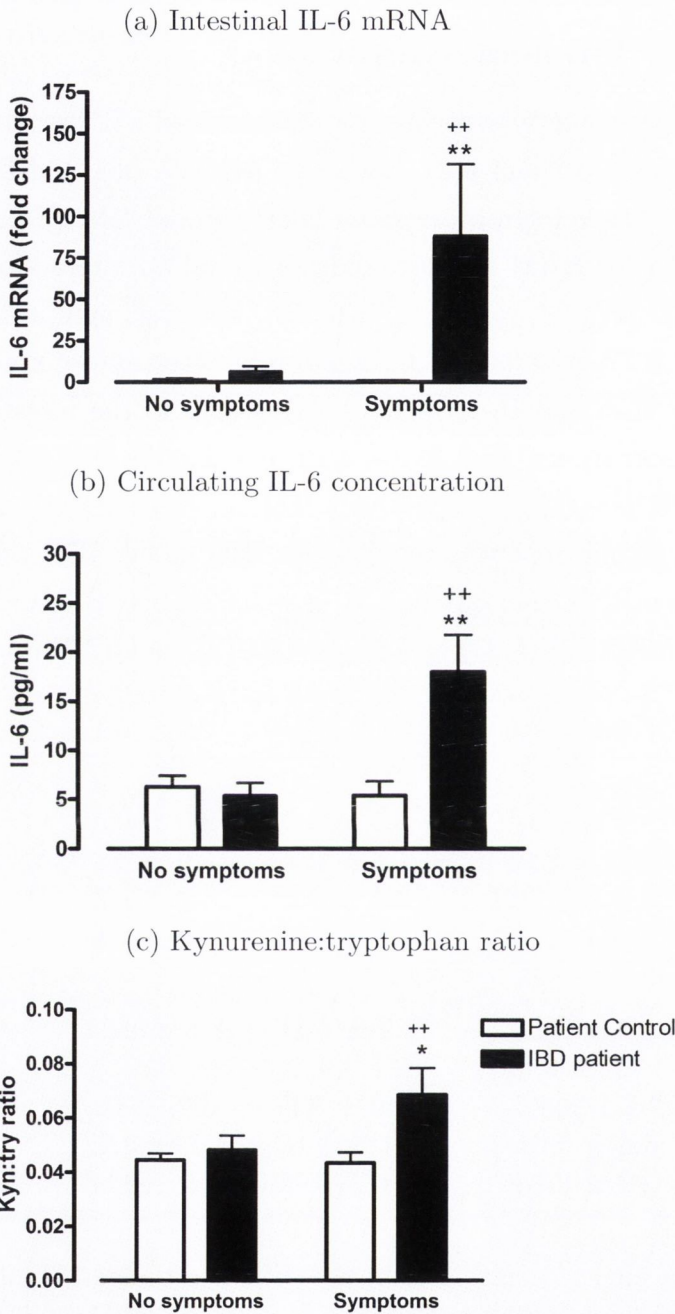


Figure 5.1: Intestinal IL-6 mRNA, circulating IL-6 and circulating kynurenine:tryptophan ratio

Representative graphs of IL-6 biopsy mRNA, circulating IL-6 and circulating kynurenine:tryptophan ratio from Table 5.5. Two-way ANOVA revealed significant differences in intestinal expression of IL-6 mRNA (a) and circulating IL-6 (b) and kynurenine:tryptophan ratio (c) in IBD patients experiencing higher symptoms scores. Data are presented as mean \pm SEM. ** $p < 0.01$ asymptomatic patient controls, ++ $p < 0.01$ versus symptomatic patient controls (Two-way ANOVA followed by Newman-Keuls *post hoc* analysis)

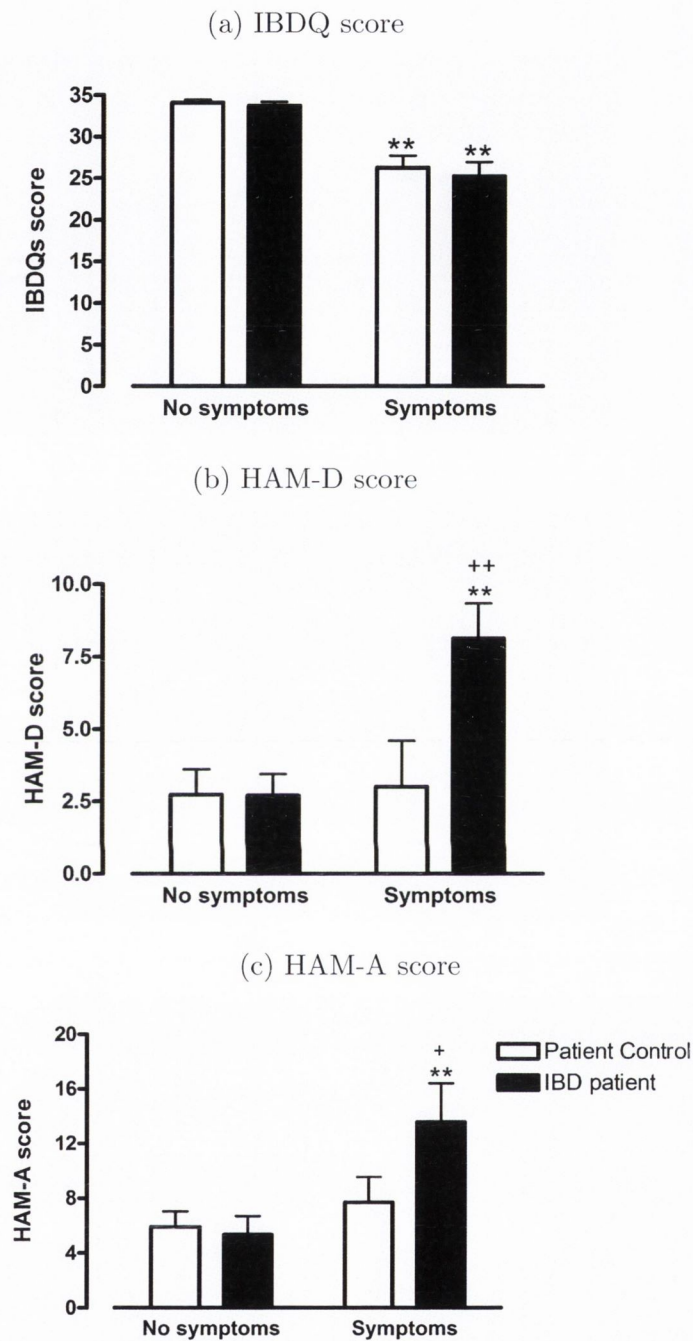


Figure 5.2: IBDQs, HAM-D and HAM-A scores during increased symptom expression

Representative graphs of IBDQs, HAM-D and HAM-A scores from Table 5.5. Two-way ANOVA revealed significant interaction between symptoms and IBD diagnosis for HAM-D scores (b). No differences in IBDQs scores were revealed between symptomatic patient controls and symptomatic IBD patients. Data are presented as mean \pm SEM. ** $p < 0.01$ asymptomatic patient controls, + $p < 0.05$ versus symptomatic patient controls (Two-way ANOVA followed by Newman-Keuls *post hoc* analysis)

Table 5.5: IBD patients experiencing bowel symptoms have increased HAM-A and HAM-D scores

	Patient Controls		IBD patients	
	No symptoms	Symptoms	No symptoms	Symptoms
M:F	7:4	3:5	5:5	2:6
Age	53.9 ± 3.4	54.9 ± 3.0	45.2 ± 4.1	53.8 ± 2.7
n	11	8	10	8
<i>Biopsy</i>				
<i>(Fold change)</i>				
IDO	1.0 ± 0.2	1.4 ± 0.3	32.2 ± 14.9	21.2 ± 9.7
IFNγ	1.0 ± 0.2	1.7 ± 0.8	11.2 ± 5.3	19.8 ± 12.4
IL-6	1.0 ± 0.7	0.4 ± 0.2	4.2 ± 2.6	61.4 ± 30.3 **++
IL-1β	1.0 ± 0.7	0.7 ± 0.3	2.3 ± 0.9	45.6 ± 22.7 **+
TNFα	1.0 ± 0.2	0.9 ± 0.2	1.9 ± 0.5	2.8 ± 1.3
MMP9	1.0 ± 0.3	1.9 ± 0.8	6.1 ± 2.6	24.2 ± 11.4 *+
iNOS	1.0 ± 0.2	1.0 ± 0.2	8.4 ± 2.6 *	6.9 ± 3.1
<i>Plasma</i>				
IL-6	6.3 ± 1.1	5.4 ± 1.5	5.3 ± 1.4	18.0 ± 3.8 **++
IFNγ	2.7 ± 0.6	1.2 ± 0.4	5.5 ± 1.8	11.6 ± 4.4 *
CRP	2.5 ± 0.8	2.3 ± 0.7	3.4 ± 1.4	7.7 ± 2.5 *
kyn:try	4.4 ± 0.3	4.3 ± 0.4	4.8 ± 0.5	6.8 ± 1.0 *+
<i>Scores</i>				
POMS	8.2 ± 4.7	19.4 ± 7.8	10.7 ± 10.5	11.8 ± 5.5
HAM-A	5.4 ± 1.2	7.3 ± 1.7	5.0 ± 1.3	12.9 ± 2.6 **+
HAM-D	2.7 ± 0.9	3.0 ± 1.6	2.7 ± 0.8	8.1 ± 1.2 **++
IBDQ	55.5 ± 0.9	47.1 ± 2.0	57.3 ± 0.7	45.4 ± 2.2 **
IBDQs	34.1 ± 0.3	26.3 ± 1.4	33.7 ± 0.5	25.3 ± 1.7 **

Data represent mRNA fold change for biopsy samples, circulating concentrations of IL-6 (pg/ml), IFN γ (pg/ml), and CRP (mg/L), the circulating total kynurenine:tryptophan ratio, and psychological scores. Data are expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$ vs non symptomatic patient controls, + $p < 0.05$, ++ $p < 0.01$ vs symptomatic patient controls (Two-way ANOVA followed by Newman-Keuls *post hoc* test). C-reactive protein (CRP), Crohn's disease (CD), female (F), Hamilton anxiety (HAM-A), Hamilton depression (HAM-D), indoleamine 2,3-dioxygenase (IDO), inducible nitric oxide synthase (iNOS), inflammatory bowel disease (IBD), inflammatory bowel disease questionnaire (IBDQ), interferon (IFN), interleukin (IL), male (M), matrix metalloproteinase (MMP), profile of mood states (POMS), tumour necrosis factor (TNF) ulcerative colitis (UC)

5.4.5 No differences in PAXgene mRNA expression of IDO, IFN γ , iNOS, or MMP9

PCR analysis of whole blood mRNA expression of IDO, IFN γ , iNOS, and MMP9 revealed no significant differences between patient controls and IBD patients, UC and CD patients, or based on DAI scores or the IBDQs index. This suggests that the differences in circulating IFN γ and kynurenine:tryptophan ratios are due to intestinal expression of these markers rather than synthesis of these mediators within the circulation (see Table 5.6). Further analysis of MMP9 activity within the circulation confirms the lack of differences in patients versus patient controls (see Table 5.7).

Table 5.6: No differences in whole blood mRNA expression of MMP9, iNOS, IDO or IFN γ compared to patient controls

	MMP9	iNOS	IDO	IFN γ
P. Ctrl	1.0 \pm 0.1	1.0 \pm 0.1	1.0 \pm 0.1	1.0 \pm 0.2
IBD	1.4 \pm 0.4	1.0 \pm 0.2	1.5 \pm 0.3	1.1 \pm 0.3
UC	0.9 \pm 0.1	1.1 \pm 0.3	1.7 \pm 0.5	1.0 \pm 0.3
CD	2.2 \pm 0.8	0.8 \pm 0.1	1.5 \pm 0.3	1.3 \pm 0.5
Inactive	0.9 \pm 0.2	1.2 \pm 0.3	1.4 \pm 0.6	1.1 \pm 0.4
Mild	1.7 \pm 0.6	1.0 \pm 0.2	1.2 \pm 0.4	1.7 \pm 0.8
M/S	1.9 \pm 1.0	0.7 \pm 0.1	1.6 \pm 0.5	0.7 \pm 0.3
low DAI	1.1 \pm 0.3	1.1 \pm 0.2	1.5 \pm 0.4	1.5 \pm 0.3
high DAI	2.1 \pm 1.0	0.8 \pm 0.2	1.4 \pm 0.5	0.4 \pm 0.1
P. Ctrl asym	1.1 \pm 0.2	1.0 \pm 0.1	1.1 \pm 0.2	1.3 \pm 0.3
P. Ctrl sym	0.9 \pm 0.2	1.0 \pm 0.01	0.8 \pm 0.1	0.5 \pm 0.1
IBD asym	0.8 \pm 0.2	1.0 \pm 0.3	1.5 \pm 0.4	1.3 \pm 0.3
IBD sym	2.0 \pm 0.7	0.9 \pm 0.1	1.4 \pm 0.4	0.8 \pm 0.4

Data represent mRNA fold change for whole blood samples. Data are expressed as mean \pm SEM. No significant differences were revealed (One-way ANOVA followed by Newman-Keuls *post hoc* test). Asymptomatic (asym), Crohn's disease (CD), disease activity index (DAI), indoleamine 2,3-dioxygenase (IDO), inducible nitric oxide synthase (iNOS), interferon (IFN), interleukin (IL), matrix metalloproteinase (MMP), moderate/severe (M/S), patient control (P. Ctrl), symptomatic (sym), ulcerative colitis (UC)

Table 5.7: No differences in circulating MMP2 and MMP9 enzyme activity versus patient controls

Patient group	pro MMP9/lipocalin complex	pro MMP9	pro MMP2
P.ctrl	12.0 ± 1.0	105.1 ± 10.9	349.9 ± 30.8
IBD	13.2 ± 1.7	128.1 ± 10.3	355.0 ± 31.5
UC	15.4 ± 2.7	133.3 ± 17.1	372.0 ± 39.9
CD	10.5 ± 1.3	121.6 ± 10.0	333.0 ± 52.3
Inactive	14.3 ± 3.7	148.6 ± 17.6	404.2 ± 62.2
Mild	10.4 ± 1.6	93.0 ± 11.7	359.5 ± 47.2
M/S	14.3 ± 2.4	133.3 ± 15.8	294.1 ± 43.8
low DAI	13.1 ± 2.2	130.5 ± 12.9	373.8 ± 41.2
high DAI	13.5 ± 2.7	123.2 ± 18.5	317.5 ± 47.3
P. Ctrl asym	12.6 ± 1.3	120.1 ± 16.1	389.0 ± 42.6
P. Ctrl sym	11.2 ± 1.5	84.5 ± 10.6	286.0 ± 35.3
IBD asym	14.0 ± 2.7	135.7 ± 17.0	364.8 ± 48.8
IBD sym	12.2 ± 1.9	118.5 ± 9.8	342.9 ± 39.4

Data represent MMP9 and MMP2 enzyme activity in the circulation. Data are expressed as mean intensity (arbitrary units) ± SEM. No significant differences were revealed (One-way ANOVA followed by Newman-Keuls *post hoc* test). Asymptomatic (asym), Crohn's disease (CD), disease activity index (DAI), inflammatory bowel disease (IBD), matrix metalloproteinase (MMP), moderate/severe (M/S), patient control (P. Ctrl), symptomatic (sym), ulcerative colitis (UC)

5.4.6 Pain is associated with increased psychological disturbances

Pain is a common symptom of IBD. The possibility that pain can influence the normal psychological functioning is therefore relevant to treatment options. In this patient cohort pain was significantly associated with increased HAM-A [$t = 2.64$; $df\ 35$; $p = .012$], HAM-D [$t = 2.73$; $df\ 35$; $p = .01$] and POMS [$t = 2.1$; $df\ 35$; $p = .043$] scores, and with decreased IBDQ [$t = 2.08$; $df\ 35$; $p = .045$] index, see Table 5.8, and figure 5.3.

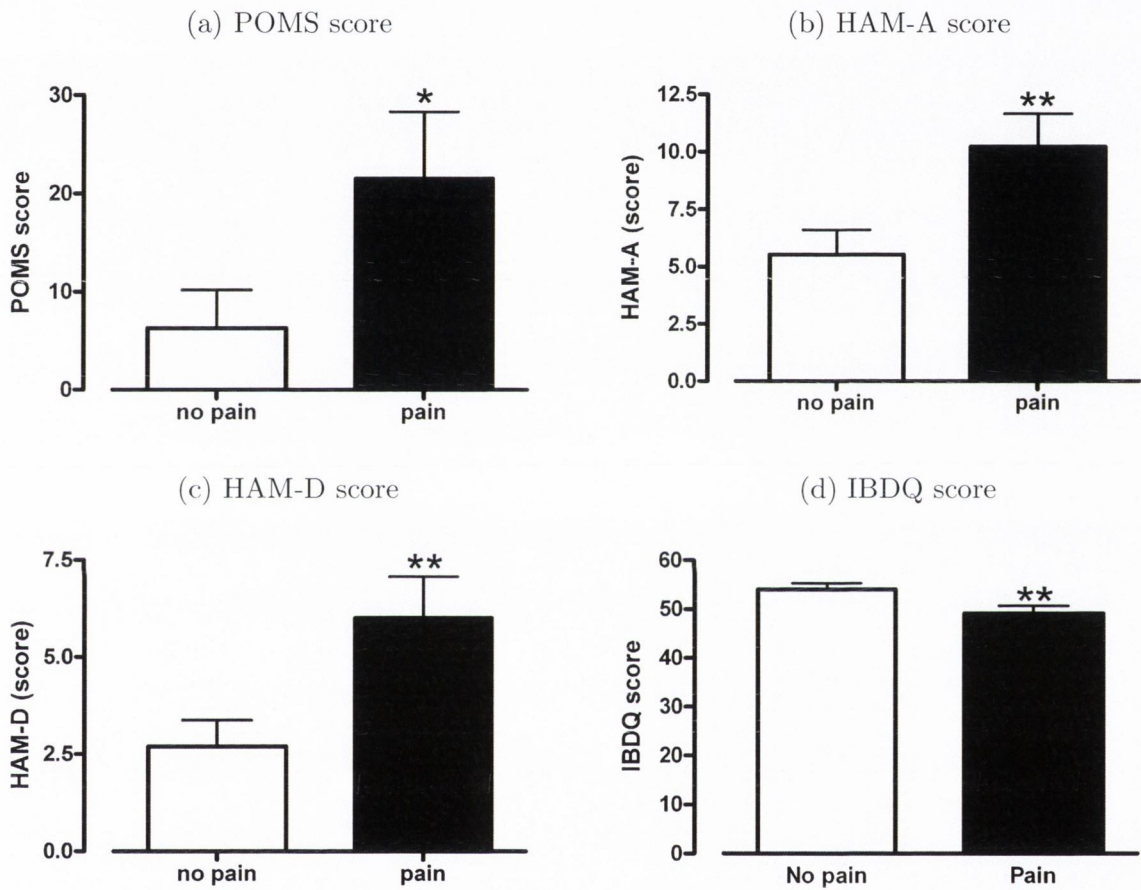


Figure 5.3: POMS, HAM-D, HAM-A and IBDQ scores for perceived pain versus non pain patients

Student's *t* test analysis revealed significant increases in scores for POMS (a), HAM-A (b), HAM-D (c) and a significant decrease in IBDQ (d) in patients suffering from pain versus patients with no pain. Data are presented as mean \pm SEM ($n_1 = 23$, $n_2 = 14$) * $p < 0.05$, ** $p < 0.01$ versus no pain (Pooled variance Student's *t* test)

Table 5.8: Increased POMS, HAM-D, HAM-A and decreased IBDQ scores for perceived pain versus non pain patients

	No pain	pain
M:F	11:12	6:8
Age	49.6 ± 2.4	55.2 ± 2.5
n	23	14
(P.ctrl:IBD)	(11:12)	(8:6)
<i>Biopsy</i>		
<i>(Fold change)</i>		
IDO	15.4 ± 6.3	6.9 ± 4.7
IFNγ	6.3 ± 2.3	6.7 ± 5.8
IL-6	16.6 ± 7.1	38.6 ± 25.8
IL-1β	7.1 ± 5.0	23.6 ± 15.9
TNFα	1.6 ± 0.3	1.7 ± 0.8
MMP9	4.7 ± 2.2	5.9 ± 3.7
iNOS	4.7 ± 1.4	3.4 ± 1.7
<i>Plasma</i>		
IL-6	7.4 ± 1.5	9.9 ± 2.4
IFNγ	4.4 ± 1.0	6.1 ± 2.3
CRP	3.6 ± 0.9	4.2 ± 1.4
kyn:try	5.1 ± 0.4	4.9 ± 0.6
<i>Scores</i>		
POMS	6.3 ± 3.9	21.5 ± 6.8 *
HAM-A	5.5 ± 1.1	10.2 ± 1.4 *
HAM-D	2.7 ± 0.7	6.0 ± 1.1 **
IBDQ	54.5 ± 1.3	49.1 ± 1.5 *
IBDQs	32.0 ± 0.8	27.9 ± 1.5 ***

Data represent mRNA fold change for biopsy samples, circulating concentrations of IL-6 (pg/ml), IFN γ (pg/ml), and CRP (mg/L), the circulating total kynurenine:tryptophan ratio, and psychological scores. Data are expressed as mean ± SEM. * p < 0.05, *** p < 0.001 compared to no pain (Pooled variance Student's *t* test). C-reactive protein (CRP), Crohn's disease (CD), female (F), Hamilton anxiety (HAM-A), Hamilton depression (HAM-D), indoleamine 2,3-dioxygenase (IDO), inducible nitric oxide synthase (iNOS), inflammatory bowel disease (IBD), inflammatory bowel disease questionnaire (IBDQ), interferon (IFN), interleukin (IL), male (M), matrix metalloproteinase (MMP), profile of mood states (POMS), tumour necrosis factor (TNF), ulcerative colitis (UC)

5.5 Discussion

In the present study the psychological scores and protein concentration or mRNA expression of inflammatory markers in IBD patients versus patient controls were compared. To our knowledge this study is the first to begin to investigate whether symptoms of IBD or inflammatory mediators could be responsible for the increased risk of depression and or anxiety in IBD patients. The major finding of this investigation is that the symptomatic phase of IBD represents the disease phase with the highest depression and anxiety scores, and this phase of IBD is also highly associated with increased intestinal expression of IL-6, IL-1 β , and MMP9, as well as increased circulating concentrations of IL-6. Of particular note is that the patient controls experiencing the same level of symptom expression, as per the modified IBDQ, do not experience the same psychological disturbance suggesting that the acute psychological impact of the symptoms themselves, are not responsible for the increased HAM-D score in IBD patients.

5.5.1 Increased intestinal and circulating inflammatory mediators in IBD patients

In terms of immune activation in the gut, increases in the inflammatory markers were revealed in the colon. PCR analysis of the intestinal biopsies of the IBD patient group revealed increases in IFN γ , IDO, IL-6, IL-1 β , TNF α , MMP9, and iNOS versus the patient controls. These markers have previously been shown to be involved in both UC and CD (McClane and Rombeau, 1999; Moriconi et al., 2007; Meijer et al., 2007). There is also an increase in circulating IL-6, IFN γ , and kynurenine:tryptophan ratio. The kynurenine:tryptophan ratio correlated with the intestinal IDO and IFN γ mRNA expression, and circulating IFN γ concentrations.

5.5.2 Psychological scores were not different in IBD patients versus patient controls

A substantial amount of recent research has confirmed that there is an increased risk of depression and anxiety in IBD patients versus healthy controls (see previous review of the recent literature chapter. 1). Here we see no differences between IBD patients and patient controls in terms of the depression (HAM-D) or anxiety scores (HAM-A), or the well-being scores (POMS, IBDQ), this is not surprising however, as the control population are patients being screened due to gastrointestinal issues, rather than healthy controls. The

lack of differences in the IBDQ scores between both groups indicates that the IBD patients and patient controls are suffering from a similar degree of gastrointestinal symptoms at the time of testing. Previous research investigating depressive and anxious symptoms in IBD patients versus patients suffering from other gastrointestinal complaints shows variability in the risk for IBD above the rest, with IBS patients being at a higher risk of the affective symptoms in most cases (Simrén et al., 2002; Crane and Martin, 2004; Tkalcic et al., 2010). While in IBD versus gastro-esophageal reflux disease (GERD) an increased risk for IBD patients in developing affective symptoms has been reported (Brandi et al., 2009), there is also a similar number of patients reporting pain in both the IBD and patient control groups, and pain was found to influence HAM-A, HAM-D and POMS scores.

5.5.3 No psychological differences between Crohn's disease or Ulcerative colitis

No significant differences were found between the UC patients and CD patients in terms of the inflammatory markers in the gut or the circulation. Division of the groups based on disease type did not reveal any differences in terms of increased risk for anxiety or depression in HAM-A and HAM-D respectively. This is in agreement with previous research, which also found no difference between risk of depression in UC and CD (Ben Thabet et al., 2012; Häuser et al., 2011; Vidal et al., 2008; Guthrie et al., 2002). The same was also reported in paediatric IBD using the CDI depression score (Szigethy et al., 2004).

5.5.4 Psychological disturbances are associated with higher symptom expression in IBD patients

One of the consistently reported factors influencing the depressive or anxious phenotype in IBD patients is the level of disease activity (see 1.5.4). A number of investigators have reported positive correlations between psychological scores and measures of disease activity such as the CDAI (Brandi et al., 2009; Mardini et al., 2004). Fernández et al. (2010) also report that the BDI depression scores are associated with increased extraintestinal symptoms, suggesting that the risks are higher when the inflammation is not maintained in the gut but becomes systemic.

In this study we examined the risk for depression, anxiety and the inflammatory markers based on different measures of activity. The first is based on the clinician's assessment of severity, following the colonoscopy, on inflammatory activity within the intestine which range from inactive, slight activity, moderate activity and severe activity. Patients were

then separated into non-IBD patient controls, inactive, mild, and moderate to severe inflammation. The PCR analysis of the biopsy markers confirms the clinical diagnosis of intestinal inflammatory state, whereby differences exist between the moderate/severe IBD group and the patient controls for $\text{IFN}\gamma$, IDO, IL-6, IL-1 β , TNF α , and MMP9, whereas no differences in the mRNA expression of any of the markers was found between inactive IBD and patient controls following *post hoc* analysis. Separation of the patients based on colonoscopy examination and clinician's assessment also revealed significant differences between the active IBD patients and patient controls as well as the inactive cohort in terms of circulating IL-6, CRP and the kynurenine:tryptophan ratio. This is consistent with previously reported increases in CRP, IL-6 and kynurenine:tryptophan ratio in active IBD (Zilberman et al., 2006; Brown et al., 2002; Gupta et al., 2012). No significant differences in the psychological scores for depression, anxiety or well-being were revealed in inflamed versus non-inflamed groups.

The second method of dividing patients in terms of disease activity level was based on the disease activity index (DAI) scores: CDAI for CD and Mayo score for UC, as per previous studies. A score of ≥ 150 for the CDAI was considered active, and a score of ≥ 4 for the Mayo index. Unlike the colonoscopy evaluation of activity, separating the patients using the DAI scores was associated with the psychological scores. This supports previous investigations into psychological disturbances in IBD patients, which have also used these DAI scores. What is also clear from the Mayo and CDAI scores is that these patients are largely in an inactive disease phase, as only 6 patients scored above the threshold set. Separating the groups based on DAI also reveals differences in circulating IL-6 concentrations, CRP concentrations and the kynrenine:tryptophan ratio in the high DAI scorers versus the patient controls and low DAI scores, indicating associations between the psychological scores and circulating markers of inflammation.

The differences between active and inactive disease based on colonoscopy evaluation only, versus DAI, may be due to the inclusion of questions based on clinical symptoms as well as physician's evaluation of disease state. Although the increase in psychological disturbances in the higher scoring DAI patients confirms the findings of previous reports it was not possible to take into account the symptom expression in the patient control group using the CDAI and Mayo index as they are disease specific. The IBDQ questionnaire is less specific than the Mayo or CDAI scores, therefore, it could be administered to the patient control group as well as the IBD patients. A slightly modified version of the IBDQ, which only includes the questions related to bowel symptoms (IBDQs) revealed a lack of association between higher symptom scores and psychological disturbances in the patient control group, whereas in the IBD patients experiencing symptoms there is an

increase in HAM-D scores. This suggests that although the acute psychological impact of these symptoms is not causing the depressive symptoms, the phase of IBD in which bowel symptoms are most prevalent represents the phase of disease in which patients are also more likely to experience mild psychological disturbances.

Association between pain perception and psychological well-being

Pain is an important manifestation of inflammation and IBD (Schirbel et al., 2010; Bielefeldt et al., 2009). Schirbel et al. (2010) report pain in up to 87.9 % of patients, they also found that pain was correlated with HRQOL. In CD patients pain was independent of CDAI scores, however, it was correlated with CAI in UC patients in this study. Bielefeldt et al. (2009) also describes high levels of pain in IBD patients, but more importantly suggests that up to 20% of IBD patients in remission will continue to experience symptoms of pain, based on clinical and endoscopic evaluation. They also suggest that visceral pain is associated with affective dimensions. In the current study, separation of patients based on perceived pain versus no current pain, as per question 5 of the EQ-5D, and irrespective of IBD diagnosis, reveals a decrease in psychological well-being within the pain group. Patients suffering from current perceived pain had increased POMS score, HAM-A and HAM-D and a decreased IBDQ score compared to the non-pain group. No differences between inflammatory markers or tryptophan concentrations was found in perceived pain versus non-pain.

Association between IL-6 and psychological well-being

IL-6 is an important cytokine in the psychoneuroimmunology literature linking inflammation to CNS disturbances. Both depression and clinical anxiety have been associated with IL-6 concentrations (Meyer et al., 2011; O'Donovan et al., 2010; Hiles et al., 2012). Previous research has revealed positive correlations between IL-6 and HAM-D depression scores in patients suffering from major depression (Hughes et al., 2012). In their study, major depressed patients also had decreased tryptophan compared to healthy controls. In animal studies which allow for greater manipulations to experimentally determine the neurobiological function of IL-6 it has been shown to induce a number of behavioural effects. It is reported to be involved in 'sickness behaviour' in rodents, is upregulated in the CNS in response to psychological stressors, and affects hypothalamic-pituitary-adrenal (HPA) axis responses (Harden et al., 2006, 2008; You et al., 2011; Chesnokova and Melmed, 2002).

5.5.5 Limitations

Although this is a low patient number exploratory study, it does provide valuable evidence for increased HAM-A and HAM-D scores during the phase of disease involving increased inflammatory cytokines and symptoms, and that for HAM-D scores, this is independent of the acute psychological impact of these symptoms. One limitation of the study, however, is that the IBDQ questionnaire asks respondents to focus on the previous two weeks only. This eliminates the ability to examine whether the increased depression scores in the symptomatic IBD patients arise from years of repeated flares, and therefore, represents a response to chronic symptom expression rather than the acute impact of the current flare. Future studies should attempt to match IBD patients with patients suffering from GI symptoms in terms of years since diagnosis, regularity of symptoms, and current symptom expression.

5.5.6 Conclusion

There is evidence to suggest an association between the symptomatic phase of IBD and psychological disturbances. Although it is not yet clear whether this is due to inflammatory mediators or the chronic impact of years of repeated symptoms, it again highlights the need for gastroenterologists to be aware of the psychological risks associated with IBD.

Chapter 6

Gut-Brain interactions and influence of DSS-induced colitis on behaviour and central inflammatory biomarkers

6.1 Introduction

Inflammatory bowel disease (IBD) is a chronic relapsing and remitting disorder of the GI tract, which affects as many as 1.4 million persons in the United States and 2.2 million persons in Europe (Loftus, 2004). Common symptoms of IBD include diarrhea with blood and mucus, constipation, vomiting, abdominal pain, fever and weight loss. IBD can be divided into two main disorders: ulcerative colitis (UC) and Crohn's disease (CD). Inflammation in UC usually remains in the rectum and colon, while CD can affect any part of the GI tract. Historically there has been significant interest in the co-morbidity of psychological well-being and disorders of the gastrointestinal tract (Murray, 1930; Sullivan, 1935; Daniels, 1942; Straker, 1960). Recent research has confirmed that there is an increased risk of depression/anxiety in patients suffering from IBD, and these psychological symptoms are most often present during the active disease phase (see Chapter 1 and Ben Thabet et al. (2012); Häuser et al. (2011); Mardini et al. (2004); Calvet et al. (2006)). Despite this finding and the recent surge in psychoneuroimmunology research there is a lack of investigation into the potential association between inflammatory mediators and psychological disturbances during the active phase.

Experimental human research directed to study the neurobiological effects of inflammation are limited to studies on post-mortem brains, neuroimaging studies and functional drug challenges. However these studies can be difficult to compare due to differences in

duration of inflammation, severity of inflammation, type of drug treatment, age of patients, and life stress. Animal studies are designed in a way that they are interrelated with clinical observations. They can provide new insights into the biological processes involved in psychological effects of peripheral inflammation that can not be investigated in humans for ethical reasons. Evidence of disturbances to CNS functioning following induction of intestinal inflammation in models of IBD has been reported. Reported disturbances to date can be classified into five subgroups: BBB permeability (Natah et al., 2005; Hathaway et al., 1999, 2000; Sans et al., 2001), c-Fos expression (Sinniger et al., 2005; Cattaruzza et al., 2010; Porcher et al., 2004; Welch et al., 2005, 2010; Miampamba and Sharkey, 1999), HPA axis activation (Greenwood-Van Meerveld et al., 2006; Kresse et al., 2001; Kojima et al., 2002; Porcher et al., 2004), and Inflammatory marker activation (Riazi et al., 2008; Medhi et al., 2009; Wang et al., 2010; Baticic et al., 2011; Alhouayek et al., 2011; Villaran et al., 2010).

Neuroinflammation in the TNBS-induced model of colitis has received a certain degree of investigation (Riazi et al., 2008; Medhi et al., 2009; Wang et al., 2010; Baticic et al., 2011; Alhouayek et al., 2011). These studies report increased $\text{TNF}\alpha$, IL-6 and IL-1 β in the brains of colitic animals. However, the TNBS model involves fasting, anaesthesia, and partial restraint prior to injections, all of which can impact on the CNS. The DSS induction process is less distressing as it involves DSS administration in the animals drinking water, with no need for anaesthesia or fasting, thus minimising confounding factors and making it a preferable model for studying behaviour and molecular changes within the CNS. To date only one study has analysed cytokine expression in the brains of rats exposed to DSS (Villaran et al., 2010). Villaran et al. (2010) report an increased mRNA expression of $\text{TNF}\alpha$, IL-6, IL-1 β and iNOS in the substantia nigra, following 4 days of 5% DSS administration. No other brain regions were investigated and cytokine induction before or after this timepoint was not analysed.

6.1.1 Aims and objectives

Analysis of IBD patients revealed that increased DAI is associated with increased intestinal and systemic inflammation, as well as increased HAM-A and HAM-D scores. With the clinical analysis and the general introduction in mind the aims of this study were to determine whether behavioural changes occur in the DSS model of colitis and whether colonic inflammation induces molecular disturbances within the CNS. The main objectives were to:

1. characterise the DSS-induced colitis model in terms of DAI, histology, MMP ex-

pression and activity, behaviour, and cortical expression of inflammatory cytokines, iNOS, and GFAP.

2. determine if changes in tryptophan or its metabolites occur in the circulation and CNS
3. compare results from TNBS-induced colitis model to DSS-induced colitis.

6.2 Methods

6.3 Animal husbandry

Male Wistar rats (175-210 grams) were obtained from the Bioresources Unit in Trinity College Dublin, and housed in hard-bottomed polypropylene cages with wood shavings as bedding. Animals were housed 4 per cage on arrival, under standard laboratory conditions, with an ambient temperature of 20-24° Celsius (C) and a 12 h light: 12 h dark cycle (lights on 08.00, lights off: 20.00). Animals had free access to food and water and were fed a standard laboratory diet (Red Mills, Ireland). A record of body weight was maintained as a general indication of the health and well-being of each animal throughout each study. All in-vivo work was approved by the Animal Ethics Committee Trinity College Dublin. Prior to beginning each experiment animals were weight matched across groups. Food intake, fluid intake and weight were recorded daily in each experiment. A number of DSS-induced colitis (5% DSS in the drinking water for 7 days, vehicle consisted of normal drinking water) studies and two TNBS-induced (1 ml TNBS enema, vehicle consisted of 1 ml 30% ethanol solution) colitis experiments were carried out:

1. In the first experiment 16 Wistar rats were randomly assigned to either control or DSS administration groups. Rats were housed 2 per cage for the duration of the investigation. DSS was administered as described previously (see chapter 4.2.1). Following 7 days DSS exposure all rats were euthanised between 14.00 and 17.00 hr.
2. A second study was carried out for behavioural characterisation. In this study 32 rats were randomly divided into two groups, DSS and control (2 per cage), of 16 rats each: Each rat was exposed to variety of behavioural tests on day 6, day 11, day 12, day 13, and day 14 of the experiment (as per figure 6.5).
3. A further 12 rats were again exposed to either water or DSS (2 per cage). In this study however, following DSS administration rats were returned to water for 7 days in order to examine induction of inflammatory mediators following a recovery period. Following 7 days recovery rats were euthanised between 14.00 and 17.00 hr.
4. A final DSS study with 8 rats per group included a control group, acute DSS group, and 2 day recovery group (4 per cage). All rats were euthanised between 10.00 and 12.00 hr to allow for immediate preparation of central HPLC samples for tryptophan and monoamine analysis.

5. Two TNBS experiments were carried out: a timecourse study and a dose-response study (as per section 4.2.2). All rats were singly housed for the duration of the study and were euthanised between 14.00 and 17.00 hr.

6.3.1 Disease Activity Index

A Disease Activity Index (DAI) was constructed to examine DSS-induced colitis severity using the criteria of weight loss, stool consistency and rectal bleeding. Weight loss was scored on a scale of 0-4; 0 for no weight loss, 1 for 1-5%, 2 for 6-10%, 3 for 11-15% and 4 for >20%. Stool consistency was scored 0-4; 0 for normal stools, 1-2 for loose stools and 3-4 for diarrhoea. Rectal bleeding was scored 0-4; 0 for none, 1 for light bleeding, 2 for moderate bleeding, 3 for heavy bleeding and 4 for very heavy bleeding. Scores for each of the 3 criteria were averaged to give the Disease Activity Index score (Cooper et al., 1993).

6.3.2 Behavioural tests

Saccharin preference testing: This was carried out over the full course of the TNBS time-course experiment. Two water bottles, one containing tap water, the other containing a 0.01% Saccharin solution were placed with each cage. Fluid intake from each bottle was monitored every 24 h and daily percentage saccharin preference was calculated. Decreased saccharin preference was used as an indicator of anhedonia.

Open field testing: The open field consisted of a cylindrical plastic arena (1 m diameter). The base of the open field was black, and the walls were mirrored. A video camera was mounted on the ceiling directly above the centre of the open field. Room lights were dimmed during the testing phase. Rats were placed in the open field for 10 min each. Automated video recording of the rats movement between zones, speed, path length, time still and thigmotaxis were recorded using the HVS image software (HVS image Ltd.) Time spent grooming was also observed and recorded. Rats were considered to be grooming when they start by licking the paws, then rubs them over the head, this is often followed by licking and rubbing the side of the body, the anogenital region, and the tail. In order to eliminate the effect of learning if rats were re-exposed to the open field test slight adaptations were made. The open field itself was identical to previously described however a small black rectangle (6 cm x 6 cm x 12 cm) was placed in the centre of quadrant SW and room lights were left on. Rats were left to explore for 10 min and recorded using the HVS image software. An observer also monitored the number of approaches the rat made to the object.

Elevated Plus maze: The Elevated plus maze (EPM) consisted of a grey plus maze elevated 50 cm off the ground. 50cm high black plexiglass surrounded two of the opposing open arms and the other two remained open. Each arm measured 50 cm x 10 cm (length by width respectively). The central junction measured 10 cm x 10 cm. Regular light-phase room lighting was maintained during the testing phase. Rats were placed in the centre of the EPM facing an open arm and tested for 5 min each. Automated video recording of the rats entries between arms, path length within open and closed arms, and time spent in each arm was recorded using the HVS image software (HVS image Ltd.)

Marble burying: Small hard-bottomed polypropylene cages (45 cm x 27 cm x 15 cm, length x width x height respectively) were filled 4.5-5 cm deep with regular wood shaving bedding. Two rows of marbles (2.3 cm diameter) were lined approximately 2 cm apart along the same short wall of the cage. Room lighting was dimmed and rats were placed in the cage and left alone for 10 min, following this rats were removed and marbles were counted. Marbles covered at least two thirds with bedding were considered buried.

Light Dark Box: The light chamber consisted of a white walled, clear glass top with a bright light in the centre, measuring 30 x 30 cm, the dark chamber had black walls and a solid black top (30 x 30 cm). The two chambers were connected via a rectangular passage (10 cm). Rats were placed in the light chamber and observed for 10 min. Number of entries to each arm, time spent in each arm (s), and fecal boli count was recorded. Head poking was also recorded as an indication of the rats investigatory tendencies (Marino et al., 2005).

6.4 Histology

Distal colon samples (1-2 cm) were dissected and placed in Camoys Solution (60% alcohol; 30% chloroform; 10% glacial acetic acid) for 2-3 h, then transferred to 100% ethanol until embedding. After embedding (as per Section 4.3), samples were sectioned with a microtome at a thickness of 10 μm (Leica Microtome). Microtomed slices were stained using hematoxylin and eosin stain. Slides were mounted with cover slips using Di-N-Butyl Phthalate in Xylene (DPX) mounting medium. Slides were digitally recorded and analysed using an Olympus BX51 video-camera microscope for histological scoring. Histological scoring was based on modified version of the protocol outlined by Cooper et al. (1993).

6.4.1 Processing of blood and tissue samples for tryptophan, kynurenine and KYNA HPLC

Following euthanasia blood and tissue samples were collected. Serum was obtained after centrifugation for 10 minutes at 2,000 x rpm. The resulting supernatant was immediately transferred into a clean polypropylene tube and the samples were stored at -80°C until required for analysis. Tissue samples were snap frozen on dry ice and stored at -80°C until analysis. Serum samples were defrosted on ice and added to an equal volume of 50nM glacial acetic acid, 100 mM zinc acetate, 3% acetonitrile dissolved in double-distilled HPLC grade H_2O , pH 4.9 with diluted NaOH, while tissue samples were weighed and added to 500 μl of the same buffer. This buffer was also used as the mobile phase used in the HPLC assay. In order to extract the analytes of interest de-proteinisation was carried out by the addition of perchloric acid (final concentration 6% and 200 ng/20 μl of internal standard (N-methyl-serotonin) was added as a quality control measure for subsequent HPLC analysis. Serum samples were vigorously vortexed for approximately 10 s, and then centrifuged at 4°C and 14,000 rpm for 20 min, while tissue samples were sonicated for approximately 10 sec before centrifugation. The supernatants were subsequently filtered into eppendorf tubes by 0.45 μm filter tipped syringe (Phenomenex).

6.4.2 HPLC for determination of tryptophan and kynurenine

Tryptophan, kynurenine, and KYNA were analysed using the Shimadzu ADVP HPLC system coupled to PDA-UV (Shimadzu SPD-M10A VP set to integrate at 230 and 250 nm) and fluorescence (Shimadzu RF-20A XS prominence set at excitation 254 nm and emission 404 nm) detectors. A reverse phase analytical column (Kinetex Core Shell Technology, Phenomenex UK) with specific area of 100 mm x 4.6 mm and particle size of 2.6 μm and fitted with a guard column was used to separate the analytes in the sample. Standards (100 ng/20 μl) of tryptophan, kynurenine and KYNA were prepared in mobile phase and standards and samples were injected in a volume of 20 μl with a continuous flow rate of 0.8 ml/min and acquisition time of 20 min. The signal to noise ratio was 3:1. Chromatograms were generated using Class VP software and peak heights and retention times of tryptophan, kynurenine, KYNA, and internal standard (N-methyl-5-HT) were used to calculate the concentrations in ng/ml plasma.

6.4.3 Processing of tissue samples for 5ht and 5-HIAA HPLC

Following euthanasia tissue samples were collected. Tissue samples were snap frozen on dry ice and stored at -80°C until analysis. Samples were defrosted on ice and added 500 μl of homogenisation buffer (0.1 M citric acid monohydrate (Sigma), 0.1 M sodium dihydrogen phosphate monohydrate (Merck), 1.4 mM octane-1 sulphonic acid (BDH) and 0.1 M EDTA disodium salt dihydrate (Sigma) dissolved in double-distilled NANOpure HPLC grade water (Sigma). The pH was adjusted using a pH meter (Jenway) to 2.8 by the addition of 5 M NaOH). This buffer was also used as the mobile phase used in the HPLC assay. A 5 ng/20 μl of internal standard (N-methyl-serotonin) was added to the samples as a quality control measure for subsequent HPLC analysis. Tissue samples were then sonicated for approximately 10 sec before centrifugation. The supernatants were subsequently filtered into eppendorf tubes by 0.45 μm filter tipped syringe (Phenomenex).

6.4.4 HPLC for determination of 5ht and 5-HIAA

5ht and 5-HIAA were analysed using the Shimadzu ADVP HPLC system coupled to an electrochemical detector (Digital Electrochemical Amperometric Detector, Antech ECD). A reverse phase analytical column (Kinetex Core Shell Technology, Phenomenex UK) with specific area of 100 mm x 4.6 mm and particle size of 2.6 μm and fitted with a guard column was used to separate the analytes in the sample. Standards (5 ng/20 μl) of 5ht and 5-HIAA were prepared in mobile phase and standards and samples were injected in a volume of 10 μl with a continuous flow rate of 0.8 ml/min and acquisition time of 60 min. The signal to noise ratio was 3:1. Chromatograms were generated using Class VP software and peak heights and retention times of 5ht, 5-HIAA and internal standard (N-methyl-5-HT) were used to calculate the concentrations in ng/ml plasma.

6.4.5 Zymography

Tissue MMP2 and MMP9 protein activity was assessed by zymography as described previously in section 4.6. Briefly plasma samples were added to 2ml round-bottomed tubes containing lysis buffer (150mM NaCl, 50mM tris-HCl pH 8.0, 1% v/v NP-40, 50 μl /10 ml Phosphatase Inhibitor Cocktail I (Sigma-Aldrich)). Samples were then vortexed vigorously, homogenised with a hand held polytron, and centrifuged at 14,000 rpm at 4°C for 15 min. Supernatants were transferred to clean Eppendorf tubes and stored at -80°C . Prior to analysis samples were equalised to 2000 $\mu\text{g}/\text{ml}$. 18 μl of sample and 6 μl of loading buffer were added to each well, and electrophoresis (voltage was set to 150V, current was

set to 300 mA) was run for 2 h and 30 min. Gels were washed and left to incubate for 2 nights in zymography buffer at 37°C. Following incubation gels were stained for 3 h, then destained in destaining solution. Gelatinase activity of MMP9 and MMP2 was analysed by detection of intensity of band clearing at the same position as in the positive control. A calibrated densitometer (GS-800 Bio-Rad) and Quantity One analysis software (Version 4 Bio-Rad) was used to measure intensity x mm.

6.4.6 Real-time PCR analysis of mRNA expression of target genes in colonic biopsies and whole blood samples

RNA was isolated from tissue samples using a Nucleospin RNA II kit (Macherey-Nagel, Germany) according to the kit protocol. Following RNA quantification and equalisation, cDNA was synthesised using a cDNA archive kit (High capacity cDNA reverse transcription kit, Applied Biosystems, UK). Gene expression analysis was conducted using real-time PCR employing Taqman® Gene Expression Assays (Applied Biosystems, UK). To quantify expression of target genes of interest Taqman Gene Expression Assays containing FAM-labelled probes were used (Assay IDs: MMP9 Rn00579162_m1, IDO Rn00576778_m1, IFN γ Rn00594078_m1, TDO Rn00574499_m1, MMP9 Rn00579162_m1, IL-1 β Rn00580432_m1, IL-6 Rn00561420_m1, TNF α Rn00579162_m1, GFAP Rn00566603_m1, and iNOS Rn00561646_m1, Applied Biosystems, UK). PCR reactions were in a duplex format also containing a Taqman Gene Expression Assay (primer-limited) containing a VIC-labelled probe for the endogenous control gene β -actin (Assay ID: 4352340EE). Samples were assayed using ABI's universal cycling conditions using a fast protocol on the StepOnePlus Real-time PCR system (Applied Biosystems, UK). Fold change in gene expression from the control group was calculated using the $\Delta\Delta C_t$ method (see section 4.5), and β -actin served as endogenous control in the amplification system. Data are expressed as fold change in gene expression relative to the control group.

6.5 NF κ B p65 detection

NF κ B p65 activation was measured using the Thermo Scientific Pierce NF κ B p65 Transcription Factor Kit. The kit contains a streptavidin-coated 96-well plate with the bound biotinylated-consensus sequence for NF κ B p65 which specifically binds only the active forms of NF κ B p65. This chemiluminescent ELISA-based assay provides greater sensitivity than traditional methods for measuring active NF κ B p65 including gel-shift or electrophoretic mobility shift assays (EMSA) and colorimetric ELISA-based assays.

The assay was performed according to manufacturer's instructions. Briefly, a working binding buffer (supplied with kit) was prepared according to the manufacturer's recommendations, and 50 μl was added to each well along with 10 μl of the nuclear extract which was prepared from the NE-PER nuclear and cytoplasmic extraction kit. The plate was incubated for 1 h at RT with mild agitation after which the well contents were discarded and the plate was tapped to remove all liquid. The plate was washed three times with 200 μl of wash buffer. 100 μl of the diluted primary antibody (1:1000) was added to each well and incubated for 1 h at RT without agitation. The plate was washed three times with 200 μl of wash buffer. 100 μl of diluted secondary antibody (1:10,000) was added to each well and incubated for 1 hour at RT without agitation. Finally, the plate was washed four times with 200 μl of wash buffer and 100 μl of the chemiluminescent solution was added to each well. Chemiluminescence was measured using a luminometer set to 200 ms and a sensitivity value of 135. Data was expressed as relative light units (RLU)/ μg protein.

6.6 Immunohistochemistry

Rats were anaesthetised by intraperitoneal urethane injection (12.5 g in 30 mls dH₂O), prior to intercardiac perfusion (see Section 4.9.1). Brains were cryo-protected in sucrose at 4°C, snap frozen in isopentane and stored at -80°C until sectioning using the cryostat (30 μm thickness). iNOS immunohistochemistry was performed on the sections as described in Section 4.9.2.

6.6.1 Statistical Analysis

Data are presented as mean \pm standard error of the mean (SEM). As appropriate Student's *t* test or One-way ANOVA were used to compare groups, followed by a Newman-Keuls *post hoc* test. All statistical analyses were considered to be significant when $p < 0.05$. Colon PCR data did not follow normal distribution and therefore was log transformed prior to ANOVA. Graphs and statistics were generated using GraphPad Prism Software Version 4.00 (GraphPad software, Inc) and GB-STAT v.10 (Dynamic Microsystems Inc) respectively.

6.7 Results

6.7.1 DSS-induced colitis induces symptoms of Ulcerative Colitis, affects general well being, and disturbs colonic structure in Wistar rats

DSS administration induces a significant change in the colonic ultrastructure similar to UC. Food intake, body weight changes and fluid intake are used as general indicators of sickness and the DAI was assessed to specifically determine the effect of DSS on UC-like symptoms. Histological assessment of the bowel wall and MMP activity provide indications of microscopic damage to the distal colon.

Body weight, Food and Fluid intake in DSS-induced colitis

Rats were assessed daily for weight changes (a,b), food intake (c,d) and fluid intake (e,f) (see Figure 6.1). DSS administration induced a significant decrease in total body weight gain (b) over 7 days: pooled variance Student's t test [$t = 8.007$; $df = 12$; $p = 0.0001$], DSS administration induced a significant decrease in average food intake (d) per rat, per cage, over 7 days: pooled variance Student's t test [$t = 4.245$; $df = 6$; $p = 0.0054$]. There was no significant difference in fluid (f) intake over the course of DSS administration (see Figure 6.1). Daily weight charts were also analysed; pooled variance Student's t test of the average area under the curve (AUC) from day 3 onwards was significantly reduced for body weight [$t = 2.231$; $df = 12$; $p = 0.045$] and food intake [$t = 4.826$; $df = 6$; $p = 0.0029$] (see Figure 6.2).

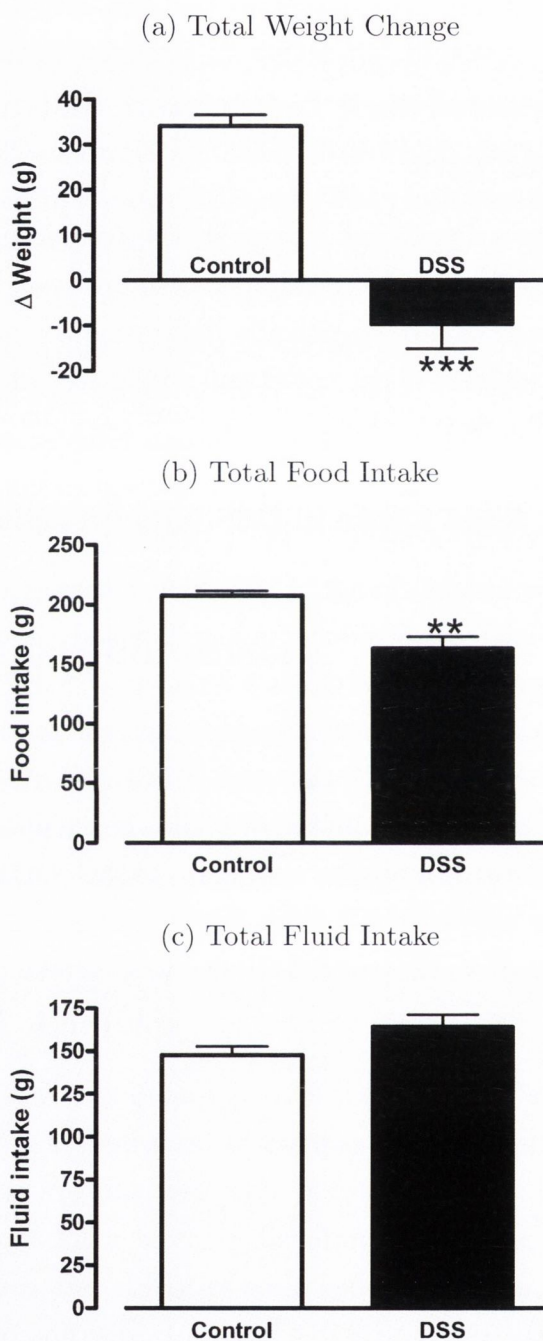


Figure 6.1: Body weight, Food and Fluid intake in DSS-induced colitis

DSS administration resulted in decreased body weight (b) and total food intake (d). No significant difference in fluid intake over the course of DSS administration was seen (f). Data are expressed as mean \pm SEM (n=6-8). ** $p < 0.01$, *** $p < 0.001$ vs controls (pooled variance Student's t test).

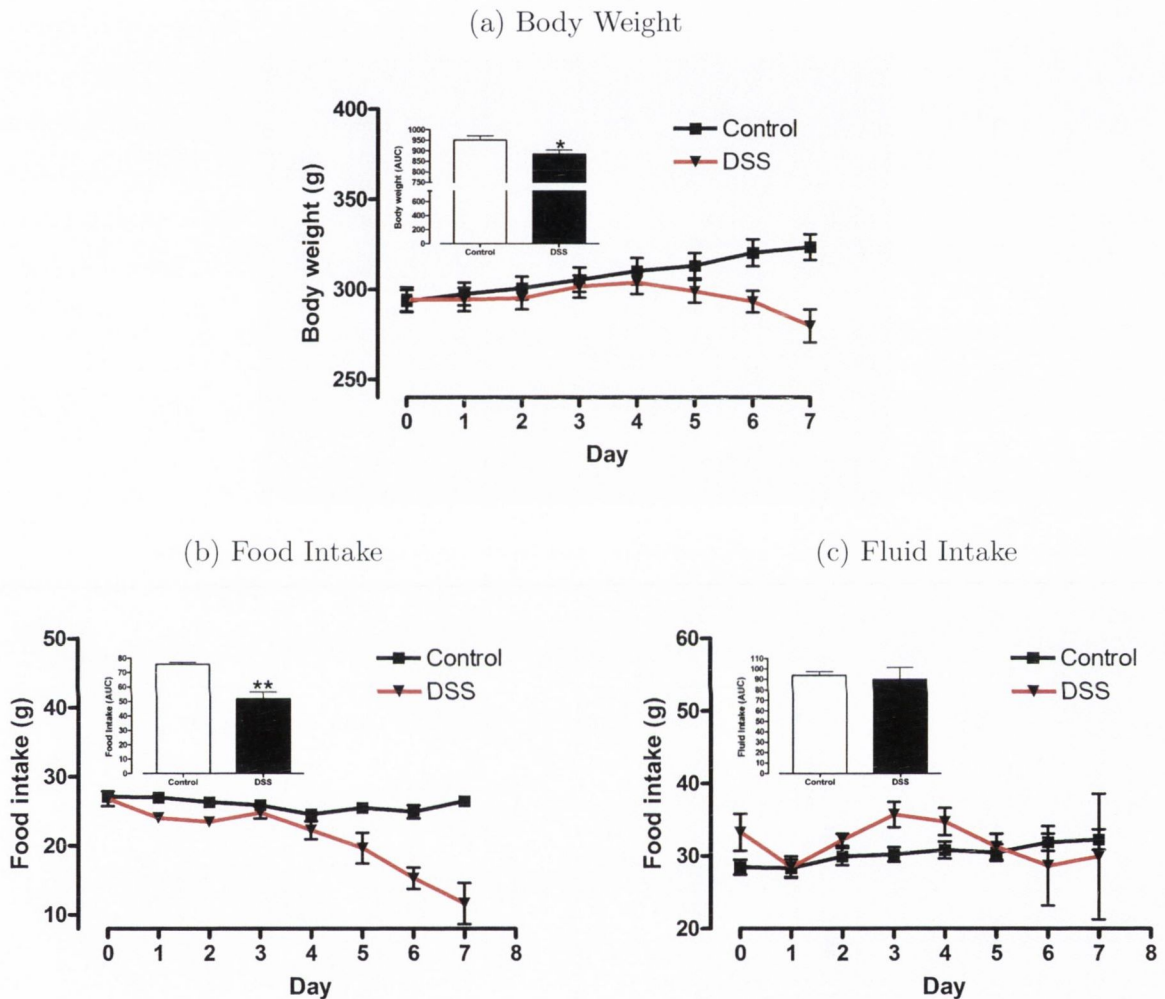


Figure 6.2: **Body weight, Food and Fluid intake in DSS-induced colitis**

Graphs represent daily body weight measurements (a), food intake (b) and fluid intake over the course of 7 days DSS administration in the drinking water. Area under the curve (AUC) analysis, from day 3 when symptoms begin, revealed a significant decreased in body weight (a)(embedded bar graph) and total food intake (d)(embedded bar graph). No significant difference in fluid intake over the course of DSS administration was seen (c). Data are expressed as mean \pm SEM (n=6-8). * $p < 0.05$, ** $p < 0.01$ vs controls (pooled variance Student's t test).

DAI and histological scores were significantly increased in DSS-induced colitis

Animals were observed daily for weight loss, stool consistency and rectal bleeding, and post-mortem histological assessment of the intestinal wall ultrastructure was carried out (see Figure 6.3). DSS administration resulted in a significant increase in DAI scores on the final day of DSS administration [$t = 10.42$; $df = 12$; $p < 0.0001$] (a). Post-mortem histological analysis of the distal colon revealed DSS significantly increased both histological criteria: crypt damage scores [$t = 15.79$; $df = 12$; $p < 0.0001$] (b) and inflammation scores [$t = 12.14$; $df = 12$; $p < 0.0001$] (c) in the colon. DSS exposure induces structural changes in the colon. Representative image of transverse sections of a normal colon (d) (showing intact colonic crypts, sparse mononuclear infiltration in the lamina propria and intact epithelial layer) and a DSS-induced colitis colon (e) (showing crypt destruction, dense infiltration of mononuclear cells into the lamina propria and significant erosion of the epithelial layer).

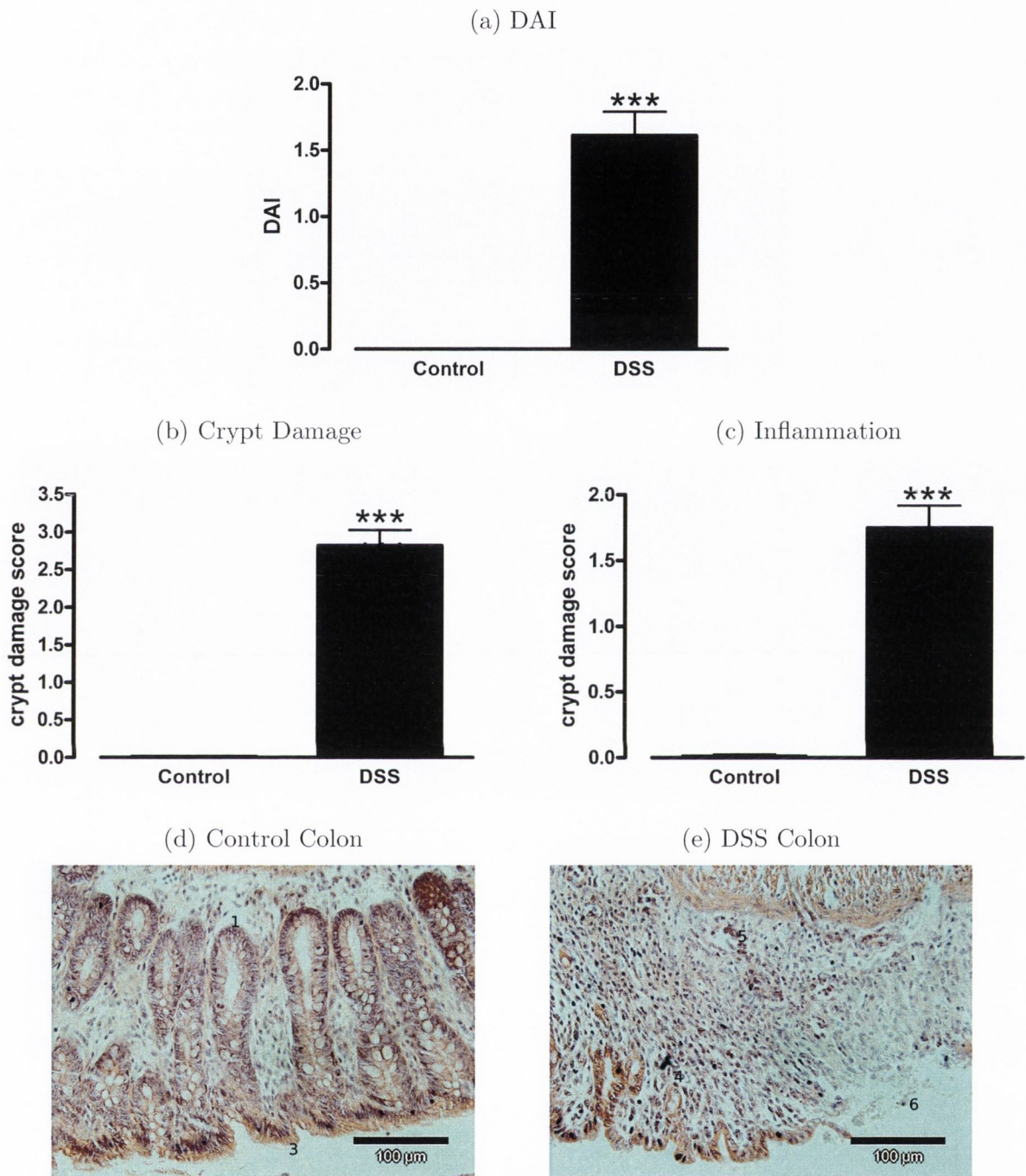


Figure 6.3: Disease Activity Index (DAI) and histological scores were significantly increased in DSS-induced colitis

DSS resulted in a significant increase in the DAI (a), crypt damage (b) and inflammation (c) scores in the colon. Data expressed as mean \pm SEM (n=6-8). *** $p < 0.001$ vs controls (pooled variance Student's *t* test). DSS exposure induces ultrastructural changes in the colon. Representative images of transverse sections of a control colon (d) showing intact colonic crypts (1), sparse mononuclear infiltration in the lamina propria and intact epithelial layer (3) and a colitic (e) colon showing crypt destruction (4), dense infiltration of mononuclear cells (5) into the lamina propria and significant erosion of the epithelial layer (6) (40x magnification).

DSS-induced colitis induced significant increases in MMP activity

MMP9 is not specifically a marker of inflammation however it does provide significant evidence of damage to colonic epithelial layers. DSS administration significantly increased MMP9 mRNA expression [$t = 8.232$; $df = 12$; $p < 0.0001$] (a), MMP2 mRNA expression [$t = 4.957$; $df = 12$; $p < 0.001$] (b), MMP9 protein [$t = 5.991$; $df = 12$; $p < 0.0001$] (c), and MMP2 protein expression [$t = 3.005$; $df = 10$; $p = 0.0132$] (d) (see Figure 6.4).

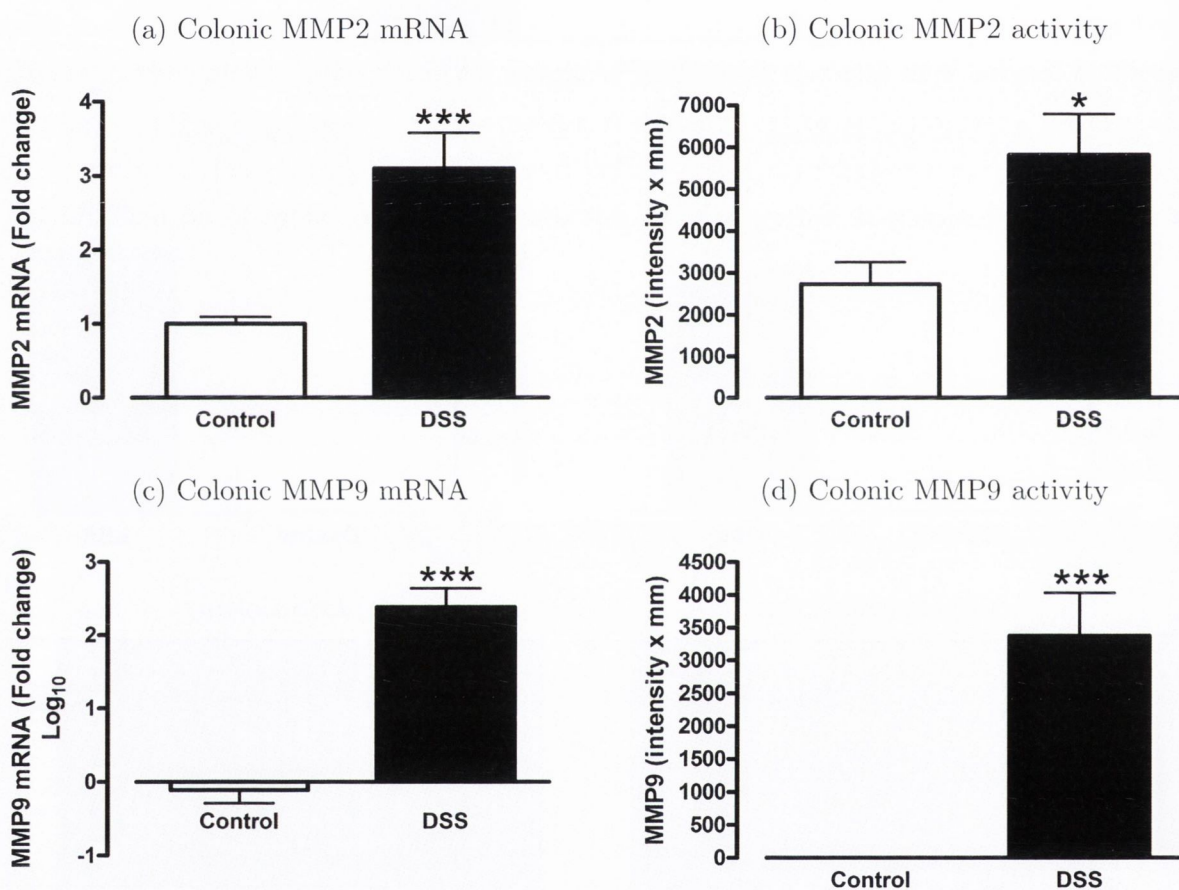


Figure 6.4: DSS-induced colitis-related changes in the expression of MMPs in the distal colon DSS administration significantly increased MMP2 (a) and MMP9 (c) mRNA expression, and MMP2 (b) and MMP9 (d) protease activity. Data expressed as mean \pm SEM ($n = 6-8$). * $p < 0.05$, *** $p < 0.001$ vs controls (pooled variance Student's t test).

6.7.2 Behavioural disturbances following DSS-induced colitis

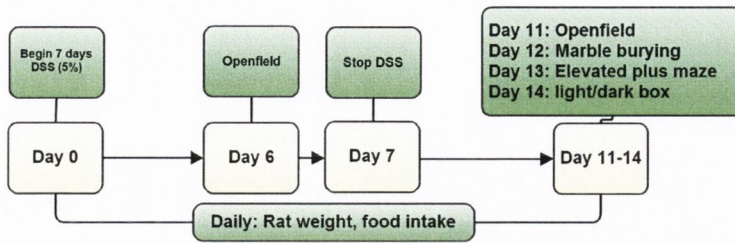


Figure 6.5: Timecourse for DSS administration and behavioural testing.

Food intake and increased weight gain during recovery from DSS administration.

DSS-induced colitis resulted in a significant decrease in rat weight gain during the acute phase of colitis (see Figure 6.6). T test analysis of average AUC revealed a significant decrease in weight gain in the DSS group from DSS day 3 on [$t = 2.784$; $df = 32$; $p = 0.009$]. Following termination of DSS administration, recovery begins and there is no significant difference in average body weight gain [$t = 0.06807$; $df = 32$; $p = 0.9462$], or average food intake [$t = 1.718$; $df = 14$; $p = 1.079$] by day 10 (recovery day 3) when compared to controls (data not shown).

(a) Daily Fluid Intake

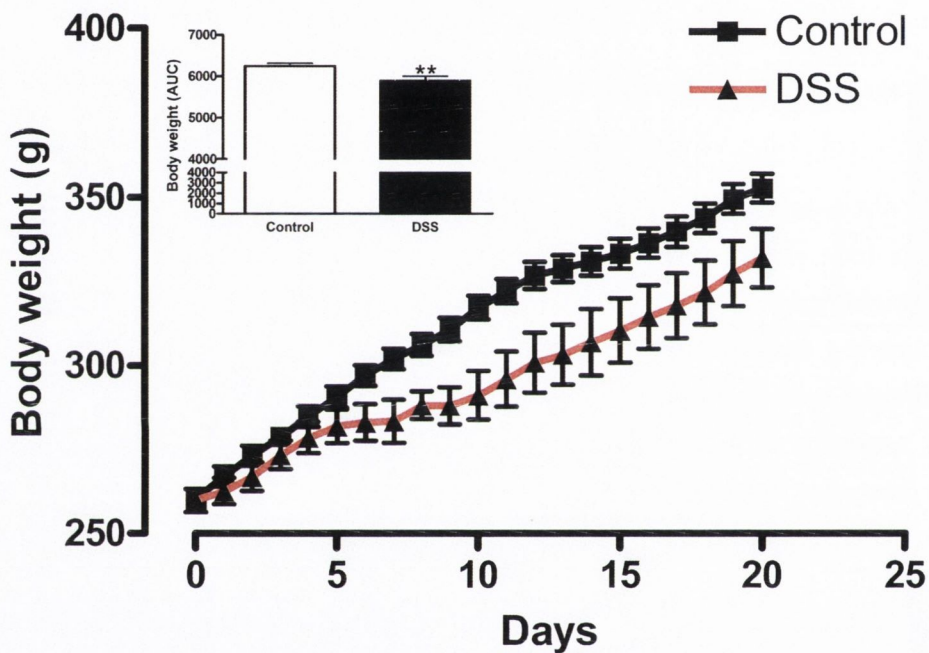


Figure 6.6: Recovery of food intake and increased weight gain during recovery from DSS administration

DSS-induced colitis resulted in a significant decrease in rat weight as per the daily weight chart. The embedded bar graph represents the average AUC from day 3 on for DSS and control rats. Weight data are expressed as mean \pm SEM (n=16-18). ** p < 0.01 vs control (pooled variance Student's *t* test)

Acute DSS-induced colitis induced behavioural changes in the openfield

DSS-induced colitis resulted in behavioural changes in the openfield during the acute colitic phase (day 6 of DSS administration)(see Figure 6.7). Analysis revealed a significant decrease in path length [$t = 2.673$; $df = 26$; $p = 0.0128$] (a) and increase in time spent grooming [$t = 2.364$; $df = 26$; $p = 0.026$] (d). Decreased speed [$t = 2.579$; $df = 26$; $p = 0.0159$] (b) and time in the centre zone of the arena [$t = 2.511$; $df = 26$; $p = 0.0186$] (c) were also revealed. Further openfield testing at recovery day 4 revealed a return to normal locomotor exploratory behaviour, path length [$t = 0.2160$; $df = 30$; $p = 0.8305$] (a) and speed [$t = 0.3391$; $df = 30$; $p = 0.7369$] (b). Increased grooming was however maintained at this timepoint [$t = 2.501$; $df = 30$; $p = 0.0181$] (c) (see Figure 6.8).

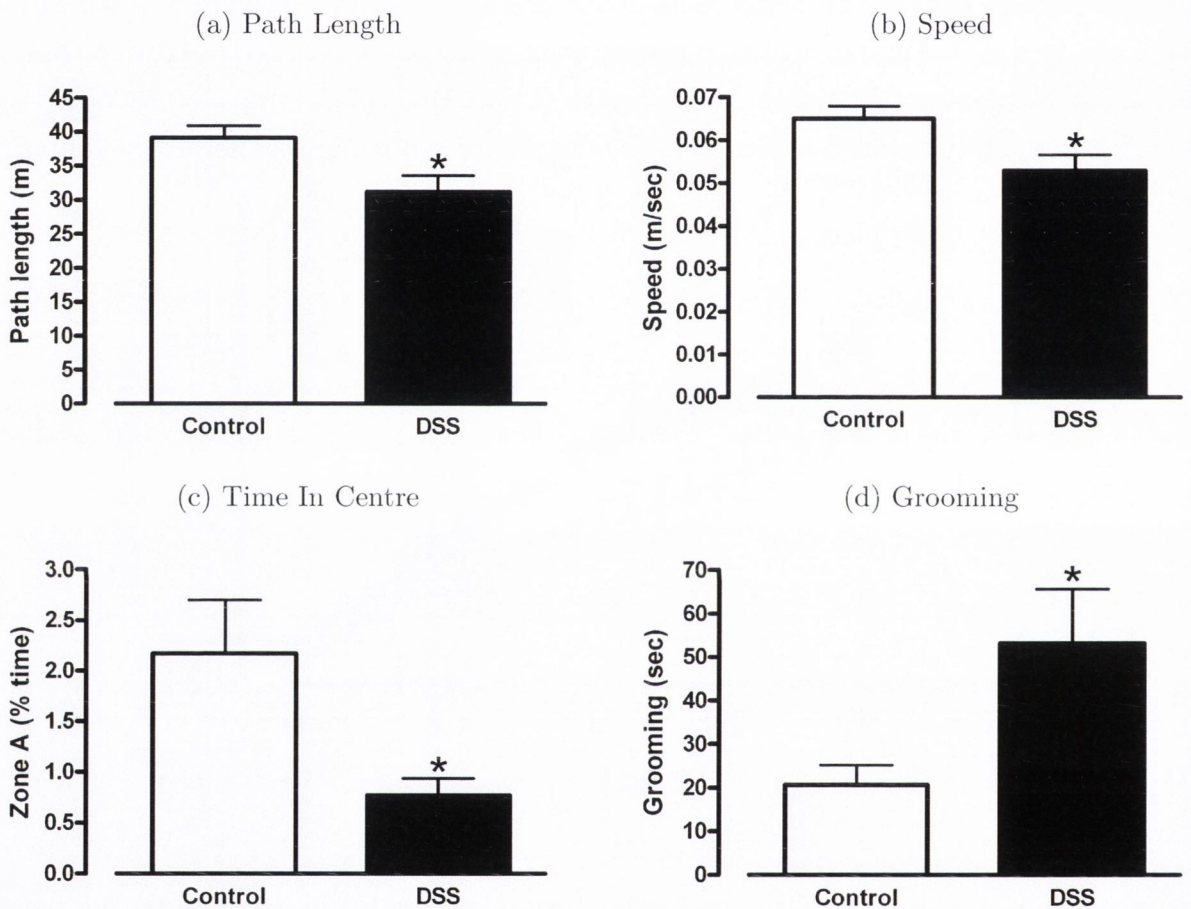


Figure 6.7: Acute DSS-induced colitis induced behavioural changes in the open-field

DSS-induced colitis significantly decreased path length (a), speed (b), time spent in the centre zone (c) and significantly increased grooming time (d) in the openfield. Data are expressed as mean \pm SEM ($n = 14$). * $p < 0.05$ vs control (pooled variance Student's t test)

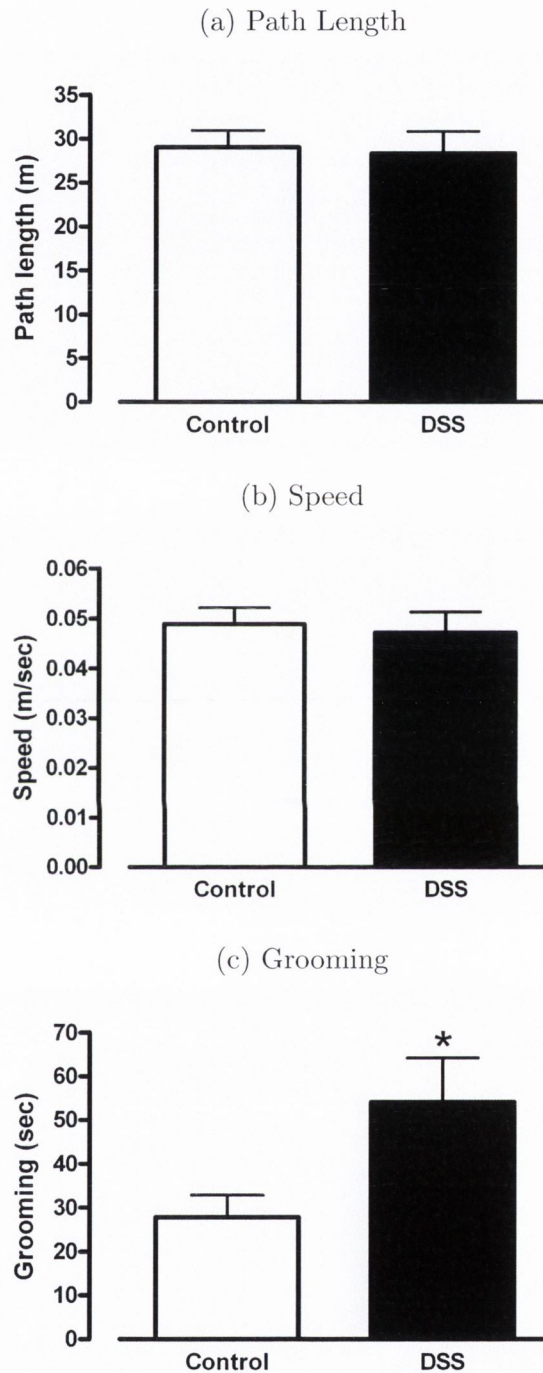


Figure 6.8: Locomotor impairments in the openfield have recovered by recovery day 4

Normal path length (a) and speed (b) have recovered by recovery day 4. Increased grooming is still present (c). Data are expressed as mean \pm SEM ($n=14$). * $p < 0.05$ vs control (pooled variance Student's t test)

Loss of normal digging/burying behaviour in the marble burying test.

Analysis revealed decreased burying/digging behaviour on day 5 of recovery from DSS administration in the Marble burying test [$t = 7.017$; $df = 30$; $p < 0.0001$] (see Figure 6.9).

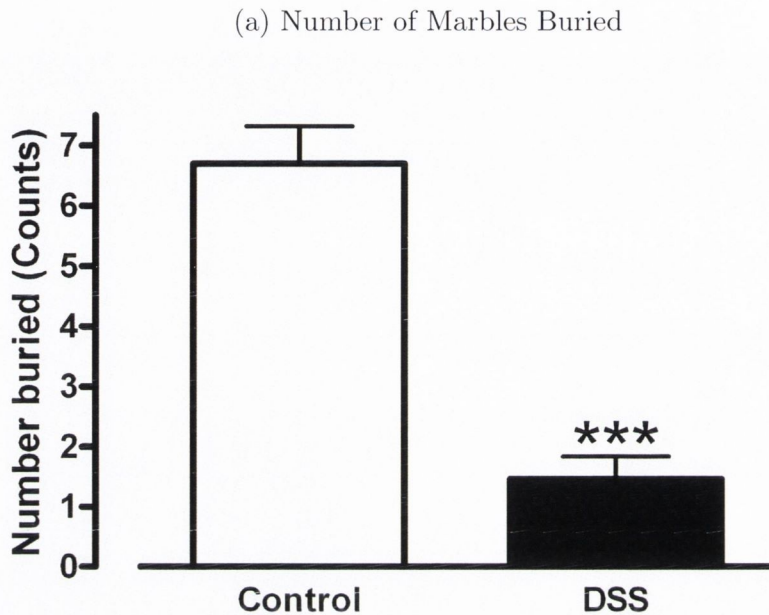


Figure 6.9: Loss of normal digging/burying behaviour in DSS-induced colitis. DSS-induced colitis significantly decreased the number of marbles buried on day 5 of recovery. Data are expressed as mean \pm SEM ($n=14$). * $p < 0.05$ vs control (pooled variance Student's t test)

Increased anxiety related behaviour in the light/dark box.

Rats were placed in the light chamber and their behaviour was observed for 10 minutes on day 6 of recovery from DSS administration. Analysis revealed no significant difference in time spent in the light chamber [$t = 1.537$; $df = 31$; $p = 0.1345$] (a), however decreased head pokes into the light chamber [$t = 4.611$; $df = 31$; $p < 0.0001$] (b) and increased fecal boli counts [$t = 2.621$; $df = 31$; $p = 0.0136$] (c) were recorded (see Figure 6.10).

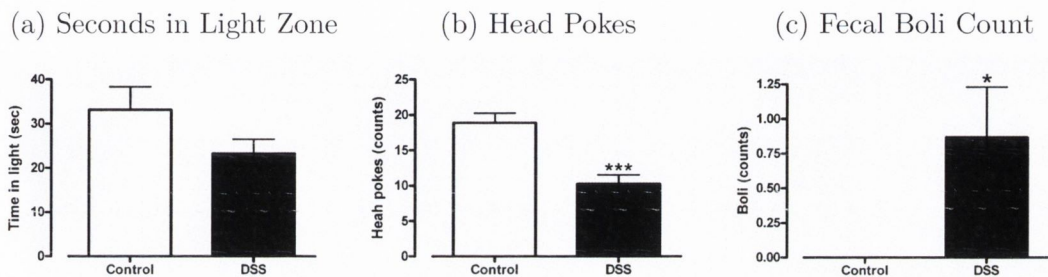


Figure 6.10: **Increased anxiogenic affect in the light chamber on day six of recovery from DSS administration.**

Analysis revealed significant decreases in number of head pokes (b) and increased fecal boli (c) in the light/dark box. No difference in time spent in the light chamber was recorded (a). Data are expressed as mean \pm SEM ($n = 15-18$). * $p < 0.05$, * $p < 0.001$ vs control (pooled variance Student's t test)

Altered behaviour in the elevated plus maze

Increased time in the 'safe' end of the closed arms (a) [$t = 2.253$; $df = 31$; $p = 0.032$], and decreased time spent in the centre zone (b) [$t = 2.355$; $df = 31$; $p = 0.026$] was observed during recovery from DSS administration. No difference in total time spent in the open arm (c) [$t = 0.4588$; $df = 31$; $p = 0.6496$] when compared to control (see Figure 6.11).

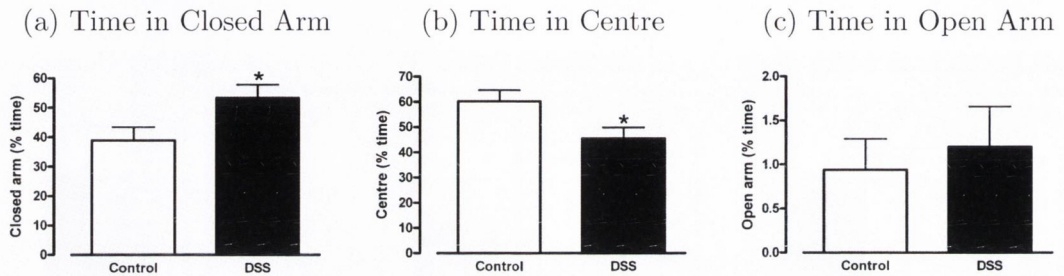


Figure 6.11: **Altered behaviour in the elevated plus maze**

Increased time in the 'safe' end of the closed arms (a) and decreased time spent in the centre zone (b) during recovery from DSS administration. No difference in total time spent in the open arms was observed. Data are expressed as mean \pm SEM ($n = 15-18$). * $p < 0.05$ vs control (pooled variance Student's t test)

6.7.3 DSS-induced colitis induces colonic and central inflammation in Wistar rats

DSS-induced colitis results in increased pro-inflammatory cytokine expression, within the bowel and cortex, during acute DSS-induced inflammation and following a seven day recovery period. Table 6.1 represents the colonic and cortical changes observed during acute DSS-induced colitis and following 7 days recovery from DSS.

Table 6.1: Cortical and colonic gene expression during acute DSS-induced colitis, and following 7 days recovery

	Cortex		Colon	
Acute	Control	DSS	Control	DSS
iNOS	1 ± 0.17	4.28 ± 0.91**	-0.003 ± 0.2	2.6 ± 0.2 ***
TNFα	1 ± 0.07	1.16 ± 0.14	-0.2 ± 0.2	0.5 ± 0.2 **
IL-1β	1 ± 0.11	0.86 ± 0.18	-0.04 ± 0.1	1.4 ± 0.2***
IL-6	1 ± 0.09	2.01 ± 0.22**	-0.04 ± 0.1	2.0 ± 0.3 ***
IFNγ	1 ± 0.17	1.27 ± 0.31	-0.1 ± 0.1	0.1 ± 0.1
GFAP	1 ± 0.07	0.49 ± 0.03***	-0.02 ± 0.5	0.7 ± 0.1 ***
Recovery	Control	DSS	Control	DSS
iNOS	1 ± 0.9	3.69 ± 0.79**	-0.3 ± 0.3	1.3 ± 0.3 ***
TNFα	1 ± 0.11	0.95 ± 0.1	-0.1 ± 0.1	0.4 ± 0.2 *
IL-1β	1 ± 0.15	1.26 ± 0.14	-0.1 ± 0.1	0.9 ± 0.2 **
IL-6	1 ± 0.09	1.44 ± 0.17*	-0.04 ± 0.1	0.8 ± 0.3 *
IFNγ	1 ± 0.23	1.13 ± 0.33	-0.1 ± 0.1	0.8 ± 0.3 ***
GFAP	1 ± 0.13	1.04 ± 0.08	1 ± 0.12	1.64 ± 0.55

Data representing mRNA fold changes for cortical and colonic gene expression during acute DSS-induced inflammation and following seven days of recovery. Data are expressed as mean ± SEM (n=5-8). * p < 0.05, ** p < 0.01, *** p < 0.001 compared to controls (Pooled variance Student's *t* test). Colon samples did not follow a normal distribution, values were log transformed prior to statistical analysis. Dextran Sulfate Sodium (DSS), Glial fibrillary acidic protein (GFAP), inducible nitric oxide synthase (iNOS), interferon (IFN), interleukin (IL), tumor necrosis factor (TNF).

Peripheral and central iNOS expression following 7 days DSS administration and following 7 days recovery

A one-way ANOVA demonstrated a significant effect of DSS-induced colitis on iNOS expression in the periphery: colon [F(2;14) = 10.58, p = 0.0016] (a) spleen [F(2;14) = 30.25, p < 0.0001] (c), and liver [F(2;14) = 10.36, p = 0.0017] (e). A one-way ANOVA also demonstrated a significant effect of DSS-induced colitis on iNOS expression in the brain samples: hypothalamus [F(2;14) = 12.98, p = 0.0006] (b), hippocampus [F(2;14) = 33.73, p < 0.0001], and cortex [F(2;14) = 18.28, p < 0.0001] (e)(see Figure 6.12). Significant correlations between peripheral and central iNOS expression were also revealed by Spearman r correlation (g).

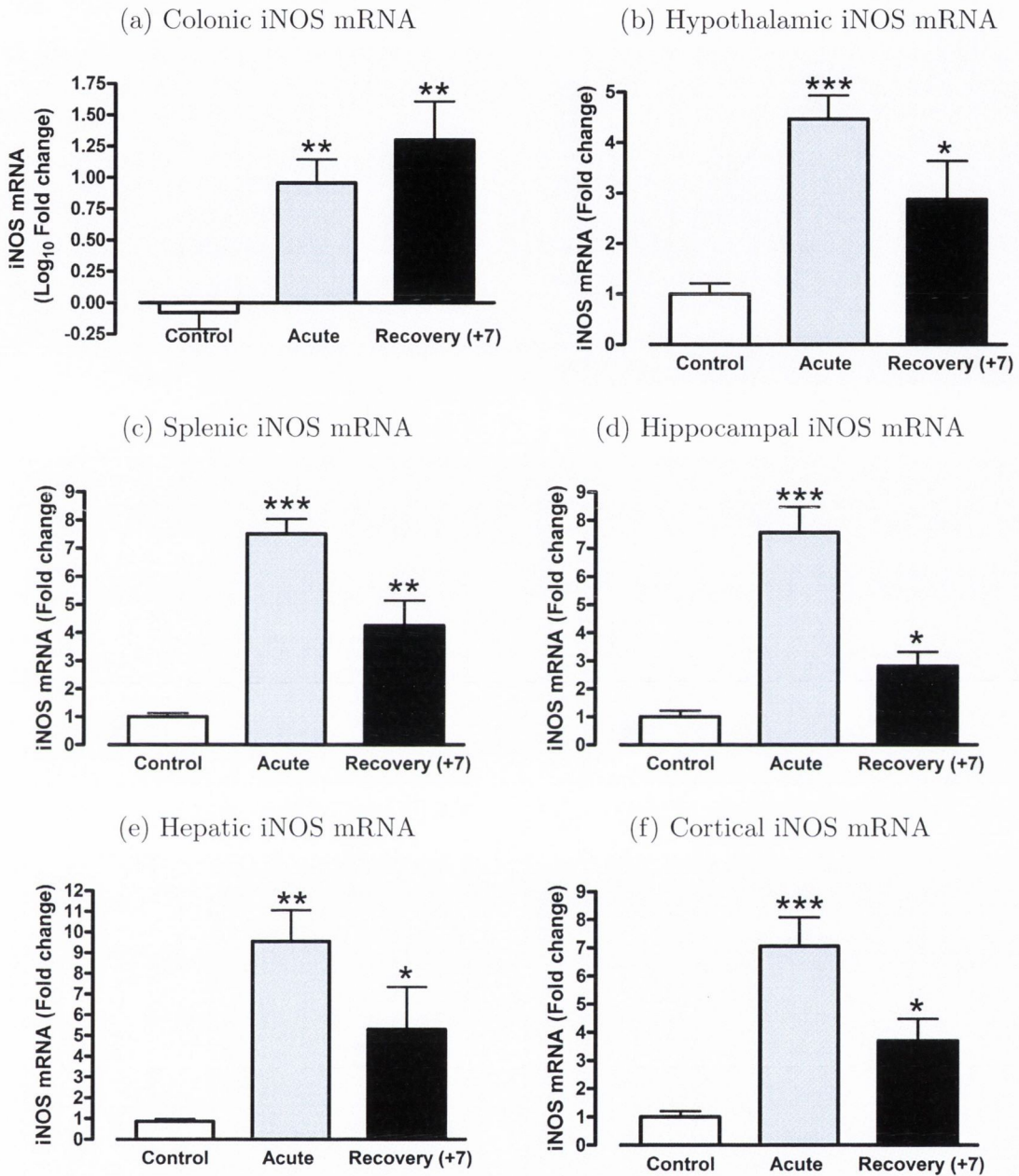


Figure 6.12: Peripheral and central expression of iNOS following 7 days DSS administration and following 7 days recovery. A one-way ANOVA demonstrated a significant effect of DSS-induced colitis on iNOS expression in the colon (a), hypothalamus (b), spleen (c), hippocampus (d), liver (e), and cortex (f). Data are expressed as mean fold change, or Log transformed fold change, in iNOS expression \pm SEM of 5-6 animals per treatment group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control (Newman-Keuls *post hoc* test).

Nuclear NF κ B activity, and IRF1 and HIF-1 α mRNA expression are not increased in parallel with cortical iNOS mRNA expression

The molecular pathway involved in the increase in iNOS mRNA expression is unclear as no increases in NF κ B, IRF1, or HIF-1 α were revealed in the frontal cortex during this acute inflammatory phase. This was despite a highly significant increase in HIF-1 α mRNA expression in the colon [$t = 5.169$; $df = 12$; $p = 0.0002$] (graph not shown).

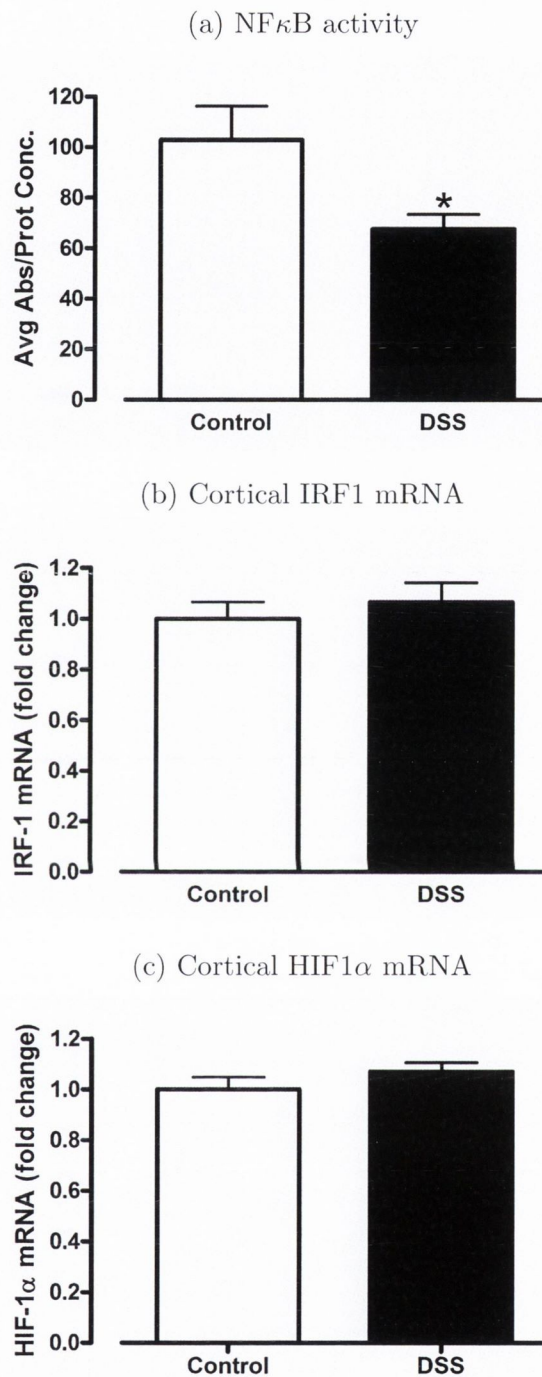


Figure 6.13: Increased iNOS expression is not mediated by NF κ B, IRF1 or HIF-1 α during acute DSS-induced colitis

Analysis revealed a significant decrease in nuclear NF κ B activity (a) in the frontal cortex. No differences in mRNA expression of IRF1 (b) or HIF-1 α (c) was revealed. Data are expressed mean \pm SEM (n = 6-8). * p < 0.05, *** p < 0.001 vs control (pooled variance Student's *t* test).

Increased iNOS protein expression surrounding the third ventricle

Immunohistochemical analysis of rat brain slices for iNOS expression revealed significant increases in iNOS in cells surrounding the third ventricle. Images of rat brain atlas figure 24 (a) and figure 28 (b) represent the brain slices used for counting iNOS positive cells in the LHb and SFO respectively. Student's *t* test revealed significant increases in the number of iNOS positive cells in the area surrounding the third ventricle (c). As per central iNOS mRNA expression this increased cell count was significantly correlated with hepatic iNOS mRNA expression (d) (see Figure 6.14). Representative images of SFO brain region for control rat (e) and DSS rat (f) are presented showing the increased iNOS staining in the DSS-induced colitis rat. This increased iNOS expression is most obvious directly around the blood vessels surrounding the SFO.

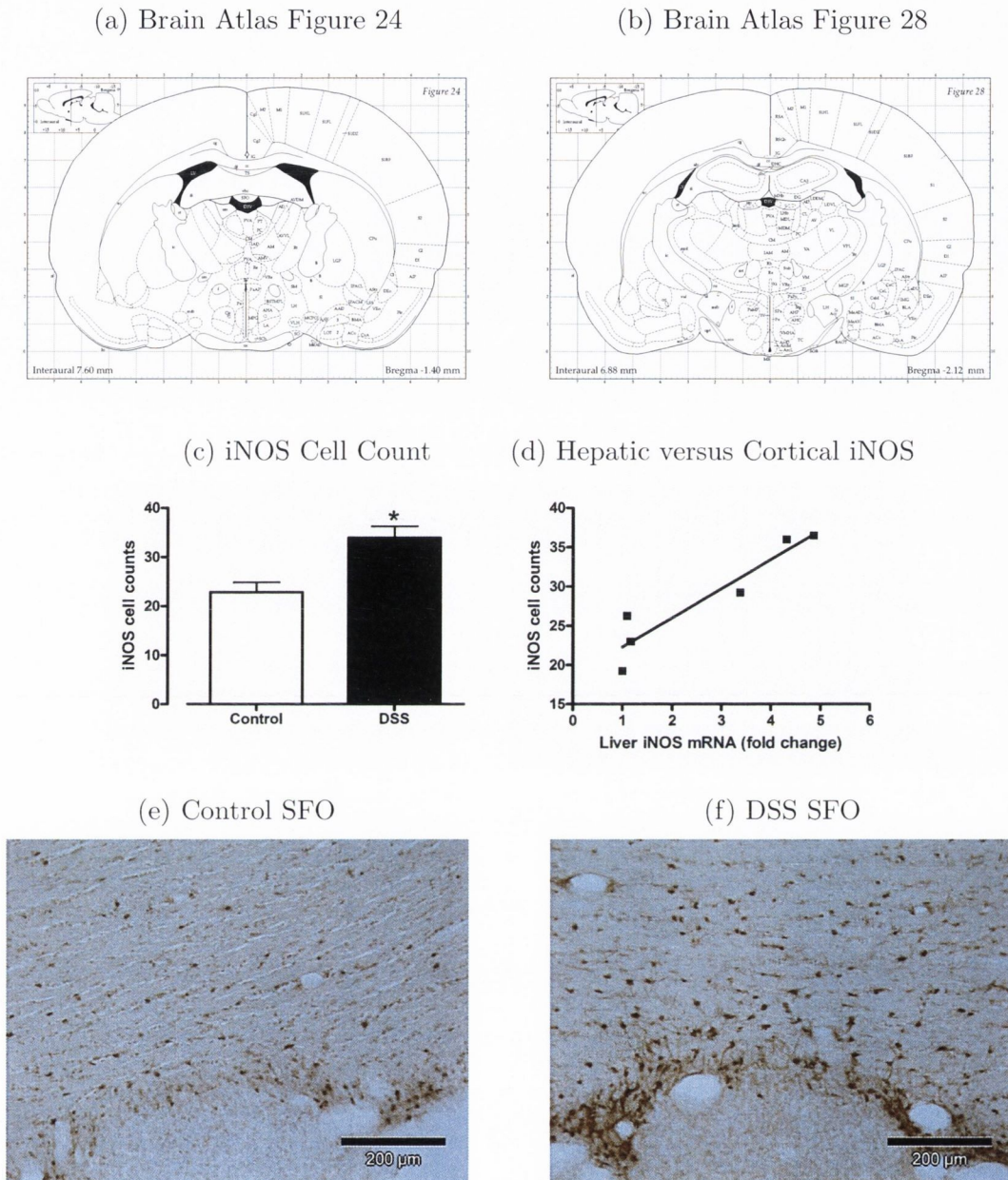


Figure 6.14: **Significant increases in iNOS cell counts surrounding the third ventricle**

Images of rat brain atlas figure 24 (a) and figure 28 (b) represent the brain slices used for counting iNOS positive cells in the subformal organ (SFO) and lateral habenula (LHb). Analysis revealed significant increases in the number of iNOS positive cells (brown) in the area around these regions surrounding the third ventricle (c). This increased cell count was significantly correlated with hepatic iNOS mRNA expression (d). Representative images of SFO brain region for control rat (e) and acute DSS-induced colitis rat (f). The DSS-induced colitis rat has increased iNOS staining around the SFO, in particular directly around the blood vessels. Data are expressed as mean \pm SEM ($n = 3$). * $p < 0.05$ vs control (pooled variance Student's t test).

6.7.4 DSS-induced colitis is associated with altered tryptophan concentrations

Analysis of clinical IBD patient samples revealed a significant increase in the kynurenine to tryptophan ratio in the circulation (see chapter 5). This increase was most significant in the IBD group with moderate to severe colonic inflammation, but was also associated with high DAI scores, and within the symptomatic IBD group who also had higher HAM-D scores. In the IBD patients the increase in ratio was mainly due to a decrease in circulating tryptophan. The implications this might have on central kynurenine, tryptophan or serotonin concentrations in IBD patients is unknown. A third DSS experiment was carried out to examine the implications of experimental colitis on circulating and central tryptophan concentrations. Based on initial pilot work the 7 day timepoint used in the previous experiments was too long to observe changes. Therefore, a shorter 2 day recovery period was allowed in this experiment. These results demonstrate a similar decrease in circulating tryptophan in the DSS model of colitis. This was associated with an increase in central tryptophan and a decrease in hippocampal serotonin.

DSS induced significant weight loss in Wistar rats following seven days administration

As per previous studies DSS induced a significant decrease in weight gain compared to control animals. A one-way ANOVA demonstrated a significant effect of DSS-induced colitis on weight gain [$F(2;20) = 5.71, p = 0.01$]. The acute phase was also associated with a decrease in average food intake per cage: average intake of 28.7g per rat per day for controls versus 23.4g per rat per day for the acute group. By two days recovery average intake had returned to 27.2g per rat per day (as there were only two cages per group one-way ANOVA could not be carried out, however results are consistent with food intake decreases previously reported following DSS administration in chapter 6).

(a) Total Weight Change

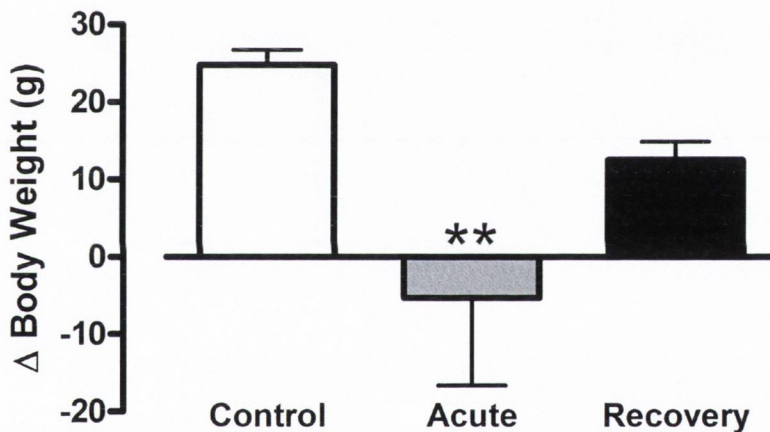


Figure 6.15: Decreased weight gain during acute DSS-induced colitis

DSS exposure induces a significant decrease in weight gain. Data are expressed as mean \pm SEM ($n = 7-8$). ** $p < 0.01$ vs control (Newman-Keuls *post hoc* test)

Circulating concentrations of kynurenine, tryptophan and KYNA following DSS-induced colitis

A one-way ANOVA demonstrated a significant effect of DSS-induced colitis on circulating concentration of tryptophan [$F(2;20) = 9.372$, $p = 0.0013$] (a), kynurenine [$F(2;20) = 3.677$, $p = 0.0436$] (b), and KYNA [$F(2;20) = 11.57$, $p < 0.001$] (c). DSS also had a significant effect on (d) kyn:try ratio [$F(2;20) = 4.633$, $p = 0.0222$] (E) KYNA:kyn ratio [$F(2;20) = 36.91$, $p < 0.0001$] (F) KYNA:try ratio [$F(2;20) = 6.716$, $p = 0.0059$] (see Figure 6.16).

Colonic, splenic and hepatic tryptophan concentrations following DSS-induced colitis

DSS induced significant increases in colonic tryptophan at acute and 2 day recovery phase, and a significant decrease in splenic tryptophan following 2 days recovery (see Figure 6.17). DSS administration had no effect on hepatic tryptophan. A one-way ANOVA demonstrated a significant effect of DSS-induced colitis on tryptophan concentrations in the colon (a) [$F(2;20) = 33.58$, $p < 0.0001$], and the spleen (b) [$F(2;20) = 5.717$, $p = 0.0109$]. No significant change in hepatic (c) tryptophan was observed [$F(2;20) = 2.592$, $p = 0.0998$].

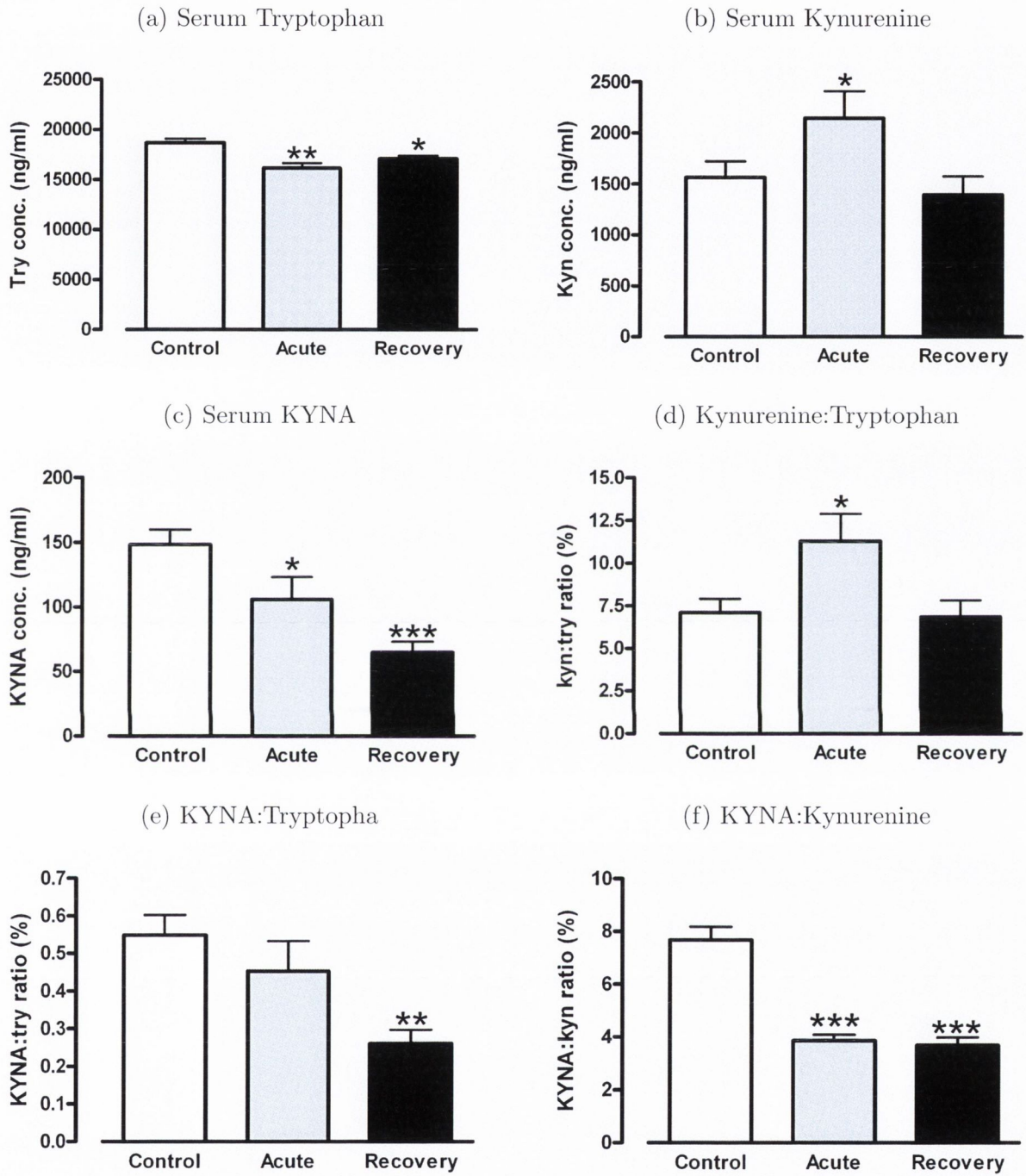


Figure 6.16: Circulating concentrations of kynurenine, tryptophan and KYNA following DSS-induced colitis

DSS induced a significant decrease in circulating tryptophan (a) and KYNA (c) and a significant increase in kynurenine (b). This resulted in a significant increase in the kynurenine:tryptophan ratio during acute colitis (d) and decreased KYNA:tryptophan (e) and KYNA:kynurenine ratios (f). Data are expressed as mean \pm SEM ($n = 7-8$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control (Newman-Keuls *post hoc* test)

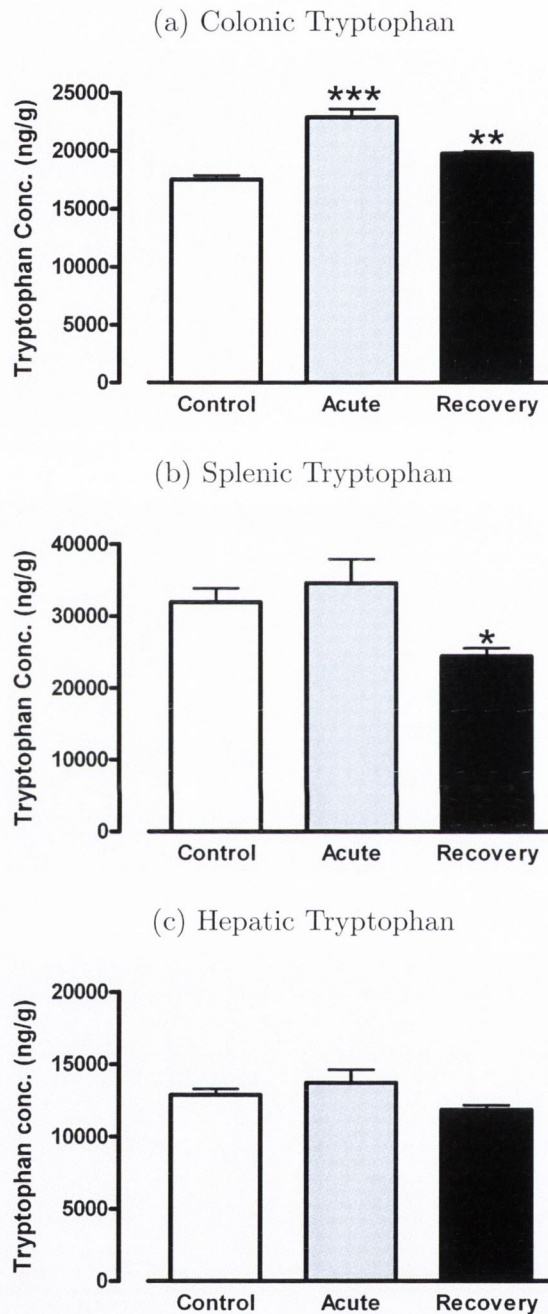


Figure 6.17: Colonic, splenic and hepatic tryptophan concentrations following DSS-induced colitis

DSS induced significant increases in colonic tryptophan (a) and a significant decrease in splenic tryptophan following 2 days recovery (b). DSS administration had no effect on hepatic tryptophan (c). Data are expressed as mean \pm SEM ($n = 7-8$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control (Newman-Keuls *post hoc* test)

Hippocampal and hypothalamic tryptophan concentrations following DSS administration

DSS administration induced significant increases in cortical, hippocampal and hypothalamic tryptophan concentrations during the acute inflammatory phase (see Figure 6.18). A one-way ANOVA demonstrated a significant effect of DSS-induced colitis on tryptophan concentrations in the hippocampus (a) [$F(2;20) = 7.714$, $p = 0.0033$] and hypothalamus (b) [$F(2;20) = 7.305$, $p = 0.0042$]

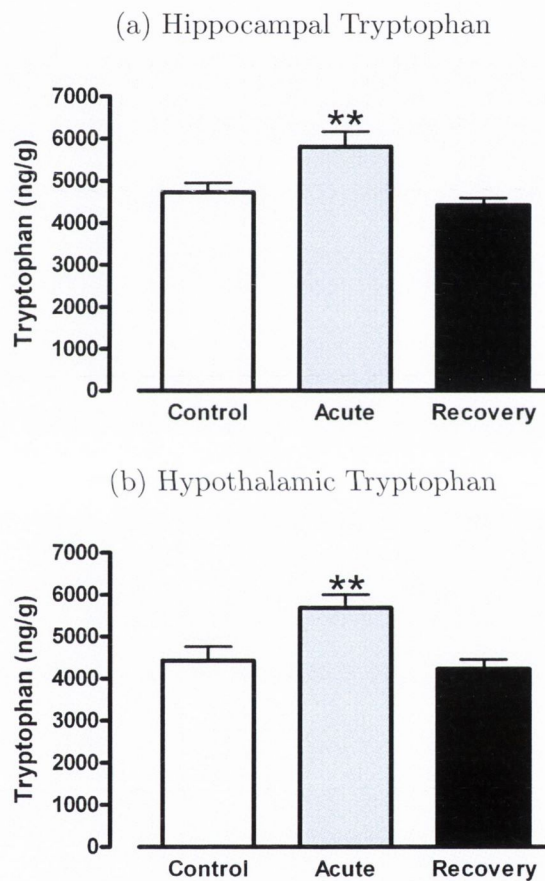


Figure 6.18: **Hippocampal and hypothalamic tryptophan concentrations following DSS-induced colitis**

DSS administration induced significant increases in hippocampal (a) and hypothalamic (b) tryptophan concentrations during the acute inflammatory phase. Data are expressed as mean \pm SEM ($n = 7-8$) ** $p < 0.01$, *** $p < 0.001$ vs control (Newman-Keuls *post hoc* test)

Increased cortical kynurenine following two days recovery from DSS administration

Further HPLC analysis of cortical tissue revealed DSS-induced increases in kynurenine concentrations following two days recovery from DSS administration (see Figure 6.19). A one-way ANOVA demonstrated a significant effect of DSS-induced colitis on concentrations of tryptophan (a) [$F(2;20) = 11.77$, $p = 0.0004$], kynurenine (b) [$F(2;20) = 5.08$, $p = 0.016$] and the kynurenine:tryptophan ratio in the cortex (c) [$F(2;20) = 5.63$, $p = 0.012$].

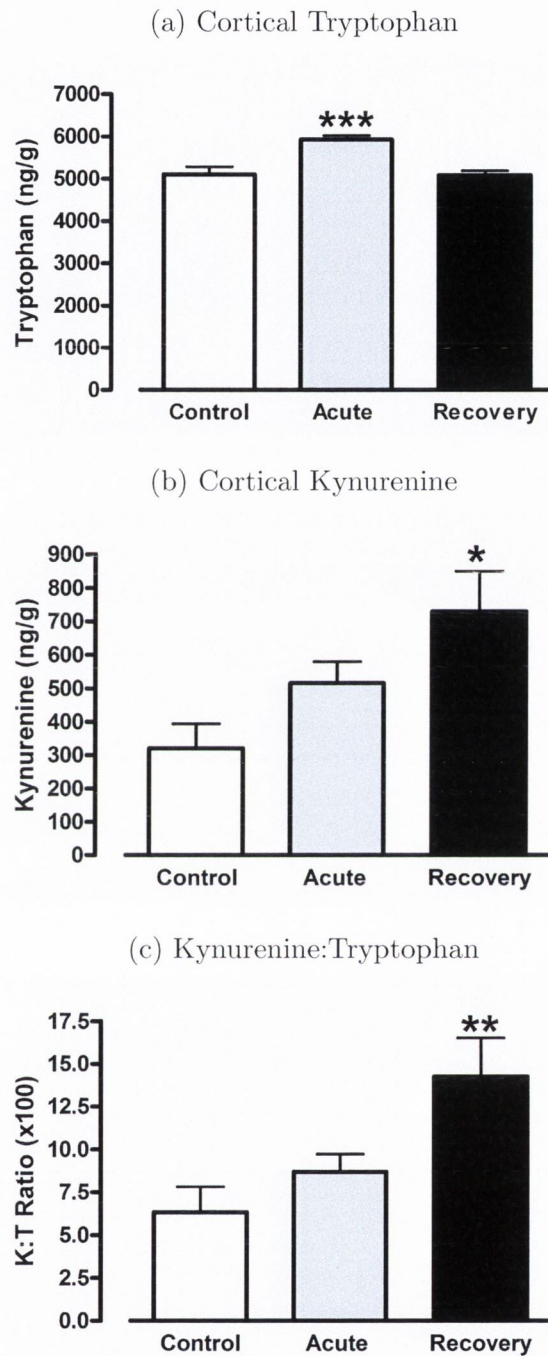


Figure 6.19: Cortical tryptophan, kynurenine and kynurenine:tryptophan ratio concentrations following DSS-induced colitis

DSS administration induced significant increases in cortical tryptophan during acute colitis (a), kynurenine after 48 hours recovery (b) and the kynurenine:tryptophan ratio after 48 hours recovery (c). Data are expressed as mean \pm SEM. (n = 7-8) * p < 0.05, ** p < 0.01, *** p < 0.001 vs control (Newman-Keuls *post hoc* test)

Hippocampal 5-HT and 5-HIAA following DSS-induced colitis

DSS administration induced a significant decrease in hippocampal 5-HT during acute sickness. A one-way ANOVA demonstrated no significant effect of DSS-induced colitis on cortical concentrations of 5-HIAA [$F(2;20) = 1.697$, $p = 0.2086$] (a), however significant effects of DSS-induced colitis on hippocampal 5-HT (b) [$F(2;20) = 3.660$, $p = 0.0442$], and the ratios of 5-HIAA:5-HT (c) [$F(2;20) = 3.270$, $p = 0.0591$] were revealed (see Figure 6.20). 5-HT concentrations were also measured in the posterior cortex and no differences between groups were found (data not shown). A one-way ANOVA also revealed significant differences in IL-6 and IL-1 β mRNA expression in the hippocampus: increased IL-6 (a) [$F(2;21) = 6.113$, $p = 0.0081$] and increased IL-1 β [$F(2;21) = 5.123$, $p = 0.015$] (see Figure 6.21).

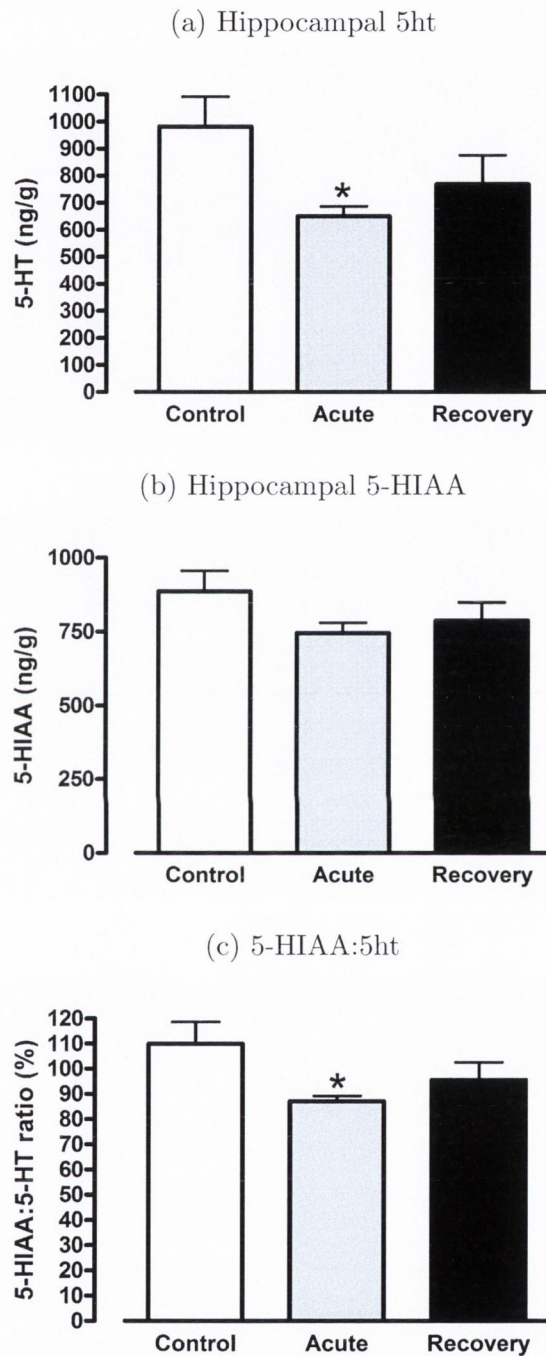


Figure 6.20: **Hippocampal 5-HT and 5-HIAA following DSS-induced colitis**
DSS administration induced a significant decrease in hippocampal 5-HT (a) during acute sickness. This was paralleled by a decrease in the 5-HT:5-HIAA ratio (c). Data are expressed as mean \pm SEM ($n = 7-8$). * $p < 0.05$, ** $p < 0.01$ vs control (Newman-Keuls *post hoc* test)

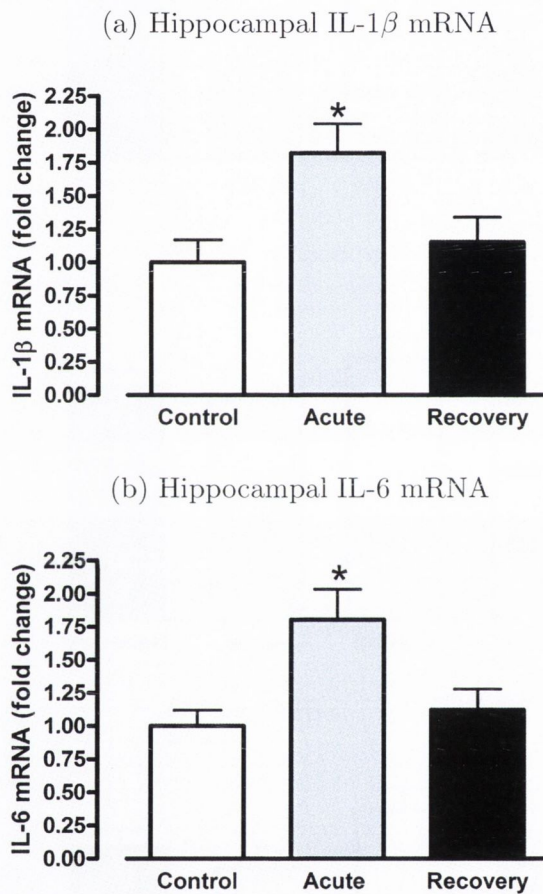


Figure 6.21: Hippocampal IL-1 β and IL-6 mRNA expression following DSS-induced colitis

DSS administration induced a significant increase in hippocampal IL-1 β (a) and IL-6 (b) mRNA expression during the acute phase of colitis. Data are expressed as mean \pm SEM (n = 7-8). * p < 0.05, ** p < 0.01 vs control (Newman-Keuls *post hoc* test)

6.7.5 TNBS-induced colitis was examined for comparative purposes

A number of investigations have reported extra-intestinal DSS distribution following oral administration (for review see Perse and Cerar (2012)). Kitajima et al. (1999) report increased histochemical detection in the liver, spleen, kidney, MLN. Although they do not see evidence of DSS in the brain, lung, heart or thymus samples it was important to ensure that the effects seen in the DSS studies presented here were not DSS specific. The TNBS-model of colitis was used to ensure that DSS itself was not responsible for any of the neuroinflammatory changes reported in the DSS model. An initial TNBS-timecourse study was carried out to analyse behaviour with respect to 3, 8 and 21 days recovery from the TNBS-enema. Due to the severity of the colitis induced by the 6% TNBS dose a further dose-response investigation was carried out to determine a more suitable dose for induction of milder colitis.

Behavioural changes in the open field, and evidence of anhedonia

Openfield testing was carried out 24 hrs prior to euthanasia. A one-way ANOVA revealed a significant effect of TNBS on pathlength (a) [$F(3;14) = 8.179$, $p = 0.0022$] and speed (b) [$F(3;14) = 10.26$, $p = 0.0005$] in the openfield arena. Although one-way ANOVA revealed a significant effect on exploration of the centre of the arena (c) [$F(3;14) = 6.657$, $p = 0.0051$], *post hoc* analysis found this to be between the 3 and 8 day TNBS groups and the 21 day group. TNBS also influenced the rats preference for saccharin solution over water (d).

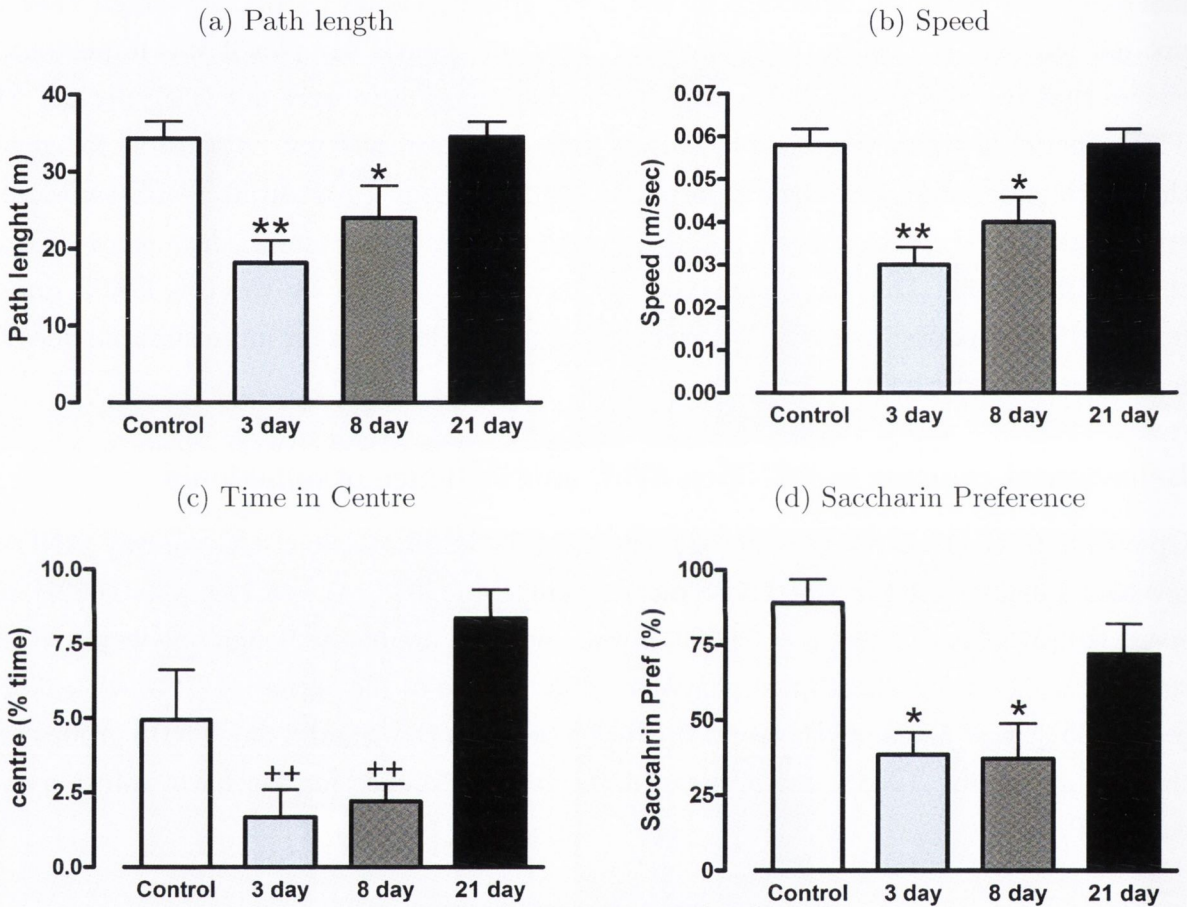


Figure 6.22: TNBS-induced behavioural changes in the open field test, and decreased saccharin preference

TNBS induced significant behavioural disturbances in the openfield: decrease in path-length (a) and speed (b) in the 3 and 8 day groups. No significant decrease in time spent in the centre of the openfield (c) was found versus controls. A significant decrease in saccharin preference was also revealed (d). Data are expressed mean \pm as SEM ($n = 4-5$ per group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control, ++ $p < 0.01$ vs 21 day TNBS group (One-way ANOVA followed by Newman-Keuls *post hoc* analysis)

TNBS induces a significant increase in colonic expression of inflammatory mediators

TNBS-induced colitis is associated with weight loss in the initial days post-enema (see Figure 6.23). TNBS administration is also associated with a significant inflammatory gene upregulation within the colon. Inflammatory marker expression returns to control expression levels by 21 days post-enema. A one-way ANOVA demonstrated a significant effect of TNBS-induced colitis on $\text{TNF}\alpha$, IL-6, IL-1 β , IFN γ and MMP9 mRNA expression in the colon (see Table 6.2). Increased $\text{TNF}\alpha$ [F(3;12) = 28.56, $p < 0.0001$], IL-6 [F(3;12) = 3.73, $p = 0.042$], IL-1 β [F(3;12) = 10.59, $p = 0.0011$], IFN γ [F(3;12) = 5.010, $p = 0.0177$] and MMP9 [F(3;12) = 10.95, $p = 0.0009$] mRNA expression was observed. There were no significant differences in colonic IDO mRNA expression.

Table 6.2: TNBS-induces a significant increase in colonic gene expression of $\text{TNF}\alpha$, IL-6, IL-1 β , IFN γ , and MMP9

	Control	3 day	8 day	21 day
TNFα	1 \pm 0.18	4.31 \pm 0.41 *	10.26 \pm 1.41 ***	2 \pm 0.33
IL-6	1 \pm 0.17	74.80 \pm 31.01	84.03 \pm 16.87	23.57 \pm 21.71
IL-1β	1 \pm 0.28	24.15 \pm 5.22 *	43.07 \pm 9.08 **	7.2 \pm 4.98
IFNγ	1 \pm 0.45	0.92 \pm 0.43	7.22 \pm 2.62 *	1.45 \pm 0.46
IDO	1 \pm 0.18	2.8 \pm 1.47	2 \pm 0.61	1.62 \pm 0.23
MMP9	1 \pm 0.54	70.43 \pm 19 **	44.45 \pm 6.88 *	3.93 \pm 0.89

Data representing mRNA fold change for colonic gene expression 3, 8, and 21 days post TNBS enema administration.. Data are expressed as mean \pm SEM (n=4-5). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs controls (One-way ANOVA with Newman-Keuls *post hoc* analysis). Indoleamine 2,3-dioxygenase (IDO), interferon (IFN), interleukin (IL), matrix metalloproteinases (MMP), tumor necrosis factor (TNF)

(a) Daily Body Weight

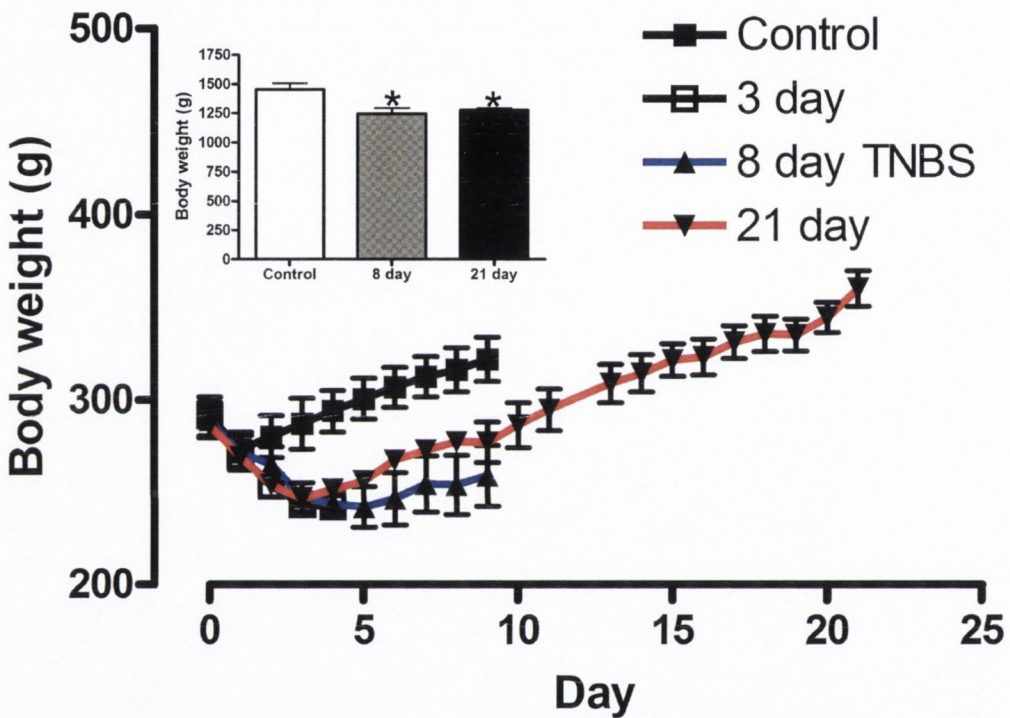


Figure 6.23: Decreased weight gain in the days immediately following 6% TNBS enema. Data represent average daily body weight per group. Day 0 represents the day of fasting, therefore a decrease in weight gain was seen for all groups. Following TNBS enema administration, on day 1, colitic rats have a prolonged decrease in weight gain for approximately 5 days before gradually recovering. The embedded bar graph represents the average AUC from day 1 to 6 on for TNBS and control rats. Data are expressed as mean \pm SEM (n=4-5). * $p < 0.05$ vs controls (One-way ANOVA with Newman-Keuls *post hoc* analysis).

TNBS-induced changes in tryptophan and the kynurenine:tryptophan ratio.

A one-way ANOVA demonstrated a significant effect of TNBS-induced colitis on the circulating concentration of tryptophan [$F(3;14) = 10.77$, $p = 0.0006$] (a) and the kynurenine:tryptophan ratio [$F(3;14) = 10.37$, $p = 0.0007$] (b) at 3 days post TNBS administration. As per DSS-induced colitis TNBS also induced a significant increase in cortical tryptophan. A one-way ANOVA demonstrated a significant effect of TNBS-induced colitis on the cortical concentration of tryptophan [$F(3;14) = 7.938$, $p = 0.0025$] (see Figure 6.24d). Cortical sample weight in this study was too small to allow for kynurenine detection.

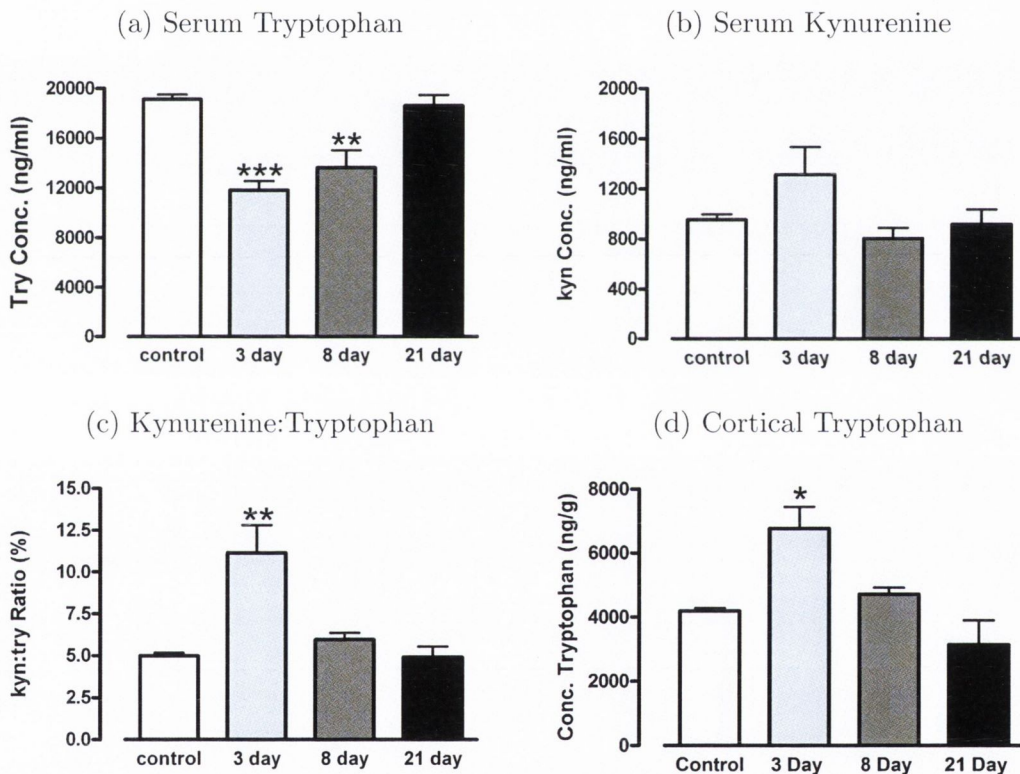


Figure 6.24: **Circulating tryptophan and kynurenine in TNBS-induced colitis** TNBS administration significantly decreased circulating tryptophan (a), increased the kynurenine:tryptophan ratio (c) and increased cortical tryptophan (d). No effect of TNBS on kynurenine (b) concentrations was seen. Data are expressed as mean \pm SEM ($n = 4-5$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control (Newman-Keuls *post hoc* test)

Upregulation of inflammatory gene expression in the cortex and colon

A dose-response study was also carried out to determine a dose which would induce a less severe colitis. As per DSS-induced colitis there was evidence of cortical inflammatory gene upregulation in the TNBS-induced colitis model. A one-way ANOVA revealed a significant increase in colonic IL-6 [$F(3;16) = 3.96$, $p = 0.028$] and $IFN\gamma$ [$F(3;15) = 4.17$, $p = 0.025$] mRNA expression following 6% TNBS administration. 6% TNBS was also associated with a significant increase in cortical IL-1 β [$F(3;16) = 5.56$, $p = 0.0083$] and IL-6 [$F(3;16) = 4.37$, $p = 0.0198$] mRNA expression (see Table 6.3). A one-way ANOVA demonstrated a significant effect of TNBS-induced colitis on iNOS expression in the liver (d) [$F(3;16) = 18.71$, $p < 0.0001$], and in the cortex [$F(3;16) = 6.516$, $p = 0.0144$] following the 6% TNBS enema (see Figure 6.25).

Table 6.3: TNBS-induces a significant increase in colonic and cortical inflammatory gene expression

	Control	1.5%	3%	6%
Colon				
IL-1β	1 \pm 0.26	2.91 \pm 1.75	11.49 \pm 8.21	52.08 \pm 31.75
IL-6	1 \pm 0.04	13.62 \pm 11.64	18.64 \pm 11.19	169.5 \pm 77.43 *
IFNγ	1 \pm 0.188	1.86 \pm 0.67	2.91 \pm 0.84	10.98 \pm 4.9 *
Cortex				
IL-1β	1 \pm 0.12	1.25 \pm 0.21	1.55 \pm 0.34	2.55 \pm 0.36 *
IL-6	1 \pm 0.1	1.38 \pm 0.11	1.84 \pm 0.2	2.81 \pm 0.69 *
IFNγ	1 \pm 0.35	0.63 \pm 0.79	0.45 \pm 0.08	0.54 \pm 0.25

Data representing mRNA fold changes for colonic and cortical gene expression 8 days post 1.5%, 3% and 6% TNBS enema administration. Data are expressed as mean \pm SEM (n=4-5). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs controls (One-way ANOVA with Newman-Keuls *post hoc* analysis). interferon (IFN), interleukin (IL)

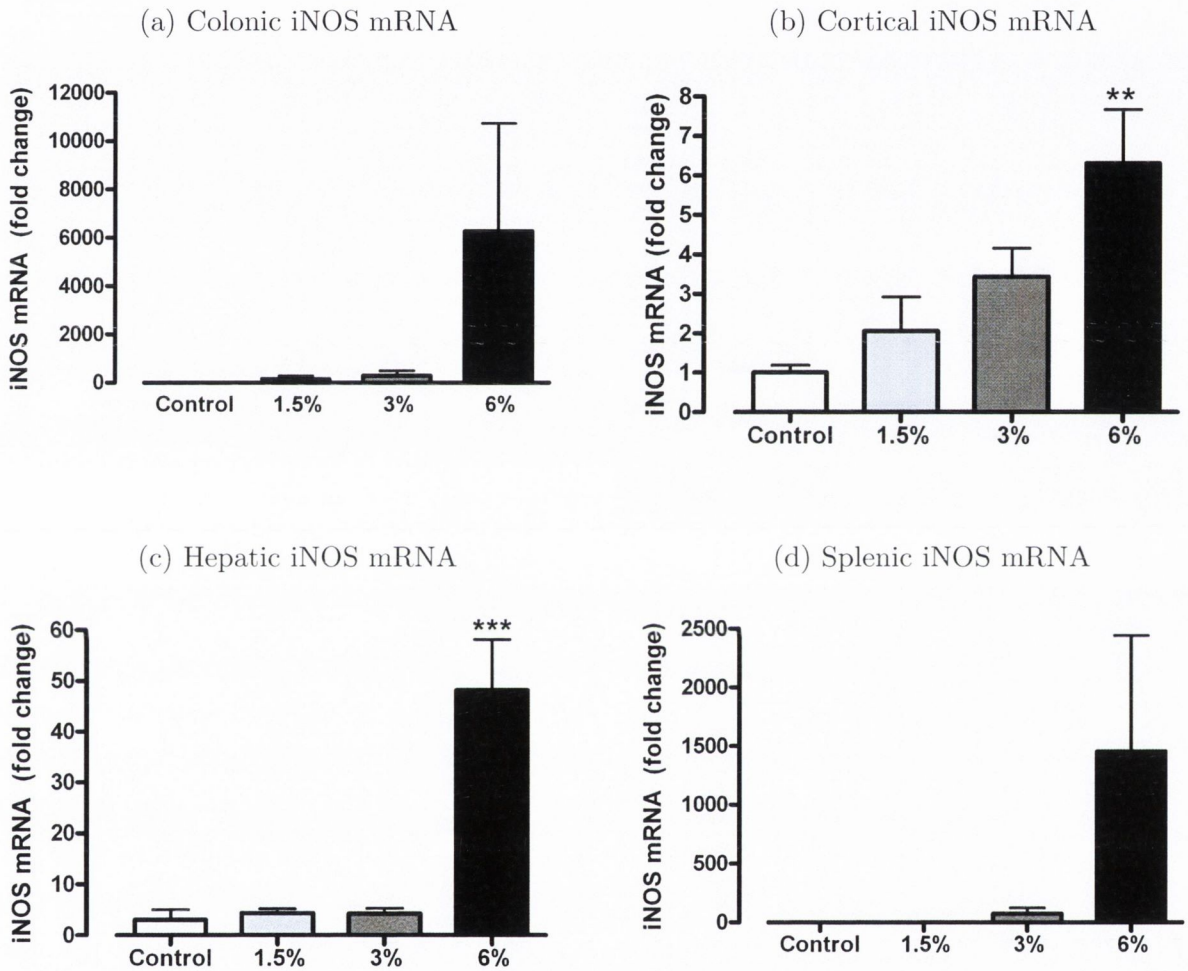


Figure 6.25: **Increased iNOS expression following TNBS-induced colitis**

TNBS-induced colitis results in increased iNOS mRNA expression. TNBS induced a significant increase in cortical (b) and hepatic (c) iNOS expression following the 6% dose only. Colonic (a) and splenic (d) iNOS expression did not reach significance. Data are expressed as mean \pm SEM (n = 4-6 per group). * $p < 0.05$ vs control (One-way ANOVA followed by Newman-Keuls *post hoc* analysis)

6.8 Discussion

6.8.1 Characterisation of the DSS model of colitis

DSS administration induced UC-like symptoms including loose stools, diarrhea, and evidence of fecal or rectal bleeding, as described in the initial paper describing the DSS model (Okayasu et al., 1990). Previous investigations have described similar symptoms, however the extent of colitis was more severe following 7 days DSS administration. Kullmann et al. (2001) reported similar symptoms and weight loss as presented here, however symptom expression occurs earlier, and by day 7 over 50% mortality had occurred. The DSS administration protocol was identical, however a higher molecular weight DSS was administered which most likely accounts for accelerated colitis reported. These overt clinical symptoms were also accompanied by decreased food intake which was associated with decreased weight gain in the colitic animals. Anorectic symptoms have also previously been described in animal models of IBD and in IBD patients (El-Haj et al., 2002; Guariso et al., 2010; Ballinger et al., 1998; Karmiris et al., 2008).

Post-mortem histological analysis of the distal colon revealed significant damage to the ultrastructure of the intestinal wall during this acute colitic phase. These ultrastructural changes, which include crypt damage and inflammatory cell infiltration, are consistent with previous reports of histological examination of DSS-induced intestinal damage (Gaudio et al., 1999). Gaudio et al. (1999) report that structural changes following DSS administration are more closely representative of UC than CD. MMPs have previously been reported to be induced in both DSS-induced colitis and patients suffering from IBD (Santana et al., 2006; Medina et al., 2001; Medina and Radomski, 2006; Medina et al., 2003). Although MMP2 and MMP9 are not specifically markers of inflammation, they are indicative of damage to colonic epithelial layers. These MMPs are members of the gelatinase family of MMPs and are therefore capable of breaking down the extracellular matrix thus rendering the bowel leaky. A leaky bowel increases the risk of bacterial translocation or movement of intestinal toxins across the intestinal wall, which can increase vulnerability to systemic inflammation. Consistent with previous investigations increased MMP9 and MMP2 mRNA and enzymatic activity were observed in the colon during acute DSS-induced colitis.

Following euthanasia, colonic samples were examined to compare inflammatory cytokine expression in acute DSS rats to the cytokine profile reported in colonic biopsies from IBD patients (see chapter 5). Specifically we examined the expression of the pro-inflammatory cytokines IL-1 β , TNF- α , IL-6 and IFN γ in the colon. Increased expression

of IL-1 β , TNF- α , iNOS, and IL-6 was found (* $p < 0.05$). In their review, Rogler and Andus (1998) also report increased IL-1 β and IL-6 in mucosal biopsies of IBD patients, however they report contradictory evidence for the induction of TNF- α . Unlike in human IBD where increased IFN γ is reported, no induction of IFN γ expression was seen in these animals. The lack of IFN γ expression does however correspond with previous research by Dieleman et al. (1998) where no increase in IFN γ , in organ cultures from colons of acute DSS treated animals, was found.

Together these results demonstrate that seven days of 5% DSS administration induces clinical symptoms *in vivo* and evidence of structural damage and inflammatory activation similar to that experienced during moderate to severe IBD in patients. It is therefore a suitable administration protocol and timepoint for investigating behavioural and central nervous system effects associated with acute intestinal inflammation.

6.8.2 Behavioural disturbances in DSS-induced colitis

In the current investigation DSS-induced colitis is achieved through 5% DSS administration in the rat's drinking water over a period of 7 days. This method of administration makes the colitis induction process relatively stress-free for the rats, compared to the TNBS model which require anaesthetic administration and fasting. For this reason it was chosen as the most appropriate model for studying behavioural changes. A second experiment involving DSS exposure, with behavioural analysis during the acute inflammatory phase, and then a recovery period with further behavioural testing was carried out. At day 6 once food intake had decreased and symptom expression was present, rats were exposed to a standard circular openfield test in which exploratory and locomotor behaviour was recorded. At this acute inflammatory stage colitic rats expressed both locomotor and exploratory deficits. This is consistent with sickness behaviour previously reported in LPS and poly I:C studies (Dantzer et al., 2011; Gibney et al., 2012). Dantzer et al. (2011) suggest that for inflammatory-induced behavioural assessment following LPS administration there is an initial sickness behaviour response in the first two to six hours post injection, which is followed by a later depressive-like phenotype. Colitic rats also spent significantly more time grooming. Increased grooming in the openfield test has previously been reported following exposure to acute stress (Daniels et al., 2008). However, due to the nature of symptoms in this model it is possible that the increased grooming is directly related to the increase in diarrhea or rectal bleeding.

Further characterisation of behavioural abnormalities during the recovery phase of colitis was carried out. On day 4 of recovery, once food intake had returned to control

levels and rats were gaining weight, further behavioural testing commenced. Rats were once again exposed to the openfield test and locomotor and exploratory behaviour was assessed. The openfield was modified slightly, with the inclusion of an object, to minimise the possibility of acclimatisation to influence behavioural exploration. Previous differences in speed and pathlength had returned to normal levels, however grooming remained higher in the DSS rats when compared to the control group. It should be noted here that this is despite no evidence of diarrhea or rectal bleeding persisting at this time. One day post openfield testing rats were tested for marble burying behaviour. Marble burying is a commonly used behavioural test in mice. It is often used to assess anxiety-like behaviour, whereby increased burying is suggestive of increased anxiety, and anxiolytic drugs can reverse increased marble burying (Deacon, 2006). In this study a modified version of the marble burying test for rats was carried out. Unexpectedly rats previously exposed to DSS buried significantly less marbles. This is despite locomotor having returned to normal when the rats were tested in the openfield on the previous day. The reason for such a decrease in the number of marbles buried is not clear however a number of previous studies have also reported decreased marble burying related to functional changes within the CNS (Mosienko et al., 2012; Browne et al., 2012; Deacon and Rawlins, 2005). Mosienko et al. (2012) report decreased marble burying, independent of locomotor activity deficits in the openfield, associated with decreased serotonin in tryptophan hydroxylase negative mice (Thp-/-). Chronic dietary tryptophan depletion, which is associated with decreased circulating and central tryptophan concentrations, also reduced marble burying (Browne et al., 2012). Finally, hippocampal lesions result in decreased marble burying (Deacon and Rawlins, 2005). It is therefore possible that functional changes within the CNS, or within the hippocampus, of colitic rats are responsible for a reduction in normal rat digging/burying activity. One day following the marble burying test rat behaviour in the light/dark box was examined. No difference in time spent in the light chamber was seen, however the DSS rats did have less head pokes into the light chamber, which is possibly indicative of decreased interest in the novelty of it (Marino et al., 2005). A significant increase in the number of fecal boli in the DSS group was seen. Increased boli count is usually indicative of increased anxiety, however, again due to the nature of the model, it is difficult to draw any behavioural conclusions based on fecal boli counts (Ennaceur et al., 2006). Finally, rats were exposed to an EPM for 5 min. This was carried out under normal room bright lighting. Both control and DSS rats demonstrated an equal fear of the open arms of the EPM. No differences in time spent in any of the zones was observed between the two groups when whole open and closed arms were analysed. However the HVS software allows for further subdivision of the maze allowing the end corners of the closed

arms (25% of the arm) to be analysed separately. When these 'safe zones' were analysed a significant difference in the time spent by DSS rats versus controls was observed, whereby colitic rats spent over 10% more time in the corners of the closed arm.

Overall the behavioural analysis demonstrates behavioural differences in rats exposed to DSS. This is especially true during the acute sickness phase of DSS administration but some mild behavioural differences persist throughout the recovery days once feeding and locomotor measures have returned to normal.

6.8.3 Evidence for neuroinflammation during acute DSS-induced colitis

Based on the symptomatic and colonic similarities observed in the DSS model and moderate to severe UC, along with the confirmation that behavioural disturbances are present cortical samples were examined for gene expression changes. Recent literature has shown that induction of peripheral cytokines can lead to central cytokine changes. Previous literature has examined IL-1 β , TNF- α , IL-6 and IFN γ for their involvement in depression and as possible inducers of sickness behaviour in animals (Harden et al., 2006, 2008). As per the colon samples, cortical samples were examined for gene expression changes of IL-1 β , TNF- α , IL-6, IFN γ , and iNOS. No significant differences in IL-1 β , TNF α or IFN γ were found in the cortex, however significant increases in IL-6 and iNOS mRNA expression were revealed. Both IL-6 and iNOS have been implicated in CNS pathologies.

Abnormalities in gene expression of GFAP was also revealed following PCR analysis. GFAP is present in astrocytes within the CNS and previous research has demonstrated decreased GFAP expression to be representative of decreased astrocytic activation. Decreased astrocytic activation is particularly interesting here as decreased GFAP is emerging as a potential marker of depression, with reported reductions in the density and number of glial cells reported in fronto-limbic brain regions in major depression (Rajkowska, 2003; Rajkowska and Miguel-Hidalgo, 2007).

6.8.4 Evidence for persistent inflammation following seven days recovery from DSS

Due to the persistence of behavioural differences beyond the acute DSS-induced inflammatory phase a further study was carried out to investigate whether prolonged mRNA expression differences exist between the colitic and control rats. Rats were allowed seven days recovery and then euthanised to match the timing of the final behavioural test.

As with the acutely colitic rats, pro-inflammatory cytokines IL-1 β , TNF- α , IL-6, IFN γ , and iNOS were examined in the colon and frontal cortex following recovery. In contrast to the acute phase where no induction of IFN γ mRNA was seen, it was significantly increased in the colon of the Wistar rats following seven days recovery. This delayed increase in IFN γ corresponds with research by Dieleman et al. (1998) in which they observed no increase in IFN γ in organ cultures from colons of acute DSS treated animals, but reported significant production of IFN γ in organ cultures from colons of animals 14 days after stopping DSS treatment. Following seven days recovery pro-inflammatory cytokines IL-1 β and IL-6 remained increased in the colon. Prolonged increases in cortical iNOS and small increases in IL-6 expression were also revealed following seven days recovery. However, the decreased GFAP expression found during the acute phase of DSS-induced colitis had returned to control levels.

6.9 TNBS-induced colitis induces similar behavioural and neuroinflammatory changes as DSS-induced colitis

Despite the potential stress-induced brain changes that could result from the TNBS colitis induction method this model was briefly assessed to ensure that the behavioural and neuroinflammatory changes observed in the DSS model were in fact due to intestinal inflammation and not to the DSS itself. Openfield behaviour for the 3 day and 8 day TNBS groups revealed similar changes in pathlength and speed to those in acute DSS-induced colitis. Although the time spent in the centre of the arena was not decreased compared to the control group. As TNBS is not administered in the drinking water, it was possible to measure saccharin preference. Saccharin preference is used as an indication of anhedonia in rodents. TNBS-induced inflammation resulted in a significant decrease in saccharin preference compared to the vehicle controls. As per DSS-induced colitis TNBS also resulted in a significant increase in inflammation within the colon. The most significant increases in mRNA expression were observed at 3 and 8 days post enema (IL-6, IL-1 β , TNF α , IFN γ) This is also consistent with previous reports on inflammatory gene expression in the TNBS model of IBD (Alex et al., 2009). Due to the severity of colitis induced by 6% TNBS a second dose response study was carried out to determine a suitable dose for future intervention studies. In this study increased pro-inflammatory gene expression was also examined in the cortex. Analysis revealed a significant increase in

cortical IL-1 β and IL-6 mRNA expression only after the highest 6% dose. Increased central IL-1 β and IL-6 expression in the TNBS model of colitis is consistent with previous studies investigating TNBS-induced colitis (Baticic et al., 2011; Wang et al., 2010; Alhouayek et al., 2011).

6.9.1 Evidence for persistent iNOS expression in the hippocampus and hypothalamus, and significant correlation between iNOS mRNA expression in the brain and periphery

The largest DSS-induced increase in central mRNA expression found following the recovery period was in cortical iNOS expression, which remained over 3 fold higher in the colitic rats. iNOS has previously been reported to be increased in a number of pathologies of the CNS and in the substantia nigra of colitic rats during the acute inflammatory phase (Villaran et al., 2010). Due to the prolonged expression of iNOS in the frontal cortex, other brain regions relevant to depression and sickness were examined: hippocampus and hypothalamus. iNOS mRNA expression analysis revealed prolonged increases in both these regions. Further analysis of hepatic and splenic iNOS mRNA expression revealed increases at both timepoints. Significant correlations between iNOS expression levels in each of these regions versus the cortex and colon were also revealed, and correlations between colonic and cortical iNOS and the spleen and liver were also present.

The main mechanisms of inflammatory-induced iNOS induction described in the literature are through NF κ B translocation to the nucleus, and through IFN γ -induced IRF1 which subsequently induces iNOS gene transcription. Both nuclear NF κ B activity and IRF1 mRNA expression were analysed in cortical samples from acutely colitic rats. Neither of these were upregulated at this time, therefore do not appear to be responsible for the increased iNOS mRNA expression. Hypoxia is also reported to be involved in iNOS upregulation. HIF-1 α is an inducer of iNOS gene expression during hypoxia and therefore was examined as a potential mechanism of iNOS upregulation following DSS administration. Again no differences in HIF-1 α were seen despite significant increases in colonic HIF-1 α being present. Future studies will therefore be required to determine the molecular mechanism involved in increased central iNOS expression during DSS-induced colitis.

Immunohistochemical analysis of brain slices for iNOS protein expression revealed a significant increase in iNOS surrounding the ventricles rather than throughout the brain structures. The most significantly stained areas were around the blood vessels, which is suggestive of mediators within the circulation leading to increases in the iNOS expression in these cells. In support of this suggestion, a previous study has demonstrated the ability

of IBD patient serum to induce iNOS expression in human umbilical vein endothelial cells (Palatka et al., 2006). Although iNOS is not significantly upregulated throughout the brain, its ability to produce very high levels of NO and the ability of NO to diffuse freely between cells makes even low level increases a potential threat to normal CNS function.

Implications of iNOS expression for anxiety/mood disturbances

Previous investigations report very high NO production following iNOS induction. This can be neurotoxic, and its expression has been implicated in a number of neuropathologies (see section 2.3.4). Recent evidence also indicates that iNOS is implicated in some of the depressive behaviours reported in various animal models. One mechanism of inducing depression/anxiety related symptoms in rodents is through exposure to chronic stress. Commonly used approaches are the chronic unpredictable mild stress (CUMS), repeated restraint stress, maternal separation, or social defeat protocols. As well as inducing depressive symptomology including weight changes, anhedonia, exploration changes, increased immobility in FST and TST (Wang et al., 2008), these chronic stress protocols induce iNOS expression in many brain regions (Khovryakov et al., 2010; Yamaguchi et al., 2010). Wang et al. (2008) demonstrated that CUMS-induced body weight and behavioural disturbances are inhibited by repeated injections of aminoguanidine (AG) directly into the hippocampus. Montezuma et al. (2012) also demonstrated antidepressant-like effects of the preferential iNOS inhibitors AG and 1400W in the forced swim test (FST) in C57BL/6 mice. In further stress studies Harvey et al. (2004) demonstrated both increased acute and prolonged NOS activity in the hippocampus of rats exposed to various stressors. They also showed that this increased NOS activity could be inhibited in full by administration of the inhibitor of NOS AG, but not by the nNOS specific inhibitor 7-NI.

Zeni et al. (2011) demonstrated that administration of plant extract *Aloysia Gratissima* to mice resulted in antidepressant like effects in the FST and tail suspension tests (TST). This effect was inhibited by pre-treatment with L-arginine, and analysis of hippocampal slices demonstrated inhibitory effects of *Aloysia Gratissima* on glutamate-induced iNOS protein expression. In a model of gastric ulcers induced by cold restraint stress (CRS) Mei et al. (2011) demonstrated both gastroprotective and antidepressant effects of zinc(II)-curcumin complex in rats. Zinc(II)-curcumin dose-dependently decreased the Ulcer index and iNOS mRNA expression following CRS, as well as inducing antidepressant effects comparable to fluoxetine at 34mg/kg and 68mg/kg in the TST.

The NMDA receptor antagonist ketamine is currently used in veterinary medicine during general anaesthesia and as a recreational drug of abuse. Aside from its illicit use it

has gained much attention in terms of its apparent antidepressant properties. In major depressed patients ketamine infusion induced a significant decrease in HAM-D scores in following 72 hours (Berman et al., 2000; Murrough, 2012). Its antidepressant effects in animal models have also been investigated and shown to induce both behavioural and molecular changes (Murrough, 2012). Interestingly in rodents ketamine has also been shown to inhibit LPS-induced iNOS in peripheral tissues including the duodenum, jejunum, ileum, colon, kidney, lung, liver, spleen, and stomach (Helmer et al., 2006; Suliburk et al., 2005b,a). Unfortunately, to my knowledge no studies have determined the potential of ketamine to inhibit central iNOS expression.

The hypothesis that iNOS mediates some aspects of depression, or anxiety, is further supported by behavioural changes following immune activation; LPS administration induces 'sickness behaviour' which mirrors many depressive symptoms. LPS also induces an inflammatory response involving cytokine expression and increased peripheral and systemic iNOS (Helmer et al., 2006; Fu et al., 2010). Conventional antidepressants such as the SSRI fluoxetine can suppress the intracranial LPS induction of iNOS, as well as decreasing ROS generation and oxidative stress (Chung et al., 2010). iNOS mRNA and protein inhibition was also reported with a tricyclic antidepressant, Amitriptyline, in the cerebellum and hippocampus in a model of neuropathic pain (Farghaly et al., 2012).

6.9.2 A role for the kynurenine pathway?

Tryptophan, an essential amino acid required by the body for protein synthesis, and precursor to serotonin and the kynurenines, has been implicated in both depression and IBD. Tryptophan and kynurenine pathway metabolites have been poorly investigated in human IBD patients or animal models, however some circulating changes have been reported. In CD patients there is evidence of decreased circulating tryptophan and increased kynurenine (Torres et al., 2007). In this DSS study, during the acute inflammatory phase of DSS the same profile was seen: total tryptophan concentration was significantly decreased, this was associated with increased kynurenine concentrations and an overall increase in the kynurenine to tryptophan ratio. Although these results match results seen in the patient study, they are not consistent with results published by Schicho et al. (2010), in which increased serum tryptophan was increased. Although it is not specified in the paper it is likely that free tryptophan is being reported by Schicho et al. (2010), as the sample preparation involves a double filtration step through 3-KDa filters for protein removal. As tryptophan exists in a largely albumin-bound state in the circulation, this preparation step is completely eliminating the bound tryptophan pool, and explains the difference with the

current investigation.

A number of mechanisms can result in decreased tryptophan. Diegelmann et al. (2012) demonstrated that decreased tryptophan availability causes decreased bacterial growth in cells co-cultured with E-coli. Therefore it is possible that the prolonged decrease in tryptophan is a mechanism to prevent sepsis in the DSS-induced colitis group. PCR analysis of colonic and splenic IDO expression did not reveal any differences between controls and colitic rats which might account for this. However, TDO expression was increased in both the DSS and TNBS models. Increased TDO expression may therefore account for some of the observed differences however further analysis of TDO activity at various timepoints post DSS or TNBS administration will need to clarify this. It is also possible however, that the decreased circulating tryptophan is simply due to decreased tryptophan absorption through the intestinal wall as there is an increased tryptophan concentration in the colonic samples during acute colitis and following the 2 day recovery period. Finally, it is possible that decreased circulating tryptophan is due to increased tryptophan utilisation in the production of acute phase proteins by the liver. HPLC analysis of the peripheral organs revealed that during the acute inflammatory phase, when colonic tryptophan concentrations are highest, there is no deficit in the splenic or hepatic tryptophan concentrations. However, there is a significant decrease in splenic tryptophan following two days recovery from DSS. Positive acute phase proteins, such as CRP, are formed during the acute phase response. This acute phase response is associated with immune activation, as well as anorexia and changed metabolism. The increased protein synthesis can put a strain on the demands of a number of amino acids including tryptophan. If the increased demands are not being met by dietary tryptophan intake the body mobilises protein stores from within muscles (Gruys et al., 2005). The decrease in splenic tryptophan following two days recovery, therefore indicates a possible strain on tryptophan availability due to the prolonged immune activation and decreased food intake induced by DSS.

After two days recovery the kynurenine concentrations and ratio return to control levels however, the circulating tryptophan concentrations remain significantly decreased compared to controls. Interestingly, decreased circulating levels of tryptophan have also been associated with depression, which is suggested to be due to decreased tryptophan availability for serotonin synthesis in the CNS or due to kynurenines themselves (Williams et al., 1999; Capuron et al., 2002; Neumeister, 2003). Associations between major depression and decreased tryptophan were also reported by Hughes et al. (2012) without increased circulating kynurenine concentrations. This suggests that the decreased tryptophan could be as important in terms of behavioural changes as increased kynurenine. Although decreased circulating tryptophan was found in acute DSS-induced colitis, un-

like the suggestion in the literature that this may decrease the availability tryptophan for serotonin production in the CNS, here we revealed an unexpected increase in tryptophan in the frontal cortex. This increased central tryptophan was confirmed in the hippocampus and the hypothalamus in the same rats. Increased central tryptophan concentrations have been frequently reported in rodents exposed to psychological and immune stressors (Dantzer et al., 2008; Curzon et al., 1972; Dunn and Welch, 1991). Previous reports of hypothalamic-pituitary-adrenal axis activation and increased circulating corticosterone have been reported in animal models of colitis (Greenwood-Van Meerveld et al., 2006; Kojima et al., 2002; Porcher et al., 2004). Therefore it is possible that the increased central tryptophan concentration is related to induction of a stress response, as well as being due to immune activation. This may be as a result of increased free tryptophan concentrations within the circulation, as reported by (Schicho et al., 2010)

Further analysis of the posterior cortex following DSS administration also revealed a significant increase in kynurenine following two days recovery. This increased kynurenine could have implications for altered CNS function as kynurenine metabolites are known neuromodulators. PCR analysis of cortex did not reveal any significant increase in IDO at either timepoint, however the majority of the samples had undetectable IDO levels (data not shown). It is possible that the timepoint for IDO upregulation occurs between the acute and two day recovery timepoint, with the increased ratio following recovery being evidence of increased IDO protein activity. Gibney et al. (2012) report increased tryptophan and increased kynurenine following poly I:C administration to Wistar rats. In their investigation IDO upregulation occurred 18 hours prior to the significant increase in kynurenine concentrations within the cortex. It is therefore possible that in DSS-induced colitis there is an increase in IDO mRNA expression following 24 hrs recovery which has returned to undetectable levels by 2 days recovery.

Again for comparative purposes the TNBS-induced colitis model was examined for circulating and cortical tryptophan concentrations. Analysis revealed a similar decrease in circulating tryptophan and increase in kynurenine concentrations suggesting that any changes seen in the DSS model are in fact related to the colonic inflammation and are not a result of low level absorption of DSS itself. As per the DSS model there was also an increase in cortical tryptophan 3 days post TNBS administration. Unfortunately the cortical tissue weight was too low to measure the low levels of kynurenine expressed in the brain.

6.9.3 Decreased hippocampal 5-HT during acute DSS-induced colitis

Tryptophan is the precursor to 5-HT in the serotonin pathway. Therefore following on from circulating and central tryptophan analysis monoamine concentrations were investigated in the CNS. Serotonin is of particular importance as it is relevant to both aspects of this study due to its presence in both the enteric nervous system and the central nervous system. In the intestinal mucosa it is produced by the enterochromaffin cells and mast cells. Kadowaki et al. (1996) demonstrated that it is responsible for increased fluid secretion which leads to diarrhea in 85% to 100% of treated mice. Previous research has demonstrated increased serotonin levels in the distal colon of DSS-treated animals (Oshima et al., 1999). Their results also demonstrated significant differences in serotonin concentrations in different parts of the bowel and showed that significant changes in serotonin concentrations only occurred in the distal and proximal colon of acute DSS rats.

In the brain monoamines are widely believed to be associated with mood. In particular decreased availability of serotonin is thought to be a cause of depressive symptoms. Decreased hippocampal serotonin concentrations were found during the acute phase of DSS. Altered serotonin within the hippocampus is highly relevant to depression as the hippocampus represents part of the cortical-limic neurocircuitry which is implicated in mood disorders (Piser, 2010). Rasheed et al. (2008) report a large decrease in total hippocampal serotonin (approximately 700 ng/g in controls vs approximately 200 ng/g following stress) in mice following 7 days exposure to two stressors of various intensity daily in an unpredictable manner. The decrease in serotonin was associated with an increased hippocampal concentration of IL-6 (approximately 4 fold increase) and IL-2 (approximately 2 fold increase). They suggest that the increased interleukin levels affected by physical and psychological stressors might provoke monoamine alterations in brain regions which mediate CNS-immune interactions. As per their reports, decreased serotonin in the current investigation was associated with increased hippocampal IL-6 and IL-1 β mRNA expression.

6.9.4 Conclusion

In Chapter 5, as per previous reports, a significant association between the DAI index and HAM-A and HAM-D scores was revealed. High DAI was also associated with increased pro-inflammatory cytokine mRNA expression in tissue biopsies and increased circulating concentrations of IL-6 and IFN γ . In the current investigation both DSS and TNBS

induced inflammatory cytokine expression within the colon and cortex, decreased circulating tryptophan, and behavioural disturbances. Behavioural characterisation of the DSS-induced colitis model revealed prolonged behavioural deficits beyond the sickness-induced decreased locomotion and feeding. These behavioural changes are indicative of a depressive or anxious response, however this will require further characterisation and pharmacological interventions to confirm. DSS-induced colitis was also associated with significant increases in IL-6 and iNOS, and decreased hippocampal serotonin concentrations, each of which represent potential mechanisms mediating behavioural changes.

Chapter 7

Brain-Gut interactions and influence of stress on DSS-induced colitis and related systemic and central inflammatory biomarkers

7.1 Introduction

In his pioneering research on stress Selye (1936) demonstrated that rats develop a typical response to a wide range of noxious stimuli, which is independent of the nature of the damaging agent. He exposed rats to cold shock, surgical injury, production of spinal shock, excessive muscular exercise, or intoxications with sub-lethal doses of a range of drugs including adrenaline, atropines, and morphine. In each case within 48 hr a consistent physiological response occurs which includes: decreased spleen, thymus, liver, and lymph gland size, intestinal erosion formation, and loss of cortical lipoids and chromaffin substance from the adrenals. Within this first report Selye (1936) also recognises the existence of acute stress and chronic stress and describes three stages of response following exposure to noxious stimuli. The first involves acute stress exposure which results in the physiological changes listed above, following repeated exposure to stress a second phase occurs which renders the rats resistant to the stressor and physiological changes return to normal, finally after chronic exposure for one to three months resistance is lost, and physiological changes similar to those described following initial acute stress return.

Since 1936 the neuroendocrine mechanisms involved in the physiological response to psychological and physical stress have been widely investigated leading to the discovery of

the HPA-axis. The HPA axis is activated in response to stressors, and involves communication between the hypothalamus, pituitary gland and the adrenals in the kidney. Upon stimulation the hypothalamus releases corticotrophin releasing hormone (CRH), this in turn stimulates the secretion of adrenocorticotrophic hormone (ACTH) from the pituitary glands which enters the circulatory system and ultimately leads to the release of glucocorticoids from the adrenal glands (Tasker and Herman, 2011).

Previous literature suggests that as psychological stress represents a threat to the normal functioning of the enteric nervous system it therefore could have a negative affect on IBD progression and relapse (Hart and Kamm, 2002; Sajadinejad et al., 2012; Levenstein et al., 2000; Mawdsley et al., 2006). Intestinal barriers including mucus/fluid barriers, microbiological barriers and immunological barriers are all vulnerable to stress and their breakdown could therefore increase invasion of noxious agents and lead to inflammation (for review see Hart and Kamm (2002)). Based on this it has also been suggested that stress could be damaging for IBD patients, however experimental results to date remain unclear. In a human study of 62 patients diagnosed with ulcerative colitis Levenstein et al. (2000) concluded that long term perceived stress but not short term stress increases the risk of exacerbation in UC. While Mawdsley et al. (2006) demonstrated in UC patients that acute psychological stress induced systemic and mucosal pro-inflammatory responses, which they suggest could lead to stress induced exacerbation of UC in ordinary life. However in disagreement with these two studies a follow-up study of national registers reported no association between major psychological stress (the loss of a child) and the development of IBD in young-to-middle-aged Danish adults. The impact of psychological stress therefore remains controversial, and may be more heavily weighted in favour of the patients own perception of stress, and/or ability to adapt to stress, than the severity of the stressors themselves.

A number of investigations have examined the impact of stress on the severity, prolongation, and relapse of colitis. The general agreement throughout the animal literature indicates that chronic psychological stress negatively impacts on chemical-induced colitis (see chapter 3). The majority of these studies have focused on the influence of stress on symptom expression, histological examination of the damaged colon, weight changes, and colonic MPO and MDA expression. As stress is reported to be immunomodulatory this study aims to specifically investigate the influence of repeated mild stress on inflammatory cytokine expression within the colon, and whether this affects the previously reported increased iNOS or IL-6 expression in the brain (as reported in chapter 6).

7.2 Aims and Objectives

The aim of this chapter was to investigate the immunomodulating properties of stress on the development of colitis in the DSS model of IBD. The main objectives were to:

1. determine the effect of repeated restraint stress on the severity of DSS-induced colitis.
2. determine the effect of repeated restraint stress on recovery following DSS administration
3. determine the ability of restraint stress prior to DSS administration to accelerate symptom expression and enhance inflammation

7.2.1 Methods: Restraint stress in combination with colitis

7.3 Animal husbandry

Male Wistar rats (175-210 grams) were obtained from the Bioresources Unit in Trinity College Dublin, and housed in hard-bottomed polypropylene cages with wood shavings as bedding. Animals were housed 4 per cage on arrival, under standard laboratory conditions, with an ambient temperature of 20-24° Celsius (C) and a 12 h light: 12 h dark cycle (lights on 08.00, lights off: 20.00). Animals had free access to food and water and were fed a standard laboratory diet (Red Mills, Ireland). A record of body weight was maintained as a general indication of the health and well-being of each animal throughout each study. All in-vivo work was approved by the Animal Ethics Committee Trinity College Dublin. Prior to beginning each experiment animals were weight matched across groups. Food intake, fluid intake and weight were recorded daily in each experiment.

7.3.1 Chronic restraint stress

Male Wistar rats were exposed to 2 h restraint stress for 7 consecutive days. Rats were restrained in well ventilated, adjustable, plexiglass restrainers (35 cm length x 7 cm internal diameter), normally used for collecting tail blood (Harvard Apparatus, UK). The restrainer was then put back in the rats home cage under standard laboratory conditions, brightly lit with an ambient temperature of 20-24°, for 2 h.

DSS experiment 1: DSS and repeated restraint stress

Male Wistar rats were housed two per cage and divided into four groups of 6 rats each. Groups 3 and 4 received 5% DSS, MW 36,000-44,000) in their drinking water (tap water) for seven consecutive days, while groups 1 and 2 received normal tap water over the course of the study (Vehicle). Groups 2 and 4 were exposed to 2hr daily restraint stress over the course of the study. The first stress exposure occurred 24 hours following commencement of 5% DSS (see Figure 7.1a). Rats were observed daily for fluid intake, weight changes, and for major symptoms of colitis such as loose stools, diarrhea, and rectal bleeding. Rats were euthanised 6hr following the final exposure to restraint stress.

DSS experiment 2: DSS followed by repeated restraint stress during recovery

Male Wistar rats were housed two per cage and divided into four groups of 6 rats each. Groups 3 and 4 received 5% DSS, MW 36,000-44,000) in their drinking water (tap water)

for seven consecutive days, while groups 1 and 2 received normal tap water over the course of the study (Vehicle). Following the 7 days DSS administration Groups 2 and 4 were exposed to 2hr daily restraint stress for a further 7 days (see Figure 7.1b). Rats were observed daily for fluid intake, weight changes, and for major symptoms of colitis such as loose stools, diarrhea, and rectal bleeding. Rats were euthanised 6 hr following the final exposure to restraint stress.

DSS experiment 3: repeated restraint stress and DSS administration

This study involves a slightly modified timeline to DSS experiment 1 (see section 7.3.1). Male Wistar rats were housed two per cage and divided into four groups of 6 rats each. Groups 3 and 4 received 5% DSS, MW 36,000-44,000) in their drinking water (tap water) for seven consecutive days, while groups 1 and 2 received normal tap water (Vehicle) over the course of the study (see Figure 7.1c). Groups 2 and 4 were exposed to 2hr daily restraint stress over the course of the study. Unlike DSS experiment 1, here the first two hour stress exposure was immediately prior to DSS administration beginning. As per previous studies rats were observed daily for fluid intake, weight changes, and for major symptoms of colitis such as loose stools, diarrhea, and rectal bleeding. Rats were euthanised 6hr following the final exposure to restraint stress.

7.3.2 Disease Activity Index

A Disease Activity Index (DAI) was constructed to examine DSS-induced colitis severity using the criteria of weight loss, stool consistency and rectal bleeding. Weight loss was scored on a scale of 0-4; 0 for no weight loss, 1 for 1-5%, 2 for 6-10%, 3 for 11-15% and 4 for >20%. Stool consistency was scored 0-4; 0 for normal stools, 1-2 for loose stools and 3-4 for diarrhoea. Rectal bleeding was scored 0-4; 0 for none, 1 for light bleeding, 2 for moderate bleeding, 3 for heavy bleeding and 4 for very heavy bleeding. Scores for each of the 3 criteria were averaged to give the Disease Activity Index score (Cooper et al., 1993).

7.3.3 Histology

Distal colon samples (1-2 cm) were dissected and placed in Camoys Solution (60% alcohol; 30% chloroform; 10% glacial acetic acid) for 2-3 h, then transferred to 100% ethanol until embedding. After embedding (as per Section 4.3), samples were sectioned with a microtome at a thickness of 10 μm (Leica Microtome). Microtomed slices were stained using hematoxylin and eosin stain. Slides were mounted with cover slips using Di-N-Butyl

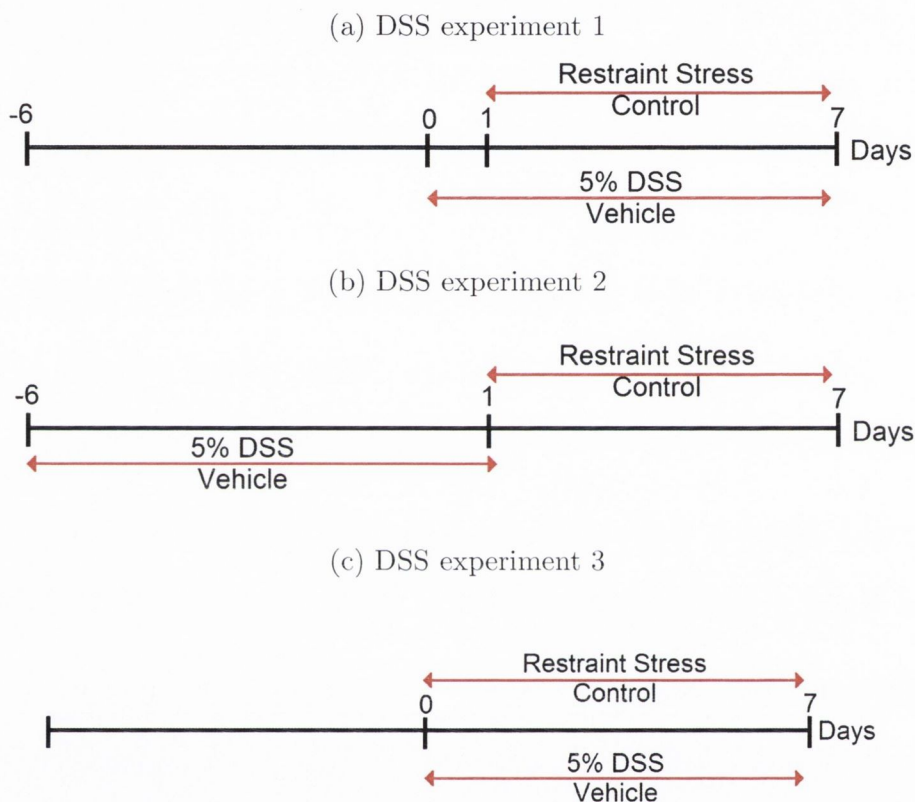


Figure 7.1: **Experimental design for examining the influence of stress on DSS-induced colitis**

Experimental design for DSS experiment 1 (a), DSS experiment 2 (b), and DSS experiment 3 (c). In each experiment rats were exposed to 7 days 5% DSS in their drinking water. In each study two groups of rats were exposed to 2 hr daily restraint stress as per timecourse above. Rats were euthanised on day 7 in each of the experiments. Euthanasia occurred 6 hr following the final exposure to restraint stress.

Phthalate in Xylene (DPX) mounting medium. Slides were digitally recorded and analysed using an Olympus BX51 video-camera microscope for histological scoring. Histological scoring was based on modified version of the protocol outlined by Cooper et al. (1993).

7.3.4 Zymography

Tissue MMP2 and MMP9 protein activity was assessed by zymography as described previously in section 4.6. Briefly plasma samples were added to 2ml round-bottomed tubes containing lysis buffer (150mM NaCl, 50mM tris-HCl pH 8.0, 1% v/v NP-40, 50 μ l/10 ml Phosphatase Inhibitor Cocktail I (Sigma-Aldrich)). Samples were then vortexed vigorously, homogenised with a hand held polytron, and centrifuged at 14,000 rpm at 4°C for 15 min. Supernatants were transferred to clean Eppendorf tubes and stored at -80°C. Prior to analysis samples were equalised to 2000 μ g/ml. 18 μ l of sample and 6 μ l of loading buffer were added to each well, and electrophoresis (voltage was set to 150V, current was set to 300 mA) was run for 2 h and 30 min. Gels were washed and left to incubate for 2 nights in zymography buffer at 37°C. Following incubation gels were stained for 3 h, then destained in destaining solution. Gelatinase activity of MMP9 and MMP2 was analysed by detection of intensity of band clearing at the same position as in the positive control. A calibrated densitometer (GS-800 Bio-Rad) and Quantity One analysis software (Version 4 Bio-Rad) was used to measure intensity x mm.

7.3.5 Real-time PCR analysis of mRNA expression of target genes in colonic biopsies and whole blood samples

RNA was isolated from tissue samples using a Nucleospin RNA II kit (Macherey-Nagel, Germany) according to the kit protocol. Following RNA quantification and equalisation, cDNA was synthesised using a cDNA archive kit (High capacity cDNA reverse transcription kit, Applied Biosystems, UK). Gene expression analysis was conducted using real-time PCR employing Taqman® Gene Expression Assays (Applied Biosystems, UK). To quantify expression of target genes of interest Taqman Gene Expression Assays containing FAM-labelled probes were used (Assay IDs: MMP9 Rn00579162_m1, IDO Rn00576778_m1, IFN γ Rn00594078_m1, TDO Rn00574499_m1, MMP9 Rn00579162_m1, IL-1 β Rn00580432_m1, IL-6 Rn00561420_m1, TNF α Rn00579162_m1, GFAP Rn00566603_m1, and iNOS Rn00561646_m1, Applied Biosystems, UK). PCR reactions were in a duplex format also containing a Taqman Gene Expression Assay (primer-limited) containing a VIC-labelled probe for the endogenous control gene β -actin (Assay ID: 4352340EE). Sam-

ples were assayed using ABI's universal cycling conditions using a fast protocol on the StepOnePlus Real-time PCR system (Applied Biosystems, UK). Fold change in gene expression from the control group was calculated using the $\Delta\Delta\text{Ct}$ method (see section 4.5), and β -actin served as endogenous control in the amplification system. Data are expressed as fold change in gene expression relative to the control group.

7.3.6 Statistical Analysis

Data are presented as mean \pm standard error of the mean (SEM). As appropriate Student's *t* test or Two-way ANOVA were used to compare groups, followed by a Newman-Keuls *post hoc* test. Colon PCR data did not follow normal distribution and therefore was log transformed prior to ANOVA. All statistical analyses were considered to be significant when $p < 0.05$. Graphs and statistics were generated using GraphPad Prism Software Version 4.00 (GraphPad software, Inc) and GB-STAT v.10 (Dynamic Microsystems Inc) respectively.

7.4 Results

7.4.1 Determination of the effect of restraint stress on DSS-induced colitis

Previous publications indicate that there is an exacerbating effect of chronic stress on the severity of colitis in the DSS animal model. This body of literature focuses on the effects of stress on the colon but has not investigated the implications of stress beyond the GI tract. Repeated restraint stress acts as a mild stressor which attempts to replicate the effect of mild daily life stress.

DSS-induced colitis significantly decreased weight gain and food intake

Two-way ANOVA of weight gain, food intake and fluid intake revealed significant effects of DSS on weight gain and food intake (see Figure 7.2). DSS induced a significant decrease in weight gain [$F(1,23) = 57.64$, $p < 0.0001$], a significant interaction between stress and DSS was also revealed [$F(1,23) = 12.43$, $p = 0.0018$] (figure 7.2a). DSS also resulted in significantly decreased average food intake per cage [$F(1,12) = 13.60$, $p = 0.0031$]. Fluid intake was not affected by stress or DSS.

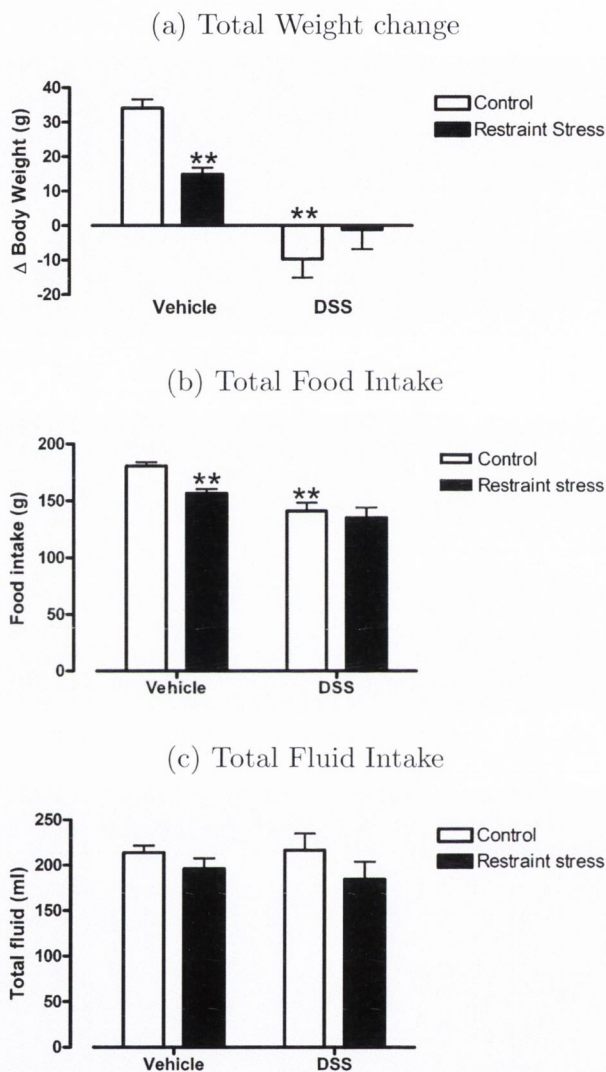


Figure 7.2: **Body weight, Food and Fluid intake in DSS experiment 1**

Graphs for body weight (a), food intake (c), and fluid intake (e) represent the average daily results over the course of the experiment. Bar graphs represent the average total weight gain (b), food intake (d) and fluid intake (e) over the 7 days. DSS administration and exposure to restraint stress resulted in decreased body weight (b) and total food intake (d). No significant stress interaction was revealed. Data are expressed as mean \pm SEM (n=6-8). **p < 0.01 vs vehicle control (Two-way ANOVA followed by Newman-Keuls *post hoc* test).

DSS induced symptoms of colitis and damage to colonic ultrastructure

Two-way ANOVA of disease activity scores (DAI) and histological scores showed a significant effect of DSS on DAI, crypt score and inflammation score (see Figure 7.3). DSS-induced colitis resulted in a significant increase in DAI (a) [$F(1,23) = 346.68$, $p < 0.0001$] on the final day of DSS administration, crypt score (b) [$F(1,23) = 668.77$, $p < 0.0001$], and inflammation score (c) [$F(1,23) = 437.76$, $p < 0.0001$]. Stress alone had no influence on DAI score or histological damage scores. No interaction between stress and colitis were found.

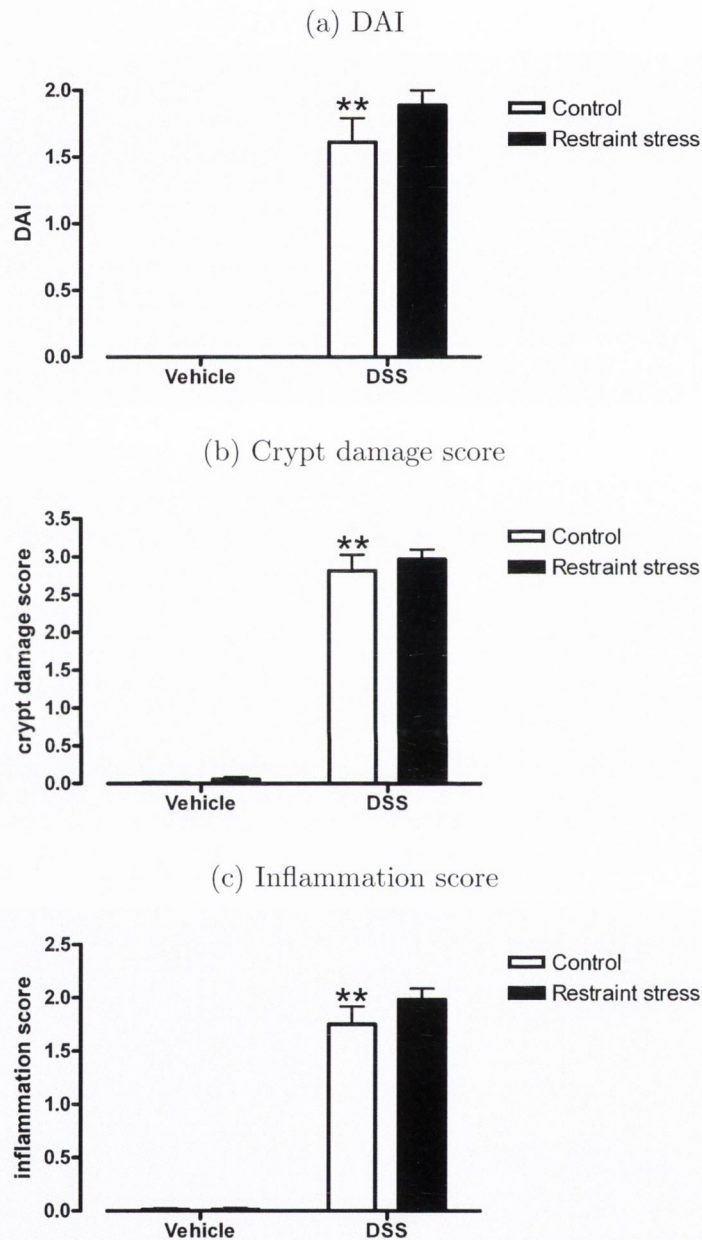


Figure 7.3: DAI, and histology scores for DSS experiment 1

On the final day of DSS exposure there is an increase in DAI (a). Post-mortem histological analysis revealed DSS induced ultrastructure disruption: crypt damage score (b) and inflammation score (c). Data are presented as mean \pm SEM (n=6-8). ** $p < 0.01$ vs vehicle control (Two-way ANOVA followed by Newman-Keuls *post hoc* test)

DSS increased colonic MMP9 mRNA expression and protein activity

Two-way ANOVA of colonic MMP9 expression showed a significant effect DSS (see Figure 7.4). DSS resulted in a significant increase in colonic MMP9 mRNA expression [$F(1,23) = 108.51, < 0.0001$], and MMP9 activity [$F(1,22) = 75.72, p < 0.0001$]. No stress effect or interaction between DSS and stress were revealed.

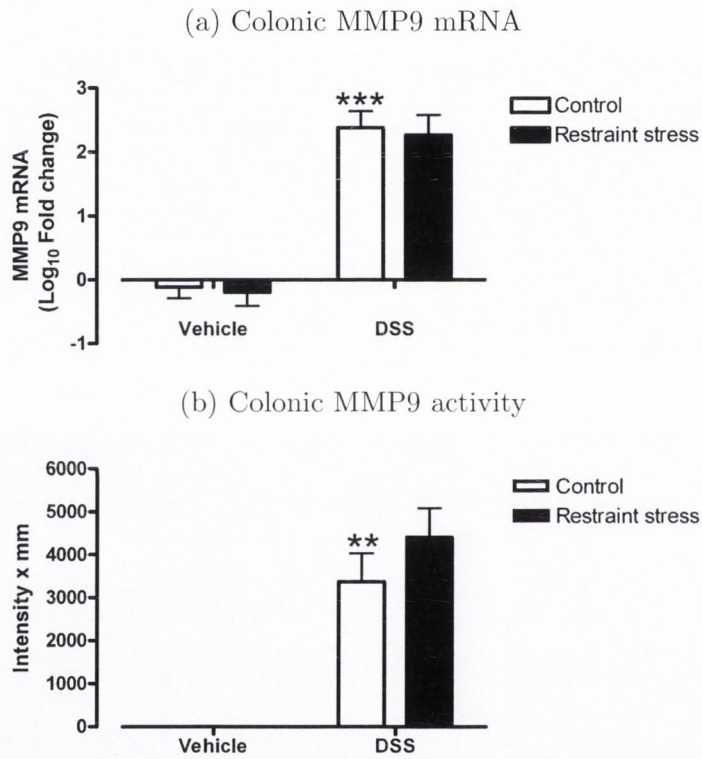


Figure 7.4: Colonic MMP9 expression and activity in DSS experiment 1

Colonic MMP9 mRNA expression (a), and MMP9 protein activity in the colon (b) were increased following DSS exposure. Data are presented as mean \pm SEM (n=6-8). ** $p < 0.01$ vs vehicle control (Two-way ANOVA followed by Newman-Keuls *post hoc* test)

DSS-induced increases in colonic inflammatory markers

Two-way ANOVA of colonic mRNA expression of IL-6, IL-1 β , TNF α , and iNOS revealed a significant effect of DSS (see Figure 7.5). DSS resulted in a significant increase in all markers examined: IL-6 [F(1,23) = 133.76, < 0.0001], IL-1 β [F(1,23) = 98.80, p < 0.0001], TNF α [F(1,23) = 26.36, p = 0.0001], and iNOS [F(1,23) = 212.73, p < 0.0001]. No significant effects of stress or interaction between stress and DSS were found for mRNA expression of these inflammatory markers in the colon.

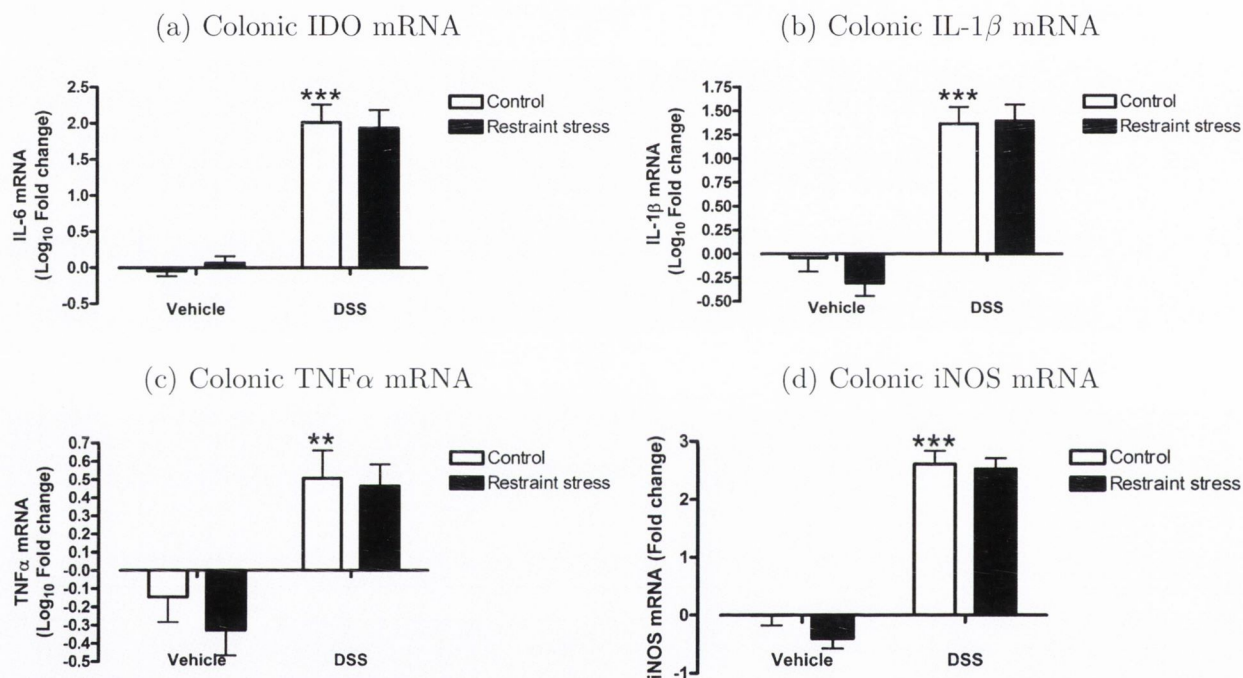


Figure 7.5: Colonic IL-6, IL-1 β , TNF α , and iNOS expression in DSS experiment 1

DSS exposure resulted in increased colonic mRNA expression of IL-6 (a), IL-1 β (b), TNF α (c), iNOS (e). Data are presented as mean \pm SEM (n=6-8). * p < 0.05, ** p < 0.01 vs vehicle control (Two-way ANOVA followed by Newman-Keuls *post hoc* test)

DSS increased hepatic iNOS mRNA expression

Two-way ANOVA of hepatic iNOS mRNA expression, and TDO mRNA expression revealed a significant effect of DSS on iNOS and stress on TDO expression (see Figure 7.6). DSS resulted in a significant increase in hepatic iNOS mRNA expression [$F(1,22) = 9.57$, $p = 0.0053$], and stress increased hepatic TDO mRNA expression [$F(1,22) = 5.82$, $p = 0.0246$]. However, Newman keul *post hoc* analysis did not reveal any intergroup differences. No interaction between DSS and stress was found in the liver.

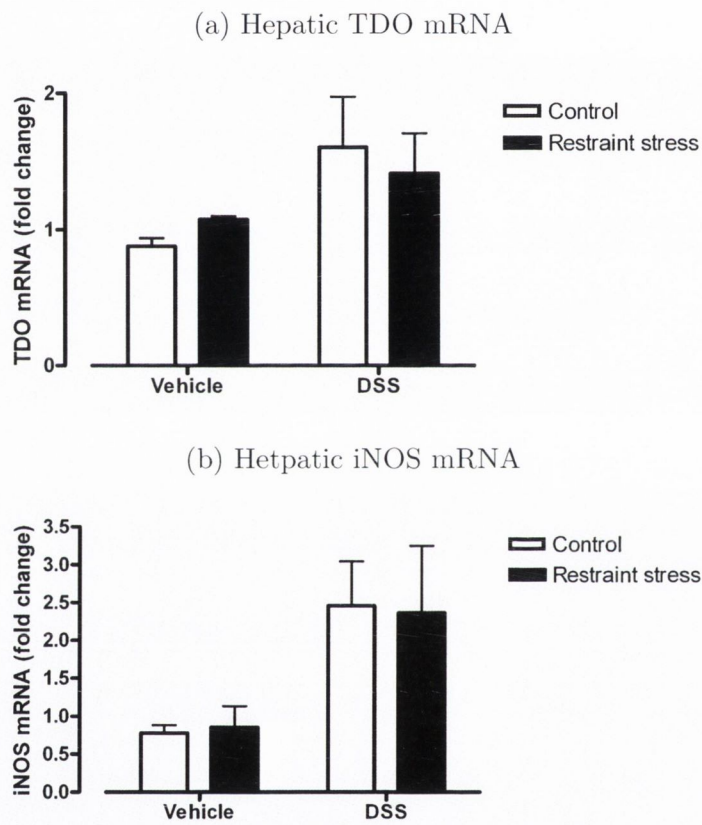


Figure 7.6: Hepatic TDO and iNOS in DSS experiment 1

Hepatic TDO (a) and iNOS (b) mRNA expression following exposure to DSS. Data are presented as mean \pm SEM ($n=6-8$). (Newman-Keuls *post hoc* test did not reveal any significant differences between groups.)

DSS and stress-induced effects in the cortex

Two-way ANOVA of iNOS (b) and IL-6 (a) mRNA expression in the cortex revealed a significant effect DSS (see Figure 7.7). DSS resulted in a significant increase in cortical iNOS mRNA expression [$F(1,23) = 19.29$, $p = 0.0002$]. A significant interaction between stress and DSS was also revealed for cortical iNOS mRNA expression [$F(1,23) = 5.77$, $p = 0.0248$]. DSS also significantly increased cortical IL-6 mRNA expression [$F(1,22) = 25.80$, $p < 0.0001$]. No interaction between DSS and stress was found for IL-6 mRNA expression.

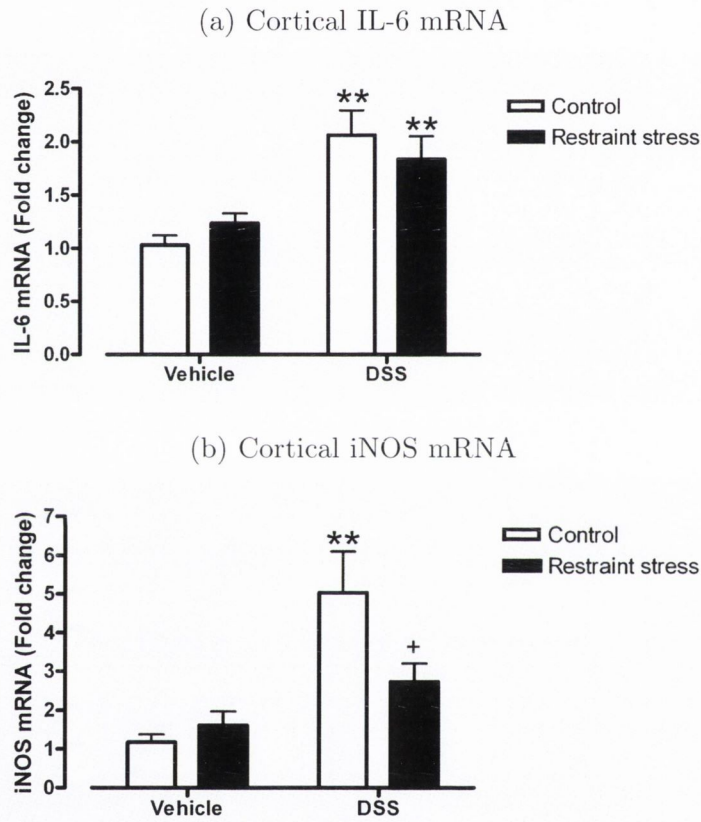


Figure 7.7: Cortical IL-6 and iNOS in DSS experiment 1

DSS exposure induced a significant increase in cortical IL-6 (a) and iNOS (b) mRNA expression. Data are presented as mean \pm SEM ($n=6-8$). ** $p < 0.01$ vs vehicle control, + $p < 0.05$ vs non-stressed counterparts (Two-way ANOVA followed by Newman-Keuls *post hoc* test)

7.4.2 Determination of the effects of restraint stress on recovery from DSS-induced colitis

Exposure to repeated restraint stress following seven days DSS administration had no exacerbating effect on DSS-induced weight loss, food intake, fluid intake or the expression of inflammatory markers in the colon, liver, or cortex. Evidence of prolonged recovery time from symptoms was evident.

DSS-induced colitis significantly decreased weight gain and food intake

Two-way ANOVA of weight gain (b), food intake (d) and fluid intake (e) revealed significant effects of DSS and stress on total weight gain over the course of the experiment (see Figure 7.8). Both DSS and stress resulted in a significant decrease in weight gain: [$F(1,20) = 8.11, p = 0.0099$] and [$F(1,20) = 7.04, p = 0.0152$] respectively. Average fluid intake per cage was also significantly increased during the recovery phase following DSS administration [$F(1,8) = 28.76, p = 0.0007$]. Total food intake over the course of the study was not significantly effected by stress or DSS, however as per previous investigations, total food intake over the course of DSS administration period was significantly reduced in this experiment [$F(1,8) = 8.96, p = 0.0173$] (graph not shown).

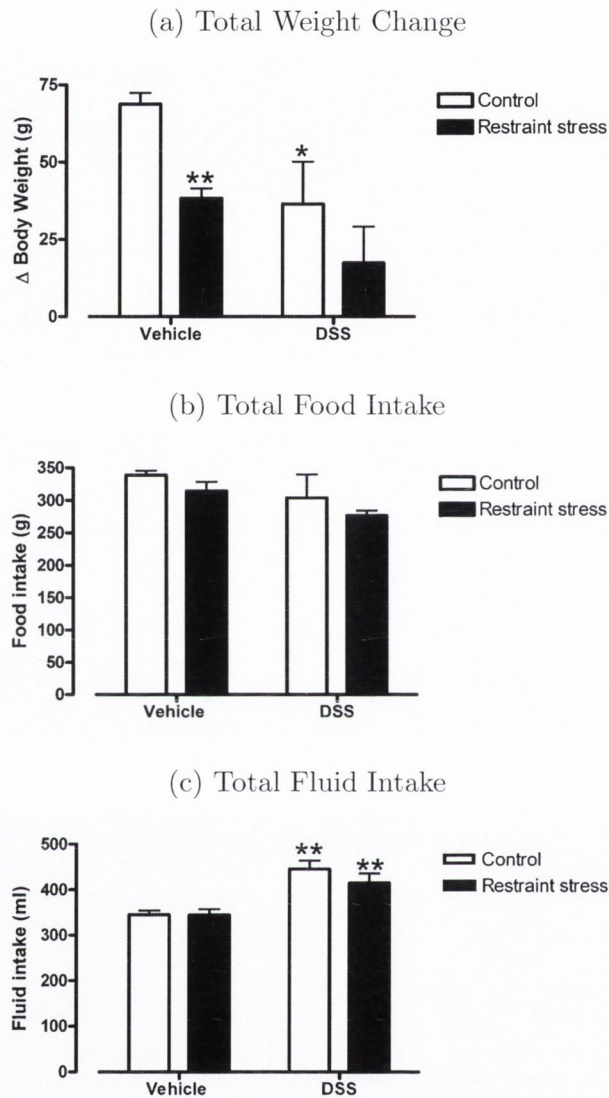


Figure 7.8: **Body weight, Food and Fluid intake in DSS experiment 2**

Graphs for body weight (a), food intake (c), and fluid intake (e) represent the average daily results over the course of the experiment. Bar graphs represent the average total weight gain (b), food intake (d) and fluid intake (e) over the 13 days. DSS administration and stress resulted in decreased body weight (b) and total food intake (d). Fluid intake increased during the recovery phase after DSS administration. No significant stress interaction was found. Data are expressed as mean \pm SEM (n=6). **p < 0.01, ***p < 0.001 vs controls (Two-way ANOVA followed by Newman-Keuls *post hoc* test).

Stress prolongs recovery time following DSS induced colitis

Restraint stress induces loose stool and diarrhea, which therefore DAI was examined prior to restraint stress. Analysis of symptoms prior to exposure to restraint stress on day 4 demonstrated a prolonged time to recovery in the DSS group exposed to restraint stress. Student's *t* test revealed the DAI score to be significantly increased on day 4 of recovery [$t = 3.051$; $df = 10$; $p = 0.0122$], when the non-stressed group symptom expression had returned to baseline (see Figure 7.9). On the following day only one rat from the stress and DSS group still had diarrhea when examined prior to stress exposure.

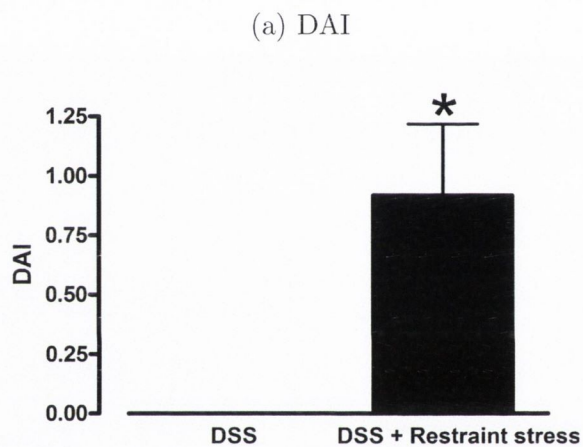


Figure 7.9: **DAI analysis for DSS experiment 2**

On recovery day 4 rats were examined for symptoms of colitis prior to exposure to restraint stress. A significant prolongation of mild symptoms of colitis was evident in the stressed group versus non-stressed counterparts. Data are expressed as mean \pm SEM (n=6). *p < 0.05 vs vehicle control (Pooled variance Student's *t* test).

DSS increased colonic MMP9 mRNA expression and protein activity

Two-way ANOVA of MMP9 mRNA expression and MMP9 activity in the colon following 7 days recovery from DSS-induced colitis revealed a significant effect of DSS (see Figure 7.10). DSS resulted in a significant increase in colonic MMP9 mRNA expression (a) [$F(1,20) = 47.47, p < 0.0001$], and MMP9 activity (b) [$F(1,19) = 22.30, p < 0.0001$]. No stress effect or interaction between DSS and stress were found.

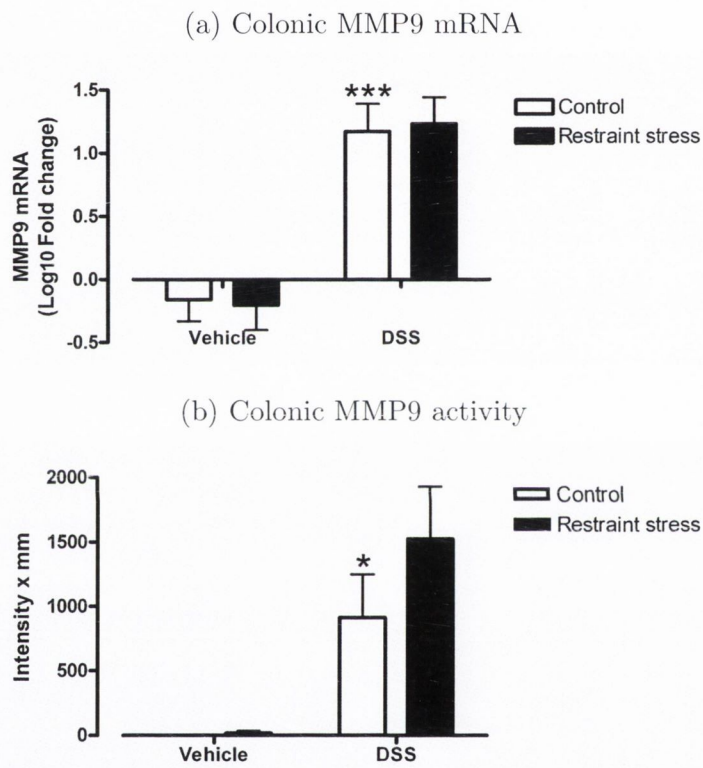


Figure 7.10: Colonic MMP9 expression and activity in DSS experiment 2

DSS administration resulted in increased MMP9 mRNA expression (a) and MMP9 activity (b). No significant stress interaction was found. Data are expressed as mean \pm SEM (n=5-6). * $p < 0.05$ vs vehicle controls (Two-way ANOVA followed by Newman-Keuls *post hoc* test).

DSS-induced increases in colonic inflammatory markers

Two-way ANOVA of colonic mRNA expression of IL-6, iNOS, IL-1 β , and TNF α following DSS-induced colitis revealed a significant effect of DSS (see Figure 7.11). DSS resulted in a significant increase in the following markers: iNOS [F(1,20) = 55.33, p < 0.0001], IL-6 [F(1,20) = 21.62, p = 0.0002], IL-1 β [F(1,20) = 33.81, p < 0.0001], and TNF α [F(1,20) = 17.62, p = 0.0004]. However, Newman keul *post hoc* analysis did not reveal any intergroup differences.

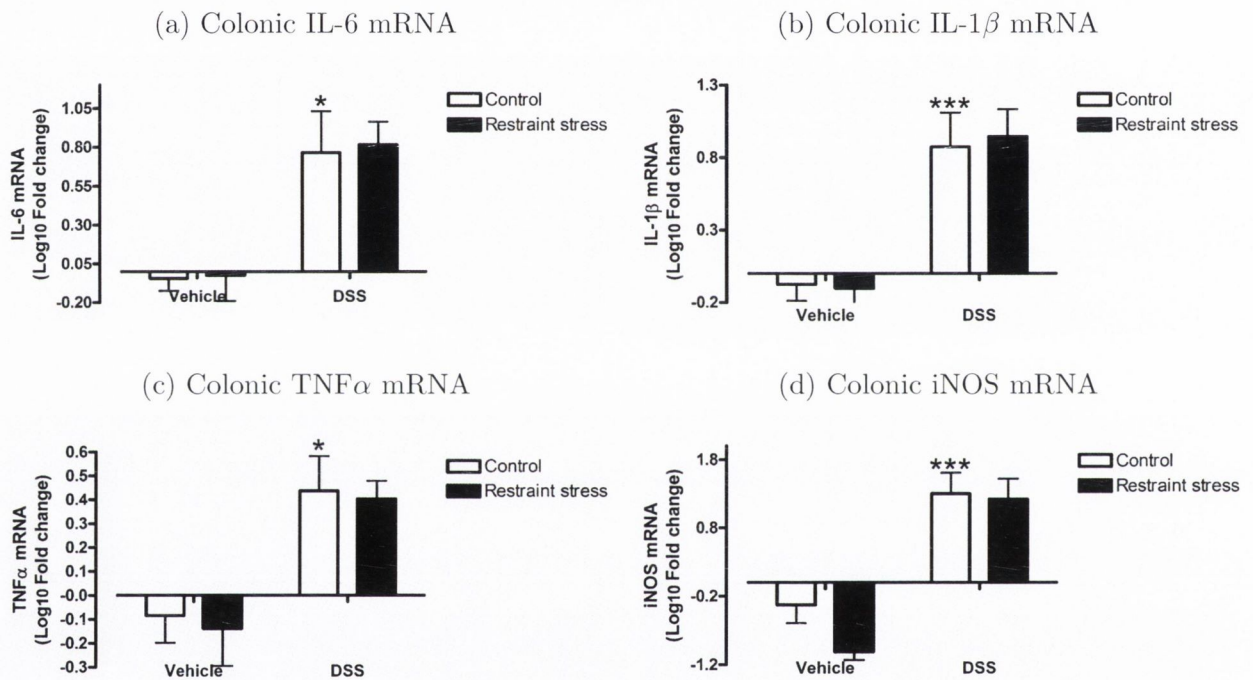


Figure 7.11: Colonic IL-6, IL-1 β , TNF α and iNOS expression in DSS experiment 2

Effects of DSS administration on colonic mRNA expression IL-6 (a), IL-1 β (b), TNF α (c), and iNOS (d). No significant stress interaction was found. Data are expressed as mean \pm SEM (n=5-6). (Two-way ANOVA followed by Newman-Keuls *post hoc* test).

DSS increased hepatic iNOS mRNA expression

Two-way ANOVA of iNOS and TDO mRNA expression in the liver following 7 days recovery from DSS-induced colitis revealed a significant effect of DSS (see Figure 7.12). DSS resulted in a prolonged increase in hepatic iNOS mRNA expression [$F(1,20) = 7.80$, $p = 0.0112$], and a trend towards stress-induced increase in hepatic TDO mRNA expression [$F(1,18) = 4.07$, $p = 0.0589$]. However, Newman keul *post hoc* analysis did not reveal any intergroup differences. No interaction between DSS and stress was found in the liver.

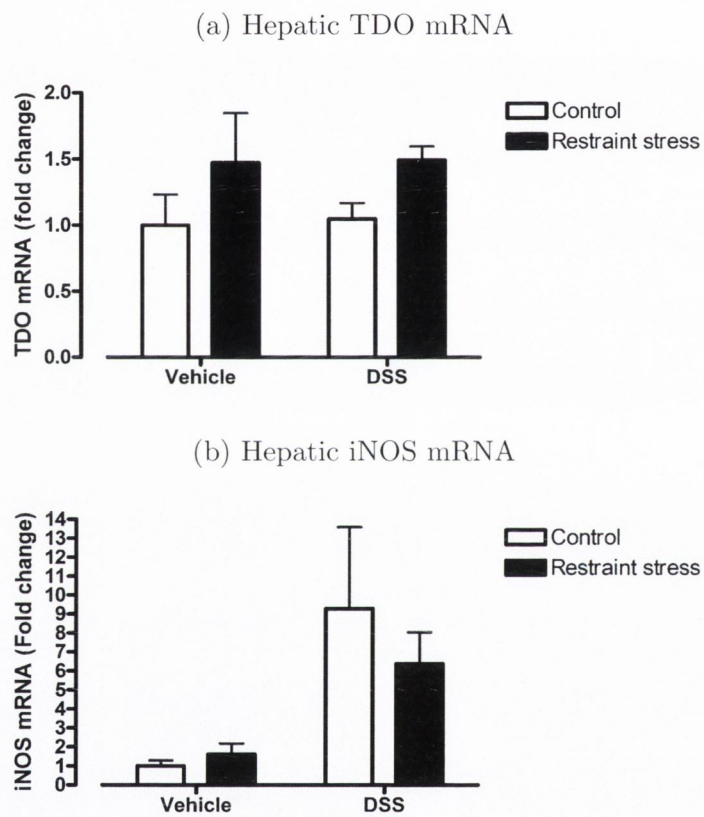


Figure 7.12: **Hepatic TDO and iNOS in DSS experiment 2**

Two-way ANOVA revealed a significant DSS-induced increase in hepatic iNOS mRNA expression. Data are presented as mean \pm SEM (n=5-6). (Two-way ANOVA followed by Newman-Keuls *post hoc* test)

DSS and stress-induced effects in the cortex

Two-way ANOVA of iNOS and IL-6 mRNA expression revealed a significant effect of DSS (see Figure 7.13). DSS resulted in a significant increase in cortical iNOS mRNA expression [$F(1,20) = 21.25$, $p = 0.0002$] and IL-6 mRNA expression [$F(1,19) = 4.69$, $p = 0.0433$]. However, Newman keul *post hoc* analysis did not reveal any intergroup differences. No interaction between DSS and stress was found.

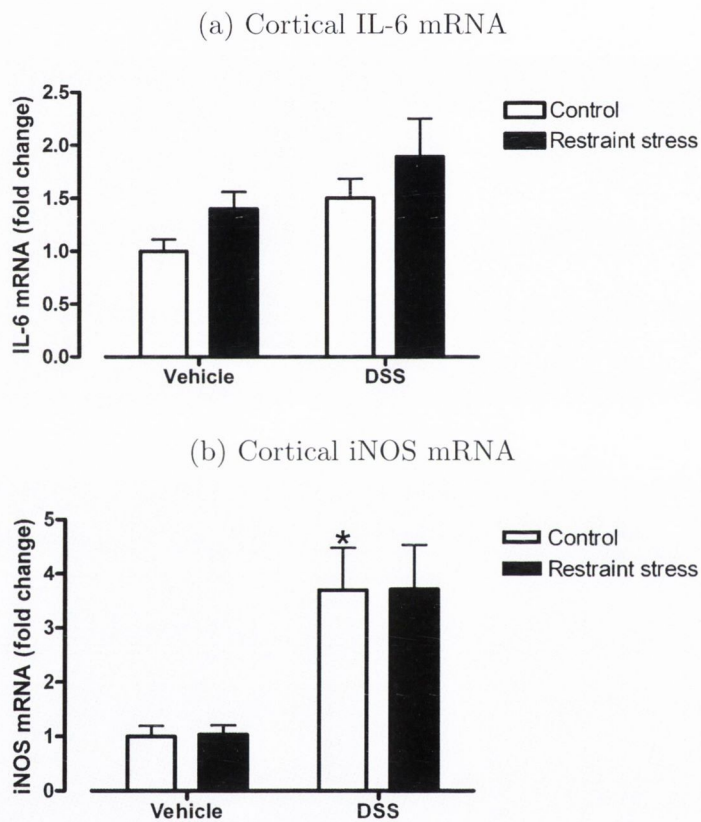


Figure 7.13: Cortical IL-6 and iNOS in DSS experiment 2

Two-way ANOVA revealed significant DSS-induced increases in cortical IL-6 and iNOS. Data are presented as mean \pm SEM (n=5-6). * $p < 0.05$ vs vehicle control (Two-way ANOVA followed by Newman-Keuls *post hoc* test)

7.4.3 Determination of the effect of stress exposure immediately prior to initiating DSS administration

DSS-induced colitis had a similar symptomology to UC including rectal bleeding, fecal blood, and diarrhea. Symptoms were more severe in the colitic rats exposed to restraint stress compared to their non-stressed counterparts. By day 5 the DSS rats exposed to stress had very severe rectal bleeding. Body weight was measured daily as an indication of the severity of colitis. Significant weight changes were seen in all groups compared to the control, however the most significant weight loss was in the DSS group exposed to repeated restraint stress.

Decreased food intake and weight gain following DSS and restraint stress

Two-way ANOVA of weight gain (b), food intake (d) and fluid intake (e) revealed significant effects of both stress and DSS on weight and food intake (see Figure 7.14). DSS induced a significant decrease in weight gain (b) [$F(1,19) = 18.72, p < 0.001$]. Restraint stress also significantly decreased weight gain (b) [$F(1,19) = 26.76, p < 0.001$]. Total average food intake (d) per cage over the course of the study was also significantly decreased following DSS administration [$F(1,8) = 24.66, p = 0.0011$], and exposure to repeated restraint stress [$F(1,8) = 19.95, p = 0.0021$]. No significant effects of either DSS or stress on fluid intake (f) were found. DAI score was increased in the the DSS group also exposed to restraint stress (see Figure 7.15a). Student's *t* test revealed the DAI score to be significantly increased on the final day of DSS administration [$t = 4.586; df = 10; p = 0.001$].

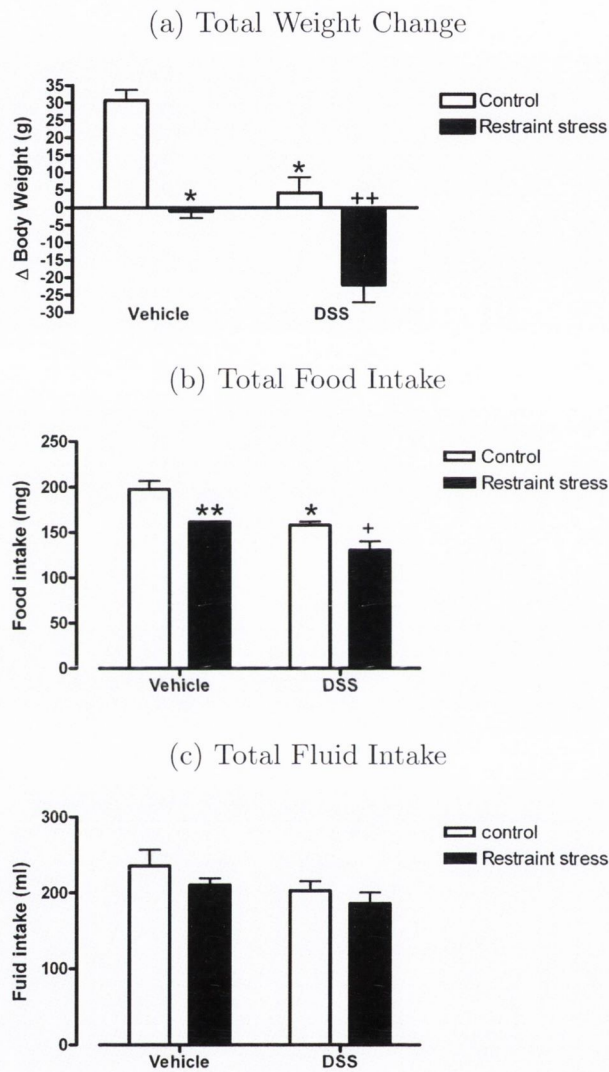


Figure 7.14: **Body weight, Food and Fluid intake in DSS experiment 3**

Bar graphs represent the average body weight change (b), food intake (d) and fluid intake (e) over the 7 days. A significant DSS-induced decrease in body weight (b) and total food intake (d) was revealed. Fluid intake was not affected by stress or DSS and no significant interaction was revealed. Data are expressed as mean \pm SEM (n=5-6). *p < 0.05, **p < 0.01 vs vehicle controls, +p < 0.05 vs non stressed DSS counterparts (Two-way ANOVA followed by Newman-Keuls *post hoc* test)

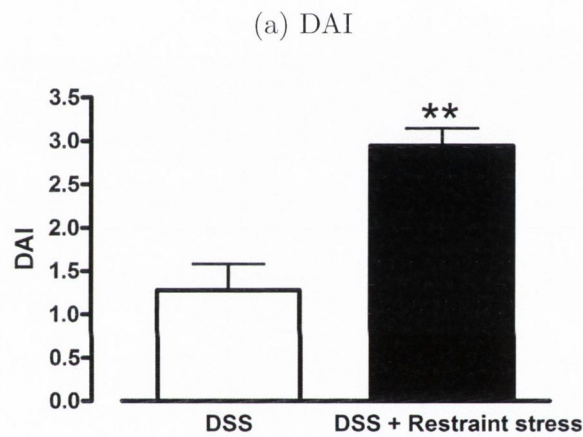


Figure 7.15: **DAI analysis for DSS experiment 3**

Increased DAI scores in the DSS group exposed to restraint stress versus the non-stressed DSS group. Both the vehicle control group and the vehicle stress groups scored 0 (not shown on graph). Data are expressed as mean \pm SEM (n=6). **p < 0.01 vs non-stressed DSS (Pooled variance Student's *t* test).

Interactive effect of DSS and stress on colonic MMP9 expression and activity

Two-way ANOVA of MMP9 expression and activity in the colon revealed a significant interaction between stress and DSS (see Figure 7.16). Interaction of DSS and stress resulted in a significant increase in MMP9 mRNA expression (a) [$F(1,17) = 54.49$, $p < 0.0001$]. Interaction between stress and DSS also resulted in a significant increase in both MMP9 activity (b) [$F(1,18) = 11.10$, $p = 0.0037$] and MMP9 mRNA expression [$F(1,17) = 7.71$, $p = 0.013$].

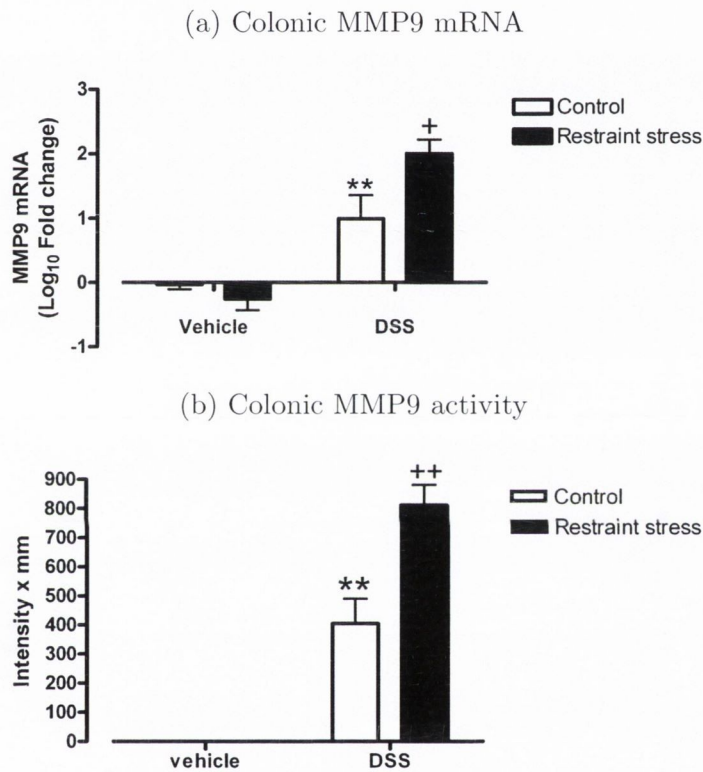


Figure 7.16: Colonic MMP9 expression and activity in DSS experiment 3

Two way ANOVA revealed a DSS-induced increase in colonic MMP9 mRNA (a) and MMP9 protein activity (b). A significant interaction between DSS and stress was also revealed. Data are expressed as mean \pm SEM ($n=5-6$) ** $p < 0.01$ vs vehicle controls, ++ $p < 0.01$ vs non stressed DSS counterparts (Two-way ANOVA followed by Newman-Keuls *post hoc* test).

Interactive effect of DSS and stress on colonic mRNA expression of IL-6

Two-way ANOVA of colonic IL-6 (a), IL-1 β (b), TNF α (c) and iNOS (d) mRNA expression revealed a significant interaction between stress and DSS on colonic IL-6 [F(1,17) = 12.18, p = 0.0028] and iNOS [F(1,17) = 7.96, p = 0.012] (see Figure 7.17). Two-way ANOVA also revealed a significant DSS-induced increase in IL-1 β [F(1,17) = 35.91, p = 0.0001], TNF α [F(1,19) = 8.90, p = 0.0008], IL-6 [F(1,18) = 52.44, < 0.0001] and iNOS [F(1,17) = 55.67, p < 0.0001].

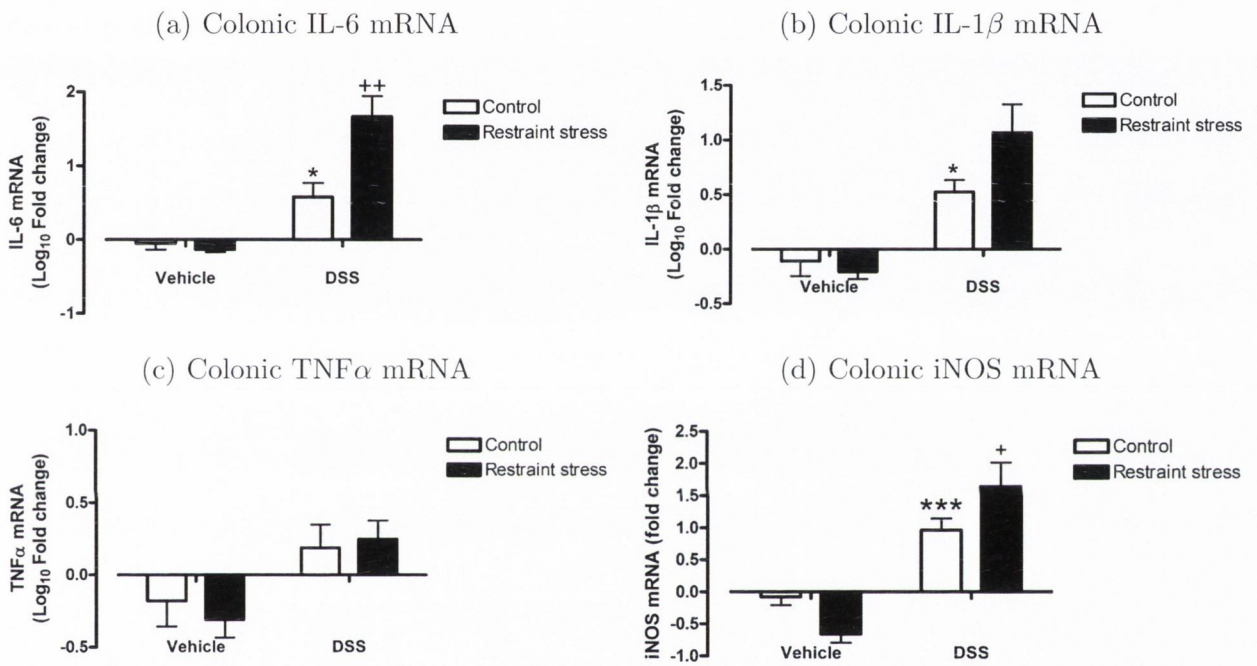


Figure 7.17: Colonic IL-6, IL-1 β , TNF α and iNOS expression in DSS experiment 3

Two way ANOVA revealed a significant DSS-induced increase in colonic IL-6 (a), IL-1 β (b) and iNOS (d) mRNA expression. A significant interaction between DSS and stress was also revealed for IL-6 and iNOS. Data are expressed as mean \pm SEM (n=5-6) Data are presented as mean \pm SEM (n = 5-6). *p < 0.05, ***p < 0.001, +p < 0.05, ++p < 0.01 vs non stressed counterparts (Two-way ANOVA followed by Newman-Keuls *post hoc* test).

No interaction between DSS and stress on hepatic or cortical mRNA markers examined

Two-way ANOVA of hepatic and cortical mRNA expression did not reveal any interactive effect of DSS and stress on hepatic TDO or iNOS, or cortical IL-6 or iNOS mRNA expression. As per previous DSS studies there was a significant DSS-induced increase in iNOS in both the liver [$F(1,19) = 13.27, p = 0.0017$] and cortex [$F(1,19) = 15.10, p = 0.001$] (see Figures 7.18b and 7.19a respectively). A significant stress induced increase in cortical IL-6 [$F(1,19) = 8.95, p = 0.0075$] was also revealed (see Figure 7.19). A trend towards stress-induced increases in hepatic TDO [$F(1,19) = 4.05, p = 0.059$] was also revealed (see Figure 7.18a).

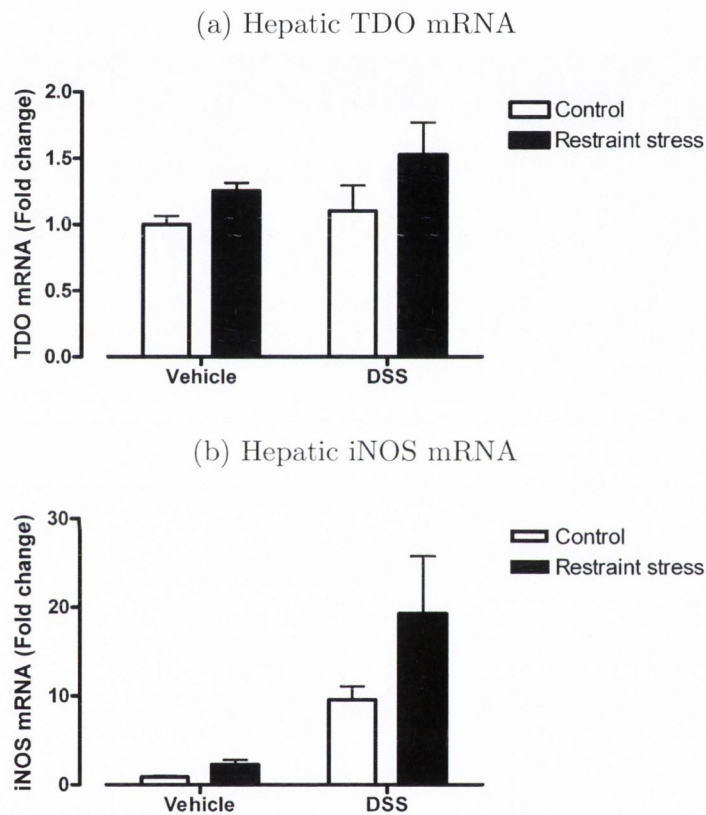


Figure 7.18: **Hepatic TDO and iNOS in DSS experiment 3**

Two way ANOVA revealed a significant DSS-induced increase in hepatic iNOS (b) mRNA expression. hepatic TDO mRNA expression was not significantly increased by stress or DSS. No significant interaction between DSS and stress was revealed. Data are expressed as mean \pm SEM (n=5-6) (Two-way ANOVA followed by Newman-Keuls *post hoc* test).

Neuroinflammatory differences extend to the hippocampus and hypothalamus

Two-way ANOVA of hippocampal and hypothalamic IL-6, IL-1 β and iNOS mRNA expression revealed significant effects of stress or DSS (see Table 7.1). DSS significantly increased hippocampal IL-1 β [F(1,17) = 12.22, p = 0.0028], IL-6 [F(1,17) = 5.07, p = 0.038], and iNOS [F(1,17) = 19.53, p = 0.0004] mRNA expression. DSS also significantly increased hypothalamic IL-1 β [F(1,19) = 6.09, p = 0.023], and iNOS [F(1,19) = 25.58, p < 0.0001] mRNA expression. Stress significantly influence increased IL-6 mRNA expression in the hippocampus [F(1,17) = 9.32, p = 0.0072] and hypothalamus [F(1,19) = 21.17, p = 0.0002]. No significant interactions between stress and DSS were found.

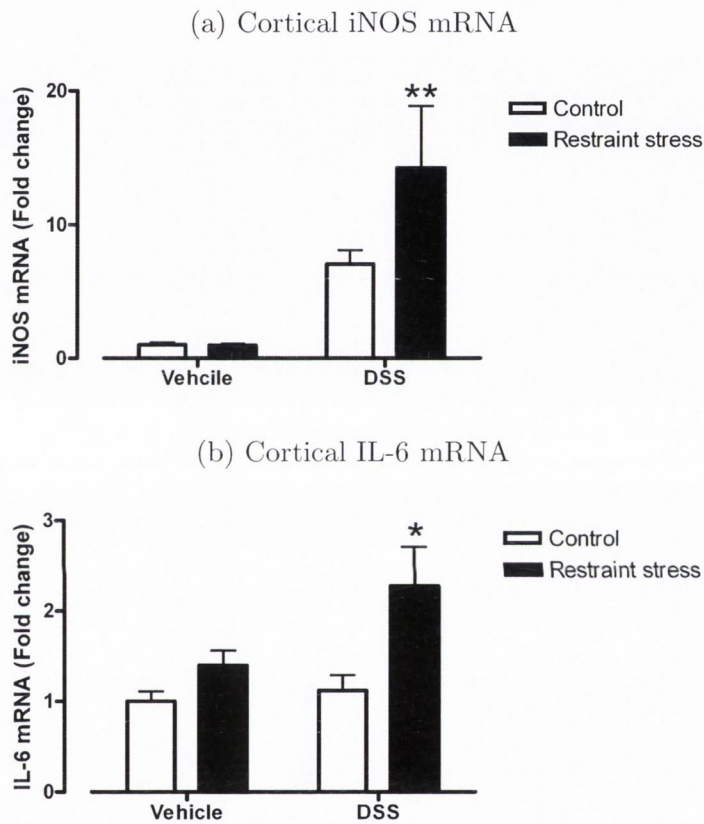


Figure 7.19: Cortical IL-6 and iNOS in DSS experiment 3

Significant DSS-induced increase in cortical IL-6 (a), and iNOS (b) mRNA expression. No significant interaction between DSS and stress was revealed. Data are expressed as mean \pm SEM ($n=5-6$) (Two-way ANOVA followed by Newman-Keuls *post hoc* test did not reveal any inter-group differences). Student's *t* test revealed a significant increase in IL-6 expression versus the non-stressed DSS group * $p < 0.05$ [$t = 2.495$; $df = 10$; $p = 0.032$].

Table 7.1: Hypothalamic and hippocampal iNOS, IL-6 and IL-1 β in DSS experiment 3

	V & Ctrl	V & R. stress	DSS & Ctrl	DSS & R. stress
Hypothalamus				
iNOS	1 \pm 0.25	1.36 \pm 0.20	5.21 \pm 0.55	9.82 \pm 2.3
IL-6	1 \pm 0.11	1.54 \pm 0.13	1.06 \pm 0.13	1.91 \pm 0.20
IL-1β	1 \pm 0.13	0.89 \pm 0.49	1.43 \pm 0.18	1.56 \pm 0.34
Hippocampus				
iNOS	1 \pm 0.23	1.13 \pm 0.31	7.56 \pm 0.91	16.34 \pm 5.09
IL-6	1 \pm 0.08	1.5 \pm 0.11	1.33 \pm 0.16	2.15 \pm 0.4
IL-1β	1 \pm 0.18	0.8 \pm 0.09	1.27 \pm 0.17	1.73 \pm 0.22

Data represent fold change in mRNA expression for iNOS, IL-6 and IL-1 β in the hypothalamus and hippocampus. Data are expressed as mean \pm SEM (n=5-6). (Two-way ANOVA followed by Newman-Keuls *post hoc* test). dextran sulfate sodium (DSS), control (ctrl), Restraint stress (R. stress), vehicle (V)

7.5 Discussion

Stress is thought to have a significant impact on the course and severity of IBD (Levenstein et al., 2000; Mawdsley et al., 2006). In this investigation three DSS experiments were carried out: male Wistar rats were housed 2 per cage and administered 5% DSS in their drinking water for 7 days, a control group and DSS group were also exposed to repeated restraint stress during the DSS administration phase, during the seven days recovery immediately following DSS administration or the first stress exposure pre DSS administration.

7.5.1 Restraint stress did not enhance changes in food intake, fluid intake, weight changes or Disease Activity Index associated with DSS-induced colitis (experiment 1 and 2)

Fluid and food intake were recorded as an indication of the general well-being of each rat. No change in fluid intake was revealed following DSS or repeated restraint stress. Stress and DSS both induced a decrease in food intake, however there was no interaction between the two (DSS experiment 1). Previous research has also demonstrated a decrease in food consumption following restraint stress (Rybkin et al., 1997; Torres et al., 2002) and anorexia is a common clinical symptom of severe IBD. Both stress and DSS also reduced weight gain. No difference between DSS and DSS combined with restraint stress was revealed. Mortality rate was also equal across the two DSS groups, with a mortality rate of two rats in each group. A number of studies previously report increased colonic damage in colitic animals also exposed to stress (Reber et al., 2006a; Cetinel et al., 2010; Colón et al., 2004). In this study, although DSS induced colitis, no differences in crypt damage score or inflammatory score following histological analysis was revealed when compared to the DSS plus stress group. In agreement with this no significant difference in DAI was seen in the DSS group compared to the DSS group exposed to stress.

Crohn's disease and Ulcerative colitis are relapsing and remitting diseases which may be influenced by stress (Bouma and Strober, 2003). A second DSS experiment, which again involved 5% DSS for seven days, followed by a 7 day recovery phase was carried out. As stress is thought to have a significant impact on the course of IBD (Levenstein et al., 2000; Mawdsley et al., 2006), in this study male Wistar rats were exposed to two hours daily restraint during the recovery phase in order to determine the impact of stress on recovery from DSS-induced colitis (DSS experiment 2). Again DSS induced a similar symptomology to UC including rectal bleeding, fecal blood, and diarrhea. Symptoms of colitis were more

evident in colitic rats directly following each 2 hour restraint stress exposure than in the non-stressed counterparts; the acute effect of stress revealed fecal bleeding and diarrhea for a number of days. More clinically relevant however was the pre-stress examination on recovery day 4 which revealed persistent evidence of colitis, diarrhea, while the non-stress counterparts had no remaining symptoms. Day 4 was used for comparison as it represents the day in Chapter 6 when food intake and weight gain had returned to control levels. Body weight was measured daily as an indication of the severity of colitis, again both stress and DSS induced a significant decrease in weight gain. This decreased weight gain was most severe in the DSS rats exposed to stress, but this was an additive effect of stress and DSS rather than interactive effect. Again stress and DSS also reduced appetite as decreased food intake was present in both groups compared to controls. Unlike during the DSS administration phase, during the recovery phase from DSS there was a significant increase in fluid intake. The large intestine is largely responsible for water absorption and sodium regulation, it is therefore possible that dysregulation of this system occurs following the DSS induced colonic damage.

The lack of mild stress-induced affect on recovery is similar to previous literature. Milde and Murison (2002) found no significant effects of exposure to restraint stress on recovery from colitis in time to first hemocult negative stool, time to second hemocult negative stool, body weight recovery, water consumption, or number of colonic erosions. In their investigation the average days to first hemocult negative stool was 3.62 (\pm 0.82) in the DSS group versus 5 (\pm 0.85) in the DSS followed by stress group (Milde and Murison, 2002), Although the difference was not statistically significant it is similar to the prolongation of symptom expression reported in the current experiment. Larsson et al. (2009) also failed to demonstrate any interaction between recovery from DSS and exposure to water avoidance stress: no difference in weight loss, fecal output, or macroscopic score was found.

7.5.2 Restraint stress failed to impact on DSS-induced colonic MMP9 or cytokine expression

MMP's are a family of zinc-dependent endopeptidases involved in the degradation of the extra cellular matrix (ECM). The gelatinase B, MMP9, is one of the major MMPs and has a key role to play in the proteolytic cascade leading to ECM cleavage (Damodharan et al., 2011). Upregulation of MMP9 mRNA and protein activity has previously been reported in colonic mucosa and is associated with disease activity in IBD patients, increased MMP9 is also reported in animal models of IBD (Bailey et al., 1994; Santana et al., 2006; Ishida

et al., 2008; Garrido-Mesa et al., 2011). In the current investigation MMP9 is increased in the distal colon following DSS administration. Increased MMP9 activity is still present in the colon following 7 days recovery from DSS in DSS experiment 2. As per the food, fluid and weight measurements, repeated restraint stress had no impact on MMP9 expression during acute colitis or following seven days recovery.

IBD is also associated with induction of inflammatory genes, increased expression of TNF α , IL-6, IL-1 β , IFN γ , IDO, and iNOS has also been reported in animal models of IBD (Ferdinande et al., 2008; Márquez et al., 2010; Benight et al., 2011; Alex et al., 2009). In agreement with previous reports DSS induced a significant upregulation of inflammatory genes in the colon. Again however restraint stress did not impact on the fold change in these markers versus the non-stressed counterparts during the acute inflammatory phase or following seven days recovery.

7.5.3 Effects of restraint stress and DSS-induced colitis on gene expression changes in the liver and cerebral cortex

Previous pilot work has demonstrated that the restraint stress protocol used induces a significant increase in cortical IL-6 and hepatic TDO mRNA expression 2 hr following removal from the restrainer. At 6 hr post restraint cortical IL-6 was no longer significantly increased and TDO mRNA had returned to control levels (see appendix B). Based on this data a six hr time point was chosen for the current experiments to investigate whether the impact of sickness and inflammation could prolong these stress induced increases, at a time when they have otherwise returned to control levels. Again no interaction between stress and DSS was revealed. Although DSS itself had some minor inducing effects on cortical IL-6 and hepatic TDO again there was no interaction between DSS and restraint stress in DSS experiment 1. Following 7 days recovery DSS-induced increase in hepatic TDO mRNA and IL-6 mRNA expression have returned to controls levels, and no interaction between stress and DSS occurred.

In the previous chapter significant increases in peripheral and central iNOS mRNA expression were reported (see chapter 6). In the current experiments we investigated the impact of repeated restraint stress pre, post, and during exposure to DSS administration. As per previous investigations iNOS was increased during the acute phase of colitis. Restraint stress during the recovery phase also failed to influence the prolonged increase in iNOS mRNA expression. However in DSS experiment 1 a significant interaction between stress and DSS was seen, whereby stress significantly reduced the DSS-induced increase in cortical iNOS mRNA expression. Curtin et al. (2009) have previously reported that

restraint stress exposure suppresses LPS-induced iNOS expression in the spleen. Curtin et al. (2009) suggest that this is due to the stress-induced suppression of the innate IFN γ response via glucocorticoid receptor activation. As per their investigation future studies should investigate the potential effects of stress exposure on splenic inflammatory markers following DSS.

Although this is the only interaction between stress and DSS revealed in the 3 experiments (DSS experiment 1, DSS experiment 2, and DSS experiment 3), it suggests that stress may be having some immune modulating effects that are not evident in the colon. Future investigations into the immunomodulating influence of stress in the DSS model should focus on the implications of stress on glucocorticoid production and innate IFN γ production.

7.5.4 Possible reasons for the lack of interaction between chronic stress and colitis

1. **The restraint stress protocol may be too mild to exaggerate colitis** Although this restraint stress protocol induced significant weight loss and reduced appetite as reported previously in immobilisation studies, here there was no evidence of restraint stress affecting DSS induced cytokine expression in the gut. Similar restraint stress protocols have shown immunomodulating properties in other models of disease, however it is possible that it is too mild to affect the severe immune response induced by DSS. Previous investigations on the effect of restraint stress on colitis have often exposed rats to a more severe restraint stress protocol: Pfeiffer et al. (2001) only exposed rats to 1 hr restraint, however it was carried out at 4°C, Israeli et al. (2008) exposed rats to 4 hr restraint rather than the 2 hr restraint used here, Milde and Murison (2002) also exposed rats to 2 hr restraint, however rats were singly housed which may act as an additional stressor, Colón et al. (2004) exposed rats to 6 hr restraint stress for 10 days, Qiu et al. (1999) exposed mice to a combination of restraint and sonic stress based on previous work which suggested that the most predictable response required combined stressors. Prolonging the stress in future studies, or combining restraint stress with other mild stressors may have a greater immunomodulatory affect on colitis.
2. **Timing of the stress relative to induction of colitis: pre, during or post** Qiu et al. (1999) have reported that acute stress (restraint and sonic stress) can reactivate quiescent colitis in TNBS mouse models. They suggest that this reacti-

vation could be due to a reduction in the protective barrier function of the colon. This also suggests that a major cause of stress induced exacerbation of colitis could be due to the increased intestinal permeability to the chemicals, DSS or TNBS, rather than through stress induced immune alterations. In a number of investigations stress induced reactivation of previous colitis was increased with exposure to a subthreshold dose of DNBS or DSS, this indicates that this stress induced permeability to the chemicals may be a vital factor (Saunders et al., 2006; Melgar et al., 2008; Qiu et al., 1999). Both DSS and restraint stress have previously been reported to increase intestinal permeability (Venkatraman et al., 2000; Saunders et al., 1994, 1997; Soderholm and Perdue, 2001; Collins, 2001). Venkatraman et al. (2000) examined the effect of oral DSS administration on ^{14}C -mannitol permeability across the distal colon on days 1 to 7 of DSS administration. They report almost a three fold increase in colonic permeability from as early as day 1 of DSS administration, this is despite no detectable changes in histology at this point. Psychological stress also results in impaired barrier function, which increases the permeability to noxious materials including antigens, toxins and other pro-inflammatory molecules (for review see Collins (2001); Soderholm and Perdue (2001)). Saunders et al. (1994, 1997) report that restraint stress results in overt intestinal barrier dysfunction as shown by increase permeability to mannitol. They also demonstrated that this effect was more pronounced in the genetically more stress susceptible Wistar-Kyoto rats than in regular Wistars, and suggest that the altered epithelial function is acetylcholine dependent. Pfeiffer et al. (2001) demonstrated that increased permeability to mannitol was most significant (2 fold increase) following 1 day of cold restraint stress. Together this suggests that the timing of the stress with respect to DSS administration might be vital to the enhancing properties of the stressor, whereby exposure to acute stress immediately prior to beginning DSS administration may exacerbate colitis due to enhanced permeability.

7.5.5 Exposure to stress prior to DSS administration increased the severity of colitis

A final experiment was carried out which involved exposing rats to their first 2 hr restraint stress session directly before beginning the DSS administration to investigate the implications of pre-stress on the development of DSS-induced colitis. Again fluid and food intake were recorded in this study. As per previous experiments there was no effect of either DSS or restraint stress on fluid intake, and both stress and DSS induced a significant

decrease in food intake, although there was no interactive effect between stress and DSS the largest decrease in food intake was in the colitis group exposed to stress. Body weight was measured daily as an indication of the severity of colitis. Significant weight changes were seen in all groups compared to the control, however the most significant weight loss was in the DSS group also exposed to repeated restraint stress. These stress-induced weight difference between the DSS and DSS plus stress group are very similar to weight changes previously reported by Reber et al. (2006a) following chronic stress prior to colitis. DSS-induced colitis had a similar symptomology to previous experiments including rectal bleeding, fecal blood, and diarrhea. Symptoms were more severe in the DSS-colitis rats compared to their non-stressed counterparts. By day 5 the DSS rats exposed to stress had very severe rectal bleeding.

7.5.6 Exposure to stress prior to DSS administration enhanced the expression of inflammatory mediators

Unlike the previous two experiments, when stress exposure began 24 hrs post initiation of DSS or 24 hr after stopping DSS, in this experiment stress significantly aggravated the expression of inflammatory markers within the colon. A significant interaction between stress and DSS was revealed for colonic MMP9 mRNA expression, which was also found for MMP9 activity. This was accompanied by a significant interaction between stress and DSS for IL-6 mRNA expression in the colon. Although a statistical interaction was not achieved for colonic IL-1 β or iNOS expression, *post hoc* analysis revealed the combination group to be the only significantly increased group versus control. This was also the case for hepatic iNOS and central iNOS and IL-6 expression, whereby the DSS group also exposed to repeated restraint stress had the most significant increase in gene expression. This is consistent with a number of investigations which examined the effect of chemically-induced colitis 1 day after stress exposure (Gue et al., 1997; Milde and Murison, 2002; Reber et al., 2006a). Reber et al. (2006a) reported an exaggerated response to DSS administration following exposure to chronic psychosocial stress (social defeat and overcrowding for 19 days). Increased severity of colitis was reflected by increased body weight loss, histological damage scores, decreased survival rate and increased secretion of proinflammatory cytokines from stimulated mesenteric lymph nodes. Gue et al. (1997) observed increased macroscopic and histological disruption in rats exposed to 4 days restraint stress prior to TNBS administration. They also demonstrate that these exacerbating effects of stress are independent of CRF or arginine vasopressin as antagonists for each of these did not inhibit the stress-induced exacerbation. Finally, Milde and Murison (2002) report that exposure

to restraint stress for 4 days prior to DSS administration also resulted in a shorter latency to development of colitic symptoms (decreased time to first hemoglobin positive fecal sample).

7.5.7 Conclusions

Together these results demonstrate a significant influence of stress on DSS-induced colitis which is highly dependent on the time of the stressor in relation to the time of DSS administration. Stress exposure immediately prior to commencing the DSS administration procedure resulted in an accelerated response to DSS and an exaggerated inflammation within the colon. However there was no significant effect of stress exposure post commencing DSS administration on any of the inflammatory markers within the GI tract. Some evidence of stress induced prolongation of recovery time was seen however by seven days recovery this was no longer present. This indicates a possible acute stress induced effect on recovery from DSS-induced colitis, which may be associated with increased inflammatory markers at an earlier stage of recovery. As glucocorticoids have anti-inflammatory properties it is unlikely that the stress-induced acceleration and aggravation of colitis seen is due to HPA-axis-induced adrenal activation, although future work will require analysis of corticosterone concentrations to confirm this observation. This suggestion is consistent with previous work by Gue et al. (1997) which found that aggravation of TNBS-induced colitis following stress was not mediated by brain levels of CRH. They also suggest that another mechanism than the immunosuppressive HPA-axis must be involved. Saunders et al. (1994, 1997) report acetylcholine-induced increased intestinal barrier permeability following cold restraint stress which may be responsible for the stress-induced exacerbation seen in the current investigation, and requires further investigation.

Chapter 8

General Discussion and Future Directions

There is a great deal of literature to support the hypothesis that the risk of depression increases in patients suffering from IBD (Burke et al., 1989; Addolorato et al., 1997; Guthrie et al., 2002; Farrokhyar et al., 2006) and that anxiety, depression or stress can influence relapse and severity of symptoms (Mardini et al., 2004; Mittermaier et al., 2004; Persoons et al., 2005; Levenstein et al., 2000; Mawdsley et al., 2006). Biomolecular mechanisms for this increased risk of depressive and anxious symptoms have not yet been explored in IBD patients or animal models. Cytokine changes, increased tryptophan metabolism or altered serotonin have previously been associated with increased psychological disorders, and were therefore suggested as potential risk factors for depression or anxiety in IBD. The first part of the thesis examined the links between disease activity and psychological scores for psychological disturbances in IBD patients. The second part of the thesis involved establishing two animal models of IBD, in order to determine the impact of colonic inflammation on behaviour, and to examine whether there is evidence of neuroinflammation or tryptophan/serotonin changes within the CNS. Thirdly, the effect of exposure to repeated restraint stress, at various time points with respect to DSS administration, was examined.

8.1 Clinical data: Depression and anxiety associated with higher disease activity

IBD patients (18) and patient controls (19) were recruited for the study. Medical examination was carried out on each patient including: colonoscopy, whole blood samples, serum

samples, intestinal biopsies, disease activity scores, psychological scores, and general well-being scores. As per previous investigations the patient group with increased DAI was associated with increased HAM-A and HAM-D scores (Mardini et al., 2004; Calvet et al., 2006; Ben Thabet et al., 2012). Further analysis of the data indicated that increased HAM-D scores are independent of the acute psychological impact of symptoms as the patient control group experiencing the same degree of intestinal symptoms did not have increased HAM-D scores. Unlike HAM-D, HAM-A scores were not statistically independent of symptoms, this indicates a relationship between the psychological experience of symptoms and anxiety. Together, these results suggest that in IBD patients inflammatory mediators, such as cytokines, which are not increased in the patient controls could influence depression score. Depression has previously been associated with decreased HRQOL and poor drug compliance in IBD patients (Hyphantis et al., 2010; Kiebles et al., 2010; Walker et al., 2008; Vidal et al., 2008; Mittermaier et al., 2004; Guthrie et al., 2002; Selinger et al., 2011). Further studies also report that depression scores are predictive of future changes in disease activity or decreased remission rates (Mardini et al., 2004; Mittermaier et al., 2004; Persoons et al., 2005). It is becoming apparent from the clinical data that bidirectional communication is at play in IBD: active disease negatively impacts on psychological well-being, and *visa versa*, psychological well-being can impact the course of disease.

Improved understanding of the mechanisms underlying psychological disturbances in IBD patients may lead to the development of novel treatments, or treatment with current antidepressant may offer IBD patients a better quality of life. The negative impact of depression on the course of IBD also indicates that treating the depression may be beneficial in treating both the mood disturbances and the course of disease. The effects of antidepressants or anxiolytics on IBD has received very little attention, however, some recent investigations suggest that they may in fact offer therapeutic benefit (Goodhand et al., 2012a; Mikocka-Walus et al., 2012). Goodhand et al. (2012a) examined the influence of antidepressant treatment on the number of relapses, courses of steroids, and number of endoscopies in the year after introduction. A significant decrease in all these disease related factors was seen following antidepressant treatment. In an interview based investigation (Mikocka-Walus et al., 2012) report IBD patients views on their current antidepressant treatment. One third of the the patients reported improvements in their IBD, again this indicates possible benefits of antidepressant treatment beyond improving patients mood.

The clinical improvements offered by antidepressant treatment may be due to improved patient mood, and improved ability to deal with their illness. However, it is also possible that improvement of the intestinal manifestations of IBD is due to the anti-inflammatory

properties of antidepressants (Kenis and Maes, 2002). Both *in vitro* and preclinical studies have demonstrated anti-inflammatory effects of selective serotonin reuptake inhibitors (SSRI), serotonin noradrenaline reuptake inhibitors (SNRI) and tricyclic antidepressants (Tynan et al., 2012; Yang et al., 2010; Sacre et al., 2010). Tynan et al. (2012) report potent inhibitory effects of SSRIs on TNF α and NO production from microglial cells. This inhibitory effect on cytokine production may therefore be responsible for the benefits offered by antidepressant treatment in IBD patients, however, further preclinical and clinical studies will be needed to confirm this suggestion.

8.2 Animal models: translational implications of this work

The second aim of the thesis was to establish DSS and TNBS animal models of IBD, and to characterise their suitability for studying the link between IBD and depression. Consistent with other investigations the results in this thesis have revealed many similarities between this animal model of colitis and human characteristics of the disease. Initial analysis was to ensure that the DSS protocol used induced histological and symptoms similar to clinical UC. Firstly, it was confirmed that 7 days exposure to 5% DSS induced weight loss, decreased appetite, increased DAI, structural damage within the distal colon, and similar intestinal inflammatory marker expression as seen in the patient samples. Overall the increased inflammatory marker expression in the DSS model was in line with the clinical samples. During the symptomatic phase of DSS-induced colitis the most significant increases were in IL-6, IL-1 β , and MMP9 mRNA expression. Low increases in TNF α mRNA was also revealed. These symptomatic, structural and inflammatory changes are also consistent with previous analysis of the DSS colitis model (Okayasu et al., 1990; Alex et al., 2009). The TNBS model was used for comparative purposes to ensure that any changes observed following DSS administration were due to the colitis and not a pharmacological effect of DSS itself. Although this model was not characterised to the same extent as the DSS model, similar increases in colonic pro-inflammatory cytokine and iNOS expression were found.

Behavioural changes were also apparent in both IBD models. DSS-induced behavioural changes were analysed using a range of tests for depressive or anxiety like behaviours: open field test, marble burying test, light dark box and EPM. DSS-induced colitis resulted in behavioural changes in the open field test during the acute inflammatory phase including increased grooming and decreased time spent exploring the centre, however rats also

had decreased locomotion making it difficult to interpret the other changes as depression-like. Further behavioural testing once food intake and locomotor deficits in the open field had returned to normal indicate a prolonged behavioural disturbance in the DSS-induced colitis rats. The most significant disturbance was decreased digging/burying behaviour in the marble burying test, thus making this a valuable test for future pharmacological intervention studies in these models. The TNBS-induced colitis model also induced behavioural changes in the open field test similar to the DSS model. The nature of TNBS administration also made it possible to do saccharin preference testing. Saccharin preference was significantly decreased in TNBS-induced colitis rats, which is indicative of anhedonia (Harkin et al., 2002; Gibney et al., 2012). Overall these results suggest an initial sickness-like behaviour in the models, however prolonged behavioural abnormalities may be indicative of a depressive or anxious state. This finding further supports testing antidepressants in these models, which may improve both the behavioural disturbances observed and the colonic inflammation.

One of the main benefits of using animal models in neuroscience research is access to brain samples for molecular analysis. This provides valuable information not available from clinical studies. In the current thesis the frontal cortex, hippocampus, and hypothalamus were the focus of all biomolecular analysis due to their reported involvement in depression and sickness. Tissue analysis revealed a number of gene expression changes in the cortex (IL-6, GFAP, and iNOS), prolonged iNOS upregulation in the cortex, hippocampus and hypothalamus, tryptophan increases in these 3 regions during acute inflammation, and importantly decreased serotonin in the hippocampus which is highly relevant to depression. Beyond confirming that molecular disturbances are present following induction of colitis which could induce mood disturbances this further justifies testing the effect of antidepressants and their ability to reverse some of these central changes.

Although the TNBS model has been invaluable here for comparative purposes, the TNBS administration protocol involves overnight fasting and anesthetic administration. The potential psychological impact of these stressors could confound results, and therefore make the DSS model a superior model for investigating the impact of colitis on CNS function and behaviour. It is therefore suggested that any initial pharmacological interventions be carried out in the DSS model of IBD in future.

8.2.1 Effects of stress on models of colitis

The final aim of the thesis was to investigate the implications of stress on DSS-induced colonic inflammation. As described in chapter 3 previous investigations report a number

of stress-induced effects on healthy bowel function: altered GI motility, increased visceral perception, altered GI secretion, increased intestinal permeability, altered GI mucosa and mucosal blood and altered intestinal microbiota (for recent review of the literature see Konturek et al. (2011)). The restraint stress experiments presented in this thesis support the hypothesis that the CNS can impact on the gastrointestinal tract. Mild restraint stress exposure prior to commencing DSS with continued stress exposure over the course of DSS-administration accelerated the course of colitis and resulted in a more severe inflammatory response. The underlying mechanism behind this stress induced exacerbation of colitis is unclear. Due to the lack of interaction between restraint stress and DSS when the first stress exposure occurs one day post commencing DSS it is possible that the exacerbation observed is due to a reduction in intestinal barrier function resulting in increased permeability to the DSS.

Stress exposure also prolonged the time to recovery when exposure started in parallel with the beginning of the recovery phase. However, although restraint stress slowed the recovery process it did not inhibit it completely. Therefore, to examine the effects this might have on inflammatory marker expression an earlier time point should be examined. At recovery day 4 colitic rats are asymptomatic, however in the DSS group also exposed to stress there was a significantly increased DAI. Recovery day 3 or 4 may therefore represent a more suitable day for examining the effects of restraint stress on prolonging recovery. From a clinical perspective both of these stress effects are very important, as they suggest that even mild stress may accelerate the time to relapse or prolong the time to remission. The potential benefit anxiolytics may offer at minimising stress induced exacerbation should be examined.

More than just models of IBD?

In the current thesis the implications of these models was examined specifically from an IBD prospective. From a more general outlook these models could represent a tool for studying the impact of systemic inflammation on the CNS. DSS-induced colitis resulted in iNOS in the liver spleen and throughout the CNS, as well as IL-6 upregulation in the brain in the current investigation. Previous studies have also reported increased circulating inflammatory cytokines in the TNBS and DSS models of colitis (Alex et al., 2009).

The ease of induction of DSS-induced inflammation and reproducibility means that the it may be possible to establish this model. It also allows for easy manipulation of the dose administered and length of administration, allowing for milder inflammation to be achieved if necessary. The rapid recovery from DSS-induced 'sickness behaviour',

colitic symptoms, and severe intestinal inflammation after returning to tap water allows for repeated administration over a number of cycles (Ghia et al., 2007). This repeated administration protocol would closely mimic the relapsing remitting presentation of a number of chronic inflammatory diseases.

Due to the high molecular weight of DSS very little passes through the intestinal barrier and although a number of investigations have reported extra-intestinal DSS distributions throughout the liver, spleen, kidney following oral DSS administration, DSS was not increased in the brain (Perse and Cerar, 2012; Kitajima et al., 1999). Confirmation of poor DSS translocation and the similarity of results between DSS and TNBS-induced colitis allows us to infer, with some certainty, that any brain changes are due to immune or endocrine signals rather than as a pharmacological effect of the DSS itself. This makes the DSS model an interesting alternative to commonly used models of inflammation such as endotoxin injection. In terms of behavioural assessment, the recovery period is also longer in these models than following a single dose of LPS, allowing for increased behavioural characterisation within the one animal and in turn minimising the use of animals.

Possible risk of neurodegeneration

The aim of this thesis was to examine the potential molecular effects which could induce depressive symptoms. However, the most significant gene upregulation throughout the periphery and CNS was iNOS, and iNOS remained upregulated following 7 days recovery in the CNS. As described previously iNOS may have some implications in stress and depression-like behaviour in rodents, however, the high levels of NO that are generated following iNOS activity are also highly neurotoxic. This requires further investigation as increased iNOS has also been implicated in many pathologies of the CNS including: Alzheimer's disease patients and models (Lüth et al., 2001; Haas et al., 2002; Nathan et al., 2005; Medeiros et al., 2007), Huntington's disease patients and models (Thomas et al., 2004; Ryu et al., 2004; Aguilera et al., 2007), and Parkinson's disease (Hunot et al., 1996; Knott et al., 2000; Hunot and Hirsch, 2003). In light of the the recent MRI study in CD patients, where decreased grey matter volume was reported in the frontal cortex and in the midcingulate cortex, and reports of white matter lesions in IBD patients, these models could prove useful for examining structural brain following systemic inflammation (Agostini et al., 2013; Rothfuss et al., 2006). In their investigation, Agostini et al. (2013) also report a correlation between the decreased gray matter volume in the frontal cortex and disease duration. Animal MRI studies would allow for close comparison with patient data, and post-mortem analysis of the same brain tissue could elucidate the underlying

mechanisms involved in the morphological differences reported (Agostini et al., 2013).

8.2.2 Conclusion

The potentially self-perpetuating effect that the gut-brain axis may have in IBD is difficult to ignore. This does not indicate that IBD is psychologically induced, however, the implications of depression or stress in genetically predisposed individuals may accelerate the onset of illness or exaggerate the systemic/extraintestinal symptoms. The psychological impact of the chronic sickness and symptoms could also, at least theoretically, result in relapse of the inflammatory condition, and biological mediators of inflammation may negatively impact on the CNS resulting in depression or anxiety, which in turn could leave individuals more sensitive to stress and to relapse of IBD.

Beyond exploring the impact of colonic inflammation on the CNS, and the impact of the CNS on inflammation, these IBD models should prove to be valuable tools in examining the potential benefit of current antidepressant and anxiolytic drugs in minimising this cycle. If treatment with psychoactive drugs could dampen the effects of stress on the GI tract, they may be therapeutically beneficial for patients.

Summary of important findings in this thesis

1. Patients suffering from IBD have increased HAM-D scores during the symptomatic phase. This is independent of the acute psychological impact of current symptoms. Increased HAM-D scores are associated with increased inflammatory marker expression (circulating IL-6, IFN γ , CRP, and kynurenine:tryptophan ratio)
2. The DSS model of colitis has increased iNOS expression in the CNS, decreased hippocampal serotonin, increased central tryptophan, and increased central IL-6/IL-1 β during the acute inflammatory.
3. Stress-induced enhancement of colitis is highly dependent on the timing of the stress exposure with respect to DSS-administration

8.3 Future Directions

Following the experiments presented in this thesis, a number of studies could be carried out to determine the implications of, and mechanisms involved in, the changes reported

8.3.1 Further evaluation of Gut-Brain interactions

1. IBD is a relapsing and remitting condition. In this thesis, rats were only exposed to one cycle of DSS. Although this provides valuable insight into the acute impact of colonic inflammation on the brain, it would be essential for future work to determine whether or not the CNS response to the DSS becomes more or less prominent after two or more cycles of DSS administration.
2. The mechanisms involved in decreased circulating tryptophan concentrations have not been determined. A number of factors can influence tryptophan concentrations including TDO upregulation, IDO upregulation, increased protein synthesis, or dietary intake. In the case of colitis models, each of these factors could be at play, or a number could be affecting tryptophan concentrations at any one time. To determine, which has the strongest impact on the decreased tryptophan concentration a number of experiments should be carried out: TDO and IDO inhibitors should be administered in combination with DSS, control groups diet restrictions to investigate decreased dietary intake, and analysis of acute phase protein mRNA expression within the liver, could provide an indication of the tryptophan usage rate during inflammation. Central tryptophan concentrations are increased in the acute phase of colitis. Increased central tryptophan is often reported due to increased free tryptophan within the circulation. It would therefore be of interest to also measure the circulating free tryptophan concentrations following induction of colitis.
3. In the current thesis MMP9 mRNA expression is increased in patient biopsies during active IBD, and both MMP9 expression and activity was increased during acute DSS-induced colitis. As MMP9 is capable of breaking down the extracellular matrix, it represents a possible mechanism by which the noxious contents of the gut can gain access to the systemic circulation and ultimately influence the CNS. Therefore, the potential ability of MMP9 inhibitors to minimise peripheral and central inflammatory markers, mainly iNOS expression, should be investigated. These inhibitors could have important therapeutic benefits, as extraintestinal symptoms are often associated with IBD.

4. In the DSS-model of IBD iNOS expression in the CNS was significantly increased, and was correlated with increased iNOS in the colon, liver and spleen. iNOS protein expression during the acute phase was significantly increased around the third ventricle. However, the mechanisms behind increased iNOS expression remain unclear. Previous research has demonstrated increased iNOS expression in HUVEC cells following exposure to IBD patient serum. The ability of serum from colitic rats to induce iNOS expression within primary neuronal and microglial cell cultures should be investigated as it could provide a valuable *in vitro* model for determining the mechanisms involved in iNOS upregulation. It would also be important to determine the cell source for iNOS expression within the CNS. iNOS expression has previously been reported in neurons, microglia and astrocytes in rodent brains. Colocalisation immunostaining for iNOS with markers of neurons (NeuN), Astrocytes (GFAP), or microglia (CD11b) would indicate whether one or more of these cells are responsible for increased iNOS expression.

5. Due to the relapsing remitting nature of IBD and to the damaging properties of overproduction of NO this also requires further attention in IBD patients. In IBD patients, as peripheral organs or brain samples are not accessible, buccal samples may represent a non invasive option for analysing iNOS mRNA expression. The sublingual mucosa of the mouth is thin and highly vascularised, it therefore provides an accessible region, which is potentially representative of iNOS expression throughout the body. In support of examining buccal cell samples as a potential biomarker of IBD-induced systemic iNOS mRNA, increased exhaled NO levels have previously been reported in IBD patients (Koek et al., 2002).

8.3.2 Further evaluation of Brain-Gut interactions

1. Stress prior to DSS administration resulted in an acceleration of symptom expression and an increased DSS-induced inflammatory marker expression. This stress-induced exacerbation of colitis represents a model for testing the potential benefit of pre-treatment with anxiolytics. The mechanism behind the enhanced response also needs to be determined. Although it appears unlikely to be due to glucocorticoid induction via HPA-axis activation, as glucocorticoids have anti-inflammatory properties, this should be confirmed by analysing the various markers of HPA-axis activation including CRH in the hypothalamus, ACTH and glucocorticoid concentrations in the circulation.

2. The influence of antidepressants and anxiolytic drugs on the behavioural changes reported in the models of IBD should be investigated. As fluoxetine is both antidepressant and anxiolytic, and has been shown to have some iNOS inhibiting properties it would be of primary interest. However, based on the small body of available clinical literature, bupropion has shown some benefit in terms of TNF α concentrations in IBD patients and therefore, also represents an interesting drug for examining both the behavioural and inflammatory aspects of DSS-induced colitis (for review see Mikocka-Walus et al. (2006)).

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Appendix A

Patient Consent form and questionnaires

A.1 Patient consent form

Name of the study:

Implication of TLR4 in the regulation of MMP-9 in inflammatory bowel disease (IBD)

I: (Name in capital letters)

.....

- have read the information I have been given
- could ask questions about the study
- have received enough information about the study
- have spoken with: (name of researcher obtaining consent in capital letters)

.....

I understand that my participation is voluntary

I understand that I can withdraw from the study:

- whenever I want
- without having to give an explanation
- without it impacting on my medical care

I freely consent to participate in the study Date (Signature of Participant)

.....

Date (Signature of Physician)

.....

A.2 Inflammatory bowel disease questionnaire

Answer the following questions with respect to how you have been feeling over the last two weeks. Answer each question to the best of your ability, without consulting with anyone. Pick the response which most closely suits your situation and do not leave any questions unanswered.

1. * How frequently have you had bowel movements during the last weeks?
 - (a) More frequent than ever
 - (b) Extremely frequently
 - (c) With a lot of frequency
 - (d) Moderate increase in the frequency of bowel movements
 - (e) Small increase in the frequency of bowel movements
 - (f) Minimal increase in the frequency of bowel movements
 - (g) Normal, without any increase in frequency of bowel movements

2. How often has the feeling of fatigue or of being tired and worn out been a problem for you during the last 2 weeks?
 - (a) All of the time
 - (b) Most of the time
 - (c) A good bit of the time
 - (d) Some of the time
 - (e) A little of the time
 - (f) Hardly any of the time
 - (g) None of the time

3. How much energy have you had over the last two weeks
 - (a) No energy
 - (b) Very little energy

- (c) Little energy
 - (d) Some energy
 - (e) A good bit of energy
 - (f) A lot of energy
 - (g) Full of energy
4. How often during the last 2 weeks have you had to delay or cancel a social engagement because of your bowel problem?
- (a) All of the time
 - (b) Most of the time
 - (c) A good bit of the time
 - (d) Some of the time
 - (e) A little of the time
 - (f) Hardly any of the time
 - (g) None of the time
5. * How frequently have you had abdominal cramps over the last two weeks?
- (a) All of the time
 - (b) Most of the time
 - (c) A good bit of the time
 - (d) Some of the time
 - (e) A little of the time
 - (f) Hardly any of the time
 - (g) None of the time
6. * How frequently have you felt generally unwell during the last two weeks
- (a) All of the time
 - (b) Most of the time
 - (c) A good bit of the time
 - (d) Some of the time

- (e) A little of the time
 - (f) Hardly any of the time
 - (g) None of the time
7. * How frequently have you felt nauseous or felt like vomiting over the last two weeks?
- (a) All of the time
 - (b) Most of the time
 - (c) A good bit of the time
 - (d) Some of the time
 - (e) A little of the time
 - (f) Hardly any of the time
 - (g) None of the time
8. * Overall, in the last 2 weeks, how much of a problem have you had with passing large amounts of gas?
- (a) A major problem
 - (b) A big problem
 - (c) A significant problem
 - (d) Some trouble
 - (e) A little trouble
 - (f) Hardly any trouble
 - (g) No trouble
9. How satisfied, content or happy have you felt about your personal life over the last two weeks?
- (a) Very unsatisfied, unhappy
 - (b) Quite unsatisfied, unhappy
 - (c) A bit unsatisfied, unhappy
 - (d) A bit satisfied, happy
 - (e) Quite satisfied, happy

- (f) Very satisfied, happy
- (g) Extremely satisfied, could not feel happier

* Questions for the modified IBDQ used in this study. Questions relating to fatigue, energy, social activities and satisfaction/happiness were excluded as they would affect the depression or anxiety scores.

A.3 Crohn's disease activity index (CDAI)

Please, fill out the following details to calculate the CDAI. Questions 1 to 3 are to be filled in daily for the 7 days prior to the next visit. Questions 4 - 8 are filled in at the visit.

1. Number of liquid stools per day
2. Abdominal pain (0. None, 1. Mild, 2. Moderate, 3. Severe)
3. General well-being (0. Normal, 1. Slightly affected, 2. Poor, 3. Very poor, 4. Terrible)
4. Extraintestinal manifestations: 1. arthritis/arthralgia, 2. inflammation of the iris or uveitis, 3. erythema nodosum, pyoderma gangrenosum, or aphthous ulcers, 4. anal fissures, fistulae or abscesses, 5. Other fistulas, 6. Fever $>38^{\circ}\text{C}$ during the previous week, 7. None.
5. Use of antidiarrheals: 1. No 2. Yes
6. Abdominal Mass: 1. No, 2. Questionable, 3. Definite
7. Hematocrit: %
8. Weight: kg.

A.4 Mayo Index. Ulcerative Colitis

Table A.1: IBD patient clinical characteristics

Stool Frequency		Points
Normal amount of stools for this patients	<input type="checkbox"/>	0
1-2 stools more than normal	<input type="checkbox"/>	1
3-4 stools more than normal	<input type="checkbox"/>	2
5 or more stools more than normal	<input type="checkbox"/>	3
Rectal Bleeding*		
No blood seen	<input type="checkbox"/>	0
Streaks of blood less than half the time	<input type="checkbox"/>	1
Obvious blood with stools most of the time	<input type="checkbox"/>	2
Only blood	<input type="checkbox"/>	3
Endoscopic Findings		
Normal or inactive disease	<input type="checkbox"/>	0
Mild disease	<input type="checkbox"/>	1
Moderate disease	<input type="checkbox"/>	2
Severe disease	<input type="checkbox"/>	3
Physician Global Assessment**		
Normal	<input type="checkbox"/>	0
Mild disease	<input type="checkbox"/>	1
Moderate disease	<input type="checkbox"/>	2
Severe disease	<input type="checkbox"/>	3

* Scoring for rectal bleeding represents the most severe bleeding of the day.

** The medics global assessment takes into account the other 3 factors, the presence of abdominal pain, and patients general well-being

A.5 Profile of Mood States (POMS)

State of well-being questionnaire (Spanish version of the POMS)

Patients are asked to rate the following feelings on a scale of 0 to 4 where 0 represents never feel like this and 4 represents feel extremely like this.

1. **State of tension:** tense, shaky, on edge, relaxed, uneasy, restless, nervous, anxious
2. **State of depression:** unhappy, sorry for things done, sad, blue, hopeless, discouraged, lonely, gloomy, desperate, hopeless, helpless, worthless, frightened, guilty
3. **State of anger-hostility:** angry, peeved, grouchy, spiteful, annoyed, resentful, bitter, ready to fight, rebellious, deceived, furious, bad tempered
4. **State of vigor-activity:** active, lively, energetic, cheerful, alert, full of joy, worry free, vigorous,
5. **State of fatigue-inertia:** worn out, listless, fatigued, exhausted, sluggish, weary, exhausted,
6. **State of confusion-bewildement:** confused, unable to concentrate, muddled, bewildered, inefficient, forgetful, indecisive,
7. **State of friendliness:** friendly, clear headed, considerate of others, sympathetic, helpful, friendly, trusting

A.6 Hamilton anxiety score (HAM-A)

Table A.2: Reports of psychological disturbance in IBD

	0	1	2	3	4
Anxious mood: Worries, anticipation of the worst, fearful anticipation, irritability.	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Tension: Feelings of tension, fatigability, inability to relax, moved to tears easily, trembling, feelings of restlessness, .	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Fears: Of the dark, of strangers, of being left alone, of animals, of traffic, of crowds.	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Insomnia: Difficulty in falling asleep, broken sleep, unsatisfying sleep and fatigue on waking, dreams, nightmares, night terrors.	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Intellectual function: Difficulty in concentration, poor memory.	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Depressed mood: Loss of interest, lack of pleasure in hobbies, depression, early waking, diurnal swing.	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Somatic (muscular): muscle pain and fatigue, muscle stiffness, shocks, myoclonic jerks, grinding of teeth, unsteady voice	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Somatic (sensory): Tinnitus, blurring of vision, hot and cold flushes, feelings of weakness, pricking sensation.	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Cardiovascular symptoms: Tachycardia, palpitations, chest pain, throbbing of vessels, extrasystoles	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Respiratory symptoms: Pressure or constriction in chest, choking feelings, sighing, shortness of breath	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Gastrointestinal symptoms: Difficulty in swallowing, dyspepsia, pain before or after eating, burning, bloating, heartburn, nausea, vomiting, feeling empty stomach, abdominal cramps, borborygmi, diarrhea, constipation	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Genitourinary symptoms: Amenorrhoea, menorrhagia, development of frigidity, frequent urination, urgency of urination, premature ejaculation, lack of erection, impotence	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Autonomic symptoms: dry mouth, flushing, pallor, excessive sweating, dizziness, tension headache, shuddering	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Behaviour during interview: Tense, uncomfortable, nervous agitation: hands, rubs fingers, clenched fists. Instability, changing posture. Hand tremors. frowning. facies tense, increased muscle tone (physiological). Swallows saliva, belching, resting tachycardia. Respiratory frequency of more than 20 min. Strong tendon reflexes. Tremor, dilated pupils, exophthalmos sweating, eyelid myoclonia.	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

A.7 Hamilton depression score (HAM-D)

1. Depressed Mood (sadness, hopeless, helpless, worthless)

0 Absent

1 These feeling states indicated only on questioning

2 These feeling states spontaneously reported verbally

3 Communicates feeling states non-verbally i.e., through facial expression, posture, voice, and tendency to weep

4 Patient reports these feeling states in his spontaneous verbal and non-verbal communication

2. Feelings of Guilt

0 Absent.

1 Self reproach, feels he has let people down

2 Ideas of guilt or rumination over past errors or bad deeds

3 Present illness is a punishment. Delusions of guilt

4 Hears accusatory or denunciatory voices and/or experiences threatening visual hallucinations

3. Suicide

0 Absent

1 Feels life is not worth living

2 Wishes he were dead or any thoughts of possible death to self

3 Suicidal ideas or gesture

4 Attempts at suicide (any serious attempt rates 4)

4. Insomnia Early

0 No difficulty falling asleep

1 Complains of occasional difficulty falling asleep, for example more than 1/2 hour

2 Complains of nightly difficulty falling asleep

5. Insomnia Middle

0 No difficulty

1 Patient complains of being restless and disturbed during the night

2 Waking during the night, any getting out of bed rates 2 (except for purposes of voiding)

6. Insomnia Late

0 No difficulty

1 Waking in early hours of the morning but goes back to sleep

2 Unable to fall asleep again if he gets out of bed

7. Work and Activities

0 No difficulty

1 Thoughts and feelings of incapacity, fatigue or weakness related to activities, work or hobbies

2 Loss of interest in activity, hobbies or work, either directly reported by patient, or indirect in listlessness, indecision and vacillation (feels he has to push self to work or activities)

3 Decrease in actual time spent in activities or decrease in productivity.

4 Stopped working because of present illness

8. Retardation: Psychomotor

0 Normal speech and thought

1 Slight retardation at interview

2 Obvious retardation at interview

3 Interview difficult

4 Complete stupor

9. Agitation

0 None

1 Fidgetiness

2 Playing with hands, hair, etc.

3 Moving about, cant sit still.

4 nail biting, biting of lips. Hand wringing, hair-pulling...

10. Anxiety (psychological)

0 No difficulty

1 Subjective tension and irritability

2 Worrying about minor matters

3 Apprehensive attitude apparent in facial expressions or speech

4 Fears expressed without questioning

11. Anxiety Somatic: Physiological concomitants of anxiety, such as: gastrointestinal (dry mouth, flatulence, indigestion, diarrhea, cramps, belching); cardiovascular (palpitations, headache); respiratory (hyperventilation, sighs) ;urinary frequency, sweating

0 Absent

1 Mild

2 Moderate

3 Severe

4 Incapacitating

12. Somatic Symptoms (gastrointestinal)

0 None.

1 Loss of appetite but eating without encouragement from others. sensation of abdominal heaviness

2 Difficulty eating without urging from others. Asks for or needs laxatives or intestinal medication for gastrointestinal symptoms.

13. Somatic Symptoms General

0 None

1 Heaviness in limbs, back or head. Backaches, headache or muscle aches. Loss of energy and fatigability.

2 Any clear-cut symptom rates 2

14. Genital Symptoms (symptoms such as loss of libido; menstrual disturbances)

0 Absent

1 Mild

2 Severe

3 Incapacitating

15. Hypochondriasis

0 Not present

1 Self-absorption (bodily)

2 Preoccupation with health

3 Frequent complaints, requests for help...

4 Hypochondriacal delusions

16. Loss of Weight

0 No weight loss

1 Probable weight loss associated with present illness

2 Definite (according to patient) weight loss

17. Insight

0 Acknowledges being depressed and ill

1 Acknowledges illness but attributes cause to bad food, climate, overwork, virus, need for rest...

2 Denies being ill at all

Appendix B

Restraint stress

B.1 Evidence for restraint stress induced changes in the brain and liver

The graphs presented here demonstrate that the repeated restraint stress protocol used is sufficient to induce increased hepatic TDO expression, which is increased in response to corticosterone, and IL-6 expression within the cortex and hippocampus. Time represents the time post removal from the restrainer after the final stressor: 0 hr rats were removed from the restrainer and euthanised immediately, 2 hr and 6 hr groups were returned to their home cage for 2 and 6 hr respectively before being euthanised.

B.2 Results

B.2.1 Restraint stress induced central IL-6 expression 2 h post stress

Two-way ANOVA revealed a significant effect of repeated restraint stress on IL-6 mRNA expression in the cortex and hippocampus (see figure B.1). Stress significantly increased cortical (A) IL-6 expression [$F(1,25) = 39.56, < 0.0001$], and hippocampal (B) IL-6 expression [$F(1,26) = 36.36, P < 0.0001$]. There was also a significant interactive effect of stress and time for cortical IL-6 expression, whereby IL-6 had significantly decreased by 6 hr post restraint [$F(1,25) = 4.72, P = 0.186$].

B.2.2 Restraint stress induced hepatic TDO expression 2 h post stress

Two-way ANOVA revealed a significant effect of stress on hepatic TDO mRNA expression (see figure B.2). stress resulted in a significant increase in TDO at 2 hr post restraint [$F(1,19) = 21.98, P = 0.0002$], and an interaction between stress and time, whereby TDO expression had recovered by 6 hr post stress [$F(1,19) = 6.63, P = 0.0186$].

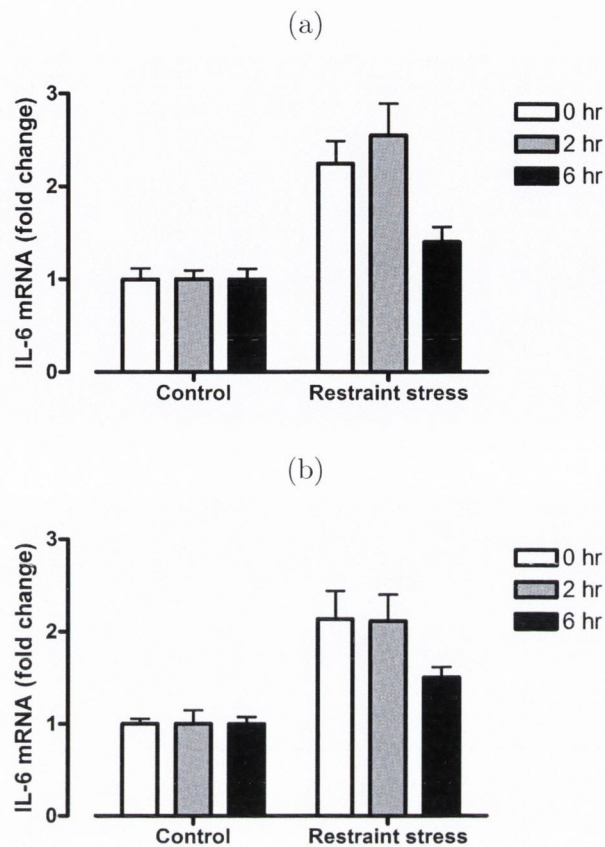


Figure B.1: **Increased CNS IL-6 mRNA expression post restraint stress**

Two-way ANOVA revealed significant effect of stress on cortical (A) and hippocampal (B) IL-6 mRNA expression. Data are presented as mean \pm SEM ($n = 3-6$). ** $P < 0.01$ versus control, + $P < 0.05$ versus 2 hr group (Two-way ANOVA followed by Newman-Keuls post hoc test)

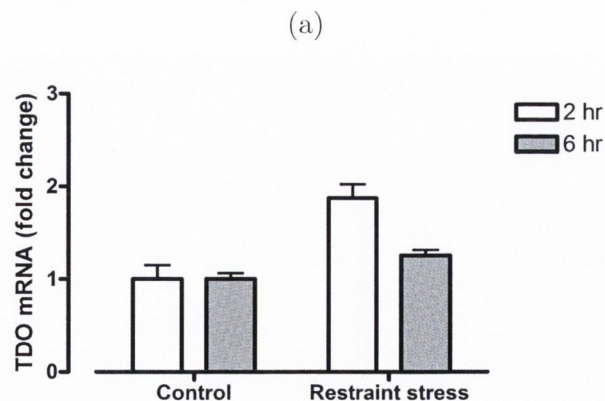


Figure B.2: **Increased hepatic TDO mRNA expression post restraint stress**

Two-way ANOVA revealed significant differences in hepatic TDO 2 hr post restraint stress. Data are presented as mean \pm SEM ($n = 5-6$). ** $P < 0.01$ versus control, + $P < 0.05$ versus 2 hr group (Two-way ANOVA followed by Newman-Keuls post hoc test)

Appendix C

National and International Conference Publications

- A. Abautret-Daly, C. Medina, T.J. Connor, A. Harkin, Altered peripheral, circulating and central tryptophan concentrations in the dextran sodium sulfate model of colitis in Wistar rats, *Proceedings of Neuroscience Ireland*, September, 2012.
- A. Abautret-Daly, N. Murphy, N. Docherty, C. Medina, T.J. Connor, A. Harkin, Behavioural changes in the dextran sodium sulfate model of colitis are associated with long-term systemic and central induction of inducible nitric oxide synthase in wistar rats, *Proceedings of Neuroscience Ireland*, September, 2012.
- A. Abautret-Daly, C. Medina, T.J. Connor, A. Harkin, Anxiety related behaviour in an animal model of experimental colitis is associated with a long-term systemic and central inflammatory response, *Brain, Behavior, and Immunity*, Volume 24, Supplement 1, August 2010, Page S55
- A. Abautret-Daly, C. Medina, T.J. Connor, A. Harkin, Behavioural disturbances observed in experimental colitis are associated with activation of the kynurenine pathway and expression of hepatic tryptophan 2,3-dioxygenase, *Brain, Behavior, and Immunity*, Volume 24, Supplement 1, August 2010, Page S50
- W. Beumer, A. Abautret-daly, C.G. van Helden-Meeuwsen, A. Harkin, H. Drexhage, M.A. Versnel, The NOD mouse model for immune induced depressive-like behavior, *Brain, Behavior, and Immunity*, Volume 24, Supplement 1, August 2010, Pages S50-S51.

- A. Abautret-Daly, C. Medina, T. J. Connor, A. Harkin, Evidence of depression and anxiety related behavioural disturbances associated with activation of the kynurenine pathway and expression of hepatic Tryptophan 2, 3 dioxygenase in an animal model of inflammatory bowel disease, *FENS Abstr.* vol.5, 050.1, 2010. (Also presented at the Drugs and the Brain Workshop, March 21st-26th 2010 where it won a poster prize)

