

**The Gut-Brain Axis in
Inflammatory Bowel Disease:
Assessment of the Central Inflammatory
Response to Dextran Sulfate Sodium-Induced
Colitis in Rats**



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by

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Declaration

I, the author, declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work except for sections 3.3.2 to 3.3.4 which were conducted in conjunction with Dr. Áine Abautret-Daly.

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April 2018

Summary

Inflammatory bowel disease (IBD) is the term used to describe disorders involving chronic recurrent bouts of inflammation of the gastrointestinal (GI) tract including Crohn's disease (CD) and ulcerative colitis (UC). IBD is thought to be caused by a genetic predisposition to immune system over-activation prompted by an environmental trigger leading to inflammation and dysfunction of the intestinal mucosa. Symptoms of IBD include abdominal pain, blood/mucus in stools and diarrhoea. IBD has also come to be associated with changes to psychological wellbeing including symptoms of anxiety and depression. It is thought that GI inflammation may be signalling to the brain via the gut-brain axis leading to changes in the brain and eventually resulting in the psychological alterations seen in IBD. Using dextran sulfate sodium (DSS)-induced colitis to model active and recovered phases of IBD, this thesis aims to identify behavioural changes associated with colitis and to link these to alterations in central inflammation, magnetic resonance imaging (MRI) measures of brain structure and tissue composition, and to use Fos markers to map neuronal activity in the brain.

Symptoms of colitis were induced in male Wistar rats by the addition of DSS (5%) to their drinking water. Following 6/7 days of DSS intake, symptoms of acute colonic inflammation developed, including decreased body weight, diarrhoea, and rectal bleeding. Similar to human UC, the inflammation was limited to the colon, where damage to crypts and epithelial cells were observed along with immune cell infiltration. The colon also exhibits a pro-inflammatory cytokine profile with increased IL-1 β , TNF- α , IL-6, iNOS, GFAP and MMP mRNA expression and MMP activity. At 7 days following cessation of DSS exposure, increases in IL-1 β , IL-6, iNOS, and IFN γ mRNA were measured in the colon. Similarly, a pro-inflammatory profile was observed in the cortex with increased IL-6 and iNOS mRNA in acute colitis and recovery. In acute colitis, sickness behaviour was observed in the open field test, and this recovered by recovery day 4. However, rats in recovery showed symptoms of anhedonia in the saccharin preference test, behavioural despair in the forced swim test, and anxiety in the elevated plus maze, light/dark box and marble burying test. As it showed the greatest increases relative to control, the inflammatory marker iNOS was investigated further and iNOS mRNA was seen to be increased in colon, liver, spleen, cortex, hippocampus, and hypothalamus in acute colitis and recovery. Levels of iNOS in all of these regions correlated with iNOS measures in the colon and in the cortex.

Central inflammation was examined further using immunohistochemistry and revealed that DSS-induced colitis leads to neuroinflammation in two regions of the brain in particular. These regions are located surrounding the subfornical organ (SFO) and the median eminence (ME) both of which are circumventricular organs (CVO). CVOs are brain regions with a more permeable blood brain barrier (BBB). Exposure to DSS-induced colitis was associated with increases in iNOS immunoreactivity in the parenchyma surrounding the SFO and the ME. iNOS is an enzyme induced in response to inflammation, which is capable of generating neurotoxic levels of nitric oxide and of altering protein structure by nitration. The nitration marker 3-NT confirms an increase in iNOS activity at the CVOs. Being the resident immune cells of the brain, microglia were assessed, using the marker IBA1, following DSS-induced colitis, and found to be increased around the SFO and ME. Morphological analysis confirms that they are in an amoeboid, immunologically activated state. Dual immunohistochemical staining of iNOS and IBA1 indicates that iNOS is produced by microglia in response to DSS-induced colitis as the markers are co-localised. In terms of astrocytes, no alterations in immunoreactivity of the astrocyte marker GFAP were observed.

To address a potential mechanism by which peripheral inflammation may be communicated to the brain, BBB integrity was investigated following DSS-induced colitis. An increase in immunoreactivity for endogenous circulating immunoglobulin IgG, was observed at the SFO following DSS-induced colitis, however this was not observed at the ME or in recovery groups. Anatomical MRI analysis revealed an increase in ventricular volumes in both acute and recovered colitis. Changes to T2 but not T1 relaxometry times in cortical regions were observed in the recovery period indicating a change in tissue composition.

Immunohistochemical analysis of neuronal activation using the immediate early gene Δ FosB revealed an increase in neuronal activity at the NAc and DRN in acute and recovered colitis, potentially indicating activation of compensatory mechanisms in the reward and serotonergic centres of the brain to counteract the anhedonia and despair observed in this model.

These experiments provide further evidence in support of the link between peripheral GI inflammation to central inflammation, and anxiety and depression-related behaviours. Future studies are required to further elucidate the pathway by which peripheral inflammation is communicated to the brain and the links between the observed brain changes and altered behaviour. Future intervention studies should explore whether treatment of inflammation, for example with iNOS inhibitors or NSAIDs, is effective in reversing the behavioural changes and the inflammatory symptoms of colitis.

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List of Abbreviations

Δ CT	CT difference
3-NT	3-nitrotyrosine
ACTH	adrenocorticotrophic hormone
ANOVA	analysis of variance
AP	area postrema
AP-1	activator protein-1
APS	ammonium persulfate
ASL	arterial spin labelling
ATF-2	activating transcription factor -2
ATG16L1	autophagy-related protein 16-1
BAX	BCL2-associated-X-protein
BBB	blood brain barrier
BCL2	B-cell lymphoma 2
BCSFB	blood CSF barrier
BDI	Beck Depression Inventory
BDNF	brain-derived neurotrophic factor
BOLD	blood-oxygen level dependent
BSA	bovine serum albumin
btASL	bolus tracking arterial spin labelling
CA	cornu ammonis
CAI	Clinical Activity Index
CD	Crohn's disease
CD11b	cluster of differentiation 11b
cDNA	complementary DNA
CeA	central amygdala
C/EBP	CCAAT-enhancer binding protein
cGMP	cyclic guanosine monophosphate
CNS	central nervous system
COX-2	cyclo-oxygenase-2
CPE	choroid plexus epithelial cell
CRC	colorectal cancer
CRF	corticotropin-releasing-factor
CSF	cerebrospinal fluid
CT	cycle number
CTT	capillary transit time
CUMS	chronic unpredictable mild stress
CVO	circumventricular organ
CXCL1	chemokine ligand 1
Da	Dalton
DAB	3,3'-di-aminobenzidine
DAI	disease activity index

DG	dentate gyrus
DNA	deoxyribonucleic acid
DNBS	di-nitrobenzene sulfonic acid
DPX	di-N-butyl phthalate in xylene
DRN	dorsal raphe nucleus
DSS	dextran sulfate sodium
DTI	diffusion tensor imaging
EAE	experimental autoimmune encephalomyelitis
ECM	extracellular matrix
EFCCA	European Federation of Crohn's and Ulcerative Colitis Associations
eNOS	endothelial nitric oxide synthase
EPM	elevated plus maze
FITC	fluorescein isothiocyanate
fMRI	functional MRI
FMT	faecal microbiota transplantation
FOS	fructo-oligosaccharide
FOV	field of view
FRET	fluorescence resonance energy transfer
FST	forced swimming test
GBA	gut-brain axis
GF	germ-free
GFAP	glial fibrillary acidic protein
GI	gastrointestinal
GOS	galacto-oligosaccharide
GRO- α	growth regulated oncogene- α
HADS	Hospital Anxiety and Depression Scale
HAM	Hamilton
HPA	hypothalamic-pituitary-adrenal
HRQOL	health-related quality of life
HSP70	heat shock protein 70
i.p.	intraperitoneal
i.v.	intravenous
IBA1	ionized calcium-binding adapter molecule 1
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
ICAM	intercellular adhesion molecule
IDL	interactive data language
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
IFNR	IFN receptor
IgG	immunoglobulin G
IKK	I κ B kinase
IL	interleukin
iNOS	inducible nitric oxide synthase
IRAK	interleukin-1 receptor-associated kinase
IRF	IFN regulatory factor

IRGM	immunity-related GTPase family M
ISCC	Irish Society for Colitis and Crohn's Disease
JAK	janus kinase
L-AAA	L-alpha aminoadipic acid
LHb	lateral habenular nucleus
LPS	Lipopolysaccharide
LS	lateral septum
LSC	lumbosacral spinal cord
LTD	long-term depression
LTP	long-term potentiation
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MCP1	monocyte chemoattractant protein 1
MDB	membrane desalting buffer
MDMA	3,4-methylenedioxymethamphetamine
ME	median eminence
meA	medial amygdala
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MLCK	myosin light-chain kinase
MLN	mesenteric lymph node
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
mRNA	messenger RNA
MSME	multi-slice multi-echo
MTT	mean transit time
MW	molecular weight
myD88	myeloid differentiation primary response 88
NAc	nucleus accumbens
NADPH	nicotinamide adenine dinucleotide phosphate
NF	nuclear factor
NFQ	non-fluorescent quencher
NGS	normal goat serum
NHS	normal horse serum
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
-NO ₂	nitro group
NOD2	nucleotide-binding oligomerization domain-containing protein 2
NOS	nitric oxide synthase
NPY	neuropeptide-Y
NS	normal serum
NSAID	non-steroidal anti-inflammatory drug
NST	nucleus of the solitary tract
ONOO ⁻	peroxynitrite
OVLT	organum vasculosum of the lamina terminalis
pAb	polyclonal antibody

PAG	periaqueductal grey
PAMP	pathogen-activated molecular pattern
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PFC	pre-frontal cortex
PMN	polymorphonuclear
PRR	pattern recognition receptor
PVN	paraventricular nucleus
RARE	Rapid Acquisition with Relaxation Enhancement
RF	radiofrequency
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROI	region of interest
ROS	reactive oxygen species
rs-fMRI	resting state fMRI
SA	signal amplitude
SCID	severe combined immunodeficiency
SEM	standard error of the mean
SFO	subfornical organ
STAT	signal transducer and activator of transcription
T	Tesla
T1	longitudinal relaxation time
T2	transverse relaxation time
TE	echo time
Th	helper T cell
TIMP	tissue inhibitor of metalloproteinase
TJ	tight junction
TLR	toll-like receptor
TNBS	tri-nitrobenzene sulfonic acid
TNF	tumour necrosis factor
TNFR	TNF receptor
TR	repetition time
TRAF6	TNF receptor associated factor 6
TRPV1	transient receptor potential vanilloid 1
TST	tail suspension test
UC	ulcerative colitis
VBM	voxel-based morphometry
VCAM	vascular cell adhesion molecule
VEGF-A	vascular endothelial growth factor A
VTE	variable echo time
VTR	variable repetition time
w/v	weight per volume
WAS	water-avoidance stress
WKY	Wistar-Kyoto

Chapter 1

Introduction

1.1 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a chronic relapsing-remitting idiopathic condition with a complex pathogenesis characterised by chronic inflammation of the gastrointestinal (GI) tract. Patients suffering from active IBD experience symptoms including abdominal pain and cramping, bloating, blood or mucus in the stool, increased faecal urgency and persistent diarrhoea; along with systemic symptoms which can persist into the remitting disease phase and include loss of appetite or food avoidance, malnutrition, weight loss, fever and fatigue (Strober et al., 2007). Extra-intestinal manifestations of IBD can affect joints, the skin and eyes (Vavricka et al., 2015) and the presence of one extra-intestinal manifestation appears to predispose patients to others, indicating a systemic disease course (Vavricka et al., 2011). Neuromuscular and neurological complications have also been reported in IBD, though there is a wide variation in terms of reported incidence (Zois et al., 2010). Furthermore, IBD patients are at a higher risk of developing colorectal cancer compared to the general population (Kim and Chang, 2014).

1.1.1 Classification of IBD

Crohn's disease (CD) and ulcerative colitis (UC) are the two main classifications of IBD. Although closely related in terms of symptomatology, UC and CD can be clinically differentiated by their histology and according to where the inflammation is situated in the GI tract. UC is characterised by continuous inflammation of the gut epithelium, with the formation of ulcers and crypt abscesses, which is restricted to the rectum and colon. In contrast, CD is a transmural disease affecting all layers of the gut wall with the formation of focal lesions such as intestinal granulomas and fistulae. In CD, the GI tract becomes inflamed in a discontinuous fashion, with multiple points of inflammation situated at any point from rectum to mouth, though it is most commonly the terminal ileum that is affected (Kim and Cheon, 2017).

1.1.2 Impact of IBD

The disease burden of IBD is challenging for patients and not only includes the therapy, hospitalisations, and surgery caused by the physiological manifestations of the disease but also psychological and social burden. IBD therefore has serious quality of life implications for

patients. It impedes social situations thereby negatively impacting relationships with family, friends, partners and co-workers while also limiting work ability and restricting diet. The unpredictable nature of relapse is a further stressor experienced by patients throughout remission. Although IBD can occur at any age, the disease has a peak in incidence rates in younger people between the ages of 15 and 30 years, meaning that most patients are faced with this diagnosis during the most productive years of life. In CD, incidence declines sharply following this peak, however in UC, peak incidence typically occurs five years later than in CD and plateaus, particularly in males, in whom incidence does not significantly decrease until the 7th decade of life (Johnston and Logan, 2008).

An IMPACT survey commissioned by the European Federation of Crohn's and Ulcerative Colitis Associations (EFCCA) in late 2010 assessed the impact that these conditions can have on sufferers in terms of medical implications, emotional well-being, education and work, and overall quality of life (Table 1.1) (Lonnfors et al., 2014). Among Irish respondents, a majority (97%) were on medication for IBD, with 74% saying that they had used steroids to treat their condition, and 90% being hospitalised within the past five years. Almost half (48%) of IBD patients surveyed said that even between flare-ups their lives are still negatively affected by symptoms of IBD. In terms of education and work, 53% believed that IBD has affected their ability to reach their educational potential, 64% said that career prospects had been impacted by their condition, and 49% said that they have lost, had to leave, or resign from a job because of IBD.

Though incurable, IBD is a treatable disease typically managed with drugs (aminosalicylates, systemic corticosteroids and immunosuppressants) and surgery (bowel resection, colectomy with ileostomy or ileo-anal pouch anastomosis) if necessary (Carter et al., 2004, Mowat et al., 2011). Surgery will generally be required in 70-80% of CD patients and up to 30% of UC patients (Hwang and Varma, 2008). Surgery may be curative for UC whereas inflammation usually recurs following surgery in CD (Sica and Biancone, 2013). Mortality for IBD is slightly higher than the general population with a UK study of over 16,000 IBD patients with age and sex - matched controls indicating 54% excess mortality associated with IBD diagnosis (Card et al., 2003).

Table 1.1 Impact of IBD on patients in Ireland and Europe

Percentage of people with IBD who:	Ireland	Europe
Are fully employed	46%	47%
Say their diagnosis took five or more visits	18%	8%
Have used steroids for the condition	74%	52%
Are concerned about long-term steroid use	72%	49%
Regularly use pain pills	46%	28%
Are satisfied with their treatment plan	66%	70%
Have been hospitalised in the past five years due to IBD	90%	85%
Have experienced one or more flare-ups in the past two years	97%	87%
Have changed/cancelled plans during a recent flare-up	71%	53%
Feel life is significantly impacted by IBD	48%	49%
Feel IBD prevented pursuing intimate relationships	47%	40%
Feel IBD prevented them from making/keeping friends	37%	57%
Feel IBD affected their ability to reach their educational potential	53%	52%
Have taken time off work due to IBD in the past year	77%	74%
Feel their work prospects are negatively affected by IBD	64%	51%
...and feel this very strongly	41%	36%
Have lost or have had to leave/resign from a job because of IBD	49%	44%
...among those who were unemployed	not available	93%
Are optimistic due to contact with others with IBD	60%	39%

This table summarises the impact IBD has on patients in Ireland compared to overall figures for those in Europe. Data in bold indicates a higher level of negative impact reported by Irish respondents compared to the European average. Data from Lonnfors et al. (2014). Table adapted from the pamphlet 'The True Impact of Inflammatory Bowel Disease in Ireland' (IBD Action Group, 2012)

1.1.3 Epidemiology of IBD

There is a high level of epidemiological variability associated with IBD, which has its highest observed incidence rates in westernised nations such as those of Northern Europe, North America and Australia. Notably, these are regions which share a high level of similarity in terms of environmental factors and genetic background. The mean prevalence of IBD in these regions is estimated to be 1 in 1000, roughly evenly distributed between men and women (Molodecky et al., 2012, Gasparetto and Guariso, 2013). Although data for developing countries is not as readily obtainable, it has been observed that incidence of the disease has been rising globally for the past 50-60 years (Molodecky et al., 2012).

It has been estimated that IBD affects approximately 2.5 million people throughout Europe (Kaplan, 2015). However, Northern European countries, including Ireland, have a higher prevalence of the disease than that observed in the rest of Europe. According to the Irish Society for Colitis and Crohn's Disease (ISCC) it is thought that at least 20,000 people are living with IBD in Ireland; a prevalence value of 0.4% of the population. Figures for the incidence of the disease in Ireland in 2011 stood at 8.24 per 100,000 population (5.9 per 100,000 for CD and 14.9 per 100,000 for UC). Cases of IBD in children are also on the rise in Ireland, with a marked increase in incidence of paediatric IBD in recent years (Hope et al., 2012).

1.1.4 Aetiology of IBD

The aetiology of IBD remains unknown, however, the consensus is that immune dysfunction and inflammation occur as a response to an environmental trigger in a genetically susceptible host leading to the activation and release of factors that result in intestinal injury and continued intestinal barrier dysfunction (Boyapati et al., 2015, Kim and Cheon, 2017).

1.1.4.1 Genetics

There is a strong genetic component to IBD with population-based studies revealing that 5–10% of IBD patients have a first-degree family member with the disease. It is thought that some susceptibility genes for IBD are specific to UC or CD, as relative risk of developing the disease among siblings of sufferers is 30-40-fold higher for CD with a 10-20-fold higher relative risk in siblings of UC patients (Peeters and Rutgeerts, 1996, Binder and Orholm, 1996). The concordance rate as revealed by monozygotic twin studies is significantly greater for CD (30-

35%) compared to UC (10-15%) and is indicative of a greater influence of genetic factors in CD (Spehlmann et al., 2008). Over 100 distinct risk alleles have been described for IBD (Peloquin et al., 2016). There are multiple genes known to be associated with both CD and UC including *IL23R*, *IL12B*, *STAT3* and *IBD5* (Glas et al., 2012). Interestingly, genetic linkage studies have revealed that mutations in *ECM1* (extra-cellular matrix protein 1) are specific only to patients with UC (Fisher et al., 2008) whereas, alterations in certain genes of the innate immune system, including nucleotide-binding oligomerisation domain-containing protein (NOD)-2, autophagy related protein-16-1 (*ATG16L1*) and immunity-related GTPase family M (*IRGM*), are specific to patients with CD (Liu and Anderson, 2014). Polymorphisms in *NOD2*, a gene involved in initiating nuclear factor (NF)- κ B signalling through altered host interactions with the bacterial component lipopolysaccharide (LPS), and other autophagy-associated genes suggests a role played by autophagy deficiency in the immunopathogenesis of CD patients as mutations in the recognition and intracellular processing of bacterial components results in weakened and ineffectual autophagic response to intracellular pathogens (Travassos et al., 2010). This potentially offers an explanation as to why smoking, which is thought to impair autophagy, has harmful effects on CD patients but is recommended as a last resort treatment for treatment-resistant UC patients (Mahid et al., 2006).

1.1.4.2 Immunology

Given that the GI tract is considered an external surface of the body, in order for dietary antigens or commensal bacteria to enter the host they must first breach the intestinal epithelial barrier. The integrity of this physical barrier, which is made up of a single layer of intestinal epithelial cells (IEC), including specialised cells such as goblet cells and Paneth cells, is joined by tight junctions, and is a frontline defence in protection from disease (Peterson and Artis, 2014). Goblet cells produce mucus which forms a layer covering the intestinal epithelium and is the first physical obstacle to translocating luminal contents. This mucus layer is therefore critical to colonic protection (Van der Sluis et al., 2006). Paneth cells form part of the barrier in the small intestine and produce anti-microbial peptides such as lysozymes and α -defensins. IECs can also activate other innate immune responses to infection as they express toll-like receptors (TLR) and NOD-like receptors which drive induction of pro-inflammatory cytokines, chemokines and anti-microbial peptides (Kobayashi et al., 2005). Cytokines and chemokines are small regulatory proteins which act as cell signalling molecules and are an important part of the hosts response to infection. The link between an increase in

translocation of bacteria and IBD indicates the importance of epithelial barrier dysfunction in IBD aetiology or pathology. Leakiness of the intestinal barrier as seen in IBD means that the integrity of the mucosal epithelium is breached by microbial and dietary antigens which access the gut-associated lymphoid tissue e.g. mesenteric lymph nodes (MLN) and initiate an immune response. The initial immune response will also involve activation of the adaptive immune system which will promote helper T cell (Th) immune signalling. Histological examination of IBD patient biopsies in times of active colitis demonstrates a presence of monocytes, lymphocytes and polymorphonuclear leukocytes.

While CD involves submucosal inflammation, in UC, inflammation is limited to the mucosa. Inflammatory infiltrate is composed of lymphocytes, plasma cells and neutrophils. This is diagnosed as cryptitis or crypt abscesses and these are defined as the presence of neutrophils in the crypt epithelium or within the crypt lumina, respectively. Crypt abscesses are more common in UC than CD. A notable difference between CD and UC is the adaptive immune response they generate. In CD, a Th1 / Th17 response is favoured leading to increases in interleukin (IL)-12, IL-18, interferon (IFN) γ and tumour necrosis factor (TNF)- α coupled with an increase in IL-17. In contrast, a Th2 response, as observed in UC, has a different inflammatory profile involving induction of IL-4, IL-5 and IL-13. This variation between diseases is believed to stem from increased levels of mature dendritic cells in inflamed regions of CD patient biopsies compared to basal levels of dendritic cells seen in UC patients. These dendritic cells promote the differentiation of pre-helper T cells into Th1 or Th2 cells (Middel et al., 2006). In IBD, the chronic issues with intestinal barrier integrity coupled with the loss of oral tolerance to commensal gut bacteria result in a prolonged inflammatory response which can migrate to the systemic circulation initiating production of cytokines, eicosanoids, nitric oxide (NO) and proteolytic enzymes all of which serve to further increase intestinal permeability leading to a self-sustaining cycle of inflammation.

Matrix metalloproteinases (MMP) are a family of zinc-dependant endopeptidases responsible for breaking down the extracellular matrix (ECM) of connective tissue. MMPs and their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMP), are produced in the GI tract by structural cells and, under physiological conditions, basal levels of these proteins have functional roles in many physiological processes in the gut. However, under pathologic conditions, an imbalance between MMPs and TIMPs can result in excessive ECM breakdown and tissue injury and is likely responsible for the breakdown in mucosal integrity in inflamed

sites of the GI tracts of IBD patients (Heuschkel et al., 2000). MMPs can be released from connective tissue cells in response to pro-inflammatory cytokines including TNF- α and IL-1 β (Sternlicht and Werb, 2001). The gelatinase MMP9 is the most up-regulated MMP in inflamed tissue from IBD patients with levels of MMP9 correlating to histologic and endoscopic disease scores, and morphological tissue damage (Pedersen et al., 2009, Meijer et al., 2007, Gao et al., 2005, Baugh et al., 1999). Furthermore, animal models of IBD show increased MMP2 and MMP9 mRNA in the colon (Suzuki et al., 2011), while double knockout of these gelatinase enzymes confer protection against experimental colitis induction (Garg et al., 2009) and inhibition of MMP9 activity in rats has been shown to decrease pathological colitis scores and modify the expression of inflammatory genes in the colon (O'Sullivan et al., 2017). In a mouse model of intestinal fibrosis, an anti-MMP9 antibody has shown therapeutic potential for CD patients with penetrating disease (Goffin et al., 2016).

1.1.4.3 Environment

Smoking is one of the most studied risk factors for IBD, likely due to the fact that smoking is associated with increased severity and triggering of CD while cessation of smoking is associated with an increased risk for UC (Mahid et al., 2006). Apart from smoking there are numerous other environmental factors that are believed to be implicated in the pathogenesis of IBD including diet, microbiome, antibiotic medications, stress, childhood hygiene, and appendectomy, along with geographic, social, economic, educational and occupational status (Molodecky and Kaplan, 2010, Danese et al., 2004, Ponder and Long, 2013, Ananthakrishnan, 2015). The higher incidence of IBD in northern developed nations highlights the importance of the role played by the hygiene hypothesis in the pathogenesis of IBD. Coupled with this, is the increase in IBD cases in the developing nations, which correlates with the westernisation and industrialisation of these regions (Ng et al., 2013). Along with improved hygiene, westernisation of a region brings many other environmental differences including increased access to antibiotics and a change in diet, which becomes more processed with increased sugar and fat and less natural fibre. As diets become less natural they contain higher amounts of foreign dietary antigens which have the ability to trigger the immune system in the GI tract of IBD patients and can also have implications in upsetting the delicately balanced microflora of the gut (Statovci et al., 2017).

1.1.4.4 Microbiome

In a healthy individual, the human GI tract contains more than 10^{14} micro-organisms of more than 1000 species which exist in a symbiotic relationship with the host and are essential for the maintenance of healthy gut function along with various other important physiological roles (D'Argenio and Salvatore, 2015, Gill et al., 2006). As humans have built up an 'oral tolerance' to these bacteria, their presence does not initiate a host immune response. In IBD however, there is a loss of oral tolerance meaning the immune system is activated in response to normal commensal bacteria, resulting in inflammation and a decrease in the amount of bacterial species present in the gut (Sartor and Mazmanian, 2012). There is significant evidence to suggest that the GI tracts of IBD sufferers are populated with a different microbial flora than those of healthy controls. Patients with IBD are described as having loss of normal anaerobic bacteria, increased number of pathogenic bacteria, or fewer *Firmicutes* (Shih and Targan, 2008). The impact that a dysregulated microbiome (dysbiosis) can have on the host has been a topic of much interest, with many suggesting links to psychological conditions such as autism spectrum disorders, obsessive compulsive disorder, schizophrenia and depression (Macfabe, 2012, Forsythe et al., 2010). Some studies have reported that neuropsychiatric disorders, which are coupled with dysbiosis and intestinal barrier defects, can be overcome with the application of certain probiotics (Hsiao et al., 2013). Due to the inflammatory nature of IBD, the epithelial cell layer of the GI tract can become damaged, thereby increasing the permeability of the intestinal wall and allowing substances such as bacterial and dietary antigens to infiltrate deeper into the mucosa. Normally sterile tissues such as mesenteric lymph nodes, spleen and liver can be compromised by this bacterial translocation and the immune response is further enhanced (Caso et al., 2009).

1.1.5 Animal models of IBD

As the exact aetiology of IBD is still largely unknown, there are many possible factors which contribute to different aspects of the pathophysiology of the disease, including immune system dysfunction, dysregulation of the microbiota, genetics, inflammation and oxidative stress. Animal models of IBD have been developed to allow for investigation of aetiological factors in terms of understanding the mechanisms of disease pathogenesis and developing therapeutic strategies for intervention. These models may be grouped into chemical- or microbial-induced models, spontaneous models, genetically engineered or transgenic models, and adoptive transfer (T cell) models [for review see Goyal et al. (2014)]. Despite the

range of models of IBD, the main body of behavioural and brain research has been carried out in the chemically-induced models, particularly the dextran sulfate sodium (DSS) and trinitrobenzene sulfonic acid (TNBS) models. Both DSS and TNBS result in immune activation in the gut, are histologically representative of IBD, and, despite their limitations in terms of studying disease aetiology, are valuable tools for studying mechanisms of IBD pathogenesis. These models are simple, inexpensive, reproducible and valid for examining the potential interaction between intestinal immune activation and the central nervous system (CNS).

1.1.5.1 DSS-induced colitis

Okayasu et al. (1990) were the first to describe the DSS-induced colitis model which involves oral administration of DSS in the drinking water of the animals. DSS-induced colitis is well-regarded as a good model of colitis due to the similarities it shares with human IBD aetiology, pathology, pathogenesis and therapeutic response (Solomon et al., 2010). This model also has practical benefits including ease of induction, and duration and reproducibility of the induced inflammation. DSS is a sulphated, low molecular-weight (MW) polysaccharide which can be administered orally, dissolved in the drinking water made available in the home cage. It can be used to invoke either acute or chronic colitis involving hemocult positive stools, loose stools progressing to diarrhoea, and weight loss (Kullmann et al., 2001). DSS is considered to be cytotoxic to gut epithelial cells thus it has the ability to interfere with intestinal barrier tight junctions (TJ) enabling DSS, luminal antigens and bacteria to translocate across the barrier. When this happens they stimulate the immune system invoking production of pro-inflammatory cytokines TNF- α , IL-1 β , IL-6, IL-10, IL-12 and IFN γ as well as the chemokines macrophage inflammatory protein (MIP)-2 and c-x-c motif ligand 1 (CXCL1). This leads to localised colonic damage restricted to the mucosa and characterised histologically by infiltration of inflammatory cells, cryptitis and crypt loss, with extensive mucosal thinning and colonic lesions (Lombardi et al., 2012). Gaudio et al. (1999) assessed the structural, ultrastructural, immunohistochemical and symptomatic aspects of DSS-induced colitis in Sprague Dawley rats in both acute and chronic colitis describing weight loss in acute DSS-induced colitis and slowed weight gain in chronic colitis. They also report decreased colon length and demonstrate that the distal colon is primarily affected. They concluded that the DSS model is more representative of UC than CD. In addition, acute colitis following DSS exposure induces a Th2 cytokine response, which in human IBD is associated with UC.

1.1.5.2 *TNBS-induced colitis*

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 ethanol. TNBS-induced colitis results in a T cell (Th1) - mediated inflammatory response which is described as CD-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 with varying severity, strictures of the lumen, and fistulae formation. However, due to the stressful induction process for TNBS colitis, there may be confounding factors associated with this model when studying the effects of colitis on behaviour.

1.1.5.3 *Other colitis models*

Other animal models used to investigate the association between GI disturbance and psychological manifestations include infection models. Bacteria such as *Citrobacter rodentium* and *Campylobacter jejuni* colonise and disrupt tissue in the GI tract of mice and are effective models of acute colitis. *C. rodentium* uses attaching and effacing lesions to colonise the GI tract resulting in ulcerative intestinal lesions, reduced barrier integrity and production of pro-inflammatory cytokines, manifesting as weight loss and diarrhoea (Nell et al., 2010). *C. jejuni* produces and secretes toxins to aid in its intestinal colonisation leading to an increase in mucosal barrier damage, translocation of commensal bacteria across the intestinal epithelium and induction of a Th1 immune response (Mansfield et al., 2007). Stress-induced models may be used to study the effects of psychological stress on GI function. Maternal separation as a model of early life stress (O'Mahony et al., 2011), chronic subordinate colony housing (Reber et al., 2007, Reber et al., 2008), and overcrowding stress (Vicario et al., 2010) have been shown to induce spontaneous GI dysfunction or to increase susceptibility to chemically-induced colitis. Although not wholly valid for studies on IBD specifically, these models demonstrate the link between stress and GI disturbance and are functional models for assessing irritable bowel syndrome (IBS) -like symptoms.

1.1.6 IBD and the gut microbiota

In terms of gut-brain axis (GBA) research, the gut microbiota and its associations with brain and behaviour is currently one of the most promising areas of study. The gut microbiota comprises the largest collection of micro-organisms in the body, existing in a symbiotic

relationship with the host, and in the colon reaching a concentration of 10^{11} or 10^{12} cells/g of luminal contents (Dave et al., 2012). Collectively, the human gut microbiota is thought to be composed of between 15,000 to 36,000 bacterial species (Frank et al., 2007a). The major bacterial phyla found in the gut are *Firmicutes* and *Bacteroidetes* though other phyla including *Actinobacteria* and *Verrucomicrobia* are also present (Jandhyala et al., 2015). The gut microbiota is believed to play a role in IBD pathogenesis. Dysbiosis of the commensal gut bacteria is commonly observed in IBD, generally as a decrease in diversity of *Firmicutes* and an increase in *Proteobacteria* (Frank et al., 2007a). Furthermore, many of the susceptibility genes for IBD are related to microbial recognition and processing (Jostins et al., 2012), and antibiotics are known to be effective in reducing IBD symptoms (Nitzan et al., 2016). This is also supported by the finding that germ-free (GF) mice do not develop severe colitis (Sellon et al., 1998).

A strong link has been proposed between a healthy gut microbiota and the CNS. The microbiota has been implicated in several neuropsychiatric disorders including depression, anxiety, autism and schizophrenia [for review see Sherwin et al. (2016)]. The bacterial flora of the gut can communicate with the brain via vagal pathways and immune mediators as previously discussed, as well as by the production of microbial metabolites. In a healthy individual this bi-directional communication maintains host homeostasis, whereas in IBD patients, this balance may be disturbed with potential consequences for the CNS.

The imbalance observed in the gut microbiota of IBD patients presents a potential therapeutic target. Manipulation of the gut microbial flora can be achieved using probiotics, prebiotics or a combination of both (synbiotics). A probiotic is a live microbial food supplement that beneficially affects the host by improving their intestinal microbial balance (Fuller, 1993). A prebiotic is a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth, activity, or both, of one of a limited number of bacterial species already resident in the colon (Gibson and Roberfroid, 1995). Faecal microbiota transplantation (FMT), an extremely effective treatment for *Clostridium difficile* infection, is another method of gut microbiota manipulation being explored as a potential therapy for IBD patients. The procedure involves transplant of a faecal preparation from a healthy donor into the colon of the patient via naso-enteric tube, colonic enema or in capsule form. In *C. difficile* infection, this works by restoring balance to the gut microbial environment. Although IBD is a

more complicated condition with many genetic, environmental and immune factors at play, the restoration of a healthy gut microbiota may be advantageous at least for some patients (Rossen et al., 2015, Moayyedi et al., 2015, Steed et al., 2010, Sood et al., 2009).

1.1.6.1 Microbiota modulation in IBD and associated psychological symptoms

To date, clinical studies investigating the effects of probiotics or prebiotics in IBD have focused on the GI aspects of IBD. Probiotics show efficacy in inducing remission and increasing remission times in UC (Mallon et al., 2007), which was not the case in CD (Rahimi et al., 2008). Despite promising results in TNBS- and DSS-induced colitis in animals, prebiotics have been tested in few clinical trials. Like probiotics, results show efficacy in reducing inflammation and inducing remission in UC (Casellas et al., 2007, Bamba et al., 2002, Kanauchi et al., 2002) while, although there are conflicting reports, has been shown to be effective at reducing disease activity and inflammation in CD (Benjamin et al., 2011, Lindsay et al., 2006). Although no work has explored the potential therapeutic benefit for probiotics or prebiotics in reducing anxiety or depression specifically in IBD patients, this therapeutic strategy has been tested in other cohorts. Messaoudi et al. (2011) administered a probiotic mixture of *Bifidobacterium longum* and *Lactobacillus helveticus* for 30 days to both rats, and healthy human volunteers, and showed a decrease in anxiety-like behaviour in rats and a decrease in anxiety and depression scores in humans measured by the Hospital Anxiety and Depression Scales (HADS) and a 90 item Hopkins symptom checklist. Similarly, Rao et al. (2009) administered a strain of *L. casei* to chronic fatigue patients resulting in a reduction in anxiety scores. In terms of prebiotics, Schmidt et al. (2015) administered a three week course of galacto-oligosaccharide (GOS) or fructo-oligosaccharide (FOS) prebiotic to healthy human volunteers. The GOS resulted in a decrease in waking cortisol levels and decreased attentional vigilance to negative versus positive emotional stimuli indicating an anxiolytic effect compared to placebo. FOS had no effects in either test. Considering these encouraging data, the use of microbiota modulation by pre/probiotics as a simple, non-invasive therapy for the psychological, as well as GI, symptoms of UC and potentially CD is an area which should be further explored in future. In terms of FMT as a treatment for IBD, a systematic review of the literature from case reports and cohort studies showed a modest increase in remission rates in IBD patients receiving FMT therapy (Colman and Rubin, 2014). More recently, two randomised, controlled trials have been carried out in UC patients with conflicting results suggesting a beneficial outcome that may be dependent on donor stool, route of administration, dosage, time since diagnosis and

whether patients are also receiving immunosuppressive therapy (Moayyedi et al., 2015, Rossen et al., 2015). Interestingly, Irish researchers have recently postulated that FMT may be of therapeutic benefit in depression after showing that FMT from depressed human patients into microbiota-deficient rats induces a depressive phenotype in the rats with symptoms of anhedonia and anxiety (Kelly et al., 2016).

1.1.6.2 *Animal models of IBD and the gut microbiota*

In animal studies, the modulation of the microbiota has been used to influence behaviour. GF mice display decreased anxiety-like symptoms in the elevated plus maze (EPM), open field and light/dark box compared to specific-pathogen-free mice (Heijtz et al., 2011). GI microbial infection and inflammation, including exposure to DSS-induced colitis, results in an increased anxiety-like profile (Bercik et al., 2011a, Goehler et al., 2008, Lyte et al., 2006). Modulation of the gut microbiota using probiotics can alter the behavioural response. Anxiety and/or depression-related behaviours in the EPM and forced swimming test (FST) in Balb/c mice (Bravo et al., 2011) and in the FST in maternally separated rats (Desbonnet et al., 2010) are rescued by probiotics. Probiotics also reduce anxiety-like behaviours induced in rats in response to DSS-induced colitis (Bercik et al., 2011a). This points to a potential therapy for neuropsychiatric disturbance in IBD patients. In the aforementioned study by Bercik et al. (2011a), results demonstrated that increased anxiety measured in the step-down test in DSS-exposed mice was reversed by the probiotic *B. longum* NCC3001 without affecting gut inflammation (as measured by myeloperoxidase activity and histological scores). They found that the anxiolytic effect of *B. longum* was lost in mice vagotomised before the third cycle of DSS potentially due to the modulatory effects of the fermentation products of *B. longum* on enteric neuron excitability. In another previously mentioned behavioural study, Emge et al. (2016) reported that the deficits in recognition memory and anxiety-like behaviour during active inflammation on day eight post-DSS were ameliorated by administration of a probiotic mixture containing *Lactobacillus rhamnosus* and *L. helveticus* (Emge et al., 2016). This study also observed a significant decrease in c-Fos expression in the *cornu ammonis* (CA)1 region of the hippocampus in mice at day eight post-DSS which is similarly rescued by administration of the *L. rhamnosus* and *L. helveticus* probiotic combination. It has recently been observed that intestinal inflammation may impact upon hippocampal neurogenesis (Zonis et al., 2015). Following four cycles of DSS (3%) in mice, Zonis and colleagues observed a down-regulation in markers for stem/early progenitor cells, with a concomitant increase in p21, a suppressor

of cell proliferation, indicating a reduction in neurogenesis in the hippocampus. As p21 can be stimulated directly by pro-inflammatory cytokines it has been proposed that this decrease in hippocampal neurogenesis may occur as a consequence of the increase in activated microglia and astrocytes observed in the hippocampus at the acute phase of colitis and in chronic colitis respectively.

1.2 Comorbid anxiety and depression in IBD

Historically, there has been a long-standing interest in the comorbidity of psychological symptoms, psychiatric illness and personality differences associated with IBD (Straker, 1960, Daniels, 1942, Sullivan and Chandler, 1932, Murray, 1930) in patients of all age groups. Many early studies in adults and children concluded that both UC and CD are related to higher incidence of psychological symptoms (Farrokhyar et al., 2006, Guthrie et al., 2002, Addolorato et al., 1997). In a recent systematic review of the comorbidity of psychological disorders with IBD, Mikocka-Walus et al. (2016) evaluated 66 articles published between 2005 and 2014. Rates of anxiety and depression were estimated to be greater in IBD patients compared to healthy individuals, with rates of both being higher during the active IBD phase compared to remission. They also found that mean rates of anxiety and depression were significantly higher in CD compared to UC.

Validated questionnaires including the HADS, Beck Depression Inventory (BDI), State Trait Anxiety Inventory, and Hamilton (HAM) anxiety and depression scales have been used to confirm an increased risk of psychological disturbances in IBD patients (Gandhi et al., 2014, Goodhand et al., 2012b, Addolorato et al., 2008, Calvet et al., 2006). Differences between studies suggest that factors such as the level of disease activity may influence anxiety and depression scores. Depression and/or anxiety are consistently reported to be increased in the active phase of IBD (Clark et al., 2014, Gandhi et al., 2014, Bokemeyer et al., 2013, Ben Thabet et al., 2012), with some reports in remission (Vidal et al., 2009).

Further details of reports documenting anxiety and depression symptoms in IBD patients are summarised in Table 1.2.

Table 1.2 Reports of anxiety- and depression-related symptoms in IBD patients

Study	Participants	Measures	Results & Author's Conclusions
(Walter et al., 2016) USA	adolescents: UC, CD, IC	FDI, RCADS	13% of sample reported clinically elevated anxiety or depression, associated with perceived functional disability but not clinical disease activity (74% of sample had inactive disease).
(Cawthorpe and Davidson, 2015) Canada	adults & children: UC, controls	medical records: ICD mental disorder & UC	Mental disorder present in 82% of UC sample. Neuroses/depressive disorders are most likely mental disorders to arise before UC diagnosis which indicates aetiological relationship between mental disorder and UC or between their treatments.
(Horst et al., 2015) USA	adults: UC, CD	HBI, SCCAI, PHQ-9, CRP	Depressive scores are decreased in IBD patients receiving immunosuppressive therapy. Significant decrease in UC and CD patients at risk of moderate to severe depression following therapy. Changes in CRP scores correlate with PHQ-9 (depression) scores.
(Keeton et al., 2015) Australia	adults: UC, CD	concerns & worries, HADS, DASS-21	Although 74% of respondents were in remission, 96% had significant disease-related concerns, 21% reported symptoms of depression, 40% of anxiety.
(Kinsinger et al., 2015) USA	adults: UC, CD, other GI disorders	demographic/psychosocial checklist, BSI, IBS-QOL, medical records	Psychological intervention can reduce healthcare burden for patients with GI conditions and is associated with reduced outpatient visits, medical procedures and medications required.
(Trindade et al., 2015) Portugal	adults: UC	DASS-21	Depressive symptoms were associated with UC-related symptom expression.
(Clark et al., 2014) USA	children & adolescents: CD	CDI, Paris Classification, KSADS, PCDAI, ESR, medication use	Infliximab use was not associated with decreased depressive symptoms. SES and disease activity were the strongest predictors of depression on the PCDAI.
(Gandhi et al., 2014) USA	adults: UC, CD	HBI, IBDQ, BDI, PHCS, CISS, CAMBI	Patients with inactive IBD had less depressive symptoms, and improved perceived health competence, task-oriented coping, and QOL compared to those with active IBD.
(Long et al., 2014) USA	adults ≥ 65 years: UC, CD	short-GDS, short -CDAI, SCCAI, short-IBDQ	22.6% of patients had GDS scores consistent with a diagnosis of major depression. Point prevalence of depression did not differ between UC and CD. Depressed patients had significantly higher disease activity scores and decreased QOL.

(Maconi et al., 2014) Italy	adults: CD-R	CDAI, HADS	Anxiety and/or depressive symptoms were present in 36.9% of patients, 58% of whom were not being treated for anxiety or depression. Anxiety significantly correlated with female sex, history of perianal disease and perianal surgery.
(Szigethy et al., 2014a) USA	children & adolescents: UC-D, CD-D	CDI, KSADS, CDRS-R, IMPACT-III, CGAS, PCDAI, PUCAI	Following three months of psychological therapy, 65% of all participants no longer met the DSM-IV-TR criteria for depression. IBD activity improved over time for both CBT and SNTD with a slightly larger decrease for CBT.
(Szigethy et al., 2014b) USA	children & adolescents: UC, CD	CDI, CDRS-R, SCARED, IMPACT III, PCDAI, PUCAI, API, BIPQ, medical records	Greater disease activity in depressed versus non-depressed youth with IBD. Evidence for 3 depressive profiles in youth with concurrent IBD and depression.
(Mirta and Kolho, 2014) Finland	children & adolescents: UC, CD, controls	analysis of national drug registers	Significant increase in anti-depressant drug use in IBD youth compared to peers up to 3 years following diagnosis.
(Machos et al., 2014) Greece	adults: UC	CDAI, ZDRS, STAI, HADS	Inducible HSP70 induction in PMN cells of UC patients correlates with depression and anxiety scores in ZDRS, STAI and HADS-D but not HADS-A. This could potentially be a biomarker for depression and anxiety in UC.
(Ananthakrishnan et al., 2013) USA	adult females: UC, CD, controls	MHI-5	Women with recent depressive symptoms had an increased risk of CD but not UC.
(Bokemeyer et al., 2013) Germany	adults: UC, CD, IC	CAI, CDAI, PGA, SF-36, IMET-IS	UC and CD patients with active disease more often reported severe depressive symptoms and sexual problems.
(Guloksuz et al., 2013) Netherlands	adults: CD	HBI, CDAI, MFI, IBDQ, HAM-D-17, BDI, SCL-90, SCID, APP, zinc, TRP	Anti-TNF- α treatment increased IBDQ scores and reduced SCL-90 depression scores for CD patients independent of disease activity. Depression scores were associated APP-y over time but not with TRP availability.
(Knowles et al., 2013) Australia	adults: CD	BIPQ, HADS, SQLS	48% of CD patients had anxiety and 42% depression according to the HADS. Of these only 20% and 31% respectively were receiving psychological care.
(Langhorst et al., 2013) Germany	adults: UC	endoscopy, histology, CAI, PSQ, HADS, CPSS	Short-term stress and male gender, but not long-term stress, depression or mucosal healing, were predictive of relapse in UC patients.
(Schuman et al., 2013) USA	adolescents: UC, CD	CDI, FAD, CBCL, LCAL, short-PCDAI	20% of patients scored above the cut-off for depression on the CDI with no difference between UC and CD. Disease severity was a significant predictor of patient-reported but not parent-reported depressive symptoms

(Selinger et al., 2013) Australia	adults: UC, CD	steroid medication self-report, CCKnow, short-IBDQ, HADS	High levels of anxiety (41.8%) and depression (14%) in IBD patients with no difference in anxiety between UC and CD. Anxiety was correlated with level of disease-related knowledge, worse depression scores and lower QOL.
(Shiga et al., 2013) Japan	adults: UC, CD	hospital records, Mayo disease scores (UC), HBI	Significant increase in relapse rate observed among UC but not CD patients following Great East Japan Earthquake compared to the same period of time the previous year. Life event stress is associated with relapse in UC, not CD.
(Ben Thabet et al., 2012) Tunisia	adults: UC, CD, patient controls	CDAI, HADS, TAS-20	More IBD patients than controls had high HADS-D (22%) and HADS-A (26%) scores. Approximately 73% of high HADS-D and HADS-A scores were within an active disease phase.
(Besharat et al., 2012) Iran	adults: UC, CD, IC	SCCAI, BDI	Depression was seen in 32% of patients with IBD despite all patients being in remission. A non-significant correlation was observed between SCCAI, BDI, age and BMI.
(Goodhand et al., 2012a) UK	all ages: UC, CD, controls	medical records	IBD patients on anti-depressants had fewer relapses and courses of steroids in the year following anti-depressant treatment compared to the previous year with fewer endoscopies in year two compared to year one.
(Goodhand et al., 2012b) UK	adults: UC, CD, healthy controls	SCCAI, CDAI, HADS, general-PSQ, FC, CRP, endoscopy	Anxiety and depression scores were significantly higher in IBD versus healthy controls. No differences in mean HADS scores were found between UC and CD. Active UC was associated with higher HADS scores but CD was not.
(Loftus et al., 2011) USA	children: CD, paediatric controls adults: CD-R	ICD-9-CM, epidemiology, medical records, prescription medication CDAI, CRP, ELISA, HADS	The risk of developing an anxiety or depressive disorder following CD diagnosis is greater for CD patients compared to non-CD patients. CD also increases the risk of developing persistent anxiety and depression. Despite clinical remission, 39% of CD patients had anxiety symptoms and 24% had depressive symptoms. Infliximab therapy is the only factor associated with anxiety, however it is inversely associated with depression.
(Vidal et al., 2009) Spain	adults: UC-R, CD-R	SCID, HBI, SCAI	IBD patients in remission had at least one psychiatric disorder (31.1%). Anxiety (17.9%) and depression (11.6%) were the most prevalent.
(Addolorato et al., 2008) Italy	adults: UC, CD, other GI disorders	STAI, ZDRS	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.

(Vidal et al., 2008) Spain	adults: UC, CD	IBDQ, CDAI, HADS, TCI	44.6% scored > 8 in HADS-A or HADS-D. Psychological distress and disease activity were predictors of low QOL.
(Walker et al., 2008) Canada	adults: UC, CD, community controls	CIDI, CPSS, HAQ, PWB, IBDQ	Social anxiety was decreased in IBD versus controls. Increased major depression in IBD versus controls. Anxiety or mood disorders lead to decreased QOL perception and earlier onset of IBD symptoms.
(Calvet et al., 2006) Spain	adults: CD, healthy controls	SF-36, HAM-A, HAM-D-17	Decreased SF-36 score and significantly increased HAM-A and HAM-D scores in active disease versus healthy controls and CD patients in thiopurinic-induced remission.
(Janke et al., 2005) Germany	adults: UC, CD	GLS, HRLS, FLZ, HADS, GIBDI	Increased psychiatric illness, medical comorbidity, 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	Major depression in CD is predictive of lower remission rates.
(Mardini et al., 2004) USA	adults: CD	CDAI, BDI, BAI, BHS, RLC	Depression scores were significantly correlated with disease activity scores at baseline and 8-12 weeks later. Depressive symptoms were positively associated with future changes in disease activity.
(Mittermaier et al., 2004) Austria	adults: UC, CD	BDI, STAI, IBDQ, PSQ, RFIPC	Depression scores significantly correlated with the number of relapses after 12 or 18 months and with the time-to-disease. Anxiety and HRQOL were also related with more frequent relapses during 18 months.
(Kurina et al., 2001) UK	adults: UC, CD	ORLS medical records	Depression and anxiety preceded UC but not CD more often than expected in control groups. Depression and anxiety were more common following CD diagnosis. UC was followed by anxiety, but not depression.

Table 1.2 Reports of anxiety- and depression-related symptoms in IBD patients

Abdominal Pain Index (API), acute phase protein-gamma (APP γ), Beck Anxiety Inventory (BAI), Beck Depression Inventory (BDI), Beck Hopelessness Scale (BHS), Brief Illness Perception Questionnaire (BIPQ), Brief Symptom Inventory (BSI), C-reactive protein (CRP), Center for Epidemiologic Studies-Depression Scale (CES-D), Child Behaviour Checklist (CBCL), Children's Depression Inventory (CDI), Children's Depression Rating Scale-Revised (CDRS-R), Children's Global Assessment Scale (CGAS), Clinical Activity Index (CAI), Cognitive Behavioural Therapy (CBT), Cohen Perceived Stress Scale (CPSS), Complementary and Alternative Medicine Beliefs Inventory (CAMBI), Composite International Diagnostic Interview (CIDI), Coping Inventory for Stressful Situations (CISS), Crohn's and Colitis Knowledge score (CCKnow), Crohn's disease / in remission / with depression (CD / CD-R /-D), Crohn's Disease Activity Index (CDAI), Depression Anxiety Stress Scale (DASS), Diagnostic and Statistical Manual of Mental Disorders-IV-Text Revision (DSM-IV-TR), Depression Anxiety and Stress Scale (DASS), enzyme-linked immunosorbent assay (ELISA), erythrocyte sedimentation rate (ESR), faecal calprotectin (FC), Family Assessment Device (FAD), Fragen zur Lebenszufriedenheit - German questionnaire on life satisfaction (FLZ), Functional Disability Index (FDI), gastrointestinal (GI), General Life Satisfaction (GLS), general population (GP), Geriatric Depression Scale (GDS), German Inflammatory Bowel Disease activity Index (GIBDI), Hamilton rating scale for Anxiety / Depression (HAM-A / -D), Harvey Bradshaw Index (HBI), Health Anxiety Questionnaire (HAQ), Health-Related Life Satisfaction (HRLS), Health Related Quality Of Life (HRQOL), heat-shock protein 70 (HSP70), Hospital Anxiety and Depression Scale / -Anxiety subscale / -Depression subscale (HADS / HADS-A / HADS-D), Illness Behaviour Questionnaire (IBQ), immunofluorescent (IF), IMET-Impairments in Sexuality (IMET-IS), indeterminate colitis (IC), International Classification of Diseases / -Clinical Modification (ICD / ICD-CM), inflammatory bowel disease (IBD), Inflammatory Bowel Disease Questionnaire (IBDQ), irritable bowel syndrome (IBS), Lichtiger Colitis Activity Index (LCAI), Mental Health Index-5 (MHI-5), Multidimensional Fatigue Inventory (MFI), Oxford Record Linkage Study (ORLS), Patient Health Questionnaire (PHQ), Paediatric Crohn's Disease Activity Index (PCDAI), Paediatric Ulcerative Colitis Activity Index (PUCAI), Perceived Health Competence Scale (PHCS), Perceived Stress Questionnaire (PSQ), perinuclear anti-neutrophil cytoplasmic antibodies (pANCA), Physician Global Assessment (PGA), polymorphonuclear cells (PMN), Psychological Well-Being manifestations scale (PWB), Quality Of Life (QOL), Rating Form of IBD Patient Concerns (RFIPC), Revised Children's Anxiety and Depression Scale (RCADS), Holmes Recent

Life Changes (RLC), Screen for Child Anxiety Related Disorders (SCARED), Short-Form Health Survey (SF), Simple Clinical Colitis Activity Index (SCCAI), Social Support List - Interactions (SSL-I), State Trait-Anxiety Inventory (STAI), Stoma Quality of Life Scale (SQLS), Structured Clinical Interview for Axis-I DSM-IV Disorders (SCID), Supportive Non-Direct Therapy (SNDT), Symptom Distress Checklist (SCL), Toronto Alexithymia Scale (TAS), tryptophan (TRP), ulcerative colitis / in remission / with depression (UC / UC-R /-D), Zung Depression Rating Scale (ZDRS).

Appending number on abbreviations in table, if present, indicates number of items on test

1.2.1 Gastrointestinal pathology and psychological symptoms: cause and effect

When studying the comorbidity of psychological symptoms with IBD it is necessary to question how these may be linked and whether one may predispose to the other. Recent work has concluded that this relationship is bi-directional such that dysregulation of the microbiome and immune system in the GI tract can impact on psychological health, as well as psychological stress having negative implications for gut function (Mayer, 2011). Although there has been a substantial amount of literature published concerning the prevalence of depression and anxiety in IBD, less investigation has been carried out into the effect of such symptoms on the development of IBD or on the course of IBD. This may be due to the longitudinal and more protracted nature of this kind of study. 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 succeeded the diagnosis of IBD. Results showed that with both CD and UC there is a greater chance of suffering from depression, however, 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. Therefore, they suggest that the onset of depression in UC might be causally related to UC, a result of living with an undiagnosed bowel condition. For CD, they suggest that depression might be a result of the disease symptoms or treatment of the illness. Cawthorpe and Davidson (2015) also found that neuroses or depressive disorders were most likely to arise before UC for men and women. They suggest that psychotropic medication used to treat anxiety and depression may play a role in the aetiology of UC. Concerning paediatric literature on this topic, a 2011 study, which analysed medical and prescription claims of children with CD and patient controls, observed a 74% increased risk of developing an anxiety disorder after CD diagnosis, with an increased risk of developing persistent anxiety or depression following diagnosis, and a significantly greater likelihood of being prescribed psychotropic medication (Loftus et al., 2011).

Walker et al. (2008) investigated the lifetime risk of depression in the Manitoba IBD patient cohort (Canadian IBD cohort), and carried out a long-term analysis of these patients over 12 months. They reported 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. Contrary to Kurina et al. (2001), Ananthkrishnan et al. (2013) found that depressive symptoms increase the risk for CD, but not UC, among women. The reasons for the differences in outcome between these studies are unclear and likely reflect the complexity of IBD and the many factors which contribute to development of psychological symptoms. Further research into the potential impact of psychological disorders on the development of IBD is needed.

1.2.2 Risk of relapse and impact of psychologically oriented therapy

A small number of studies have investigated the influence of anxiety or depression on the risk of relapse in IBD. In a study of 112 patients with inactive IBD, Vidal et al. (2009) reported that neither depression nor anxiety increased the risk of relapse in UC or CD patients. Langhorst et al. (2013) also failed to demonstrate a predictive effect of depression on the risk of relapse in patients with UC. This is contradictory to two older studies where BDI scores were predictive of future changes in IBD activity (Mardini et al., 2004, Mittermaier et al., 2004). In a paediatric study of children and adolescents with IBD, Szigethy et al. (2014b) reported greater disease activity in depressed compared to non-depressed youth with IBD. Persoons et al. (2005) also reported decreased remission rates in patients with major depressive disorder.

Goodhand et al. (2012a) have reported improvement in IBD in patients who have been prescribed anti-depressants, compared to matched patients who did not receive treatment for depression. Another study by Szigethy et al. examined the effect of therapy on depressed youth with IBD (Szigethy et al., 2014a). They found that cognitive behavioural therapy and supportive non-direct therapy improved health-related quality of life (HRQOL) and psychosocial functioning, and were associated with an improvement in IBD activity over time. In a recent study concerning psychological intervention for patients with a range of GI disorders, including IBD, functional bowel disorder, dyspepsia, and oesophageal symptoms, Kinsinger et al. (2015) reported that psychological intervention can reduce healthcare burden. These studies suggest that psychological assessment may help to identify patients at risk of disease exacerbation or decreased rates of remission and may be an effective way to improve HRQOL. Importantly, these factors suggest that treating the psychological symptoms is beneficial in terms of the overall course and management of IBD.

1.2.3 Effects of stress on risk of relapse

As well as anxiety and depression, short-term and long-term stress may influence the course of IBD. Evidence of bi-directional communication between the gut and brain is emerging, indicating that psychological stress and/or depression has negative implications for normal gut function. It is also hypothesised that stress may be a risk factor for relapse in patients suffering from IBD, although this remains controversial, with some studies reporting no effect of stress on development of IBD or increased risk of relapse (Vidal et al., 2008, Li et al., 2004). However, Langhorst et al. (2013) found that short-term stress, but not long-term stress, was predictive of relapse in UC patients. The impact of life-event stress on UC, but not CD, was highlighted after the Great East Japan Earthquake in 2011 (Shiga et al., 2013). Results from twelve hospitals, found that the disease activity scores of UC patients increased significantly in the two months following the earthquake. Dietary changes and anxiety regarding family finance were independent predictors of relapse.

1.2.3.1 The hypothalamic-pituitary-adrenal axis

The hypothalamus is the part of the brain which controls our response to stress. The short-term response to stress, the 'fight or flight' response, occurs via the sympathomedullary pathway which involves activation of the adrenal medulla and the secretion of adrenaline leading to arousal of the sympathetic nervous system and dampening of the parasympathetic nervous system. Activation of this pathway therefore results in an increase in heart rate, breathing, release of glucose, and a decrease in digestion. Once this stress passes, the parasympathetic system takes over again and the body returns to normal. The hypothalamic-pituitary-adrenal (HPA) axis is a major neuroendocrine axis which functions to control the long-term response to stress and to regulate physiological processes including digestion, the immune system, mood and emotions, as well as energy storage and expenditure (Stephens and Wand, 2012). HPA axis activation involves production and secretion of vasopressin and corticotrophin-releasing factor (CRF) from the paraventricular nucleus of the hypothalamus (PVN). Vasopressin and CRF stimulate the pituitary gland to secrete adrenocorticotrophic hormone (ACTH) which in turn stimulates the adrenal gland to release cortisol. Levels of cortisol dictate activation of the sympathetic nervous system. Cortisol may act on the PVN and pituitary gland to inhibit further hormone release and as cortisol levels decrease the parasympathetic nervous system dampens the stress response. CRF may also be released peripherally from the adrenal cortex in response to stress and directly influences stress-

induced alterations in gut motility. Cortisol is the main stress hormone in humans which acts on the brain to regulate the response to stress. Cortisol released from the adrenal cortex can inhibit the activation of the pro-inflammatory transcription factor NF- κ B. In times of chronic stress, for example, in IBD, it is possible that the sustained increase in cortisol can have a dampened effect on NF- κ B inhibition, thereby influencing inflammatory responses (Tian et al., 2014).

1.2.3.2 Effects of stress on the gut

HPA axis dysfunction has been implicated in mood disorders including anxiety, depression, bipolar disorder, post-traumatic stress disorder and attention-deficit-hyperactivity-disorder, along with other illnesses including IBS (de Kloet et al., 2006, Chang et al., 2009, Keller et al., 2017, Ma et al., 2011, Watson et al., 2004, Arborelius et al., 1999). Depressed patients have an increase in cortisol levels in urine, blood and CSF and an increase in CRF in the CSF (Roy et al., 1988, Varghese and Brown, 2001). A year-long study by Bitton et al. (2003) showed that of 60 UC patients in remission, those who relapsed had more stressful life events in the month preceding relapse than those who did not relapse. A further study by Bitton et al. (2008) showed that, of a group of patients in remission from CD, those who were the least likely to relapse were those who had the least stressful lives and who were least engaged in social diversion/distraction.

Konturek et al. (2011) describes several stress-induced disturbances to normal GI physiology: including alterations to GI motility, GI secretion, GI mucosa and mucosal blood flow, intestinal microbiota and also increased visceral perception and intestinal permeability. The chronic unpredictable mild stress (CUMS) model augments levels of colonic cytokines (IL-6, IL-1 β and IL-17A) and neutrophil infiltration observed in DSS-exposed rats (Deng et al., 2016). Previous investigations have shown that when 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 psychosocial stress on histological changes in the murine colon and reported that exposure to chronic subordinate colony housing leads to colonic inflammation resulting in macroscopic damage to the mucosal layers of the colon, and an increased secretion of pro- and anti-inflammatory markers by the MLN.

Milde and Murison (2002) report decreased time-to-symptom-expression in DSS rats previously exposed to restraint stress, and 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). The genotoxic agent azoxymethane predisposes mice to develop colorectal cancer (CRC) when challenged with DSS. Peters et al. (2012) report an increased risk of inflammation-related CRC when azoxymethane mice were also exposed to chronic subordinate colony housing. 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 immune dysregulation, increased intestinal permeability and disturbed intestinal microbiota. One caveat when studying the effect of psychological stress or maternal separation on models of 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.

1.2.3.3 GI effects on the HPA axis

Due to the bi-directionality of the GBA not only will a disturbance such as stress influence the GI system but a disturbance in the GI system may also influence stress by activation of the HPA axis. In a water avoidance stress (WAS) paradigm, DSS treatment increased basal and post-stress (90 minute) levels of circulating corticosterone, indicating an index for increased HPA axis activity (Reichmann et al., 2015). Greenwood-Van Meerveld et al. (2006) studied the long-term effects of acute colitis on the expression of central CRF in rats. They found a significant increase in CRF mRNA expression in the PVN of the hypothalamus three days post-TNBS enema, which persisted up to 30 days post-enema. The increased CRF expression was also present in the central amygdala (CeA) three days post-enema, however it had returned to basal levels in this region at 30 days post-enema. Porcher et al. (2004) reported increased 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. Kojima et al. (2002) report the opposite effect following TNBS: decreased CRF expression in the PVN at three and seven days post-enema. They do however report increased circulating corticosterone on days 1, 3, 7 and 14 post-enema. 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 indicating that a higher dose of TNBS may provoke a more severe colitis necessary for increased CRF expression in the brain.

1.2.4 Factors influencing anxiety and depression in IBD

Some socioeconomic/environmental/physiological factors such as education, socioeconomic status, gender, diet, pain, perceived stress, etc. may be predictive of, or have an effect on, psychological disturbance in IBD and are worth studying to increase our understanding of disease pathogenesis and psychological comorbidity. These factors may account for some of the variation observed across studies of depression and anxiety in IBD and are noted in Table 1.2 where applicable.

Various studies have observed that lower socioeconomic status and lower educational level are associated with depression and anxiety in IBD patients (Ennaifer et al., 2014, Schuman et al., 2013, Nahon et al., 2012). Such patients are also shown to have lower HRQOL than the general population; however, it is difficult to draw conclusions from this link as this may be a result of other non-disease-related factors (Sainsbury and Heatley, 2005).

Generally, there is little difference in IBD occurrence between men and women (Zelinkova and der Woude, 2014). Interestingly, gender does appear to be linked to differences in psychosocial manifestations of the disease. The majority of studies indicate that female gender is a predictor of anxiety and depression in IBD (Ennaifer et al., 2014, Panara et al., 2014, Maconi et al., 2014). Females are also believed to be more susceptible to the impact of IBD on HRQOL (Casellas et al., 2002) potentially due to increased symptom perception in women (Hauser et al., 2011). Females with IBD are also more likely to have IBS-like symptoms concurrent with IBD (Berrill et al., 2013b), greater levels of fatigue (Norton et al., 2015), and show a higher incidence of mood swings among those with CD (Lima et al., 2012), all of which may impact on HRQOL and psychological health.

Although diet is not a causative factor in IBD it is thought to be a potential trigger for IBD flares, and it is believed that the Westernised diet, rich in processed foods, is a factor in the increased incidence of the disease in these regions (Hou et al., 2011). A modified diet, limiting excess fat, carbohydrates, fibre and lactose, and encouraging intake of prebiotic and probiotic foods may be helpful as an adjunct therapy in IBD for decreasing symptoms and reducing medication requirements (Olendzki et al., 2014). Although it is still unclear whether dietary modification would have therapeutic effects in treating the psychological aspects of IBD, considering the

links between diet, stress, and microbiota on psychological well-being, it is reasonable to suggest that diet may be influential.

The literature indicates that active IBD is associated with an increase in psychological manifestations (Long et al., 2014, Clark et al., 2014) with disease severity being an independent predictor of depressive symptoms (Panara et al., 2014, Schuman et al., 2013). UC patients with pain have been shown to have significantly higher depression scores than UC patients without pain (Deberry et al., 2014), with higher pain scores being a predictor of depression in both UC and CD (Deberry et al., 2014, Srinath et al., 2014). As well as pain, the nature of other symptoms experienced by IBD patients can be extremely stressful. As previously discussed, stress can affect visceral sensitivity, gut motility, and the immune system in IBD, and as previously noted, may be a trigger for IBD flares (Langhorst et al., 2013). Perceived stress has been associated with mood disturbance in both UC and CD (Goodhand et al., 2012b), indicating a link between stress, symptom-related or otherwise, and psychological disturbance in IBD.

1.2.5 Inflammatory origins of mood disorders

Mounting evidence indicates that inflammation plays a critical role in the pathophysiology of mood disorders. Patients with schizophrenia, major depression and bipolar disorder have been shown to have elevated levels of pro-inflammatory cytokines such as IL-6 and TNF- α (Goldsmith et al., 2016, Dowlati et al., 2010). Reciprocally, many inflammatory conditions including IBD, rheumatoid arthritis, psoriasis, cardiovascular disease and diabetes (Margaretten et al., 2011, Tying et al., 2006, Hare et al., 2014, Andreoulakis et al., 2012) have been linked to a higher risk of mood disorders. Elevated cytokine levels associated with peripheral inflammation have also been suggested to contribute to brain inflammation and pathogenesis associated with Alzheimer's disease, Parkinson's disease and schizophrenia (Reale, 2015, Reale et al., 2009, Müller et al., 2015). Evidence for this link is also found in studies of cancer patients and hepatitis C patients receiving immune-based therapy. These patients display increases in depressive tendencies while receiving IFN or IL-2 treatment, which subside once treatment finishes (Bonaccorso et al., 2002, Capuron et al., 2000). Furthermore, both clinical and pre-clinical studies have shown that the induction of a pro-inflammatory state in otherwise healthy subjects results in poor mood and 'sickness behaviour'; a behavioural phenotype resembling depression with symptoms including lethargy, anxiety, social withdrawal, anhedonia and anorexia (Grigoleit et al., 2011, Reichenberg et al., 2001,

Kent et al., 1992, Eisenberger et al., 2010). Though they share many symptoms and are both thought to have a basis in inflammation, sickness behaviour is distinct from clinical depression. Sickness behaviour is evolutionarily intended to confer benefit by allowing for rest and isolation thus conserving energy, enabling an effective inflammatory response, and preventing the spread of infection to others. It has been proposed that for clinical depression to occur there is a transition from sickness behaviour resulting in sensitisation of immune-inflammatory pathways, progressive damage by oxidative and nitrosative stress and an autoimmune response directed against self-epitopes with the latter processes leading to neural tissue damage and functional and cognitive deficits over repeated depressive episodes (Maes et al., 2012). Considering that IBD is a lifelong disorder involving chronic relapsing and remitting inflammation (Zhu and Li, 2012) it is likely that the depressive symptoms observed in IBD patients is not simply sickness behaviour but a comorbid depression (Maes et al., 2011). Furthermore, sickness behaviour is evolutionarily intended as an adaptive response to sickness, whereas comorbid depression exacerbates the original illness as has been observed in IBD patients (Persoons et al., 2005, Mardini et al., 2004, Mittermaier et al., 2004, Szigethy et al., 2014b), and treatment for depressive symptoms improves IBD course (Szigethy et al., 2014a, Goodhand et al., 2012a). In response to the above findings, the use of anti-inflammatories as an adjunct to conventional therapy for depression has been explored and a beneficial effect is reported though further research is required in this area (Köhler et al., 2014). Potential mechanisms for the inflammatory induction of behavioural changes may include effects of cytokines on HPA axis dysregulation, monoamine changes and induction of the kynurenine pathway, over-activation of microglia, impairments in neuroplasticity, and structural and functional changes in the brain.

1.2.6 Gut-brain axis and inflammation

For the brain and GI tract to influence and react to each other, pathways of communication exist between them and are known as the GBA. The bi-directional communication between brain and gut is an important factor in regulating many essential functions in health and disease. Signals relaying information about visceral sensation to the brain influence reflex-regulation, anxiety, and mood states, while the brain can exert changes pertaining to motility, secretion and immune function in the GI tract (O'Mahony et al., 2011). The major routes of GBA communication involve neural pathways i.e. the vagus nerve, short-chain fatty acids, tryptophan and cytokines, and humoral pathways i.e. the HPA axis. The inflammatory

reactions which occur in the GI tract during IBD produce large quantities of soluble inflammatory mediators such as cytokines. Cytokines are produced by cells of the immune system and may act in an autocrine, paracrine or endocrine fashion. They are therefore important mediators between the immune system and brain. Cytokines can communicate peripheral inflammation to the brain, and may also induce cytokine expression by neurons and glia in the CNS. Their ability to precipitate an inflammation-like response in the brain has linked them to certain psychiatric disorders such as depression (Leonard and Maes, 2012).

1.2.7 Inflammation and depression in IBD

Despite the recent surge in psychoneuroimmunology research, there is a lack of investigation into the inflammatory mediators and mechanisms underlying psychological disturbances during active IBD. There may be psychoneuroimmunological components that predispose some people to the development of psychological disorders in UC. Vlachos et al. (2014) assessed levels of constitutive and inducible heat shock protein 70 (HSP70) at various sites in the colon of UC patients. They found that inducible HSP70 was strongly expressed in polymorphonuclear (PMN) leukocytes in the colonic mucosa of most patients. They also report that the induction of HSP70 significantly correlated with anxiety and depression scores in various psychometric tests, including HADS-D, State Trait Anxiety Inventory, and Zung Depression Rating Scale, and with the Rachmilewitz Clinical Activity Index (CAI) but not with HADS-A scores. This group suggest HSP70 induction in PMN cells as a possible biomarker for depression and anxiety in UC.

In addition to clinical investigations, experimental models of IBD in animals allow for the study of interactions between the gut and the brain during acute colitis and in recovery from colitis in order to decipher the mechanisms by which IBD interacts with the CNS and to develop potential therapies to best manage comorbid symptoms. Due to the paucity of biomarkers (molecular and cellular) reported in human studies, arising from the difficulty in assessing impact of stress and / or psychological disturbance in IBD in a clinical setting, data available from animal models are the best available source to obtain insight into gut-brain interactions underlying psychological comorbidity in IBD.

1.2.8 Behavioural disturbances in animal models of IBD

It is important to be mindful that methods for the study of affective behaviours in animals do not give direct insight into the manifestations and complications of these disorders in humans, as the majority of behavioural tests have been designed around the study of pharmacological agents. Caution is therefore recommended when making conclusions about behavioural tests of anxiety or depression in rodents in the context of humans. Another caveat in terms of these studies lies with the tendency for researchers to use male animals only, under the incorrect assumption that they behave the same as female rodents minus the complications of the female oestrous cycle (Bale and Epperson, 2017). Given that the prevalence, incidence and morbidity risk of depression is higher in women than men (Piccinelli and Wilkinson, 2000) and evidence for behavioural sex differences in animal models of psychiatric disorders (Kokras and Dalla, 2014) it is important to consider possible sex-dependant differences in the behavioural tests with the colitis model.

Heydarpour et al. (2016) have shown an increase in immobility in the FST, a depression-related behaviour, in male mice three days post-TNBS administration. This effect is attenuated using a specific inducible nitric oxide synthase (iNOS) inhibitor (aminoguanidine) administered 30 minutes prior to the FST indicating the potential involvement of nitric oxide in the provocation of this behaviour. In the DSS model of colitis, Chen et al. (2015) performed anxiety- and depression-related behavioural tests in male rats following DSS (5%)-induced colitis and reported that DSS exposure caused a decrease in open arm entries and time spent in the open arms of the EPM, indicating anxiety, with an increase in immobility time in the FST, indicating behavioural despair. DSS exposure also decreased sucrose preference in the sucrose preference test, interpreted as anhedonic behaviour, and reduced social interaction between animals suggestive of social withdrawal. Interestingly, this study also found that the anxious and depressive-like behaviours were reversed by prolonged desensitisation of transient receptor potential vanilloid 1 (TRPV1) -expressing colonic afferent neurons using a colonic infusion of the potent activator of TRPV1, resiniferatoxin. 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. Painsipp et al. (2011) analysed female and male mouse behaviour in the open field, EPM, and FST on days 8, 9 and 11 respectively of an 11-day DSS (2%) exposure regimen. Colitis had some sex-dependant behavioural effects: 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.

Lyte et al. (2006) examined anxiety-like behaviour on a hole-board open field apparatus in mice infected with *Citrobacter rodentium*. In this test, mice are allowed five minutes to freely explore the open field, in which there are 9 shallow holes with a sugary treat contained in the central hole. Male mice were tested 7-8 hours post-infection and results provide evidence for an anxiety-like phenotype: decreased exploration of the central zone of the open field, decreased number of head pokes into the holes as well as a preference for the first corner hole next to which the animal is initially placed compared to control mice. A more recent study using the same colitis mouse model in females assessed anxiety-like behaviours in the light/dark box and found no behavioural alterations ten days post-infection when inflammation was at its peak (Gareau et al., 2011). However, Emge et al. (2016) using a DSS colitis model in male and female mice, reported that during active inflammation (eight days post-DSS) mice exhibited anxiety-like behaviour in the light/dark box while recognition memory was impaired in the novel object recognition test. These behavioural alterations had normalised by 14 days post-DSS when the colitis had resolved. In an investigation by Bercik et al. (2011b), male mice who received 3% DSS in drinking water during three 1-week cycles demonstrated increased anxiety compared to controls in the step-down test which measures latency to step down from a raised (4 cm high) platform onto the tabletop below.

The di-nitrobenzene sulfonic acid (DNBS) -induced colitis model is similar to the TNBS model in terms of induction method and symptom progression. Haj-Mirzaian et al. (2017) and colleagues assessed male mice administered with DNBS 3 days post-enema in terms of inflammation and anxious/depressive behaviour. They report an increase in immobility in the FST and tail suspension test (TST), decreased grooming in the splash test, decreased sucrose preference in the sucrose preference test, as well as a decrease in time spent in the centre of the open field and the open arms of the EPM and a decreased number of exploratory head pokes in the hole board test. Assessment of the inflammatory state of the hippocampus at day 3 post-enema revealed an increase in IL-6 and TNF- α and a decrease in neurogenesis measured by brain-derived neurotrophic factor (BDNF).

1.3 Blood-brain communication

Although the brain was long considered to be an immune-privileged organ sealed off from the periphery by the blood brain barrier (BBB), considerable evidence to the contrary has demonstrated that the brain is highly immunologically active and can initiate its own, primarily innate, response to immune system activation (Rivest, 2009). The BBB is itself an adaptive site of selective exchange and an important regulator of brain homeostasis (Lampron et al., 2013, Muldoon et al., 2013). Although the precise mechanisms are yet to be established, systemic inflammation or infection communicates with the brain to cause a fever and/or behavioural responses, known as sickness behaviours. These sickness behaviours which manifest as anorexia, fatigue, lethargy and malaise exist to preserve energy and to prevent heat loss and the spread of infection. It has been established with the use of animal models that sickness behaviours can be induced by peripheral administration of either TNF- α or IL-1 β (Bluthe et al., 1994, Dantzer, 2001) while fever is induced by IL-6. In severe cases however, the system can become dysregulated and these behavioural symptoms can present as depression or anxiety.

1.3.1 The blood brain barrier

At the beginning of the 20th century, Lewandowsky demonstrated that strychnine and sodium ferrocyanide injected in lower doses into the brains of various animals were more effective than if injected subcutaneously (Lewandowsky, 1900). He is generally considered the first to attribute the disparity between brain and periphery to a difference in the properties of the blood vessels in the brain compared to other organs (Saunders et al., 2014). Furthermore, around this time scientists including Franke (1905) and Goldmann (1909, 1913) observed that soluble dyes injected parenterally did not stain brain tissue. Thus, the concept of a physical barrier between the blood and the brain was proposed.

Considering the fragile nature of neurons and the complexity of neuronal signalling that occurs within the brain, the importance of brain homeostasis and strict regulation of the entry and exit of potentially damaging substances can be appreciated. However, neuronal cells are particularly sensitive to loss of oxygen or glucose, therefore the brain must also be highly vascularised. The brain receives approximately 15% of basal cardiac output (Clarke and Sokoloff, 1999), contains a network of capillaries estimated to be 400 miles long (Begley and Brightman, 2003) and it has been estimated that each neuron in the brain has its own capillary

(Zlokovic, 2005). Fluctuations in plasma constituents including ions, amino acids, proteins or potentially harmful toxins can occur during normal conditions, after exercise, during illness and following food intake, therefore, in order to maintain brain homeostasis, it is essential that a protective barrier exists within the brain.

In line with Lewandowsky's hypothesis, the endothelial cells which line cerebral capillaries are different from peripheral capillaries. These specialised capillaries have a decreased permeability to soluble blood components and thereby form the BBB (Figure 1.1 A). Specialisations of BBB endothelial cells include: a lack of fenestrations and increased TJs limiting passive paracellular movement, high expression levels of transporters to allow specificity of transcellular movement, high trans-endothelial electrical resistance to repel ionic substances, low levels of pinocytosis to limit non-specific transcytosis, and increased mitochondria compared to peripheral endothelial cells representative of higher energy expenditure due to maintenance and functioning of transporters (Weiss et al., 2009, Mayhan and Arrick, 2016). As well as the barrier created by brain endothelial cells, other cells in proximity to the endothelial cells also contribute to the structure and maintenance of the BBB. This is collectively referred to as the neurovascular unit and includes neurons, astrocytes, pericytes, basal lamina and ECM (Varatharaj and Galea, 2017). *In vitro* it has been confirmed that brain endothelial cells respond to pro-inflammatory cytokines IL-1 β and TNF- α by secreting chemokines, adhesion molecules, cytokines and growth factors (O'Carroll et al., 2015).

1.3.2 Blood-cerebrospinal fluid barrier

Another important interface between the brain and the blood is located at the choroid plexus. This brain structure is situated inside ventricles in the brain and is primarily composed of choroid plexus epithelial (CPE) cells. These cells form the physical blood cerebrospinal fluid (CSF) barrier (BCSFB) with the presence of TJ proteins preventing paracellular transport, however, in contrast to the BBB, these cells surround fenestrated capillaries (Figure 1.1 C). This allows for free communication between the blood and the stroma of the choroid plexus. The function of these CPE cells is to aid the maintenance of brain homeostasis by secreting CSF, which contains nutrients, growth factors and neurotrophins, protects the brain from blood pressure changes, and removes toxic molecules and drugs from the brain (de Lange, 2004). CPE cells express polarised and specific transporter proteins on their membranes to enable them to control the trafficking of ions, amino acids and peptide hormones. The choroid

plexus is also a site of selective leukocyte entry into the brain, acting as an inflammation sensor which detects signals from both the central and peripheral nervous systems (Vandenbroucke et al., 2012, Brkic et al., 2015). When such a signal is detected it can be transmitted by the CPE cells by production of cytokines and chemokines. The choroid plexus can also secrete chemoattractants, such as adhesion molecules, to facilitate entry of peripheral immune cells into the brain (Demeestere et al., 2015). Indeed, the presence of T cells has been reported in the CSF and the choroid plexus has been shown to contain CD4⁺ memory T cells which respond specifically to CNS antigens (Baruch et al., 2013). Although leukocytes enter the CSF, they do not enter the parenchyma under healthy conditions (Shechter et al., 2013a). Inflammatory activation by the choroid plexus can be beneficial, for example in cases of spinal cord injury, where leukocyte activation facilitates repair (Shechter et al., 2013b). However, in cases of neuroinflammation such as in multiple sclerosis, CNS inflammation is exacerbated, damaging the parenchyma, and immune cell infiltration is believed to augment the damage (Aubé et al., 2014, Shechter et al., 2013a).

1.3.3 Circumventricular organs

Certain regions of the brain lack a BBB, due to their need for rapid communication with the periphery for control of autonomic functions, and are therefore potentially more vulnerable to harmful substances in the circulation. They function to permit polypeptide hypothalamic hormones to leave the brain and permit substances that would not normally enter the brain to trigger changes in brain function (Ganong, 2000). These areas are known as the circumventricular organs (CVO), and include: the area postrema (AP; a sensory organ known as the vomiting centre), the subfornical organ (SFO) and organum vasculosum lamina terminalis (OVLT; which are both sensory organs involved in fluid and electrolyte balance), the median eminence (ME; a secretory organ which releases hypophysiotropic hormones including CRF, thyrotropin-releasing hormone, growth hormone-releasing hormone and dopamine), the neurohypophysis (a secretory organ which releases oxytocin and vasopressin), and the pineal gland (which secretes melatonin and is therefore involved in circadian rhythm regulation) (Weiss et al., 2009).

CVOs are located along the midline of the brain within the ependymal walls lining the third and fourth ventricle. CVOs contain a plexus of fenestrated capillaries which are separated from the CSF by a barrier of specialised glial cells called tanycytes (Figure 1.1 B). The ME is located at the base of the third ventricle and contains the terminations of axons from

hypothalamic neurons. The SFO hangs from the roof of the third ventricle, below the ventro-hippocampal commissure, at the level of the interventricular foramina. It appears that CVOs may be immunologically active, as a study demonstrating the involvement of circumventricular organs (OVLT, SFO, ME and AP) in the induction of CNS inflammation during experimental autoimmune encephalomyelitis (EAE) showed significantly increased numbers of CD45⁺ leukocytes, upregulation of adhesion molecules [vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1)] on fenestrated capillaries, induction of major histocompatibility complex (MHC) class I and II and microglial activation in all CVOs studied (Schulz and Engelhardt, 2005).

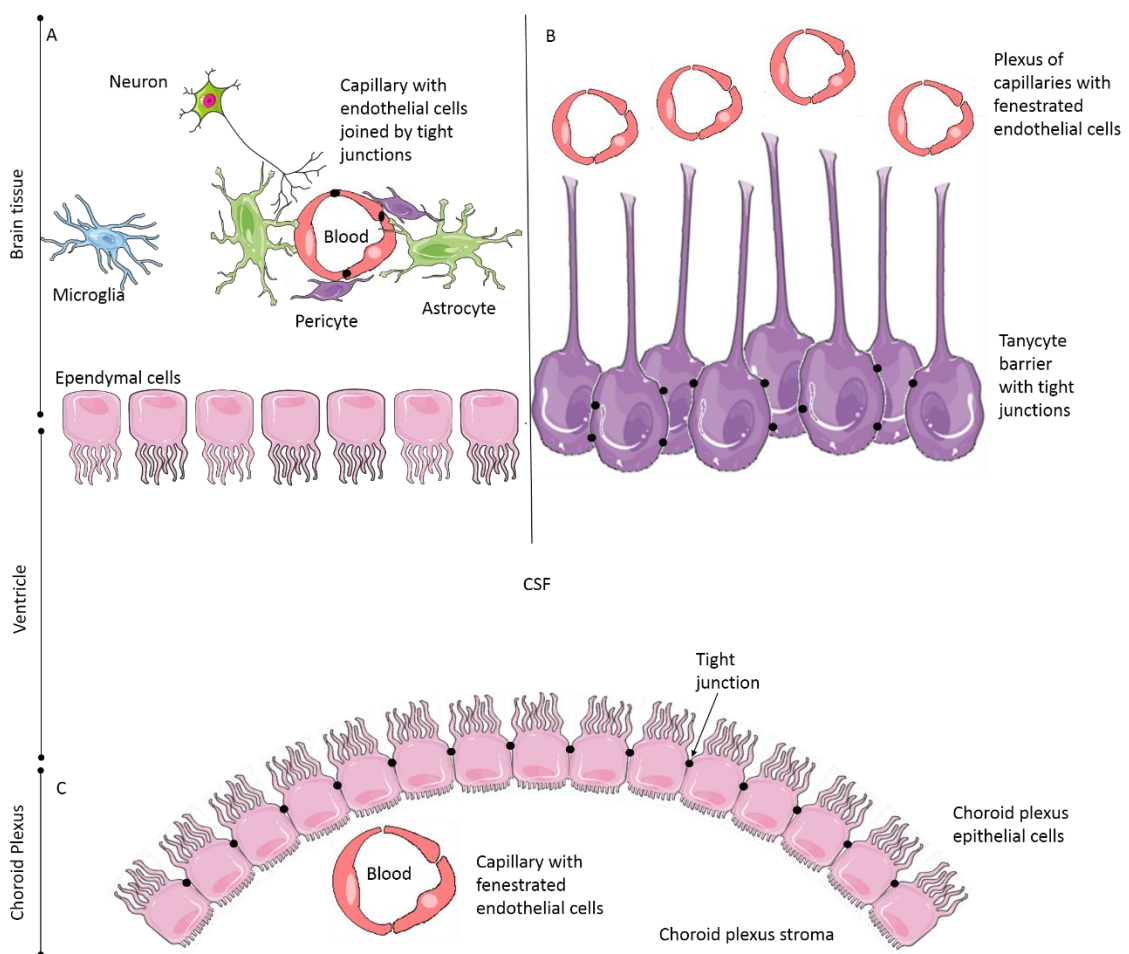


Figure 1.1 Diagram of BBB, CVO and BCSFB structure

The BBB (A) is composed of brain capillaries which are made up of specialised endothelial cells held together closely by TJ proteins. The neurovascular unit surrounding and strengthening this includes astrocytes and pericytes. CVOs (B) contain a plexus of fenestrated capillaries made of endothelial cells. Tanyctyes are specialised glial cells containing TJs which form a

barrier between CVOs and the CSF. The BCSFB (C) comprises CPE cells, which contain fenestrated capillaries composed of endothelial cells without TJs. CPE cells contain TJs and are polarized such that the basal side is in contact with the inner choroid plexus stroma and the apical side is in contact with the CSF.

1.3.4 BBB response to inflammation

It has been observed that cytokines, in particular, IL-1 β , IL-6, TNF- α and IFN γ , can cause an increase in paracellular transport in GI epithelium (Al-Sadi et al., 2012) and in the brain vasculature (Argaw et al., 2006, Rochfort et al., 2014).

In vitro, rat cerebral endothelial cells show increased BBB disruption in response to IL-1 β , IL-6 and TNF- α related to decreases in the electrical resistance of the cells indicating breakdown of TJ integrity (de Vries et al., 1996). More recently, it has been reported that pathological over-activation of myosin light chain kinases (MLCK) by cytokines leads to actin-myosin contraction and thereby retraction of the plasma membrane resulting in re-organisation and destabilisation of the TJ complex and an increase in paracellular permeability. A meta-analysis performed in 2016 focused on BBB changes in response to systemic inflammation caused by LPS (Varatharaj and Galea, 2017). This study found that LPS led to disruptive BBB alterations including disturbance of TJs, endothelial damage, breakdown of the ECM and structural and functional changes to astrocytes, including astrocyte proliferation and activation, followed by astrocyte loss, structural alterations to astrocytic end feet, and altered gene transcription, with induction of pro-inflammatory cytokines and cytotoxic pathways. It has also been reported that the cytokines IL-1 β , TNF- α and IFN γ , when co-cultured with astrocytes, decreased trans-endothelial electrical resistance in brain endothelial cells *ex vivo* indicating increased permeability (Chaitanya et al., 2011).

1.3.5 BBB permeability in models of IBD

Systemic inflammation can disrupt the BBB and has been linked to syndromes such as sickness behaviour and delirium, and neurological disorders such as Alzheimer's disease and multiple sclerosis (Varatharaj and Galea, 2017). The presence of intestinal inflammation in IBD models and the increase in permeability of the gut-blood barrier has been linked to an increase in the permeability of the BBB. Hathaway et al. (1999) investigated potential disruption to the BBB in rabbits exposed to TNBS. Barrier disruption was assessed following

i.v. administration of sodium fluorescein (MW = 376 Da) or a larger molecule fluorescein isothiocyanate (FITC)-dextran (MW = 71 kDa) 48 hours post-TNBS administration. Results demonstrated a significant increase in the permeability of the BBB to fluorescein, however no difference in permeability to the larger FITC-dextran was found. In a later investigation, they confirmed these findings and conclude that free radical damage is not responsible for the BBB disruption (Hathaway et al., 2000). 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 (MW = 156 kDa) as markers of increased permeability. As per Hathaway et al. (1999, 2000), they revealed an increased permeability to sodium fluorescein but not to the larger IgG molecules. The regions of higher permeability were located at the CVOs: specifically, OVLT, SFO and ME during days one and two following TNBS administration.

VCAM and ICAM are endothelial cellular adhesion molecules of the immunoglobulin family, which are responsible for the adhesion of leukocytes in various inflammatory diseases. Sans et al. (2001) measured expression of ICAM-1 and VCAM-1 in the brain, using a dual radiolabelled antibody technique in four established colitis models: mice with DSS-induced colitis, rats with TNBS-induced colitis, IL-10^{-/-} mice, and severe combined immunodeficiency (SCID) mice which are deficient in functional B and T lymphocytes but are reconstituted with CD45RB^{high} T cells leading to pathogenicity. This study demonstrated that there is a significant increase in VCAM expression in the brain of all four models of colitis, which corresponded with colonic VCAM expression and colon weight. They also reported that TNBS-induced colitis induces ICAM expression, although this is not the case in the DSS model. 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.

The consequences of BBB disruption in inflammatory conditions such as IBD is that it leaves the CNS vulnerable to inflammatory mediators and gut-derived bacterial or viral antigens. In the case of IBD, these inflammatory mediators are likely to be at higher concentrations in the circulation stemming from the increased permeability of the intestinal barrier in this condition.

1.4 Mediators of central neuroinflammation

1.4.1 Cytokines

Cytokines are soluble regulatory proteins released by immune cells, which act as intercellular mediators of inflammation. They can also interact with the CNS either via the vagus nerve or by directly interacting with receptors on the BBB, thus providing a means of communication between the immune system and the brain. Following peripheral immune activation, cytokines can also be produced within neurons and glial cells in the brain, and their involvement has also been proposed in the pathophysiology of a number of psychiatric disorders including depression (Leonard and Maes, 2012) [For a more detailed review of the role of pro-inflammatory cytokines in neuroinflammation and depression see Kim et al. (2016)]. With a pivotal role in regulating intestinal inflammation, cytokines are implicated in the pathogenesis and symptoms of IBD (Neurath, 2014). The use of TNF- α antagonists as a standard therapy for IBD highlights the crucial role of cytokines in this disease. Although different cytokine profiles exist between UC and CD, some are common to both diseases: IL-1 β , TNF α , IL-6, and IFN γ (McClane and Rombeau, 1999, Moriconi et al., 2007). As an increased risk of psychological disturbances exists for UC and CD, these are potential cytokine candidates responsible 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 (Harden et al., 2008, Harden et al., 2006, Schiepers et al., 2005).

1.4.1.1 Effects of cytokines on the brain

Cytokine receptors located on the BBB allow for non-barrier-disruptive communication between the periphery and brain, though in some cases cytokines may cross the BBB either via transporters or via a compromised BBB (Varatharaj and Galea, 2017). Diapedesis of leukocytes across the BBB may also lead to immune activation and cytokine production in the brain (Becher et al., 2017). Microglia can respond to these cytokine signals in paracrine and autocrine fashion to facilitate tissue repair, initiate immune responses and recruit immune cells, however, sustained activation of microglia can result in neurotoxicity and production of reactive oxygen species (ROS) (Hanisch, 2002).

Astrocytes also communicate using the cytokine network to influence immune responses in the CNS and there is also evidence to suggest that activation of astrocytes by inflammatory mediators modulates astrocyte signalling, thereby influencing synaptic and neural function

and potentially playing a role in the behavioural effects of inflammation such as sickness behaviour and depression (Sofroniew, 2014).

Cytokines can directly affect neuronal activity, influencing neuronal excitability, neuronal plasticity, neuronal development and synaptogenesis (Dantzer et al., 2008, Khairova et al., 2009, Stellwagen and Malenka, 2006). Cytokines have been shown to affect neurotransmitter metabolism, specifically glutamate, serotonin, and dopamine, in brain regions associated with emotional regulation namely the nucleus accumbens, amygdala and hippocampus (Miller et al., 2013). Cytokines can also affect the kynurenine pathway in the brain by stimulating indoleamine 2,3-dioxygenase (IDO) production. As the IDO enzyme is responsible for conversion of tryptophan to kynurenine, the amount of tryptophan available for serotonin production may be limited, and depressive-like behaviours related to reduced serotonin may develop (O'Connor et al., 2009). Pro-inflammatory cytokines may also increase kynurenine-3-mono-oxygenase enzyme activity. This enzyme degrades kynurenine into 3-hydroxykynurenine, shifting the kynurenine pathway from neuroprotection towards the production of metabolites that promote oxidative stress and excitotoxicity.

Cytokines can influence the HPA axis impacting glucocorticoid receptor function, HPA feedback regulation, and activation of the HPA axis (Dunn, 2000, Miller et al., 1999). HPA axis activation results in an increase in glucocorticoids, and dysregulation of the axis has been implicated in depression (Pariante and Lightman, 2008).

1.4.1.2 Central cytokines in TNBS-induced colitis

Riazi et al. (2008) investigated the influence of TNBS-induced colitis on hippocampal TNF- α concentrations and microglial activation in male Sprague-Dawley rats. They found an increase in both hippocampal TNF- α protein concentrations and microglial activation at four 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 increases circulating TNF- α concentrations which are paralleled by increased brain TNF- α 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 (Medhi et al., 2009). 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 reported an increase in IL-6 mRNA expression and IL-6 protein concentration in the hypothalamus and cerebral cortex which peaks at seven days post-enema. Concentrations of brain IL-6 were also increased in

mice exposed to TNBS, however peak IL-6 protein concentrations were at two days post-enema and remained increased seven and 15 days post-TNBS administration (Baticic et al., 2011). In this study, a decrease in protein concentration of the anti-inflammatory cytokine IL-10 in the brain at two and seven days post-TNBS is also reported. Alhouayek et al. (2011) also found increased central inflammatory cytokine expression following TNBS-induced colitis. Three days post-TNBS administration there was an increase in IL-1 β , TNF- α and monocyte chemoattractant protein 1 (MCP1) mRNA expression in the brains of C57BL6 mice, which was associated with an increase in circulating endotoxin concentrations attributable to extensive histological damage to the colon.

1.4.1.3 Central cytokines in DSS-induced colitis

Villaran et al. (2010) reported a significant increase in TNF- α , IL-6, IL-1 β , and iNOS mRNA expression in the substantia nigra of male Wistar rats during acute DSS-induced colonic inflammation. More recently, Reichmann et al. (2015) measured levels of IL-1 β , IL-6, IL-17A, IL-18, TNF- α , and growth regulated oncogene (GRO)- α protein in the circulation and in the hypothalamus, hippocampus and amygdala of mice following seven days DSS administration (2%) and in combination with WAS. Prolonged immobility of C57BL/6 mice with DSS-induced colitis during WAS was associated with brain region-dependent alterations in the expression of genes associated with energy homeostasis [neuropeptide-Y (NPY), NPY receptor Y1], stress pathway activation [CRF, CRF1 receptor and glucocorticoid receptor] and neurogenesis [BDNF]. They reported increased GRO- α in the hypothalamus as a result of DSS alone. The combination of DSS and WAS induced increases in IL-6 in all three brain regions and in GRO- α in the hippocampus and hypothalamus. Cytokine concentrations in the brain did not correlate with plasma cytokine levels suggesting that WAS is required to effect a brain inflammatory response in DSS-exposed mice. The authors propose that alterations in gut-brain signalling may be responsible for the observed behavioural changes in response to stress in DSS animals. As well as demonstrating a decrease in hippocampal neurogenesis in DSS (3%) - treated mice, Zonis and colleagues (2015) also show an increase in circulating IL-6 and an increase in hippocampal ionised calcium-binding adapter molecule 1 (IBA1) and glial fibrillary acidic protein (GFAP), markers for activated microglia and astrocytes respectively, in acute colitis. Following three more rounds of DSS, increases were observed in hippocampal TNF- α , IL-1 β and GFAP mRNA expression and IBA1 and IL-6 protein simultaneously with the reductions in neurogenesis.

1.4.2 Nitric oxide synthase

Nitric oxide (NO) is a small, highly diffusible, free-radical gaseous messenger molecule expressed by almost all cells, and which, at low concentrations, has various important physiological roles including neurotransmission, vasodilation and host cell defence. Nitric oxide synthases (NOS) are enzymes responsible for the production of NO via the formation of L-citrulline from L-arginine. Three isoforms of NOS have been found to exist in mammals: neuronal NOS (nNOS), cytokine inducible NOS (iNOS), and endothelial NOS (eNOS) (Förstermann and Sessa, 2012). These isoforms are structurally similar and may be broadly divided into two distinct domains: the reductase domain at the carboxyl terminus and the oxidative domain at the amine terminus (Figure 1.2). The central oxygenase domains bind haemoglobin, tetrahydrobiopterin and arginine. The reductase domains contain binding sites for nicotinamide adenine dinucleotide phosphate (NADPH), flavin-adenine dinucleotide and flavin mononucleotide. The calmodulin binding site lies between the oxygenase and reductase domains (Alderton et al., 2001).

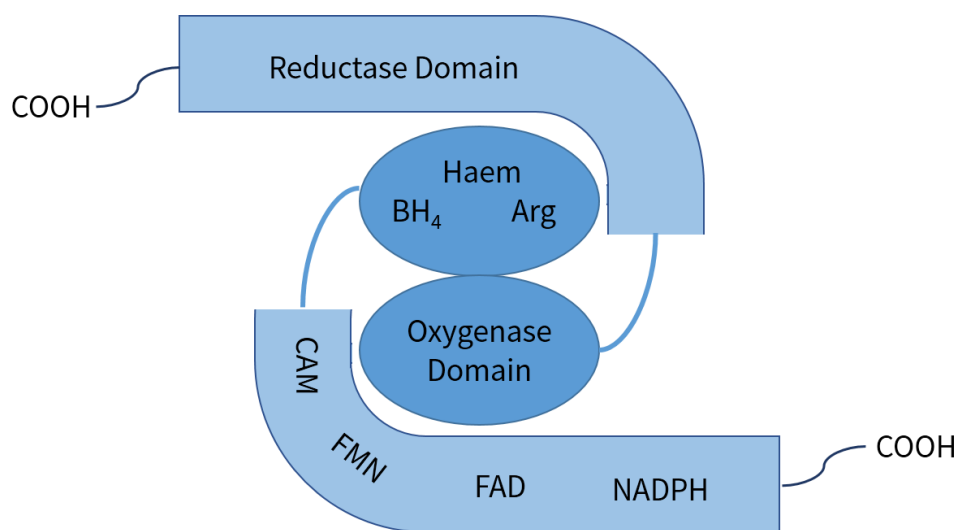


Figure 1.2 Diagram of NOS structure

Amine group (-NH₂), arginine (Arg), calmodulin (CAM), carboxyl group (-COOH), flavin-adenine dinucleotide (FAD), flavin mononucleotide (FMN), haemoglobin (haem), nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin (BH₄).

nNOS and eNOS are constitutive calcium (Ca^{2+})-dependent enzymes while iNOS is calcium independent and is not constitutively produced. nNOS is expressed in central and peripheral neurons and functions in long-term synaptic plasticity in the CNS, the central regulation of blood pressure and smooth muscle dilation in the periphery. Hyperactive nNOS, stimulated by massive calcium influx into neuronal cells, has been implicated in NMDA-receptor-mediated neuronal death in cerebrovascular stroke (Lipton et al., 1993). As well as excitotoxicity following stroke, it is widely believed that abnormal NO contributes to a variety of neurodegenerative pathologies such as multiple sclerosis, Alzheimer's disease and Parkinson's disease (Steinert et al., 2010). eNOS is primarily expressed in endothelial cells and functions in blood vessel dilation, blood pressure control as well as having other vasoprotective and anti-atherosclerotic roles. Cardiovascular disease risk factors can lead to eNOS dysfunction. Expression of eNOS is increased in vascular disease as is the production of superoxide (O_2^-) by NADPH oxidase. This leads to O_2^- reduction by eNOS uncoupling from NO formation, thus a functional NOS is converted into a dysfunctional superoxide-generating enzyme that contributes to vascular oxidative stress (Förstermann and Sessa, 2012). As it is a long-lasting source of excessive, cytotoxic levels of NO, iNOS expression is tightly transcriptionally regulated and must be induced by bacterial products like LPS, cytokines such as $\text{TNF-}\alpha$ and $\text{IFN}\gamma$, or by hypoxia. The NO formed by NOS can act on a number of target enzymes and proteins. The most important physiological signalling pathway stimulated by NO is the activation of soluble guanylyl cyclase and the generation of cyclic guanosine monophosphate (cGMP). In the CNS, nNOS is the principal NO-producing NOS isoform in neurons whereas iNOS mainly contributes to NO production in glia. However, LPS and cytokines including $\text{IL-1}\beta$, $\text{TNF-}\alpha$ and $\text{IFN}\gamma$ have the capability to initiate signal transduction pathways for iNOS production in both neuronal and glial cell types (Saha and Pahan, 2006, Heneka and Feinstein, 2001). iNOS induction has been demonstrated in neurons, astrocytes and microglia in a variety of brain regions (Alderton et al., 2001, Heneka and Feinstein, 2001, Wang and Marsden, 1995). Upon stimulation, the $\text{NF-}\kappa\text{B}$ pathway is activated, and downstream signalling events lead to the transcription of the iNOS gene (Figure 1.3) (Lowenstein and Padalko, 2004). In terms of pathophysiology, the high levels of NO produced by macrophages and microglia against pathogens can also be harmful to surrounding healthy cells either directly by NO, by the combined effects of NO with hypoxia, or by the combination of NO with superoxide forming peroxynitrite (ONOO^-). [The pathophysiological effects of iNOS will be discussed further in Chapter 4.]

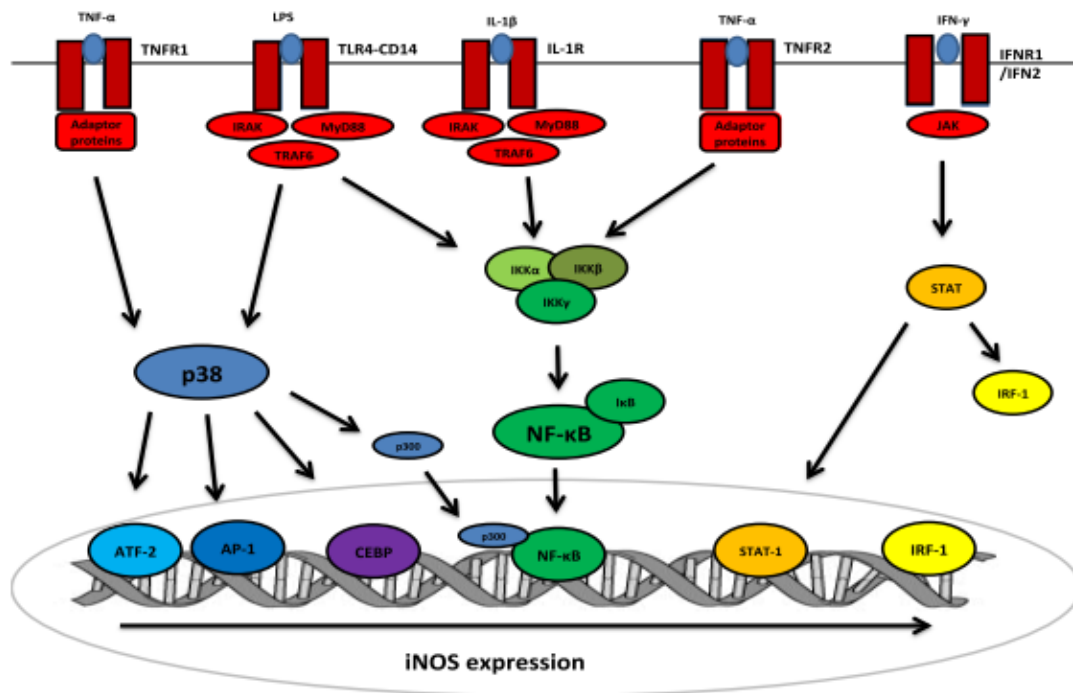


Figure 1.3 Diagram of various pathways of iNOS expression

Pro-inflammatory stimuli bind to receptors to activate signalling pathways that promote iNOS expression. Binding of TNF- α to TNF receptor (TNFR)-1 leads to activation of the mitogen-activated protein kinase (MAPK) pathway, resulting in the production of p38 that leads to activation of transcription factors: activating transcription factor (ATF)-2, activator protein (AP)-1, CCAAT-enhancer binding protein (C/EBP), and p300 involved in regulating iNOS expression. P300 forms a complex with NF- κ B in the nucleus enhancing *iNOS* transcription. LPS binds to the TLR4-CD14 complex activating the NF- κ B pathway, recruiting adaptor proteins IL-1 receptor-associated kinase (IRAK), myeloid differentiation primary response 88 (myD88) and TNF receptor associated factor 6 (TRAF6). The three I κ B kinases (IKK) phosphorylate NF- κ B, which allow it to be transcribed to the nucleus where it can induce iNOS expression. Binding of IL-1 β to IL-1R and TNF- α to TNFR-2 activates the NF- κ B pathway. IL-1R recruits the same adaptor proteins as TLR-4, and TNFR-2 recruits its own specific adaptor proteins. Production of transcription factors STAT-1 and IFN regulatory factor (IRF)-1 are activated and transcribed to the nucleus through the activation of the janus kinase - signal transducer and activator of transcription (JAK-STAT) pathway, induced by IFN- γ binding to IFN receptor (IFNR)-1/2.

1.4.2.1 *iNOS in IBD*

Although NO is known to play a role in intestinal host-defence and has protective anti-microbial properties, iNOS production is thought to have an important role as part of the inflammatory pathology in IBD patients. The *NOS2* gene, which encodes for iNOS, has been linked to very early onset IBD in genetic association studies (Dhillon et al., 2014). Other studies have reported increased iNOS protein expression measured by immunohistochemical staining in rectal biopsies from CD and UC patients relative to controls (Leonard et al., 1998, Kolios et al., 1998). An increase in iNOS mRNA expression has also been reported in the GI tract of animal models of IBD (Beck et al., 2004, McCafferty et al., 1999). Outside of the GI tract, elevated circulating nitrate levels in patients with active IBD are thought to occur as a result of increased iNOS activity, potentially contributing to the pathology of the disease (Oudkerk Pool et al., 1995). Palatka et al. (2006) exposed human endothelial cells to IBD patient serum which resulted in increased iNOS expression compared to control serum. Interestingly, iNOS mRNA expression has been observed in the substantia nigra in the DSS model of colitis in rats (Villaran et al., 2010).

1.5 Aims and objectives

There is accumulating evidence strengthening the concept of a link between chronic inflammatory illnesses of the periphery and alterations to typical brain functioning which may manifest as psychiatric conditions or neurodegenerative diseases (such as depression or anxiety, Alzheimer's disease, Parkinson's disease and multiple sclerosis). However, the mechanisms by which these conditions develop, or are exacerbated, remain unclear.

Previous data from this lab has focused on the link between IBD and psychological disorders including depression and anxiety (Abautret-Daly et al., 2017b). There is a general consensus that IBD is associated with increased vulnerability to symptoms of depression and anxiety particularly during the active disease state.

The aim of this work was to investigate changes occurring in the brain during both active and inactive states of colitis using the DSS-induced colitis model in Wistar rats. In general, published literature on this topic has focused on either behavioural or inflammatory effects of colitis. This work proposed to study behavioural alterations in DSS-induced colitis concurrently with changes in inflammation, brain magnetic resonance biomarkers and regional neuronal activation patterns with the overall goal being to gain additional insights into the impact of colitis-induced changes in the brain.

The specific objectives for this thesis were to:

1. Establish DSS- and TNBS-induced colitis models in rats and investigate the implications of colonic inflammation for behaviour; furthermore, to examine the association between systemic and central inflammatory markers which may account for behavioural changes.
2. Further characterise the neuroinflammatory response following DSS-induced colitis by mapping the expression and activity of iNOS, microglia and astrocytes and associated changes to the integrity of the BBB.
3. Investigate associated patterns of neuronal activation, functional brain connectivity and regional cerebral blood flow following DSS-induced colitis.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Anaesthetics

Isoflurane	Abbott
Ketamine (Vetalar)	Bio-Resources TCD
Medetomidine Hydrochloride (Domitor)	Pfizer
Urethane	Sigma Aldrich
Xylazine (Chanazine)	Bio-Resources TCD

2.1.2 Animals

Dextran Sulfate Sodium (DSS; MW approx.40 kDa)	TdB Consultancy AB
Male Wistar Rats	Bio-Resources TCD
Rat Diet	Red Mills
Saccharin	Sigma Aldrich
Trinitrobenzene Sulfonic Acid (TNBS)	Sigma Aldrich

2.1.3 Antibodies

3-NT (mAb; mouse)	Santa Cruz Biotech.
Alexa Fluor Anti-Mouse 488 nm (goat)	Life Technologies
Alexa Fluor Anti-Rabbit 488 nm (goat)	Life Technologies
Anti-Rat IgG (biotinylated; goat)	Vector Laboratories
CD45 (mAb; mouse)	BD Biosciences
cFos (pAb; rabbit)	Santa Cruz Biotech.
Δ FosB (pAb; rabbit)	Santa Cruz Biotech.
GFAP (pAb; rabbit)	Dako
IBA1 (mAb; mouse)	Wako Chemicals
iNOS (mAb; mouse)	Santa Cruz Biotech.
iNOS (pAb; rabbit)	Santa Cruz Biotech.

2.1.4 General laboratory chemicals

β -mercaptoethanol	Sigma Aldrich
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3,3' - Diaminobenzidine (DAB)	Dako Laboratories
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
Chromium (III) Potassium Sulfate ($\text{CrK}(\text{SO}_4)_2$)	Fisher Scientific
Citric Acid Anhydrous ($\text{C}_6\text{H}_8\text{O}_7$)	BDH Chemicals
DPX Mountant for Microscopy	Fisher Scientific
Ethanol (EtOH)	Sigma Aldrich
Ethylene Glycol	Sigma Aldrich
Gelatin	Fluka
Glycerol	Sigma Aldrich
Hydrochloric Acid (HCl)	BDH Chemicals
Hydrogen Peroxide (H_2O_2)	Sigma Aldrich
Isopentane	Sigma Aldrich
Liquid Nitrogen (N_2)	BOC
Magnesium Chloride (MgCl_2)	Sigma Aldrich
Medical Oxygen (O_2)	BOC
Methanol	Sigma Aldrich
Molecular Grade Ethanol	Sigma Aldrich
Normal Goat Serum (NGS)	Sigma Aldrich
Normal Horse Serum (NHS)	Sigma Aldrich
Paraformaldehyde	Sigma Aldrich
Phosphatase Inhibitor Cocktail I and II	Sigma Aldrich
Potassium Chloride (KCl)	Sigma Aldrich
Protease Inhibitor	Sigma Aldrich
Sodium Chloride (NaCl)	BDH Chemicals
Sodium Hydroxide (NaOH)	Sigma Aldrich
Sodium Phosphate Dibasic (Na_2HPO_4)	Sigma Aldrich
Sodium Phosphate Monobasic (NaH_2PO_4)	Sigma Aldrich
Sterile Saline (0.9% w/v)	Fannin

Sucrose	Sigma Aldrich
Tetramethylethylene-Diamine (TEMED)	Sigma Aldrich
Tissue-Tek OCT Compound	Sakura
Triton-X-100	Sigma Aldrich
Trizma-HCl	Sigma Aldrich
Trizma Base	Sigma Aldrich
Tween-20	Sigma Aldrich
Xylene	Sigma Aldrich

2.1.5 General laboratory consumables

6-Well Plates	Fisher Scientific
Falcon Tubes (15 mL, 50 mL)	Sarstedt
Filter Paper	Whatman Ltd.
Filter Tips (10 μ L, 200 μ L, 1000 μ L)	Sarstedt
Glass Coverslips (22 mm x 50 mm)	Fisher Scientific
Microamp 96-Well Reaction Plates	Applied Biosystems
Microscope Slides (76 mm x 26 mm)	Fisher Scientific
Microtome Blades (C35)	Feather
Microtubes (0.5 mL, 1.5 mL, 2 mL)	Sarstedt
Needles (26 Gauge)	BD Biosciences
Needles (butterfly)	Abbott
Netwells	Corning Life Sciences
Optical Adhesive Covers	Applied Biosystems
Pasteur Pipettes (3.5 mL)	Sarstedt
PCR Tubes (0.2 mL)	Sarstedt
Petri Dishes	Sarstedt
Pipette Tips (10 μ L, 200 μ L, 1000 μ L)	Sarstedt
Round-Bottomed Microtubes (2 mL)	Sarstedt
RNase-Free Microtubes (1.5 mL)	Ambicon Inc.
RNase Zap Wipes	Ambicon Inc.
Scalpels	Swann-Morton
Screw-Cap Tubes	Sarstedt
Syringes (1 mL, 5 mL)	BD Biosciences

2.1.6 General laboratory equipment

Centrifuges:	Heraeus Pico 17	Thermo Scientific
	Rotina 380R	Hettich
	Z216MK	Hermle
	Legend RT ⁺	Sorvall
Cryostat (CM1850)		Leica
Gyro-Rocker		Stuart
Heating Block		Grant
Hotplate		Yellowline
Microscopes:	BX51	Olympus
	CKX41	Olympus
	Axio Imager Z1	Carl Zeiss
Mini-Centrifuge		Apollo
Peristaltic Pump (Minipuls 3)		Gilson
pH Meter		Mettler-Toledo
Pipettes (automated)		Gilson Inc.
Pipettes (manual)		Gilson Inc.
Polymerase Chain Reaction (PCR; GeneAmp 9700)		Applied Biosystems
Polytron		Kinetica
Scale (precision)		Mettler-Toledo
Spectrophotometer (Nanodrop)		Thermo Scientific
Thermal Cycler		Bio-Rad
Vortex (Vortex Genie2)		Scientific Industries
Water-Bath		Nickel Electro

2.1.7 Equipment for behavioural testing

Behavioural Recording System (Ethovision 3.1)	Noldus
Elevated Plus Maze	Custom Made
Forced Swim Test Water Tank	Custom Made
Light/Dark Box Apparatus	Custom Made
Marbles	Grafix RMS Ltd.
Open Field Arena	Custom Made

2.1.8 Immunostaining

Paintbrush (AF85 Round)	Daler-Rowney
Vectashield with DAPI	Vector Laboratories
Vectastain Elite ABC Kit (rabbit IgG)	Vector Laboratories
Vectastain Elite ABC Kit (mouse IgG)	Vector Laboratories
Vectastain Elite Standard Kit	Vector Laboratories

2.1.9 Magnetic resonance imaging (MRI) equipment

Helmholtz Transmitter Coil	Bruker BioSpin
Surface Quadrature Receiver Coil	Bruker BioSpin
Infusion Pump	KD Scientific
MRI Scanner (7 Tesla)	Bruker BioSpin
ParaVision 6.0 Software	Bruker BioSpin
Respiration Monitor	SA Instruments Inc.
Temperature Regulator	SA Instruments Inc.
Ventilator	Ugo Basile

2.1.10 Real time - polymerase chain reaction

High Capacity cDNA Archive Kit	Applied Biosystems
Nucleospin RNA II Isolation Kit	Macherey-Nagel
TaqMan Gene Expression Assays	Applied Biosystems
Taqman Universal PCR Master Mix	Applied Biosystems

2.2 Methods

Detailed descriptions of all solutions prepared may be found in Appendix 1.1.

2.2.1 Animals

All experiments were approved by the Animal Ethics Committee, Trinity College Dublin and carried out in accordance with the guidelines of the Animal Ethics Committee, Trinity College Dublin and the European Council Directive 1986 (86/806/EEC).

Male Wistar rats (175-250 g) were obtained from the Bio-Resources Unit in Trinity College Dublin and housed in hard-bottomed polypropylene cages with stainless steel lids and wood shavings as bedding. Rats were kept under standard housing conditions with an ambient temperature of 20-24°C and a 12 hour light/dark cycle (lights on at 8:00 and lights off at 20:00). Rats had free access to food and water and were fed a standard diet (Red Mills, Ireland).

2.2.2 Animal models of colitis

The DSS model was the principal model of colitis in the experiments described in this thesis. To complement the effects observed in the DSS rat model, a second model, TNBS-induced colitis, was also employed to a lesser extent.

2.2.2.1 *Dextran sulphate sodium -induced colitis*

Rats were given at least four days to acclimatise to their environment in the animal unit before being normalised by weight into control and colitis groups. Control groups received normal tap water for the duration of the study. Colitis groups received 5% (weight per volume; w/v) DSS (MW = 45 kDa; TdB Consultancy, Sweden) in their drinking bottles. For studies 1-3 rats were exposed to DSS for 7 days. However, it was observed that while some rats developed acute colitis, others only developed mild symptoms or were euthanised as a result of severe symptoms by this timepoint. In order to ensure DSS-colitis severity was standard between animals in subsequent experiments, each rat was exposed to DSS until a disease activity index (DAI) score of ≥ 2.5 (which must include scores for diarrhoea and rectal bleeding) was reached, at which point that animal was taken off DSS. This required an average of 6 days but never more than 8. Following cessation of DSS exposure, animals were given normal tap water and allowed to recover for a set number of days. Rats being assessed at the acute stage of colitis were euthanised either immediately or 2-3 days following cessation of DSS, whereas rats

being assessed in recovery were euthanised 7-10 days following the return to normal tap water. In this way rats are assessed while acutely colitic and recovered rats are assessed when their colitic symptoms have dissipated.

2.2.2.2 *Trinitrobenzene sulfonic acid -induced colitis*

Rats were given four days to acclimatise to their environment in the animal unit before being randomly distributed into control and colitis groups of equal average body weight. All rats were singly housed and fasted for 24 hours prior to administration of the enema. During this time they received a 20% sucrose solution containing a laxative (X-Prep; 15 mL/L). Following this, rats were given an i.p. injection of anaesthetic (1.5 mL ketamine and 1.5 mL xylazine). TNBS groups received a 1 mL rectal enema containing 30 mg TNBS in ethanol (30%). Control groups received a 1 mL rectal enema of vehicle only. The enema is administered slowly using a canula inserted 8 cm into the anus. Following this, the rat is suspended by the tail for 1 minute to ensure the solution is retained in the rectum. Rats were euthanized at 3, 8, or 21 days post-enema.

2.2.3 Physiological assessments

The body weight of each rat, along with food consumption and fluid intake, was recorded daily as a general day-to-day indication of their health and wellbeing. TNBS rats were monitored daily for signs of colitis (loose stools, diarrhoea, and abdominal bloating). For DSS studies, rats were monitored for signs of disease activity according to a modified version of the DAI as described by Cooper et al. (1993). This index was used to assess DSS-induced colitis severity for each rat under three criteria, each scored on a scale from 0-4, as outlined in [Table 2.1](#). Scores for all three criteria were averaged to give the daily DAI score per rat. Weight loss was assessed as the percentage difference in weight of the rat compared to the previous day. Stool consistency was measured by analysis of a fecal sample from each rat. Normal stools appear as solid pellets, a score of '1' is given to semi-solid or 'soft' stools, and a score of '2' is given following >2 days of soft stools which are progressing towards diarrhoea consistency. Liquid stool or diarrhoea is scored as '3' and a stool consistency score of '4' is given when rats fail to produce a fecal sample within 15 minutes, likely due to extreme wateriness of diarrhoea and lack of food intake as the colitis progresses. Rectal bleeding was assessed by examining the cages, bedding, the rats, and their fecal sample. A rectal bleeding score of '1' is given when a

small amount of blood is evident, typically in the rats bedding near where they were sleeping and a score of '2' is given when blood is evident in the stool. A rectal bleeding score of '3' is given when a small amount of blood is evident around the anus and a score of '4' is given when there is a greater amount of blood evident in this area.

Table 2.1: Disease activity index scoring system

Score	Weight Loss	Stool Consistency	Rectal Bleeding
0	<1%	Normal	None
1	1-5%	Semi-Solid Stools	Evidence of Blood
2	6-10%	Semi-Solid Stools for >2 Days	Blood in Stool
3	11-15%	Diarrhoea	Rectal Bleeding
4	>15%	No Stool	Severe Rectal Bleeding

2.2.4 Behavioural tests

2.2.4.1 *Open field test*

The open field test is used to assess locomotor activity and anxiety-like behaviour in rodents. Locomotor ability is assessed based on the rats movements including rearing. Anxious rats will spend less time exploring the centre of the arena. This test was carried out in a room with dim lighting to which the animals were acclimatised for at least ten minutes. The open field was a cylindrical plastic arena, 1 metre in diameter, with mirrored walls and a black floor (Figure 2.1). A video camera mounted above the open field was used to record the movement tracks for each animal using HVS Image (HVS Image Ltd.) image acquisition software and Ethovision (Noldus) video tracking software. This software also allowed the arena to be divided into inner and outer zones to enable later calculation of each rats' path length, speed, thigmotaxis, and time spent in certain zones. Animals were each placed in the centre of the open field arena and left to explore freely for 10 minutes. Rearing and grooming were manually recorded. Rats were considered to be grooming when they were observed to lick their paws and run their forepaws over their head, often followed by licking and stroking the side of the body, the anogenital region and the tail. Between each test the walls and floor of the open field were wiped clean with 70% ethanol followed by water to remove olfactory cues.

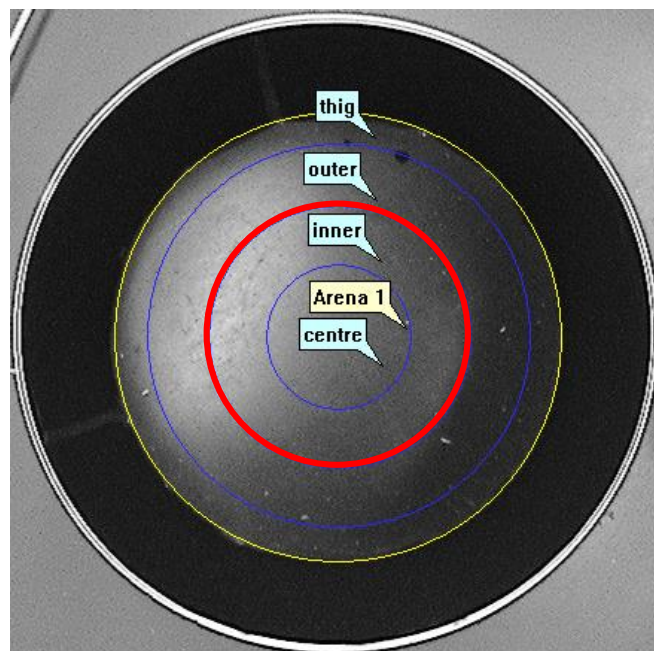


Figure 2.1 Image of an open field arena

2.2.4.2 *Marble burying test*

The marble burying test is used to assess anxiety-related behaviour in rodents. Rodents have a natural instinct to dig and to bury or sequester things such as food. However, increased burying of marbles is seen as an anxious behaviour as rodents are neophobic to the marbles and are 'removing' them from their environment. The majority of marble burying studies are carried out with mice, therefore this protocol was based on the Schneider and Popik (2007) adaptation of the protocol for female Wistar rats. Small, hard-bottomed polypropylene cages (45 cm x 27 cm x 15 cm) were filled 5 cm deep with regular woodshaving bedding (Figure 2.2). Two rows of marbles (2.3 cm diameter) were lined approximately 2 cm apart along the same short wall of the cage. The test session was carried out in a room with dim lighting to which the animals were acclimatised. For each test session a rat was placed in the cage, observed for 10 minutes and then removed. At the end of the session marbles covered at least two-thirds with bedding were scored as buried.

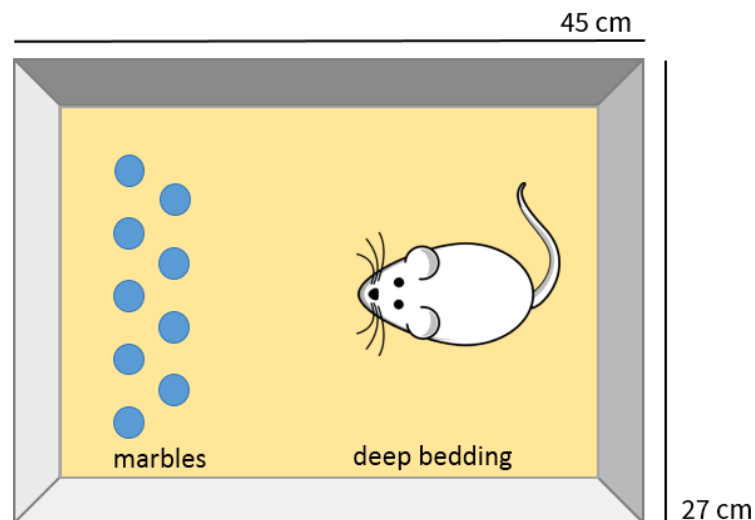


Figure 2.2 Diagram of marble burying test setup

2.2.4.3 *Forced swimming test*

The FST was used to assess depressive-like behaviour. It was performed as first described in rodents by Porsolt et al. (1977). One day prior to the test, animals were placed in a glass cylinder filled to a depth of 30 cm with water at a temperature of $23 \pm 1^\circ\text{C}$ for 15 minutes (Figure 2.3) and were then removed and dried. Clean water was used for each animal. Twenty-four hours later rats were placed in the cylinder under the same conditions for 5 minutes. Both sessions were recorded and time spent immobile was scored by a researcher blind to treatment group. Immobility was defined as an absence of active escape-oriented behaviour such as swimming, climbing or diving. In the first session, the animals learn that escape is impossible, therefore, in the second session, an increase in immobility is interpreted as behavioural despair or depressive-like behaviour.

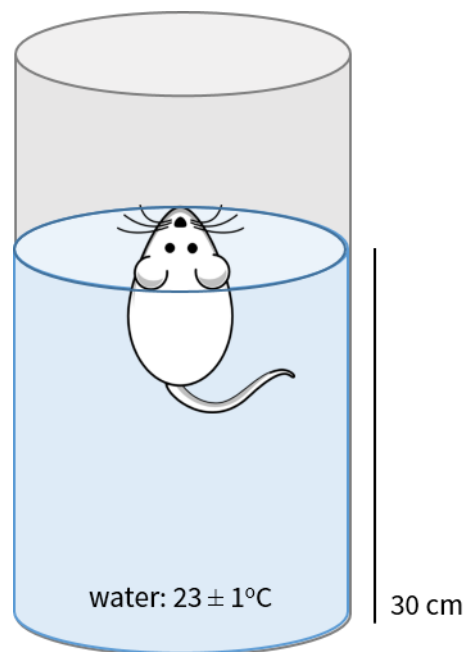


Figure 2.3 Diagram of forced swim test setup

2.2.4.4 Elevated plus maze

This test of anxiety is adapted from the protocol described by Walf and Frye (2007). Anxious rats will spend more time exploring the safe zones and spend less time in the inner zone. The EPM consisted of a grey plus maze elevated 50 cm off the ground (Figure 2.4). Black Plexiglas® (50 cm high) surrounded two of the opposing open arms and the other two remained open. Each arm measured 50 cm x 10 cm. The 'safe zones' of the closed arms were measured as the outer 25 cm of these arms. The central junction measured 10 cm x 10 cm. Normal light-phase room lighting was maintained during testing. Rats were placed in the centre of the EPM facing an open arm and tested for 5 minutes 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 HVS Image software (HVS Image Ltd.).

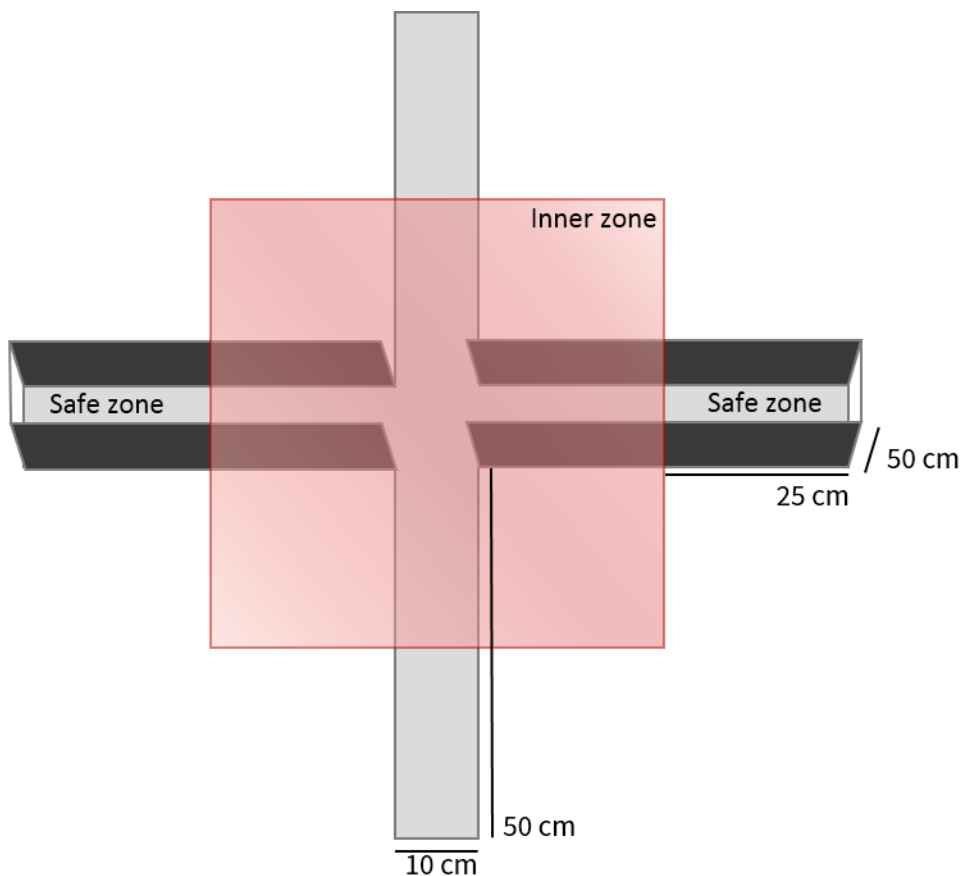


Figure 2.4 Diagram of elevated plus maze setup

2.2.4.5 Light/dark box

The light/dark box is a standard test often combined with the EPM test to assess anxiety-like behaviour. Rats prefer a dark environment, therefore exploration of the light box indicates a less anxious phenotype. In this protocol, the light chamber consisted of a white-walled box with a clear glass top (30 x 30 cm) and a bright light in the centre, whereas the dark chamber had black walls, a solid black top and no light source (30 x 30 cm) (Figure 2.5). The two chambers were connected via a rectangular passage (10 cm). Rats were placed in the light chamber and observed for 10 minutes. Number of entries to each area, time spent in each area and fecal boli count were recorded. Head poking was also recorded as an indication of investigatory tendencies (Marino et al., 2005).

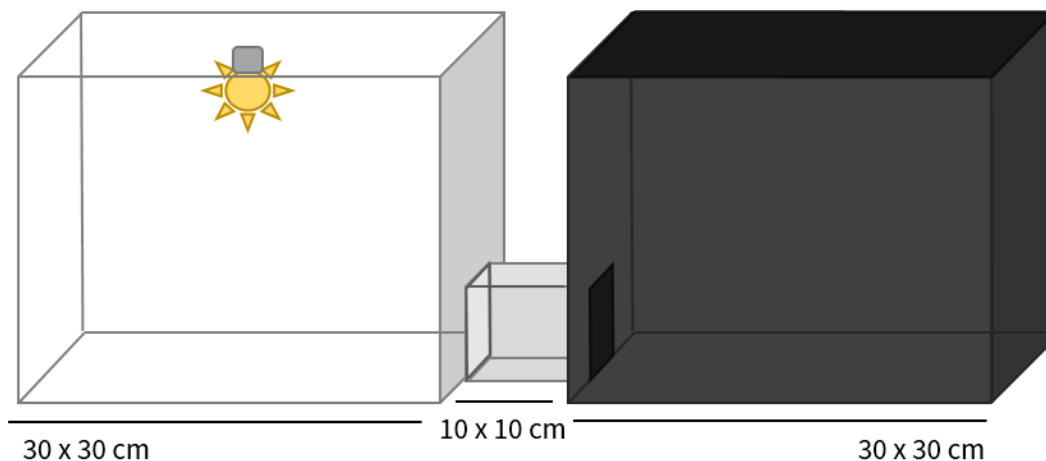


Figure 2.5 Diagram of light/dark box setup

2.2.4.6 Social interaction test

This test is used to assess sociability in rats and is adapted from the protocol by Crumeyrolle-Arias et al. (2014). Two unfamiliar rats from the same group not differing in weight by > 15 g, were placed in the open field arena as described in Section 2.2.4.1, to which they have previously been acclimatised, and allowed to explore and interact freely for 12 minutes. This session was recorded and later analysed by a researcher blind to treatment group to give a social interaction score per pair. Various types of social interaction were measured including time spent sniffing, playing, grooming, fighting etc. to give a combined total interaction time.

2.2.4.7 Saccharin preference test

The saccharin preference test is used to assess anhedonic behaviour in rodents by measuring their preference for a palatable saccharin-containing solution compared to normal drinking water. Normally, rats will prefer the sweet-tasting solution however, anhedonic animals will show no preference or will display neophobia to the sweet solution. For DSS studies, the test took place 24 hours after being returned to water from DSS for acutely colitic rats, and on recovery day 8 for recovered rats. For TNBS studies, saccharin preference was monitored daily throughout the study. Rats were habituated to drinking from two equally accessible water bottles throughout the study and were singly housed for the duration of the test. At the start of the test, one of the drinking bottles was replaced with 10 mM saccharin solution (0.01%) (Figure 2.6). To avoid side preference the saccharin bottle was placed on the left for half of the cages and on the right for the other cages. The bottles were weighed at the start and end of each test which lasted for 24 hours. Saccharin preference was calculated as weight-change in the saccharin bottle as a percentage of the total weight-change of both bottles.

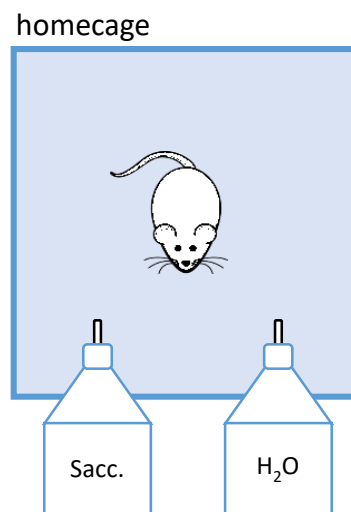


Figure 2.6 Diagram of saccharin preference test setup

2.2.5 Magnetic resonance imaging - theory

Magnetic resonance imaging (MRI) is a medical imaging technique which uses the principle of nuclear magnetic resonance to generate high-quality images of inner anatomy [for a complete overview of the principles and applications of MRI see Dale et al. (2015)]. The non-invasive nature of MRI, coupled with its ability to generate both structural and functional data, make it a formidable translational tool in brain research both clinically and pre-clinically.

The basic principle for MRI makes use of the directional magnetic field associated with charged particles in motion. Hydrogen atoms exist naturally in biological organisms primarily in the form of water and fat, and have a characteristic motion, producing a small magnetic field (Figure 2.7 A). This property is referred to as 'spin'. When placed in a strong magnetic field the magnetic field of many of the hydrogen atoms in a biological organism will align with the applied field resulting in a small net magnetisation in the direction of the applied magnetic field (Figure 2.7 B). The hydrogen atoms resonate at a specific frequency depending on the strength of the magnetic field known as the Larmor frequency. For MRI, this frequency is within the radiofrequency (RF) range. When RF energy is applied at the Larmor frequency it is absorbed by the nuclei which then flip to a different plane (Figure 2.7 C). When this RF energy is removed, the nuclei return to their natural alignment within the magnetic field and release a photon of energy which is received as a small current by the receiver coil (Figure 2.7 D). By transmitting multiple RF pulses while applying a gradient magnetic field, data may be obtained, converted via Fourier transformation, and used to gather three-dimensional information on the location of hydrogen nuclei.

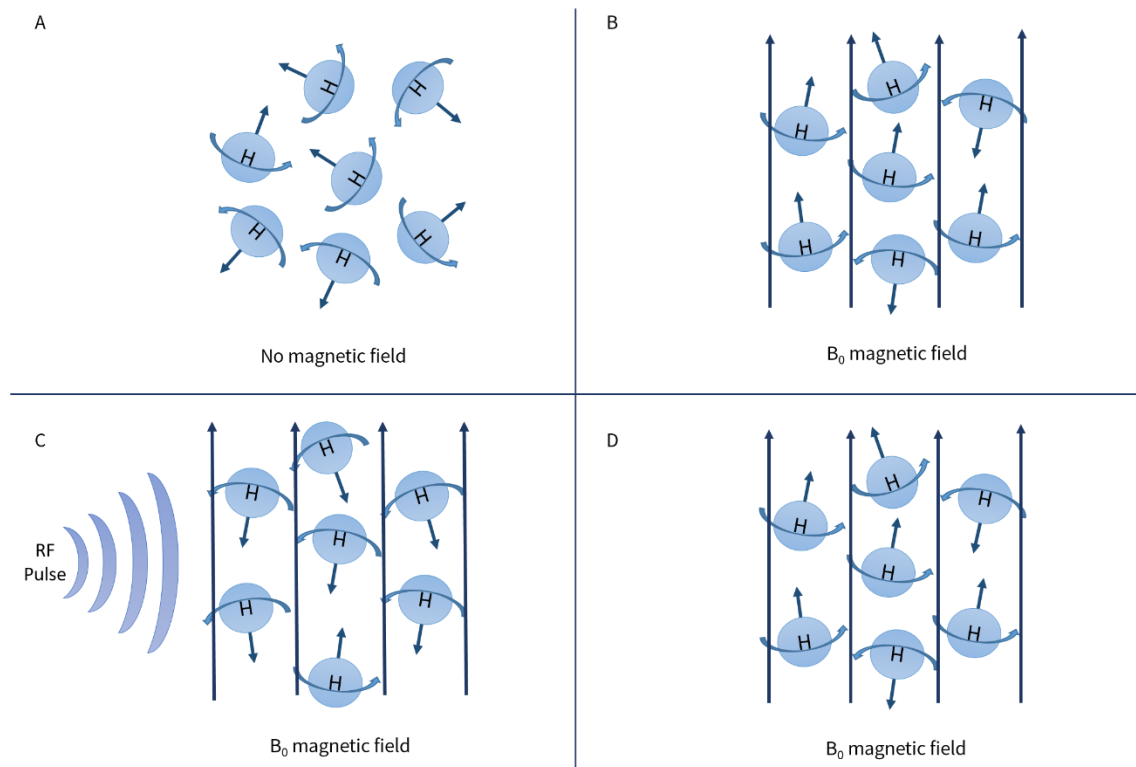


Figure 2.7 Diagram of the basic principles of MRI

In the absence of a magnetic field hydrogen nuclei axes are randomly aligned (A). A strong magnetic field (B_0) is applied causing a uniform alignment of protons (B). An RF pulse is applied perpendicular to B_0 pulling protons into the transverse plane (C). When the RF pulse stops, protons spin to return to equilibrium, parallel to B_0 , resulting in emission of a radiofrequency photon (D).

2.2.5.1 *T1 and T2 relaxometry*

As described previously, protons in a magnetic field oscillate in such a way that the frequency of oscillation depends on magnetic field strength. Nuclei absorb the energy from an RF pulse and release this energy returning to their original state of equilibrium. When the RF pulse is stopped, two types of relaxation occur and emit what are referred to as T1 and T2 signals [see Deoni (2010) for review of the uses of tissue relaxometry measures in the brain]. Longitudinal relaxation time (T1) is the time in milliseconds taken for the protons to realign to their original magnetic field-induced orientation following the pulse. More specifically, the time taken for the signal to recover to 63% of its initial value after flipping to the transverse plane. Transverse relaxation time (T2) results from the de-phasing of the spins and is the time taken for the signal to decay to 37% of its initial value after flipping to the transverse plane.

T1 and T2 are independent values and are intrinsic properties of the tissue being scanned with T2 values generally being smaller than T1. The time between application of the RF pulse and acquisition is referred to as the echo time (TE). The time between individual RF pulses is referred to as repetition time (TR). Image acquisition with short TE and TR will produce T1-weighted images whereas acquisition with long TE and TR will produce T2-weighted images. Tissues with longer T1 times will appear hypo-intense (dark) on T1-weighted images whereas tissues with longer T2 times will appear hyper-intense (bright) on T2-weighted images. T1-weighting provides good structural brain images as there is strong contrast between grey matter and white matter (due to differences in the levels of fat) which appear brightly as a dark and light grey respectively, while fluid such as CSF appears dark. T2-weighting is generally used to determine pathology such as demyelination or inflammation, which increase fluid content in the tissue, as in these images fluid such as CSF appears bright and fat appears dark.

2.2.5.2 *Bolus tracking arterial spin labelling*

Arterial spin labelling (ASL) works on the basis of neurovascular coupling which refers to the close relationship between neural activity and blood flow (Kelly et al., 2010). This technique magnetically tags inflowing arterial blood and uses this as a tracer to enable the quantification and tracking of cerebral blood flow. Water molecules within the blood are tagged in the neck via either adiabatic continuous inversion or repeated saturation pulses to invert or saturate the magnetisation of the hydrogen nuclei (Figure 2.8 A). An image is then taken, and altered tissue magnetisation in this slice is due to the labelled arterial blood and is therefore proportional to blood flow (Figure 2.8 B). A control image in which inflowing arterial blood has not been tagged (Figure 2.8 C) is then acquired (Figure 2.8 D) and is subtracted from the tagged image to provide a contrast between perfused and unperfused tissue. Alternative techniques to measure blood flow require the administration of an exogenous tracer molecule. ASL therefore provides a less invasive option which allows repeated measurements to be taken without concern for the effects of contrast agents.

Bolus tracking ASL (btASL) requires a series of continuous ASL acquisitions which use a constant labelling duration but varied labelling delays. This allows the data to be interpreted as a bolus of arterial blood flowing through the imaging plane. It enables the calculation of mean transit time (MTT), capillary transit time (CTT) and signal amplitude (SA). MTT represents the time taken for the bolus to reach the imaging plane, while CTT represents the diffusion of the labelled arterial blood within the imaging plane. Both MTT and CTT are

inversely proportional to cerebral blood flow. SA is directly proportional to the area under the signal-time curve and is directly related to cerebral blood volume (Kelly et al., 2010, Kelly et al., 2009).

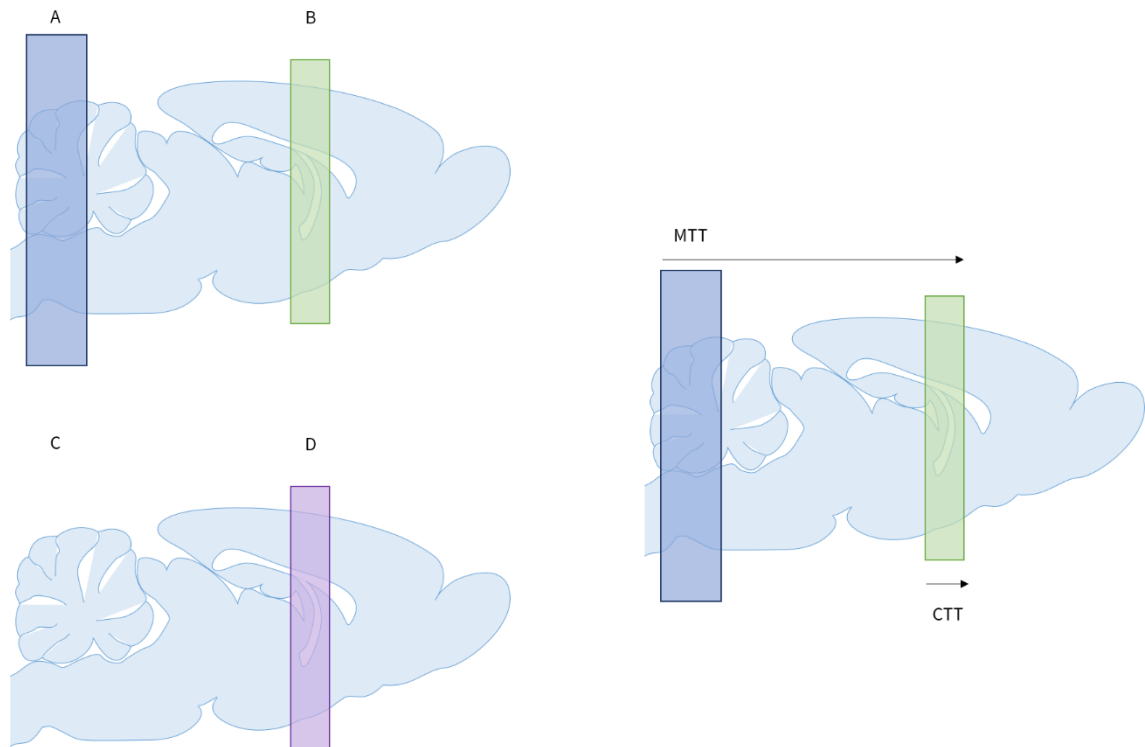


Figure 2.8 Diagram of btASL technique

The inflowing arterial blood water is magnetically labelled (A), an image of this magnetically labelled blood is taken at the imaging plane (the labelled image) (B), the inflowing arterial blood is not magnetically labelled (C) and an image is taken at the same imaging plane (the control image) (D). Subtraction of the label from the control image generates a perfusion weighted image. By varying the delay between labelling and image acquisition, a time series of perfusion weighted images is acquired. Signal time curves for regions of interest can be constructed from this time series which are then fitted to a non-compartmental model of cerebral perfusion as previously described (Kelly et al., 2009).

2.2.5.3 Functional MRI

Functional magnetic resonance imaging (fMRI) uses the principle of neurovascular coupling, as described above, to map neuronal activity on the basis of alterations to metabolism and blood flow in the brain. The most common fMRI modality relies on the blood oxygen level

dependent (BOLD) contrast signal. This signal depends on the change between haemoglobin oxygenation levels and deoxyhaemoglobin which is paramagnetic. Decreases in levels of deoxyhaemoglobin due to increased levels of localised blood flow in response to neuronal activation results in a change in contrast on a T2-weighted scan (Detre and Wang, 2002) displaying a high degree of spatial sensitivity (Silva, 2005). However, it has been suggested that the btASL technique outlined above is a superior tool to BOLD for tracking cerebral blood flow and consequently, for measuring regional neuronal activity. The btASL technique has demonstrated changes in somatosensory cortex activation in response to rodent forepaw stimulation (Griffin et al., 2010) and has been shown to be sensitive to blood perfusion changes in response to 3,4-methylenedioxymethamphetamine (MDMA) administration in rats (Rouine et al., 2015, 2013).

2.2.6 Magnetic resonance imaging - protocol

All MRI was carried out on a dedicated rodent Bruker Biospec system (Bruker BioSpin, Germany) with a 7 Tesla (T) magnet and a 30 cm diameter bore, equipped with a 20 cm actively-shielded gradient system. A pair of actively decoupled 12 cm Helmholtz transmitter and 3 cm surface quadrature receiver coils (Bruker BioSpin, Germany) were used for signal transmission and reception, respectively. The machine was connected to a workstation running ParaVision 6.0 software (Bruker BioSpin, Germany) for data reconstruction and analysis. Total scanning time was not greater than 90 minutes per rat.

2.2.6.1 *Anaesthesia and animal preparation*

Animals were initially anaesthetised using 5% isoflurane (Isoflo; Abbott Animal Health, Maidenhead, UK) in 100% oxygen and after induction received a subcutaneous injection of 1 mg/kg medetomidine (0.07 mg/mL in 0.9% w/v sterile saline). Sedation was maintained throughout scanning with continuous infusion of medetomidine at 0.14 mg/kg/hr beginning ten minutes after the initial bolus dose. Animals were subsequently placed onto a custom-built fibreglass cradle and temperature was maintained using a warming surface controlled by a water-pump-driven temperature regulator (SA Instruments Inc., Stony Brook, NY, USA). A rectal temperature probe coated in petroleum jelly was used to monitor body temperature throughout scanning. A mechanical ventilator (Ugo Basile, Comerio, VA, Italy) was used to deliver adequate inflowing gas to the facemask and the respiration signal was monitored

using custom hardware and software (SA Instruments Inc., Stony Brook, NY, USA). Anaesthetic depth was controlled by maintaining respiration rate in the range of 60 to 75 breaths per minute. The receiver coil was placed over the skull of the animal and fixed with adhesive tape. The cradle was then inserted into the bore of the scanner. Accurate positioning was ensured by acquiring an initial pilot image using a fast gradient echo scan and a single-slice high contrast scan taken at the isocentre of the magnetic field as described below. To maintain consistent positioning throughout all experiments, the animal was repositioned and scanned until the required slice was determined to be in the isocentre.

2.2.6.2 MRI sequences

2.2.6.2.1 Animal positioning

To ensure accurate positioning of the animal at the centre of the B_0 magnetic field, an initial pilot image was acquired using a fast gradient echo scan and a single-slice high contrast scan (T2-weighted Rapid Acquisition with Relaxation Enhancement; RARE) taken at the isocentre of the magnetic field to ensure that the imaging region was centred at 3.60 mm from bregma according to a rat brain atlas (Paxinos and Watson, 2007). The pilot scan was taken using the following acquisition parameters: slice thickness = 1.5 mm, TR = 3134.511 ms, TE = 12 ms, RARE factor = 8, RF flip angle = $90^\circ / 180^\circ$, field of view (FOV) = 30 x 30 mm, image matrix = 128 x 128 and total scan time was 50 seconds.

2.2.6.2.2 High resolution anatomical sequence

High resolution anatomical images were acquired using T2-weighted MR axial images that were collected using a RARE sequence. The following acquisition parameters were used: FOV = 4.00 x 3.00 cm, image matrix = 266 x 200, 64 x 0.5 mm slices, TR = 6.26 s, TE = 36.00 ms.

2.2.6.2.3 Echo planar imaging sequence – Bolus tracking arterial spin labelling

An echo planar imaging sequence was used to provide signal-time curves of the passage of a 3 second bolus through a region of interest. The following acquisition parameters were used: slice thickness = 2 mm, TR = 1300 ms, TE = 12.287 ms, RF flip angle = 90° , FOV = 30 x 30 mm, image matrix = 128 x 128. Four repetitions of each image type were acquired for signal averaging.

2.2.6.2.4 Relaxometry sequences

T1 relaxation times were calculated from a RARE with variable repetition time (RARE-VTR) image. The following acquisition parameters were used: slice thickness = 1.5 mm, varying echo time (VTE) = 25.3 ms, RF flip angle = 180°, FOV = 3.0 x 3.0 cm, image matrix = 128 x 128 with varying repetition time, using values of 300.0, 589.12, 942.3, 1396.1, 2032.0, 3103.1 and 8000.0 ms.

T2 relaxation times were calculated from a multi-slice multi-echo (MSME) image. The following acquisition parameters were used: slice thickness = 1.5 mm, VTR = 2000 ms, RF flip angle = 180°, FOV = 3.0 x 3.0 cm, image matrix = 128 x 128 with VTE, using values of 8.06, 16.12, 24.18, 32.24, 40.29, 48.35, 56.41, 64.47, 72.53, 80.59, 88.65 and 96.71 ms. An echo train of 12 values was used to ensure signal was at noise level by the last echo times to ensure accuracy of T2 estimation. Only the central slice of the MSME scan was used for analysis of T2 relaxation times.

2.2.6.3 MRI data analysis

2.2.6.3.1 Bolus tracking arterial spin labelling

Data were analysed using ParaVision 6.0 (Bruker Biospin, Germany) data acquisition and analysis software, and scripts were written using Interactive Data Language 7.0 (IDL; ITT Visual Information Systems, USA). In addition to the inbuilt functions of IDL, use was also made of the Coyote IDL Library (Fanning Software Consulting, USA; downloaded from <http://www.dfanning.com>) to generate perfusion-weighted maps. ImageJ (Rasband, USA) software was used to select regions of interest (ROI) for analysis with reference to a standard rat stereotactic atlas (Paxinos and Watson, 2007). Analysis of one coronal brain section (at bregma) comprised cingulate, motor and somatosensory cortices as well as the SFO and striatum (Figure 2.9). IDL was used to perform a subtraction of the labelled from the control images generated by the ASL sequence.

MTT, CTT and SA values were generated by fitting the non-compartmental model of cerebral perfusion to the experimental data (Kelly et al. 2009). The curve-fitting routine in Mathematica (Wolfram Research Inc, Version 5.1, Champaign, IL, USA) was used to calculate MTT and CTT from the first and second statistical moments of the signal-time curves respectively. The amplitude of the fitted curve is also reported as it is directly proportional to the area under the curve and therefore an estimate of regional cerebral blood volume (Kelly et al., 2010). MTT and CTT are inversely proportional to cerebral blood flow.

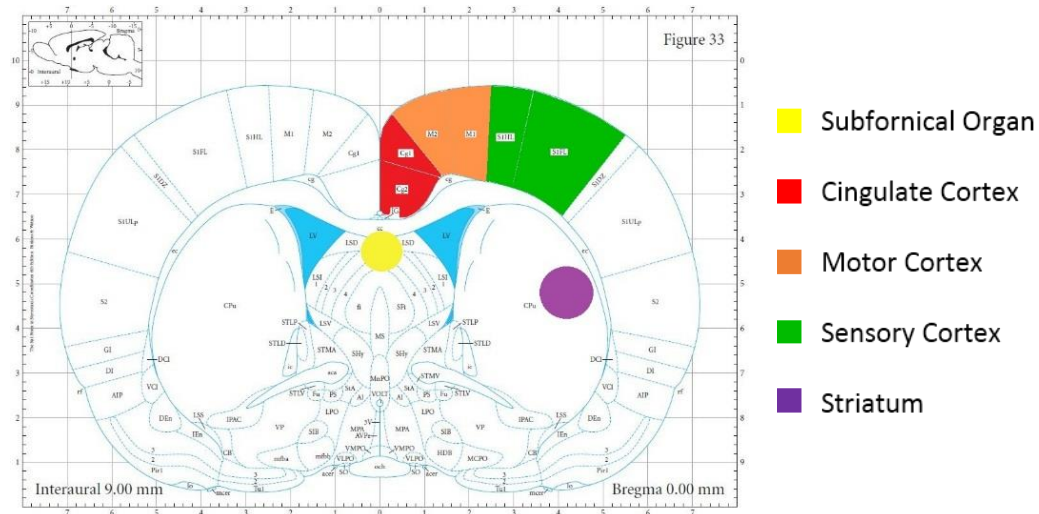
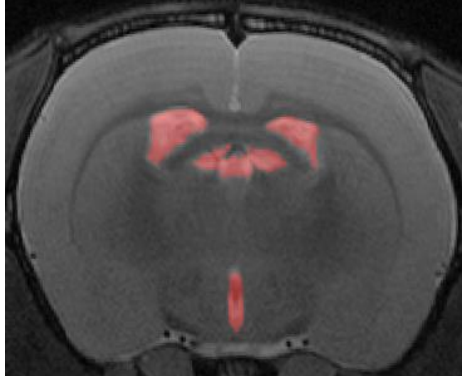
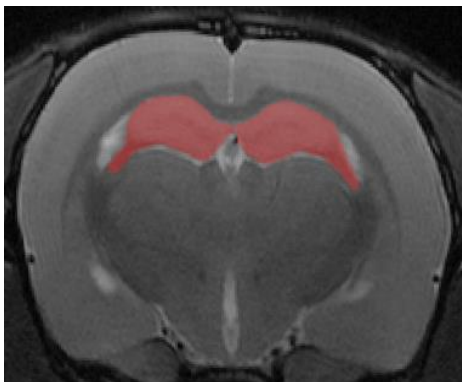


Figure 2.9 Regions of interest acquired for ASL analysis

2.2.6.3.2 Brain volume measurements

MIPAV software was used to manually define ROIs, slice by slice, along the coronal plane. ROIs analysed included both lateral and third ventricles, and hippocampus. The regions were defined with reference to anatomical landmarks set out in the rat brain atlas (Paxinos and Watson, 2007) (Table 2.2). Manual tracing of these regions along the coronal plane formed a three-dimensional mask which was then quantified using MIPAV tools. This was normalised for brain size by calculating the percentage volume of each region relative to total brain volume.

Table 2.2 Anatomical criteria for volume measurements

Brain Region	Anatomical Criteria for Measurements	Representative Image
Ventricles	Including both lateral and third ventricles and defined from brain areas of intensive CSF contrast	
Hippocampus	Defined with reference to the corpus callosum	

2.2.6.3.3 Relaxometry analysis

Analysis of T1 and T2 relaxation times was performed on the RARE-VTR and MSME images respectively. All analysis was performed using the image sequence analysis tool in the Bruker ParaVision 6.0 software package. ROIs were selected using the ROI tool and included the SFO, the cingulate cortex, the motor cortex, the somatosensory cortex and the striatum (caudate putamen) 0.72 mm posterior to bregma (Figure 2.10). The same regions in each animal were used for the analysis of both T1 and T2 relaxation times. Relaxation times were measured bilaterally and averaged for each ROI except for the SFO due to the central location of this structure.



Figure 2.10 Regions of interest acquired for relaxometry analysis

Labelled circles indicate the ROIs used for measurement of T1/T2 relaxation times. For bilateral structures, identical ROIs were placed on each hemisphere and values were averaged. Each ROI measured an area of 0.879 mm^2 except for the SFO where the area of the ROI measured was 0.562 mm^2 .

Cingulate cortex (cing), caudate putamen (cpu), sensory cortex (sensory), subfornical organ (sfo), motor cortex (motor).

2.2.7 Tissue collection

For DSS studies 1-3 and TNBS studies, rats were decapitated and tissue samples of interest (colon, liver, spleen, and brain tissue) were retrieved, snap-frozen in liquid nitrogen, and stored at -80°C until use. In later DSS studies, rats were perfused to allow preparation of brains for immunohistochemical assessment of brain sections.

2.2.7.1 *Transcardial perfusion*

Rats were anaesthetised by intraperitoneal (i.p.) urethane injection (urethane 1.5 g/kg). A median incision from underneath the chin to below the sternum was made and the diaphragm was cut to gain entry to the thoracic cavity. The front of the ribcage was removed to expose the heart. A small incision was made at the apex of the left ventricle and a gavage connected via tubing to a perfusion pump was inserted into the aorta. A right atrial incision was also made to prevent blood from re-entering the systemic circulation. Rats were transcardially perfused via perfusion pump (Gilson Mini Pulse 3) with phosphate buffered saline (PBS; 10 mM; pH 7.4) for a minimum of 5 minutes to clear the vasculature of blood and then with ice-cold paraformaldehyde (PFA; 4% w/v in PBS; pH 7.4) for a minimum of 10 minutes to fix the tissue. Brains were extracted and post-fixed in 4% PFA at 4°C for 48 hours prior to cryoprotecting in sucrose solution (sucrose 30% w/v in 10 mM PBS) at 4°C for 72 hours and were then snap-frozen in isopentane on dry ice. Snap-frozen brains were stored at -80°C until sectioning.

2.2.7.2 *Brain sectioning*

Olfactory bulbs were removed and frozen brains were sliced from frontal lobes to mid cerebellum. Brain sections (30 µm thick) were cut using a Leica CM1850 cryostat at -21°C. Ten series of slices were collected per brain with the slices in each series being taken at approximately 300 µm apart. Each series of slices were stored in freezing storage solution (30% v/v ethylene glycol, 30% w/v sucrose in 10 mM PBS) at -80°C until use.

2.2.8 Histology

Distal colon samples (1-2 cm) were embedded in paraffin wax. Slices were sectioned with a microtome (Leica) at a thickness of 10 μm and placed on microscope slides. Slides were put in an oven at 60°C for 30 minutes to set before being placed in a Leica Autostainer and stained using haematoxylin and eosin. Slides were mounted with coverslips using Di-N-Butyl Phthalate in Xylene (DPX) mounting medium. Slides were digitally recorded and analysed using an Olympus BX51 video-camera microscope. Histological scoring was performed using a modified version of a protocol to examine DSS damage in the mouse colon (Cooper et al., 1993). Crypt damage was rated on a scale of 0-4 and inflammation was scored on a scale of 0-3 (Table 2.3). Crypt damage and inflammation scores were recorded for ten randomly selected areas of each colon sample; scores were averaged to give approximate scores of colonic crypt damage and inflammation respectively. Histological scoring was not performed in TNBS rats.

Table 2.3 Histological scoring system for crypt damage and inflammation

Score	Crypt Damage	Inflammation
0	No visible shortening/damage of crypts	No visible enlargement of lamina propria
1	Mild destruction of crypts	Mild enlargement of lamina propria
2	Moderate destruction of crypts	Moderate enlargement of lamina propria with increased infiltration of leukocytes
3	Near/complete destruction of crypts with presence of surface epithelial cells	Substantial enlargement of lamina propria with heavy infiltration of leukocytes into surrounding areas
4	Complete destruction of crypts with absence of surface epithelial cells	-

2.2.9 Immunohistochemistry

Immunostaining protocols were carried out at room temperature with gentle agitation and each rinse step consisted of two 5-minute washes in 10 mM PBS (pH 7.4) unless otherwise specified. The principles of immunostaining protocols are summarised in [Figure 2.11 – 2.13](#).

2.2.9.1 *Single Immunostaining*

Free-floating sections were rinsed and incubated in 0.75% H₂O₂ and 5% MeOH in PBS for 20 minutes to block endogenous peptidases and rinsed again. [At this point tissue being stained for 3-NT was subject to an antigen-retrieval step where slices were incubated in sodium citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0) in a water bath at 80°C for 20 minutes then left on the bench to cool for 20 minutes and rinsed.] All slices were incubated in 10% normal serum (NS) in 10mM PBS for 30 minutes to block non-specific proteins. Slices were washed once in PBS and incubated overnight in primary antibody solution [(iNOS: rabbit anti-iNOS pAb 1:350; 3-NT: mouse anti-3-NT mAb 1:75; ΔFosB: rabbit anti-ΔFosB pAb 1:2500; cFos: rabbit anti-cFos pAb 1:6,500; Santa Cruz Biotechnology, Santa Cruz, CA, USA; IBA1: rabbit anti-IBA1 pAb 1:5000; Wako Pure Chemical Industries Ltd., Osaka, Japan) in 10mM PBS with 5% NS and 0.05% Triton-X-100]. The following day, slices were rinsed and incubated for 90 minutes in a biotinylated secondary antibody from a Vectastain Elite ABC kit (anti-rabbit IgG or anti-mouse IgG; 1:200; Vector Laboratories, Burlingame, CA, USA) in 10 mM PBS with 1% NS. Sections were rinsed and Vectastain ABC reagent (Vector Laboratories) was applied for 90 minutes. A peroxidase reaction was performed to visualize immunolabelling by incubating sections with 3,3-diaminobenzidine tetrahydrochloride (DAB; 0.5 mg/ml; Sigma) in 0.01% H₂O₂ in PBS for 7 minutes before stopping the reaction rinsing in dH₂O followed by 10 mM PBS. Sections were then mounted onto gelatin-coated slides, air-dried, de-salted in dH₂O, dehydrated in ethanol, cleared in xylene and coverslipped in DPX. To assess antibody specificity, incubation with the primary antibody was omitted for some sections and no significant staining was observed in this case.

2.2.9.2 *Double Immunostaining*

For dual immunostaining with IBA1 and iNOS, the single immunostaining protocol was followed up to the first primary antibody incubation which was altered to a 2.5 hr incubation (rabbit anti-IBA1 pAb 1:5000; Wako Pure Chemical Industries Ltd., Osaka, Japan) at 37°C. Subsequent secondary antibody, ABC and DAB steps were carried out as described previously.

Following post-DAB washes the single immunostaining protocol was then repeated from, and including, the non-specific protein blocking step as follows. Slices were incubated in 10% NS in 10 mM PBS for 30 minutes. The slices were washed for 5 minutes in 10 mM PBS and incubated in a solution of the second primary antibody overnight at room temperature (mouse anti-iNOS mAb 1:350; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Slices were then rinsed and incubated for 90 minutes in secondary antibody solution using a Vectastain Elite ABC kit (anti-mouse IgG; 1:200; Vector Laboratories, Burlingame, CA, USA) in 10 mM PBS with 1% NS. Sections were rinsed and Vectastain ABC reagent (Vector Laboratories) in 10 mM PBS was applied for 90 minutes. A peroxidase reaction was performed to visualize iNOS immunolabelling by incubating sections with SG solution (Vector Labs) for 7 minutes. Slices were rinsed in dH₂O and in 10 mM PBS. Slices were mounted onto gelatin-coated slides, air-dried, de-salted in dH₂O, dehydrated, cleared and coverslipped as previously described. To assess antibody specificity, incubation with each primary antibody was omitted for some sections, and no significant staining was observed in either case.

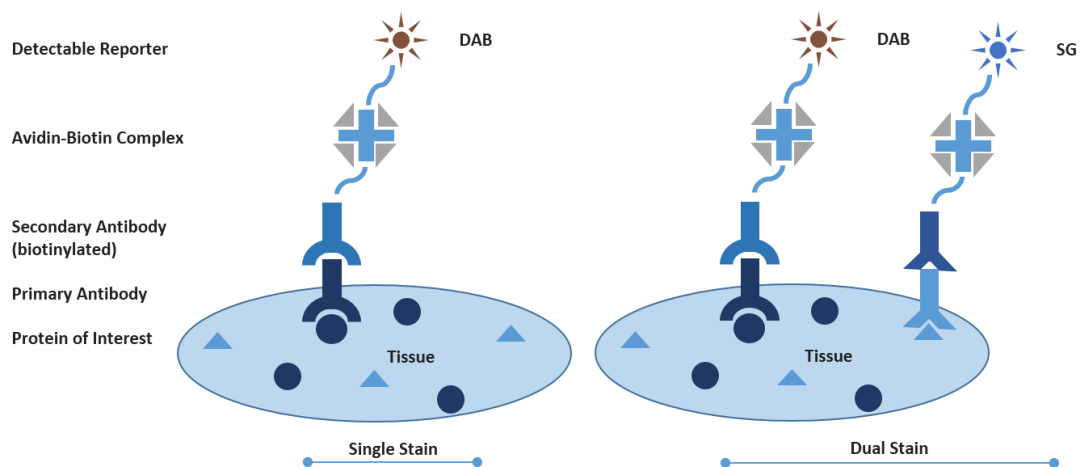


Figure 2.11 Diagram of immunohistochemical staining

2.2.9.3 IgG Immunostaining

Free-floating sections were rinsed and incubated in 0.75% H₂O₂ and 5% MeOH in PBS for 20 minutes to block endogenous peptidases and rinsed again. All slices were incubated in 10% NS in 10mM PBS for 30 minutes to block non-specific proteins. Slices were washed once in PBS and incubated overnight at 4°C in primary antibody solution [(Biotinylated Goat Anti-Rat IgG; 1:1000; Vector Laboratories, Burlingame, CA, USA) in 10mM PBS with 5% NS and 0.05% Triton-X-100]. The following day sections were rinsed and Vectastain ABC reagent (Vector Laboratories) was applied for 90 minutes. A peroxidase reaction was performed to visualize immunolabelling by incubating sections with DAB (0.5 mg/ml; Sigma) in 0.01% H₂O₂ in PBS for 7 minutes before stopping the reaction rinsing in dH₂O followed by 10 mM PBS. Sections were then mounted onto gelatin-coated slides, air-dried, de-salted in dH₂O, dehydrated in ethanol, cleared in xylene, and coverslipped in DPX. To assess antibody specificity, incubation with the primary antibody was omitted for some sections, and no significant staining was observed in this case.

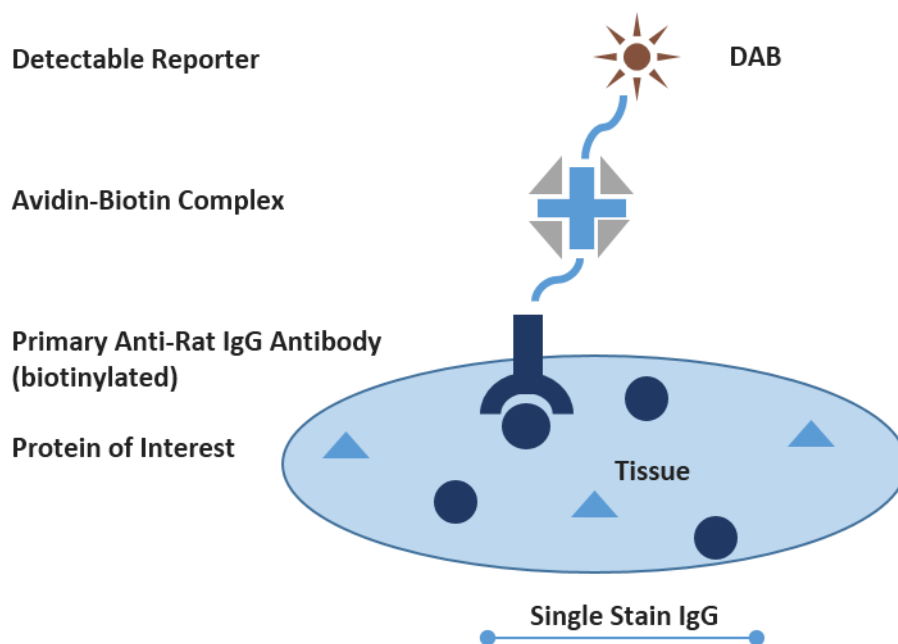


Figure 2.12 Diagram of IgG immunohistochemical staining

2.2.9.4 Immunofluorescent Staining

Free-floating sections were rinsed and incubated in 10% NS in 10mM PBS for 40 minutes to block non-specific proteins. Slices were washed once in PBS and incubated overnight at 4°C in primary antibody solution [(rabbit anti-GFAP pAb; 1:1000; Dako or mouse anti-CD45 mAb; 1:1000; BD Biosciences) in 10mM PBS with 1% bovine albumin serum (BSA) and 0.1% Triton-X-100]. (Primary antibody solution was kept for re-use for up to 10 days or 4 uses.) The following day, sections were rinsed and incubated in secondary antibody solution [(Alexa-Fluor 488 nm fluorescent secondary anti-rabbit/anti-mouse; 1:1000; Invitrogen) and 1% BSA in PBS] for 2 hours. This incubation and all following steps took place in the dark. Sections were rinsed and mounted onto gelatin-coated slides, any excess liquid was removed and once dry, slides were coverslipped in Vectashield mounting medium containing DAPI (Vector laboratories) and sealed with clear nail varnish. To assess antibody specificity, incubation with the primary antibody was omitted for some sections, and no significant staining was observed in this case. The sections were stored at 4°C until ready for imaging up to 7 days later.

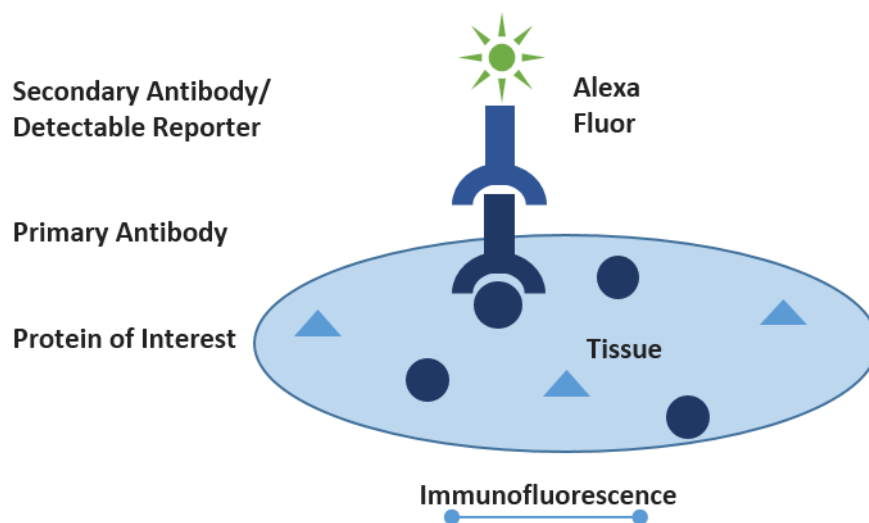


Figure 2.13 Diagram of immunofluorescent staining

2.2.10 Microscopy, cell quantification and analysis

Images were first observed at low magnification and co-ordinates of coronal planes and limits of the various structures analysed were defined according to a rat brain atlas (Paxinos and Watson, 2007). Structures were analysed through multiple coronal sections to confirm that staining was localised to a specific structure. One section was chosen per structure for quantification purposes. The locations from bregma of analysed structures are listed below and illustrated in Figure 2.15: lateral septum (LS), nucleus accumbens (NAc) and organum vasculosum of the lamina terminalis (OVLT) at 0.60 mm, choroid plexus (CP) and subfornical organ (SFO) at -1.08 mm, lateral habenular nucleus (LHb) and paraventricular nucleus of the hypothalamus (PVN) at -1.80 mm, central amygdala (CeA), cingulate, motor and sensory cortices, hippocampus (CA regions 1-3 & dentate gyrus ; DG), median eminence (ME) and striatum at -2.76 mm, and dorsal raphe nucleus (DRN) at -7.44 mm. Bilateral structures were analysed on each hemisphere where available and counts were averaged. Due to the size of the structure, cell counts for the LHb were measured per region, however, cell counts for all other regions were measured as follows: three identical boxes of known area ($200 \mu\text{m}^2$) were placed at random on the image, cells of interest were counted per box, and the number of cells was then averaged. All cell counting was performed by researchers blind to the treatment group.

2.2.10.1 *Brightfield Microscopy*

Brain slices stained with DAB and/or SG chromophores were imaged using an Olympus BX-51 microscope attached to an Olympus DP72 digital camera which allowed them to be visualised on a computer by the Olympus Cell-D software program. ROIs were imaged at a magnification of 100x and further analysed using the ImageJ software program.

DAB-positive staining was defined as a dark brown cell stain, SG staining was defined as dark blue stain and co-localised staining was counted as black coloured staining.

For analysis of microglial perimeter, all microglia with a visible cell body were isolated from an image of the ROI at 400x magnification and converted to individual 8-bit images (Figure 2.14). One image was used per rat per region. Threshold was adjusted (up to 17%) to remove background noise. The 'analyse particle' tool on ImageJ software was used to calculate the perimeter of each microglia which was then averaged per region.

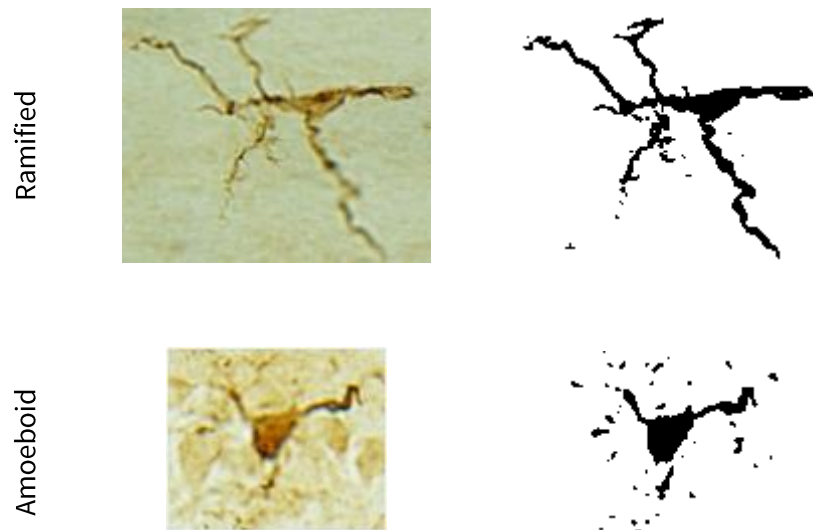


Figure 2.14 Representative images from microglial perimeter analysis

Representative ramified and amoeboid microglia isolated at 400x magnification and their corresponding 8-bit conversions.

2.2.10.2 Confocal Microscopy

Slices stained using Alexa Fluor fluorophores were imaged using confocal microscopy. Image stacks (consisting of 10 images, 3 μm apart) of the ROIs were acquired at 200x magnification using the Axiovert 200M microscope and Zeiss M Image software. ImageJ was used to convert image stacks into single images allowing for the quantification of cells throughout the thickness of the slice.

GFAP-positive cells were quantified using a method adapted from Gosselin et al. (2009). GFAP immunoreactive cells were only quantified if they met three criteria: (1) the astrocyte must show continuous labelling with at least three distinct processes, (2) the astrocyte must be contained within the limits of the quantification field, (3) the astrocyte must not exist in association with a blood vessel, as astrocytes surrounding vessels are often not individually distinguishable. The total number of GFAP-positive cells to meet these criteria were counted in three boxes (200 μm^2) per region. Counts were taken from the right and left hemisphere of each slice and averaged to give the final number of GFAP immunoreactive cells present.

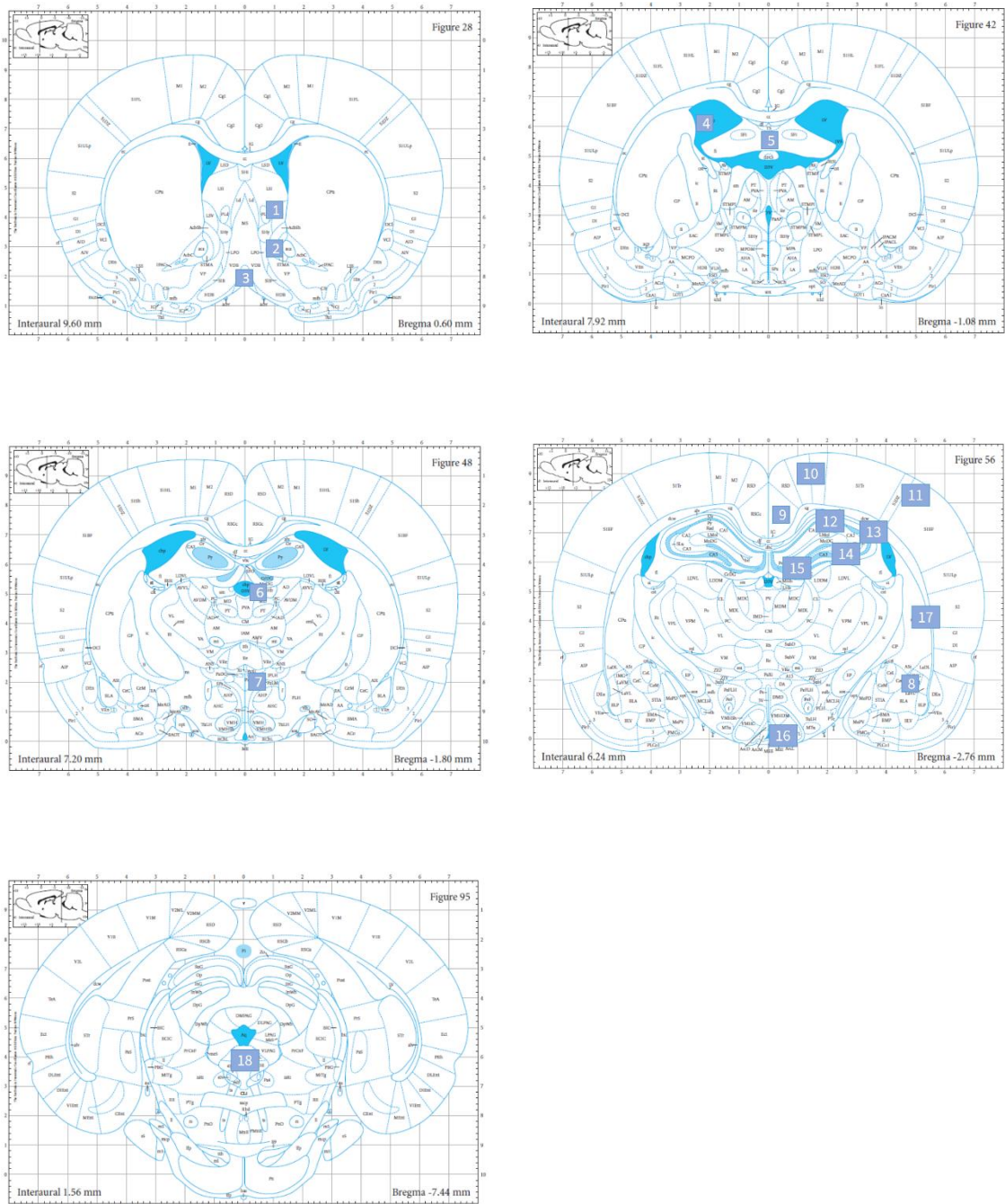


Figure 2.15 Representative images of sections used for quantification

Cell numbers were quantified in the following regions: lateral septum (1), nucleus accumbens (2), organum vasculosum of the lamina terminalis (3), choroid plexus (4), subfornical organ (5), lateral habenular nucleus (6), paraventricular nucleus of the hypothalamus (7), central amygdala (8), cingulate (9), motor (10), and sensory (11) cortices, CA1 hippocampus (12), CA2 hippocampus (13), CA3 hippocampus (14), dentate gyrus (15), median eminence (16), striatum (17), and dorsal raphe nucleus (18). Adapted from the rat brain atlas 6th edition (Paxinos and Watson, 2007).

2.2.11 Zymography

Gelatin zymography was performed to investigate MMP-2 and MMP-9 activity. Zymography is an electrophoretic technique that is used to measure proteolytic activity. An SDS gel is co-polymerised with a gelatinase substrate which is degraded by the loaded protease during the incubation period. Coomassie blue staining reveals zones of proteolysis on the gel as white bands on a blue background. The intensity of these bands is directly associated to the amount of protease loaded.

2.2.11.1 Tissue preparation

Colon tissue (15-20 mg) was added to lysis buffer (150 mM NaCl, 50mM Trizma-HCl pH 8.0, 1% v/v NP-40) containing Phosphatase Inhibitor Cocktail I (50 μ L/10 mL ;Sigma-Aldrich), Phosphatase Inhibitor Cocktail II (50 μ L/10 mL; Sigma-Aldrich) and Proteinase Inhibitor Cocktail (100 μ L/10 mL; Sigma-Aldrich) in a round-bottomed tube. Samples were homogenised using a polytron and centrifuged at 14,000 rpm at 4°C for 15 minutes and stored in clean eppendorf tubes at -80°C. Protein concentrations of each sample were determined via Bradford assay as described below (Section 2.2.11.2) and samples were equalised in dH₂O (2000 μ g/mL).

2.2.11.2 Bradford assay

The Bradford assay was used as a method of protein detection and quantification. A serial dilution of a 1000 μ g/mL solution of BSA in TBS-T (1x) was carried out to give 8 solutions with final protein concentrations of 1000, 500, 250, 125, 62.5, 31.25, 15.625 and 0 μ g/mL. Protein standards and samples (10 μ L) were pipetted in duplicate into a 96 well plate, Bio-Rad solution (200 μ L) was added and incubated for 5 minutes. Absorbance at 595 nm was read using an ELX 800 Universal Microplate Reader (Bio-Tek Instruments). A standard curve was constructed by plotting the standards against the absorbance and protein concentrations were measured as microgram per millilitre.

2.2.11.3 Gel preparation and electrophoresis

Glass plates and 1 mm spacers (Bio-Rad) were cleaned with ethanol (70%), assembled and checked for leaks. Separating gels (30% acrylamide, 1.5 M 4x Trizma-HCl pH 8.8, 20 mg/mL gelatin containing 1% sodium dodecyl sulphate (SDS), 10% ammonium persulfate (APS), and

TEMED in ddH₂O) were prepared and vortexed before being loaded gently between the glass spacers and left to solidify for 45 minutes. A layer of ddH₂O was added to level the gel and promote gel polymerisation and was removed before adding the stacking gel. Loading buffer (6 µL; 0.5M 4X Trizma-HCl pH 6.8, glycerol, SDS, ddH₂O) was added to each equalised sample (18 µL). Conditioned media from HT-1080 human fibrosarcoma cells which contains high amounts of proMMP-2, MMP-2, proMMP-9 and MMP-9 was kindly provided by Dr. Carlos Medina and was used as a positive control. The control was prepared by adding loading buffer and Bromophenol blue (0.02 mg/mL in ddH₂O) to 18 µL HT-1080 standard. Stacking gels (30% acrylamide, 1.5M 4x Trizma-HCl pH 6.8, 10% APS and TEMED in ddH₂O) were prepared and vortexed before loading onto the separating gels. Combs were inserted to form wells in the gels, the gels were left to solidify for 30 minutes and then the combs were removed. The glass plates containing the gels were placed into the electrophoresis stand in an electrophoresis box. Ice-cold tank buffer (10x: 30.3 g Trizma base, 144 g glycine, 10 g SDS, in 1 L ddH₂O, pH 8.3) was poured into the electrophoresis box between the two glass plates to ensure current could pass through the gels. Samples and standards (20 µL) were then loaded carefully into the wells. The electrophoresis box was placed into a tank containing ice and the electrophoresis electrodes were connected to the power supply (Bio-Rad). The electrophoresis was run (150 V; 300 mA) until samples had migrated to the bottom of the gel (approx. 2 hours). Separating gels were extracted and washed (3 x 20 minutes) in Triton buffer with mild agitation. They were then washed (2 x 20 minutes) in zymography buffer (2 M Trizma-HCl, NaCl, CaCl₂ and NaN₃ in ddH₂O) before being incubated in fresh zymography buffer overnight at 37°C. The next day, the gels were incubated in staining solution (MeOH, acetic acid, ddH₂O, and Coomassie Brilliant Blue G-250) for 3 hours followed by de-staining solution (acetic acid and MeOH in ddH₂O). Gels were stored in de-staining solution until analysis. Gelatinase activity of MMP9 and MMP2 was analysed by detection of intensity of band clearing at the same position as the positive control. A calibrated densitometer (GS-800 Bio-Rad) and Quantity One analysis software (Version 4 Bio-Rad) were used to measure intensity x mm.

2.2.12 Real time polymerase chain reaction

2.2.12.1 RNA extraction

All RNA extractions were carried out in an RNase-free fume hood. Prior to extraction, all instruments and work surfaces were wiped down with RNase Away™ wipes (Invitrogen). Isolation of total RNA from all tissue samples (brain, colon, liver and spleen) was carried out using Nucleospin RNA II kits (Macherey-Nagel) as per the kit protocol. Briefly, 20 mg of tissue was dissected using a sterile scalpel and placed in an RNase free tube. The tissue was then homogenised in lysis buffer using a polytron. The polytron blade was washed with RNase Away™ and RNase-free water between samples to minimise cross-contamination. Lysates were then filtered through purple NucleoSpin Filter Units in collection tubes by centrifuging for 1 minute at 13,000 rpm. The filter was discarded. 350 µL ethanol (70%) was added to the filtrate and mixed by slowly pipetting up and down. The samples were then transferred into blue NucleoSpin RNA II columns in collection tubes and were centrifuged for 30 seconds at 13,000 rpm. The filtrate was disposed of and the blue column containing the RNA and DNA contaminants was de-salted by adding 350 µL membrane desalting buffer (MDB) and centrifuging at 13,000 rpm for 1 minute. The filtrate was discarded. DNase reaction mix, which will remove any possible DNA contaminants, was prepared according to the number of samples. DNase reaction mixture (95 µL) was added directly onto the centre of the silica membrane of the column and left to incubate at room temperature for 15 minutes. The silica membrane was then washed using 200 µL RA2 buffer and centrifuged for 30 seconds at 13,000 rpm. Filtrate was discarded and the membrane was washed with 600 µL RA3 buffer and centrifuged for 30 seconds at 13,000 rpm. The filtrate was discarded and the membrane was washed using 250 µL RA3 buffer and centrifuged for 2 minutes at 13,000 rpm. Columns were then transferred into RNase-free collection tubes and the RNA was eluted by adding 60 µL RNase-free water and centrifuging at 13,000 rpm for 1 minute. RNA was stored at -80°C.

2.2.12.2 RNA quantification

Yields were assessed by spectrophotometry using a NanoDrop© ND-1000 spectrophotometer (Thermo Scientific). RNA sample (1 µL) was pipetted onto the Nanodrop detector and RNA concentration and quality was determined based on an absorbance value of 260 nm. Detection was run in duplicate and the detector was wiped down between samples to avoid contamination. The RNA was then equalised to the concentration of the sample containing the lowest amount of RNA by the addition of the appropriate volume of RNase free water

2.2.12.3 cDNA synthesis

Complementary DNA (cDNA) synthesis was performed using a High Capacity cDNA reverse transcription kit (Applied Biosystems, UK). A master-mix of solution was prepared according to the number of samples, and stored on ice. Master mix was added to diluted RNA in a 1:1 ratio in PCR mini-tubes. The mini-tubes were then placed in a thermocycler (MJ Research, PTC-200, Peltier Thermal Cycler) and an automatic amplification program consisting of two cycles was set up. The first cycle ran for 10 minutes at 25°C and the second cycle ran for 120 minutes at 37°C.

2.2.12.4 Real-time PCR

Real-time PCR was performed using Taqman Gene Expression Assays (Applied Biosystems, UK), which contain forward and reverse primers, and a FAM-labelled MGB Taqman probe to each gene of interest (Table 2.4). β -actin was used as an endogenous control to normalise gene expression data. β -actin expression was measured using gene expression assays containing forward and reverse primers and a VIC-labelled MGB Taqman probe (Applied Biosystems, UK).

Table 2.4 List of genes used in PCR studies with the gene expression assay and genbank reference sequence numbers.

Gene Symbol	Gene Name	Taqman Gene Assay ID
MMP9	Matrix metalloproteinase 9	Rn00579162 m1
IFN γ	Interferon-gamma	Rn00594078 m1
IL-1 β	Interleukin-1 β	Rn00580432 m1
IL-6	Interleukin-6	Rn00561420 m1
TNF- α	Tumour necrosis factor- α	Rn99999017 m1
GFAP	Glial fibrillary acidic protein	Rn00566603 m1
CD11b	Cluster of differentiation 11b	Rn00709347_m1
β -actin	β -actin	4352340E

A 1 in 5 dilution of cDNA was prepared using RNase free water (Applied Biosystems). Diluted cDNA (4 μ L) was pipetted into a PCR plate, along with 0.5 μ L target primer 0.5 μ L β -actin primer and 5 μ L Taqman Universal PCR Master Mix. To avoid loss of sample through evaporation, the

PCR plate was sealed with an adhesive parafilm sheet (Applied Biosystems). The plate was then centrifuged at 800 rpm for 15 seconds to spin down the contents.

The sealed plate was inserted into Step One Plus real time PCR system (Applied Biosystems) and assayed in one run composed of two stages. The first stage being 50°C for a period of 2 minutes, followed by 95°C for 20 seconds. The second step was repeated 40 times and consisted of a temperature of 95°C for 1 second, followed by a temperature of 60°C for 20 seconds, at which point the fluorescence is read by the machine. During this second step, the double-stranded DNA is denatured and then as the temperature falls to 60°C, the target probe binds to the single stranded cDNA first, owing to its higher melting point in comparison to the target primers. This probe contains the FAM/VIC dye and a proprietary non-fluorescent quencher (NFQ) dye. This prevents the dye from emitting a fluorescence signal by fluorescence resonance energy transfer (FRET) technology (Applied Biosystems). When the temperature reaches 60°C the target primers anneal and the strand is extended at the 5' end by the nuclease activity of Taq polymerase. This displaces the FAM/VIC labelled probe which in turn causes the FRET between the dye and quencher to be broken, leading to the generation of a fluorescent signal. Due to the specificity of the probe and the primers of the cDNA sequence, one fluorescent signal is generated for each new cDNA copy.

2.2.12.5 Real-time PCR analysis

Gene expression for all real time PCR analysis was measured using the $\Delta\Delta CT$ method (Applied Biosystems RQ software, Applied Biosystems, UK). This method compares relative gene expression in the sample to the β -actin endogenous control to demonstrate up or down-regulation of genes of interest. Changes in fold-difference can be used to compare samples. Fold-difference is determined using the cycle number (CT) difference between samples. To determine the fold-difference relative to control, the CT of the endogenous control (β -actin) was subtracted from the CT of the target gene for each sample to explain for any differences in cDNA quantity that exist. This gives the normalised CT value. The CT difference (ΔCT) of the control is subtracted from itself to give 0, and subtracted from all of the other samples giving the $\Delta\Delta CT$ value. These $\Delta\Delta CT$ values (cycle difference corrected for β -actin) are then converted into fold-difference values. As a one-cycle difference corresponds to a two-fold increase or decrease relative to control, 2 to the power of $\Delta\Delta CT$ (difference in control and sample CT corrected for β -actin) gives the fold-difference in gene expression between control and treated samples.

2.2.13 Statistical analysis

Data was analysed and graphs were generated using GraphPad Prism v6.0 (GraphPad Software). Data are presented as mean \pm standard error of the mean (SEM). The Shapiro-Wilks normality test was used to test normality of data and the Grubbs outlier test was used to test for outliers. Data were analysed by student's *t*-test, Kruskal-Wallis test, one-way analysis of variance (ANOVA) or two-way repeated measures ANOVA as appropriate. If any statistically significant differences were revealed by ANOVA, *post hoc* comparisons were performed using a Newman-Keuls test following a one-way ANOVA, and a Bonferroni comparison following a two-way repeated measures ANOVA. Spearman's *r* correlation statistic was used for all correlations. In all cases, a *p* value of < 0.05 was considered significant.

2.2.14 Study design

A number of DSS-induced colitis experiments were carried out in order to analyse the effects of DSS colitis during the acute and recovery phase, to allow for collection of both perfused whole brains and snap-frozen brain and peripheral tissue, and also to perform various behavioural tests during the same period of recovery (approx. recovery days 5-7). Two TNBS studies were carried out. A detailed summary of each experiment follows below (see Figure 2.16 for a breakdown of all *in vivo* experiments and Figure 2.17 for an overall timeline of behavioural tests).

2.2.14.1 DSS Studies

1. Sixteen Wistar rats were randomly divided into control and acute colitis groups ($n = 8$) and were housed in pairs. The colitis group were exposed to DSS for seven days.
2. Thirty-two Wistar rats were randomly divided into control and colitis groups ($n = 16$) and were housed in cages of four. DSS rats were exposed to DSS from day 1 to 7 and following this, returned to water for seven days. Each rat was exposed to a variety of behavioural tests: day 6 of DSS exposure - open field, day 11 - open field, day 12 - marble burying, day 13 - elevated plus maze, and day 14 - light/dark box.

3. Twenty-four Wistar rats were randomly divided into control ($n = 12$), acute colitis ($n = 6$) (0 days recovery) and recovered colitis ($n = 6$) (7 day recovery) groups and were housed in groups of four. Colitis groups were exposed to DSS for seven days. Following DSS administration, DSS rats were returned to water for seven days in order to examine induction of inflammatory mediators following a recovery period.

4. Twenty-four rats were randomly divided into control, acute colitis (2 day recovery), and recovered colitis (7 day recovery) groups ($n = 8$) and were singly housed. Colitis rats were exposed to DSS until a DAI score of ≥ 2.5 was reached. Control and recovered colitis groups were exposed to an open field test (recovery day 5). Rats were perfused on the final day of the study to allow for immunohistochemical examination of brain tissue.

5. Thirty-six rats were randomly divided into control ($n = 14$), acute colitis ($n = 8$) (3 day recovery) and recovered colitis ($n = 14$) (10 day recovery) groups and were housed in pairs (except for saccharin preference testing when they were singly housed for 24 hours). Colitis rats were exposed to DSS until a DAI score of ≥ 2.5 was reached. Control and recovered colitis groups were exposed to open field (recovery day 5) and forced swim test (recovery day 6) while all groups underwent saccharin preference testing (acute: recovery day 1 and recovered: recovery day 8) and MRI scans (acute: recovery day 2 and recovered: recovery day 9). Rats were perfused on the final day of the study to allow for immunohistochemical examination of brain tissue.

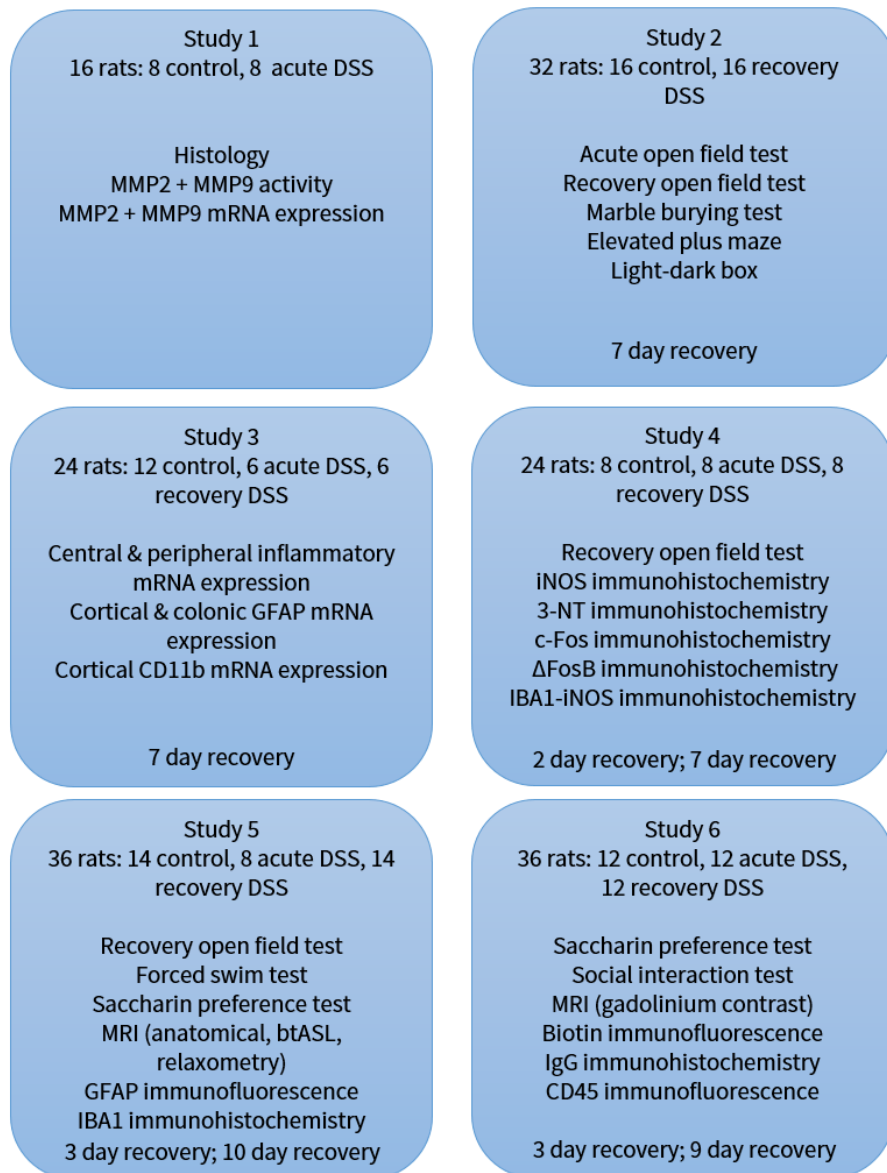
6. Thirty-six rats were randomly divided into control, acute colitis and recovered colitis groups ($n = 12$) and were housed in pairs (except for saccharin preference testing when they were singly housed for 24 hours). Colitis rats were exposed to DSS until a DAI score of ≥ 2.5 was reached. Rats were subject to saccharin preference testing ($n = 6$) (acute: recovery day 1 and recovered: recovery day 8) and social interaction test ($n = 12$) (control and recovered only: recovery day 8 prior to the saccharin preference test). Rats also underwent MRI scans – gadolinium enhanced ($n = 6$) (acute: recovery day 3 and recovered: recovery day 9) (Appendix 2). All rats were perfused on the final day of the study to allow for immunohistochemical examination of brain tissue.

2.2.14.2 *TNBS Studies*

1. Twenty rats were randomly divided into four groups: control, and three TNBS groups ($n = 5$) and were singly housed. Saccharin preference was assessed throughout the study. Rats were tested in the open field (control: day 8, and TNBS groups on day 3, 8, and 21 post-enema. Following the open field test the control group were euthanized at day 8 post-enema and the TNBS (30 mg/mL) groups were euthanized at 3, 8, or 21 days post-enema.

2. Ten rats were randomly divided into control and TNBS groups ($n = 5$) and singly housed. Both control and TNBS (30 mg/mL) groups were euthanized at 8 days post-enema.

DSS: Total rats = 168 (70 control; 42 acute; 56 recovery)



TNBS: Total rats = 30 (10 control; 5 TNBS 3 day; 10 TNBS 8 day; 5 TNBS 21 day)

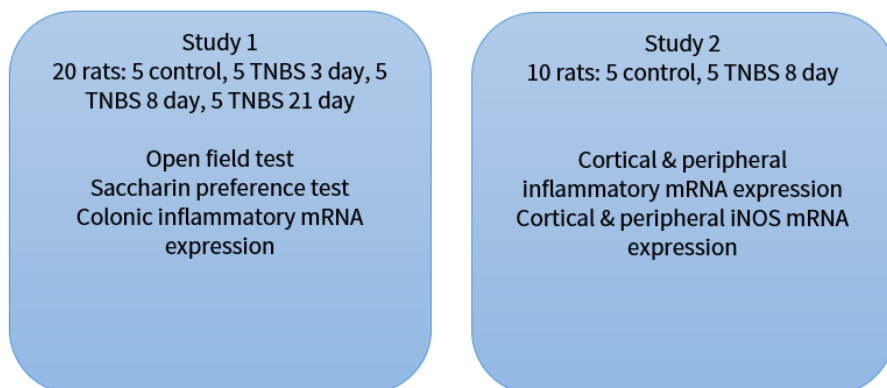


Figure 2.16 Summary of *in vivo* experiments

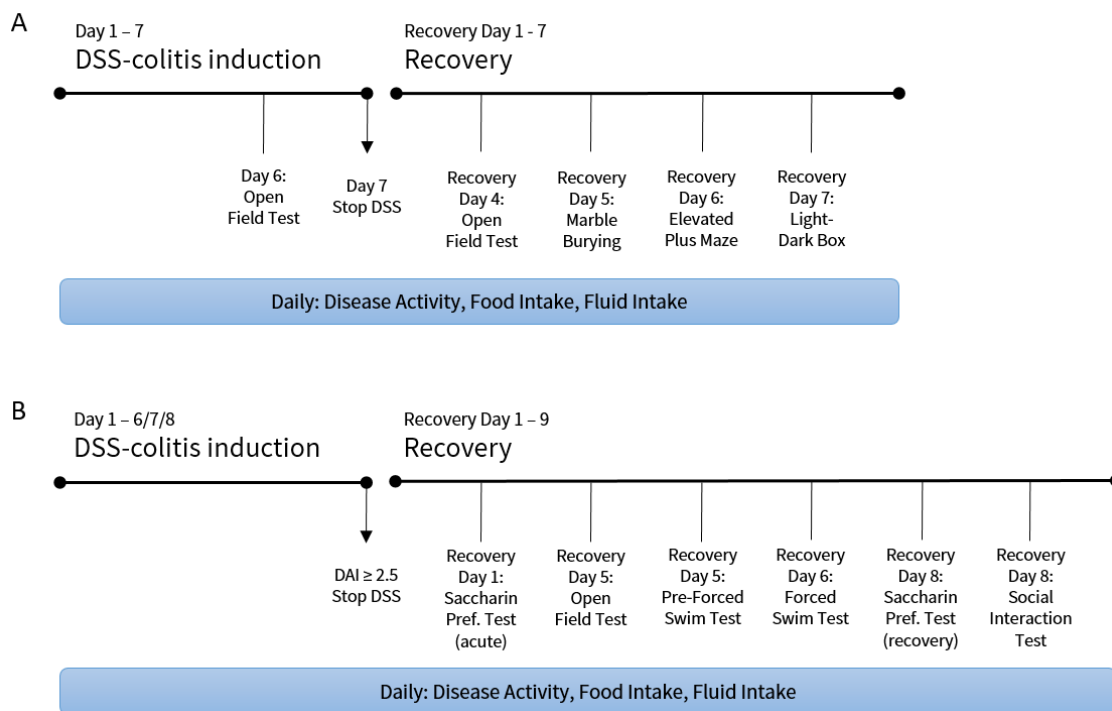


Figure 2.17 Overall experimental timeline for behavioural tests

For the first battery of behavioural tests (A) DSS was given for 7 days and behavioural tests were performed on the same set of animals. The open field test was performed on DSS day 6. On day 7 the animals were returned to tap water and allowed to recover. Following an open field test on recovery day 4 which confirmed the return of locomotor capability, a marble burying test, elevated plus maze test and light/dark box test were performed on recovery day 5, 6, and 7 respectively.

Following this study, to decrease variability of GI symptoms in DSS-exposed animals, animals received DSS until symptoms reached a pre-determined level of colitis according to the DAI (6-8 days). Recovery days were counted following return to water post-DSS. Multiple studies were carried out in this way and contributed to the behavioural assessment of DSS-exposed rats in recovery (B). As saccharin preference testing cannot be performed at the same time as DSS administration, this test was used to measure anhedonia in acutely colitic rats on day 1 of recovery. At this timepoint rats are still exhibiting symptoms of colitis. For behavioural tests in recovered rats an open field test was performed on recovery day 5, an FST pre-test and FST on day 5 and 6 respectively, and social interaction test and saccharin preference test on recovery day 8 (in different cohorts).

Chapter 3

DSS-Induced Colitis: Inflammation and Behaviour

Assessment of GI inflammation, behavioural alterations and peripheral and central inflammatory cytokines

3.1 Introduction

Mounting research indicates that further to the physiological symptoms of IBD, patients are also at an increased risk of psychological manifestations such as depression and anxiety (Mikocka-Walus et al., 2016, Abautret-Daly et al., 2017b). This is particularly significant, considering that depression and anxiety are the first and sixth leading causes of disability worldwide respectively (WHO, 2017). Furthermore, as symptoms comorbid to IBD, extra-intestinal manifestations have been seen to reduce HRQOL and medication adherence, and to increase the risk of relapse in IBD patients (Persoons et al., 2005, Goodhand et al., 2013, Iglesias-Rey et al., 2014, Long et al., 2014, Szigethy et al., 2014b).

MMPs are small molecules capable of cleaving most components of the intestinal ECM. As gelatinase enzymes capable of disrupting the ECM, they may be used as indicators of damage to colonic epithelial layers. MMP9 in particular is increased in tissue biopsies from IBD patients and in intestinal samples from animal models of IBD (Medina and Radomski, 2006, Medina et al., 2001, Medina et al., 2003, Harden et al., 2008). MMP9 is also capable of cleaving ECM proteins at the BBB causing barrier disruption (Turner and Sharp, 2016). Previously, an increase in MMP9 and in pro-inflammatory mediators including TNF- α , IL-1 β , IL-6, IFN γ and iNOS were shown in the colon and in circulating plasma of IBD patients with moderate symptoms (Abautret-Daly et al., 2017b). These mediators of inflammation are capable of disseminating to the brain and other organs and potentially play a role in the induction of psychological symptoms.

In order to fully uncover the mechanisms by which gastrointestinal inflammation may lead to neuroinflammation, and the link between this and psychological symptoms, a multi-systemic approach is required. However, clinical research on the neuropsychological aspects of IBD is limited to *post-mortem* brain studies, psychological tests, neuroimaging studies and functional drug challenges. When comparing these studies, one must remain wary of differences in type, severity and duration of inflammation, drug treatment, and environmental life stress between patients. Animal studies allow for the removal of much of this variability while still taking the lead from clinical observations. Animal models of IBD have been developed to allow for investigation of etiological factors in terms of understanding the mechanisms of disease pathogenesis and developing therapeutic strategies for intervention. Animal models of IBD also allow for the required multi-systemic approach to the study of gut-

brain interactions and aim to uncover the mechanisms by which IBD interacts with the CNS, thereby developing strategies/interventions to manage comorbid symptoms. Progress in establishing biomarkers of comorbidity in IBD through clinical data has been limited to date. This is primarily due to the difficulty in assessing the impact of stress and/or psychological disturbance in IBD in a clinical setting which can vary due to individual thresholds and personal/environmental factors. Thus, data available from animal models are the best available source to obtain insight into gut-brain interactions underlying comorbidity in IBD.

Multiple studies have to date investigated the neuroinflammatory aspects of TNBS-induced colitis model (Riazi et al., 2008, Medhi et al., 2009, Wang et al., 2010, Alhouayek et al., 2011, Baticic et al., 2011). Overall, these studies report increased TNF- α , IL-6, IL-1 β and MCP-1 in the brains of TNBS-colitis rodents. However, although the TNBS model is a valid, commonly used tool for studying the mechanisms of IBD pathogenesis, it requires fasting, anaesthesia, and partial restraint prior to TNBS enema. These aspects of TNBS-colitis induction are stressful for the subjects and may have an influence on the CNS. Therefore a preferable model for studying the neurobiological and behavioural impact of gastrointestinal inflammation in rodents is the DSS model. DSS is administered via home-cage drinking water thus no stress is caused by the colitis induction process itself. However, only three studies have to date analysed the cytokine expression profile in the brains of animals exposed to DSS. Villaran et al. (2010) report increased TNF- α , IL-6, IL-1 β and iNOS mRNA expression in the substantia nigra of rats following four days of 5% DSS administration. Zonis et al. (2015) focused on the hippocampus and showed an increase in IL-1 β , and TNF- α mRNA in this region following a seven-day acute DSS (3%) colitis induction protocol in mice. Reichmann et al. (2015) measured levels of IL-1 β , IL-6, IL-17A, IL-18, TNF- α , and GRO- α (also known as CXCL1) mRNA in the hypothalamus, hippocampus and amygdala of mice following seven days of DSS (2%) administration. They report increased GRO- α in the hypothalamus with stress-induced increases in IL-6 in all three regions and in GRO- α in the hippocampus and hypothalamus. CXCL1 is a chemokine which is expressed by the brain endothelium in response to IL-6 (Roy et al., 2012) and plays a role in neutrophil infiltration to the brain (Marro et al., 2016).

Behavioural tests can be used as indicators of anxiety- or depressive-like phenotypes in animal models of IBD. Considering the moderate physiological effects of chemically-induced and infection-induced colitis, the validity of behavioural tests, particularly those which

passively measure depressive-like behaviour during acute sickness should be questioned. In behavioural tests such as the open field test and FST, where lack of activity may be interpreted as anxious- or depressive-like behaviour, one should not discount the impact of abdominal pain experienced by animals with active colitis. Symptoms of diarrhoea and rectal bleeding associated with colitis may affect grooming behaviour and may also impact social interaction tests. Few studies, if any, report disease activity scores or take disease activity into account in behavioural tests of colitic animals. Additionally, many studies do not report the timepoint during colitis at which each behavioural test was carried out. In the DSS model of colitis, Painsipp et al. (2011) analysed mouse behaviour during DSS (2%) exposure and demonstrated a decrease in time spent in the open arms of the EPM indicative of anxiety-like behaviour, and increased immobility in the FST, a measure of learned helplessness or despair, indicative of a depressive-like phenotype. However these tests were carried out on days 8 and 11, respectively, of an 11 day DSS exposure protocol and, as such, sickness is a confounding factor in these results. Chen et al. (2015) performed anxiety- and depression-related behavioural tests in rats following a DSS (5%) colitis induction period and reported that DSS exposure caused a decrease in open arm entries and time spent in the open arm of the EPM, with an increase in immobility time in the FST. In this study DSS exposure also decreased sucrose preference in the sucrose preference test indicating reduced responsivity to a rewarding stimulus, an anhedonic behaviour symptomatic of depression, and reduced social interaction between animals suggestive of social avoidance and withdrawal. Very few studies have investigated the effects of colitis on measures of cognition, learning or memory. Three days following DNBS colitis induction via enema, Gharedaghi et al. (2015) tested mice in the Y-maze, a measure of spatial recognition memory and memory acquisition, and in the passive avoidance test, a measure of associative long term memory. DNBS has similar induction methods, mechanism of action and symptoms to TNBS-induced colitis which means that these mice had acute colitis symptoms when tested. This study found that DNBS-induced colitis decreased spatial recognition memory in the Y-maze but did not affect associative memory in the passive avoidance paradigm. Furthermore, it was demonstrated that these deficits in spatial memory could be attenuated using NMDA antagonists or an iNOS inhibitor.

3.1.1 Aims

The aim of this chapter is to examine the peripheral inflammatory and pathological effects, as well as central inflammatory and behavioural effects, of DSS-induced colitis, to determine whether colonic inflammation induces inflammation or alterations within the CNS.

Specifically, the main objectives are to characterise the DSS-induced colitis model in terms of disease activity, colonic inflammation, behavioural alterations in the open field test, marble burying test, FST, EPM, light/dark box, social interaction test and saccharin preference test, and peripheral and central expression of IL-1 β , IL-6, TNF- α , IFN γ , GFAP, CD11b and iNOS. Finally, the TNBS model of colitis is examined for comparison. This model involves fasting, anaesthesia and direct application of TNBS into the colon, and results in acute colitis approximately three days following application, unlike the DSS model which is administered via drinking water and allows colitis to slowly develop over approximately seven days. Examination of the TNBS model of colitis allows us to confirm that the inflammatory effects observed in the brain occur as a result of colitis and are not only related to the model induced by DSS.

3.2 Methods

3.2.1 Study design

A number of DSS-induced colitis experiments were carried out in order to analyse the effects of DSS colitis during the acute phase (when animals are still symptomatic) and the recovery phase (when symptoms have gone and animals are no longer exhibiting sickness behaviours). Multiple behavioural tests were carried out during the same period of recovery, to avoid familiarisation that would occur by repeating behavioural tests at differing timepoints in the same animals and to allow for collection of both perfused whole brains and snap-frozen brain and peripheral tissue. A detailed breakdown of each experiment and the *in vivo* and *ex vivo* work carried out within each experiment is provided in Chapter 2 (Section 2.2.14 and summarised in Figure 2.16). Although behavioural tests were performed with different cohorts of animals, the overall timelines for behavioural tests are summarised in Chapter 2 (Figure 2.17).

3.2.2 Experimental protocols

All procedures in this chapter were conducted as described in detail in Chapter 2 and are outlined briefly below.

3.2.2.1 *Animals*

Male Wistar rats (175-250 grams) were obtained from the Bio-Resources Unit in Trinity College Dublin and housed under standard conditions with access to food and water *ad libitum*. The body weight, food consumption and fluid intake of each rat were recorded as a daily indication of welfare.

3.2.2.2 *DSS-colitis induction*

A 5% w/v DSS solution was used to induce symptoms of colitis. The rats were monitored for signs of disease activity according to a modified version of the DAI (Table 2.1). DSS was administered for seven days or until a rat reached a DAI score of ≥ 2.5 at which point the rat was given normal tap water.

3.2.2.3 *TNBS-colitis induction*

Rats were singly housed and fasted for 24 hours during which time they were given a 20% sucrose solution containing a laxative (X-Prep; 15 mL/L). Following this, rats were anaesthetised (1.5 mL ketamine and 1.5 mL xylazine) and given a 1 mL rectal enema. TNBS groups received 30 mg TNBS in ethanol (30%) and control groups received a vehicle enema. The rats were monitored for signs of colitis including loose stools, diarrhoea and abdominal bloating. Rats were euthanized at 3, 8, or 21 days post-enema.

3.2.2.4 *Behavioural tests*

3.2.2.4.1 Open field test

The open field was a cylindrical plastic arena 1 metre in diameter with mirrored walls and a black floor (Figure 2.1). A video camera mounted above the open field was used to record the movement tracks for each animal using image acquisition software (HVS Image) and video tracking software (Ethovision). Animals were each placed in the centre of the open field arena and left to explore freely for 10 minutes. Rearing and grooming were manually recorded. Between each test the walls and floor of the open field were wiped clean with 70% ethanol followed by H₂O to remove olfactory cues.

3.2.2.4.2 Marble burying test

Small, hard-bottomed polypropylene cages (45 cm x 27 cm x 15 cm) were filled 5 cm deep with regular woodshaving bedding (Figure 2.2). Two rows of marbles (2.3 cm diameter) were lined approximately 2 cm apart along the same short wall of the cage. For each test session a rat was placed in the cage, observed for 10 minutes and then removed. At the end of the session, marbles covered at least two-thirds with bedding were scored as buried.

3.2.2.4.3 Forced swimming test

One day prior to the test, animals were placed in a glass cylinder filled to a depth of 30 cm with water at a temperature of $23 \pm 1^\circ\text{C}$ for 15 minutes (Figure 2.3). Clean water was used for each animal. Twenty-four hours later rats were placed in the cylinder under the same conditions for 5 minutes. Both sessions were recorded and time spent immobile was scored by a researcher blind to treatment group. Immobility was defined as an absence of active escape-oriented behaviour such as swimming, climbing or diving.

3.2.2.4.4 Elevated plus maze

The EPM consisted of a grey plus maze elevated 50 cm off the ground (Figure 2.4). Black Plexiglas® (50 cm high) surrounded two of the opposing open arms and the other two remained open. Normal light-phase room lighting was maintained during testing. Rats were placed in the centre of the EPM facing an open arm and tested for 5 minutes 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 HVS Image software (HVS Image Ltd.).

3.2.2.4.5 Light/dark box

In this protocol the light chamber consisted of a white walled box with a clear glass top (and a bright light in the centre; the dark chamber had black walls and a solid black top, and both chambers were connected via a rectangular passage (Figure 2.5). Rats were placed in the light chamber and observed for 10 minutes. Number of entries to each area, time spent in each area and fecal boli count were recorded. Head poking was also recorded as an indication of investigatory tendencies (Marino et al., 2005).

3.2.2.4.6 Social interaction test

Two unfamiliar rats from the same group, not differing in weight by >15 g, were placed in the open field arena as described in Chapter 2 (Section 2.2.6.1), to which they have previously been acclimatised, and allowed to explore and interact freely for 12 minutes. This session was recorded and later analysed by a researcher blind to treatment group to give a social interaction score per pair. Various types of social interaction were measured including time spent sniffing, playing, grooming, fighting etc. to give a combined total interaction time.

3.2.2.4.7 Saccharin preference test

Rats were habituated to drinking from two equally accessible water bottles throughout the study and were singly housed for the duration of the test. At the start of the test, one of the drinking bottles was replaced with 10 mM saccharin solution (0.01%). To avoid side preference the saccharin bottle was placed on the left of half of the cages and on the right of the other cages. The bottles were weighed at the start and end of each test which lasted 24 hours. Saccharin preference was calculated as weight-change in the saccharin bottle as a percentage of the total weight-change of both bottles.

3.2.2.5 *Tissue collection*

Rats were decapitated and tissue samples of interest (colon, liver, spleen, and brain tissue) were retrieved, snap-frozen in liquid nitrogen and stored at -80°C until use.

3.2.2.6 *Histology*

Distal colon samples were embedded in paraffin wax, sectioned with a microtome at a thickness of 10 µm, and placed on microscope slides. Slides were put in an oven at 60°C for 30 minutes to set, and stained using haematoxylin and eosin in a Leica Autostainer before being coverslipped in DPX mounting medium. Slides were digitally recorded and analysed using an Olympus BX51 video-camera microscope. Crypt damage was rated on a scale of 0-4 and inflammation was scored on a scale of 0-3 (Table 2.3). Crypt damage and inflammation scores were recorded for ten randomly selected areas of each colon sample and averaged to give approximate scores of colonic crypt damage and inflammation respectively.

3.2.2.7 *Zymography*

Colon samples were homogenised using a polytron in lysis buffer containing Phosphatase Inhibitor Cocktail I & II and Proteinase Inhibitor Cocktail, before being centrifuged at 14,000 rpm at 4°C for 15 minutes. Protein concentrations of each sample were determined via Bradford assay as described in Chapter 2 (Section 2.2.11.2) and samples were equalised in dH₂O (2000 µg/mL). Loading buffer was added to each equalised sample. A positive control was prepared by adding loading buffer and Bromophenol blue to 18 µL HT-1080 standard. Samples and standards were loaded into the wells of an SDS gel in an electrophoresis box. The electrophoresis box was placed into a tank containing ice. The electrophoresis was run (150 V; 300 mA), until samples had migrated to the bottom of the gel. Separating gels were extracted and washed in Triton buffer with mild agitation. They were then washed in zymography buffer before being incubated in fresh zymography buffer overnight at 37°C. The next day, the gels were incubated in staining solution containing Coomassie Blue for 3 hours followed by washing in de-staining solution. Gelatinase activity of MMP9 and MMP2 was analysed by detection of intensity of band clearing at the same position as the positive control (HT-1080 standard). A calibrated densitometer and Quantity One analysis software were used to measure intensity x mm.

3.2.2.8 *Real Time Polymerase Chain Reaction*

Isolation of RNA from all tissue samples was carried out using Nucleospin RNA II kits (Macherey-Nagel) according to the kit protocol. Following RNA quantification and equalisation, cDNA was synthesised using a High Capacity cDNA reverse transcription kit (Applied Biosystems, UK). Gene expression analysis was conducted using real-time PCR to quantify expression of target genes employing a Taqman Gene Expression Assay (Applied Biosystems, UK) containing a FAM-labelled MGB Taqman probe (see Table 2.4 for genes of interest). β -actin was used as an endogenous control to normalise gene expression data. β -actin expression was measured using gene expression assays containing a VIC-labelled MGB Taqman probe (Applied Biosystems, UK). Samples were assayed using universal cycling conditions using a fast protocol on the StepOnePlus PCR system (Applied Biosystems, UK). Gene expression for all real time PCR analysis was measured using the $\Delta\Delta$ CT method (Applied Biosystems, UK). This method compares relative gene expression in the sample to the β -actin endogenous control to demonstrate up- or down-regulation of genes of interest. Changes in fold-difference can be used to compare samples.

3.3 Results

3.3.1.1 *DSS-induced colitis induces symptoms of UC, affects general well-being, and disturbs colonic structure in Wistar rats*

3.3.1.2 *Body weight, food intake and fluid intake in DSS-induced colitis*

Body weight changes, food intake and fluid intake were measured daily as general indicators of wellbeing (Figure 3.1). This analysis includes an amalgamation of data from animals in all studies. A two-way repeated measures ANOVA of body weight change shows an effect of DSS ($F_{1,91} = 73.68; p < 0.0001$), day ($F_{6,546} = 28.36; p < 0.0001$), and an interaction effect ($F_{6,546} = 18.11; p < 0.0001$) (A) during the DSS exposure phase. A Bonferroni *post hoc* test shows a decrease in weight change in acute colitis on days 5, 6 and 7 of DSS exposure compared to controls ($p < 0.001$). A two-way repeated measures ANOVA of body weight change shows an effect of DSS ($F_{1,77} = 14.02; p = 0.0003$), day ($F_{6,462} = 3.684; p = 0.0014$), and an interaction effect ($F_{6,462} = 8.365; p < 0.0001$) (B) during the recovery phase. A Bonferroni *post hoc* test shows a decrease in weight change in recovering colitis on days 1, 2 and 3 of recovery compared to controls ($p < 0.001$). A two-way repeated measures ANOVA of food intake shows an effect of DSS ($F_{1,48} = 30.19; p < 0.0001$), day ($F_{6,288} = 11.52; p < 0.0001$), and an interaction effect ($F_{6,288} = 11.42; p < 0.0001$) (C) during the DSS exposure phase. A Bonferroni *post hoc* test shows a decrease in food intake in acute colitis on days 4, 5, 6 and 7 of DSS exposure compared to controls ($p < 0.05$). A two-way repeated measures ANOVA of food intake shows an effect of DSS ($F_{1,43} = 26.12; p < 0.0001$), day ($F_{6,258} = 2.807; p = 0.0116$), and an interaction effect ($F_{6,258} = 3.360; p = 0.0033$) (D) during the recovery phase. A Bonferroni *post hoc* test shows a decrease in food intake in recovering colitis on days 1, 2, 3 and 4 of recovery compared to controls ($p < 0.01$). A two-way repeated measures ANOVA of fluid intake shows an effect of day ($F_{6,246} = 13.09; p < 0.0001$), with no effect of DSS ($F_{1,41} = 0.1946; p = 0.6614$), and no interaction effect ($F_{6,246} = 1.845; p = 0.0909$) (E) during the DSS exposure phase. A Bonferroni *post hoc* test shows no change in fluid intake during DSS exposure compared to controls. A two-way repeated measures ANOVA of fluid intake show an effect of DSS ($F_{1,45} = 5.075; p = 0.0292$), day ($F_{6,270} = 2.450; p = 0.0253$), with no interaction effect ($F_{6,270} = 1.022; p = 0.4114$) (F) during the recovery phase. A Bonferroni *post hoc* test shows no change in fluid intake during recovery compared to controls.

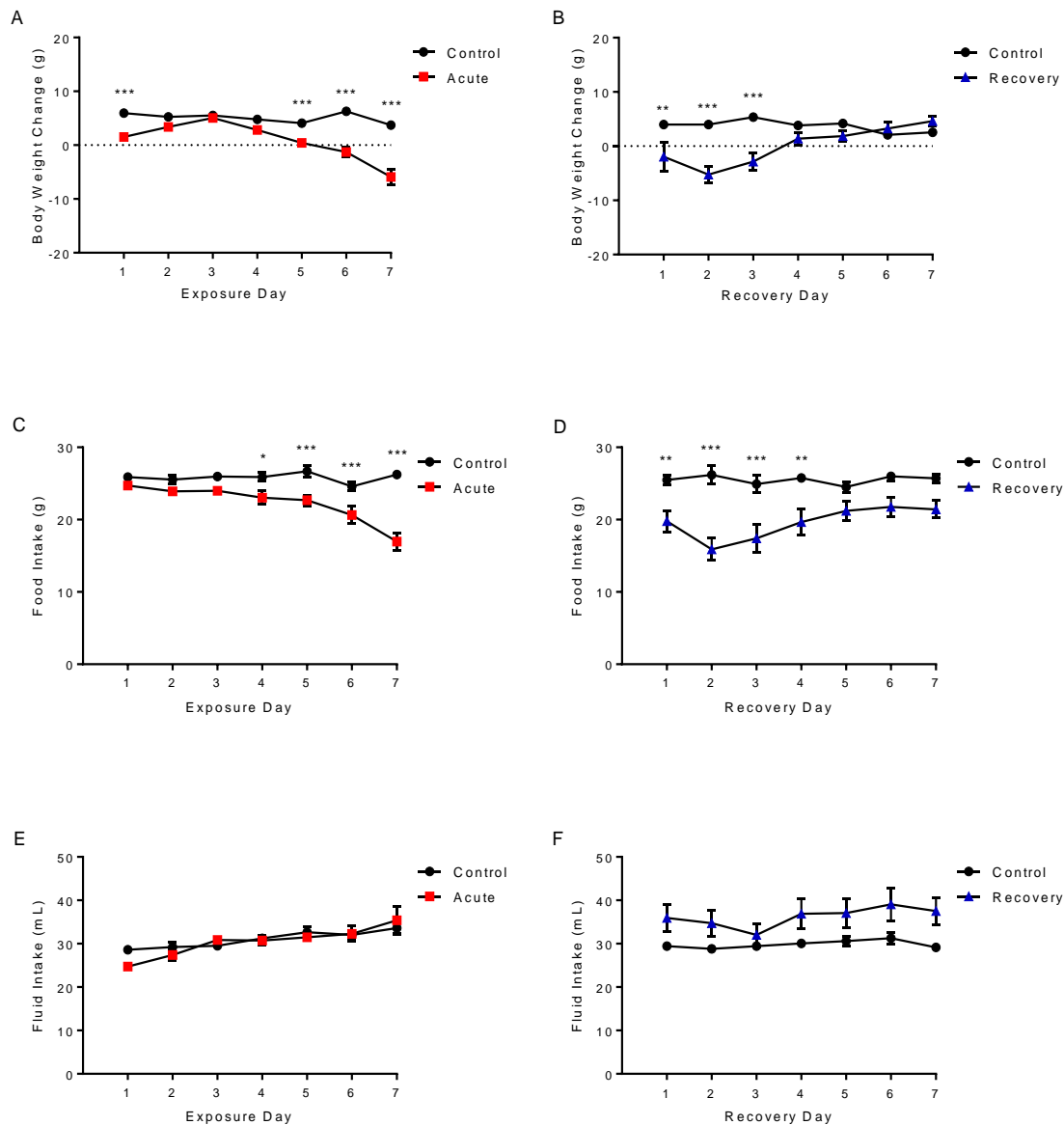


Figure 3.1 Body weight, food intake, and fluid intake during DSS-induced colitis exposure and recovery in rats

Rats were weighed and measurements of food and fluid intake were taken daily throughout each study. During DSS exposure body weight decreases (A); body weight continued to decrease until midway through the recovery phase (B). Food intake begins to decrease during the DSS exposure phase (C) and recovers towards the end of the recovery period (D). Fluid intake is similar to control during the DSS exposure phase (E) and recovery (F), although there is a trend toward increased fluid intake during recovery from DSS-induced colitis. The data presented here is an amalgamation of data from all DSS-experiments performed.

Data are presented as mean \pm SEM ($n = 15-58$). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ relative to control (two-way repeated measures ANOVA with Bonferroni *post hoc* test).

3.3.1.3 Disease activity, histological scores and MMP activity in DSS-induced colitis

Disease activity was assessed to specifically determine the effect of DSS on UC-like symptoms. Animals were scored daily for weight loss, stool consistency and rectal bleeding according to the DAI (see Table 2.1). DSS administration induced an increase in DAI over 7 days, which decreases during the recovery phase (Figure 3.2). A two-way repeated measures ANOVA of DAI scores shows an effect of DSS ($F_{1,79} = 192.1; p < 0.0001$), day ($F_{5,395} = 66.38; p < 0.0001$), and an interaction effect ($F_{5,395} = 68.52; p < 0.0001$) (A) during the DSS exposure phase. A Bonferroni *post hoc* test shows an increase in DAI score in acute colitis on days 3, 4, 5 and 6 of DSS exposure compared to controls ($p < 0.001$). A two-way repeated measures ANOVA of DAI scores show an effect of DSS ($F_{1,66} = 102.5; p < 0.0001$), day ($F_{6,396} = 24.19; p < 0.0001$), and an interaction effect ($F_{6,396} = 23.92; p < 0.0001$) (B) during the recovery phase. A Bonferroni *post hoc* test shows an increased DAI score in recovering colitis on days 1-7 of recovery compared to controls ($p < 0.01$). It was observed that DSS administration resulted in increased DAI scores as measured on the final day of DSS administration which returns to control levels at the recovery timepoint ($F_{2,146} = 32.30; p < 0.0001$) (Figure 3.2). *Post-mortem* histological assessment showed that DSS administration induces a change in colonic ultrastructure similar to human UC. Analysis of the distal colon revealed DSS increased both histological criteria (see Table 2.3): crypt damage scores ($t = 15.79; df = 12; p < 0.0001$) (A) and inflammation scores ($t = 12.14; df = 12; p < 0.0001$) (B) (Figure 3.3). DSS administration increased MMP9 mRNA expression ($t = 2.533; df = 12; p = 0.0263$) (A) and MMP2 mRNA expression ($t = 4.957; df = 12; p < 0.001$) (B), MMP9 activity ($t = 5.991; df = 12; p < 0.0001$) (C) and MMP2 activity ($t = 3.005; df = 10; p = 0.0132$) (D) in the colon (Figure 3.4).

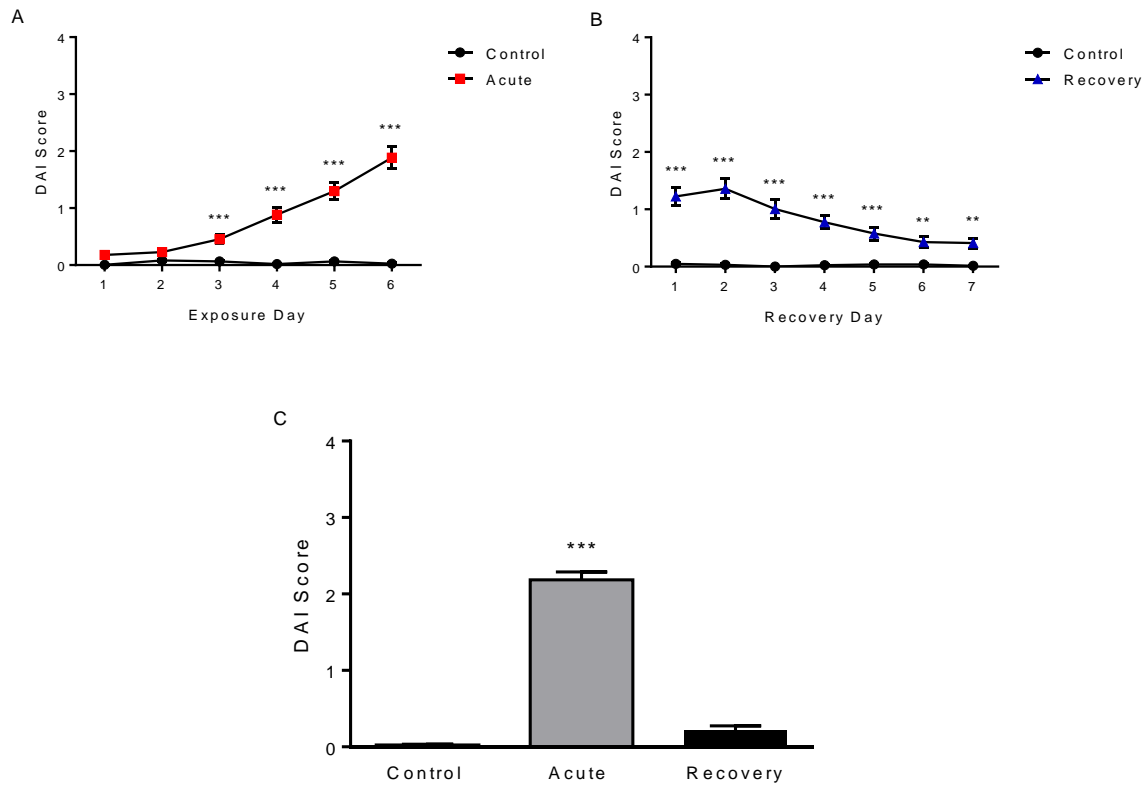


Figure 3.2 Disease activity index scores during DSS-induced colitis exposure and recovery in rats

Rats were scored daily according to the DAI throughout each study. The DAI increases during DSS administration (A) and decreases during recovery (B). DAI as measured on the final day of DSS administration (acute) is increased relative to control, however after recovery the DAI score has returned to control levels (C). The data presented here is an amalgamation of data from all DSS-experiments performed.

Data are presented as mean \pm SEM ($n = 38 - 57$). *** $p < 0.001$, ** $p < 0.01$ relative to control (two-way repeated measures ANOVA with Bonferroni *post hoc* test [A&B] and one-way ANOVA with Newman-Keuls *post hoc* test [C]).

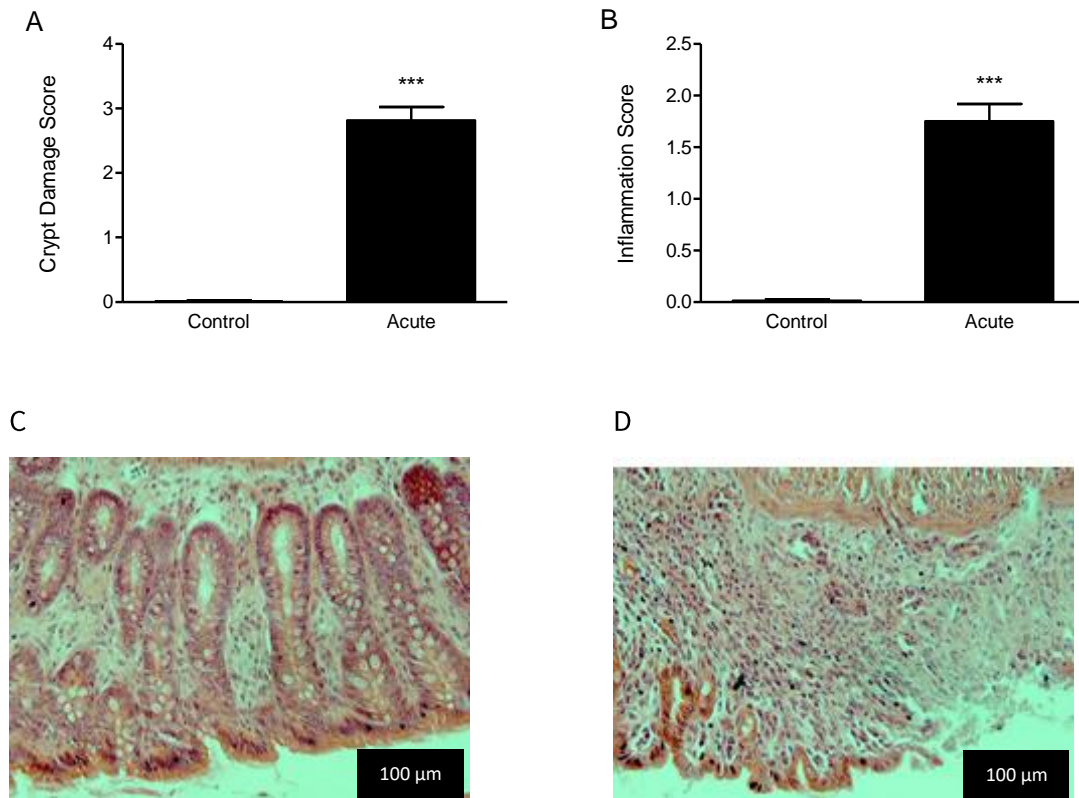


Figure 3.3 Histological scores of the bowel wall following DSS-induced colitis in rats

DSS resulted in an increase in crypt damage (A) and inflammation (B) scores in the colon. Representative images show transverse sections of a control colon (C) demonstrating intact colonic crypts, sparse mononuclear infiltration in the lamina propria and intact epithelial layer and a colitic colon (D) demonstrating crypt destruction, dense infiltration of mononuclear cells into the lamina propria and erosion of the epithelial layer (400x magnification). The data presented here comes from rats in DSS study 1.

Data are presented as mean \pm SEM ($n = 6-8$). *** $p < 0.001$ relative to control (unpaired student's t -test).

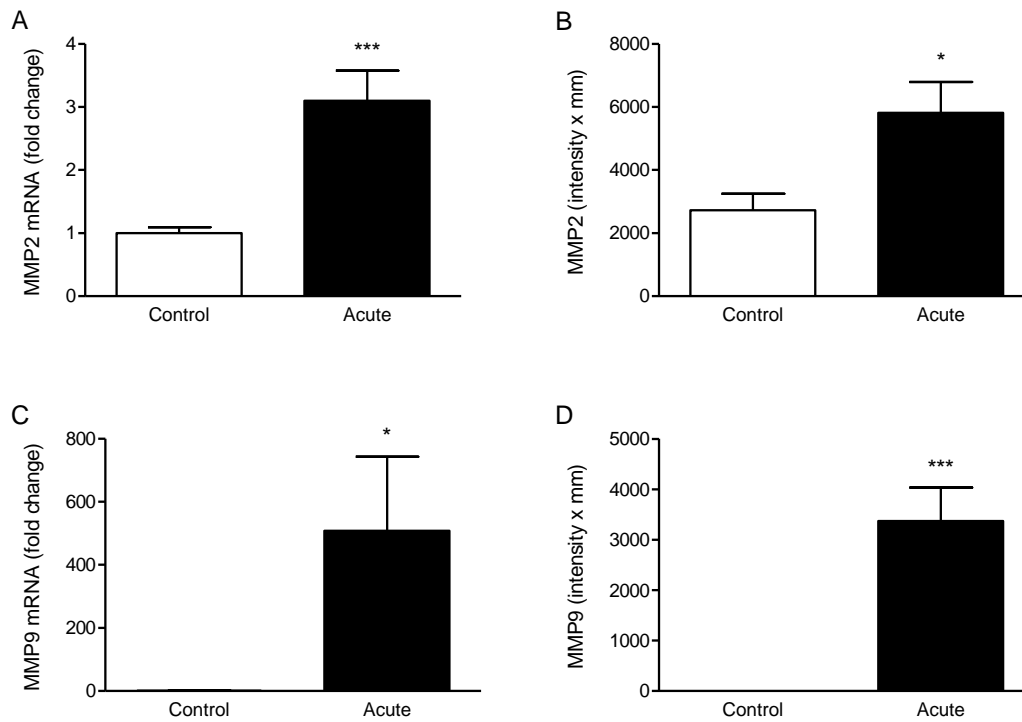


Figure 3.4 Colonic MMP2/9 expression and activity following DSS-induced colitis in rats

DSS administration increased MMP2 (A) and MMP9 (C) mRNA expression, and MMP2 (B) and MMP9 (D) protease activity as measured by zymography. The data presented here comes from rats in DSS study 1.

Data are presented as mean \pm SEM ($n = 6 - 8$). *** $p < 0.001$, * $p < 0.05$ relative to control (unpaired student's t -test).

3.3.2 Effect of DSS-induced colitis on performance in open field, marble burying, forced swimming, elevated plus maze, light/dark box, social interaction and saccharin preference tests in rats

3.3.2.1 *Behavioural changes in the open field following DSS-induced colitis in rats*

DSS-induced colitis resulted in behavioural changes in the open field during the acute colitic phase (day 6 of DSS administration; Figure 3.5). Analysis revealed a decrease in path length ($t = 2.673$; $df = 26$; $p = 0.0128$) (A), speed ($t = 2.579$; $df = 26$; $p = 0.0159$) (B), percentage of time spent in the centre zone of the arena ($t = 2.511$; $df = 26$; $p = 0.0186$) (C) and total number of rears ($t = 2.695$; $df = 26$; $p = 0.0122$) (D) when compared to vehicle-treated controls. Open field testing on recovery day 4 in these animals showed a return to normal locomotor exploratory behaviour (Figure 3.6) in terms of path length ($t = 0.4242$; $df = 32$; $p = 0.6743$) (A), speed ($t = 0.5451$; $df = 32$; $p = 0.5895$) (B), percentage of time spent in the centre zone ($t = 0.3417$; $df = 32$; $p = 0.7348$) (C) and total number of rears ($t = 0.4131$; $df = 32$; $p = 0.6823$) (D) when compared to vehicle-treated controls. Open field testing on recovery day 5 in a separate study showed a return to normal locomotor exploratory behaviour (Figure 3.7) in terms of path length ($t = 1.500$; $df = 31$; $p = 0.1436$) (A), speed ($t = 1.787$; $df = 32$; $p = 0.0835$) (B), percentage of time spent in the centre zone ($t = 0.9071$; $df = 30$; $p = 0.3716$) (C) and total number of rears ($t = 0.4414$; $df = 30$; $p = 0.6621$) (D) when compared to vehicle-treated controls.

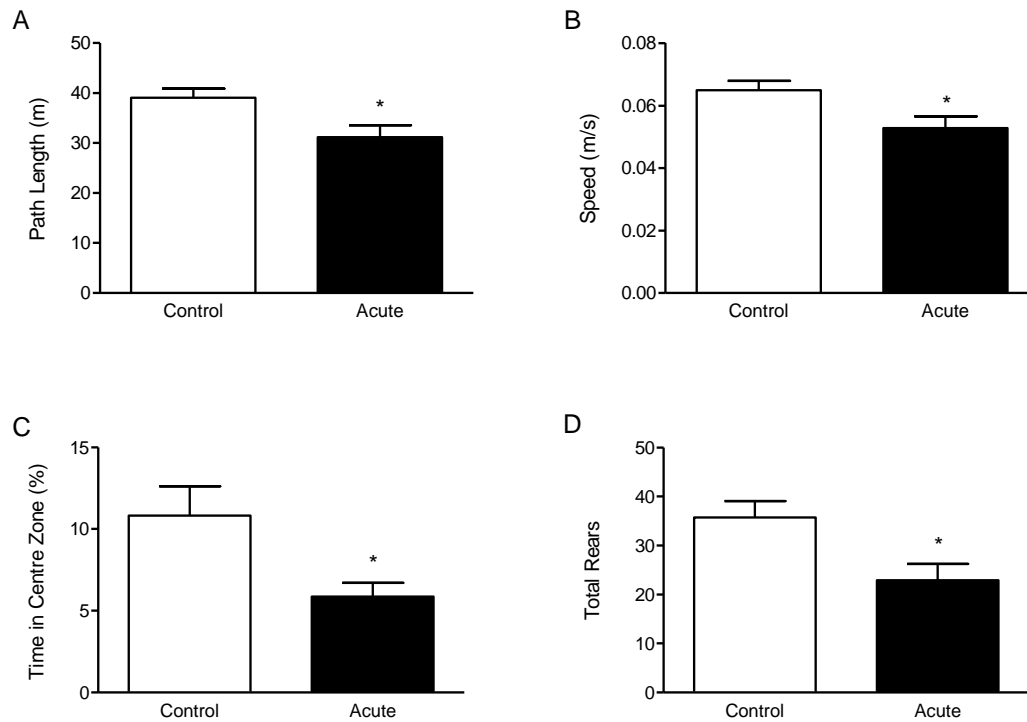


Figure 3.5 Performance in the open field test following DSS-induced colitis in rats

Rats were assessed in the open field test on day 6 of DSS exposure. DSS-induced colitis decreased path length (A), speed (B), percentage of time spent in the centre zone (C) and total number of rears (D) in the open field test. The data presented here comes from rats in DSS study 2.

Data are presented as mean \pm SEM ($n = 14$). * $p < 0.05$ relative to control (unpaired student's t -test).

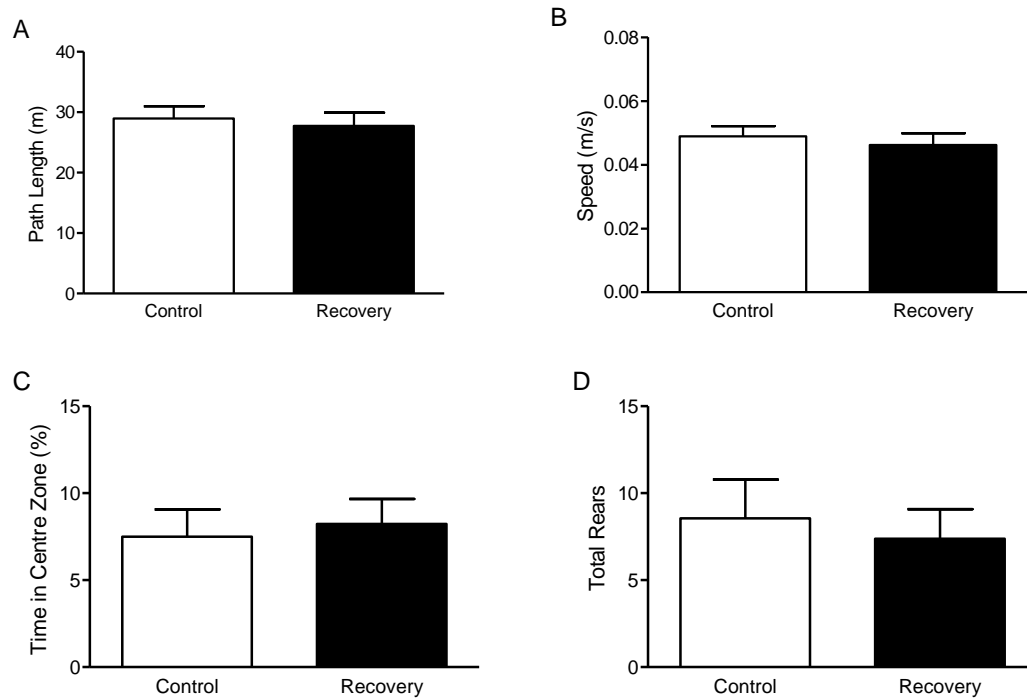


Figure 3.6 Performance in the open field test in recovery (day 4) from DSS-induced colitis in rats

Rats were assessed in the open field test on day 4 of recovery from DSS exposure. Normal path length (A), speed (B), percentage of time spent in the centre zone (C) and total rears (D) are observed in the open field test on recovery day 4. The data presented here comes from rats in DSS study 2.

Data are presented as mean \pm SEM ($n = 15 - 18$). (unpaired student's t -test).

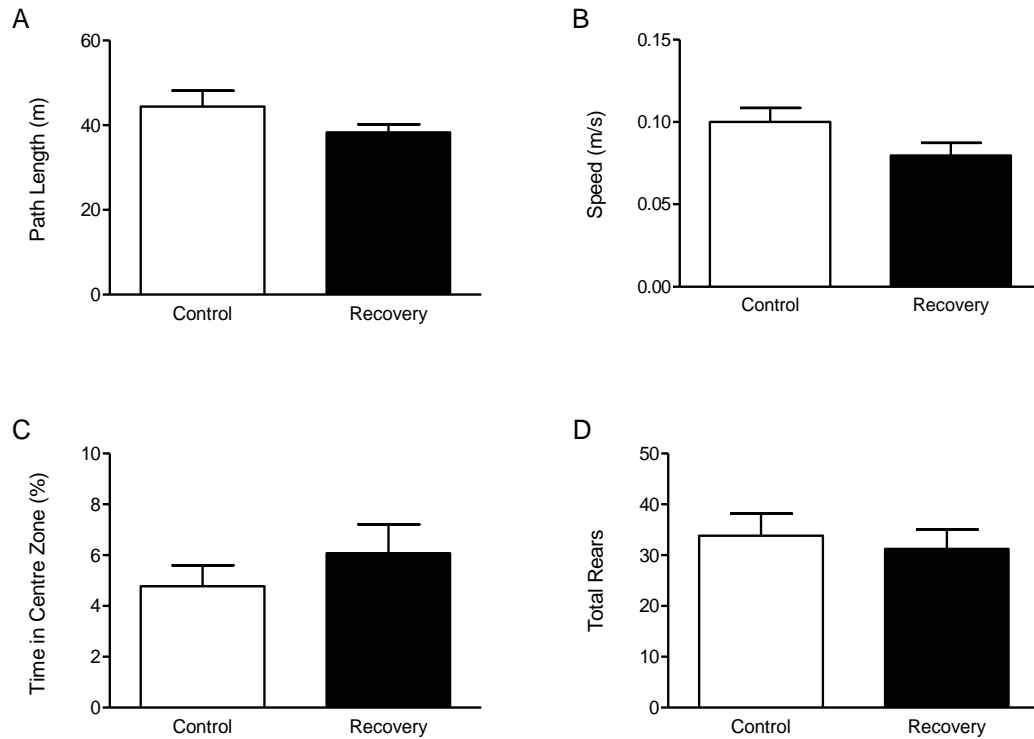


Figure 3.7 Performance in the open field test in recovery (day 5) from DSS-induced colitis in rats

Rats were assessed in the open field test on day 5 of recovery from DSS exposure. Normal path length (A), speed (B), percentage of time spent in the centre zone (C) and total rears (D) are observed in the open field test on recovery day 5. The data presented here comes from rats in DSS studies 4 & 5.

Data are presented as mean \pm SEM ($n = 15 - 18$). (unpaired student's t -test).

3.3.2.2 Behavioural changes in recovery from DSS-induced colitis

3.3.2.2.1 Marble burying

On day 5 of recovery from DSS exposure, DSS-exposed animals showed decreased marble burying behaviour when compared to non-DSS-exposed controls ($t = 6.789$; $df = 14$; $p < 0.0001$) (Figure 3.8).

3.3.2.2.2 Forced swimming test

An FST was performed on recovery day 6 and showed an increase in immobility time for rats in recovery from DSS-induced colitis (Figure 3.9) ($t = 3.059$; $df = 12$; $p = 0.0099$) (B) when compared to vehicle-treated controls. Twenty-four hours previously, rats in recovery from colitis showed lower immobility times in the first two 5 minute intervals of the 15 minute pre-test swim relative to controls (Figure 3.9) ($t = 3.831$; $df = 12$; $p = 0.0024$) and ($t = 3.849$; $df = 12$; $p = 0.002$) respectively (A).

3.3.2.2.3 Elevated plus maze

Rats were tested in the EPM on recovery day 6 (Figure 3.10). Increased time in the 'safe' end of the closed arms ($t = 2.445$; $df = 26$; $p = 0.0216$) (A), and decreased time spent in the inner zone was observed ($t = 2.567$; $df = 26$; $p = 0.0163$) (B) in DSS-exposed animals compared to controls. There was no difference between the groups in total time spent in the outer ends of the open arm compared to control ($t = 0.5413$; $df = 32$; $p = 0.5920$) (C) and no difference in path length between the groups ($t = 1.257$; $df = 32$; $p = 0.2179$) (D).

3.3.2.2.4 Light/dark box

On day 7 of recovery from DSS administration rats were tested in the light/dark box (Figure 3.11). There was no difference between the groups in time spent in the light chamber ($t = 1.537$; $df = 31$; $p = 0.1345$) (A), however decreased head pokes into the light chamber ($t = 4.492$; $df = 32$; $p < 0.0001$) (B) and increased fecal boli counts ($t = 2.877$; $df = 32$; $p = 0.0071$) (C) were recorded in DSS-exposed animals compared to controls.

3.3.2.2.5 Social interaction test

On day 8 of recovery from DSS administration pairs of rats were subject to a social interaction test. There was no difference in social interaction time between the groups (Figure 3.12) ($t = 0.2263$; $df = 8$; $p = 0.8267$).

3.3.2.2.6 Saccharin preference

Saccharin preference testing was carried out on day 1 and day 8 of recovery for acutely colitic and recovered rats respectively (Figure 3.13). One-way ANOVA of saccharin preference showed an effect of DSS ($F_{2,48} = 37.9; p < 0.0001$) (A). A Newman Keuls *post hoc* test revealed a decrease in saccharin preference as a percentage of total fluid intake in acute and recovered colitis compared to controls ($p < 0.05$). One way ANOVA of total fluid intake show no effect of DSS ($F_{2,48} = 2.259; p = 0.1154$) (B).

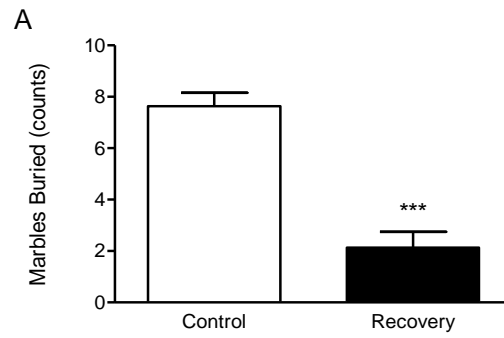


Figure 3.8 Performance in the marble burying test in recovery (day 5) from DSS-induced colitis in rats

On day 5 of recovery, rats were assessed in the marble burying test and those in recovery from DSS-induced colitis buried fewer marbles (A). The data presented here comes from rats in DSS study 2.

Data are presented as mean \pm SEM ($n = 8$). *** $p < 0.001$ relative to control (unpaired student's t -test).

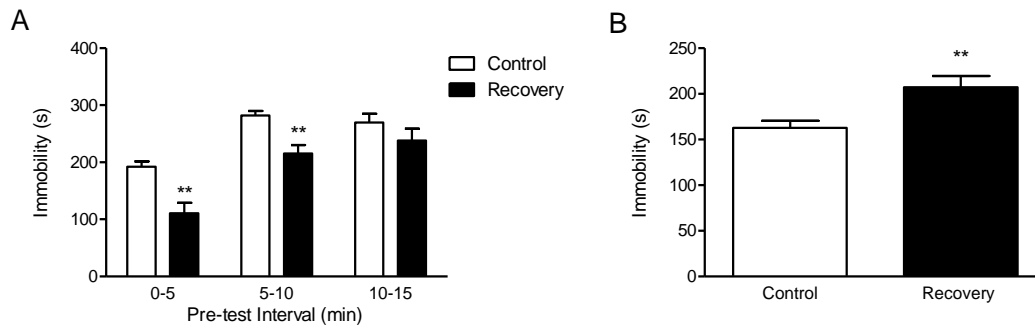


Figure 3.9 Immobility in the forced swim pre-test and test in recovery (day 5&6) from DSS-induced colitis

Rats recovering from DSS-induced colitis showed decreased immobility in the first two 5 minute intervals of the pre-FST (A). Twenty four hours later in the test session immobility was increased relative to control (B). The data presented here comes from rats in DSS study 5.

Data are presented as mean \pm SEM ($n = 6$). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ relative to control (unpaired student's t -test).

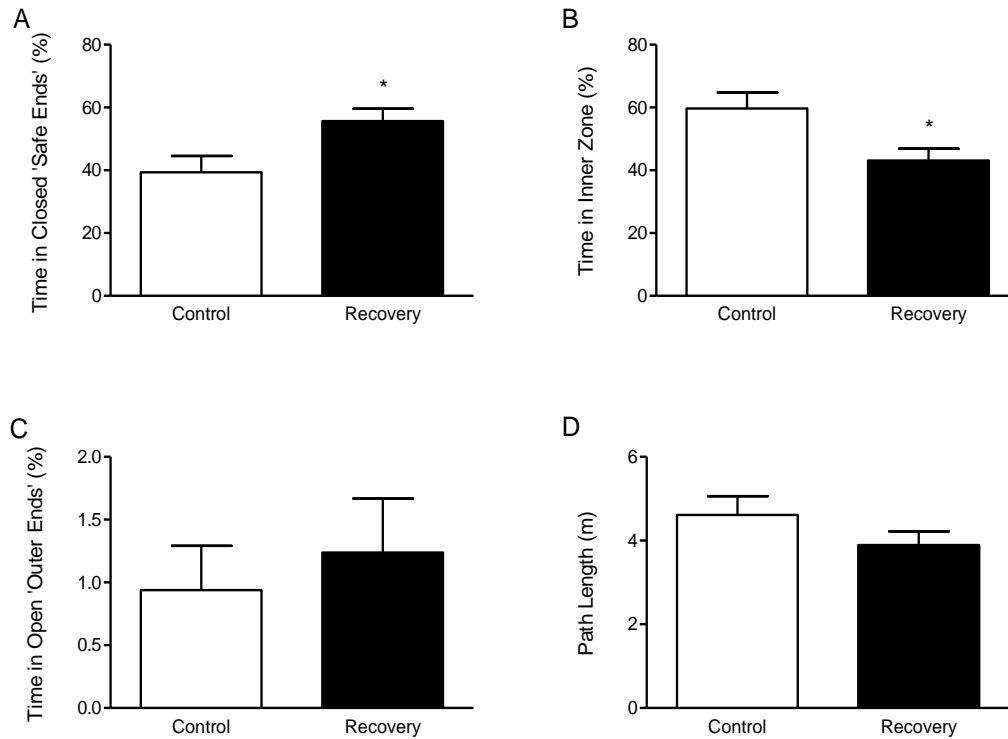


Figure 3.10 Performance in the elevated plus maze in recovery (day 6) from DSS-induced colitis in rats

Rats recovering from DSS-induced colitis showed an increase in the percentage of time spent in the safe ends of the closed arm (A) and decreased percentage of time spent in the inner zone (B) of the EPM. There is no increase in percentage of time spent in the outer ends of the open arm (C) and no difference in path length was observed (D). The data presented here comes from rats in DSS study 2.

Data are presented as mean \pm SEM ($n = 13 - 18$). * $p < 0.05$ relative to control (unpaired student's t -test).

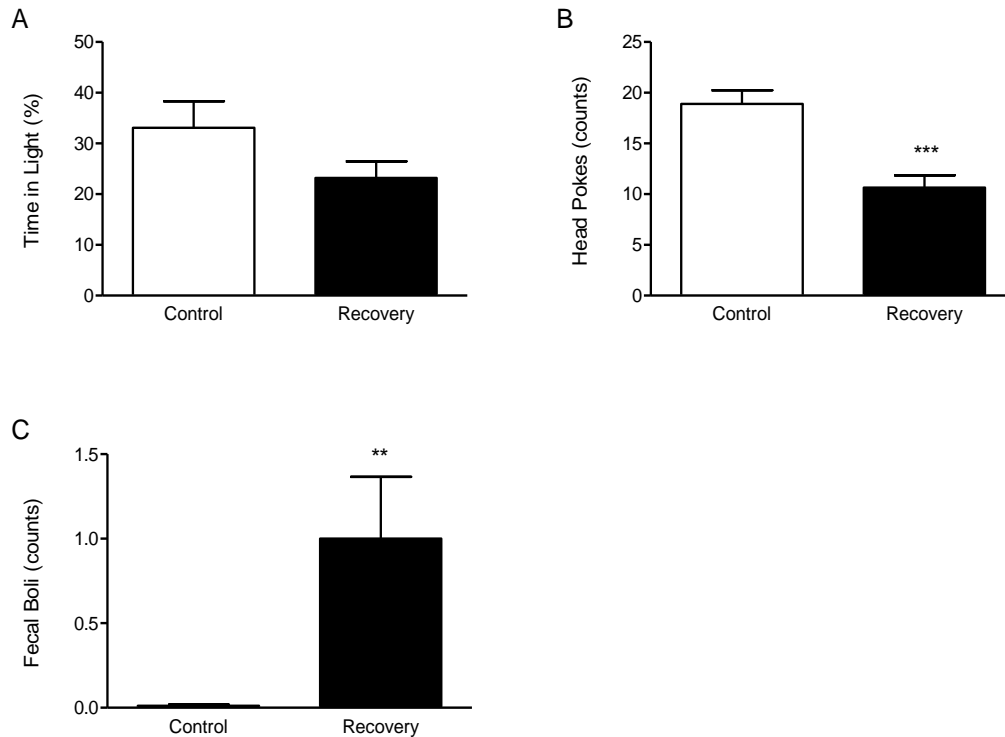


Figure 3.11 Performance in the light/dark box in recovery (day 7) from DSS-induced colitis in rats

Rats recovering from DSS-induced colitis showed no alterations in percentage of time spent in the light in the light/dark box (A), however they display a decrease in investigatory head pokes into the light side (B) and an increase in fecal boli deposited in the box (C). The data presented here comes from rats in DSS study 2.

Data are presented as mean \pm SEM ($n = 15 - 18$). *** $p < 0.001$, ** $p < 0.01$ relative to control (unpaired student's t -test).

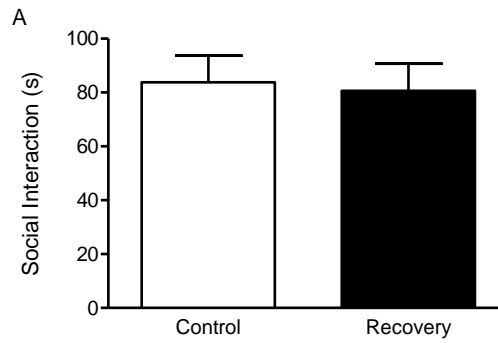


Figure 3.12 Performance in the social interaction test in recovery (day 8) from DSS-induced colitis in rats

Rats recovering from DSS colitis displayed similar levels of social interaction time to control rats (A). The data presented here comes from rats in DSS study 6.

Data are presented as mean \pm SEM ($n = 6$; where $n = 1$ pair). (unpaired student's t -test).

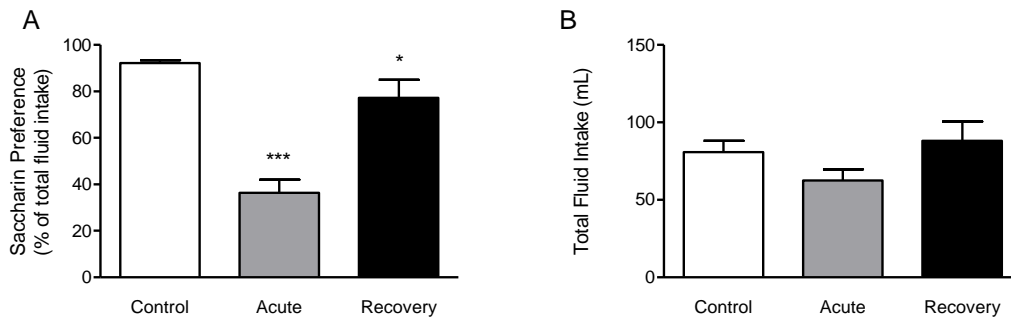


Figure 3.13 Saccharin preference test and total fluid intake in acute and recovery (day 8) DSS-induced colitis in rats

Saccharin preference was decreased in acute and recovered colitis compared to control (A) with no difference in fluid consumption (B). The data presented here comes from rats in DSS studies 5 & 6.

Data are presented as mean \pm SEM ($n = 13 - 20$). *** $p < 0.001$, * $p < 0.05$ relative to control (unpaired student's t -test).

3.3.3 Expression of inflammatory cytokines, iNOS, GFAP and CD11b in colon, spleen, liver and brain following DSS-induced colitis in Wistar rats

3.3.3.1 *Central and peripheral alterations in inflammatory markers*

Changes in the expression of pro-inflammatory cytokines in the bowel and brain during acute DSS-induced inflammation and following a seven day recovery period are summarised in Table 3.1. Following 7 days of DSS exposure there are increases in expression of colonic iNOS, TNF- α , IL-1 β , IL-6 and GFAP and in expression of cortical iNOS and IL-6 with decreases in cortical GFAP and CD11b. After 7 days of recovery from DSS-induced colitis increases in colonic expression of IL-1 β , IL-6 and IFN γ mRNA are observed. Cortical increases in iNOS and IL-6 mRNA also persist after 7 days recovery.

As iNOS was the most significantly increased inflammatory marker which is increased in brain and colon during colitis this was investigated in further detail (Figure 3.14). One-way ANOVA of iNOS expression in the periphery showed an effect of DSS: in the colon ($F_{2,11} = 24.42$; $p < 0.0001$) (A), spleen ($F_{2,15} = 29.10$; $p < 0.0001$) (B), and liver ($F_{2,14} = 10.36$; $p = 0.0017$) (C), and in the cortex ($F_{2,15} = 16.15$; $p = 0.0002$) (D), hippocampus ($F_{2,14} = 33.73$; $p < 0.0001$) (E), and hypothalamus ($F_{2,15} = 5.445$; $p = 0.0167$) (F). Newman Keuls post hoc test revealed an increase in iNOS expression in acute and recovered DSS-induced colitis in all regions relative to control ($p < 0.05$). Correlations were also revealed between cortical and colonic iNOS expression and all other regions (Table 3.2).

Table 3.1 Colonic and cortical gene expression following DSS-induced colitis in rats

		Colon		Cortex	
		Control	DSS	Control	DSS
Acute	iNOS	1 ± 0.5	420.2 ± 200.38*	1 ± 0.17	4.28 ± 0.91**
	TNF- α	1 ± 0.29	4.72 ± 1.96*	1 ± 0.07	1.16 ± 0.14
	IL-1 β	1 ± 0.28	27.64 ± 11.47*	1 ± 0.11	0.86 ± 0.18
	IL-6	1 ± 0.17	200.9 ± 86.18*	1 ± 0.09	2.01 ± 0.22**
	IFN γ	1 ± 0.02	1.66 ± 0.46	1 ± 0.17	1.27 ± 0.31
	GFAP	1 ± 0.12	6.18 ± 1.34***	1 ± 0.07	0.49 ± 0.03***
	CD11b	-	-	1 ± 0.02	0.77 ± 0.08*
Recovery	iNOS	1 ± 0.43	7.24 ± 0.77***	1 ± 0.9	3.69 ± 0.79**
	TNF- α	1 ± 0.31	2.37 ± 0.59	1 ± 0.11	0.95 ± 0.1
	IL-1 β	1 ± 0.26	6.25 ± 2.52*	1 ± 0.15	1.26 ± 0.14
	IL-6	1 ± 0.24	4.48 ± 1.64*	1 ± 0.09	1.44 ± 0.17*
	IFN γ	1 ± 0.34	4.09 ± 0.67**	1 ± 0.23	1.13 ± 0.33
	GFAP	1 ± 0.12	1.64 ± 0.55	1 ± 0.13	1.04 ± 0.08
	CD11b	-	-	1 ± 0.28	0.78 ± 0.06

Data representing fold changes in mRNA expression for colonic and cortical gene expression during acute DSS-induced inflammation and following seven days of recovery. The data presented here comes from rats in DSS study 3.

Data are presented as mean \pm SEM ($n = 4-6$). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ relative to control (unpaired student's t -test).

Cluster of differentiation molecule 11b (CD11b), dextran sulfate sodium (DSS), glial fibrillary acidic protein (GFAP), inducible nitric oxide synthase (iNOS), interferon (IFN), interleukin (IL), tumour necrosis factor (TNF).

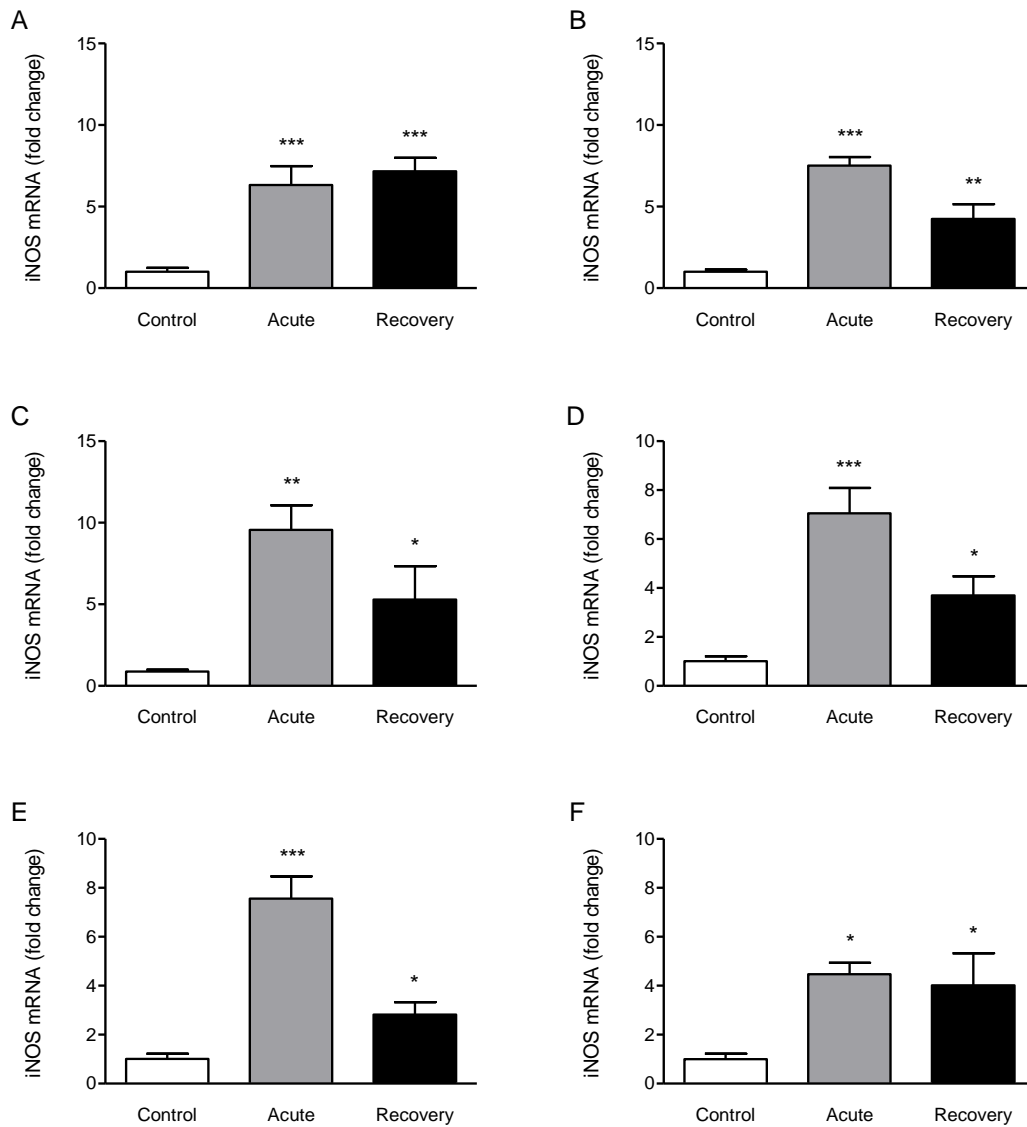


Figure 3.14 Peripheral and central iNOS expression following DSS-induced colitis in rats

iNOS expression was increased in the colon (A), spleen (B), liver (C), cortex (D), hippocampus (E) and hypothalamus (F) in acute and recovered DSS-induced colitis. The data presented here comes from rats in DSS study 3.

Data are presented as mean fold change in iNOS expression \pm SEM ($n = 4 - 6$). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ relative to control (one-way ANOVA with Newman-Keuls *post hoc* test).

Table 3.2 Correlation of peripheral and central iNOS mRNA expression following DSS-induced colitis in rats

	Colonic iNOS	Cortical iNOS
Colonic iNOS	-	0.65 *
Hepatic iNOS	0.74 **	0.89 ***
Splenic iNOS	0.62 *	0.92 ***
Cortical iNOS	0.65 *	-
Hippocampal iNOS	0.71 **	0.79 ***
Hypothalamic iNOS	0.66 **	0.81 ***

iNOS mRNA expression was correlated in colon and cortex to liver, spleen, hippocampus and hypothalamus. The data presented here comes from rats in DSS study 3. ($n = 14 - 18$) *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ (Spearman r correlation statistic).

3.3.4 TNBS-induced colitis in rats

The presence of extra-intestinal DSS has been previously reported (for review see Perse and Cerar (2012)). Kitajima et al. (1999) report increased histochemical detection of DSS in the liver, spleen, kidney and MLN. No DSS was detected in brain, lung, heart, or thymus samples. In order to confirm that the extra-intestinal inflammation and behavioural effects seen in the DSS model generalise to another IBD model and are not caused by DSS itself, a study was performed using the TNBS method of colitis induction. Rats were assigned to control or TNBS (6%) groups. All rats were fasted and were administered a laxative on day 0 of the study, and received an enema of 6% TNBS or ethanol (vehicle) on day 1. Controls were euthanised on day 8, and TNBS groups were euthanized on day 3, day 8, or day 21 post-enema.

3.3.4.1 *Body weight and measures of colonic inflammation following TNBS-induced colitis in rats*

Body weight is decreased on day 1 in all animals, likely due to fasting and administration of a laxative, however TNBS rats show continued weight loss in the following two days (Figure 3.15). Two-way repeated measures ANOVA of body weight change up to day 8 post-enema shows an effect of TNBS ($F_{1,16} = 22.88; p < 0.001$), day ($F_{7,112} = 33.59; p < 0.001$) and an interaction effect ($F_{7,112} = 9.545; p < 0.001$). A Bonferroni post hoc test revealed a decrease in body weight change at day 2 and 3 in TNBS-induced colitis ($p < 0.001$).

TNBS is associated with an up-regulation of inflammatory gene expression in the colon at 3 and 8 days post-enema which has recovered by day 21 post-enema (Figure 3.16). A one-way ANOVA revealed an effect of TNBS-induced colitis on IL-1 β ($F_{3,14} = 6.688; p = 0.0050$) (A), TNF- α ($F_{3,14} = 9.771; p = 0.0010$) (B), IL-6 ($F_{3,13} = 3.456; p = 0.0483$) (C), iNOS ($F_{3,14} = 6.957; p = 0.0043$) (D), IFN γ ($F_{3,11} = 4.807; p = 0.0224$) (E) and MMP9 ($F_{3,13} = 9.962; p = 0.0011$) (F) fold change mRNA expression. Newman-Keuls *post hoc* tests show an increase in IL-1 β , TNF- α and IFN γ mRNA in the colon at 8 days post-enema ($p < 0.01$) and increases in iNOS and MMP9 mRNA at day 3 and 8 post-enema ($p < 0.01$). However no effect on IL-6 mRNA was observed in the Newman-Keuls *post hoc* test.

3.3.4.2 *Behavioural changes in TNBS-colitis*

Open field testing was carried out 24 hours prior to euthanasia for all groups. TNBS-induced colitis is associated with alterations in exploratory behaviour in the open field at day 3 and 8 post-enema with changes in total rears being the only parameter to remain altered at 21 days

post-enema (Figure 3.17) compared to vehicle-treated controls. A one-way ANOVA revealed an effect of TNBS-induced colitis on path length ($F_{3,14} = 8.179$; $p = 0.0022$) (A), speed ($F_{3,14} = 10.26$; $p = 0.0008$) (B), percentage of time spent in the centre zone ($F_{3,13} = 8.824$; $p = 0.0019$) (C) and total number of rears ($F_{3,17} = 10.76$; $p = 0.0003$) (D). Newman-Keuls *post hoc* tests show a decrease in path length, speed and percentage of time spent in the centre zone at day 3 and 8 post-enema ($p < 0.01$) with decreases in number of rears at day 3, 8 and 21 post-enema. A Newman-Keuls *post hoc* test shows a decrease in path length at day 3 and 8 post-enema ($p < 0.01$).

Saccharin preference testing was carried out daily following TNBS-induced colitis and results are presented for controls at day 8 and TNBS groups at day 3, 8, and 21 post-enema (Figure 3.18). A one-way ANOVA revealed an effect of TNBS-induced colitis on saccharin preference ($F_{3,17} = 4.530$; $p = 0.0165$) (A) with no change in total fluid intake ($F_{3,15} = 1.002$; $p = 0.4189$) (B). A Newman-Keuls *post hoc* test showed a decrease in saccharin preference at day 3 and day 8 post-enema compared to controls ($p < 0.01$).

3.3.4.3 Evidence of peripheral and central inflammation in TNBS colitis

For comparison with findings in DSS-induced colitis, inflammatory mediator mRNA expression was measured in the cortex in TNBS colitis at 8 days post-enema (Figure 3.19). Increases were observed in IL-1 β ($t = 3.675$; $df = 7$; $p = 0.0079$) (A) and iNOS ($t = 3.406$; $df = 7$; $p = 0.0113$) (D) mRNA expression. IL-6 ($t = 2.323$; $df = 7$; $p = 0.0532$) (B) mRNA expression was also increased but did not reach significance. IFN γ ($t = 1.115$; $df = 7$; $p = 0.3016$) (C) mRNA expression was not altered in TNBS colitis. Correlations were also revealed between cortical and peripheral (colonic and splenic) iNOS mRNA expression (Table 3.3). However, the correlation with hepatic iNOS mRNA expression did not reach significance ($p = 0.0503$).

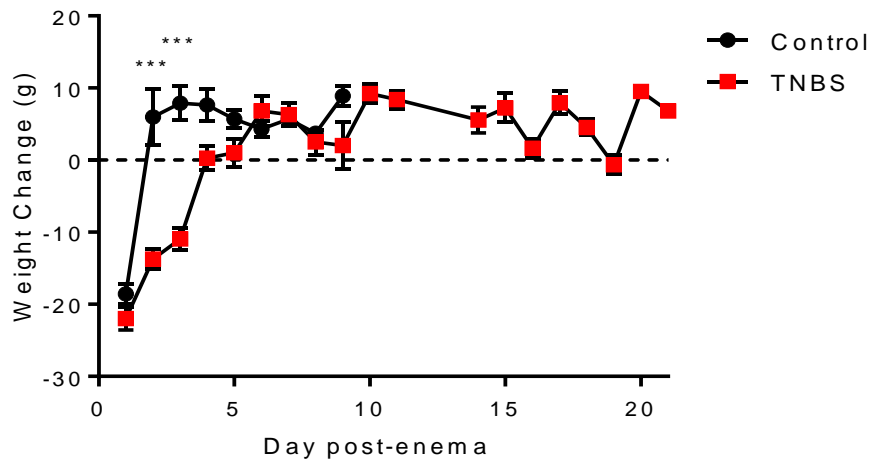


Figure 3.15 Body weight following TNBS-induced colitis in rats

Rats were weighed daily throughout the study. Rats were fasted on day 0 and a single enema of vehicle or TNBS (6%) was given on day 1. TNBS rats regain weight more slowly than controls. The data presented here is an amalgamation of data collected from rats in TNBS studies 1 & 2 at all timepoints post-enema.

Data are presented as mean \pm SEM ($n = 9$ control; 20 TNBS). *** $p < 0.001$ relative to control (two-way repeated measures ANOVA with Bonferroni *post hoc* test on data up to 8 days post-enema; $n = 9$).

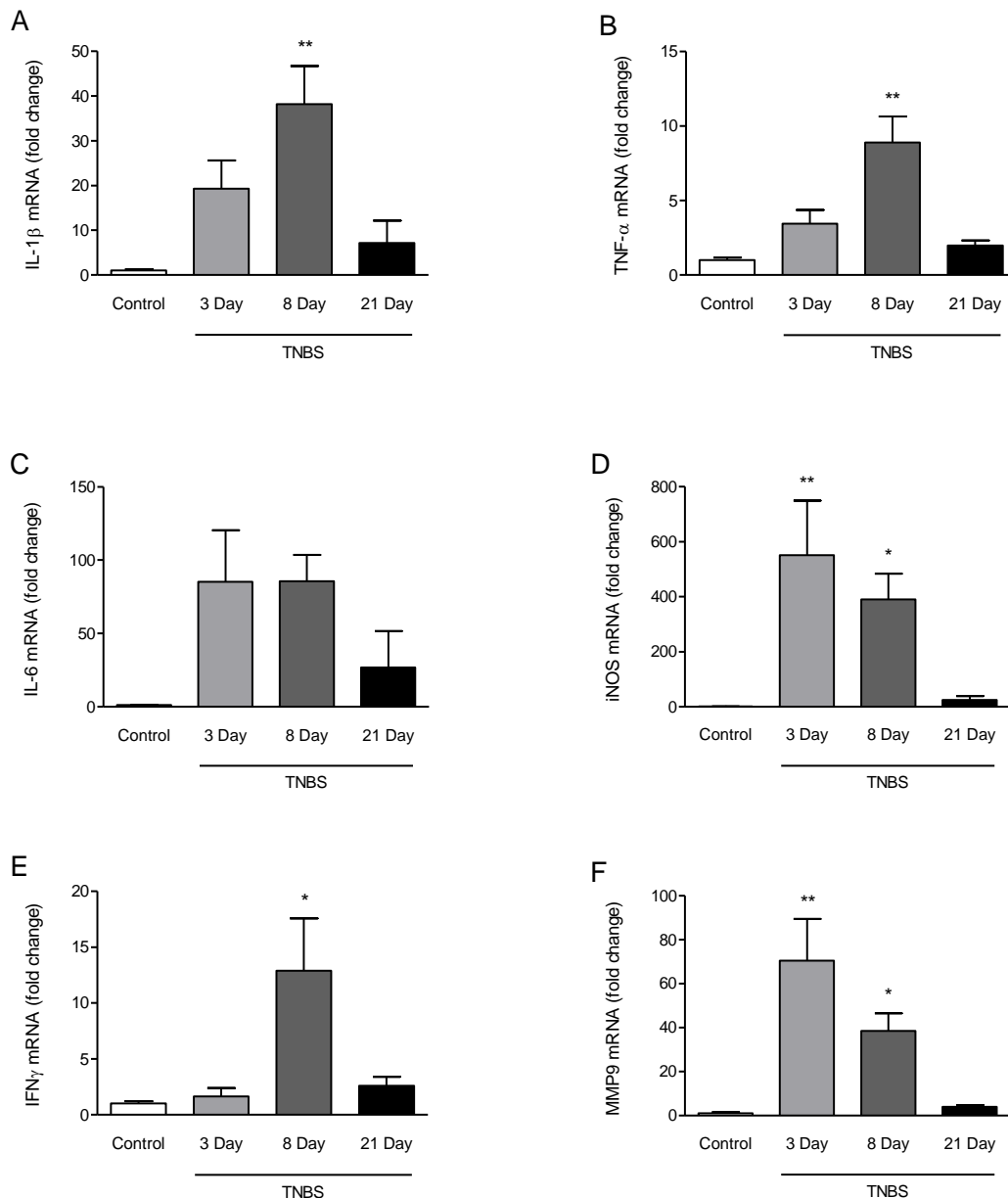


Figure 3.16 Expression of inflammatory cytokines, iNOS and MMP9 in the colon following TNBS-induced colitis in rats

Rats were assessed for inflammatory mediator and MMP mRNA expression at day 3, 8 and 21 post-enema. Increases were observed in IL-1 β (A), TNF- α (B) and IFN γ (E) mRNA expression at 8 days post-enema, and in iNOS (D) and MMP9 (F) mRNA expression at 3 and 8 days post-enema. No alterations to IL-6 (C) mRNA expression were recorded at any timepoint. The data presented here comes from rats in TNBS study 1.

Data are presented as mean \pm SEM ($n=4-5$). ** $p < 0.01$, * $p < 0.05$ relative to control (one-way ANOVA with Newman-Keuls *post hoc* test).

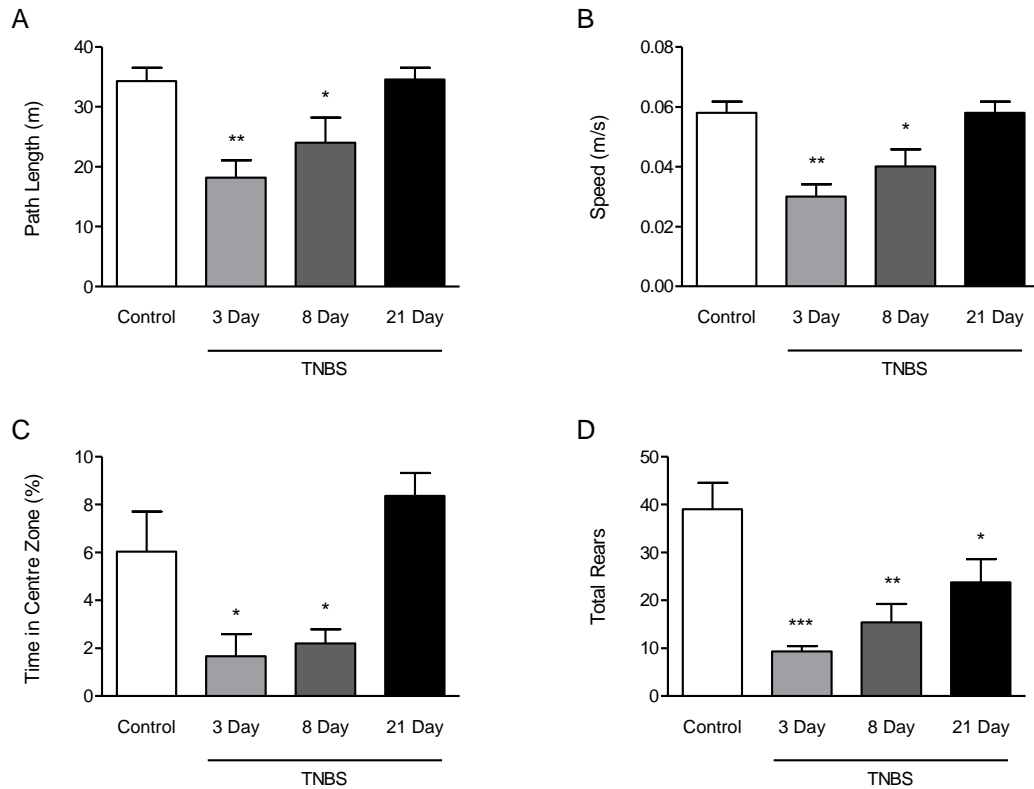


Figure 3.17 Performance in the open field test following TNBS-induced colitis in rats

TNBS-induced colitis decreased path length (A), speed (B), percentage of time spent in the centre zone (C) and total number of rears (D) in the open field on days 3 and 8 post-enema. Total rears remained decreased up to 21 days post-enema compared to control. Controls were assessed in the open field on day 8 post-enema. The data presented here comes from rats in TNBS study 1.

Data are presented as mean \pm SEM ($n=4-6$). *** $p < 0.001$; ** $p < 0.01$, * $p < 0.05$ relative to control (one-way ANOVA with Newman-Keuls *post hoc* test).

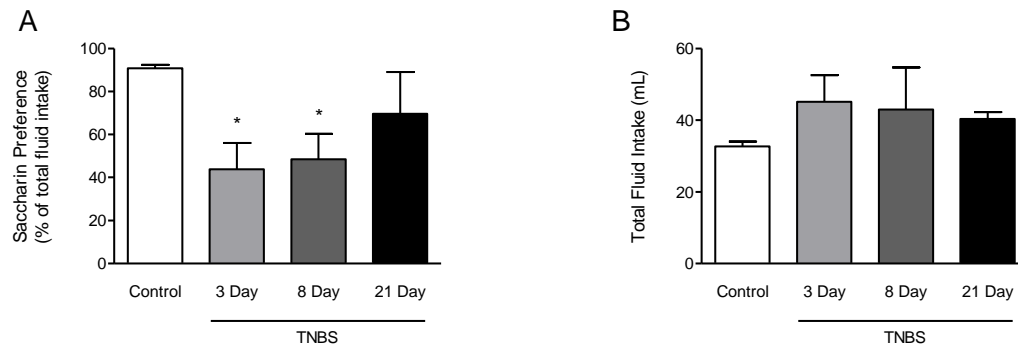


Figure 3.18 Saccharin preference following TNBS-induced colitis in rats

Rats were assessed for saccharin preference throughout the study. Data is presented from day 3, 8 and 21 post-enema for TNBS rats and from day 8 post-enema for control rats. Rats show a decreased preference for saccharin at day 3 and day 8 post-enema (A) without alterations to total fluid intake (B). The data presented here comes from rats in TNBS study 1.

Data are presented as mean \pm SEM ($n=4-7$). * $p < 0.05$ relative to control (one-way ANOVA with Newman-Keuls *post hoc* test).

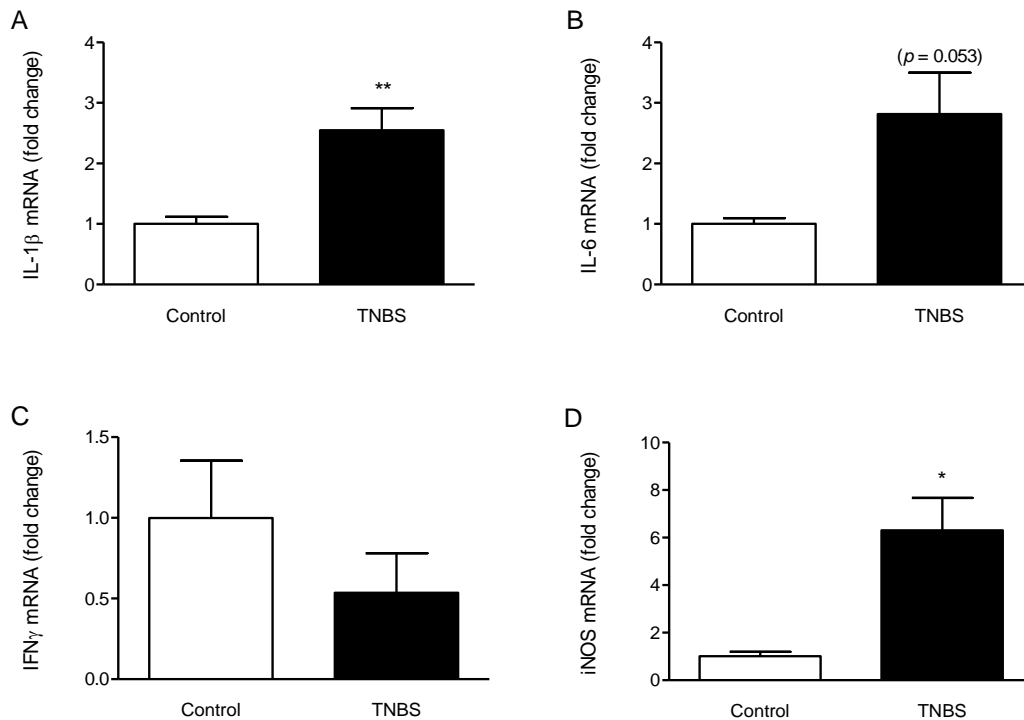


Figure 3.19 Cortical expression of inflammatory cytokines and iNOS following TNBS-induced colitis in rats

Cortical inflammatory mediator mRNA expression was assessed at 8 days post-enema. IL-1 β (A) and iNOS (D) mRNA was increased at this timepoint. IL-6 (B) mRNA expression was also increased though not significantly ($p = 0.053$). No alterations to IFN γ (C) mRNA expression were recorded. The data presented here comes from rats in TNBS study 2.

Data are presented as mean \pm SEM ($n = 4-5$). ** $p < 0.01$, * $p < 0.05$ relative to control (unpaired student's t test)

Table 3.3 Correlation of peripheral and central iNOS mRNA expression following TNBS-induced colitis in rats

	Cortical iNOS
Colonic iNOS	0.80*
Hepatic iNOS	0.68 ($p = 0.0503$)
Splenic iNOS	0.80*

Cortical iNOS mRNA was correlated to colon and spleen. The data presented here comes from rats in TNBS study 2. Data are presented as mean \pm SEM ($n = 4-5$). (Spearman r correlation).

3.4 Discussion

The results of this chapter demonstrate that DSS induces acute colonic inflammation in rats with symptoms comparable to those observed in human UC. Following 6-7 days of DSS intake rats have decreased body weight and food intake and manifest diarrhoea and rectal bleeding. The colons of these rats show damage to crypts and epithelial cells with immune cell infiltration. There is a pro-inflammatory cytokine profile in the colon relative to control with increased IL-1 β , TNF- α , IL-6, iNOS, GFAP and MMP mRNA expression and activity. Following 7 days recovery increases in IL-1 β , IL-6, and iNOS persist and IFN γ becomes increased at this timepoint. Open field test indicates the presence of sickness behaviour in acute colitis which recovers 5 days following cessation of DSS administration. Other behavioural tests carried out during recovery indicate anxiety, anhedonia and depressive-like behaviours. A pro-inflammatory profile is also observed in the cortex with acutely colitic rats showing increased iNOS and IL-6 and recovered rats showing increases in iNOS and IL-6. Decreased cortical GFAP and CD11b [astrocytic and microglial markers respectively] are observed, indicating potential glial dysfunction. iNOS mRNA is increased in colon, liver, spleen, cortex, hippocampus, and hypothalamus in acute colitis and recovery. Correlations exist between colonic and cortical iNOS and iNOS in all other regions. Comparable results in the TNBS-induced colitis model indicate that these symptoms generalise to colitis.

3.4.1 Characterisation of the DSS model of colitis in rats

Okayasu et al. (1990) were the first to describe the DSS-induced colitis model, in mice reporting that administration of a 5% solution of DSS via the drinking water leads to development of acute and chronic colitis, involving symptoms of loose stools and diarrhoea, rectal bleeding, and weight loss. Other studies have reported similar symptoms with increased duration of DSS exposure leading to more severe symptoms. Kullmann et al. (2001) confirmed these findings in rats exposed to 5% DSS solution demonstrating similar symptoms in acute and chronic bouts of DSS exposure.

As well as colitis symptoms, DSS administration was also associated with decreased food intake giving rise to decreased weight gain. Anorectic symptoms are a common finding both in experimental animal models of IBD, and in IBD patients (Ballinger et al., 1998, El-Haj et al., 2002, Karmiris et al., 2008, Guariso et al., 2010). Unexpectedly, a decrease in body weight is

observed following 1 day of DSS administration. The reason for this is unknown, however it could be related to the non-significant decrease in fluid intake also observed at this timepoint which may represent neophobia associated with the taste of DSS. Although not statistically significant, the observable increase in fluid intake once rats are returned to water following DSS is likely a response related to rehydration.

Upon *post-mortem* histological examination of the distal colon, crypt damage and epithelial damage leading to immune cell infiltration was observed following acute DSS-induced colitis. This ultrastructural damage, is in agreement with previous studies reporting histological damage in DSS-induced colitis (Gaudio et al., 1999). Gaudio et al. (1999) conclude that the ultrastructural damage and the location of gastrointestinal inflammation in DSS-induced colitis more closely resemble UC rather than CD.

MMP2 and MMP9 are peptidases which can be used as markers of epithelial damage and have previously been shown to be increased in DSS-induced colitis and in IBD patients (Medina et al., 2001, Medina et al., 2003, Medina and Radomski, 2006, Santana et al., 2006). MMP2 and MMP9 are classified as gelatinases meaning that they are capable of breaking down gelatinase and collagen. These MMPs can degrade the ECM of the intestinal wall potentially rendering the bowel leaky. A compromised intestinal wall greatly increases the risk of bacterial translocation or movement of GI toxins into the circulation, thereby increasing susceptibility to systemic inflammation. MMP2 and MMP9 mRNA expression and activity were shown to be increased in the colon following acute DSS-induced colitis. Considering that MMP9 in particular can potentially cleave ECM proteins of the BBB it would be of interest in future to measure gelatinase expression and activity in the cortex.

We have previously reported an increased pro-inflammatory cytokine profile in colonic biopsies of IBD patients (Abautret-Daly et al., 2017b). In this study, the expression of the pro-inflammatory cytokines IL-1 β , TNF- α , IL-6, IFN γ and the inflammatory marker iNOS were found to be increased in the colon ($p < 0.05$). 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 no induction of TNF- α . In this study, samples of distal colon from animals with acute DSS-induced colitis were examined for changes in the expression of pro-inflammatory markers and increased IL-1 β , TNF- α , IL-6, iNOS and GFAP were observed. In the colon, GFAP is expressed by

enteric glial cells which share morphological and functional characteristics with astrocytes (Yu and Li, 2014). In terms of inflammation, enteric glial cells are recognised as immune cells as they express MHC class I and II and TLRs and can release and respond to a wide range of cytokines and chemokines (Ochoa-Cortes et al., 2016). In contrast to human IBD, 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.

Overall, these results demonstrate that seven days of DSS (5%) exposure induces colitic symptoms *in vivo* and evidence of structural damage and inflammatory activation similar to that experienced during moderate to severe IBD in humans. It is therefore a suitable experimental protocol for the assessment of behavioural and central nervous system responses to acute intestinal inflammation.

3.4.2 Behavioural disturbances following DSS-induced colitis

DSS (5%) was chosen as the model of colitis induction in these experiments as the induction process simply involves administration via home cage drinking water. This renders the induction process relatively stress-free for animals. For this reason it was chosen as the most appropriate model for studying behavioural changes. In these behavioural studies, DSS is administered over a period of seven days or until a pre-determined standard level of colitis severity is observed (see Figure 2.17 for timeline of behavioural experiments).

At the acute inflammatory stage (day 6 of DSS administration), colitic rats expressed both locomotor and exploratory deficits in the open field. This can likely be attributed to sickness-associated behaviour and is consistent with sickness behaviour previously reported in LPS and poly I:C studies (Dantzer et al., 2011, Gibney et al., 2013). 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.

Further characterisation of behavioural abnormalities during the recovery phase of colitis was carried out once food intake had returned to control levels and rats were regaining weight. On recovery day 4 and 5, locomotor and exploratory behaviour was assessed in the open field in

the same cohort and a separate cohort of rats respectively. Previous differences in speed, path length, rearing and exploration of the centre zone had returned to control levels.

On day 5 of recovery, rats were tested for marble burying behaviour. Marble burying is a commonly used behavioural test in mice, often used to assess anxiety-like behaviour, whereby increased burying is suggestive of increased anxiety, and anxiolytics have been shown to reduce burying behaviour (Deacon, 2006). In this study a modified version of the marble burying test for rats was carried out. Rats exposed to DSS colitis had a decreased tendency to bury marbles despite their locomotor/exploratory behaviour in the open field having returned to normal at this timepoint. Marble burying is a compulsive behaviour which occurs spontaneously in rodents (Lazic, 2015). In contrast to the expected anxiety-like response of heightened awareness and arousal, the response generated is more indicative of a depressive symptomatology including lack of motivation, withdrawal and fatigue. 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: in studies using chronic tryptophan-depleted mice, in tryptophan hydroxylase knockout mice with reduced serotonin levels and in mice with hippocampal lesions (Deacon and Rawlins, 2005, Browne et al., 2012, Mosienko et al., 2012). 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 marble burying activity. The association between decreased marble burying and limitations to the serotonergic system are interesting and may provide a biological link between altered function in the CNS and depressive-like behaviour.

A separate cohort of rats underwent FST on day 6 of recovery and increased immobility was observed in rats exposed to DSS relative to controls. In the 15 minute pre-swim, 24 hours previous to the FST, DSS-exposed rats showed lower immobility compared to controls for the first two 5-minute intervals which firstly shows that their locomotive abilities are not responsible for increased immobility in the FST and also strongly indicates that the immobility observed the following day is due to behavioural adaptation or despair. Results from the FST share similarity with work by Chen et al. (2015) who found that rats recovering from DSS displayed a higher immobility time in the FST compared with a control group. This has also been reported in TNBS mice coincident with an increase in TNF- α and iNOS mRNA, and nitrite levels in the hippocampus (Heydarpour et al., 2016). It is worth noting that selective iNOS

inhibition using an i.p. injection of aminoguanidine caused immobility time in the FST to return to normal in the TNBS-exposed mice and also attenuated the increase seen in hippocampal TNF- α , iNOS and nitrite. Aminoguanidine administration did not affect inflammation in the colon. Overall these results suggest that iNOS is involved in behavioural despair in the FST.

On recovery day 6, rats were exposed to the EPM. Both control and DSS-exposed 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 (data not shown). However with further subdivision of the maze allowing the end corners of the closed arms (25% of each arm) to be analysed, a difference in the time spent in these zones by DSS-exposed rats versus controls was observed. DSS recovery rats spent over 10% more time in the corners of the closed arms. Decreased time spent in the open arms and decreased number of open arm entries have previously been reported in the EPM in DSS-colitis rats, however the timing at which this test was performed post-DSS administration is unclear (Chen et al., 2015).

On recovery day 7, behaviour in the light/dark box was assessed. No difference in time spent in the light chamber was seen, however the DSS rats had fewer head pokes into the light chamber, possibly indicative of decreased interest in the novelty of the light chamber (Marino et al., 2005). This would be in agreement with the suspected apathy observed in the marble burying test. An increase in the number of fecal boli in the DSS group was seen despite normal fecal boli counts being recorded in the open field on the previous day. Increased boli count is usually indicative of increased anxiety, however due to the gastrointestinal nature of the model, it is difficult to draw any conclusions relating to behaviour based on fecal boli counts (Ennaceur et al., 2006).

Chen et al. (2015) also performed a social interaction test during the recovery period from DSS-induced colitis, however, in contrast to the findings presented in this investigation, which show no change in social interaction between groups, they report an increase in social contact time with a decreased number of contacts in DSS-exposed rats compared to controls. This may be attributed to the fact that in this study rats were paired with unfamiliar rats, whereas Chen et al. (2015) paired familiar cagemates together for this task. Familiar cagemates may be

more likely to spend an increased amount of time in contact as a comfort-seeking mechanism. Hassan et al. (2014) tested mice in the social interaction test using a novel mouse and showed a decrease in social interaction in DSS-induced colitis mice compared to controls. These mice were tested 2 days following cessation of DSS exposure (2%) therefore it is possible that they were still suffering from symptoms of colitis.

A 24 hour saccharin preference test was carried out as a measure of anhedonia at 1 day and 8 days post-DSS exposure in order to test acutely colitic and recovered rats respectively. To avoid confounding effects of habituation, separate groups of rats were used. Results showed a decreased preference for saccharin indicative of anhedonia in both acutely colitic rats and rats recovered from colitis. Chen et al. (2015) carried out a sucrose preference test in recovered DSS rats and found a decrease in sucrose preference compared to control, although the time post-DSS exposure at which this test was carried out is unclear. A major caveat when using saccharin/sucrose preference tests to measure anhedonia in the DSS model is potential neophobia to novel tastes following DSS-induced sickness, compounded by the likelihood that DSS itself is a sweet-tasting solution. Taste is strongly associated with interoceptive illness (Garcia and Koelling, 1967); a series of experiments from the 1970s demonstrate that lithium chloride sickness in rats raised on water will suppress intake of novel saccharin solution, but not water, while the animals are still experiencing symptoms (Domjan, 1976, Domjan et al., 1977) (for review see (Reilly, 2018)). Neophobia as a result of illness must be considered for the observed decrease in saccharin preference in acutely colitic animals though it should be less of an influence at 8 days post-DSS when symptoms of colitis are gone. Replication of this finding in the TNBS model indicates that colitis and not the taste of DSS is responsible for decreased saccharin preference.

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 behavioural differences persist throughout recovery when colitic symptoms and feeding, weight gain and locomotor measures have returned to normal.

3.4.3 Evidence for neuroinflammation during acute DSS-induced colitis

As changes in behaviour are centrally mediated, it was of interest to determine if there were any related changes in the expression of inflammatory markers centrally. DSS induces

symptoms in rats similar to human UC patients in association with behavioural alterations. Previous literature has shown that induction of peripheral cytokines can lead to central cytokine changes and 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, Harden et al., 2008). In this investigation, cortical samples were examined for IL-1 β , TNF- α , IL-6, IFN γ , and iNOS mRNA expression. No differences in IL-1 β , TNF- α or IFN γ were found in the cortex, however increases in IL-6 and iNOS mRNA expression were observed. Reductions in expression of the astrocytic marker GFAP and the microglial marker CD11b were observed following DSS exposure. 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) and in the Wistar-Kyoto (WKY) animal model of depression (Gormley et al., 2016). The decrease in the microglial marker CD11b is also interesting as microglia would be expected to increase in response to inflammation. However, CD11b is associated with the M1 microglial phenotype, therefore it is possible that microglia are polarising to the immunoprotective M2 phenotype.

3.4.4 Evidence for persistent inflammation during recovery from DSS-induced colitis

In consideration of the fact that behavioural differences are observed up to 8 days following acute DSS-induced colitis, the cytokine profile of DSS-treated rats was assessed following seven days recovery. This is to determine whether the observed increases in pro-inflammatory mediators at the acute timepoint persist into recovery. For consistency, the cytokines IL-1 β , TNF- α , IL-6, IFN γ , and iNOS were examined in the colon and frontal cortex. Following seven days of recovery, the expression of inflammatory cytokines IL-1 β and IL-6 and iNOS remained increased in the colon. Persistent increases in cortical iNOS and IL-6 expression were also evident at this timepoint. However, the decreased GFAP and CD11b expression found during the acute phase of DSS-induced colitis had returned to control levels. In contrast to the acute colitis timepoint, IFN γ mRNA expression was increased in the colon 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 production of IFN γ in organ cultures from colons of animals 14 days after cessation of DSS exposure.

3.4.5 Generalisation of gut-brain inflammation in the TNBS-induced colitis rat model

Although the TNBS model of colitis involves fasting and anaesthesia as potential confounds, particularly in terms of stress-induced changes, this model was assessed to determine if the findings observed in the DSS-colitis model generalise to colitis and not to DSS exposure itself.

Body weight, as well as clinical symptoms of colitis were measured daily as an indication of disease activity in each rat. TNBS rats showed symptoms of intestinal inflammation including diarrhoea and loose stools while control rats displayed no symptoms. All rats were fasted and a laxative was administered on day 0 of the study to facilitate the TNBS/vehicle enema on day 1. Therefore all rats display decreased body weight on day 1 of the study, however, TNBS rats displayed continued weight loss for the following 2 days whereas control rats began regaining weight on day 2. These observations have been reported previously in this model of colitis (Kresse et al., 2001).

Open field behaviour in TNBS rats on day 3 and day 8 post-enema show similar alterations to DSS-exposed rats in terms of decreased path length, speed, percentage of time spent in the centre of the arena and total number of rears relative to control. Rats exposed to TNBS-induced colitis also show a decreased preference for saccharin in the saccharin preference test on day 3 and day 8 post-enema with no concurrent alterations in total fluid intake. Similar to the results observed in DSS-induced colitis, this decreased preference for saccharin is an indicator of anhedonic behaviour. Unlike DSS, TNBS is not taken orally therefore the potential confounding effect of a sweet-tasting DSS solution influencing saccharin intake does not apply in this model. It can be proposed that the effects of colitis itself are responsible for the decrease in saccharin preference observed.

TNBS-induced colitis also resulted in an increase in the expression of inflammatory mediators in the colon. This was again observed on day 3 and in particular on day 8 post-enema with

increases in IL-1 β , TNF- α , iNOS, IFN γ , and MMP9 evident compared to controls. No increase was observed in IL-6. These results are in agreement with a previous study by Alex et al. (2009) who reported similar increases in pro-inflammatory markers at the mRNA level in TNBS induced colitis. It is worth noting the previous reports have shown TNBS induces peak acute inflammation as well as both macroscopic and microscopic histological alterations in the colon approximately one week following administration (Menozzi et al., 2006).

In a separate cohort, mRNA expression of inflammatory mediators was assessed in the cortex of control and TNBS rats on day 8 post-enema. IL-1 β and iNOS mRNA were increased at this timepoint while IL-6 was increased though did not reach significance ($p = 0.0532$) when compared to controls. IFN γ was not increased at this time. This is in agreement with other studies measuring inflammatory gene expression in the TNBS model (Alhouayek et al., 2011, Baticic et al., 2011). For comparison with findings in the DSS-induced colitis model, iNOS mRNA was measured peripherally in the colon, liver and spleen and was found to correlate with cortical iNOS mRNA in colon and spleen though the correlation did not reach significance in the liver ($p = 0.0503$).

Overall, compared to the DSS model, the TNBS model induces colitis symptoms including weight loss, and colonic expression of inflammatory cytokines, iNOS and MMP9. IFN γ is increased in TNBS but not DSS colitis. Similar deficits are seen in both models in the open field test during active colitis in terms of locomotor ability and time spent in the centre zone. Saccharin preference is similarly decreased in both models, indicating anhedonia as a result of colitis. In the brain, both the DSS and TNBS models of colitis have a similar profile of persistent inflammation in terms of iNOS, IL-1 β and IL-6, and both models also show a correlation in iNOS expression between the brain and periphery.

3.4.6 Conclusion

DSS induces symptoms of colitis in Wistar rats similar to human UC. Intestinal damage occurs and inflammatory mediators are increased in the colon. Some inflammatory mediators are still up-regulated 7 days into the recovery period. These inflammatory increases are also observed in the brains of colitic and recovered animals. Acutely colitic rats display sickness-related behaviours while recovered animals also show behavioural alterations despite a lack of symptoms and a return of normal weight gain, food intake, and normal exploratory and

locomotor behaviour. These results are broadly replicated in the TNBS-induced colitis model, demonstrating that they are likely caused by the effects of intestinal inflammation and not occurring as a result of DSS exposure itself.

Chapter 4

DSS-Induced Colitis: Central

Inflammation

Assessment of iNOS, 3-NT, microglia, peripheral macrophage and astrocyte immunoreactivity in the brain

4.1 Introduction

As discussed in Chapter 1 (Section 1.2), there is a well-documented increase in the risk of psychological disturbances, such as depression or anxiety, in patients with IBD. Although the reason for this remains unclear, increased disease activity has been associated with increased psychological disturbances (Abautret-Daly et al., 2017b, Ben Thabet et al., 2012, Panara et al., 2014, Gandhi et al., 2014). Furthermore, immunomodulation therapy leads to a reduction in disease activity scores and psychological symptom scores in IBD patients (Calvet et al., 2006, Horst et al., 2015). During active IBD the protective intestinal barrier can become compromised, which can lead to increased intestinal permeability. This can in turn induce a local inflammatory response and/or systemic immune activation. It is proposed that increased gut epithelial permeability due to chronic inflammation and the close inter-relationship between the GI tract, immune system and the nervous system could lead to inflammatory mediators from the inflamed gut entering the brain via the circulation and inducing neuroinflammation (de Theije et al., 2011).

Microglia are the predominant immune cells in the brain, considered akin to other tissue-resident macrophages like liver Kupffer cells, being the only cells of the CNS with hematopoietic origins (Hickey and Kimura, 1988). At resting or native state, microglia are highly ramified in shape with many processes possessing pathogen recognition receptors (PRR) to allow them to sample the environment and sense any changes indicative of neuronal injury or signs of infection. Microglia respond to tissue damage or infection much like macrophages of the periphery; they express TLR receptors and respond to TLR ligands, producing cytokines to activate and recruit other microglia, and phagocytosing debris. In response to a stimulus, microglia are rapidly activated entering activation state M1 or M2 depending on the nature of the stimulus (Crain et al., 2013, Franco and Fernández-Suárez, 2015). *In vitro* stimulation with LPS induces the M1 activation state in which microglia secrete pro-inflammatory cytokines such as IL-1 β and TNF- α , produce iNOS and ROS and are primed for phagocytosis or proteolysis (Chhor et al., 2013). M2 activated microglia, activated by IL-4 or IL-10, are primed toward a tissue repair role, secreting chemokines and ECM proteins (Cherry et al., 2014). The activated phenotype, which promotes a further activation of the immune system in order to eliminate the threat and encourage tissue repair, is usually beneficial and self-limiting, resolving itself once the infection has been eliminated or the

tissue repaired. Sustained microglial activation promoting inflammation can however lead to tissue pathology.

Of interest in terms of IBD, is the fact that microglia may become activated in response to peripheral infections, even without compromising the BBB, as increased permeability at CVOs facilitates infiltration of cytokines or antigens, and microglial detection of PAMPs. In this instance it is thought that microglia enter the M1 activation state as animal studies have shown that microglial cells in the parenchyma respond to peripheral (i.p. and i.v.) LPS administration by production of IL-1 β in choroid plexus, CVOs, cortex and hypothalamus (Li et al., 2014, van Dam et al., 1995). The evidence shows that acute LPS administration is not directly damaging to neurons, however chronic inflammatory stimulation of microglia by LPS *in vitro* leads to oligodendrocyte cell death, the effects of which are heightened if the microglia are in a mixed glial preparation with astrocytes. IL-1 β and TNF- α produced by microglia are thought to activate astrocytes which then contribute to the neurotoxic inflammatory response by production of NO and ROS (Saijo et al., 2009).

In humans, it is known that mood disorders can arise following perturbations to normal brain function, and in particular it has been observed that stress can precipitate depression in IBD (Goodhand et al., 2012b). In various animal models of chronic stress including restraint stress, maternal separation, and CUMS, the classic depressive symptoms are accompanied by an increase in iNOS expression in many brain regions including PVN and hippocampus (Yamaguchi et al., 2010, Khovryakov et al., 2010). The psychosocial stress associated with IBD may also have an impact on microglia, as pre-clinical studies have shown that both acute and chronic stress cause microglia to multiply and adopt an activated state, referred to as priming (Tynan et al., 2010, Frank et al., 2007b), which is a liability factor in the development of psychiatric disorders (Frank et al., 2016). Chronic stress studies have also recently shown that stress-induced microglial activation in mice leads to the recruitment of bone marrow-derived microglia to infiltrate the PVN (Ataka et al., 2013).

The activation of microglia and polarization to the M1 activation state has been associated with the development of depressive symptoms (Hinwood et al., 2012, Wohleb et al., 2013, Kreisel et al., 2014). In support of this, the microglial inhibitor minocycline has been shown to have therapeutic effects on working memory deficit and depressive behaviours in rodent stress models (Kreisel et al., 2014, Hinwood et al., 2012). Little is known regarding microglial

activation in terms of DSS-induced colitis, however, as previously mentioned, a study by Riazi et al. (2008) investigated microglial activation in TNBS-induced colitis in the brains of rats. They found an increase in microglial activation in the hippocampus at 4 days post-enema which returned to baseline by 10 days post-enema. This study was recently repeated using minocycline, which demonstrated that changes to LTP and LTD in the hippocampus during TNBS-induced neuroinflammation are mediated by microglia (Riazi et al., 2015). This group postulate that microglial-mediated synaptic alterations may be the cause of behavioural comorbidities seen in patients with peripheral inflammation.

It has been reported that peripheral blood inflammatory cells (neutrophils and monocytes) may infiltrate the CNS and contribute to neuroinflammation (Ji et al., 2007). The antibody IBA1 may be used to distinguish neutrophils from monocytes and microglia as, unlike monocytes and microglia, neutrophils do not express IBA1, and furthermore, unlike monocytes and microglia, neutrophils tend to be polymorphonuclear (Jeong et al., 2013). In order to distinguish monocytes from microglia in terms of morphology, monocytes are observed as round cells whereas microglia have short or long processes. In addition, the marker CD45 may be used to distinguish between these cells as it is more highly expressed in monocytes than microglia (Jeong et al., 2013, Shah et al., 1988).

Astrocytes are another major glial cell in the brain which serve various functions including structural support of neurons, vasodilation, and regulation of synaptic concentrations of neurotransmitters (Kimelberg and Nedergaard, 2010). As mentioned above, these cells may also play a role in inflammation as they may be activated by microglia, being both a target and a source of pro-inflammatory cytokines and other inflammatory mediators including iNOS (Rajkowska and Stockmeier, 2013, Askalan et al., 2006). The typical hallmarks for astrocyte reactivity also called astrogliosis are increased astrocyte proliferation/density, astrocyte hypertrophy and increased GFAP expression (Formichella et al., 2014). The link between astrocytes and psychological disorders stems from the finding that there is a decrease in astrocytic activity and/or astrocytic number in regions of the brain (such as the anterior cingulate gyrus) in *post-mortem* studies of depressed patients (Rajkowska and Miguel-Hidalgo, 2007, Rajkowska and Stockmeier, 2013, Gosselin et al., 2009), and in animal models of depression (Li et al. 2013). Glial dysfunction achieved via administration of an astrocyte-

specific toxin such as L-AAA has been shown to induce anxiety- and depression-like behaviours in rodents (Banasr and Duman, 2008, Gormley et al., 2016).

Previous data from Chapter 3 regarding inflammatory markers in DSS-induced colitis (Section 3.3.3), showed increases in iNOS mRNA in the brain which were sustained through the recovery period. As introduced in Chapter 1 (Section 1.3.9), iNOS is produced following stimulation by endotoxins or cytokines and generates a large amount of NO as an innate immune mechanism to prevent the growth of invading micro-organisms. Although it was originally characterised in macrophages, iNOS is present in numerous cell types in the brain, including endothelial cells, neurons, microglia and astrocytes. Over-expression of iNOS is a common feature of chronic inflammatory disorders including IBD. However, excessive levels of NO can be toxic to neurons. The deleterious effect of iNOS on host cells is NO-mediated tissue damage and cell death achieved through mechanisms including inhibition of mitochondrial complex I and II, lipid peroxidation, DNA fragmentation, tyrosine nitrosylation and by combining with superoxide ions to form cytotoxic peroxynitrite (ONOO⁻).

Immunologically, NO has many different functions (Figure 4.1). As previously mentioned, iNOS-produced NO is involved in the inflammatory response during infection and NO produced by iNOS can activate cGMP-dependent and independent pathways, but its main contribution to the immune response is seen through the formation of RNS. RNS are produced when iNOS-induced NO combines with superoxide produced by NADPH-oxidase to form peroxynitrite. Peroxynitrite is a destructive free radical that has the ability to oxidize several lipoproteins, and nitrate tyrosine residues in many proteins (Aktan, 2004). Superoxide can also form hydrogen peroxide, which leads to the formation of hypochlorous acid and other oxidants (Lowenstein and Padalko, 2004). RNS and ROS work alongside each other to fight infection. Formation of peroxynitrite is favoured when equivalent amounts of superoxide and NO are present in the cell, during the immune response (Zhao, 2007). Cellular or tissue injury occurs due to the effects of excessive production of peroxynitrite, which include inhibition of mitochondrial respiration, depletion of cellular energetics, DNA damage, necrosis and apoptotic cell death (Ghasemi and Fatemi, 2014). Peroxynitrite also has the ability to nitrate proteins.

Nitration is the addition of a nitro group (-NO₂) to a protein. This nitration modulates catalytic activity, cytoskeletal organization and cellular signalling (Souza et al., 2008). 3-NT residues are identifiable markers of nitrosative stress and are detectable in a large number of pathological conditions (Calcerrada et al., 2011). As peroxynitrite has a very short half-life (< 1 second), it is extremely difficult to detect endogenously (Herce-Pagliai et al., 1998). An alternative approach to measuring NO-mediated inflammation is detection of 3-NT levels. The half-life of 3-NT is relatively longer. Due to the stability of 3-NT residues they are used as a suitable marker for NO-mediated tissue damage (Lorch et al., 2000, Herce-Pagliai et al., 1998). The formation of 3-NT residues in proteins of inflamed tissues as a result of peroxynitrite nitration is a hallmark of NO-mediated inflammation. Nitration of proteins has been shown to affect many different cellular processes crucial for cell proliferation and differentiation, such as phosphorylation of proteins by protein tyrosine kinases (Herce-Pagliai et al., 1998). In many inflammatory and neurodegenerative diseases, peroxynitrite has been attributed to, and shown to play a role in, the underlying pathology of these conditions. Atherosclerosis, cerebral ischemia, Alzheimer's disease and septic shock are among the diseases in which peroxynitrite has been implicated to play a contributing role, resulting in inflammation and cell death (Mihm et al., 2001, Tabrizi-Fard et al., 1999).

S-nitrosylation is a post-translational, reversible, covalent chemical reaction involving the addition of a NO moiety to the thiol group of cysteine residues on a target protein to regulate its function. This process plays a crucial role in a variety of biological processes. It can trigger conformational changes of proteins, activate or inhibit protein activity, alter protein-protein interactions, affect protein aggregation, or influence protein localisation (Nakamura et al., 2013). S-nitrosylation occurs through direct interaction of NO with proteins. S-nitrosylation has both protective and neurotoxic functions, depending on the target protein and levels of NO produced. As a result of the high levels of NO produced during inflammation, proteins are nitrosylated and contribute to neuroinflammation in the brain. S-nitrosylation as a result of iNOS has been implicated in the production of prostaglandins by activating COX-2. A single cysteine residue located beside the arachidonic acid binding site is nitrosylated, leading to increased prostanoid synthesis (Foster et al., 2009). Neurons are extremely NO-sensitive and respond to sustained NO generation by undergoing apoptosis illustrating the harmfulness of NO over-expression in the CNS. NO causes cell death of brain parenchymal cells *in vitro* and *in*

vivo via down-regulation of anti-apoptotic BCL2 and up-regulation of pro-apoptotic BAX coupled with LPS/IFN- γ induced neuronal apoptosis (Semmler et al., 2005).

The damage induced by such oxidative and nitrosative stress and the ensuing immune activation is well-documented as playing a role in the progression of neurodegeneration via lipid peroxidation and oxidative damage to proteins in neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease and multiple sclerosis (Li et al., 2013), and also in mediating sickness- and depression-related behaviours (Montezuma et al., 2012, Khovryakov et al., 2010, Yamaguchi et al., 2010). It is thought that peripheral inflammation may signal to the brain, triggering glial cell activation and leading to consequent NO, RNS and ROS production, and potentially driving the development or progression of neurodegenerative disorders (Perry et al., 2007, Qin et al., 2007, Träger and Tabrizi, 2013).

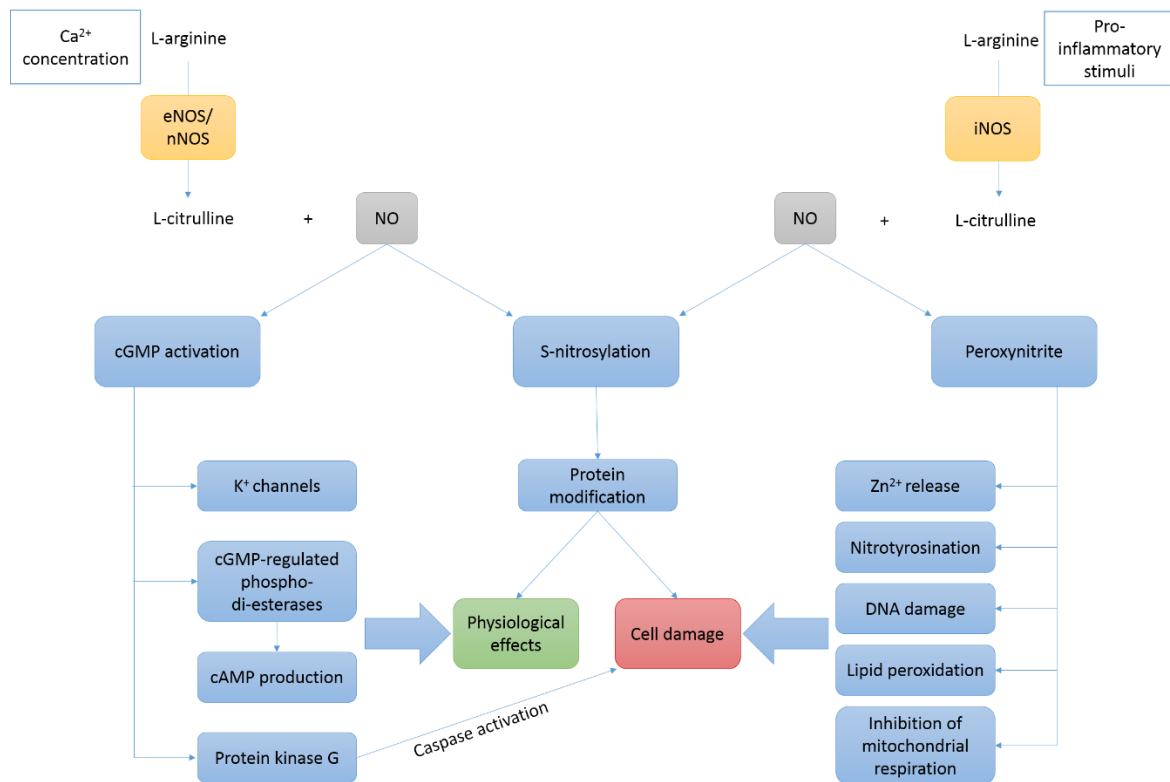


Figure 4.1 Diagram of the production and effects of NO

Both eNOS and nNOS produce NO through a calcium-dependent mechanism. Once produced, this NO is involved in cGMP production or s-nitrosylation. cGMP activation leads to changes that account for NO-mediated physiological effects. In response to pro-inflammatory stimuli iNOS produces NO which is involved in s-nitrosylation, and in the presence of superoxide produces RNS such as peroxynitrite. Nitration also occurs as a result of RNS production. S-nitrosylation leads to protein modification, and the production of peroxynitrite leads to cell damage through nitration, lipid peroxidation, zinc release, inhibition of mitochondrial respiration and DNA damage.

4.1.1 Aims

The aim of this chapter is to further examine the inflammatory response in the brain following exposure to DSS-induced colitis. The main objectives are to characterise this model in terms of iNOS production and activity within the brain, and to assess the involvement of glial cells in brain inflammation associated with DSS-induced colitis.

4.2 Methods

4.2.1 Study design

A number of experiments were carried out in order to analyse the effects of DSS-induced colitis, during acute colitis and recovery from colitis, in a range of behavioural tests in rats as described previously (Chapter 3). Perfused whole brains and snap-frozen brain and peripheral tissues were obtained from these experiments and analysed for evidence of peripheral and central inflammation. A detailed breakdown of each experiment and the *in vivo* and *ex vivo* work carried out within each experiment is provided in Chapter 2 (Section 2.2.14 and summarised in Figure 2.16).

4.2.2 Experimental protocols

All procedures in this chapter were conducted as described in detail in Chapter 2 and are outlined briefly below.

4.2.2.1 DSS-colitis induction

Male Wistar rats (175-250 grams) were obtained from the Bio-Resources Unit in Trinity College Dublin and housed under standard conditions with access to food and water *ad libitum*. The body weight, food consumption and fluid intake of each rat were recorded as a daily indication of welfare. The rats were monitored for signs of disease activity according to a modified version of the DAI (Table 2.1). A 5% w/v DSS solution was used to induce symptoms of colitis. DSS was administered until a rat reached a DAI score of ≥ 2.5 at which point the rat was given normal tap water.

4.2.2.2 Tissue collection

Rats were anaesthetised by i.p. urethane injection (urethane 1.5 g/kg) and transcardially perfused via perfusion pump with PBS (10 mM; pH 7.4) for a minimum of 5 minutes followed by ice-cold PFA (4% w/v in PBS; pH 7.4) for a minimum of 10 minutes. Brains were removed from the skull and post-fixed in 4% PFA at 4°C for 48 hours prior to cryoprotecting in sucrose solution (sucrose 30% w/v in 10 mM PBS) at 4°C for 3-4 days followed by snap-freezing in isopentane on dry ice. Snap-frozen brains were stored at -80°C until sectioning. Brain sections (30 μm thick) were cut using a cryostat at -21°C. Ten series of slices were collected per brain

with the slices in each series being taken approximately 300 μm apart. Each series of slices were stored in freezing storage solution (30% v/v ethylene glycol, 30% w/v sucrose in 10 mM PBS) at -80°C until use.

4.2.2.3 *Immunohistochemistry*

All steps for immunostaining protocols were carried out at room temperature with gentle agitation and each rinse step consisted of two 5-minute washes in 10 mM PBS at pH 7.4 unless otherwise specified. Free-floating sections were rinsed and incubated in 0.75% H_2O_2 in 5% MeOH in PBS for 20 minutes to quench endogenous peroxidases and rinsed again. [At this point, tissue being stained for 3-NT was subject to an antigen-retrieval step where slices were incubated in sodium citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0) in a water bath at 80°C for 20 minutes then left on the bench to cool for 20 minutes and rinsed.] Slices were incubated in 10% NS in 10 mM PBS for 30 minutes to block non-specific proteins. Slices were washed once in PBS and incubated overnight in primary antibody solution [(iNOS: rabbit anti-iNOS pAb 1:350; 3-NT: mouse anti-3-NT mAb 1:75; Santa Cruz Biotechnology, Santa Cruz, CA, USA; IBA1: rabbit anti-IBA1 pAb 1:5000; Wako Pure Chemical Industries Ltd., Osaka, Japan) in 10mM PBS with 5% NS and 0.05% Triton-X-100]. The following day, slices were rinsed and incubated for 90 minutes in a biotinylated secondary antibody from a Vectastain Elite ABC kit (anti-rabbit IgG or anti-mouse IgG; 1:200; Vector Laboratories, Burlingame, CA, USA) in 10 mM PBS with 1% NS. Sections were rinsed and Vectastain ABC reagent was applied for 90 minutes. A peroxidase reaction was performed to visualize immunolabelling by incubating sections with DAB (0.5 mg/ml) in 0.01% H_2O_2 in PBS for 7 minutes before stopping the reaction rinsing in dH_2O and 10 mM PBS. Sections were then mounted onto gelatin-coated slides, air-dried, desalted in dH_2O , dehydrated in ethanol, cleared in xylene, and coverslipped in DPX. To assess antibody specificity, incubation with the primary antibody was omitted for some sections, and no significant staining was observed in this case.

4.2.2.4 *Double Immunostaining*

For dual immunostaining with IBA1 and iNOS, the single immunostaining protocol was followed up to the first primary antibody incubation which was altered to a 2.5 hr incubation (rabbit anti-IBA1 pAb 1:5000; Wako Pure Chemical Industries Ltd., Osaka, Japan) at 37°C . Subsequent secondary antibody, ABC and DAB steps were carried out as described previously. Following post-DAB washes the single immunostaining protocol was then repeated beginning

with the non-specific protein blocking step. The second primary antibody to be applied was mouse anti-iNOS mAb (1:350; Santa Cruz Biotechnology, Santa Cruz, CA, USA). A peroxidase reaction was performed to visualize iNOS immunolabelling by incubating sections with SG solution (Vector Labs) for 7 minutes. Slices were rinsed in dH₂O and in 10 mM PBS. Slices were mounted onto gelatin-coated slides, air-dried, de-salted in dH₂O, dehydrated, cleared and coverslipped as previously described. To assess antibody specificity, incubation with each primary antibody was omitted for some sections, and no significant staining was observed in either case.

4.2.2.5 *Immunofluorescent Staining*

Free-floating sections were rinsed and incubated in 10% NS in 10mM PBS for 40 minutes to block non-specific proteins. Slices were washed once in PBS and incubated overnight at 4°C in primary antibody solution [(rabbit polyclonal anti-GFAP; 1:1000; Dako) in 10mM PBS with 1% BSA and 0.1% Triton-X-100]. The following day, sections were rinsed and incubated in secondary antibody solution [(Alexa-Fluor 488 nm fluorescent secondary anti-rabbit; 1:1000; Invitrogen) and 1% BSA in PBS] for 2 hours. This incubation and all following steps took place in the dark. Sections were rinsed and mounted onto gelatin-coated slides, excess liquid was removed and once dry, slides were coverslipped in Vectashield mounting medium containing DAPI (Vector laboratories) and sealed with clear nail varnish. To assess antibody specificity, incubation with the primary antibody was omitted for some sections, and no significant staining was observed in this case. The sections were stored at 4°C until ready for imaging up to 7 days later.

4.2.2.6 *Microscopy and analysis*

Co-ordinates of coronal planes and limits of the various structures analysed were defined according to the rat brain atlas (Paxinos and Watson, 2007). The locations from bregma of analysed structures are illustrated in Chapter 2 (Figure 2.15). Bilateral structures were analysed on each hemisphere, where available, and counts were averaged. Cell counts for the LHb were measured per region, however, cell counts for all other regions were measured as the average number of cells counted in three identical boxes (200 μm²) placed at random on the ROI. All cell counting was performed by researchers blind to the treatment group.

Brain slices stained with DAB and/or SG chromophores were imaged using brightfield microscopy. ROIs were imaged at a magnification of 100x and further analysed using the ImageJ software program. DAB-positive staining was defined as a dark brown cell stain, SG staining was defined as dark blue stain and co-localised staining was counted as black coloured staining. For analysis of microglial perimeter, all microglia with a visible cell body were isolated from an image of the ROI at 400x magnification and converted to individual 8-bit images (Figure 2.14). Threshold was adjusted (up to 17%) to remove background noise. The 'analyse particle' tool on ImageJ software was used to calculate the perimeter of each microglia.

Slices stained using Alexa Fluor fluorophores were imaged using confocal microscopy. Image stacks (consisting of 10 images, 3 μm apart) of the ROIs were acquired at 200x magnification using the Axiovert 200M microscope and Zeiss M Image software. ImageJ was used to convert image stacks into single images allowing for the visualisation of cells throughout the thickness of the slice. GFAP immunoreactive cells were only quantified if they met three criteria: (1) the astrocyte must show continuous labelling with at least three distinct processes, (2) the astrocyte must be contained within the limits of the quantification field, and (3) the astrocyte must not exist in association with a blood vessel.

4.3 Results

4.3.1 DSS-induced colitis stimulates central iNOS production in paraventricular areas

4.3.1.1 *iNOS immunoreactivity in paraventricular areas following DSS-induced colitis*

Immunohistochemical staining of iNOS protein was used to determine whether iNOS mRNA expression was leading to translation of iNOS protein in the brain. iNOS immunoreactivity was observed and mapped in serial sections in two regions primarily located surrounding CVOs, namely at the ventral hippocampal commissure above the SFO and the arcuate nucleus surrounding the ME (Figure 4.2). A single central slice was chosen for each region and iNOS immunoreactivity was quantified in this slice (Figure 4.3). One-way ANOVA of iNOS immunoreactivity showed an effect of DSS ($F_{2,21} = 5.737$; $p = 0.0112$) at the SFO (A). *Post hoc* analysis revealed an increase in iNOS immunoreactivity in both acute and recovered colitis groups relative to control ($p < 0.05$). One-way ANOVA of iNOS immunoreactivity showed an effect of DSS ($F_{2,23} = 4.499$; $p = 0.0236$) at the ME (B). *Post hoc* analysis revealed an increase in iNOS immunoreactivity in both acute and recovered colitis groups relative to control ($p < 0.05$).

Other paraventricular areas including CVOs, hippocampal regions and cortical regions were examined for iNOS immunoreactivity (Figure 4.4). One-way ANOVA of iNOS immunoreactivity showed no effect of DSS at the OVLT ($F_{2,21} = 1.987$; $p = 0.1646$) (A), PVN ($F_{2,21} = 0.2505$; $p = 0.7810$) (B), LHb ($F_{2,21} = 1.086$; $p = 0.3576$) (C), CeA ($F_{2,21} = 0.6915$; $p = 0.5130$) (D), CA1 region ($F_{2,22} = 1.253$; $p = 0.3072$) (E), CA2 region ($F_{2,22} = 0.5719$; $p = 0.5734$) (F), CA3 region ($F_{2,22} = 0.6553$; $p = 0.5301$) (G), the dentate gyrus (DG) ($F_{2,22} = 0.4928$; $p = 0.6182$) (H). No quantifiable iNOS immunoreactivity was observed in the pre-limbic, cingulate, motor, or sensory cortices (data not shown).

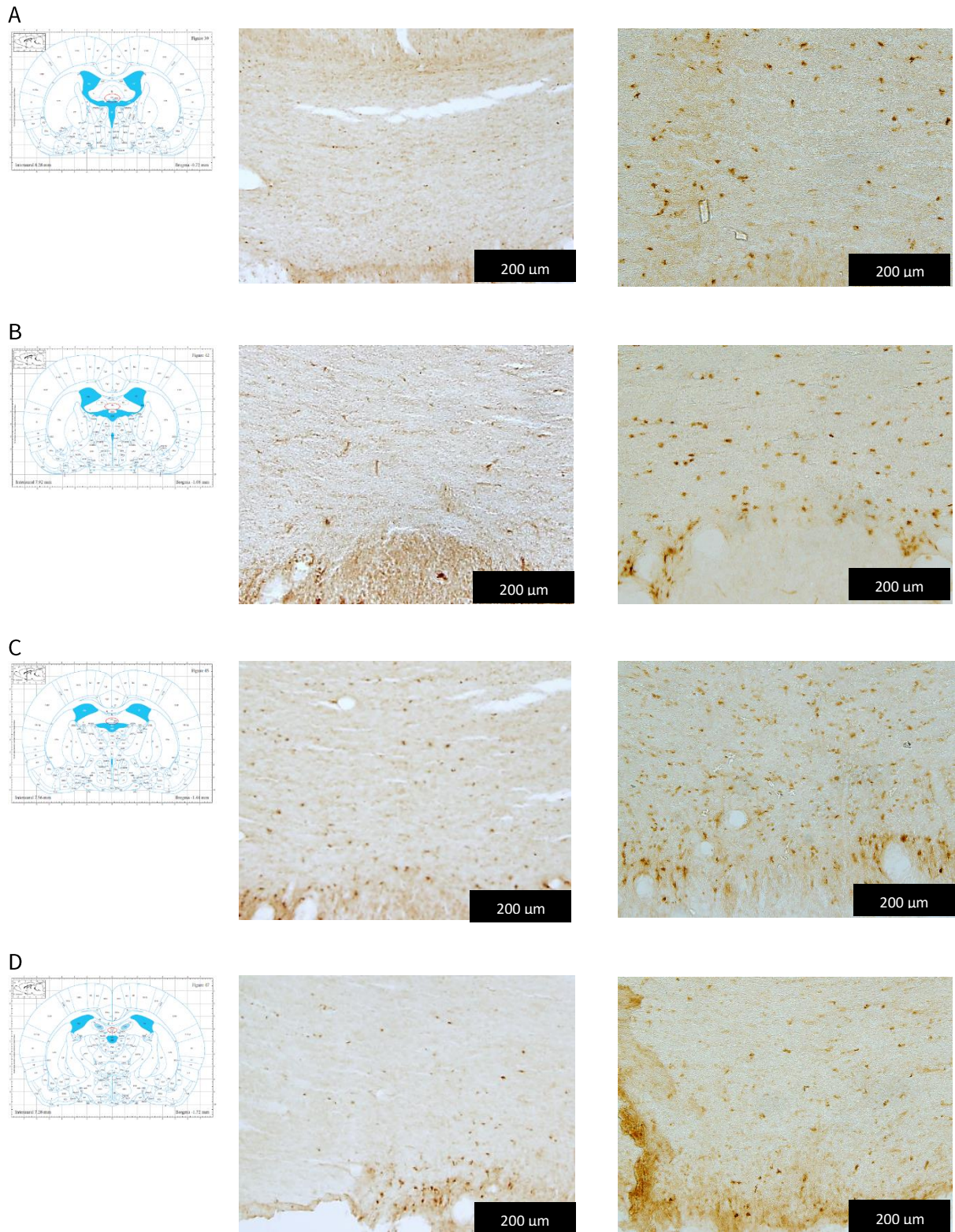


Figure 4.2 iNOS immunoreactivity in the SFO & ME following DSS-induced colitis

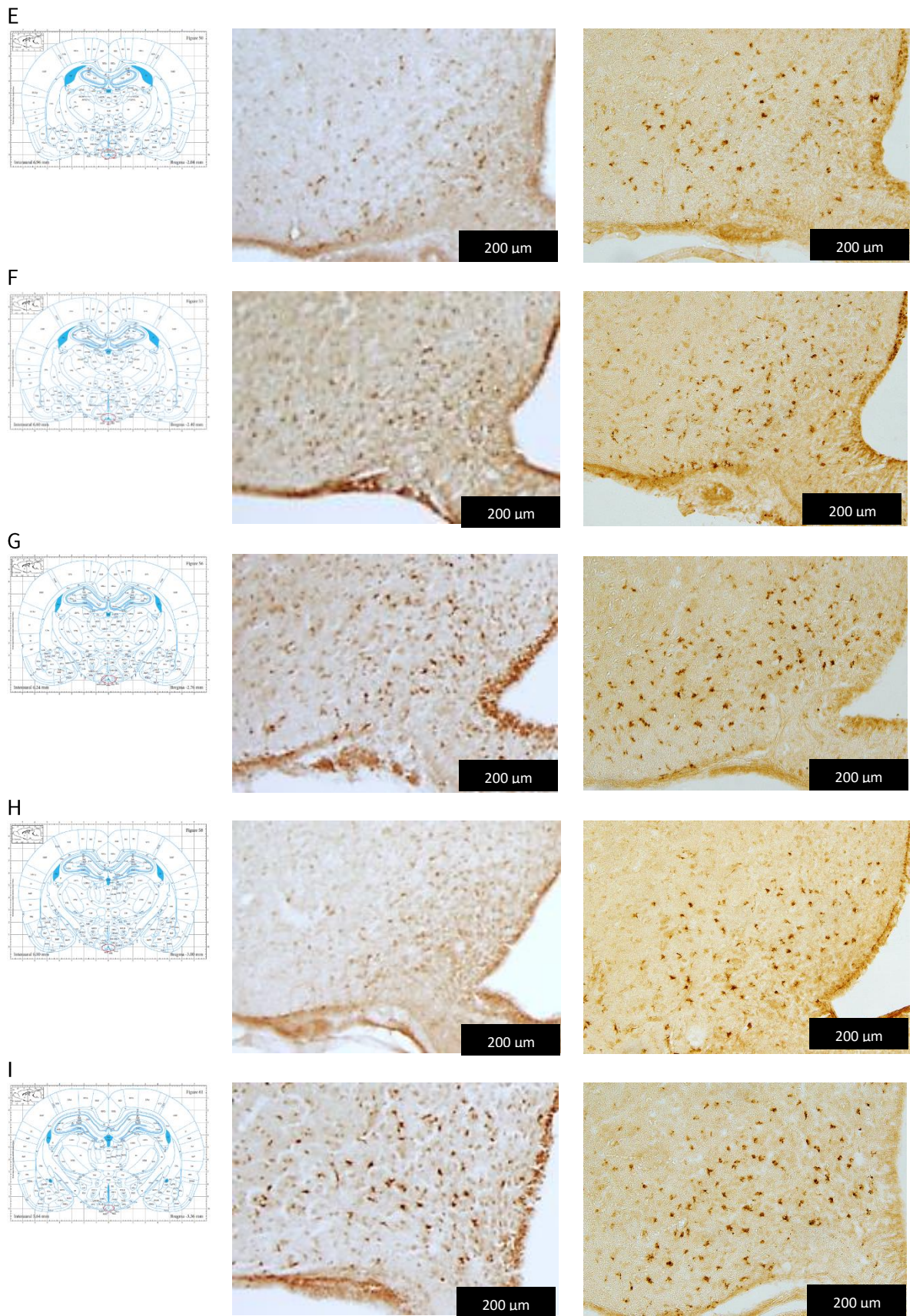


Figure 4.2 iNOS immunoreactivity in the SFO & ME following DSS-induced colitis

Figure 4.2 iNOS immunoreactivity in the SFO & ME following DSS-induced colitis

iNOS immunoreactivity, indicated by dark brown dots, was mapped on serial sections through the SFO (A -D) and ME (E - I) following DSS-induced colitis. Images show representative iNOS immunohistochemical staining in the control (centre) and acute colitis (right) groups at 200x magnification alongside figures from the rat brain atlas (left) which indicate the location of the SFO and ME circled in red (Paxinos and Watson, 2007) on the left. The images presented here come from rats in DSS study 4.

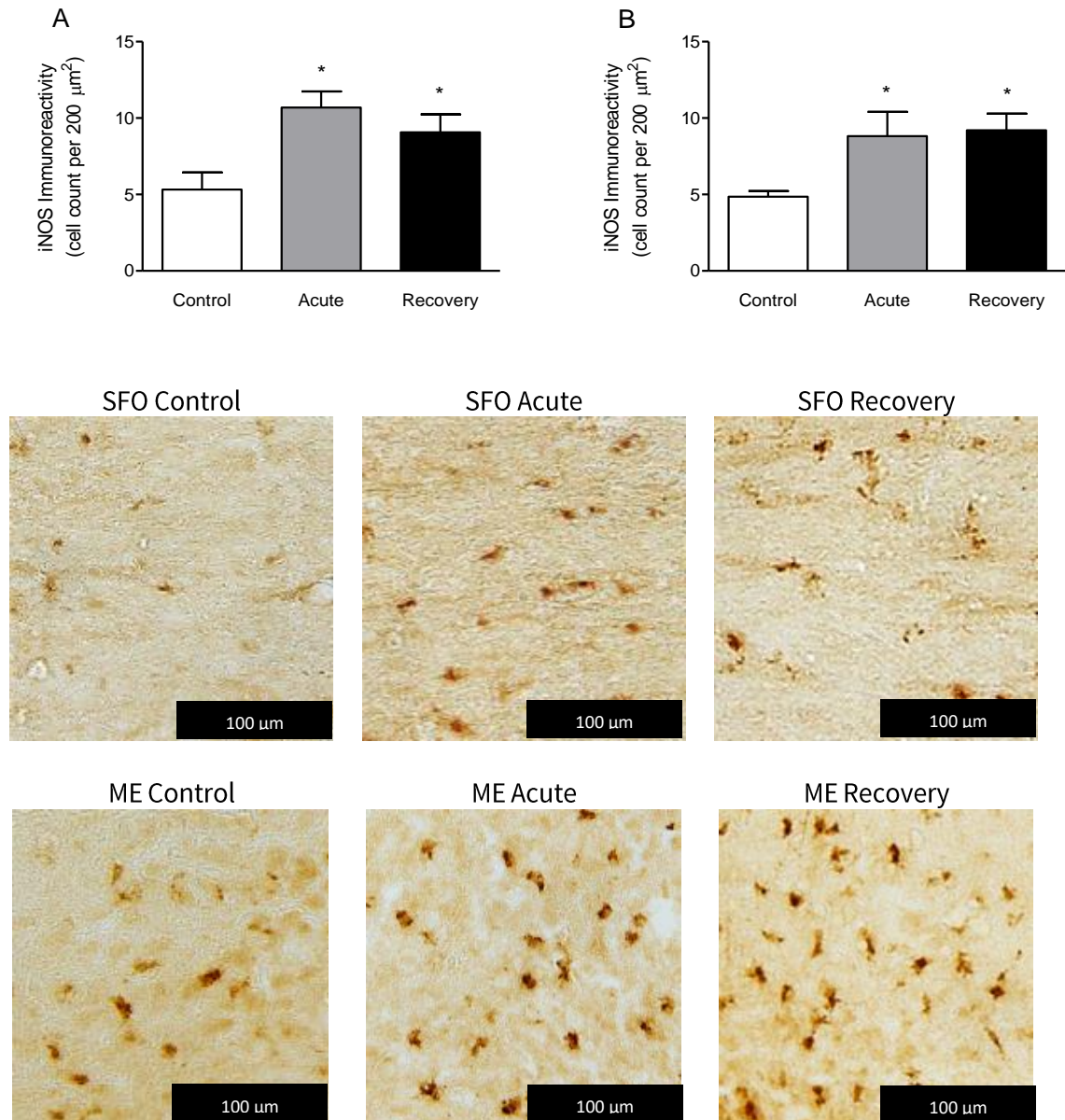


Figure 4.3 iNOS immunoreactivity at the SFO and ME following DSS-induced colitis

The ventral hippocampal commissure surrounding the SFO (A) and the arcuate nucleus surrounding the ME (B) were assessed for increases iNOS immunoreactivity per 200 μm^2 in acutely colitic and recovered rats following exposure to DSS-induced colitis. DSS-induced colitis increases iNOS immunoreactivity at the SFO and ME in acute and recovered colitis. Images show representative 200 μm^2 areas of iNOS immunohistochemical staining at the SFO and ME in each group at 200x magnification. The data presented here comes from rats in DSS study 4.

Data are presented as mean \pm SEM ($n = 7-8$). * $p < 0.05$ relative to control (one-way ANOVA with Newman Keuls *post hoc* test).

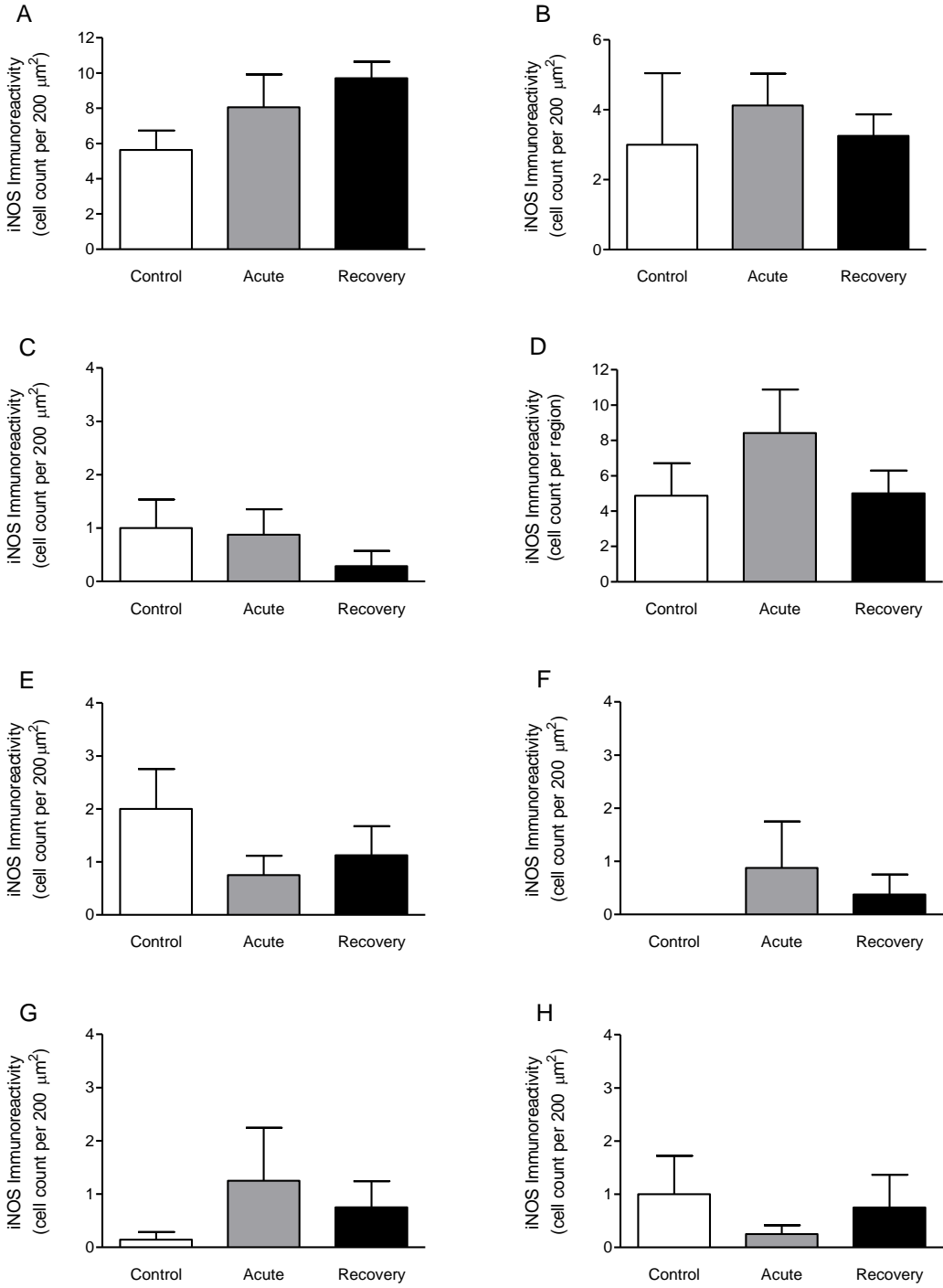


Figure 4.4 iNOS immunoreactivity in other regions following DSS-induced colitis

Figure 4.4 iNOS immunoreactivity in other regions following DSS-induced colitis

DSS colitis has no significant effect on iNOS immunoreactivity in other regions analysed: OVLT (A), PVN (B), CeA (C) or LHb (D), in the CA1 (E), CA2 (F), CA3 (G) or DG (H) of the hippocampus. No quantifiable iNOS immunoreactivity was observed in the pre-limbic, cingulate, motor or sensory cortices (data not shown). The data presented here comes from rats in DSS study 4. Data are presented as mean \pm SEM ($n = 7-8$ [A-D]; $8-16$ [E-H]). (one-way ANOVA).

4.3.1.2 3-NT immunoreactivity in DSS-induced colitis

As iNOS activity is capable of producing damaging levels of NO, *post-mortem* brain tissue was analysed for NO-induced protein nitration by immunohistochemical detection of 3-NT. In rats exposed to DSS, 3-NT immunoreactivity was observed surrounding the CVOs where iNOS reactivity was recorded, namely at the ventral hippocampal commissure above the SFO and the arcuate nucleus surrounding the ME (Figure 4.5). One-way ANOVA of 3-NT immunoreactivity showed an effect of DSS ($F_{2,19} = 4.561$; $p = 0.0260$) at the SFO (A). *Post hoc* analysis revealed an increase in 3-NT immunoreactivity in the acute colitis group only relative to control ($p < 0.05$). One-way ANOVA of 3-NT immunoreactivity showed an effect of DSS ($F_{2,22} = 6.862$; $p = 0.0054$) at the ME (B). *Post hoc* analysis revealed an increase in 3-NT immunoreactivity in both acute and recovered colitis groups relative to control ($p < 0.05$).

3-NT immunoreactivity was analysed in the other paraventricular areas and the hippocampus where no increases in iNOS were observed. No 3-NT reactivity was recorded at the OVLT, PVN, LHb, CeA, or in the CA1-3 or DG of the hippocampus (data not shown).

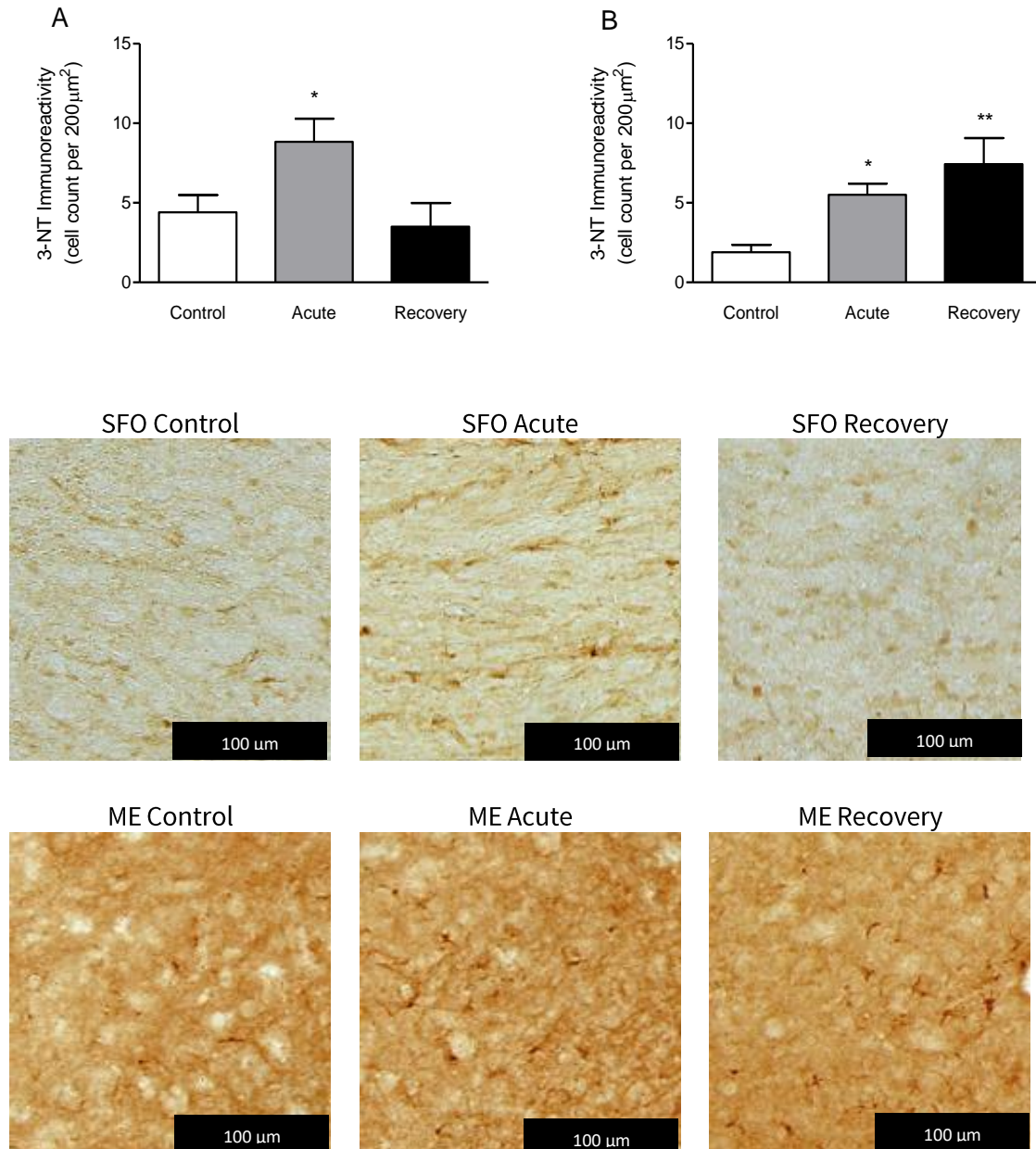


Figure 4.5 3-NT immunoreactivity at the SFO and ME following DSS-induced colitis

The ventral hippocampal commissure surrounding the SFO (A) and the arcuate nucleus surrounding the ME (B) were assessed for 3-NT immunoreactivity per 200 μm^2 in acutely colitic and recovered rats following exposure to DSS-induced colitis. DSS-induced colitis increases 3-NT immunoreactivity at the SFO in acute colitis and at the ME in acute and recovered colitis. Images show representative 200 μm^2 areas of 3-NT immunohistochemical staining at the SFO and ME at 200x magnification. The data presented here comes from rats in DSS study 4.

Data are presented as mean \pm SEM ($n = 8$). ** $p < 0.01$, * $p < 0.05$ relative to control (one-way ANOVA with Newman Keuls *post hoc* test).

4.3.2 Glial response following DSS-induced colitis

4.3.2.1 IBA1 immunoreactivity following DSS-induced colitis

Immunohistochemical staining of IBA1 was used to determine whether microglial activation was associated with central iNOS production. Basal levels of microglia displaying a ramified phenotype are observed throughout the brain. However, in rats exposed to DSS, increased IBA1 immunoreactivity was observed surrounding the CVOs where iNOS immunoreactivity was observed, namely at the ventral hippocampal commissure above the SFO and the arcuate nucleus surrounding the ME (Figure 4.6). One-way ANOVA of IBA1 immunoreactivity showed an effect of DSS ($F_{2,17} = 10.34$; $p = 0.0015$) at the SFO (A). *Post hoc* analysis revealed an increase in IBA1 immunoreactivity in the recovered colitis group only relative to control ($p < 0.01$). One-way ANOVA of IBA1 immunoreactivity showed an effect of DSS ($F_{2,11} = 20.33$; $p = 0.0005$) at the ME (B). *Post hoc* analysis revealed an increase in IBA1 immunoreactivity in both acute and recovered colitis groups relative to control ($p < 0.001$).

On activation, microglia retract their processes and become amoeboid in shape, therefore microglial morphology was analysed to determine microglial activation. Qualitative analysis indicated a ramified morphology in control rats in contrast to a more amoeboid morphology in acute and recovered DSS-induced colitis groups (Figure 4.7). The perimeter length of the microglial cells were calculated in order to quantitatively confirm their activation state identified by morphology (Figure 4.7). One-way ANOVA of microglial perimeter length showed an effect of DSS ($F_{2,17} = 10.34$; $p = 0.0015$) at the SFO (A). *Post hoc* analysis revealed a decrease in perimeter length in both acute and recovered colitis groups relative to control ($p < 0.01$). One-way ANOVA of microglial perimeter length showed an effect of DSS ($F_{2,11} = 20.33$; $p = 0.0005$) at the ME (B). *Post hoc* analysis revealed a decrease in perimeter length in both acute and recovered colitis groups relative to control ($p < 0.01$). Dual immunohistochemical staining of IBA1 and iNOS revealed that between 75-80% of all iNOS staining was co-localised with IBA1 staining (Figure 4.8).

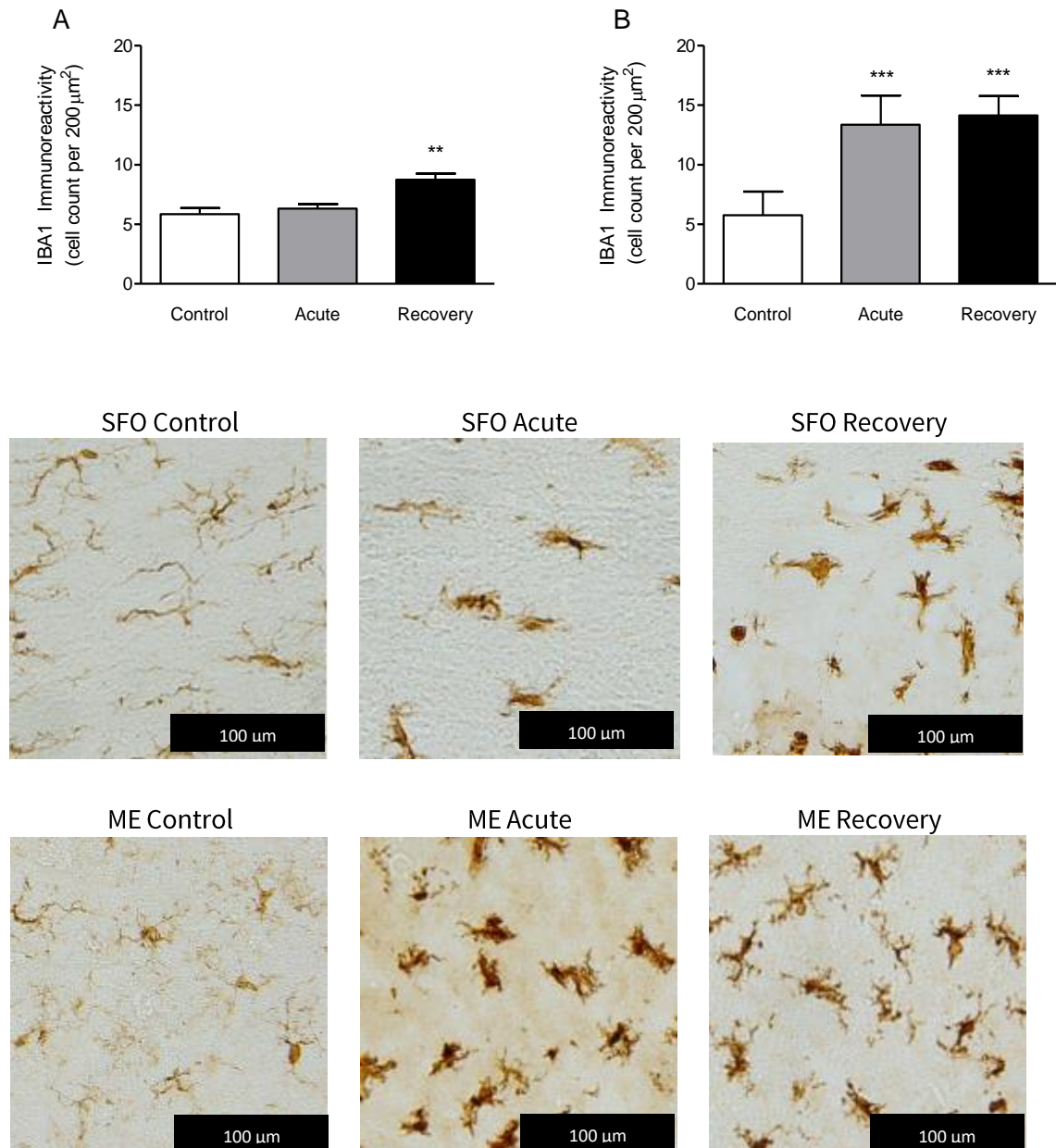


Figure 4.6 IBA1 immunoreactivity at the SFO and ME following DSS-induced colitis

The ventral hippocampal commissure surrounding the SFO (A) and the arcuate nucleus surrounding the ME (B) were assessed for IBA1 immunoreactivity per 200 μm^2 in acutely colitic and recovered rats following exposure to DSS. DSS-induced colitis increases IBA1 immunoreactivity at the SFO in recovered colitis and at the ME in acute and recovered colitis. Images show representative 200 μm^2 areas of IBA1 immunohistochemical staining at the SFO in each group at 200x magnification. The data presented here comes from rats in DSS study 5. Data are presented as mean \pm SEM ($n = 8$). *** $p < 0.001$, ** $p < 0.01$ relative to control (one-way ANOVA with Newman-Keuls *post hoc* test).

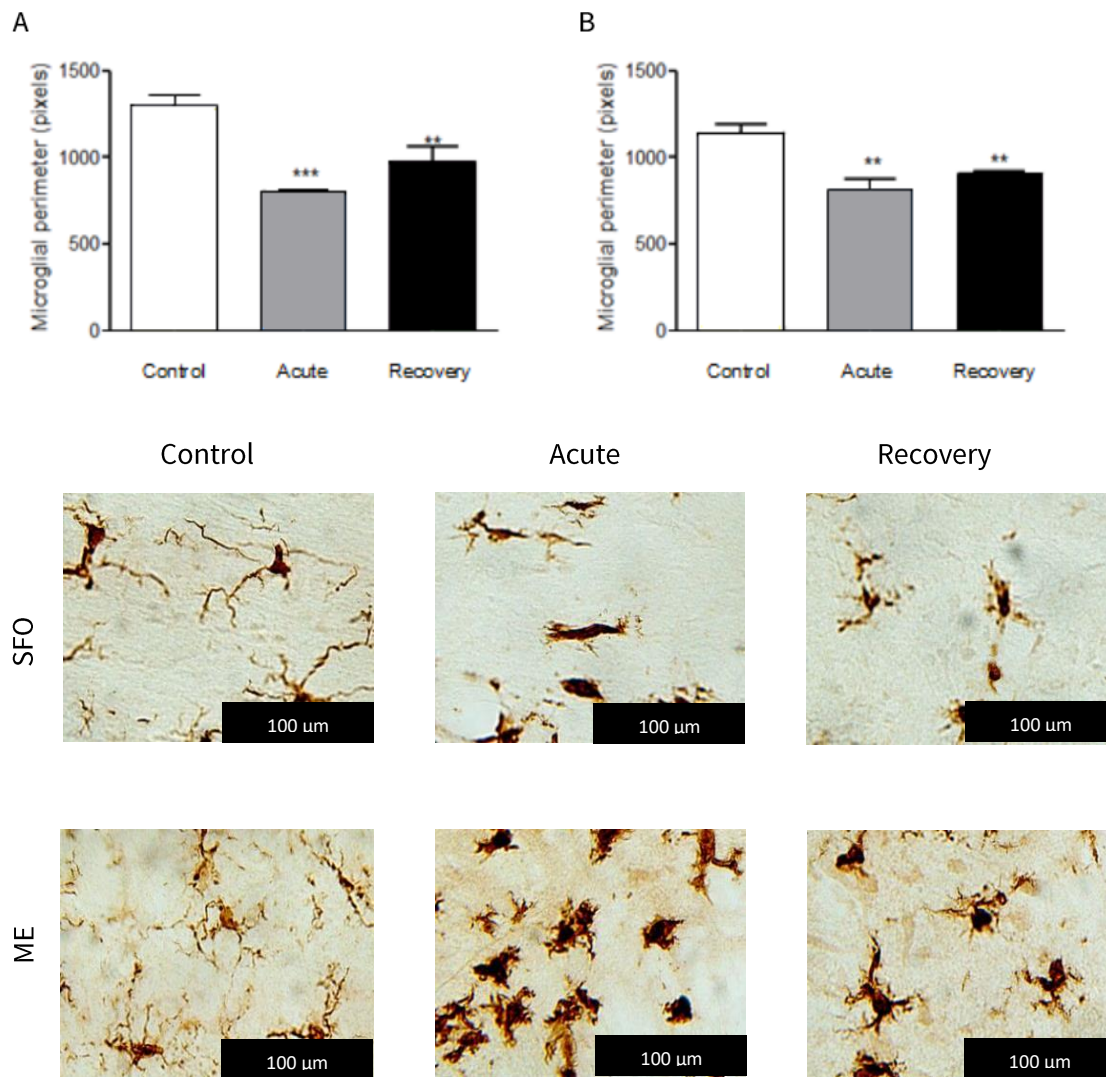


Figure 4.7 Microglial morphology and perimeter analysis at the SFO and ME following DSS-induced colitis

Microglial morphology and perimeter length were assessed in acutely colitic and recovered rats following exposure to DSS. Microglial perimeters were significantly reduced in acute and recovered DSS-induced colitis at both the SFO (A) and the ME (B), indicating activation. Representative images show IBA1 microglial staining at the SFO and ME in control, acute and recovered colitis respectively. Microglial ramifications observed in control images indicate quiescent microglia. Microglial activation is marked by transition to a more amoeboid shape observed in acute and recovered colitis. (400x magnification) The data presented here comes from rats in DSS study 5.

Data are presented as mean \pm SEM ($n = 8$). *** $p < 0.001$, ** $p < 0.01$ relative to control (one-way ANOVA with Newman-Keuls *post hoc* test).

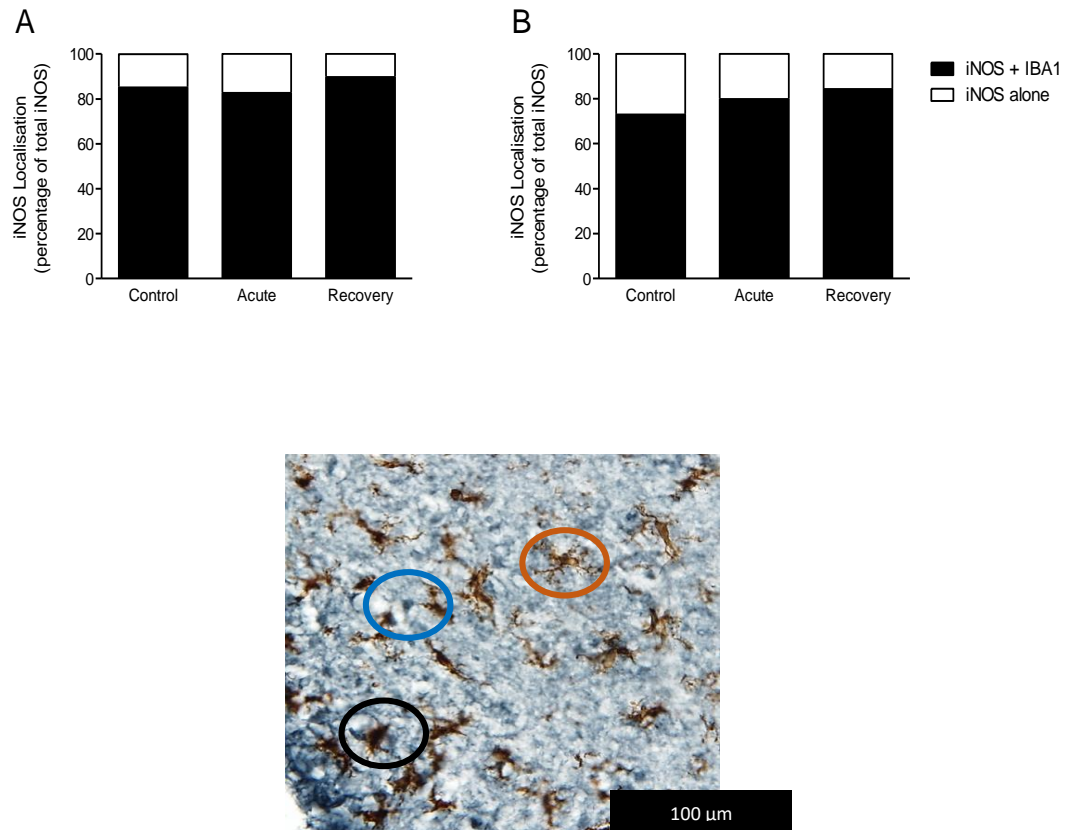


Figure 4.8 Co-localisation of iNOS with IBA1 at the SFO and ME following DSS-induced colitis

Co-localisation of iNOS and IBA1 immunoreactivity was assessed in acutely colitic and recovered rats following exposure to DSS. Immunoreactivity of iNOS-IBA1 co-localisation (navy/black) was calculated and added to immunoreactivity of iNOS alone (brown) to give total iNOS immunoreactivity. iNOS co-localisation is expressed as a percentage of total iNOS. In both the SFO and the ME, the majority (approximately 75-85%) of all iNOS immunoreactivity is co-localised with the microglial marker IBA1. The remaining iNOS immunoreactivity in these areas therefore does not have a microglial source. Representative sample image from the ME with circles indicating iNOS alone (blue), microglia alone (brown) and co-localised iNOS and microglia (black). The data presented here comes from rats in DSS study 4.

Data are presented as mean \pm SEM ($n = 8$).

4.3.2.2 *CD45 immunoreactivity following DSS-induced colitis*

Immunofluorescent labelling of CD45 was used to determine whether infiltrating peripheral monocytes accounted for IBA1 immunoreactivity observed. Both monocytes and microglia express IBA1, however monocytes express CD45, while microglia generally do not. No CD45 immunoreactivity was observed in the parenchyma surrounding CVOs although CD45 positive staining was observed within circumventricular regions themselves and within the choroid plexus in animals recovering from DSS-induced colitis (Figure 4.9).

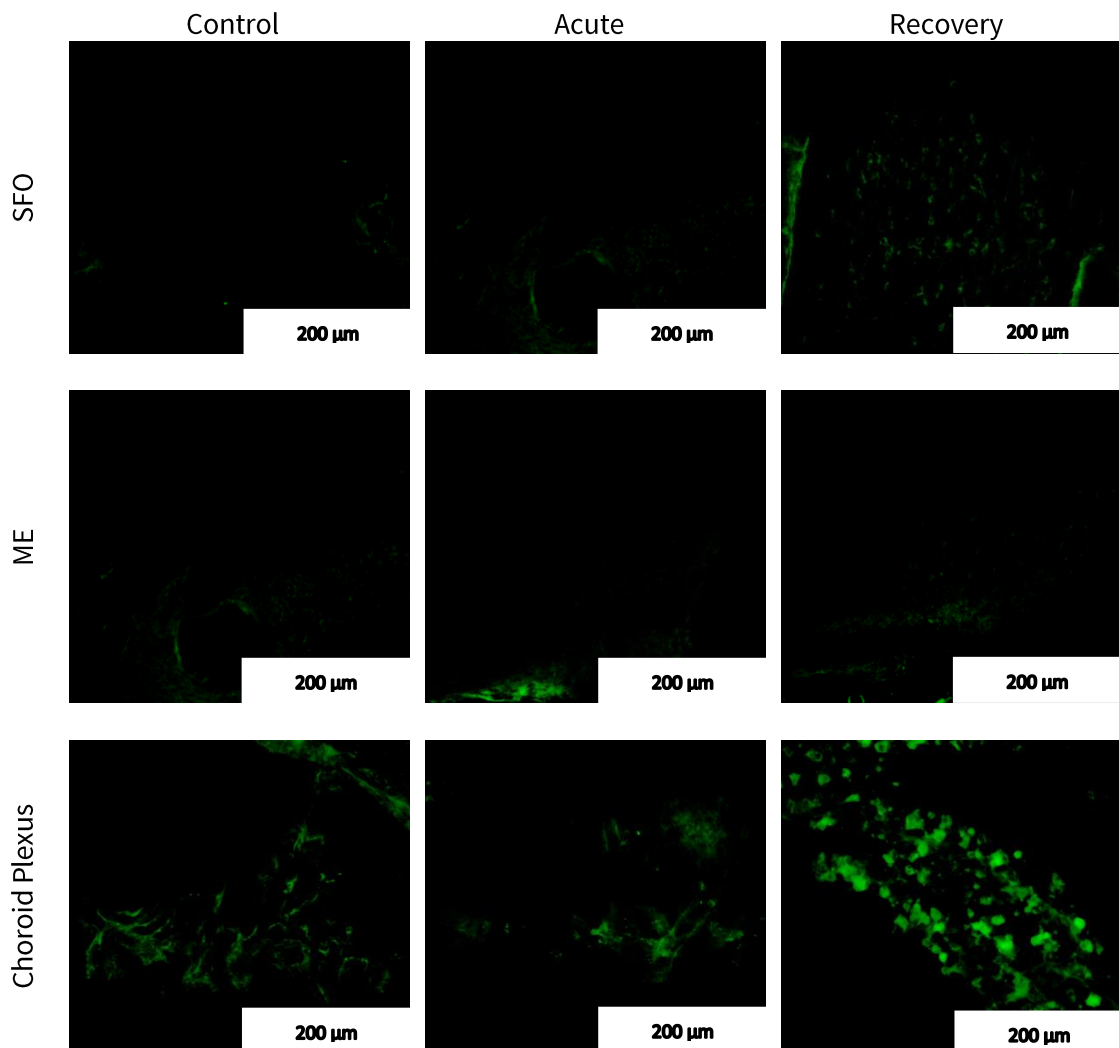


Figure 4.9 CD45 immunoreactivity at the choroid plexus, SFO and ME following DSS-induced colitis

The SFO, ME and CP were assessed for CD45 immunoreactivity in acutely colitic and recovered rats following exposure to DSS. No CD45 immunoreactive cells were visible in the parenchyma surrounding the SFO or ME in DSS-induced colitis. In recovered DSS-induced colitis, CD45 staining is visible in the choroid plexus. The images presented here come from rats in DSS study 6. Images are representative of $n = 6$.

4.3.2.3 GFAP immunoreactivity following DSS-induced colitis

Brains were stained using GFAP immunofluorescence to analyse the presence of astrocytes following DSS-induced colitis. GFAP immunoreactivity was measured at the SFO and ME (Figure 4.10). One-way ANOVA of GFAP immunoreactivity showed no effect of DSS ($F_{2,16} = 0.3859$; $p = 0.6868$) at the SFO (A). One-way ANOVA of GFAP immunoreactivity showed no effect of DSS ($F_{2,19} = 0.1464$; $p = 0.8649$) at the ME (B).

The hippocampus and cortex were assessed for GFAP immunoreactivity (Figure 4.11). In the hippocampus, one-way ANOVA of GFAP immunoreactivity showed no effect of DSS at the CA1 ($F_{2,19} = 0.09034$; $p = 0.9141$) (A), CA2 ($F_{2,17} = 0.3202$; $p = 0.7309$) (B), CA3 ($F_{2,18} = 0.4335$; $p = 0.6556$) (C), or DG ($F_{2,15} = 0.5579$; $p = 0.5855$) (D). Similarly, in cortical regions, one-way ANOVA of GFAP immunoreactivity showed no effect of DSS at cingulate ($F_{2,16} = 0.0437$; $p = 0.9574$) (E), sensory ($F_{2,15} = 0.9158$; $p = 0.4245$) (F), or motor ($F_{2,16} = 0.7797$; $p = 0.4775$) (G) cortices.

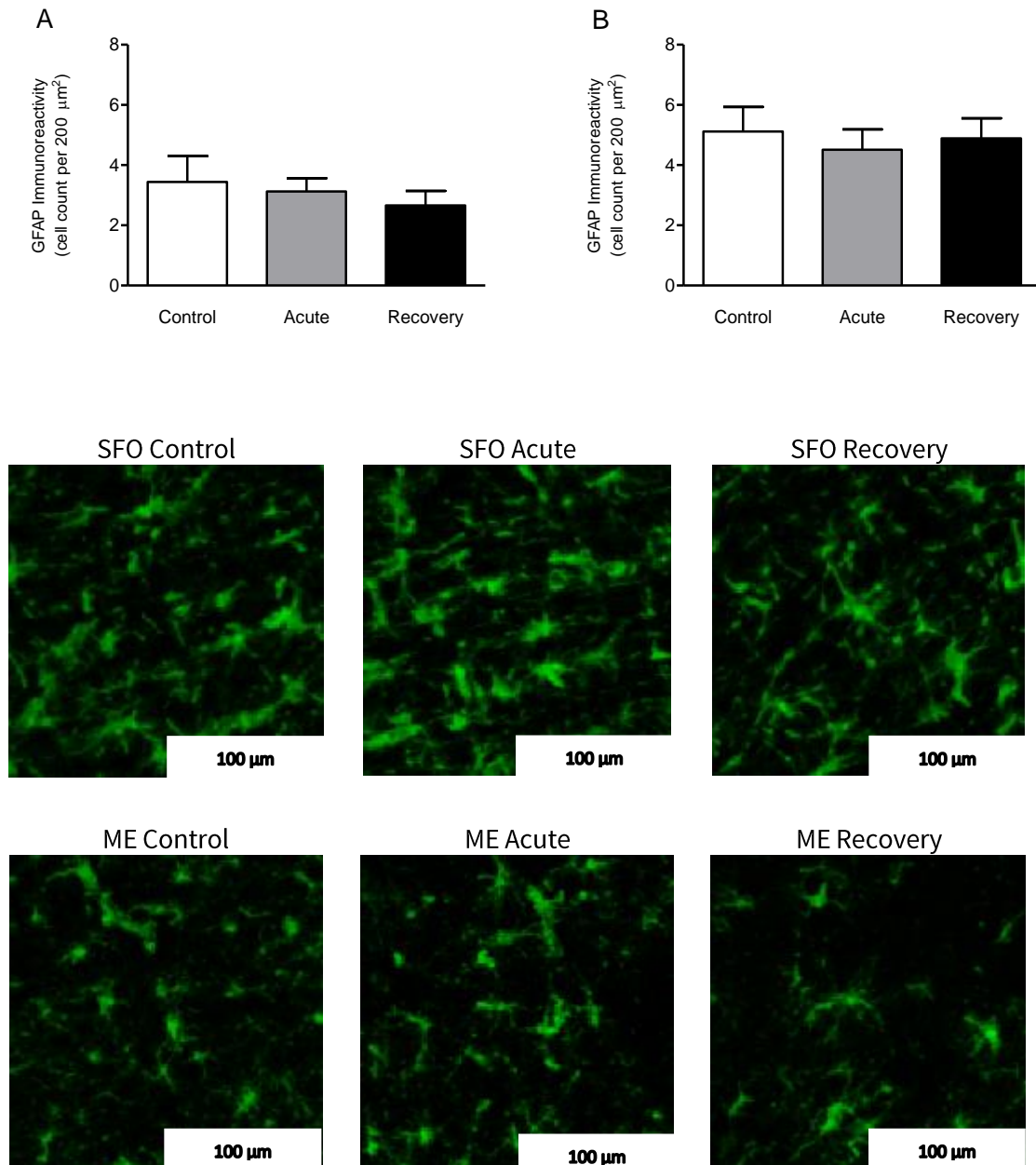


Figure 4.10 GFAP immunoreactivity at the SFO and ME following DSS-induced colitis

The ventral hippocampal commissure surrounding the SFO (A) and the arcuate nucleus surrounding the ME (B) were assessed for GFAP immunoreactivity per 200 μm^2 in acutely colitic and recovered rats following exposure to DSS. There were no differences in GFAP immunoreactivity at the SFO or ME. Images show representative 200 μm^2 areas of GFAP immunohistochemical staining at the SFO and ME in each group at 200x magnification. The data presented here comes from rats in DSS study 5.

Data are presented as mean \pm SEM ($n = 5-8$). (one-way ANOVA).

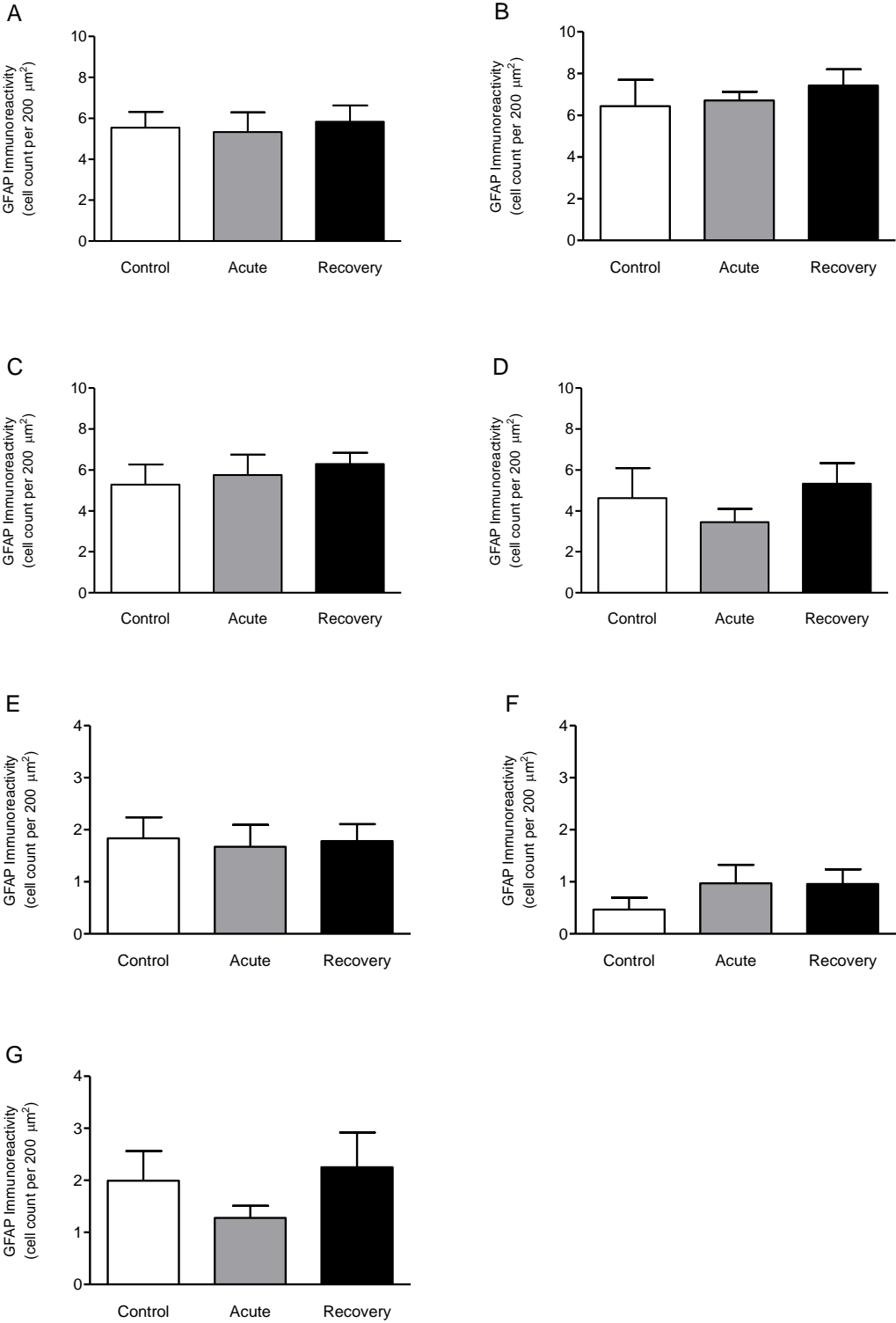


Figure 4.11 GFAP immunoreactivity in the hippocampus and cortex in DSS-induced colitis

Figure 4.11 GFAP immunoreactivity in the hippocampus and cortex in DSS-induced colitis

Hippocampal and cortical regions were assessed for GFAP immunoreactivity per 200 μm^2 in acutely colitic and recovered rats following exposure to DSS. There were no differences in GFAP immunoreactivity in the CA1 (A), CA2 (B), CA3 (C) or DG (D) of the hippocampus or in the cingulate (E), sensory (F) or motor (G) cortex. The data presented here comes from rats in DSS study 5.

Data are presented as mean \pm SEM ($n = 4-8$). (one-way ANOVA).

4.4 Discussion

The results of this chapter demonstrate that neuroinflammation is increased in association with DSS-induced colitis in paraventricular regions of the brain. Following DSS-induced colitis, increases in iNOS immunoreactivity are observed in the parenchyma surrounding two CVOs, namely the SFO and the ME. These increases are observed both with acute DSS-induced colitis and following seven days recovery. Associated increases in iNOS activity are confirmed at these regions with the nitration marker 3-NT. Increased 3-NT is recorded with acute DSS-induced colitis only at the SFO and in both acute colitis and also following seven days recovery at the ME. No increases in iNOS or 3-NT immunoreactivity are observed in other brain regions analysed including the hippocampus, PVN, CeA, LHb, or OVLT. To determine glial involvement in the induction of iNOS associated with DSS-induced colitis, microglia and astrocytes were examined. Following DSS-induced colitis, increases in immunoreactivity of the microglial marker IBA1 are observed in the parenchyma surrounding the SFO and the ME. At the SFO these increases are observed only at the recovery timepoint, however, at the ME, this increase is observed with acute DSS-induced colitis and following recovery. Dual immunohistochemical staining of iNOS and IBA1 shows co-localisation of these markers indicating that iNOS produced following DSS-induced colitis is largely localised to microglial cells. Morphological analysis, including the reduction in microglial perimeter, seen at these regions indicate that the microglia are more amoeboid in shape with less ramifications in acute colitis and following recovery. This indicates that the microglia are in an activated state. In terms of astrocytes, no alterations in GFAP immunoreactivity were observed at the CVOs or in any other regions analysed including hippocampal or cortical regions.

4.4.1 Evidence for iNOS-related inflammation at CVOs

Results from this study suggest that iNOS protein is present at basal level in the brains of both control and DSS-exposed animals as described previously (Semmler et al., 2005). However this investigation shows that there is an increase in iNOS protein in areas surrounding certain CVOs: the SFO and ME, following DSS-induced colitis and into recovery. It is believed that cytokines from the periphery can access the CNS and initiate local immune activation via CVOs where the BBB is more permeable. It is therefore likely that the increases in iNOS seen in CVOs are due to soluble immune mediators in the circulation infiltrating the brain at these vulnerable points and initiating an immune response centrally. Although the presence of

iNOS-positive cells is not observed further into other regions of the parenchyma, the ability of iNOS to produce sustained high levels of NO which can diffuse freely throughout the brain may have pathological implications for the brain at large. This would be of particular importance in the case of IBD patients who suffer chronic GI inflammation over long periods of time which may result in consistently up-regulated levels of iNOS in the CNS. Considering the results from Chapter 3 (Section 3.3.3), which show increased iNOS mRNA in hippocampus, hypothalamus and cortex in acute and recovered DSS-induced colitis, it is worth noting that the area surrounding the SFO where iNOS immunoreactivity is observed is the ventral hippocampal commissure and the area surrounding the ME where iNOS immunoreactivity is observed is the arcuate nucleus of the hypothalamus. Therefore iNOS immunoreactivity is localised to discrete areas within these brain structures. However, no iNOS immunoreactivity was observed in the cortex despite the presence of iNOS mRNA. This is potentially due to the fact that iNOS translation in the brain is tightly regulated (Lee et al., 2003) and is subject to post-transcriptional modifications and translational regulation (Saha and Pahan, 2006) even though the cortex may receive pro-inflammatory signals to drive iNOS transcription. A decrease in CD11b mRNA expression was observed in the cortex in acute colitis, which we interpreted as a potential indicator of microglial priming towards the M2 phenotype as CD11b is generally associated with M1 microglia. These microglia do not generate iNOS and are instead geared toward an immunoprotective role.

Previously, various stress-induced animal models of depression have made links between increases in iNOS expression in discrete brain regions with increases in depressive-like behaviours. Treatment of these stress-induced models of depression with iNOS inhibitors lead to lessening of the depressive phenotype as demonstrated for example by a reduction in immobility in the FST (Montezuma et al., 2012) and tail suspension test (Zeni et al., 2012). In turn, conventional anti-depressants such as fluoxetine can inhibit LPS-induced iNOS expression in the rat brain *in vivo* thereby decreasing ROS generation and oxidative stress (Chung et al., 2010). Interestingly, fluoxetine pre-treatment has been shown to ameliorate symptoms of DSS-induced colitis in mice, by inhibiting NF- κ B activation in intestinal epithelial cells (Koh et al., 2011). In terms of colitis models, Heydarpour et al. (2016) demonstrate that depression-related behaviour recorded in the FST in mice 3 days post-TNBS administration can be attenuated using a specific iNOS inhibitor (aminoguanidine) administered 30 minutes prior to the FST. Haj-Mirzaian et al. (2017) and colleagues have also demonstrated an increase

in hippocampal NO and ROS associated with anxiety- and depressive-like behaviours in a variety of behavioural tests following DNBS-induced colitis. These data indicate the potential involvement of NO in the provocation of colitis-induced anxiety/depressive-like behaviour. Thus, increases in iNOS production in the brain as a result of colitis may be an important mediator in behavioural changes reported in Chapter 3. Previous studies have implicated oxidative challenge and nitrenergic system dysfunction in modulation of anxiety- and depressive-like behaviours (Haj-Mirzaian et al., 2016), however it is still unclear how peripheral inflammation can influence the brain to induce psychological symptoms or behaviour, although most researchers agree that cytokines are key mediators in this response (Mackner et al., 2011). It is likely that an increased inflammatory profile in the brain leads to increased oxidative and nitrosative stress, however the mechanism by which this precipitates alterations in behaviour is yet to be uncovered.

Immunohistochemical mapping of the production of 3-NT residues in the brain showed that 3-NT induction is increased surrounding both CVOs analysed in acute DSS-induced colitis. This indicates that the iNOS observed in these regions is producing NO to such an extent that peroxynitrite formation is occurring and causing protein alterations. Surrounding the ME, immunoreactivity of 3-NT is also increased following recovery from DSS-induced colitis. This data correlates with increased levels in iNOS production seen, implicating increased iNOS expression in this process. However, 3-NT immunoreactivity surrounding the SFO following recovery, returned to control levels. This is interesting considering iNOS expression remains increased in this region at 7 days recovery. Perhaps a depletion of NADPH-oxidase or superoxide in this regions is responsible for the decrease in NO-mediated protein nitration.

4.4.2 Evidence for glial-related inflammation at circumventricular organs

Following DSS-induced colitis, an increase in microglial cell number, indicated by the microglial marker IBA1, was observed in the areas surrounding the SFO and ME. At the SFO, immunoreactivity of IBA1 was increased in acute colitis only, however at the ME this increase was observed at both the acute and the recovered timepoints relative to control. The continued activation of microglia from acute to the recovery stage suggests the microglia are chronically activated in this model. The increase in iNOS protein seen in these areas persists

for up to 7 days of recovery, the increase in the numbers of microglia at the ME remains increased as the numbers of activated microglia at the SFO have declined. This could indicate that inflammation in the ME may continue to propagate despite the absence of outward colitic symptoms.

This increase in microglial numbers may explain the increase in iNOS protein expression as microglia become activated in response to inflammatory stimuli and are recruited from surrounding areas. On this point, analysis of iNOS and IBA1 dual immunoreactivity demonstrates that iNOS appears mainly to be co-localised with microglial cells. It is therefore likely that microglia surrounding CVOs are the source of the increase in iNOS immunoreactivity observed in rats exposed to DSS.

It is of note that increased iNOS is observed surrounding the SFO in acute colitis despite no increase in microglial numbers. Morphological examination of the microglia surrounding the SFO shows that although there was no increase in their number during the acute phase, they adopt an amoeboid appearance, as identified by cell perimeter analysis, indicative of an active state. It is possible that the already resident microglia present at the SFO become activated and are responsible for the observed increase in iNOS into recovery. At the recovery timepoint following DSS-induced colitis, microglia at the SFO retain their amoeboid morphology accompanied by increased IBA1 immunoreactivity. At the ME, IBA1 immunoreactivity is increased both in acute colitis and following recovery. Morphologically, microglia in this region adopt an amoeboid appearance which is evident at both acute and recovered timepoints following exposure to DSS. These findings are consistent with a previous study by Radler et al. (2014) which examined LPS-induced alterations to IBA1 reactivity in the brains of mice and found increased IBA1 density at the SFO 6 hours post-injection (i.p.) without an increase in microglial numbers and increased IBA1 density and increased microglial numbers at the arcuate nucleus surrounding the ME.

At the ME, β 2-tanycytes form an efficient barrier between the ME milieu and the ventricular CSF, while β 1-tanycytes establish a lateral barrier, separating the ME from the arcuate nucleus (Peruzzo et al., 2000). As the arcuate nucleus contains feeding-control circuits, ME permeability can be altered by fasting. As seen in Chapter 3 (Section 3.3.1.1) exposure to DSS-induced colitis leads to a decrease in food intake during acute colitis which does not recover until day 5 of recovery at the earliest. Similarly, IBD patients may experience loss of appetite

and anorectic symptoms. In response to fasting indicated by a drop in glucose levels, β 1-tanycytes containing glucose sensors can increase vascular permeability via enhanced secretion of vascular endothelial growth factor -A (VEGF-A) in order to increase access of metabolic substrates to the arcuate nucleus (Langlet et al., 2013, Bolborea and Dale, 2013). This is a possible mechanism for an increase in permeability of the BBB at the ME in DSS-induced colitis which does not involve BBB dysfunction and may also partly explain differences observed in inflammation at the SFO compared to the ME.

Repeated social defeat stress models have shown that CD45^{hi} peripheral macrophages are increased in the brain as part of the immune response to stress without disruption to the BBB and are associated with long-term stress-related anxiety (Wohleb et al., 2013). These infiltrating monocytes differentiate into ramified IBA1⁺ cells, however, are morphologically less ramified than resident microglia (Elmore et al., 2014). This also occurs in response to inflammation, for example in EAE, a rodent model of multiple sclerosis (Yamasaki et al., 2014). Immunofluorescent analysis targeting CD45, a marker of peripheral monocytes, was used to determine whether peripheral immune cells were infiltrating the brain in response to DSS-induced colitis and contributing to inflammation. No CD45 staining was observed at either the SFO or the ME following acute DSS-induced colitis. Although some CD45 immunoreactivity was observed in the SFO itself at the recovery timepoint, no immunoreactivity was observed within the parenchyma in either region. Choroid plexus macrophages express CD45 and function in CSF immune surveillance (Chinnery et al., 2010). An increase in CD45 was observed in the choroid plexus at the recovery timepoint following DSS-induced colitis and is likely associated with the observed CD45 immunoreactivity in the SFO at this time. As no CD45 immunoreactivity is observed in the parenchyma, it appears monocytes are not capable of infiltrating the BBB in response to DSS-induced colitis.

Despite the decrease in cortical GFAP mRNA in acute DSS-induced colitis reported in Chapter 3 (Section 3.3.3) no alteration in the numbers of astrocytes were observed at any of the ROIs analysed at either the acute or the recovered timepoint. Considering microglial activation is confirmed at both the SFO and ME it is possible that GFAP is being down-regulated in astrocytes without a change in GFAP-positive astrocyte numbers.

4.4.3 Conclusion

Following DSS-induced colitis in rats, the increase in iNOS enzyme observed surrounding CVOs signifies that circulating inflammatory mediators are the most likely cause of induction of an immune response in the brain in response to colitis. This corresponds with data from Chapter 3 (Section 3.3.3.1) which shows that the increase in peripheral levels of iNOS mRNA in colon, liver and spleen is directly correlated with the levels of iNOS mRNA in the brain in the cortex, hippocampus and hypothalamus. The presence of 3-NT in these regions shows that the iNOS protein is actively producing NO at levels capable of nitrating and potentially altering protein function. Increased iNOS immunoreactivity at CVOs is here shown to co-localise with microglia and is accompanied by increased microglial activation and recruitment. In a chronic inflammatory condition like IBD, where cycles of inflammation and remission are continuous, the impact of successive increases in inflammatory enzymes like iNOS could potentially be more damaging over time.

Considering the fact that inflammation, indicated by the presence of iNOS and microglial activation, is observed following DSS-induced colitis in CVOs where the BBB is known to be more permeable, it is likely that peripheral inflammatory mediators are infiltrating the brain. However, no monocyte infiltration is detected despite the presence of monocytes in the choroid plexus in recovery. Alternatively, it is possible that the presence of inflammatory mediators in the circulation of these animals is enough to instigate inflammation at the CVOs. Therefore a further analysis of BBB integrity is warranted to elucidate whether the BBB is compromised in DSS-exposed animals compared to controls.

Chapter 5

DSS-Induced Colitis: CNS Structure and Function

Assessment of blood brain barrier integrity, ventricular volume, regional T1 and T2 relaxometry, blood perfusion and neuronal activation, in and beyond paraventricular zones

5.1 Introduction

The BBB is a tightly-controlled diffusion interface which regulates the transport of molecules between the peripheral circulation and the CNS. Endothelial cells of the BBB are non-fenestrated and have more extensive TJs acting as a protective barrier against pathogens and neurotoxic substances, while still permitting the influx of essential nutrients and neurotransmitters (Mayhan and Arrick, 2016). In the previous chapter, an increase in inflammatory markers is observed surrounding CVOs. These are regions of the brain which contain networks of fenestrated capillaries and therefore lack a BBB allowing them to communicate quickly with the periphery (Weiss et al., 2009). Tancycyte cells, bound together by TJs, form a barrier to separate these capillaries from the CSF and, ultimately, from the brain tissue. A loss of BBB integrity involving dysfunction or loss of TJ proteins would be likely to affect these regions most significantly.

Systemic inflammation can disrupt the BBB and has been linked to neurological disorders such as Alzheimer's disease and multiple sclerosis (Varatharaj and Galea, 2017). The gut-immune barrier and the BBB share many similarities (Daneman and Rescigno, 2009) and it is possible that mediators such as MMPs that impair barrier function in the gut during IBD could also affect barrier function at the BBB. It has been reported that the microbiota of the gut can modulate the BBB (Braniste et al., 2014), therefore dysbiosis of the microbiota, which occurs in IBD, may impact BBB integrity. The presence of intestinal inflammation and the increase in permeability of the GI barrier has been linked to an increase in the permeability of the BBB in IBD models. Rabbits show increased permeability of the BBB to sodium fluorescein (376 Da) but not to FITC-dextran (71 kDa) 48 h post-TNBS administration (Hathaway et al., 1999, Hathaway et al., 2000). Natah et al. (2005) report BBB permeability to sodium fluorescein (376 Da) but not IgG (156 kDa) at the OVLT, SFO, and ME, 24 and 48 hr following TNBS administration in rats. However, BBB integrity has yet to be investigated in the DSS colitis model.

MRI offers a non-invasive, translational tool with potential for longitudinal assessment of patients through the course of their illness, allows for comparison to healthy controls, and may be informed directly by pre-clinical studies. The clinical neuroimaging literature indicates alterations in regional brain volumes associated with depression and anxiety, including decreases in volume of cingulate, dorso-fronto-medial, and orbito-frontal cortices, the

hippocampus, and the striatum (Arnone et al., 2012, Bora et al., 2012, Sacher et al., 2012, Videbech and Ravnkilde, 2004, Grieve et al., 2013, van Tol et al., 2010). Increased lateral ventricular volume has also been reported in depression (Kempton et al., 2011). Depressed patients are also more likely to have white matter alterations with decreases in fronto-cortical white matter integrity and fibre directionality as determined by diffusion tensor imaging (Li et al., 2007, Bae et al., 2006, Nobuhara et al., 2006). BOLD-fMRI is an imaging technique used to detect local increases in blood oxygenation resulting from neuronal firing/activation. This can be measured during a task state or at resting state. Resting state is characterised by slow breathing and basal mental or physical activity. At basal conditions, resting state fMRI (rs-fMRI) can be used to analyse functional connectivity of regions, regional homogeneity and amplitude of low-frequency fluctuation (Lv et al., 2017). In terms of functional changes mapped by fMRI in IBD patients, one of the most common measures reported in depression is increased amygdalar activation in response to negative stimuli [review: Savitz and Drevets (2009)]. Resting state fMRI has also shown decreased whole-brain connectivity in depressed patients (Veer et al., 2010) as well as decreased activation in the dorsolateral prefrontal cortex at rest (Alcaro et al., 2010).

Pre-clinical MRI studies have shown similar alterations in brain structure and function in animal models of depression [for a review of the relevance of MRI as a translational tool in stress and depression see McIntosh et al. (2017)]. The WKY rat is a rodent model of anxiety and depression which endogenously expresses a strong anxious- and depressive-like phenotype. These behaviours have been linked with reductions in resting state fronto-cortical perfusion as measured by btASL (Gormley et al., 2016). Similar to clinical studies, ventricular enlargement has also been shown in animal models of depression (Gigliucci et al., 2014), along with the likely causative shrinking of other regions, such as the hippocampus, in the WKY and CUMS models (Gormley et al., 2016, Luo et al., 2014).

There has been a recent surge of interest in the clinical literature regarding the effects of IBD on brain structure and function using neuroimaging tools (Table 5.1). Literature originally focused on white matter lesions, visible in T2-weighted images, which were more prevalent in IBD patients compared to controls (Geissler et al., 1995, Hart et al., 1998, Chen et al., 2012). More recent studies have focused on showing brain morphologic changes observed in patients with IBD, although some conflicting studies show no differences in brain structure

between IBD patients and controls (Agostini et al., 2015). These morphological studies indicate that factors such as pain, disease severity, disease duration, inflammation and the presence of extra-intestinal manifestations, including anxiety/depression, influence brain volume measurements (Mrakotsky et al., 2016, Mrakotsky et al., 2017, Nair et al., 2016, Thomann et al., 2016, Bao et al., 2015). In CD patients, during remission, rs-fMRI has been used to assess neural responses to pain and regional homogeneity, and analysis has shown altered responses in the insula and medial cingulate cortex related to the severity of abdominal pain in IBD patients (Bao et al., 2016). A small study ($n = 4$) has shown that, in CD patients whose IBD symptoms respond to the anti-TNF treatment, infliximab, during the first 2 weeks ($n = 3$), pain perception in the brain is reduced within 24 hours of receiving infliximab (Hess et al., 2015). In terms of task fMRI studies, CD patients in remission show decreased neural activity in amygdala, hippocampus, insula, putamen and cerebellum in response to stress in the stroop task, used to enhance the stress response and engage brain regions associated with habituation, indicative of decreased habituation to stress (Agostini et al., 2013b). The same group tested UC patients in remission in an emotional picture task and fMRI data indicated decreased activity in the amygdala in response to positive stimuli with no alterations in response to neutral or negative stimuli (Agostini et al., 2011).

Table 5.1 MRI studies of brain alterations in IBD patients

Study	Participants	Method/Modality	Results & Authors Conclusions
(Mirakotsky et al., 2017) (oral presentation)	children: CD & healthy controls	T1-weighted MRI images, tests of IQ, attention, emotion & memory	CD patients display reduced GM (cortical thickness) in posterior regions (including supramarginal and parietal regions), and reduced subcortical volumes in hippocampus and thalamus. Decreased cognitive and memory scores were observed in CD patients. Associations were reported between circulating cytokines, NK cells and NFkB1 gene expression with GM changes.
(Bao et al., 2016)	adults: CD-R & healthy controls	rs-fMRI - regional homogeneity	Resting-state brain activities differ between remissive CD patients with and without abdominal pain. Abnormal activities in insula and medial cingulate cortex are related to the severity of abdominal pain.
(Mirakotsky et al., 2016) (oral presentation)	children: CD & healthy controls	T1-weighted MRI images, DTI images	Relative to controls, CD patients show reduced GM volumes in parietal and supramarginal regions and in the middle frontal gyrus. CD patients also had reduced subcortical volumes in hippocampus, right thalamus, and bilateral putamen. White matter fibre density was altered in limbic tracts (cingulate and uncinate fasciculus). Disease severity was negatively associated with brain structure. Inflammation in active disease was associated with cortical thinning, reduced white matter density and impaired memory and cognition.
(Nair et al., 2016)	adults: CD-R & healthy controls	T1-weighted MRI images, IPIP, CES-D BIS/BAS, PANAS, VF cognitive task, memory task	CD was associated with cortical thickening in the left superior frontal cortex and decreased subcortical volumes (putamen and pallidum). Pain and disease duration were negatively correlated with subcortical volume. CD patients differed from controls in BAS-fun-seeking, BAS-drive and BIS, and backward and total digit span memory tests. Correlations between brain measures and behavioural responses suggest that structural brain changes associated with CD may lead to alterations in processing and responding to emotional and painful stimuli.

(Thomann et al., 2016)	adults: CD-R & healthy controls	T1-weighted MRI images, MoCA, BDI, WEIMUS	No differences in GM (cortical thickness, area, or folding) between CD patients and healthy controls. CD patients with extra-intestinal manifestations showed differences in cortical area and folding but not thickness compared to healthy controls independent of psychological factors. Non-significant trend towards higher depression and fatigue scores was observed in CD.
(Agostini et al., 2015)	adults: UC-R & healthy controls	T1-weighted MRI images	No brain morphological changes were observed in GM volumes of UC patients compared to controls measured by VBM. No patients had extra-intestinal manifestations and clinical course of UC was very mild.
(Bao et al., 2015)	adults: CD-R, healthy controls	T1-weighted MRI images, HADS-A, HADS-D	Decreased GM volumes were observed in CD patients in the cerebral cortex, whereas increased GM volumes were observed primarily in subcortical regions including thalamus, hippocampus and amygdala, and in the cerebellum and brain stem. Cortical thickness was reduced in the insula, cingulate cortex, parahippocampal cortex and other regions of frontal, temporal and parietal cortices in CD patients. HADS-A and HADS-D scores were significantly higher in CD patients compared to controls. Changes in GM volume in regions involved in emotional processing were associated with anxiety and depression factors.
(Hess et al., 2015)	adults: CD (n = 4)	BOLD fMRI with/ without pain (abdominal compression)	Anti-TNF therapy (infliximab) decreased pain perception in the brain of responding CD patients within 24 hours as measured by whole brain BOLD signal.

(Zikou et al., 2014)	adults: CD, UC & healthy controls	T1-weighted MRI images, DTI images & sagittal FLAIR	Higher prevalence of hyper-intensities in white-matter in IBD patients (66%) compared to controls (45%). VBM showed decreased GM volume in IBD patients relative to control in multiple regions of the frontal, temporal and parietal cortices. Decreased axial diffusivity in right white matter corticospinal tracts in IBD.
(Agostini et al., 2013a)	adults: CD-R & healthy controls	T1-weighted MRI images	Decreased GM volume in the dorsolateral prefrontal cortex and anterior midcingulate cortex. Disease duration was negatively correlated with GM volumes in the PFC and cingulate cortex.
(Agostini et al., 2013b)	adults: CD-R & healthy controls	BOLD fMRI (stroop task)	CD patients showed decreased habituation response to stress compared to controls with differences in neural activity observed in the amygdala, hippocampus, insula, putamen, and cerebellum.
(Chen et al., 2012)	adults: CD & healthy controls	T2-weighted MRI images	Higher prevalence of T2 hyper-intensities in white-matter in CD patients (75%) compared to controls (34%).
(Agostini et al., 2011)	adults: UC-R & healthy controls	BOLD fMRI (emotional picture task)	BOLD signal was decreased in the amygdala of UC patients in response to positive stimuli. There were no differences in the amygdala in response to negative or neutral stimuli. No differences in GM volume between groups were observed using VBM.
(Hart et al., 1998)	adults: CD, UC & controls	T2-weighted MRI images	T2-weighted images showed white matter lesions in 12.5% of IBD patients and 5% of controls (non-significant).
(Geissler et al., 1995)	adults: CD, UC & healthy controls	T2-weighted MRI images	T2-weighted images showed white matter lesions in CD (42%) and UC (46%) patients and healthy controls (16%). A longer disease duration and older age were associated with an increased tendency for the lesions. The authors note that none of the patients had neurological symptoms.

Table 5.1 MRI studies of brain alterations in IBD patients

Beck Depression Inventory (BDI), Behavioural Inhibition Scale and Behavioural Activation Scale (BIS/BAS), blood-oxygen level dependant (BOLD), Centre for Epidemiologic studies-Depression scale (CES-D), Crohn's disease in remission (CD-R), diffusion tensor imaging (DTI), grey matter (GM), International Personality Item Pool (IPIP), Montreal Cognitive Assessment (MoCA), periaqueductal grey (PAG), Positive Activation/Negative Activation Schedules (PANAS), pre-frontal cortex (PFC), resting state functional magnetic resonance imaging (rs-fMRI), ulcerative colitis in remission (UC-R), Verbal Fluency (VF), voxel based morphometry (VBM), Wurzbürger Fatigue Inventory for Multiple Sclerosis (WEIMuS)

The activation of a neuron by an external stimulus will prompt a host of intracellular signalling cascades resulting in gene transcription. Immunohistochemical mapping of immediate early genes may be used as a method for assessing neuronal activation *post-mortem*. cFos is a well-known example of an immediate early gene, a family of genes induced in a neuron immediately following an action potential. The expression of these genes are therefore used to indirectly measure neuronal activity and map out regions of brain activation [for reviews see Kovacs (2008) and Okuno (2011)]. cFos was the first of the family of Fos genes to be described. Upon neuronal activation it is expressed rapidly but has a short half-life, with a peak in protein levels occurring between 90-120 minutes, and so is best used to monitor short term neuronal activity (Okuno, 2011). Δ FosB is a truncated splice variant of the FosB protein, a close relative of cFos, which has the unusual characteristic in the Fos family of having an extended half-life. Although Δ FosB is produced in smaller quantities to cFos, due to its relatively long half-life it accumulates over time and can therefore be best employed as a marker of long-term neuronal activation (McClung et al., 2004). Δ FosB heterodimerises with Jun family proteins to form AP-1 complexes which bind to AP-1 sites within gene promoters, thereby regulating gene transcription.

Original evidence of cFos activation in the nervous system following induction of colitis was published by Miampamba and Sharkey (1999). Colitis was induced following a per-endoscopic injection of formalin and rats were euthanised 2 hours later. Immunohistochemical analysis demonstrated a significant increase in cFos in the lumbosacral spinal cord (LSC), and in two CVOs: nucleus of the solitary tract (NST) and area postrema (AP). Treatment with the α -2-adrenoceptor agonist xylazine inhibited the colitis-related increase in regional cFos expression. The expression of cFos as a marker of neuronal activation has also been utilised in the TNBS model of acute colitis. Porcher et al. (2004) extensively analysed the expression of cFos 2 hours post-TNBS administration throughout the brain. They report significant increases in cFos immunostaining across a number of brain regions including nuclei involved in the autonomic, behavioural and neuroendocrine response to inflammation, in most CVOs, and in CRF pathways, particularly the PVN of the hypothalamus. At 6 hours post-TNBS administration, cFos mRNA expression in the PVN had completely returned to basal levels. Later studies focused on TNBS-induced cFos activation in periventricular gray, hypothalamic/visceral thalamic stress axes and cortical domains, and septal/preoptic/amygdalar brain areas (Hsiao, 2014). Results from this study support previous

evidence of increased cFos induction following experimentally-induced colitis, in addition to patterns of prolonged neuronal activation (Welch et al., 2005). In a later study, this group showed that subdiaphragmatic vagotomy did not inhibit the observed increase in cFos induction in the PVN of the hypothalamus, basolateral amygdala, CeA, and piriform cortex indicating the unlikely role of the vagus nerve in mediating the brain activation response in these regions (Welch et al., 2010). However, a separate study which used *C. jejuni* infection to induce intestinal inflammation in mice, showed cFos induction in vagal sensory ganglia and in the NST - the primary sensory vagal afferent nucleus indicating that intestinal inflammation signals to the brain via the vagus nerve (Goehler et al., 2005). The presence of cFos and Δ FosB have previously been mapped in the brains of animals challenged with peripheral LPS injection (Frenois et al., 2007). Initial increases in cFos are linked to the original sickness behaviour resulting from LPS, however the authors conclude that later increases in Δ FosB are related to post-sickness depressive-like behaviour.

5.1.1 Aims

The aim of this chapter is to use MRI and immunohistochemistry to further characterise the DSS-induced colitis in terms of BBB integrity, and tissue structure and function in the CNS. Specifically, the main objectives are to examine BBB integrity, blood perfusion, brain volumetrics, tissue relaxometry data, and neuronal activation following DSS-induced colitis.

5.2 Methods

5.2.1 Study design

Experiments were carried out in order to analyse the effects of DSS-induced colitis during acute colitis and recovery from colitis in a range of behavioural tests in rats as described earlier (Chapter 3). Perfused whole brains and snap-frozen brain and peripheral tissues were obtained from these experiments and assessed for peripheral and central inflammation as described earlier (Chapters 3 & 4). MRI experiments were also performed in a subgroup of rats and *post-mortem* tissue collected was used to investigate BBB integrity and neuronal activation.

A detailed breakdown of each experiment and the *in vivo* and *ex vivo* work carried out for each experiment is provided in Section 2.2.14 and summarised in Figure 2.16.

5.2.2 Experimental protocols

All procedures in this chapter were conducted as described in detail in Chapter 2 and are outlined briefly below.

5.2.2.1 DSS-colitis induction

Male Wistar rats (175-250 grams) were obtained from the Bio-Resources Unit in Trinity College Dublin and housed under standard conditions with access to food and water *ad libitum*. The body weight, food consumption and fluid intake of each rat were recorded as a daily indication of welfare. The rats were monitored for signs of disease activity according to a modified version of the DAI (Table 2.1). A 5% w/v DSS solution was used to induce symptoms of colitis. DSS was administered until a rat reached a DAI score of ≥ 2.5 at which point the rat was given normal tap water.

5.2.2.2 Tissue collection

Rats were anaesthetised by i.p. urethane injection (urethane 1.5 g/kg) and transcardially perfused via perfusion pump with PBS (10 mM; pH 7.4) for a minimum of 5 minutes followed by ice-cold PFA (4% w/v in PBS; pH 7.4) for a minimum of 10 minutes. Brains were removed from the skull and post-fixed in 4% PFA at 4°C for 48 hours prior to cryoprotecting in sucrose solution (sucrose 30% w/v in 10 mM PBS) at 4°C for 3-4 days followed by snap-freezing in

isopentane on dry ice. Snap-frozen brains were stored at -80°C until sectioning. Brain sections (30 µm thick) were cut using a cryostat at -21°C. Ten series of slices were collected per brain with the slices in each series being taken approximately 300 µm apart. Each series of slices were stored in freezing storage solution (30% v/v ethylene glycol, 30% w/v sucrose in 10 mM PBS) at -80°C until use.

5.2.2.3 Immunohistochemistry

All steps for immunostaining protocols were carried out at room temperature with gentle agitation and each rinse step consisted of two 5 minute washes in 10 mM PBS at pH 7.4 unless otherwise specified. Free-floating sections were rinsed and incubated in 0.75% H₂O₂ in 5% MeOH in PBS for 20 minutes to quench endogenous peroxidases and rinsed again. Slices were incubated in 10% NS in 10 mM PBS for 30 minutes to block non-specific proteins. Slices were washed once in PBS and incubated overnight with primary antibody (ΔFosB: rabbit anti-ΔFosB polyclonal antibody 1:2500; cFos: rabbit anti-cFos polyclonal antibody 1:6,500; IgG: biotinylated goat anti-rat IgG 1:1000) in 10 mM PBS with 5% NS and 0.05% Triton X-100. The following day, slices were rinsed and incubated for 90 minutes in a biotinylated secondary antibody (anti-rabbit IgG; 1:200) in 10 mM PBS with 1% NS [this step is omitted for IgG staining]. Sections were rinsed and Vectastain ABC reagent was applied for 90 minutes. A peroxidase reaction was performed to visualize immunolabelling by incubating sections with DAB (0.5 mg/ml) in 0.01% H₂O₂ in PBS for 7 minutes before stopping the reaction rinsing in dH₂O and 10 mM PBS. Sections were then mounted onto gelatin-coated slides, air-dried, desalted in dH₂O, dehydrated in ethanol, cleared in xylene, and coverslipped in DPX. To assess antibody specificity, incubation with the primary antibody was omitted for some sections, and no significant staining was observed in this case.

5.2.2.4 Microscopy and analysis

Co-ordinates of coronal planes and limits of the various structures analysed were defined according to the rat brain atlas (Paxinos and Watson, 2007). The locations from bregma of analysed structures are illustrated in Chapter 2 (Figure 2.15). Bilateral structures were analysed on each hemisphere, where available, and counts were averaged. Cell counts were measured as follows: three identical boxes of known area (200 µm²) were placed at random on the image and cells of interest were counted per box and averaged. All cell counting was performed by researchers blind to the treatment group.

Brain slices stained with DAB were imaged using brightfield microscopy. ROIs were imaged at a magnification of 100x and further analysed using the ImageJ software program. DAB-positive staining was defined as a dark brown cell stain.

5.2.2.5 *Magnetic resonance imaging system*

MRI was carried out on a dedicated rodent Bruker Biospec system with a 7 T magnet and a 30 cm diameter bore, equipped with a 20 cm actively-shielded gradient system. A pair of actively decoupled 12 cm Helmholtz transmitter and 3 cm surface quadrature receiver coils were used for signal transmission and reception, respectively. ParaVision 6.0 software was used for data reconstruction and analysis. Total scanning time was not greater than 90 minutes per rat.

5.2.2.6 *Animal anaesthesia and preparation*

Animals were initially anaesthetised using 5% isoflurane in 100% oxygen and after induction received a subcutaneous injection of 1 mg/kg medetomidine (0.07 mg/mL in 0.9% w/v sterile saline). Sedation was maintained throughout scanning with continuous infusion of medetomidine at 0.14 mg/kg/hr beginning ten minutes after the initial bolus dose. Animals were subsequently placed onto a custom-built fibreglass warmed cradle. Anaesthetic depth was controlled by maintaining respiration rate in the range of 60 to 75 breaths per minute. The receiver coil was placed over the skull of the animal and fixed with adhesive tape. The cradle was then inserted into the bore of the scanner.

5.2.2.7 *MRI sequences*

Animal positioning

An initial pilot positioning image was acquired using a fast gradient echo scan and a single-slice high contrast RARE scan taken at the isocentre of the magnetic field to ensure that the imaging region was centred at distance from bregma = 3.60 mm according to a rat brain atlas (Paxinos and Watson, 2007). The pilot scan was taken using the following acquisition parameters: slice thickness = 1.5 mm, TR = 3134.511 ms, TE = 12 ms, RARE factor = 8, RF flip angle = 90° / 180°, FOV = 30 x 30 mm, image matrix = 128 x 128 and total scan time was 50 seconds.

High resolution anatomical sequence

High resolution anatomical images were acquired using T2-weighted MR axial images that were collected using a RARE sequence. The following acquisition parameters were used: FOV = 4.00 x 3.00 cm, image matrix = 266 x 200, 64 x 0.5 mm slices, TR= 6.26 s, TE = 36.00 ms.

Echo planar imaging sequence – Bolus tracking arterial spin labelling

An echo planar imaging sequence was used to provide signal-time curves of the passage of a 3 second bolus through a region of interest. The following acquisition parameters were used: slice thickness = 2 mm, TR = 1300 ms, TE = 12.287 ms, RF flip angle = 90°, FOV = 30 x 30 mm, image matrix = 128 x 128. Four repetitions of each image type were acquired for signal averaging.

Relaxometry sequences

T1 relaxation times were calculated from a RARE-VTR image. The following acquisition parameters were used: slice thickness = 1.5 mm, VTE = 25.3 ms, RF flip angle = 180°, FOV = 3.0 x 3.0 cm, image matrix = 128 x 128 with varying repetition time, using values of 300.0, 589.12, 942.3, 1396.1, 2032.0, 3103.1 and 8000.0 ms.

T2 relaxation times were calculated from an MSME image. The following acquisition parameters were used: slice thickness = 1.5 mm, VTR = 2000 ms, RF flip angle = 180°, FOV = 3.0 x 3.0 cm, image matrix = 128 x 128 with VTE, using values of 8.06, 16.12, 24.18, 32.24, 40.29, 48.35, 56.41, 64.47, 72.53, 80.59, 88.65 and 96.71 ms. An echo train of 12 values was used to ensure signal was at noise level by the last echo times to ensure accuracy of T2 estimation. Only the central slice of the MSME scan was used for analysis of T2 relaxation times.

5.2.2.8 MRI data analysis

Brain volume measurements

MIPAV software was used to manually define ROIs, slice by slice, along the coronal plane. ROIs analysed included both lateral and third ventricular volume, and hippocampus. The regions were defined with reference to anatomical landmarks set out in the rat brain atlas (Paxinos and Watson, 2007) (Table 2.2). Manual tracing of these regions along the coronal plane formed a three-dimensional mask which was then quantified using MIPAV tools. This was normalised for brain size by calculating the percentage volume of each region relative to total brain volume.

Relaxometry analysis

Analysis of T1 and T2 relaxation times was performed on the RARE-VTR and MSME images respectively. All analysis was performed using the image sequence analysis tool in the Bruker ParaVision 6.0 software package. ROIs were selected using the ROI tool and included the SFO, the cingulate cortex, the motor cortex, the somatosensory cortex and the striatum (caudate putamen) 0.72 mm posterior to bregma bilaterally (Figure 2.10). The same regions in each animal were used for the analysis of both T1 and T2 relaxation times.

Bolus tracking arterial spin labelling

Paravision 6.0 and IDL software were used to generate perfusion weighted maps from the data. ImageJ software was used to select ROIs for analysis with reference to the rat stereotactic atlas (Paxinos and Watson, 2007). Analysis of one coronal brain section comprised cingulate, motor and somatosensory cortices as well as the SFO and striatum (Figure 2.9). IDL was used to perform a subtraction of the labelled from the control images generated by the ASL sequence. MTT, CTT and SA values were generated by fitting the non-compartmental model of cerebral perfusion to the experimental data (Kelly et al. 2009). Mathematica was used to calculate MTT and CTT from the first and second statistical moments of the signal-time curves respectively.

A simplified description of the principles of MRI can be found in Chapter 2 (Section 2.2.6).

5.3 Results

5.3.1 Blood brain barrier integrity in DSS-induced colitis

Evidence from Chapters 3 and 4 indicates that peripheral inflammation is communicated to the brain resulting in neuroinflammation, primarily at paraventricular areas where the BBB is more permeable. It is important to determine whether the BBB is compromised in DSS-induced colitis and to what extent. Three methods of assessing the integrity of the BBB were employed: IgG immunohistochemistry as described below, contrast (gadolinium)-enhanced MRI, and biotin immunofluorescence (Appendix 2).

5.3.1.1 *IgG immunoreactivity*

IgG is an endogenous circulating immunoglobulin which infiltrates an impaired BBB, and was therefore used to detect BBB permeability at the SFO and ME (Figure 5.1). One-way ANOVA of IgG immunoreactivity showed an effect of DSS ($F_{2,10} = 4.176$; $p = 0.0045$) (A) at the SFO. *Post hoc* analysis revealed an increase in IgG immunoreactivity at the SFO in acute colitis only relative to control ($p < 0.01$). One-way ANOVA of IgG immunoreactivity showed no effect of DSS ($F_{2,12} = 3.124$; $p = 0.0809$) (B) at the ME.

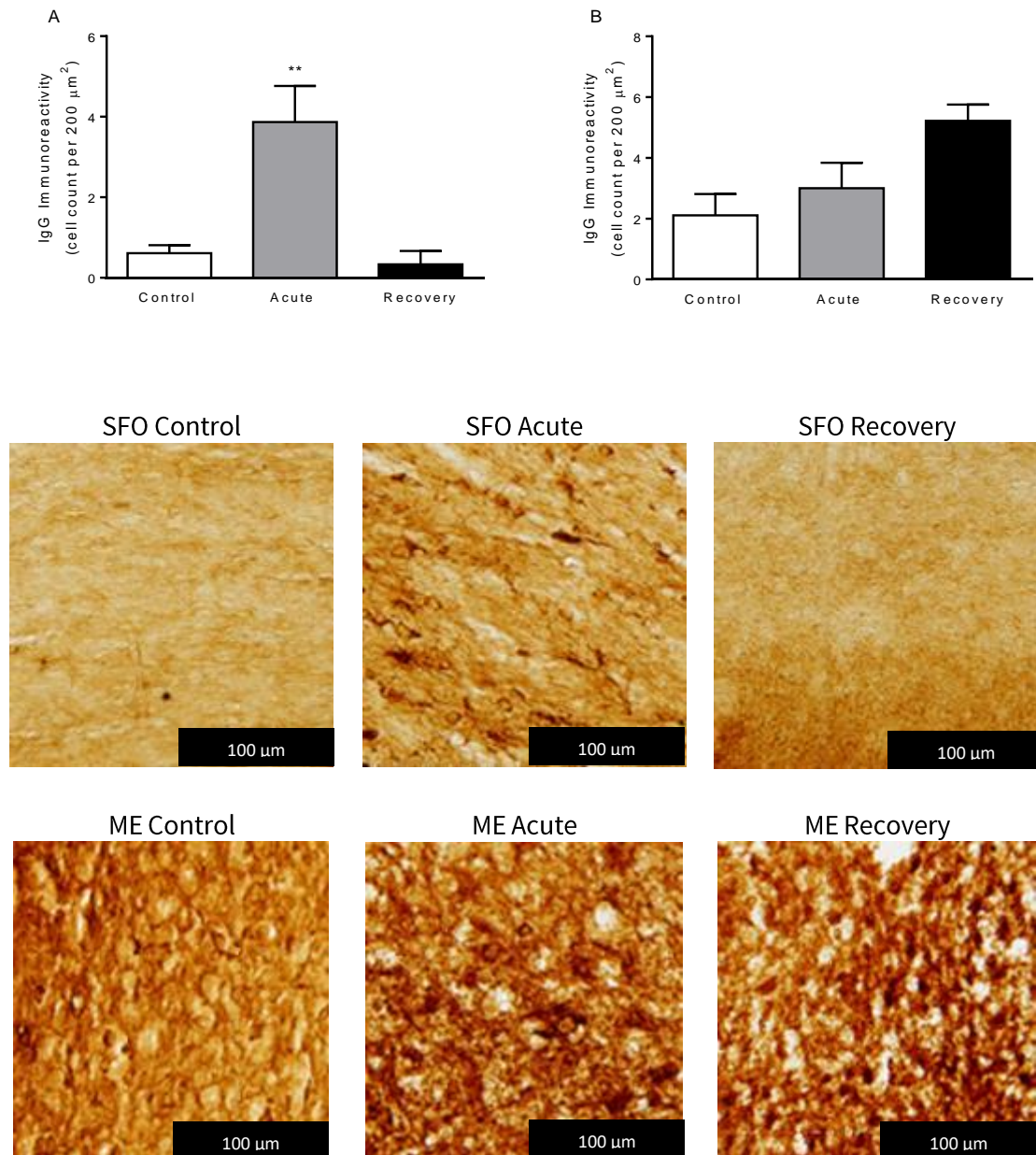


Figure 5.1 IgG immunoreactivity at the SFO and ME following DSS-induced colitis

The ventral hippocampal commissure surrounding the SFO (A) and the arcuate nucleus surrounding the ME (B) were assessed for increases IgG immunoreactivity per 200 μm^2 in acutely colitic and recovered rats following exposure to DSS-induced colitis. DSS-induced colitis increases IgG immunoreactivity at the SFO in acute only. Images show representative 200 μm^2 areas of IgG immunohistochemical staining at the SFO and ME in each group at 200x magnification. The data presented here comes from rats in DSS study 6.

Data are presented as mean \pm SEM ($n = 3-6$). ** $p < 0.01$ relative to control (one-way ANOVA with Newman Keuls *post hoc* test).

5.3.2 Alterations in brain MRI following DSS-induced colitis in rats

Rats were assessed in a 7 T rodent MRI scanner at both acute and recovered DSS-induced colitis phases. This allowed *in vivo* measurement of cortical and subcortical volumes, T1 and T2 relaxation times, and cerebral blood perfusion.

5.3.2.1 *DSS-induced colitis results in changes to ventricular and hippocampal volume*

High resolution anatomical scans were obtained at acute and recovery timepoints and used to measure ventricular (lateral and third ventricles) and hippocampal volumes as percentages of total brain volume. One-way ANOVA of ventricular volume as % of total brain volume showed an effect of DSS ($F_{2,26} = 7.459$; $p = 0.0028$) (Figure 5.2). *Post hoc* analysis revealed an increase in ventricular volume as % of total brain volume in both the acute colitis and recovered group relative to control ($p < 0.05$). One-way ANOVA of hippocampal volume as % of total brain volume showed no effect of DSS ($F_{2,15} = 0.3516$; $p = 0.7092$) (Figure 5.3). One-way ANOVA of total brain volume confirmed no effect of DSS (data not shown).

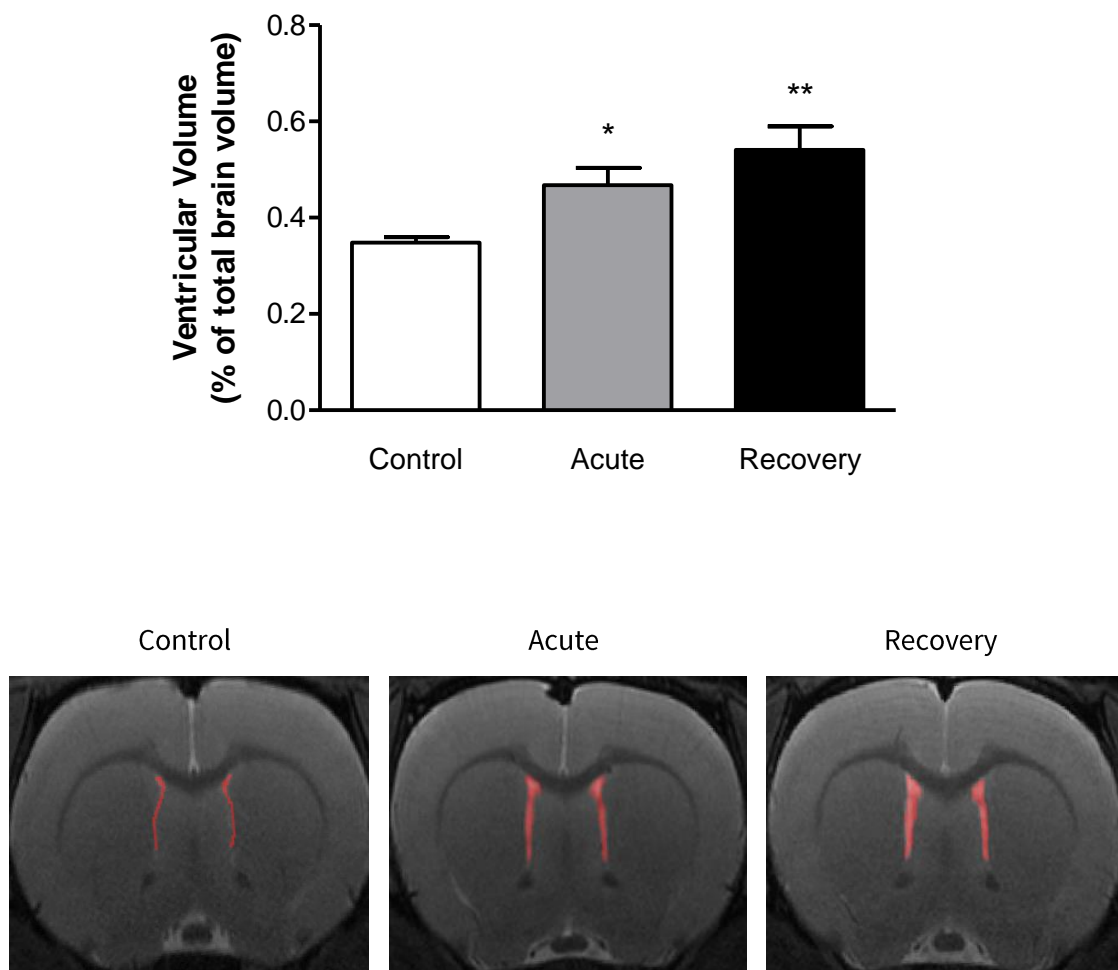


Figure 5.2 Brain ventricular volume following DSS-induced colitis

A high resolution anatomical sequence was used to enable mapping of volume changes in serial sections through the whole brain. Ventricular volume is expressed as a percentage of total brain volume and is increased in acute and recovered DSS-induced colitis compared to control. Representative images show sample slices of a control, acute and recovery brain with the lateral ventricles highlighted in red. The data presented here comes from rats in DSS study 5.

Data are presented as mean \pm SEM ($n = 8-11$). ** $p < 0.01$, * $p < 0.05$ relative to control (one-way ANOVA with Newman-Keuls *post hoc* test).

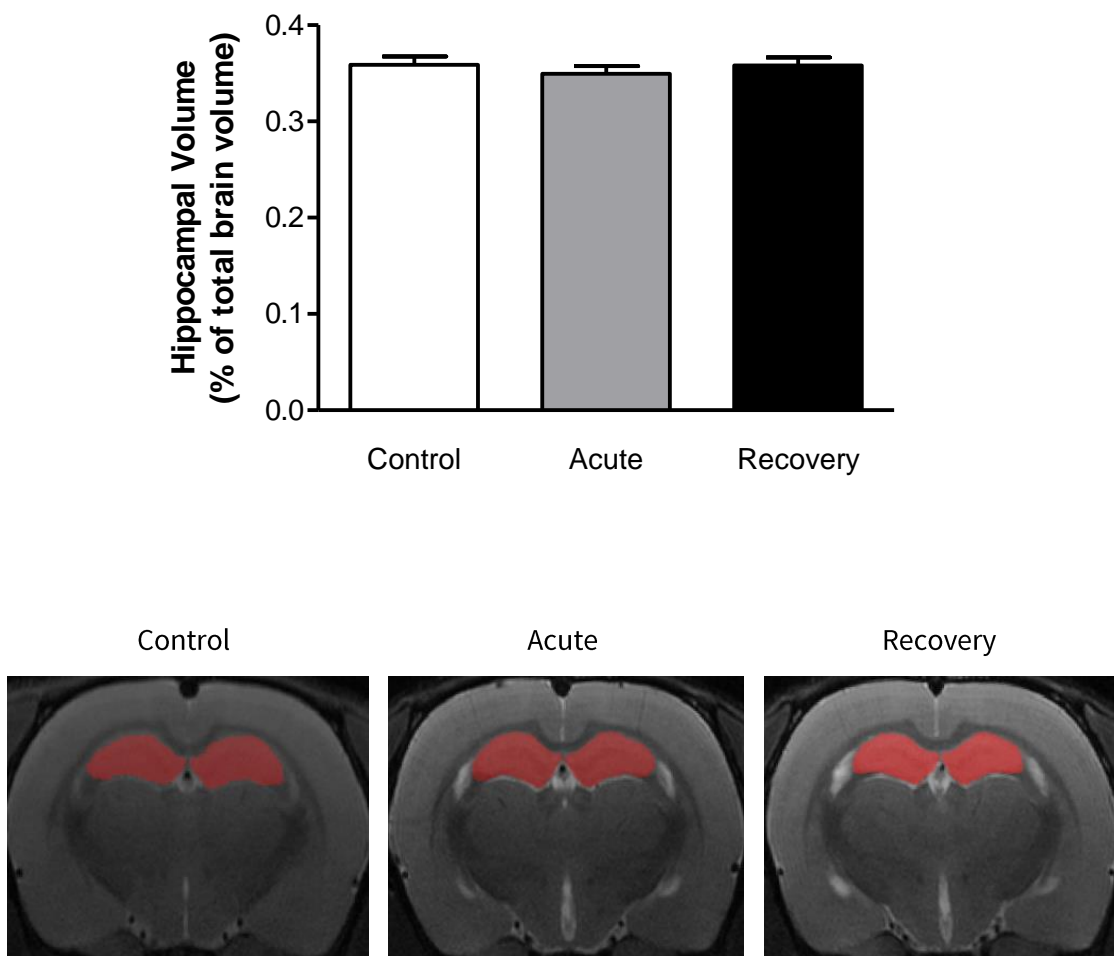


Figure 5.3 Brain hippocampal volume following DSS-induced colitis

A high resolution anatomical sequence was used to enable mapping of volume changes in serial sections through the whole brain. Hippocampal volume is expressed as a percentage of total brain volume and is not altered in acute and recovered DSS-induced colitis compared to control. Representative images show sample slices of a control, acute and recovery brain with the hippocampus highlighted in red. The data presented here comes from rats in DSS study 5. Data are presented as mean \pm SEM ($n = 5-6$). (one-way ANOVA).

5.3.2.2 *DSS-induced colitis is associated with changes in T2 but not T1 relaxation times*

T1 and T2 relaxation times were measured bilaterally at 0.72 mm posterior to bregma as described in Chapter 2 (Figure 2.10). One-way ANOVA of T1 relaxation times showed an effect of DSS in the striatum ($F_{2,29} = 4.340$; $p = 0.0232$) (D), but no effect of DSS in the cingulate ($F_{2,29} = 2.760$; $p = 0.0812$) (A), sensory ($F_{2,29} = 3.178$; $p = 0.0576$) (B), or motor ($F_{2,29} = 3.216$; $p = 0.0559$) (C) cortices, or at the SFO ($F_{2,29} = 1.099$; $p = 0.3477$) (E) (Figure 5.4). *Post hoc* analysis revealed no changes in T1 relaxation times in the striatum relative to control. One-way ANOVA of T2 relaxation times showed an effect of DSS in the cingulate ($F_{2,29} = 6.557$; $p = 0.0048$) (A), sensory ($F_{2,29} = 6.826$; $p = 0.0040$) (B), and motor ($F_{2,28} = 4.527$; $p = 0.0206$) (C) cortices, however, no effect of DSS was shown in the striatum ($F_{2,29} = 3.319$; $p = 0.0514$) (D), or at the SFO ($F_{2,29} = 2.487$; $p = 0.1020$) (E) (Figure 5.5). *Post hoc* analysis revealed a decrease in T2 relaxation times in cingulate, motor, and sensory cortices in the recovery group relative to control ($p < 0.05$).

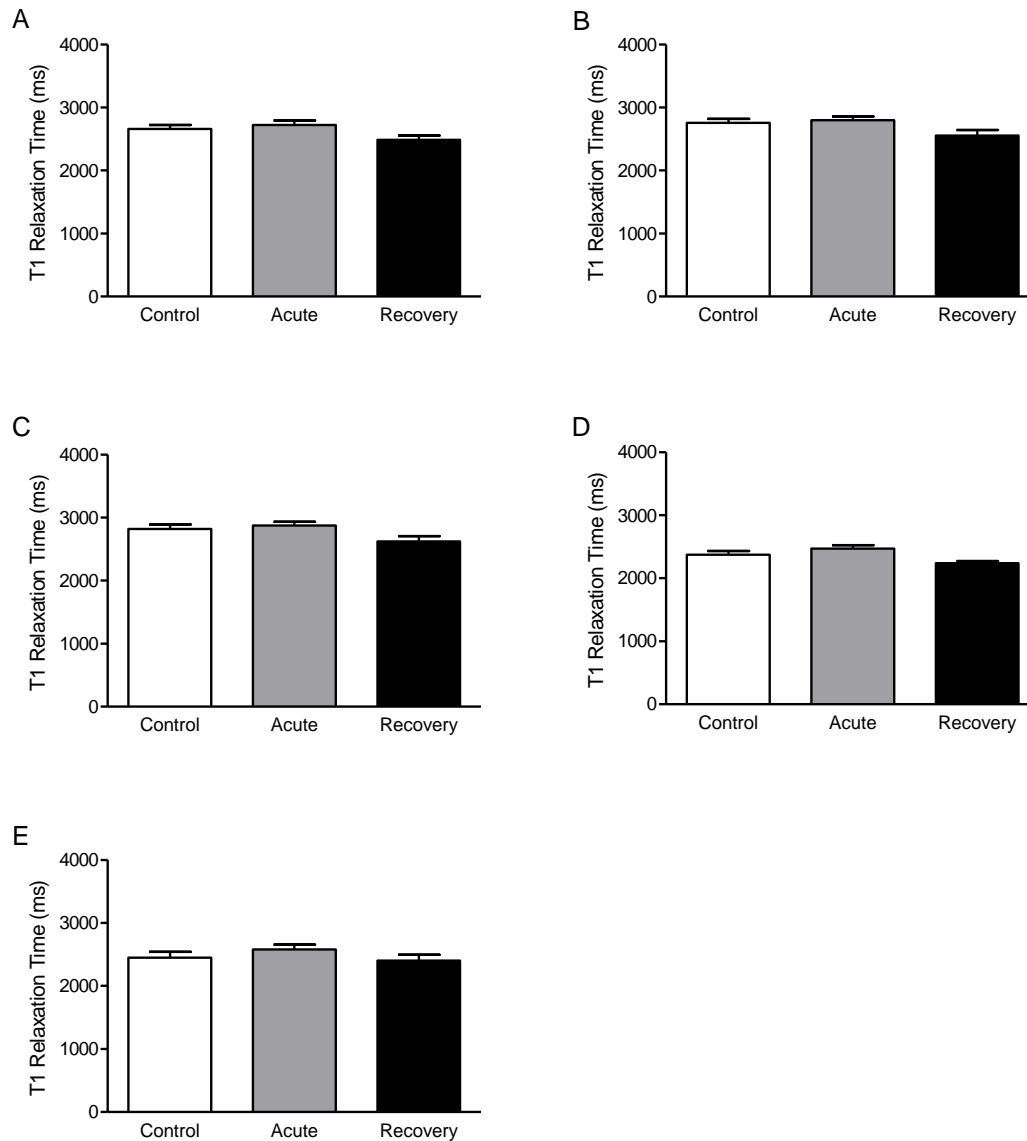


Figure 5.4 T1 relaxometry measures following DSS-induced colitis

The effects of DSS-induced colitis were assessed on T1 relaxation times in the cingulate (A), sensory (B), and motor (C) cortices, in the striatum (D) and at the SFO (E) in acutely colitic and recovered rats. No changes in T1 relaxation times were observed relative to control. The data presented here comes from rats in DSS study 5.

Data are presented as mean \pm SEM ($n = 8-11$). (one-way ANOVA).

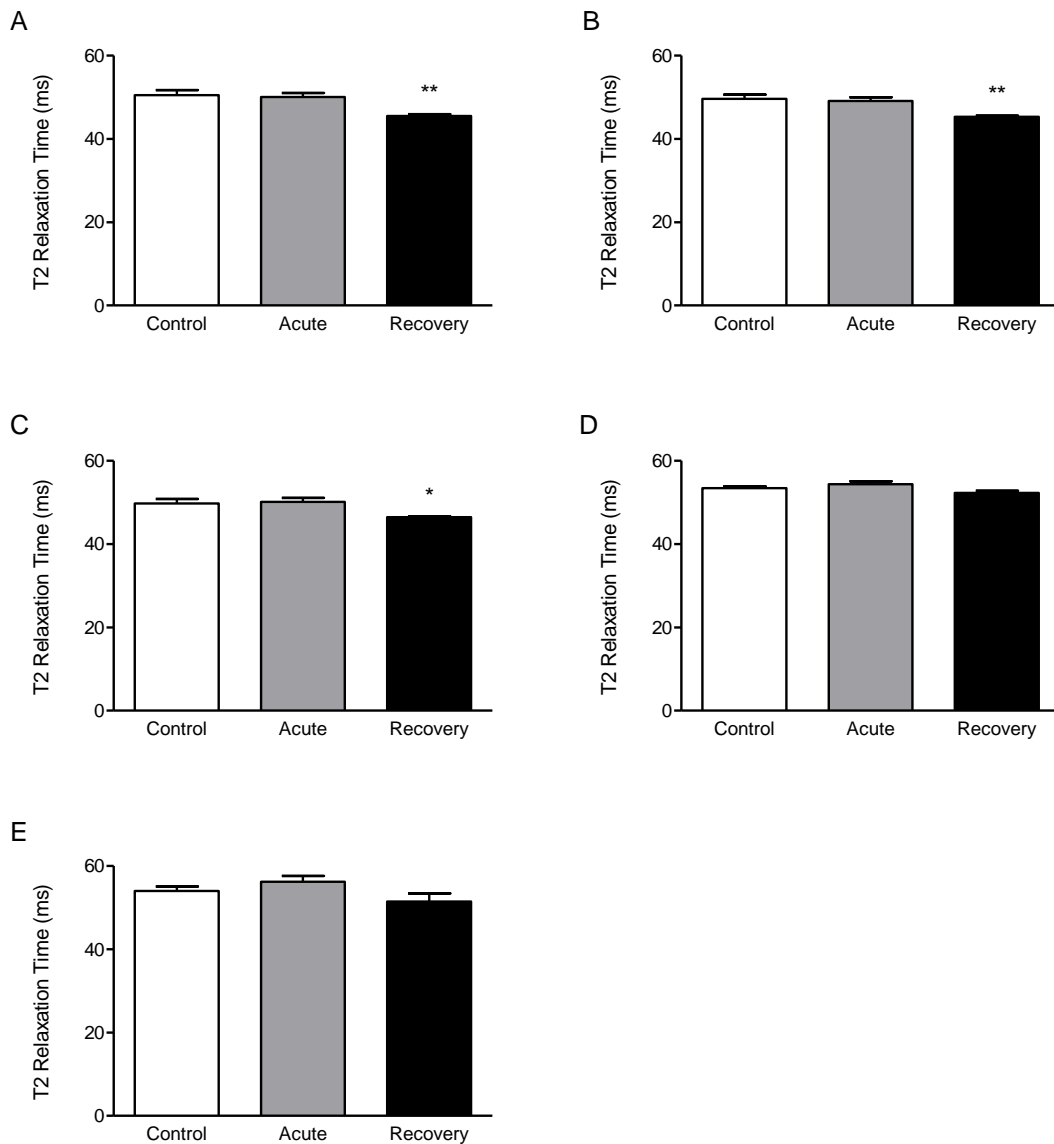


Figure 5.5 T2 relaxometry measures following DSS-induced colitis

The effects of DSS-induced colitis were assessed on T2 relaxation times in the cingulate (A), sensory (B), and motor (C) cortices, in the striatum (D) and at the SFO (E) in acutely colitic and recovered rats. T2 relaxation is decreased relative to control in the cingulate, sensory and motor cortices. The data presented here comes from rats in DSS study 5.

Data are presented as mean \pm SEM ($n = 8-11$). * $p < 0.05$, ** $p < 0.01$ relative to control (one-way ANOVA with Newman-Keuls *post hoc* test).

5.3.2.3 *DSS-induced colitis is not associated with cerebral blood perfusion alterations*

btASL was used to assess regional cerebral blood perfusion in acute and recovered DSS-induced colitis (Table 5.2). One-way ANOVA of MTT showed no effect of DSS in the cingulate ($F_{2,24} = 0.4194$; $p = 0.6622$), sensory ($F_{2,24} = 0.4976$; $p = 0.6141$), or motor ($F_{2,24} = 1.026$; $p = 0.3738$) cortices, or in the striatum ($F_{2,22} = 1.347$; $p = 0.2806$). One-way ANOVA of MTT showed an effect of DSS at the SFO ($F_{2,24} = 3.733$; $p = 0.0388$). *Post hoc* analysis revealed an increase in MTT values in the acute colitis group relative to control ($p < 0.05$). One-way ANOVA of CTT showed no effect of DSS in the cingulate ($F_{2,23} = 1.788$; $p = 0.1897$), sensory ($F_{2,24} = 0.7783$; $p = 0.4704$), or motor ($F_{2,23} = 2.338$; $p = 0.1191$) cortices, in the striatum ($F_{2,22} = 2.100$; $p = 0.1464$), or at the SFO ($F_{2,21} = 2.081$; $p = 0.1498$). One-way ANOVA of signal amplitude showed no effect of DSS in the cingulate ($F_{2,22} = 0.6634$; $p = 0.5251$), sensory ($F_{2,24} = 1.751$; $p = 0.1951$), or motor ($F_{2,24} = 2.230$; $p = 0.1293$) cortices, in the striatum ($F_{2,22} = 1.718$; $p = 0.2026$), or at the SFO ($F_{2,24} = 0.1022$; $p = 0.9033$).

Table 5.2 Cerebral blood perfusion following DSS-induced colitis as measured by btASL

		Control	Acute	Recovery
SFO	MTT (s)	1.702 ± 0.08	2.059 ± 0.12*	1.805 ± 0.07
	CTT (s)	1.404 ± 0.03	1.508 ± 0.05	1.416 ± 0.03
	Amplitude	0.09814 ± 0.01	0.09942 ± 0.01	0.09418 ± 0.004
Cingulate Cortex	MTT (s)	1.989 ± 0.09	2.062 ± 0.06	2.094 ± 0.10
	CTT (s)	1.458 ± 0.06	1.617 ± 0.06	1.653 ± 0.10
	Amplitude	0.09926 ± 0.01	0.09136 ± 0.003	0.09309 ± 0.001
Motor Cortex	MTT (s)	2.1 ± 0.07	2.006 ± 0.091	1.943 ± 0.03
	CTT (s)	1.554 ± 0.05	1.458 ± 0.04	1.444 ± 0.01
	Amplitude	0.09878 ± 0.01	0.08703 ± 0.004	0.08205 ± 0.003
Sensory Cortex	MTT (s)	2.028 ± 0.06	2.032 ± 0.09	1.937 ± 0.04
	CTT (s)	1.513 ± 0.04	1.532 ± 0.08	1.433 ± 0.01
	Amplitude	0.1021 ± 0.01	0.0929 ± 0.003	0.09117 ± 0.003
Striatum	MTT (s)	1.978 ± 0.09	1.899 ± 0.05	1.837 ± 0.04
	CTT (s)	1.58 ± 0.09	1.503 ± 0.03	1.418 ± 0.04
	Amplitude	0.1141 ± 0.01	0.1056 ± 0.003	0.1029 ± 0.001

MTT values were increased in acute DSS-induced colitis at the SFO relative to control. However, the lack of corresponding changes to CTT and signal amplitude values indicate no change in cerebral blood perfusion in this region. Overall, no differences were observed in blood perfusion in any of the regions analysed by btASL in acute or recovered DSS-induced colitis. The data presented here comes from rats in DSS study 5.

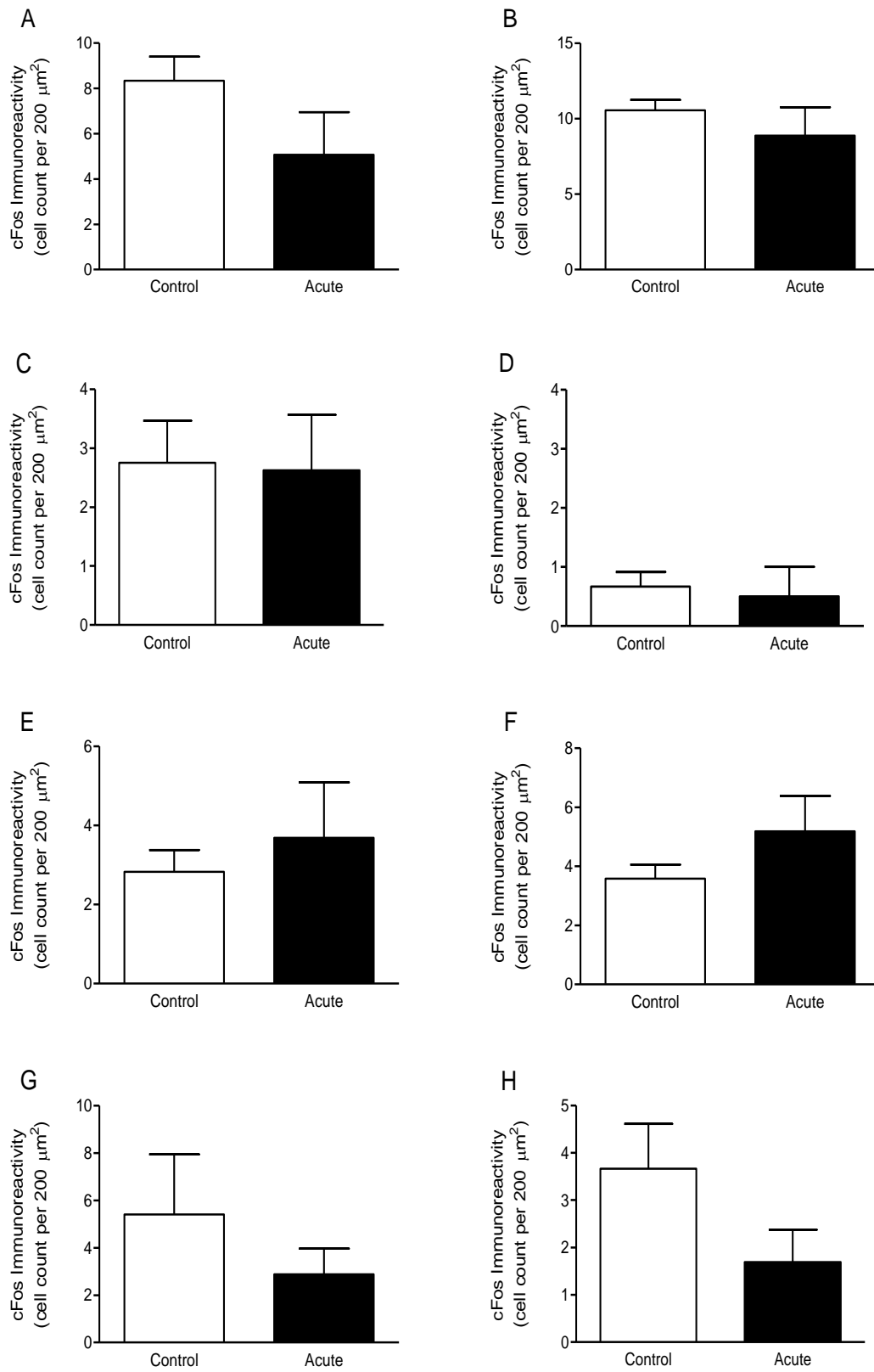
Data are presented as mean ± SEM ($n = 6-9$) (one-way ANOVA).

5.3.3 Altered neuronal activity in DSS-induced colitis in rats

The immediate early genes of the Fos family can be used as markers of neuronal activation as they are expressed in response to neuronal firing. cFos and Δ FosB were used to examine short and long term neuronal activation respectively.

5.3.3.1 *cFos as a marker for neuronal activity in acute DSS-induced colitis in rats*

To examine the effects of acute DSS-induced colitis on central neuronal activity, brain tissue was immunohistochemically assessed for cFos (Figure 5.6). There was no difference between acute colitis and control groups in any of the regions analysed: SFO ($t = 1.506$; $df = 8$; $p = 0.1705$) (A), ME ($t = 0.9663$; $df = 8$; $p = 0.3622$) (B), CA1 ($t = 0.1073$; $df = 8$; $p = 0.9172$) (C), CA2 ($t = 0.3322$; $df = 8$; $p = 0.7483$) (D), CA3 ($t = 0.6565$; $df = 8$; $p = 0.5299$) (E), DG ($t = 1.438$; $df = 8$; $p = 0.1883$) (F), OVLT ($t = 0.7743$; $df = 8$; $p = 0.4610$) (G), PVN ($t = 0.1604$; $df = 5$; $p = 0.8789$) (H), CeA ($t = 1.521$; $df = 8$; $p = 0.1667$) (I), DRN ($t = 0.6333$; $df = 6$; $p = 0.5499$) (J).



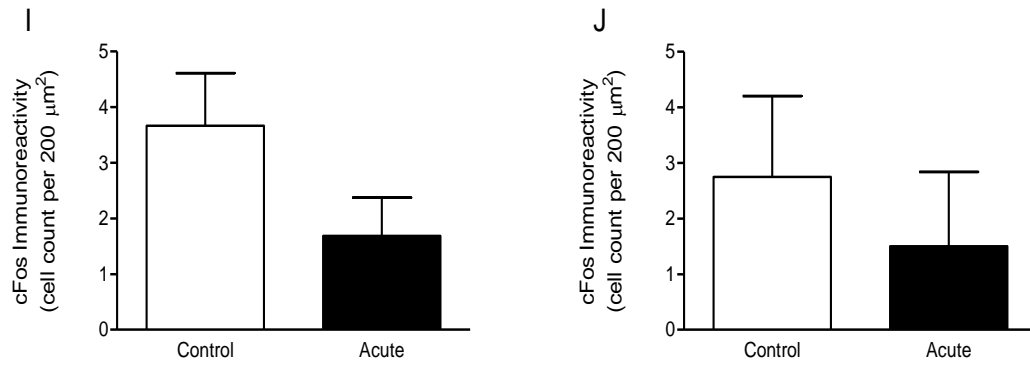


Figure 5.6 cFos immunoreactivity following DSS-induced colitis

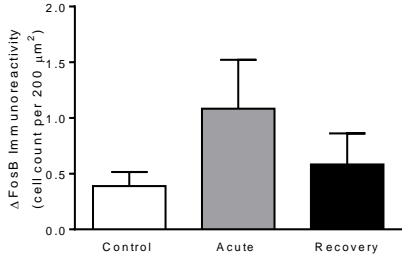
CVOs, the hippocampus and stress/anxiety-associated brain regions were assessed for cFos immunoreactivity per 200 μm^2 in acutely colitic rats. DSS-induced colitis does not alter cFos immunoreactivity at the SFO (A), ME (B), CA1 (C), CA2 (D), CA3 (E), DG (F), OVLT (G), PVN (H), CeA (I) or DRN (J). The data presented here comes from rats in DSS study 4.

Data are presented as mean \pm SEM ($n = 4 - 6$). (unpaired student's t -test).

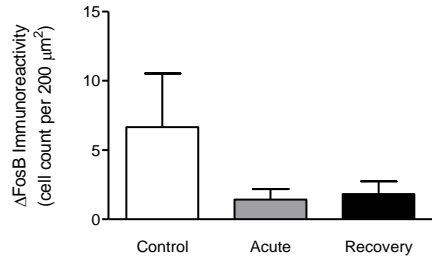
5.3.3.2 Δ FosB as a marker for neuronal activity in DSS-induced colitis in rats

As the expression of cFos is very transient, the effects of DSS-induced colitis on central neuronal activity was also immunohistochemically assessed using Δ FosB (Figure 5.7). One-way ANOVA of Δ FosB immunoreactivity showed an effect of DSS in the NAc ($F_{2,18} = 6.174$; $p = 0.0103$) (H) and DRN ($F_{2,18} = 5.148$; $p = 0.0188$) (K). *Post hoc* analysis of the NAc revealed an increase in Δ FosB immunoreactivity in acute colitis only relative to control ($p < 0.05$). *Post hoc* analysis of the DRN revealed an increase in Δ FosB immunoreactivity in acute and recovered colitis relative to control ($p < 0.05$). One-way ANOVA of Δ FosB immunoreactivity showed no effect of DSS in the ME ($F_{2,17} = 1.252$; $p = 0.3109$) (B), PFC ($F_{2,18} = 1.698$; $p = 0.2143$) (G), LS ($F_{2,21} = 0.8907$; $p = 0.4268$) (I) or PVN ($F_{2,16} = 2.241$; $p = 0.1432$) (J). No quantifiable Δ FosB immunoreactivity was recorded at the SFO, CA1, CA2, CA3 or DG (data not shown).

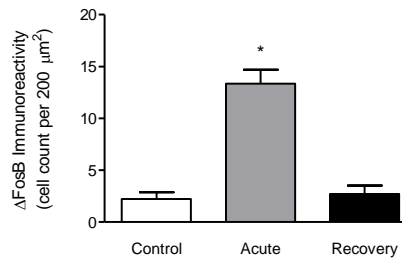
A



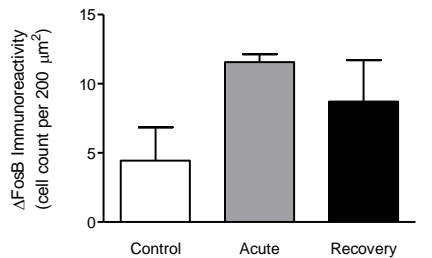
B



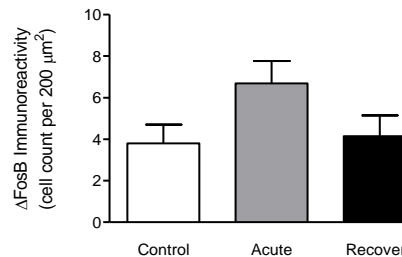
C



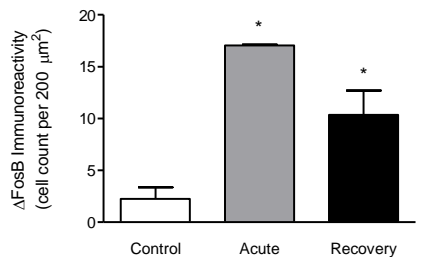
D



E



F



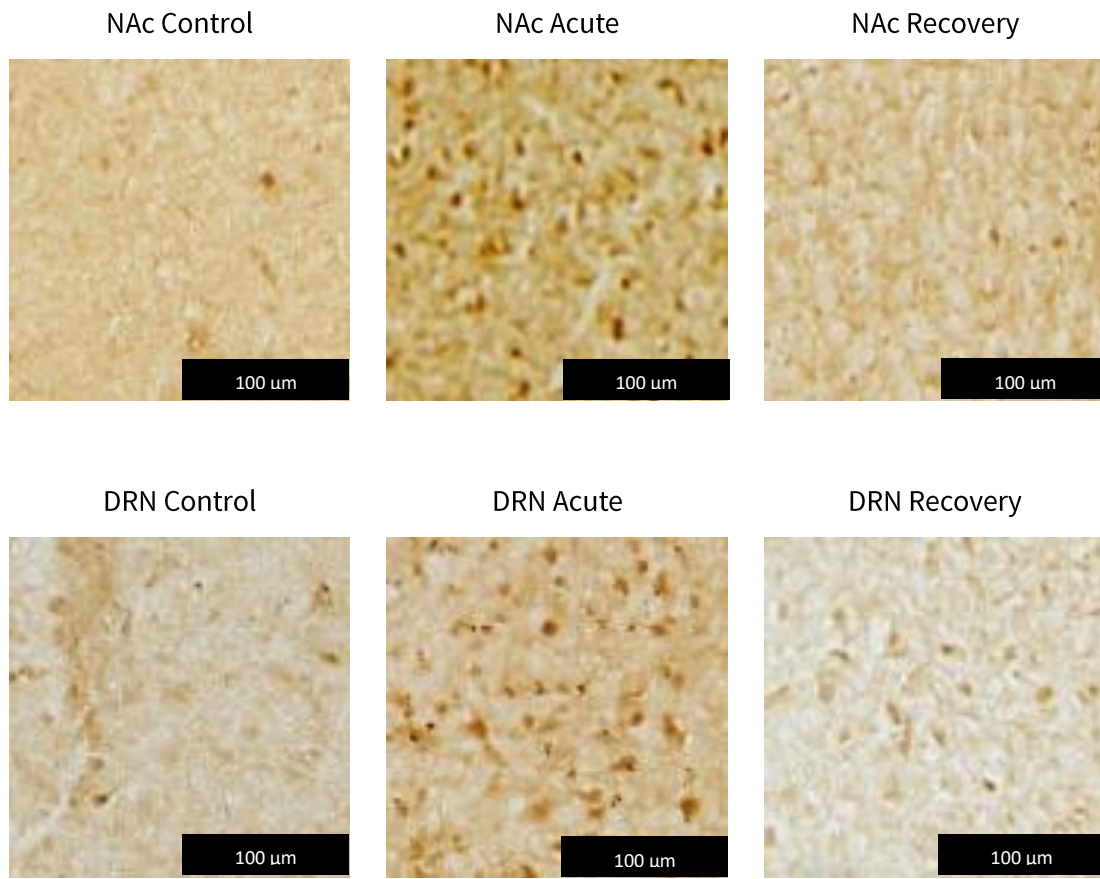


Figure 5.7 Δ FosB immunoreactivity following DSS-induced colitis

CVOs, the hippocampus and stress/anxiety-associated brain regions were assessed for Δ FosB immunoreactivity per $200 \mu\text{m}^2$ in acutely colitic and recovered rats following exposure to DSS. DSS-induced colitis alters Δ FosB immunoreactivity at the NAc (C) and DRN (F). DSS-induced colitis does not alter Δ FosB immunoreactivity at the ME (A), PFC (B), LS (D) or PVN (E). No quantifiable Δ FosB immunoreactivity was observed at the SFO, CA1, CA2, CA3 or DG (data not shown). Control, acute and recovery representative images show Δ FosB immunoreactivity per $200 \mu\text{m}^2$ at the NAc and DRN. The data presented here comes from rats in DSS study 4. Data are presented as mean \pm SEM ($n = 5 - 8$). * $p < 0.05$ relative to control (one-way ANOVA with Newman-Keuls *post hoc* test).

5.4 Discussion

The results of this chapter demonstrate some loss of BBB integrity at the SFO in acute DSS-induced colitis measured by an increase in immunoreactivity for IgG, however, this was not observed at the ME or in recovery from colitis. Brain MRI markers of DSS-induced colitis were identified and include an increase in ventricular volumes, which does not relate to changes in hippocampal volumes, and changes to T2 but not T1 relaxometry times in cortical regions. Cerebral blood perfusion as measured by btASL revealed no changes in blood perfusion in cortical regions, SFO or striatum in response to DSS-induced colitis. Immunohistochemical analysis of neuronal activation using Fos markers revealed an increase in activation in discrete brain regions following exposure to DSS-induced colitis including increases in neuronal activity at the NAc and DRN in colitis. No changes to neuronal activity were observed surrounding the SFO or ME.

5.4.1 BBB integrity at circumventricular regions

Previous experiments in this thesis (Chapter 4) have indicated that, following DSS-induced colitis, neuroinflammation is present in certain paraventricular areas of the brain. In order to determine whether the integrity of the BBB is compromised in these regions allowing for the transmission of inflammatory signals into the brain parenchyma, the SFO and ME were assessed using IgG immunohistochemistry. An increase in IgG immunohistochemistry was observed only in acutely colitic rats at the SFO. This indicates that the BBB is compromised in this region, however, as no increase was observed at the ME at either timepoint, despite evidence of inflammation, an interruption to barrier function is likely not the only means by which peripheral inflammation may be communicated to the brain. However, the MW of IgG is approximately 150 kDa (Sandin et al., 2004) and a previous study in TNBS colitis was unable to show the presence of IgG in the brain, citing the large MW, although the study did confirm perfusion of sodium fluorescein into the brain at the SFO and ME (Natah et al., 2005). Further work is required to determine the integrity of the BBB in DSS-induced colitis and to determine the alternate methods by which inflammation is communicated to the brain from the periphery.

5.4.2 Alterations to brain structure and function as measured by MRI

5.4.2.1 *Brain volumetric alterations in DSS-induced colitis*

There was a robust increase in ventricular volume (as a percentage of total brain volume) for the acute and recovered DSS-induced colitis groups relative to controls. This has not been previously analysed in patients with IBD or in animal models of colitis, however it has been previously observed in patients with depression (Kempton et al., 2011) and in animal models of depression (Gormley et al., 2016, Gigliucci et al., 2014). The WKY rat model of depression also showed a concomitant decrease in hippocampal volume (Gormley et al., 2016). However, no such changes were observed in hippocampal volume following DSS-induced colitis. Although decreases in hippocampal volume are also commonly observed in depressed patients (Kempton et al., 2011, Arnone et al., 2012), the data for IBD patients is conflicting. No alterations have been reported in grey matter (GM) volumes in UC patients in remission relative to control (Agostini et al., 2015, Agostini et al., 2011). Decreased hippocampal volumes have been reported in paediatric CD patients (Mrakotsky et al., 2017, Mrakotsky et al., 2016) however other studies report no volume change (Nair et al., 2016), or an increase in GM volume in the hippocampus (Bao et al., 2015). This may be due to the fact that these studies only included patients in remission whereas the paediatric studies also included patients with active disease. Given that GM volume decreases in limbic brain areas may be related to the development of psychological disturbances that have been observed in IBD patients, this warrants further investigation.

A decrease in GM may indicate a change in the neuropil, changes in cell number or size, increased cell packing, or changes in interstitial fluid. In CD, disease duration, disease severity, pain, anxiety and depression factors, and inflammatory mediator expression have been associated with volumetric changes in some regions (Mrakotsky et al., 2016, Mrakotsky et al., 2017, Nair et al., 2016, Bao et al., 2015, Agostini et al., 2013a). In particular, it is possible that overstimulation of the pain network in the brain by chronic pain may be related to brain structural reorganisation and plasticity and may recover following pain resolution (Rodriguez-Raecke et al., 2013). It has been reported that structural GM changes are associated with the increase in inflammatory cytokines in CD (Agostini et al., 2013a). As previously discussed, pro-inflammatory mediators may influence the brain via a leaky BBB, by active transport across the barrier, or by signalling receptors on the BBB which propagate the signal by activation of glial and endothelial cells (Bonaz and Bernstein, 2013). The neural route of the GBA may also

propagate the inflammatory signal, as cytokines bind receptors on the peripheral afferent fibres of the vagus nerve relaying the signal directly to the NST. Projections of the NST reach the cingulate cortex and the dorsolateral prefrontal cortex, as well as other cortical and subcortical areas including the DRN (Bonaz et al., 2013). This indicates a link between GI inflammation and structural changes to the cortex. Reduction of neurogenesis is a potential mechanism by which brain regions decrease in volume. Zonis et al. (2015) showed the pro-inflammatory cytokines up-regulated in the systemic circulation of DSS-induced colitis rats, to be capable of halting neurogenesis in the DG. Despite this finding, no changes were observed in hippocampal volumes following DSS-induced colitis. It is possible that had DSS exposure been performed chronically, inducing multiple cycles of colitis over a longer period of time, measurable changes may have occurred in hippocampal size. A voxel-based morphometric analysis of brain volumes may be more sensitive to smaller changes in regional brain volumes.

5.4.2.2 *T1/T2 relaxometry*

As T1 and T2 relaxometry times are generated by molecular motion and proton-proton interactions, as described in Chapter 2 (Section 2.2.5), they are influenced by the local biological structure and environment. T1 and T2 relaxometry values depend on local tissue density (water content), macromolecule, protein and lipid concentrations, and paramagnetic atom (iron) concentrations. Changes to these values therefore indicate changes associated with tissue pathology such as iron accumulation, inflammation, oedema, or necrosis, or other biological processes such as neurodegeneration, neuroplasticity and neurodevelopment (Deoni, 2010). However the exact underlying biophysical mechanisms, tissue biochemistry, and structure, that precipitate T1 and T2 relaxometry values are poorly understood. In response to DSS-induced colitis, T2 but not T1 relaxation times were reduced in the cingulate, motor and sensory cortices. Reductions in T2 relaxation times have previously been linked to microglial activation and to increased cell packing density in the brain parenchyma (Blau et al., 2012, Ding et al., 2008). This decrease in T2 relaxometry values may therefore reflect inflammation during the recovery phase of DSS-induced colitis and may be reflective of a decrease in cortical volume due to compression of the neuropil as a result of the observed increase in ventricle size.

Despite increases in microglia as measured by IBA1 surrounding the SFO in recovered colitis (Chapter 4), no reduction in T2 relaxation times were observed in this region. This is potentially due to structural attributes of the ventral-hippocampus, the parenchyma dorsal to the SFO, where relaxation times were measured, as this structure alters rostro-caudally. The fact that alterations in T2 relaxation times are observed in the recovery group rather than in acute colitis suggests that the biological alterations to the brain tissue causing relaxation changes may take time to occur or may even be cumulative, which could relate with the observation that morphometric changes are proportional to disease duration in CD patients in remission (Nair et al., 2016, Agostini et al., 2013a).

5.4.2.3 Cerebral blood perfusion determined by btASL

Although increased MTT values were observed in acute DSS-induced colitis at the SFO relative to control, the lack of corresponding changes to CTT and signal amplitude values mean that this result cannot be interpreted as a change in cerebral blood perfusion. MTT and CTT are both inversely proportional to cerebral blood flow, therefore an increase in MTT should be confirmed by a corresponding increase in CTT. In addition, SA is proportional to relative cerebral blood volume and therefore should change inversely to MTT and CTT. However, this btASL scan measured blood perfusion through a 2 mm slice thickness. As the region surrounding the SFO where inflammation is observed is quite small, this slice thickness may have masked any changes to cerebral blood perfusion in such a discrete region. No other changes in MTT, CTT or signal amplitude values were observed in any regions analysed in association with exposure to DSS-induced colitis. Therefore, no changes in cerebral blood flow were recorded following exposure to DSS-induced colitis. Cerebral blood perfusion has to date not been assessed via btASL in IBD patients or in animal models of colitis.

5.4.3 Activation of discrete stress-associated brain areas

Although no alterations to neuronal activity as measured by cFos were observed in association with acute DSS-induced colitis, immunohistochemical mapping of Δ FosB across brain regions associated with stress and anxiety showed that neuronal activity is up-regulated in the NAc and DRN. Due to the nature of Δ FosB protein, the increase in activity of these regions can be estimated to occur over an extended period of time (Nestler, 2008, McClung et al., 2004). Chronic perturbations to brain function caused by drugs of abuse, anti-depressant

treatments, stress and dopamine denervation can be mapped in discrete regions of the brain by Δ FosB expression patterns.

Other Fos family proteins such as cFos are transiently induced by acute exposure to drugs of abuse, however chronic drug administration will induce a long-lasting accumulation of Δ FosB in the NAc (Nestler, 2008). Virally-mediated over-expression of FosB in the NAc has previously been reported to protect against the long-term reward-mediated addiction behaviours in rats - decreasing cocaine self-administration and enhancing the extinction of cocaine-seeking behaviour (Zhang et al., 2014). This suggests that activation of the Δ FosB pathway might counteract positive feedback adaptations which tend to intensify drug reward. In addition, the same study found that over-expression of Δ FosB increases the response to sucrose when motivated by hunger, but decreased the sucrose response in satiated animals. It had previously been observed that anhedonic animals had dampened up-regulation of Δ FosB in the NAc following administration of sucrose (Grippo et al., 2004). This indicates that expression of Δ FosB in the NAc following acute DSS-induced colitis may have a dampening effect on the reward pathways, thus mediating anhedonic behaviour as observed in the saccharin preference test. However, increased Δ FosB is observed in the NAc following acute colitis only and does not account for decreased saccharin preference observed in the recovery group at this time. It has been theorised that Δ FosB modulates the response to reward by increasing silent synapses on D1 dopamine receptor-expressing direct pathway neurons (Grueter et al., 2013). It is therefore possible that the effects of Δ FosB expression may last longer than evidence of the protein itself. Of interest is the study by Vialou et al. (2010) which describes over-expression of Δ FosB in the NAc leading to an anti-depressant effect in the FST. In addition, this paper concludes that the anti-depressant action of fluoxetine requires Δ FosB induction in the NAc. It may be that DSS colitis-induced Δ FosB expression in the NAc is part of an adaptive mechanism to aversive colitis symptoms in an attempt to promote resilience to stress in these animals.

The DRN is commonly known as a serotonin centre of the brain with frontal projections. It is innervated by the vagal nerve which may be stimulated in the gut by inflammatory mediators, serotonin or short-chain fatty acids. Δ FosB immunostaining has previously been reported in the DRN following vagal nerve stimulation in rats in an effect similar to administration of the selective serotonin reuptake inhibitor sertraline (Furmaga et al., 2012). Interestingly, this group previously showed that vagal nerve stimulation had anxiolytic and anti-depressant

effects in the novelty suppressed feeding test and FST respectively (Furmaga et al., 2011). However increased Δ FosB staining in the DRN was not observed following administration of the tricyclic anti-depressant desipramine indicating the serotonergic basis of this pathway, despite a lack of overlap between serotonergic neurons and Δ FosB (Furmaga et al., 2012). The authors suggest that, as evidenced by Dorr and Debonnel (2006), vagal nerve stimulation may require long-term stimulation to raise the firing rate of serotonergic neurons in the DRN. In response to vagal nerve stimulation, Δ FosB staining is observed lateral to the DRN in the periaqueductal gray area. Berton et al. (2007) observe Δ FosB staining in this region in resilient mice in response to stress and conclude that Δ FosB expression occurs as an adaptive coping mechanism. It is therefore possible that, like Δ FosB expression in the NAc, Δ FosB is expressed in the DRN in response to DSS-induced colitis as a compensatory method to decrease behavioural despair. It is possible that these signals are initiated by inflammation at the arcuate nucleus surrounding the ME as projections from the PVN are known to innervate both the DRN and the NAc (Sim and Joseph, 1991).

Previous studies on stress-induced neuronal activation patterns show that Δ FosB activity is up-regulated in response to restraint, unpredictable, and foot shock stress in areas including PFC, NAc, LS and DRN (Berton et al., 2007, Perrotti et al., 2004). It is believed that Δ FosB in the DRN may function as a coping mechanism to oppose the behavioural despair that can occur when subjected to stressful situations such as a foot shock (Berton et al., 2007). The increases in Δ FosB activity in these regions during acute colitis may be expected due to the stressful nature of the symptoms experienced as a result of colitis, however previous work by (Frenois et al., 2007) has shown that increased Δ FosB activity in certain structures may be associated with depressive-like behaviours. In the study by Frenois and colleagues, rats received an acute LPS challenge and were later assessed for sickness and depressive-like behaviours. They found that 24 hours post-LPS, cytokine-induced sickness behaviours had abated but depressive-like behaviours measured by immobility in the FST and anhedonia in the sucrose preference test were still present. Immunohistochemical mapping in this study used cFos expression to study the sickness-associated neuronal changes at 6 hours post-LPS and Δ FosB expression to show depression associated neuronal alterations at 24 hours post-LPS and reported that Δ FosB expression in the PVN and hippocampus was up-regulated relative to controls correlating with the depressed phenotype.

5.4.4 Conclusion

The increase in IgG extravasation to the parenchyma at the SFO in acute colitis is an indicator that DSS-induced colitis may increase BBB permeability in this region accounting for the increase in inflammation observed here in previous chapters. However, the fact that this is not observed at any timepoint at the ME indicates that there may be other mechanisms by which inflammation is propagated from the periphery to the brain. As well as these other mechanisms it is possible that BBB integrity is compromised later at this region considering the fact that a significant increase in IgG ($p < 0.05$) is observed in the recovery group compared to control when just these groups are analysed using a *t*-test.

The increase in ventricular volume observed following DSS-induced colitis is reminiscent of that observed in depressed patients and points toward a corresponding decrease in the volume of brain tissue. As hippocampal volume is unchanged following DSS-induced colitis, further work is required to determine whether other areas such as the cortex are affected instead. Alterations to T2 relaxation times in recovery from DSS-induced colitis in cortical regions are an indication of changes to tissue composition and may occur as a result of changes to tissue structure as a result of altered brain volumetrics.

Blood perfusion is unchanged in response to DSS-induced colitis as measured by btASL. Although no changes in neuronal activity as measured by cFos immunoreactivity were observed in acute DSS-induced colitis, Δ FosB immunoreactivity revealed increased activity in certain brain regions linked with anxiety. This may be a marker of the behavioural differences outlined in Chapter 3 in recovery from colitis and may also provide a link to the increased risk for anxiety and depression symptoms in humans with IBD.

Chapter 6

General Discussion and Future Directions

6.1 General discussion

6.1.1 Introduction

In recent years, there has been a substantial increase in the number of studies investigating the increased risk for developing psychological disturbances such as depression and anxiety among patients suffering from IBD [for review see Abautret-Daly et al. (2017a)]. It has been reported that anxiety and depression can in turn affect risk of relapse and disease severity in IBD patients (Goodhand et al., 2012a, Persoons et al., 2005, Szigethy et al., 2014a). The reason for this association remains unclear, however, increased disease activity in IBD patients has been linked with increased psychological disturbance (Abautret-Daly et al., 2017b), and immunomodulation therapy has reduced disease activity and psychological disturbances (Horst et al., 2015, Calvet et al., 2006). This points to an inflammation-driven development of psychological symptoms. However, few studies have explored the underlying biological mechanisms responsible for the increased risk of depression or anxiety in IBD or in animal models. While it is possible that the psychological impact of GI symptoms could be sufficient to induce depression or anxiety, it is likely that inflammatory mediators themselves are responsible.

The first part of this thesis established the DSS-induced colitis model of IBD, exploring the inflammatory aspects of colitis in both colon and brain, assessing the impact of colitis on anxiety- and depression-related behaviours and comparing these findings to the TNBS-induced colitis model. The second part of this thesis examined the colitis-induced central inflammation in more detail in terms of iNOS and nNOS activity, the recruitment of microglia and microglial activity, leukocyte infiltration and putative involvement of astrocytes. Finally, the third part of this thesis assessed BBB integrity, brain volumetrics, regional T1 and T2 relaxometry, blood perfusion, and neuronal activation in and beyond paraventricular zones. The overall findings of this thesis are summarised in Figure 6.1.

6.1.2 GI dysfunction and inflammation following DSS and TNBS - induced colitis

The primary aim of this thesis was to establish the DSS-induced colitis model of IBD in order to investigate the link between GI inflammation and psychological symptoms such as anxiety

and depression. DSS is a well-established colitis model, and, as reported in the literature, displayed many similar characteristics to human IBD. Initially, it was confirmed that DSS produced an inflammatory profile similar to human UC. Exposure to a 5% solution of DSS for 6-7 days resulted in weight loss, reduced food intake, rectal bleeding and diarrhoea, and histological hallmarks comparable to UC patients. Increases in mRNA expression of IL-1 β , IL-6, TNF- α , MMP2, and MMP9 were observed in the colon during the acute phase of DSS-induced colitis in agreement with previous work in the DSS model (Okayasu et al., 1990) and in IBD patient samples (Abautret-Daly et al., 2017b). In recovery from DSS-induced colitis, IL-1 β and IL-6 mRNA expression remain increased in the colon despite remission of symptoms.

The TNBS-induced colitis model of IBD was also assessed, albeit to a lesser extent, in terms of symptom expression and inflammation to confirm the generalisation of symptoms to colitis rather than to DSS itself. It was established that TNBS induced symptoms of colitis including diarrhoea and decreased weight gain. Expression of pro-inflammatory mediators were also increased in the colon during acute TNBS-induced colitis however, by day 21 post-enema, the inflammatory profile in the colon had returned to normal.

6.1.3 Behaviour and central inflammation following DSS- and TNBS-induced colitis

Behavioural changes were also evident in both colitis models. Acute DSS-induced colitis was associated with changes to exploratory and locomotor behaviour in the open field test which are likely related to sickness. By recovery day 5, these exploratory and locomotor deficits return to normal. A decreased preference for saccharin was recorded in both acute and recovered DSS-induced colitis groups. Again, decreased saccharin preference in the acute colitis group may be suggestive of sickness behaviour, however in the recovery group it is likely indicative of an anhedonic phenotype. Rats in recovery from DSS-induced colitis were also tested in the marble burying test, FST, EPM, light/dark box, and social interaction test with results suggestive of anxiety and behavioural despair. TNBS rats were tested in the open field and saccharin preference test and show similar results to DSS-exposed rats, with a decrease in exploratory and locomotor behaviours as well as a decrease in saccharin preference on days 3 and 8 post-enema.

The expression of pro-inflammatory mediators was assessed in the cortex to determine any association between peripheral and central inflammation. Cortical IL-6 and iNOS expression were increased in acute and recovered DSS-induced colitis and indicate the presence of persistent inflammation in the brain resulting from GI inflammation. The central inflammation in recovery from colitis may be linked to the behavioural alterations observed at that time. TNBS rats show a similar pro-inflammatory profile in the cortex during acute colitis with increased IL-1 β and iNOS expression.

As iNOS was the most robust inflammatory marker measured in colon and cortex, in acute and recovered colitis, it was assessed further in other regions of the periphery and brain. iNOS expression was increased in acute and recovered DSS-induced colitis in the spleen, liver, hypothalamus and hippocampus. Levels of iNOS mRNA in colon and cortex correlated with iNOS mRNA in all other regions suggestive of a direct association between peripheral and central inflammation.

6.1.4 Central inflammation at CVOs following DSS-induced colitis

A further investigation of central iNOS was carried out in DSS-induced colitis using immunohistochemistry. iNOS immunostaining was primarily observed surrounding two CVOs, the SFO and ME, where it was increased in both acute and recovered colitis groups. Inflammation appears to be restricted to the SFO and ME, considering no change in iNOS immunoreactivity was detected in the OVLT, which is another CVO, or the LHb, a paraventricular structure located near the SFO but which is not a CVO. There is also no change detected in iNOS in the PVN and no iNOS is recorded in the CA1-3 or DG of the hippocampus or in cortical areas despite increased iNOS mRNA expression in these regions. However, the area surrounding the SFO where iNOS immunoreactivity is analysed in this thesis is the ventral hippocampal commissure and the region surrounding the ME where iNOS is detected is the arcuate nucleus of the hypothalamus. Therefore, these structures may be responsible for the increase in iNOS mRNA expression recorded in the hippocampus and hypothalamus. In the cortex, it is possible that although iNOS expression increases, post-transcriptional regulation prevents iNOS translation. Active iNOS can alter protein structure by nitration, therefore the presence of increased 3-NT, a measure of protein nitration, demonstrates iNOS activity at SFO and ME. Previous work in TNBS mice has demonstrated a decrease in depressive-like

behaviour in the FST in mice administered with a selective iNOS inhibitor (Heydarpour et al., 2016). It would be of interest to perform this experiment in recovery from DSS.

As further confirmation of the inflammatory state in the areas surrounding the SFO and ME, an increase in microglial numbers was also observed. Co-localisation analysis indicates that these cells are producing iNOS in these regions and are therefore likely to be M1-activated pro-inflammatory microglia. Morphological assessment determined a decrease in microglial perimeter following DSS-induced colitis indicating that the microglia are indeed amoeboid in shape and are active. The lack of CD45 immunoreactivity in the brain parenchyma confirms that these cells are in fact microglia and not peripheral infiltrating macrophages. Inflammation has been previously suggested as having a role in depression in some patients (Krishnadas and Cavanagh, 2012, Miller et al., 2009). A recent study has linked a marker of microglial activation with depressive symptoms, in particular, suicidal ideations in patients with major depressive disorder (Holmes et al., 2017). This highlights the potential importance of activated microglia in precipitating inflammation-induced depressive behaviour. In the DSS model, it would be of interest to determine whether a microglial inhibitor such as minocycline might affect the depressive-like behaviour observed in recovery.

Considering the decrease in cortical GFAP expression following acute DSS-induced colitis and the previous evidence for astrocytic dysfunction in psychological disorders (Rajkowska and Stockmeier, 2013), GFAP immunoreactivity was also analysed throughout the brain following DSS-induced colitis. No alterations in GFAP immunoreactivity were detected in any of the regions analysed. It would be of interest in future studies to measure astrocytic density, hypertrophy and GFAP reactivity as identifiers of astrocytic activity to determine whether astrocytes are also becoming immunologically activated and contributing to inflammation, perhaps accounting for the small amount iNOS not co-localised with IBA1.

6.1.5 BBB integrity and structural alterations in the brain following DSS-induced colitis

IgG immunoreactivity was increased at the SFO in acute colitis, indicating an increase in BBB permeability in this region. However no changes were observed at the ME, where inflammatory markers are also observed, or in the recovery group. Therefore it is possible that

other mechanisms are responsible for the pro-inflammatory response observed in the brain in response to DSS-induced colitis. Further work is required to conclusively assess BBB integrity and other mechanisms of gut-brain communication in this model of colitis.

The volume of ventricles as a percentage of total brain volume was increased following DSS-induced colitis. No changes to hippocampal volume were observed despite reported decreases in hippocampal volumes in paediatric CD patients (Mrakotsky et al., 2016). Other regions, particularly within the cortex, should be analysed to determine whether there are decreases in cortical volume, as has been reported in IBD patients (Bao et al., 2015). In this study, volume changes in regions associated with emotional processing were associated with anxiety and depression factors. The change in ventricular volume may contribute to volumetric changes in cortical regions as the increased volume of the ventricles puts pressure on these regions causing compression of the neuropil. It is possible that the alterations in T2 relaxation times observed in cortical regions are a result of this tissue compression. Although the biological causes of T2 relaxation changes are difficult to interpret, they are indicative of changes to tissue structure and composition.

6.1.6 Changes to neuronal activation following DSS-induced colitis

Δ FosB staining indicates alterations to neuronal activity in the brains of animals exposed to DSS-induced colitis in the NAc and DRN; regions previously shown to be activated following stress (Perrotti et al., 2004). The NAc is typically considered a reward centre and the DRN is a centre for serotonin production in the brain. It is possible that the stress associated with the sickness caused by DSS-induced colitis leads to activation of these areas as a compensatory mechanism by the brain to combat symptoms of anhedonia and despair. It remains to be seen whether central inflammation associated with DSS-induced colitis is linked to changes in neuronal activity in brain regions which are potentially responsible for some of the behavioural differences observed.

6.1.7 Conclusions

This thesis uses the DSS model of colitis to illustrate the biological changes occurring centrally caused by GI inflammation and which may manifest as psychological symptoms. Bi-directional transmission within the GBA may contribute to a cyclical comorbidity whereby GI

inflammation impacts the CNS sufficiently to precipitate psychological dysfunction, which in turn may worsen colitis symptoms or increase relapse risk. However, this also means that breaking the cycle by treating the underlying inflammation may be beneficial in terms of both GI and psychological symptoms.

The DSS model of colitis has proven an effective tool in exploring the mechanisms by which colitis promotes CNS inflammation and leads to behavioural comorbidities. The model will be useful in future investigations further examining these associations and in identifying the benefits of current or future IBD treatments in terms of treating inflammation and psychological symptoms.

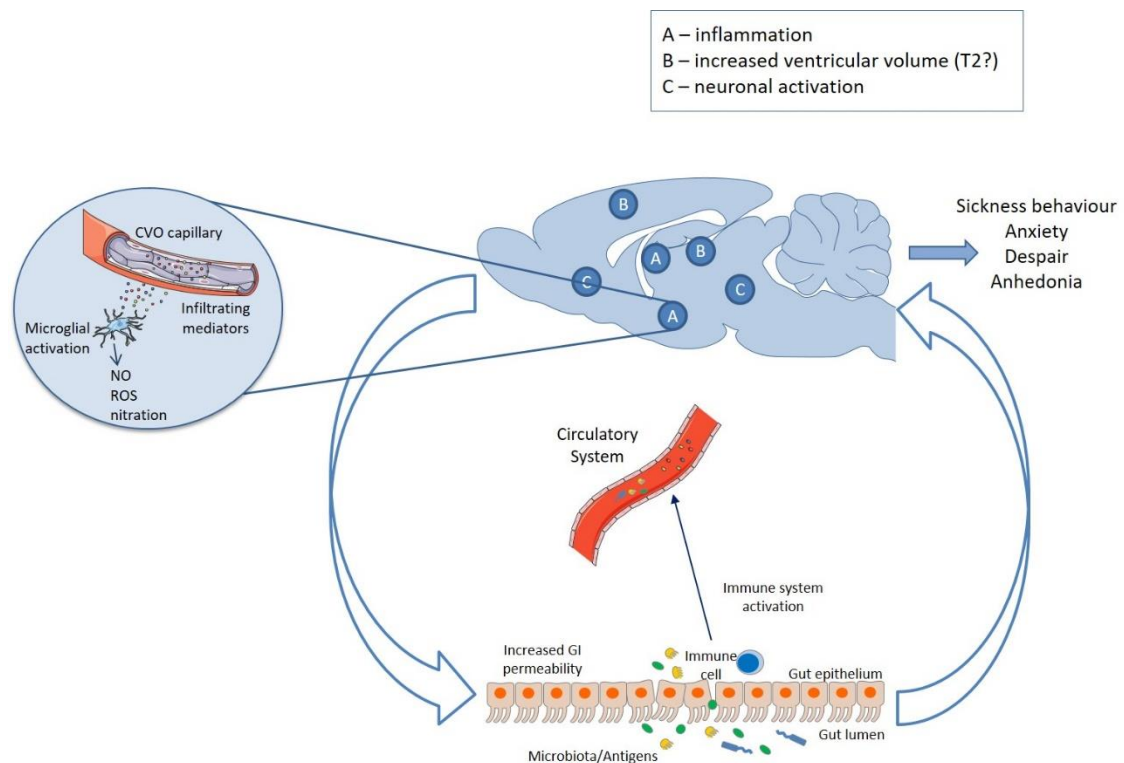


Figure 6.1 Summary of disturbances to the gut-brain axis when animals are exposed to DSS-induced colitis

Induction of inflammation in the bowel by DSS results in intestinal damage and symptoms of IBD accompanied by increased gastrointestinal permeability. Microbes/microbial products and antigens infiltrate into the mucosa of the gut where they lead to immune system activation and production of pro-inflammatory mediators. Microbial products and

inflammatory mediators may enter into the circulatory system and travel to the brain. Potentially as a result of this inflammation in the brain, evidenced by microglial activation and iNOS production and iNOS/ROS activity, is observed in circumventricular regions (A). Increased ventricular volume in the lateral and third ventricles is potentially associated with decreased T2 values measured in cortical regions (B). Increased neuronal activation, measured by Δ FosB immunoreactivity, in the dorsal raphe nucleus and nucleus accumbens (C) potentially indicates areas of the brain that are activated in response to the sickness behaviour, anxiety, despair and anhedonia observed in behavioural tests in these animals.

6.2 Future directions

In consideration of the experiments presented in this thesis, numerous future studies could be carried out to expand upon and to determine the implications of, and mechanisms involved in, the reported results.

1. IBD is a relapsing and remitting condition. For the experiments in this thesis, rats are exposed to one cycle of DSS exposure only to allow examination of the acute impact of colitis on the brain. Nonetheless, it would be important for future work to investigate the effects of chronic DSS exposure particularly given the evidence for microglial priming (Cunningham, 2013). This would give insight into whether the effects of DSS on the CNS are more or less evident following two or more cycles of DSS and would more closely resemble the chronic inflammatory state of IBD patients.
2. In addition to the increased risk for depression and anxiety in IBD, some literature also reports cognitive deficits in IBD patients which may be associated with disease activity and depressive symptoms (Golan et al., 2016, van Langenberg et al., 2016, Berrill et al., 2013a). Cognitive function in rats exposed to DSS-induced colitis remains to be assessed and it would be of interest to determine whether this is associated with measures of depressive-like behaviour and inflammation scores.
3. In this thesis, rats are assessed in behavioural tests during the recovery period when they no longer display sickness behaviour in the open field. Behavioural testing is performed from recovery day 5 to 8. It would be interesting to perform these tests later in the recovery period to determine how long symptoms of anxiety, behavioural despair and anhedonia persist.
4. It remains unclear how peripheral inflammation generated as a result of colitis communicates to the brain to precipitate inflammation and other CNS changes described in this thesis. Increased permeability of the BBB was investigated in this thesis and could not conclusively account for all CNS changes observed. It would be of interest to expose vagotomised rats to DSS to determine any vagal involvement in gut-brain communication in this model.

5. Psychological intervention - including therapy, anti-depressant and anxiolytic drugs - has been demonstrated as effective in reducing IBD symptoms in patients. Considering the anti-depressant, anxiolytic, and iNOS inhibiting properties of fluoxetine it would be a prime candidate to investigate in the DSS model.
6. Conversely, treating inflammation in DSS-induced colitis could potentially reveal more about the inflammatory link between colitis and psychological symptoms. Bearing in mind the fact that many studies report a link between disease activity or disease severity and psychological symptoms in IBD, anti-inflammatory drugs may impact the behavioural disturbances seen in the DSS model.
7. The involvement of iNOS as part of the inflammatory response regarding the development of behavioural alterations and changes to neuronal activity could be investigated using an iNOS inhibitor such as 1400W. This selective iNOS inhibitor has previously been shown to have anti-depressant properties in mice in the FST (Montezuma et al., 2012).
8. Colonic MMP2 and MMP9 mRNA expression and activity were increased in acute DSS-induced colitis. As these gelatinase enzymes are capable of cleaving ECM proteins they may be responsible for reduced barrier function and the spread of inflammation from the gut to the peripheral tissues and the brain. The ability of MMP inhibitors to minimise inflammation in DSS-induced colitis should be explored. In addition, MMP9 in particular can reduce the integrity of the BBB (Lakhan et al., 2013). It would be of interest to determine whether there is an increase in circulating or cortical MMP9 which may be affecting BBB permeability.
9. The microbiota-GBA is increasingly being explored as a link between intestinal dysfunction and psychological symptoms. Dysbiosis of commensal gut bacteria is thought to play a role in IBD. Furthermore, the microbiota has been implicated in CNS disorders including anxiety, depression, autism and schizophrenia. Prebiotics have recently been used to reduce colitis induced anxiety and recognition memory deficits in the DSS-induced colitis model in mice (Emge et al., 2016, Ferenczi et al., 2016). It would be interesting to confirm these findings in the rat model of DSS during the recovery phase and to assess the impact of prebiotics on the CNS.

10. Further work is required to definitively establish the case for loss of BBB integrity in the DSS model. It would be informative to assess other markers of BBB integrity, for example the TJ proteins claudin-5 and zona occludins-1. A capillary isolation protocol could be applied to isolate the brain vasculature for this assessment (Campbell et al., 2012). In addition, analysis of VEGF-A expression in the hypothalamus would give an indication as to whether low food intake in DSS-induced colitis is causing tanycytes to produce VEGF-A which acts on endothelial cells to increase vascular permeability.
11. An increase in activated microglia was observed in CVOs following DSS-induced colitis. Due to the co-localisation of iNOS with the microglial marker IBA1, it is likely that these are M1-activated pro-inflammatory microglia. This could be confirmed using immunohistochemistry (Barros et al., 2013).
12. Δ FosB immunoreactivity indicates alterations to neuronal activity in certain brain regions in response to DSS-induced colitis. fMRI analysis would allow mapping of neuronal activity changes in these animals at resting state and would give a fuller picture of alterations to regional brain connectivity in this model.

6.3 Concluding remarks

It is generally accepted that IBD is associated with increased vulnerability to comorbid depression and anxiety disorders, particularly during active disease. It is likely that the presence of chronic inflammation plays a role in the development of these symptoms, although the precise mechanisms for this are yet to be uncovered. The anxiety- and depression-like symptoms which manifest in the DSS-induced colitis rat model reflect the psychological symptoms experienced in human IBD, and the changes to the CNS measured in these rats as outlined in this thesis, indicate potential changes that may be occurring clinically. Further work is required in order to elucidate the impact of these alterations and their contribution to symptom development, however this work may inform on future areas for therapeutic intervention in psychological dysfunction in IBD patients. Considering the emerging evidence which indicates efficacy of psychological intervention in the management of IBD symptoms, reducing relapse rates and increasing HRQOL, patients with IBD should be routinely screened for psychological symptoms and, where indicated, treated accordingly as part of their overall treatment plan.

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Appendices

Appendix 1: Solutions prepared

*Store at 4°C

†Use fume hood

In vivo & perfusion5% DSS†

100 g DSS†

2 L tap water

Stir

10 mM Saccharin

1.8318 g saccharin

Make up to 1 L in tap water

4% PFA†

Prepare 4% PFA fresh on day of use.

40 g PFA†

100 mL PBS 10x

800 mL dH₂O

2 crystals NaOH

Stir and heat until dissolved. (~20 mins)

Make up to 1L and allow to cool on ice.

pH 7.4 with conc. HCl.

Keep on ice throughout experiment.

Urethane† (12.5 g/25 mL)

12.5 g urethane†

Make up to 25 mL with dH₂O or saline.

Shake to dissolve. (~15 mins)

Formalin† (10% neutral buffered)

100 mL Formaldehyde (37-40%)†

900 mL dH₂O4 g NaH₂PO₄6.5 g Na₂HPO₄

Stir.

pH 6.8-7.4

PBS 10X2.622 g NaH₂PO₄11.5 g Na₂HPO₄

87.66 g NaCl

Adjust to 1 L with dH₂OPBS 1X430 mL dH₂O

50 mL PBS 10X

Adjust to pH 7.4

Adjust to 500 mL with dH₂OSucrose 30%

150 g Sucrose

500 mL PBS 10 mM (pH7.4)

Stir

Zymography

BSA Solution (1000 µg/mL) *

50 mg BSA (Sigma)

50 mL TBS-T (1x)

Bio-Rad Solution*

5 mL Bio-Rad Protein Assay Dye Reagent

Concentrate (Bio-Rad)

45 mL dH₂O

Gelatin 20 mg/mL 1% SDS

2 g Gelatin

98 mL ddH₂O

Heat and stir

1 g SDS

Lysis Buffer*

100 mL dH₂O

0.788 g Trizma Base

0.876 g NaCl

1 mL Triton-X-100

Adjust to pH 8 with conc. HCl

Lysis Buffer & Inhibitors*

4 mL Lysis Buffer

4 µL Protease Inhibitor (Sigma-Aldrich)

4 µL Phosphatase Inhibitor Cocktail I
(Sigma-Aldrich)

4 µL Phosphatase Inhibitor Cocktail II
(Sigma-Aldrich)

Made fresh each day.

Ammonium Persulfate (APS; 10%)

0.1 g APS[†]

960 µL ddH₂O

Make up fresh.

Stacking Buffer* (0.5 M Trizma-HCl 4X pH 6.8)

6.05 g Trizma Base

40 mL ddH₂O

Adjust pH to 6.8 with conc. HCl

Adjust to 100 mL with ddH₂O

Filter at 0.45 µm

0.4 g Sodium Dodecyl Sulfate (SDS)

1.5 M Trizma-HCl* (pH 8.8)

91 g Trizma Base

300 mL ddH₂O

Adjust to pH 8.8 with conc. HCl

Adjust to 500 mL with ddH₂O

Filter at 0.45 µm

2 M Trizma-HCl*

242 g Trizma Base

500 mL ddH₂O

Adjust to pH 7.4-7.6

Adjust to 1 L with ddH₂O

Triton Buffer 2.5%

25 mL Triton-X-100

975 mL ddH₂O

Loading Buffer 4X

5 mL Stacking Buffer

4 mL Glycerol

0.8 g SDS

0.4 mL ddH₂O

0.2 mg Bromophenol Blue

Dissolve in 10 mL ddH₂O

Store at -20°C

Bis Acrylamide*† 30%30 g Acrylamide[†]0.8 g N,N Methylenebisacrylamide[†]Adjust to 100 mL with ddH₂O

Filter at 0.45 µm

Separating Gel (x2)4 mL 30% Bis-Acrylamide[†]

3.75 mL 1.5M Trizma-HCl 4X pH 8.8

1.5 mL Gelatin

5.75 mL ddH₂O50 µL 10% APS[†]10 µL TEMED[†]Stacking Gel (x2)650 µL 30% Bis-Acrylamide[†]

1.25 mL Stacking Buffer

3 mL ddH₂O25 µL 10% APS[†]5 µL TEMED[†]Tank Buffer (10X)*

30.2 g Trizma Base

144 g Glycine

10 g SDS

1 L ddH₂O

Adjust to pH 8.3

Tank Buffer (1X)*

100 mL Tank Buffer (10X)

900 mL ddH₂OZymography Buffer

50 mL 2M Trizma-HCl

18 g NaCl

1.47 g CaCl₂1 g NaN₃ – toxic[†]2 L ddH₂OStaining Solution650 mL ddH₂O250 mL MeOH[†]100 mL Acetic Acid[†]

0.25 g Coomassie Brilliant Blue G-250

De-staining Solution880 mL ddH₂O80 mL Acetic Acid[†]40 mL MeOH[†]

Immunohistochemistry

Freezing Storage Solution*

100 mL 10 mM PBS (pH 7.4)

75 g sucrose

75 mL ethylene glycol

Made up to 250 mL with 10 mM PBS (pH 7.4)

Gelatin Solution

500 mL dH₂O – heated to approx. 40°C

2.5 g gelatin – fully dissolve

0.25 g chromium III potassium sulfate[†]

Allow to cool fully before use.

PBS 10X

1.311 g NaH₂PO₄

5.75 g Na₂HPO₄

43.8 g NaCl

Adjust to 500 mL with dH₂O

PBS 1X

430 mL dH₂O

50 mL PBS 10X

Adjust to pH 7.4

Adjust to 500 mL with dH₂O

H₂O₂ Solution (0.75%)

18.5 mL 10 mM PBS (pH 7.4)

500 µL H₂O₂ (30%)

1 mL MeOH[†]

10% Normal Serum (NS) Solution

2 mL Normal Serum

18 mL 10 mM PBS (pH 7.4)

50 µL Triton-X-100 (10%)

Secondary Antibody Solution

20 mL 10 mM PBS (pH 7.4)

2 drops Secondary Antibody (Vectastain ABC kit) (Vector Labs)

6 drops Normal Serum (Vectastain ABC kit) (Vector Labs)

A/B Solution

15 mL 10 mM PBS (pH 7.4)

5 drops reagent A (Vectastain ABC kit) (Vector Labs)

5 drops reagent B (Vectastain ABC kit) (Vector Labs)

Di-Amino-Benzidine (DAB) Solution

19.5 mL 10 mM PBS (pH 7.4)

500 µL DAB[†]

3 µL H₂O₂ (30%)

Immunofluorescence

PBS 10X

1.311 g NaH₂PO₄

5.75 g Na₂HPO₄

43.8 g NaCl

Adjust to 500 mL with dH₂O

PBS 1X

430 mL dH₂O

50 mL PBS 10X

Adjust to pH 7.4

Adjust to 500 mL with dH₂O

Blocking Buffer*

45 mL PBS 1X

5 mL NGS (same species as 2° Ab.)

150 µL Triton-X-100

Antibody Dilution Buffer*

50 mL PBS 1X

0.5 g (1%) BSA

1°Ab – 500 µL / 2° Ab – 50 µL 0.1% Triton-X

[0.1x = diluted 1 in 10]

Remove 15 mL and use as control buffer

Antibody Dilutions

Primary*

1:1000 35 µL per 35 mL buffer

IBA1 / GFAP (rabbit)

1:300 117 µL per 35 mL buffer

iNOS (mouse)

1:350 100 µL per 35 mL buffer

NeuN (mouse)

Secondary* (keep covered)

1:1000 50 µL per 50 mL buffer

Alexa Fluor 488 nm anti-mouse (goat)

Alexa Fluor 546 nm anti-rabbit (goat)

Real-time PCR

Lysis Buffer†

350 µL RA1 buffer (supplied)

3.5 µL β-mercaptoethanol† (Sigma-Aldrich)

Master Mix (for 1 reaction)

2 µL Reverse Transcription Buffer

2 µL Random primers

0.8 µL dNTPs

4.2 µL Nuclease-free water

1 µL MultiScribe™ Reverse Transcriptase

Primer Mix (for 1 sample)

1.25 µL Primer

1.25 µL β-actin

12.5 µL Taqman Universal Master Mix

DNase Reaction Mix (for 1 sample)

10 µL reconstituted rDNase (supplied)

90 µL reaction buffer for rDNase (supplied)

Appendix 2: Additional BBB integrity data

Gadolinium contrast-enhanced MRI

Animals were subject to MRI scans to measure gadolinium perfusion through the brain as an indicator of BBB integrity. Rats were anaesthetised by isoflurane only (5% isoflurane in 100% oxygen) and the protocol for animal preparation and positioning was otherwise followed as described in Chapter 2 (Section 2.2.6.1). High-resolution T1-weighted MR images were acquired prior to, and following, i.v. administration of 1 mL gadolinium (gadobenate dimeglumine; 33% v/v; 552 Da; Bracco, Italy) via tail-vein catheter. The following acquisition parameters were used: slice thickness = 1.2 mm, TR = 312.5 ms, TE = 2.53 ms, RF flip angle = 30°, FOV = 3.0 x 3.0 cm, image matrix = 128 x 128.

ImageJ software was used to assess ROIs (SFO and third ventricle) for gadolinium flow through the brain (Figure A2.1). The rat brain atlas (Paxinos and Watson, 2007) was used to identify the image slices required for analysis of the ROIs: distance from bregma = -1.08 mm. Images were inverted and signal intensity changes were measured as changes in image density over time pre and post-injection. Corresponding to other MRI analyses in this thesis, the location of the ME in the brain makes it difficult to accurately analyse due to the poor signal:noise ratio. Therefore the SFO alone was investigated.

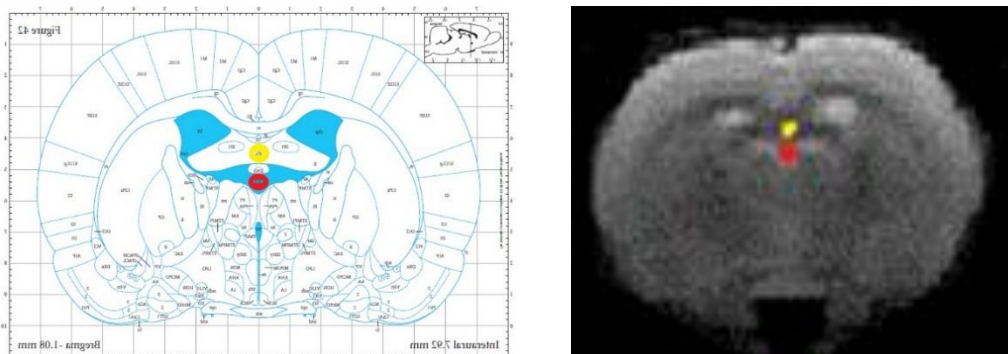


Figure A2.1 Regions of interest examined for gadolinium at the SFO

Two-way repeated measures ANOVA of image density showed an effect of time ($F_{34,204} = 19.05$; $p < 0.001$) but not DSS ($F_{1,204} = 0.27$; $p = 0.6242$) and no interaction effect ($F_{34,204} = 0.32$; $p = 0.9999$) in the third ventricle ventral to the SFO (Figure A2.2 A). Two-way repeated measures ANOVA of image density showed an effect of time ($F_{34,204} = 22.12$; $p < 0.001$) but not DSS ($F_{1,204} = 0.04$; $p = 0.8426$) and no interaction effect ($F_{34,204} = 0.41$; $p = 0.9987$) in the parenchyma dorsal to the SFO (Figure A2.2 B).

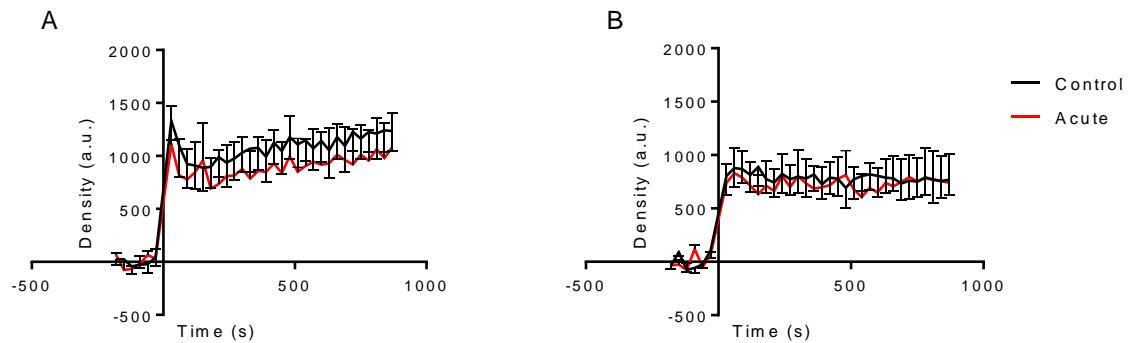


Figure A2.2 Gadolinium contrast MRI at the SFO and third ventricle

T1 images of the SFO and the third ventricle surrounding the SFO were assessed for changes in density at 30 second intervals prior to and for 15 minutes following an i.v. infusion of gadolinium. Image density peaks in these regions following gadolinium administration and plateaus signifying the influx of gadolinium to the brain. DSS-induced colitis has no effect on the perfusion of gadolinium in the third ventricle (A) or paraventricular parenchymal tissue surrounding the SFO (B). The data presented here comes from rats in DSS study 6.

Data are presented as mean \pm SEM [upper control and lower acute error bars omitted for clarity] ($n = 4$). (two-way repeated measures ANOVA).

Despite evidence for an increase in IgG (Mw = 156 kDa) immunohistochemistry at the SFO following acute DSS-induced colitis, gadolinium (MW = 552 Da) contrast-enhanced MRI failed to show any loss of BBB integrity in this region. This is despite the much smaller MW of gadolinium compared to IgG. As IgG is an endogenous circulating immunoglobulin it is a more reliable marker than gadolinium which requires carefully timed i.v. administration coupled to tracing of the MR signal generated. There may be insufficient time allowed for gadolinium to cross the BBB following a single i.v. injection prior to clearance from the body if the loss of barrier integrity is mild.

Biotin immunofluorescence

Biotin has been widely used as a tracer molecule to detect BBB integrity and determine TJ functionality (Ding *et al.*, 2011). Loss of barrier integrity would allow biotin (443 Da) to pass into the brain where it can be detected via immunostaining with a fluorescently labelled avidin probe (Figure A2.3).

Acute and recovered rats exposed to DSS-induced colitis were injected with 1.5 mL biotin (sulpho-NHS-SS-biotin; 4mg/mL; GBiosciences, USA) via tail-vein catheter 5 minutes prior to perfusion with PBS (10 mM; pH7.4). Brains were extracted from the skulls and one hemisphere was post-fixed, cryoprotected, snap-frozen and stored. Brains were sliced in 12 µm thick sagittal sections on a cryostat at -21°C and placed directly onto gelatin-coated slides. A hydrophobic circle was drawn around the brain tissue using a PAP pen (Sigma-Aldrich) to keep liquid pooled in a droplet on the slice. Tissue was blocked using 10% NGS for 40 minutes. Slides were placed into a slide rack and washed (2 x 5mins) in 10 mM PBS on a rocker. Slices were incubated in fluorescently labelled avidin (Streptavidin CY3; 1:300; Thermofisher, UK) in the dark for 2 hours. Slides were washed (1 x 5mins) in 10 mM PBS on the rocker and dried at room temperature. Once dry, slides were cover slipped in Vectashield anti-fade mounting medium with DAPI (Vector Laboratories) and sealed using nail varnish. Sagittal slices were examined using a Zeiss confocal microscope, using LSM 510 software, as described previously.

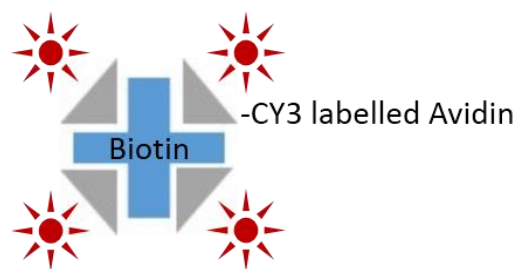


Figure A2.3 Diagram of biotin-avidin fluorescent staining

Whole brain (single hemisphere) analysis of sagittal rat brain slices revealed no positive immunofluorescent staining. This fails to confirm that the blood brain barrier is impaired in this model of colitis, as indicated by IgG immunohistochemistry, however the lack of a positive control for this analysis means that this cannot be confirmed. In future studies, the liver could be used as a positive control as it is a highly vascular organ which should contain biotin following i.v. biotin administration.

Publications

Poster presentations

Behavioural Alterations Associated with a Change in Brain Relaxometry in an Animal Model of Inflammatory Bowel Disease.

Dempsey E., McIntosh A., Kerskens C., Harkin A. Young Neuroscientists Symposium Dublin, 2016.

Evidence for Central Molecular Changes and Changes to Neuronal Activity in an Animal Model of Inflammatory Bowel Disease.

Dempsey E., Downing J., Abautret-Daly Á., Medina C., Harkin A. 28th ECNP Congress Amsterdam, 2015.

Evidence for Behavioural and Central Molecular Changes in an Animal Model of Inflammatory Bowel Disease.

Dempsey E., Abautret-Daly Á., Medina C., Harkin A. CINP Thematic Meeting on Stress, Inflammation and Depression, Dublin, 2015.

Central neuroinflammation in inflammatory bowel disease: evidence for a central increase in iNOS enzyme in an animal model of colitis.

Dempsey E., Abautret-Daly Á., Medina C., Docherty N., Harkin A. ECNP Junior Scientists Workshop on Neuropsychopharmacology, Nice, 2015.

Neuroinflammation in Inflammatory Bowel Disease: Evidence for a Central Increase in iNOS Enzyme associated with Anxiety-Related Behavioural Changes in the DSS-Induced Colitis Model of Inflammatory Bowel Disease.

Dempsey E., Abautret-Daly Á., Medina C., Docherty N., Harkin A. Moodinflamm Consortium Neuroimmunology of Health and Disease, Amsterdam, 2014.

iNOS as a Mediator of Central Inflammation in an Animal Model of Inflammatory Bowel Disease.

Dempsey E., Abautret-Daly Á., Medina C., Docherty N., Harkin A. Gut-Brain-Axis European Conference on Microbiology and Immunology (EUCMI), Berlin, 2014.

Publications

Gut-brain actions underlying comorbid anxiety and depression associated with inflammatory bowel disease. (Review)

Abautret-Daly Á., **Dempsey E.**, Parra-Blanco A., Medina C., Harkin A. *Acta Neuropsychiatrica* 2017.

Association between psychological measures with inflammatory and disease-related markers of inflammatory bowel disease. (Clinical Research Article)

Abautret-Daly Á., **Dempsey E.**, Riestra S., de Francisco-García R., Parra-Blanco A., Rodrigo L., Medina C., Connor T.J., Harkin A. *International Journal of Psychiatry in Clinical Practice* 2017.

Central neuroinflammation in inflammatory bowel disease: evidence for a central increase in iNOS enzyme in an animal model of colitis. (Conference Abstract)

Dempsey E., Abautret-Daly Á., Medina C., Docherty N., Harkin A. *European Neuropsychopharmacology* 2015.



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ORIGINAL ARTICLE

Association between psychological measures with inflammatory and disease-related markers of inflammatory bowel disease

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ABSTRACT

Objective: This study aimed at investigating the associations between inflammatory mediators, symptoms and psychological disturbances in inflammatory bowel disease (IBD) patients.

Methods: IBD patients and patient controls were examined during a single visit to a gastroenterology clinic. Disease activity was assessed using the Mayo index for ulcerative colitis (UC), inflammatory bowel disease questionnaire (IBDQ), Crohn's disease activity index (CDAI) and Crohn's disease endoscopic index of severity (CDEIS). Gene expression of inflammatory mediators were measured in intestinal biopsies and whole blood samples along with circulating concentrations of interleukin (IL)-6, interferon (IFN) γ , C-reactive protein (CRP), kynurenine and tryptophan. Validated depression, anxiety and quality of life scores were used to assess psychological well-being.

Results: Patients who were symptomatic had the highest depression and anxiety scores, together with increased intestinal expression of IL-1 β , IL-6 and matrix metalloproteinase-9, increased circulating IL-6 and CRP, and an increased circulating kynurenine:tryptophan ratio. Increased Hamilton depression (HAM-D) scores in IBD patients were observed independent of the psychological impact of acute symptoms.

Conclusions: Active IBD is associated with symptoms of depression and anxiety and with a raised circulating inflammatory mediator profile. Patients with active IBD exhibiting psychological symptoms should undergo psychological evaluation to ensure the psychological aspects of the condition are considered and addressed.

ARTICLE HISTORY

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KEYWORDS

Inflammatory bowel disease; depression; anxiety; cytokines; kynurenine

Introduction

There is a well-documented increase in the risk of psychological disturbances such as depression or anxiety in patients with inflammatory bowel disease (IBD) [reviewed by Abautret-Daly et al. (2017)]. The reason for increased psychological disturbance remains unclear; however, increased disease activity has been associated with increased psychological disturbances (Ben Thabet et al. 2012; Gandhi et al. 2014; Panara et al. 2014). Furthermore, immunomodulation therapy leads to a reduction in disease activity scores and psychological symptom scores in IBD patients (Calvet et al. 2006; Horst et al. 2015). During active IBD the protective intestinal barrier can become compromised, which can lead to increased intestinal permeability. This can in turn induce a local inflammatory response and/or systemic immune activation. Measurement of tissue matrix metalloproteinases (MMP), small molecules capable of cleaving most components of the intestinal extracellular matrix, can provide an indication of the damage within the gut wall. MMP9 in particular is increased in tissue biopsies from IBD patients and in intestinal samples from animal models of IBD (Medina et al. 2001, 2003; Medina & Radomski 2006; Harden et al. 2008). Despite this evidence for inflammation and psychological disturbances during the active inflammatory phase of IBD, few studies have investigated the association between the expression of specific inflammatory mediators and psychological scores in IBD patients.

Based on the recent literature two potential molecular mechanisms are proposed which may influence normal psychological function in IBD patients; increased cytokine expression and kynurenine pathway activation. Cytokines are soluble mediators of inflammation produced by a variety of cell types including immune cells. Cytokines can interact with the central nervous system (CNS) and 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 (Licinio & Wong 1999; Schiepers et al. 2005; Abautret-Daly et al. 2017). Different cytokine profiles exist between ulcerative colitis (UC) and Crohn's disease (CD); however, some are common to both diseases: IL-1 β , TNF α , IL-6, IFN γ (McClane & Rombeau 1999; Moriconi et al. 2007). As an increased risk of psychological disturbances exists for 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).

The amino acid tryptophan is the precursor to both serotonin and kynurenine. During immune activation, through induction of indoleamine 2,3 di-oxygenase (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 implicated in both depression and anxiety disorders (Graeff et al. 1996; Albert et al. 2014); secondly, the products of tryptophan metabolism, kynurenines, are neuromodulators and could therefore influence the CNS in their own right (Ruddick et al. 2006; Guillemin et al. 2007; Nemeth et al. 2007; O'Farrell & Harkin 2017).

Depression and anxiety are associated with decreased health related quality of life (HRQOL) in IBD patients (Hyphantis et al. 2010; Iglesias-Rey et al. 2014) and a link between psychological disorders and poor drug compliance has also been reported (Goodhand et al. 2013). As a result, treating psychological disturbances associated with IBD may improve IBD patients' quality of life and the mechanisms involved are worth investigating. The overall aim of this investigation was to determine if symptoms or inflammatory mediators are associated with psychological disturbances in IBD patients. Specifically the objectives were (1) to 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 with active or inactive inflammation; (2) to measure 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 and (3) to assess depression, anxiety and HRQOL scores in IBD patients versus patient controls.

Methods

Subjects

This study included IBD patients and patient controls inclusion criteria were an age of 30–70 years and confirmed UC or CD. Exclusion criteria included general exclusion criteria for colonoscopy, infective colitis, pregnancy, terminal illness and patients unfit to provide informed consent. Patient disease activity was assessed using the Mayo index for UC (Su et al. 2007) and the Crohn's disease activity index (CDAI) for CD (Best et al. 1976). An experienced gastroenterologist (Adolfo Parra-Blanco) performed the colonoscopies with Olympus endoscopes and immediately after the procedure graded the findings according to both Mayo endoscopic scoring for UC and Crohn's disease endoscopic index of severity (CDEIS) for CD. Both Mayo and CDEIS endoscopic scoring systems assess inflammatory severity within the intestine which can range between inactive, mild activity, moderate activity and severe activity. Patients with normal colonoscopy attending for screening of

colon cancer were included as controls (n ¼ 19). A fasting sample of blood was obtained from each of the study participants. Serial biopsies were obtained from inflamed areas during colonoscopy. Prior to the colonoscopy, patient demographics, symptoms and treatment were recorded. Most patients (15/18) were receiving anti-inflammatory medication (aminosalicylates, methotrexate, azathioprine and anti-TNF α antibodies). After collection, all biopsy specimens were washed with phosphate buffered saline then frozen in liquid nitrogen and stored at 80 C. Ethical approval, patient recruitment, clinical interviews and colonoscopy were carried out by the Department of Gastroenterology, Hospital Universitario Central de Asturias, Oviedo, Spain. All procedures involving human participants were in accordance with the 1964 Helsinki declaration and its later amendments and comparable ethical standards. Signed consent was obtained from each participant prior to assessment. All subsequent tissue analysis was carried out in Trinity College Dublin, Ireland.

Well-being, mood and anxiety scores

A profile of mood state (POMS) test was administered to all study participants by interview 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 according to how they have been feeling in the past week (Pollock et al. 1979). Participants can choose from 0 (not at all) to 4 (extremely). Seven factors have been derived from this including tension-anxiety, depression-dejection, anger-hostility, fatigue-inertia, vigour-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. Total mood disturbance is calculated by adding the raw scores from tension, depression, anger, fatigue and confusion and then subtracting the vigour score. This yields a value between 24 and 177, with lower scores indicative of people with stable mood profiles.

Quality of life was assessed using the Spanish Version of the EuroQoL-5D (EQ-5D). This is one of the most widely used HRQOL questionnaires. It assesses five dimensions, mobility, self-care, usual activities, pain/discomfort and anxiety/depression. Each dimension has three levels: no problems, some problems, or severe problems at the time of completion. For this study the dimensions were divided into two groups: no problems versus problems. The inflammatory bowel disease questionnaire (IBDQ)-9 is a shortened version of the IBDQ-36 questionnaire, it is

a widely used questionnaire 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 IBDQ-9 (IBDQs) which contains five questions of relevance to bowel symptoms was also employed in this study. 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.

Depressive symptoms were 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 depressive symptoms. Eight 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 (0–4), feelings of guilt (0–4), suicide (0–4), insomnia early, middle or late (0–2), work and activities (0–4), psychomotor retardation (0–4), agitation (0–4), psychological anxiety (0–4), somatic anxiety (0–4), loss of appetite (0–2) general somatic symptoms (0–2), genital symptoms (0–2), hypochondriasis (0–4), loss of weight (0–3), insight or denial (0–2), diurnal variation (0–2) and severity of variation (0–2), depersonalization and derealisation (0–4), paranoid symptoms (0–3), obsessional compulsive symptoms (0–2). 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 >23 is considered a very severe condition. Anxious symptomatology 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. Fourteen 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 and behaviour at interview. A score of 17 indicates mild severity, 18–24 mild to moderate severity and 25–30 moderate to severe.

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 min at 2000g. The resulting supernatant was immediately transferred into a 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 50 nM glacial acetic acid, 100 mM zinc acetate, 3% acetonitrile dissolved in double-distilled HPLC grade H₂O, 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 the samples were de-proteinated by the addition of perchloric acid (final concentration 6%); 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, Macclesfield, UK). 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 14,000g for 30 min at 4 C using AmiconUltra-0.5mL 3K centrifugal filters (Millipore, Carrigtwohill, Ireland) 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 s and vortexed vigorously prior to centrifugation and filtration as per total samples.

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 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.

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

Plasma IL-6 and IFN γ concentrations were measured using ELISA MAXVR Deluxe kits (Biolegend, UK), and plasma CRP concentrations were measured using a CRP ELISA DuoSet (R&D Systems, Abingdon, 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.

Zymography

Gelatin zymography was used to detect the activity of plasma MMP2 and MMP9 as described previously (Medina et al. 2006). Briefly plasma samples were added to 2 mL round-bottomed tubes containing lysis buffer (150 mM NaCl, 50 mM tris-HCl pH 8.0, 0.1% v/v NP-40, 50 µL/10 mL phosphatase inhibitor cocktail I [SigmaAldrich, Arklow, Ireland]). Samples were then vortexed vigorously and centrifuged at 14,000 rpm at 4 C for 15 min. Supernatants were transferred to eppendorf tubes and stored at 80 C. Prior to analysis samples were equalised to 4000 µg/mL. 18 µL of sample and 6 µL of loading buffer were added to each well. Afterwards, samples were subjected to 10% SDS-polyacrylamide gel electrophoresis with co-polymerized gelatin (0.2%; SigmaAldrich Co., Arklow, Ireland). The conditioned medium of HT-1080 human fibrosarcoma cells (that contains high amounts of MMP2 and MMP9), was used as internal control. Gels were then washed with 2.5% Triton X-100 (three times, 20 min each) and then incubated for 48 h at 37 C in enzyme assay buffer (25 mM Tris HCl, 0.9% NaCl, 5 mM CaCl₂, and 0.05% Na₃N, pH 7.5). Following incubation gels were fixed and 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, Watford, UK) and Quantity One analysis software (Version 4 Bio-Rad) was used to measure intensity mm.

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, Manchester, 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, Life Technologies, Paisley, UK). Gene expression analysis was conducted using realtime PCR employing TaqmanVR Gene Expression Assays (Applied Biosystems, Life Technologies, Paisley, 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, Life Technologies, Paisley, 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, Life Technologies, Paisley, UK). Fold change in gene expression from the control group was calculated using the $\Delta\Delta C_t$ method, and GAPDH served as endogenous control in the amplification system. Data are expressed as fold change in gene expression relative to the control group.

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 if required. All statistical analyses were considered to be significant when $p < .05$. Graphs and statistics were generated using GraphPad Prism Software Version 4.00 (GraphPad Software, Inc., La Jolla, CA) and GB-STAT v.10 (Dynamic Microsystems Inc., Silver Spring, MD), respectively.

Results

The study groups comprised of 18 patients with IBD (UC, $n = 10$; CD, $n = 8$) in addition to 19 age-balanced patient controls. For IBD patient clinical characteristics see Table 1. Of the 19 patient controls one (5.3%) had previously suffered from depression (female), while three (16.6%) of the IBD patients had a previous diagnosis of depression (two males and one female).

Table 1. IBD patient and patient control clinical characteristics.

IBD patients		Patient controls	
Variable	n	Variable	n
Female	11	Female	9
Male	7	Male	10
<i>IBD diagnosis</i>		<i>Indication for colonoscopy</i>	
UC	10	Haematochezia	6
CD	8	Screening	4
<i>Location</i>		Diarrhoea	
Left-colitis	6	Pain	3
Ileocolitis	6	Polyps	3
Pancolitis	3	<i>Result of colonoscopy</i>	
Proctitis	1	Polyps	9
Colon	1	Diverticula	8
Ileal	1	Normal	7
<i>Medication</i>		<i>Treatment</i>	
5-aminosalicylic acid	7	None	12
Azathioprine	5	Polypectomy	7
None	3	<i>Medication</i>	
Methotrexate	2	None	18
Infliximab	1	Nifedipine	1

Increased intestinal and circulating inflammatory markers in IBD

IBD patients had significant increases in the expression of inflammatory markers compared to control patients. Student's t test revealed increases in the expression of IDO, IFN γ , IL-6, MMP9, TNF α , and iNOS mRNA in intestinal biopsy samples from IBD patients versus patient controls. IL-1 β is approaching significance ($p = .06$). Increases in circulating inflammatory mediators including IL-6 and IFN γ , and a trend towards increased CRP were also revealed. Total, bound and free tryptophan and kynurenine concentrations were quantified. There were no changes observed in free tryptophan and kynurenine or in the ratio of these in IBD patients compared to patient controls. Both bound and total tryptophan and kynurenine and the ratio of these showed a similar profile of change in IBD patients and therefore only total tryptophan and kynurenine concentrations together with the corresponding ratio are reported in the Tables 2–5. Tryptophan concentrations are lower in the IBD patients and CD patients when compared to patient controls. The kynurenine:tryptophan ratio is significantly increased in IBD and CD patients compared to patient controls (Table 2). Lower tryptophan concentrations are indicative of mobilisation along the kynurenine pathway. Future studies should also quantify metabolites downstream from

kynurenine such as hydroxyanthranilic acid, kynurenic acid and index were revealed when patients were divided based on quinolinic acid to more fully profile kynurenine pathway the colonic inflammatory state. metabolism in IBD patients.

No significant differences in the HAM-D scores, HAM-A scores, POMS score or IBDQ scores were found between the IBD patient and patient control groups (see Table 2). Comparison of the UC and CD groups showed no differences in the inflammatory markers in the tissue biopsies or in the circulation with the exception of tryptophan in the CD group. Psychological scores were not significantly different in the CD patients versus the UC patients (Table 2).

PAX-gene analysis of whole blood mRNA expression of MMP9, iNOS, IDO and IFN γ , and circulating MMP enzyme activity revealed no differences between patient controls and IBD patients, UC and CD patients, or based on DAI scores or the IBDQ index (data not shown). 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.

Increase in inflammatory mediators during moderate/severe intestinal inflammation

IBD patients were subdivided based on the severity of intestinal inflammation 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 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. The Mayo CDEIS is an endoscopic score that classifies CD into inactive, mildly, moderately and severely active disease. Biopsy PCR analysis of inflammatory markers confirmed the clinical diagnosis of severity, whereby the greatest 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) $\frac{1}{4}$ 6.25, p $\frac{1}{4}$.0018], IFN γ mRNA [F(3,33) $\frac{1}{4}$ 5.28, p $\frac{1}{4}$.0044], IL-6 mRNA [F(3,33) $\frac{1}{4}$ 10.12, p < .0001], IL-1 β mRNA [F(3,33) $\frac{1}{4}$ 8.35, p $\frac{1}{4}$.0003], TNF α mRNA [F(3,33) $\frac{1}{4}$ 4.24, p $\frac{1}{4}$.012], MMP9 mRNA [F(3,33) $\frac{1}{4}$ 9.03, p $\frac{1}{4}$.0002], and iNOS mRNA [F(3,33) $\frac{1}{4}$ 10.56, p < .0001]. Significant differences in the circulating markers of inflammation were also revealed during moderate/severe inflammation: IL-6 [F(3,33) $\frac{1}{4}$ 14.58, p < .0001], IFN γ [F(3,33) $\frac{1}{4}$ 7.39, p $\frac{1}{4}$.0006], CRP [F(3,33) $\frac{1}{4}$ 30.38, p < .0001] and kynurenine:tryptophan [F(3,33) $\frac{1}{4}$ 12.0, p < .0001]. However, no significant differences in POMS, HAM-D, HAM-A or IBDQ

Increase 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 4). One-way ANOVA showed effects on HAM-D [F(2,34) $\frac{1}{4}$ 4.57, p $\frac{1}{4}$.018], HAM-A [F(2,34) $\frac{1}{4}$ 4.29, p $\frac{1}{4}$.022], circulating IL-6 [F(2,34) $\frac{1}{4}$ 21.37, p .0001], circulating IFN γ [F(2,34) $\frac{1}{4}$ 4.66, p $\frac{1}{4}$.0166], circulating CRP [F(2,34) $\frac{1}{4}$ 13.66, p .0001], and circulating kynurenine:tryptophan ratio [F(2,34) $\frac{1}{4}$ 9.70, p .001]. One-way ANOVA also showed differences in mRNA expression from the intestinal biopsy samples for IDO [F(2,34) $\frac{1}{4}$ 4.17, p $\frac{1}{4}$.024], IFN γ [F(2,34) $\frac{1}{4}$ 4.56, p $\frac{1}{4}$.018], IL-6 [F(2,34) $\frac{1}{4}$ 12.47, p .0001], IL-1 β [F(2,34) $\frac{1}{4}$ 12.13, p $\frac{1}{4}$.0001], TNF α [F(2,34) $\frac{1}{4}$ 3.83, p $\frac{1}{4}$.032], MMP9 [F(2,34) $\frac{1}{4}$ 11.03, p $\frac{1}{4}$.0002] and iNOS [F(2,34) $\frac{1}{4}$ 7.17, p $\frac{1}{4}$.003].

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

Depression was associated with increased IBDQs symptom related score (see Table 5). Two-way ANOVA of HAM-D score revealed a significant interaction between symptom score (IBDQs) and IBD [F(1,33) $\frac{1}{4}$ 5.501, p $\frac{1}{4}$.025]. Two-way ANOVA for HAM-A scores did not reveal an interaction between symptom expression and IBD [F(1,33) $\frac{1}{4}$ 3.3, p $\frac{1}{4}$.078], however, symptoms influence HAM-A score in IBD patients [F(1,33) $\frac{1}{4}$ 8.77, p $\frac{1}{4}$.006]. Interaction between symptom expression and IBD was also revealed for IL-6 and IL-1 β mRNA concentrations: [F(1,33) $\frac{1}{4}$ 4.79, p $\frac{1}{4}$.036], [F(1,33) $\frac{1}{4}$ 4.92, p $\frac{1}{4}$.034] and [F(1,33) $\frac{1}{4}$ 11.278, p $\frac{1}{4}$.002], respectively. For the other intestinal and circulating inflammatory markers significance was revealed for IBD alone: IDO mRNA [F(1,33) $\frac{1}{4}$ 7.73, p $\frac{1}{4}$.0089], IFN γ mRNA [F(1,33) $\frac{1}{4}$ 5.31, p $\frac{1}{4}$.028], iNOS mRNA [F(1,33) $\frac{1}{4}$ 11.56, p $\frac{1}{4}$.0018] and MMP9 mRNA [F(1,33) $\frac{1}{4}$ 7.14, p $\frac{1}{4}$.012].

Table2. Evidence of intestinal and circulating immune mediators in IBD patients.

	Patient control	IBD	UC	CD
M:F	10:9	7:11	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
Biopsy (fold change)				
IDO	1 ± 0.13	23.95 ± 8.047	15.75 ± 5.53	34.21 ± 16.66
IFN γ	1 ± 0.29	12.12 ± 4.96	6.46 ± 2.7	19.18 ± 10.49
IL-6	1 ± 0.58	42.46 ± 21.15	19.37 ± 15.9	71.32 ± 42.7
IL-1 β	1 ± 0.47	26.2 ± 13.39	13.81 ± 11.31	41.68 ± 26.68
TNF- α	1 ± 0.16	2.38 ± 0.65	1.83 ± 0.44	30.7 ± 1.36
MMP9	1 ± 0.26	9.36 ± 3.73	9 ± 4.84	10.27 ± 6.17
iNOS	1 ± 0.15	7.64 ± 1.91	9.88 ± 2.65	4.85 ± 2.58
Plasma				
IL-6	5.9 ± 0.88	10.96 ± 2.33	9.78 ± 2.82	12.43 ± 4.05
IFN γ	2.63 ± 0.42	8.23 ± 2.22	9.25 ± 3.57	6.96 ± 2.46
CRP	2.41 ± 0.51	5.3 ± 1.4 (0.06)	4.52 ± 1.82	6.26 ± 2.27
Kyn	734 ± 84.37	639 ± 47.11	687.7 ± 48.69	578.1 ± 85.8
Try	12492 ± 1334	9206 ± 804.3	10390 ± 581	7726 ± 1559
kyn:try	5.9 ± 0.2	7.4 ± 0.6	6.8 ± 0.6	8.3 ± 1.2
Psy scores				
POMS	12.89 ± 4.32	11.17 ± 6.16	2.6 ± 5.5	21.88 ± 11.37 (0.1)
HAM-A	6.16 ± 0.97	8.5 ± 1.6	8.9 ± 2.77	8 ± 1.28
HAM-D	2.8 ± 0.82	5.11 ± 0.9	5.5 ± 1.51	4.6 ± 1
IBDQ	51.95 ± 1.36	53 ± 1.75	55.7 ± 1.54	49.63 ± 3.16 (0.08)
IBDQs	30.63 ± 1.08	30.28 ± 1.31	32.1 ± 1.18	28 ± 2.41 (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), circulating total kynurenine, tryptophan, the circulating total kynurenine:tryptophan ratio and psychological scores. Data are expressed as mean ± SEM. $p < .05$, $p < .01$ versus patient controls (pooled variance student's t-test). Numbers in brackets represent p values approaching significance. CRP: C-reactive protein; CD: Crohn's disease; F: female; HAM-A: Hamilton anxiety; HAM-D: Hamilton depression; IDO: indoleamine 2,3-dioxygenase; iNOS: inducible nitric oxide synthase; IBD: inflammatory bowel disease IBDQ: inflammatory bowel disease questionnaire; IFN: interferon; IL: interleukin; M: male; MMP: matrix metalloproteinase; POMS: profile of mood states; psy: psychological; TNF: tumour necrosis factor; UC: ulcerative colitis.

Table3. 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	52.17 ± 5.72
N	19	7	5	6
Biopsy (fold change)				
IDO	1 ± 0.13	7.38 ± 6.58	24.43 ± 11.9	42.9 ± 19.22
IFN γ	1 ± 0.29	1.48 ± 0.77	12.35 ± 7.64	24.33 ± 12.47
IL-6	1 ± 0.58	1 ± 0.56	4.7 ± 3.13	122.35 ± 51.40
IL-1 β	1 ± 0.47	1.15 ± 0.57	4.21 ± 1.6	73.74 ± 33.87
TNF- α	1 ± 0.16	0.96 ± 0.26	2.79 ± 0.88	3.68 ± 1.68
MMP9	1 ± 0.26	1.43 ± 0.43	5.47 ± 3.16	22.46 ± 9.12
iNOS	1 ± 0.15	2.44 ± 1.45	12.82 ± 3.83	9.39 ± 3.62
Plasma				
IL-6	5.9 ± 0.88	6.65 ± 1.66	4.43 ± 1.65	21.43 ± 4.08
IFN γ	2.63 ± 0.42	8.99 ± 4.93	1.05 ± 0.42	13.32 ± 1.5
CRP	2.41 ± 0.51	1.05 ± 0.27	2.5 ± 0.97	12.59 ± 1.76
kyn	734 ± 84.37	674 ± 65.65	502.3 ± 115.9	712.2 ± 56.31
Try	12492 ± 1334	11050 ± 1012	8553 ± 2094	7602 ± 929.9
kyn:try	5.9 ± 0.2	6.2 ± 0.4	6.1 ± 0.5	10.0 ± 1.2
Psy scores				
POMS	12.89 ± 4.32	12 ± 13.95	13.2 ± 9.45	8.5 ± 7.28
HAM-A	6.16 ± 0.97	7.42 ± 1.58	8 ± 3.86	10.17 ± 3.43
HAM-D	2.8 ± 0.82	4.43 ± 1.4	4 ± 1.55	6.83 ± 1.87
IBDQ	51.95 ± 1.36	54 ± 3.1	54 ± 1.92	51 ± 3.76
IBDQs	30.63 ± 1.08	32 ± 1.78	30.4 ± 1.86	28.17 ± 3.01

Data represent mRNA fold change for biopsy samples, circulating concentrations of IL-6 (pg/mL), IFN γ (pg/mL), and CRP (mg/L), circulating total kynurenine, tryptophan, the circulating total kynurenine:tryptophan ratio, and psychological scores. Data are expressed as mean ± SEM. $p < .05$, $p < .01$ versus patient controls (one-way ANOVA followed by Newman-Keuls post hoc test). CRP: C-reactive protein; CD: Crohn's disease; F: female; HAM-A: Hamilton anxiety; HAM-D: Hamilton depression; IDO: indoleamine 2,3-dioxygenase; iNOS: inducible nitric oxide synthase; IBD: inflammatory bowel disease IBDQ: inflammatory bowel disease questionnaire; IFN: interferon; IL: interleukin; M: male; MMP: matrix metalloproteinase; POMS: profile of mood states; psy: psychological; TNF: tumour necrosis factor; UC: ulcerative colitis.

Table 4. Increased HAM-A, and HAM-D scores associated with raised inflammatory biomarkers 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
Biopsy (fold change)			
IDO	1 ± 0.13	24.03 ± 11.16	23.8 ± 10.59
IFN γ	1 ± 0.29	7.69 ± 3.72	20.97 ± 12.92
IL-6	1 ± 0.58	5.29 ± 3.06	116.8 ± 53.51
IL-1 β	1 ± 0.47	2.95 ± 0.96	72.69 ± 34.29
TNF- α	1 ± 0.16	1.84 ± 0.47	3.46 ± 1.72
MMP9	1 ± 0.26	3.64 ± 1.5	21.4 ± 9.47
NOS	1 ± 0.15	6.51 ± 2.13	9.91 ± 3.97
Plasma			
IL-6	5.9 ± 0.88	5.76 ± 1.2	21.4 ± 4.12
IFN γ	2.63 ± 0.42	6.96 ± 3.1	10.78 ± 2.47
CRP	2.41 ± 0.51	2.59 ± 0.99	10.72 ± 2.63
kyn	734 ± 84.37	607.7 ± 63.29	701.7 ± 61.89
try	12,492 ± 1334	9635 ± 1076	8349 ± 1133
kyn:try	5.9 ± 0.2	6.5 ± 0.3	9.4 ± 1.5
Psy scores			
POMS	12.89 ± 4.32	11.25 ± 8.86	11 ± 6.47
HAM-A	6.16 ± 0.97	6.25 ± 1.18	13 ± 3.74
HAM-D	2.8 ± 0.82	3.75 ± 0.91	7.83 ± 1.72
IBDQ	51.95 ± 1.36	54.75 ± 1.87	49.5 ± 3.49
IBDQs	30.63 ± 1.08	29.33 ± 1.66	31.33 ± 1.96

Data represent mRNA fold change for biopsy samples, circulating concentrations of IL-6 (pg/mL), IFN γ (pg/mL), and CRP (mg/L), circulating total kynurenine, tryptophan, 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 ± SEM. $p < .05$, $p < .001$ versus patient controls (one-way ANOVA followed by Newman-Keuls post hoc test). CRP: C-reactive protein; CD: Crohn's disease; F: female; HAM-A: Hamilton anxiety; HAM-D: Hamilton depression; IDO: indoleamine 2,3-dioxygenase; iNOS: inducible nitric oxide synthase; IBD: inflammatory bowel disease IBDQ: inflammatory bowel disease questionnaire; IFN: interferon; IL: interleukin; M: male; MMP: matrix metalloproteinase; POMS: profile of mood states; psy: psychological; TNF: tumour necrosis factor; UC: ulcerative colitis.

Table 5. IBD patients experiencing bowel symptoms have increased HAM-A and HAM-D scores.

	Patient control		IBD patients	
	Nosymptoms	Symptoms	Nosymptoms	Symptoms
M:F	7:4	3:5	5:5	2:6
Age	53.91 ± 3.39	54.88 ± 2.98	45.2 ± 4.06	53.75 ± 2.68
n	11	8	10	8
Biopsy (fold change)				
IDO	1 ± 0.15	1.35 ± 0.28	32.21 ± 14.89	21.18 ± 9.69
IFN γ	1 ± 0.28	1.66 ± 0.78	11.18 ± 5.34	19.75 ± 12.38
IL-6	1 ± 0.7	0.42 ± 0.17	4.21 ± 2.55	61.39 ± 30.33 bp
IL-1 β	1 ± 0.65	0.68 ± 0.28	2.34 ± 0.85	45.61 ± 22.67 p
TNF- α	1 ± 0.23	0.88 ± 0.2	1.9 ± 0.54	2.81 ± 1.28
MMP9	1 ± 0.32	1.87 ± 0.81	6.12 ± 2.61	24.21 ± 11.37
iNOS	1 ± 0.22	1.03 ± 0.18	8.39 ± 2.6	6.91 ± 3.08
Plasma				
IL-6	6.26 ± 1.14	5.39 ± 1.45	5.33 ± 1.35	18 ± 3.76 p
IFN γ	2.65 ± 0.61	1.18 ± 0.41	5.33 ± 1.75	11.61 ± 4.37
CRP	2.52 ± 0.76	2.26 ± 0.67	3.36 ± 1.38	7.72 ± 2.48
kyn	727.1 ± 93.07	743.5 ± 162.6	668 ± 46.96	602.8 ± 90.79
trp	12670 ± 1694	12248 ± 2283	10303 ± 725.5	7835 ± 1485
kyn:try	5.8 ± 0.25	6.0 ± 0.45	6.6 ± 0.5	8.5 ± 1.2
Psy scores				
POMS	8.18 ± 4.67	19.38 ± 7.79	10.7 ± 10.5	11.75 ± 5.49
HAM-A	5.36 ± 1.16	7.25 ± 1.66	5 ± 1.27	12.88 ± 2.55 p
HAM-D	2.73 ± 0.89	3 ± 1.59	2.7 ± 0.75	8.13 ± 1.22 p
IBDQ	55.45 ± 0.87	47.13 ± 2	57.3 ± 0.72	45.38 ± 2.22
IBDQs	34.09 ± 0.34	26.25 ± 1.43	33.7 ± 0.47	25.25 ± 1.68

Data represent mRNA fold change for biopsy samples, circulating concentrations of IL-6 (pg/mL), IFN γ (pg/mL), and CRP (mg/L), circulating total kynurenine, tryptophan, the circulating total kynurenine:tryptophan ratio, and psychological scores. Data are expressed as mean ± SEM. $p < .05$, $p < .01$ versus non-symptomatic patient controls, $\text{b}\text{p} < .05$, $\text{b}\text{b}\text{p} < .01$ versus non-symptomatic IBD patients (two-way ANOVA followed by Newman-Keuls post hoc test). CRP: C-reactive protein; CD: Crohn's disease; F: female; HAM-A: Hamilton anxiety; HAM-D: Hamilton depression; IDO: indoleamine 2,3-dioxygenase; iNOS: inducible nitric oxide synthase; IBD: inflammatory bowel disease IBDQ: inflammatory bowel disease questionnaire; IFN: interferon; IL: interleukin; M: male; MMP: matrix metalloproteinase; POMS: profile of mood states; psy: psychological; TNF: tumour necrosis factor; UC: ulcerative colitis.

Discussion

This study supports the hypothesis that raised inflammatory mediators may be responsible for the increased risk of depression and/ or anxiety-related symptoms in IBD patients (Martin-Subero et al. 2016). It is one of a limited number of studies which investigates both inflammatory profile and psychological wellbeing within the same cohort of IBD patients when compared to a patient control group. The main finding is that the symptomatic phase of IBD represents the disease phase with the highest depression and anxiety scores, and this phase of IBD is associated with increased intestinal expression of IL-6 and IL-1 β , as well as increased circulating concentrations of IL-6. Of particular note is that patient controls with the same level of symptom expression, as per the modified IBDQ, do not experience the same psychological disturbance. This finding suggests that the acute psychological impact of the symptoms themselves are not responsible for increased HAMD scores in IBD patients. In support, other studies have shown that inflammation in CD (indicated by CRP levels) is a predictor of depression (Banovic et al. 2013) and inflammation in children (indicated by erythrocyte sedimentation rate) is related to clinically significant depressive symptoms as measured on the children's depressive inventory (CDI) (Clark et al. 2014).

Psychological scores were not different in IBD patients versus patient controls

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 an increased risk for psychological symptoms with IBD compared to other gastrointestinal conditions related to disease activity and CDAI scores (Brandi et al. 2009), with only irritable bowel syndrome (IBS) patients being at a higher risk for affective symptoms in most cases (Tkalcic et al. 2010; Bengtsson et al. 2013).

No significant differences were found between the UC patients and CD patients in terms of the inflammatory markers in the gut or the circulation. Furthermore, no differences in anxiety or depression scores were seen between UC and CD patients. This is in agreement with

previous research which also found no difference between risk of depression in UC and CD (Schuman et al. 2013; Selinger et al. 2013; Long et al. 2014). The same was also reported in paediatric IBD using the CDI depression score (Szigethy et al. 2004).

Psychological disturbances are associated with higher symptom expression in IBD patients

In this study we examined the risk of depression and anxiety, and inflammatory markers based on different measures of disease activity. The first is based on the clinician's assessment of severity, following the colonoscopy, on inflammatory activity within the intestine which ranges between inactive, slight activity, moderate activity and severe activity. Patients were then separated into non-IBD patient controls, and inactive, mild and moderate/severe inflammation groups. The PCR analysis of the biopsy markers confirms the clinical diagnosis of intestinal inflammatory state, whereby significant differences exist between the moderate/ severe IBD group and the patient controls for IFN γ , IDO, IL-6, IL-1 β , TNF α , iNOS 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 IFN γ , 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 (Brown et al. 2002; Zilberman et al. 2006; Gupta et al. 2012). No significant differences in the psychological scores for depression, anxiety or well-being were revealed in moderate/severe IBD versus inactive IBD.

The second method of dividing patients in terms of disease activity level was based on the DAI scores: CDAI for CD and Mayo score for UC, as per previous studies. A score of 150 for the CDAI and a score of 4 for the Mayo index were considered active. Unlike the colonoscopy evaluation of activity, separating the patients using the DAI scores showed disease activity was significantly associated with the psychological scores. This supports previous investigations into psychological disturbances in IBD patients (Ben Thabet et al. 2012). What is also clear from the Mayo and CDAI scores is that these patients are largely in an inactive disease phase as only six patients scored above the threshold. Separating the groups based on DAI also reveals significant differences in circulating IFN γ , IL-6, CRP concentrations and the kynurenine:tryptophan ratio in patients with high DAI scores versus the patient controls and those with 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 indices 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.

Limitations

Although this is an exploratory study with low patient numbers, contains a heterogeneous IBD patient population and is unable to fully distinguish cause from correlation with regards to psychological symptoms and the measured inflammatory biomarkers, it does provide evidence for increased HAM-A and HAM-D scores during the phase of IBD involving increased inflammatory cytokines and symptoms which appear to be independent of the acute psychological impact of these symptoms. A further limitation of the study is that the IBDQ 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. There has been a growing interest and awareness of the effectiveness of anti-inflammatory medication in treating the symptoms of depression (Köhler et al. 2014; Kappelmann et al. 2016). Considering that IBD patients are routinely prescribed anti-inflammatory medication for inflammatory symptoms, these treatments may also have beneficial effects on associated psychological symptoms. This has not been studied to date in IBD patients and should be considered. Future studies should attempt to match IBD patients with patients suffering from GI symptoms in terms of years since diagnosis, regularity of symptoms, current symptom

expression and take into account the efficacy of anti-inflammatory medications.

Conclusions

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

Key points

We aim at investigating whether inflammatory mediators or IBD symptoms are responsible for the increased risk of depression/anxiety in IBD.

This study shows higher depression and anxiety scores for IBD patients who have a higher disease activity.

HAM-D depression scores are increased in IBD patients versus patient controls with the same level of symptom expression.

Symptomatic IBD patients also had higher expression of inflammatory mediators as measured in intestinal biopsy and in the circulation as well as an increased circulating kynurenine:tryptophan ratio.

Changes to inflammatory mediators and tryptophan availability are potentially involved in the increased comorbidity of anxiety/depressive disorders in these patients.

Disclosure statement

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Review Article

Gut–brain actions underlying comorbid anxiety and depression associated with inflammatory bowel disease

Abautret-Daly Á, Dempsey E, Parra-Blanco A, Medina C, Harkin A.

Gut–brain actions underlying comorbid anxiety and depression associated with inflammatory bowel disease.

Introduction: Inflammatory bowel disease (IBD) is a chronic relapsing and remitting disorder characterised by inflammation of the gastrointestinal tract. There is a growing consensus that IBD is associated with anxiety- and depression-related symptoms. Psychological symptoms appear to be more prevalent during active disease states with no difference in prevalence between Crohn's disease and ulcerative colitis. Behavioural disturbances including anxiety- and depression-like symptoms have also been observed in animal models of IBD.

Results: The likely mechanisms underlying the association are discussed with particular reference to communication between the gut and brain. The close bidirectional relationship known as the gut–brain axis includes neural, hormonal and immune communication links. Evidence is provided for a number of interacting factors including activation of the inflammatory response system in the brain, the hypothalamic–pituitary–adrenal axis, and brain areas implicated in altered behaviours, changes in blood brain barrier integrity, and an emerging role for gut microbiota and response to probiotics in IBD.

Discussion: The impact of psychological stress in models of IBD remains somewhat conflicted, however, it is weighted in favour of stress or early stressful life events as risk factors in the development of IBD, stress-induced exacerbation of inflammation and relapse.

Conclusion: It is recommended that patients with IBD be screened for psychological disturbance and treated accordingly as intervention can improve quality of life and may reduce relapse rates.

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Keywords: anxiety; colitis; depression; gut–brain axis; inflammatory bowel disease

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Summations

- Inflammatory bowel disease (IBD) patients are at an increased risk for developing an anxiety- or depression-related disorder.
- Animal models of IBD are a useful tool in understanding the physiological changes that occur in the brain in response to gastrointestinal (GI) inflammation.
- Due to the bi-directionality of the gut-brain axis stress, anxiety or depression may in turn exacerbate or trigger IBD. Treatment of psychological symptoms may improve health-related quality of life (HRQOL) and relapse rates for patients.

Considerations

- The increased risk of psychological symptoms during active IBD is generally agreed upon in the literature, however, further work is required during disease remission. It is possible that persistent psychological symptoms occur as a result of ongoing irritable bowel syndrome (IBS)-like symptoms.
- No one animal model or behavioural test can accurately represent the human condition in IBD or psychological illness. Translational tools such as magnetic resonance imaging (MRI) should be used for more direct comparisons between models and humans.
- The link between stress and IBD remains controversial and requires further study. The use of psychological treatments for IBD is unlikely to replace traditional anti-inflammatory treatments.
- Management of GI symptoms may also improve psychological outcomes.

Introduction

IBD is a chronic relapsing and remitting disorder of the GI tract. Crohn's disease (CD) and ulcerative colitis (UC) are the two main subtypes of IBD. CD and UC have similar symptomatology, however, CD symptoms can depend on the site of inflammation. Symptoms common to both CD and UC include abdominal pain/cramping, loose stools, diarrhoea, bloody stools, rectal bleeding, fatigue and a loss of appetite or food avoidance. Extra-intestinal manifestations of IBD can affect joints, the skin and eyes (1). IBD patients are also at a higher risk of developing colorectal cancer (CRC) compared with the general population (2). CD and UC differ mainly in their histology and in terms of their location within the GI tract. CD affects all layers of the gut wall, whereas in UC inflammation is usually confined to the mucosa. The formation of intestinal granulomas and fistulae are hallmarks of CD. Inflammation in UC usually remains in the rectum and colon, whereas CD can affect any part of the GI tract, however, most commonly involves ileocaecal inflammation (3,4).

The disease burden of IBD is challenging for patients and not only includes the physiological manifestations of the disease but also psychological and social burden. An IMPACT survey commissioned by the European Federation of Crohn's and Ulcerative Colitis Associations in late 2010 assessed the impact that IBD can have on patients in terms of medical implications, emotional well-being, education and work, and overall quality

of life. Almost half (48%) of European IBD patients surveyed indicated that even between flare-ups their lives are negatively affected by symptoms of IBD (5). Although IBD can occur at any age, the disease has a peak in incidence rates in younger people between the ages of 15 and 30 years, meaning that the majority of patients are faced with this diagnosis during the most productive years of life. In CD, incidence declines sharply following this peak, however, in UC, peak incidence typically occurs 5 years later than in CD and plateaus, particularly in males, in whom incidence does not significantly decrease until the seventh decade of life (6).

The aetiology of IBD remains unknown, however, the consensus is that immune dysfunction and inflammation occurs as a response to an environmental trigger in a genetically susceptible host. IBD is an incurable yet treatable disease typically managed with drugs (aminosalicylates, systemic corticosteroids and immunosuppressants) and surgery (such as bowel resection for CD or colectomy with ileostomy or ileo-anal pouch anastomosis) if necessary (7,8). Surgery will generally be required in 70–80% of CD patients and up to 30% of UC patients (9). Surgery may be curative for UC, whereas inflammation usually recurs following surgery in CD (10). Mortality for IBD is slightly higher than the general population with a UK study of over 16 000 IBD patients with age and sex-matched controls indicating 54% excess mortality associated with IBD diagnosis (11).

Table 1. Reports of anxiety- and depression-related symptoms in inflammatory bowel disease (IBD) patients

Study	Participants	Measures	Results and author's conclusions
Cawthorpe and Davidson (27): Canada	Adults and children: UC, controls	Medical records: ICD mental disorder and UC	Mental disorder present in 82% of UC sample. Neuroses/depressive disorders are most likely mental disorders to arise before UC diagnosis. Indicates aetiological relationship between mental disorder and UC or between their treatments
Horst et al. (28): USA	Adults: UC, CD	HBI, SCCAI, PHQ-9, CRP	Depressive scores decreased in IBD patients on immunosuppressive therapy. Significant decrease in UC and CD patients at risk of moderate to severe depression. Changes in CRP scores correlate with PHQ-9 (depression) scores
Keeton et al. (29): Australia	Adults: UC, CD	Concerns and worries, HADS, DASS-21	Although 74% of respondents were in remission 96% had significant disease-related concerns, 21% reported symptoms of depression, 40% of anxiety
Kinsinger et al. (30): USA	Adults: UC, CD, other GI disorders	Demographic/psychosocial checklist, BSI, IBS-QOL, medical records	Psychological intervention can reduce healthcare burden for patients with GI conditions and is associated with reduced outpatient visits, medical procedures and medications required
Clark et al. (24): USA	Children and adolescents: CD	CDI, Paris classification, KSADS, PCDAI, ESR, medication use	Infliximab use not associated with decreased depressive symptoms. SES and disease activity were the strongest predictors of depression on the PCDAI
Gandhi et al. (20): USA	Adults: UC, CD	HBI, IBDQ, BDI, PHCS, CISS, CAMBI	Patients with inactive IBD had less depressive symptoms, and improved perceived health competence, task-oriented coping, and QOL compared with those with active IBD
Long et al. (31): USA	Adults ≥65 years: UC, CD	Short-GDS, short-CDAI, SCCAI, short-IBDQ	22.6% of patients had GDS scores consistent with a diagnosis of major depression. Point prevalence of depression did not differ between UC and CD. Depressed patients had significantly higher disease activity scores and decreased QOL
Maconi et al. (32): Italy	Adults: CD-R	CDAI, HADS	Anxiety and/or depressive symptoms were present in 36.9% of patients, 58% of whom were not being treated for anxiety/depression. Anxiety significantly correlated with female sex, history of perianal disease and perianal surgery
Szigethy et al. (33): USA	Children and adolescents: UC-D, CD-D	CDI, KSADS, CDRS-R, IMPACT-III, CGAS, PCDAI, PUCAI	Following 3 months of psychological therapy 65% of all participants no longer met the DSM-IV-TR criteria for depression. IBD activity improved over time for both CBT and SMDT with a slightly larger decrease for CBT
Szigethy et al. (34): USA	Children and adolescents: UC, CD	CDI, CDRS-R, SCARED, IMPACT-III, PCDAI, PUCAI, API, BIPQ, medical records	Greater disease activity in depressed vs. non-depressed youth with IBD. Evidence for three depressive profiles in youth with concurrent IBD and depression
Virta and Kolho (35): Finland	Children and adolescents: UC, CD, controls	Analysis of national drug registers	Significant increase in anti-depressant drug use in IBD youth compared with peers up to 3 years following diagnosis
Vlachos et al. (36): Greece	Adults: UC	CDAI, ZDRS, STAI, HADS	Inducible HSP70 induction in PMN cells of UC patients correlates with depression and anxiety scores in ZDRS, STAI and HADS-D but not HADS-A. This could potentially be a biomarker for depression and anxiety in UC
Ananathkrishnan et al. (37): USA	Adult females: UC, CD, controls	MHI-5	Women with recent depressive symptoms had an increased risk of CD but not UC
Bokemeyer et al. (25): Germany	Adults: UC, CD, IC	CAI, CDAI, PGA, SF-36, IMET-IS	UC and CD patients with active disease more often reported severe depressive symptoms and sexual problems
Guloksuz et al. (38): The Netherlands	Adults: CD	HBI, CDAI, MFI, IBDQ, HAM-D-17, BDI, SCL-90, SCID, APP, zinc, TRP	Anti-TNF- α treatment increased IBDQ scores and reduced SCL-90 depression scores for CD patients independent of disease activity. Depression scores were associated APP- γ over time but not with TRP availability
Knowles et al. (39): Australia	Adults: CD	BIPQ, HADS, SCL-90	48% of CD patients had anxiety and 42% depression on the HADS. Of these only 20% and 31%, respectively, were receiving psychological care
Langhast et al. (40): Germany	Adults: UC	Endoscopy, histology, CAI, PSQ, HADS, CPSS	Short-term stress and male gender but not long-term stress, depression or mucosal healing were predictive of relapse in UC patients
Schuman et al. (41): USA	Adolescents: UC, CD	CDI, FAD, CBCL, LCAI, short-PCDAI	20% of patients scored above the cut-off for depression on the CDI with no difference between UC and CD. Disease severity was a significant predictor of patient-reported but not parent-reported depressive symptoms
Selinger et al. (42): Australia	Adults: UC, CD	Steroid medication self-report, CCKnow, short-IBDQ, HADS	High levels of anxiety (41.8%) and depression (14%) in IBD patients with no difference in anxiety between UC and CD. Anxiety was correlated with level of disease-related knowledge, worse depression scores and lower QOL
Shiga et al. (43): Japan	Adults: UC, CD	Hospital records, Mayo disease scores (UC), HBI	Significant increase in relapse rate observed among UC but not CD patients following Great East Japan Earthquake compared with the same period of time the previous year. Life-event stress is associated with relapse in UC, not CD
Ben Thabet et al. (26): Tunisia	Adults: UC, CD, patient controls	CDAI, HADS, TAS-20	More IBD patients than controls had high HADS-D (22%) and HADS-A (26%) scores.
Besharat et al. (44): Iran	Adults: UC, CD, IC	SCCAI, BDI	Approximately 73% of high HADS-D and HADS-A scores were within an active disease phase. Depression was seen in 32% of patients with IBD despite all patients being in remission. A non-significant correlation was observed between SCCAI, BDI, age and BMI
Goodhand et al. (45): UK	All ages: UC, CD, controls	Medical records	IBD patients on anti-depressants had fewer relapses and courses of steroids in the year following anti-depressant treatment compared with the previous year with a decreased number of endoscopies in year 2 compared with year 1
Goodhand et al. (21): UK	Adults: UC, CD, healthy controls	SCCAI, CDAI, HADS, general-PSQ, FC, CRP, endoscopy	Anxiety and depression scores were significantly higher in IBD vs. healthy controls. No differences in mean HADS scores between UC and CD. Active UC was associated with higher HADS scores but not CD

Table 1 (Continued)

Study	Participants	Measures	Results and author's conclusions
Loftus et al. (46): USA	Children: CD, paediatric controls	ICD-9-CM, epidemiology, medical records, prescription medication	The risk of developing an anxiety or depressive disorder following CD diagnosis is greater for CD patients compared with non-CD patients. CD also increases the risk of developing persistent anxiety and depression
Iglesias et al. (47): Spain	Adults: CD-R	CDAI, CRP, ELISA, HADS	Despite clinical remission, 39% of CD patients had anxiety symptoms and 24% had depressive symptoms. Infliximab therapy is the only factor associated with anxiety, however, it is inversely associated with depression
Vidal et al. (48): Spain	Adults: UC-R, CD-R	SCID, HBI, SCAI	In remission 31.1% of IBD patients had at least one psychiatric disorder. Anxiety (17.9%) and depression (11.6%) were the most prevalent
Addolorato et al. (22): Italy	Adults: UC, CD, other GI disorders	STAI, ZDRS	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
Vidal et al. (49): Spain	Adults: UC, CD	IBDQ, CDAI, HADS, TCI	44.6% scored >8 in HADS-A or HADS-D. Psychological distress and disease activity were predictors of low QOL
Walker et al. (50): Canada	Adults: UC, CD, community controls	CIDI, CPSS, HAQ, PWB, IBDQ	Social anxiety decreased in IBD vs. controls. Increased major depression in IBD vs. controls. Anxiety or mood disorders lead to decreased QOL perception and earlier onset of IBD symptoms
Calvet et al. (23): Spain	Adults: CD, healthy controls	SF-36, HAM-A, HAM-D-17	Decreased SF-36 score and significantly increased HAM-A and HAM-D scores in active disease vs. healthy controls and thiopurinic-induced remission
Janke et al. (51): Germany	Adults: UC, CD	GLS, HRLS, FLZ, HADS, GIBDI	Increased psychiatric illness, medical comorbidity, 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. (52): Belgium	Adults: CD	CDAI, PHQ-9, HADS, TAS-20, SSL-I, infliximab, CRP	Major depression in CD is predictive of lower remission rates
Mardini et al. (53): USA	Adults: CD	CDAI, BDI, BAI, BHS, RLC	Depression scores were significantly correlated with disease activity scores at baseline and 8–12 weeks later. Depressive symptoms were positively associated with future changes in disease activity
Mittermaier et al. (54): Austria	Adults: UC, CD	BDI, STAI, IBDQ, PSQ, RFPC	Depression scores significantly correlated with the number of relapses after 12 or 18 months and the time to disease. Anxiety and HRQOL were also related with more frequent relapses during 18 months
Kurina et al. (55): UK	Adults: UC, CD	ORLS medical records	Depression and anxiety preceded UC but not CD more often than expected in control groups. Depression and anxiety were more common following CD diagnosis. UC was followed by anxiety, not depression more often than expected by chance

API, Abdominal Pain Index; APp, acute phase protein – gamma; BAI, Beck Anxiety Inventory; BDI, Beck Depression Inventory; BHS, Beck Hopelessness Scale; BIPQ, Brief Illness Perception Questionnaire; BSI, Brief Symptom Inventory; CRP, C-reactive protein; CES-D, Center for Epidemiologic Studies-Depression Scale; CBCL, Child Behaviour Checklist; CDI, Children's Depression Inventory; CDRS-R, Children's Depression Rating Scale – Revised; CGAS, Children's Global Assessment Scale; CAI, Clinical Activity Index; CBT, Cognitive Behavioural Therapy; CPSS, Cohen Perceived Stress Scale; CAMBI, Complementary and Alternative Medicine Beliefs Inventory; CIDI, Composite International Diagnostic Interview; CISS, Coping Inventory for Stressful Situations; CCKnow, Crohn's and Colitis Knowledge score; CD/CD-R/-D, Crohn's disease/in remission/with depression; CDAI, Crohn's Disease Activity Index; DSM-IV-TR, Diagnostic and Statistical Manual of Mental Disorders-IV-Text Revision; DASS, Depression Anxiety and Stress Scale; ELISA, enzyme-linked immunosorbent assay; ESR, erythrocyte sedimentation rate; FC, faecal calprotectin; FAD, Family Assessment Device; FLZ, Fragen zur Lebenszufriedenheit – German questionnaire on life satisfaction; GI, gastrointestinal; GLS, General Life Satisfaction; GP, general population; GDS, Geriatric Depression Scale; GIBDI, German Inflammatory Bowel Disease activity Index; HAM-A/-D, Hamilton rating scale for Anxiety/Depression; HBI, Harvey Bradshaw Index; HAQ, Health Anxiety Questionnaire; HRLS, Health-Related Life Satisfaction; HRQOL, Health-Related Quality of Life; HSP70, heat shock protein 70; HADS/HADS-A/HADS-D, Hospital Anxiety and Depression Scale/-Anxiety subscale/-Depression subscale; IBD, Illness Behaviour Questionnaire; IF, immunofluorescent; IMET-IS, IMET – Impairments in Sexuality; IC, indeterminate colitis; ICD/ICD-CM, International Classification of Diseases/-Clinical Modification; IBD, inflammatory bowel disease; IBDQ, Inflammatory Bowel Disease Questionnaire; IBS, irritable bowel syndrome; LCAI, Lichtiger Colitis Activity Index; MHI-5, Mental Health Index-5; MFI, Multi-dimensional Fatigue Inventory; ORLS, Oxford Record Linkage Study; PHQ, Patient Health Questionnaire; PCDAI, Paediatric Crohn's Disease Activity Index; PUCAI, Paediatric Ulcerative Colitis Activity Index; PHCS, Perceived Health Competence Scale; PSQ, Perceived Stress Questionnaire; PGA, Physician Global Assessment; PMN, polymorphonuclear cells; PWB, Psychological Well-Being Manifestations Scale; QOL, Quality of Life; RFPC, Rating Form of IBD Patient Concerns; RLC, Holmes Recent Life Changes; SCARED, Screen for Child Anxiety-Related Disorders; SF, Short-Form Health Survey; SCCAI, Simple Clinical Colitis Activity Index; SSL-I, Social Support List – Interactions; STAI, State-Trait Anxiety Inventory; SQLS, Stoma Quality of Life Scale; SCID, Structured Clinical Interview for Axis-I DSM-IV Disorders; SNTD, Supportive Non-Direct Therapy; SCL, Symptom Distress Checklist; TAS, Toronto Alexithymia Scale; TRP, tryptophan; UC/UC-R/-D, ulcerative colitis/in remission/with depression; ZDRS, Zung Depression Rating Scale.

Appending number on abbreviations in table, if present, indicates number of items on test

Comorbid anxiety and depression in IBD

Historically there has been a long-standing interest in the comorbidity of psychological well-being, psychiatric illness and personality differences associated with IBD (12–15) in patients of all age groups. Many early studies in adults and children came to the conclusion that both UC and CD are related to higher incidence of psychological symptoms (16–18). Of particular interest is the study by Addolorato et al. (18) as only patients naïve to steroid treatment and surgical intervention were included, and again IBD patients had significantly higher expression of depressive symptoms than controls (41.9% and 50% of CD and UC patients, respectively, show depressive symptoms compared with 11.1% of controls). They also report an association between mood and disease activation.

In a recent systematic review of the comorbidity of psychological disorders with IBD, Mikocka-Walus et al. (19) evaluated 66 articles published between 2005 and 2014. Rates of anxiety and depression were estimated to be greater in IBD patients compared with healthy individuals, with rates of both being higher during the active IBD phase compared with remission. Mean rates of anxiety and depression were significantly higher in CD compared with UC but only modestly so.

Validated questionnaires including the Hospital Anxiety and Depression Scales (HADS), Beck Depression Inventory (BDI), State-Trait Anxiety Inventory (STAI) and Hamilton anxiety and depression scales have been used to confirm an increased risk of psychological disturbances in IBD patients (20–23). Differences between studies suggest that factors such as the level of disease activity may influence anxiety and depression scores. Depression and/or anxiety are consistently reported to be increased in the active phase of IBD (20,24–26).

A detailed summary of reports documenting anxiety and depression symptoms in IBD patients is summarised in Table 1.

Anxiety/depression in paediatric IBD

Increased risk for psychiatric disorders is not unique to adults with IBD; adolescents and children with IBD are also reported to have

increased risk of anxiety and depression. In a large paediatric patient study; Loftus et al. (46) compared 2144 paediatric patients with CD to 10 720 age- and sex-matched 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 anti-depressants, anti-psychotics, anxiolytics, mood stabilisers and benzodiazepines was higher in CD patients compared with controls (35). Szigethy et al. (34) have reported that three distinct profiles of depression exist in youths with IBD ($n = 217$): mild depression (in 75% of participants) encompassing diverse, low-grade depressive symptoms and possessing the highest quality of life; somatic depression (in 19%) displaying severe fatigue, appetite change, anhedonia, decreased motor activity and depressed mood with concurrent high-dose steroid therapy and the highest IBD activity; and cognitive despair (in 6%) with the highest rates of self-reported depressive symptoms, ostomy placements and anxiety. Patients in the cognitive despair group reported IBD symptoms in the relative absence of inflammation and rated as the highest of the three profile groups on measures of morbid and suicidal ideations. As a result, they suggested that subgroup-specific interventions may be needed when treating depression in youths with IBD.

Anxiety/depression in active IBD versus remission

In a small case-control study which included healthy controls and CD patients with active disease, investigators reported increased anxiety and depression during active IBD (23). A third group of CD patients, being treated with a thiopurinic immunomodulator, was also included. Interestingly, thiopurinic-induced

remission restored psychological well-being to normal range in CD patients. In a more recent retrospective study, IBD patients treated with anti-tumour necrosis factor (TNF)- α antibodies (infliximab, adalimumab or certolizumab) or immunomodulator therapy (methotrexate or azathioprine) also had significant improvements in depressive symptoms (28). Furthermore, Guloksuz et al. (38) reported changes in depression scores in patients with CD at 2, 4 and 8 weeks after an infliximab infusion (see Table 1). In contrast, a paediatric study carried out in the United States failed to show an effect of infliximab infusion on depression scores in children and adolescents with CD (24).

When taken together these studies suggest that the risk of psychological manifestations is increased during the active phase of IBD, however, the results for patients during remission remain unclear. Maconi et al. (32) reported that anxiety/depressive symptoms were present in 36.9% of CD patients in remission and strikingly that 58% of these patients were not currently receiving treatment for psychological illness. Similarly, Knowles et al. (39) reported that of CD patients with anxiety (48%) and depression (42%) only 20% and 31%, respectively, are receiving psychological care. Besharat et al. (44) also reported high rates (32%) of depressive characteristics in a group of Iranian IBD patients and patients with indeterminate colitis, despite all patients being in remission at the time of the study. This observation is echoed in an Australian cohort where despite 74% being in remission 96% had significant disease-related concerns, and symptoms of depression and anxiety were reported by 21% and 40%, respectively (29). In slight contrast to some studies mentioned previously, Iglesias et al. (47) reported that a cohort of CD patients in remission for at least 6 months on infliximab therapy were shown to have increased anxiety and a decreased frequency of depression. This study concluded that despite clinical remission a significant number of CD patients present with anxiety or depressive symptoms, and that those CD patients in remission would benefit from psychological support.

Anxiety/depression in UC versus CD

It is now widely reported that no psychological differences between UC and CD exist (21,26,31, 41,42). A recent study on the effectiveness of immunosuppressive therapy on depression in IBD reports differences in prevalence of moderate to severe depression depending on IBD diagnosis (51% CD and 18% UC) (28). Furthermore, differences have been reported in the type of anxiety observed between UC and CD patients (22) and in the risk of psychiatric factors (anxiety and depression) on health-related life satisfaction in CD compared with UC (51) (see Table 1).

Impact of psychological symptoms on the development/ course of IBD

GI and psychological pathologies: cause and effect

An important question surrounding comorbidity of psychological symptoms with IBD is how these may be linked and whether one may predispose to the other. Although there has been a substantial amount of literature on the prevalence of depression and anxiety in IBD, less investigation has been carried out into the effect of such symptoms on the development of IBD or on the course of IBD. This may be due to the longitudinal and more protracted nature of this kind of study. Kurina et al. (55) 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 succeeded the 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, whereas in patients with CD the depression followed the diagnosis of the disease. Therefore, they suggest that the onset of depression in UC might be causally related to UC, a result of living with an undiagnosed bowel condition. For CD they suggest that depression might be a result of the disease symptoms or treatment of the illness. Cawthorpe and Davidson (27) also found that

neuroses or depressive disorders were most likely to arise before UC for men and women. They suggest that psychotropic medication used to treat anxiety and depression may play a role in the aetiology of UC. Concerning paediatric literature on this topic, a 2011 study which analysed medical and prescription claims of children with CD and patient controls observed a 74% increased risk of developing an anxiety disorder after CD diagnosis with an increased risk of developing persistent anxiety or depression following diagnosis and a significantly greater likelihood of being prescribed psychotropic medication (46).

Walker et al. (50) investigated the lifetime risk of depression in the Manitoba IBD patient cohort (Canadian IBD cohort), and carried out a long-term 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. Contrary to Kurina et al. (55), Ananthakrishnan et al. (37) found that depressive symptoms increase the risk for CD, but not UC, among women. The reasons for the differences in outcome between these studies are unclear. Further research into the potential impact of psychological disorders on the development of IBD is needed.

Risk of relapse and effects of psychological treatment

A small number of studies have investigated the influence of anxiety or depression on the risk of relapse in IBD. In a study of 112 patients with inactive IBD, Vidal et al. (48) reported that neither depression nor anxiety increased the risk of relapse in UC or CD patients. Langhorst et al. (40) also failed to demonstrate a predictive effect of depression on the risk of relapse in patients with UC. This is contradictory to two older studies where BDI scores were predictive of future changes in IBD activity (53,54). In a paediatric study of children and adolescents with IBD, Szigethy et al. (34) reported greater disease activity in depressed compared with non-depressed youth with IBD. Persoons et al. (52)

also reported decreased remission rates in patients with a major depressive disorder.

Goodhand et al. (45) have reported improvement in IBD (see Table 1) in patients who have been prescribed anti-depressants, compared with matched patients who did not receive treatment for depression. In another study by Szigethy et al., this group examined the effect of therapy on depressed youth with IBD (33). They found that cognitive behavioural therapy and supportive non-direct therapy caused an improvement in HRQOL and psychosocial functioning, and were associated with an improvement in IBD activity over time (see Table 1). In a recent study concerning psychological intervention for patients with a range of GI disorders, including IBD, functional bowel disorder, dyspepsia and oesophageal symptoms, Kinsinger et al. (30) reported that psychological intervention can reduce healthcare burden (see Table 1). These studies suggest that psychological assessment may help to identify patients at risk of disease exacerbation or decreased rates of remission and may be an effective way to improve HRQOL. Importantly, these factors suggest that treating the psychological symptoms could be beneficial in terms of the overall course and management of IBD.

Effects of stress on risk of relapse

As well as anxiety and depression, short-term and long-term stress may influence the course of IBD. Evidence to suggest bidirectional communication between the gut and brain is emerging indicating that psychological stress and/or depression has negative implications for normal gut function. It is also hypothesised that stress may be a risk factor for relapse in patients suffering from IBD, although this remains controversial with some studies reporting no effect of stress on development of IBD or increased risk of relapse (49,56). However, Langhorst et al. (40) found that short-term stress, but not long-term stress, was predictive of relapse in UC patients. The impact of life-event stress on UC, but not CD, was highlighted after the Great East Japan Earthquake in 2011 (43). Results from 12 hospitals, found that UC patients activity scores increased significantly in the 2 months following the earthquake. Dietary

changes and anxiety regarding family finance were independent predictors of relapse.

Immunologic mechanisms underlying psychological disturbance in IBD

Inflammatory origins of mood disorders

Mounting evidence indicates that inflammation plays a critical role in the pathophysiology of mood disorders. Patients with schizophrenia, major depression and bipolar disorder have been shown to have elevated levels of pro-inflammatory cytokines (57). Reciprocally, many inflammatory conditions including IBD, rheumatoid arthritis, psoriasis, cardiovascular disease and diabetes (58–61) have been linked to a higher risk of mood disorders. Evidence for this link is also found in studies of cancer patients and hepatitis C patients receiving immune-based therapy. These patients display increases in depressive tendencies while receiving interferon or interleukin-2 treatment, which subside once treatment finishes (62,63). Furthermore, both clinical and preclinical studies have shown that the induction of a pro-inflammatory state in otherwise healthy subjects results in poor mood and ‘sickness behaviour’; a behavioural phenotype resembling depression with symptoms including lethargy, anxiety, social withdrawal, anhedonia and anorexia (64–67). Though they share many symptoms and are both thought to have a basis in inflammation sickness behaviour is distinct from clinical depression. Sickness behaviour is evolutionarily intended to confer benefit by allowing for rest and isolation thus conserving energy, enabling an effective inflammatory response and preventing the spread of infection to others. It has been proposed that for clinical depression to occur there is a transition from sickness behaviour resulting in sensitisation of immune-inflammatory pathways, progressive damage by oxidative and nitrosative stress, and an autoimmune response directed against self-epitopes with the latter processes leading to neural tissue damage and functional and cognitive artefacts over repeated depressive episodes (68). Considering that IBD is a lifelong disorder involving chronic relapsing and

remitting inflammation and activation of oxidative and nitrosative pathways (69), it is likely that the depressive behaviour observed in IBD patients is not simply sickness behaviour but a comorbid depression (70). Furthermore, sickness behaviour is evolutionarily intended as an adaptive response to sickness, whereas comorbid depression worsens the original sickness as has been observed in IBD patients (34,52–54), while treatment for depressive symptoms improves IBD course (33,45). In response to the above findings the use of anti-inflammatories as an adjunct to conventional therapy for depression has been explored and suggests a beneficial effect though further research is required in this area (71). Potential mechanisms for the inflammatory induction of behavioural changes may include effects of cytokines on hypothalamic–pituitary–adrenal (HPA) axis dysregulation, monoamines and the kynurenine pathway, over-activation of microglia, impairments in neuroplasticity, and structural and functional changes in the brain.

Factors influencing anxiety and depression in IBD

Some socioeconomic/ environmental/ physiological factors such as education, socioeconomic status, gender, diet, pain, perceived stress, etc. may be predictive of or have an effect on psychological disturbance in IBD and are worth studying to increase our understanding of disease pathogenesis and psychological comorbidity. These factors may account for some of the variation observed across studies of depression and anxiety in IBD and are noted in Table 1 where applicable.

Various studies have observed that lower socioeconomic status and lower educational level are associated with depression and anxiety in IBD patients (41,72,73). Such patients are also shown to have lower HRQOL than the general population, however, it is difficult to draw conclusions from this link as this may be a result of other non-disease-related factors (74).

Generally, there is little difference in IBD occurrence between men and women (75). However, gender does appear to be linked to differences in psychosocial manifestations of the disease. The majority of studies indicate that female gender is a predictor of anxiety and depression in IBD (32,72,76). Females are also

believed to be more susceptible to the impact of IBD on HRQOL (77) potentially due to increased symptom perception in women (78). Females with IBD are also more likely to have IBS-like symptoms concurrent with IBD (79), greater levels of fatigue (80), and show a higher incidence of mood swings among those with CD (81), all of which may impact on HRQOL and psychological health.

Although diet is not a causative factor in IBD it is thought to be a potential trigger for IBD flares, and it is believed that the Westernised diet rich in processed foods is a factor in the increased incidence of the disease in these regions (82). A modified diet limiting excess fat, carbohydrates, fibre and lactose, and encouraging intake of pre- and probiotic foods may be helpful as an adjunct therapy in IBD in decreasing symptoms and reducing medication requirements (83). Although it is still unclear whether dietary modification would be therapeutic in the psychological aspects of IBD, considering the link between diet, stress and the influence of gut microbiota on mood it is reasonable to think that this may be beneficial and will be addressed further later in this review.

The literature indicates that active IBD is associated with an increase in psychological manifestations (24,31) with disease severity being an independent predictor of depressive symptoms (41,76). UC patients with pain have been shown to have significantly higher depression scores than UC patients without pain (84), with higher pain scores being an accurate predictor of depression in both UC and CD (84,85). As well as pain, the nature of other symptoms experienced by IBD patients can be extremely stressful. As will be discussed in greater detail later in this review, stress can affect visceral sensitivity, gut motility and the immune system in IBD, and as previously noted may be a trigger for IBD flares (40). Perceived stress has been associated with mood disturbance in both UC and CD (21) indicating a link between stress, symptom-related or otherwise, and psychological disturbance in IBD.

Inflammation and depression in IBD

Despite the recent surge in psychoneuroimmunology research, there is a lack of investigation into the inflammatory

mediators and mechanisms underlying psychological disturbances during active IBD. There may be psychoneuroimmunological components that predispose some people to the development of UC. Vlachos et al. (36) assessed levels of constitutive and inducible heat shock protein 70 (HSP70) at various sites in the colon of UC patients. They found that inducible HSP70 was strongly expressed in polymorphonuclear (PMN) cells in the colonic mucosa of the majority of patients. They also report that the induction of HSP70 significantly correlated with anxiety and depression scores in various psychometric tests, including HADS-D, STAI and Zung Depression Rating Scale, and with the Rachmilewitz Clinical Activity Index but not with HADS-A scores. This group suggest HSP70 induction in PMN cells as a possible biomarker for depression and anxiety in UC.

In addition to clinical investigations, experimental models of IBD in animals allow for the study of interactions between the gut and the brain during and in recovery from colitis in order to decipher the mechanisms by which IBD interacts with the central nervous system (CNS) and to develop potential therapies to best manage comorbid symptoms. Due to the paucity of biomarkers (molecular and cellular) reported in human studies, arising from the difficulty in assessing impact of stress and/or psychological disturbance in IBD in a clinical setting, data available from animal models are the best available source to obtain insight into gut-brain interactions underlying comorbidity in IBD.

Animal models of IBD

As the exact aetiology of IBD is still largely unknown there are many possible factors which contribute to different aspects of the pathophysiology of the disease including immune system dysfunction, dysregulation of the microbiota, genetics, inflammation and oxidative stress. Animal models of IBD have been developed to allow for investigation of aetiological factors in terms of understanding the mechanisms of disease pathogenesis and developing therapeutic strategies for intervention. These models may be grouped into chemical- or microbial-induced models;

spontaneous models; genetically engineered or transgenic models; and adoptive transfer (T-cell) models [for review see (86)].

Despite the range of models of IBD, the main body of behavioural and brain research has been carried out in the chemically induced models, particularly the dextran sulphate sodium (DSS) and trinitrobenzenesulphonic acid (TNBS) models. Okayasu et al. (87) were the first to describe the DSS-induced colitis model which involves oral administration of DSS in the drinking water of the animals leading to the development of acute and chronic colitis. Gaudio et al. (88) assessed the structural, ultrastructural, immunohistochemical and clinical aspects of DSS colitis in Sprague-Dawley rats in both acute and chronic DSS-induced colitis and suggest that the DSS model is more representative of UC than CD. Indeed, DSS-induced colitis is histologically characterised by infiltration of inflammatory cells, crypt loss and extensive mucosal erosions, with predominance in the distal portion of the large intestine. Occasionally, crypt abscesses and regenerated epithelium are also seen. The TNBS model of colitis was first reported by Morris et al. (89). This model of IBD involves a single enema of the toxin TNBS in an ethanol solution. TNBS-induced colitis results in a T-cell (TH1)-mediated inflammatory response which is described as CD-like in nature (90). Unlike the DSS model which predominantly involves the distal colon, TNBS can induce a more widespread colitis, involving macroscopic ulceration of the large intestine with varying severity, strictures of the lumen and fistulae formation. Both of these models result in immune activation in the gut, are histologically representative of IBD, and despite their limitations in terms of studying disease aetiology are valuable tools for studying mechanisms of IBD pathogenesis. Both models are simple, inexpensive, reproducible and valid for examining the potential interaction between intestinal immune activation and the CNS.

Other animal models used to investigate the association between GI disturbance and psychological manifestations include infection models by which bacteria such as *Citrobacter rodentium* and *Campylobacter jejuni*, which colonise and disrupt tissue in the GI tract of mice and are effective models of acute colitis. *C. rodentium* uses attaching and effacing lesions to colonise the GI tract resulting in ulcerative

intestinal lesions, reduced barrier integrity, production of pro-inflammatory cytokines and manifesting as weight loss and diarrhoea (91). *C. jejuni* produces and secretes toxins to aid in its intestinal colonisation and increase mucosal barrier damage, translocation of commensal bacteria across the intestinal epithelium and induction of a Th1 immune response (92). Stress-induced models may be used to study the effects of psychological stress on GI function. Maternal separation as a model of early life stress (93), chronic subordinate colony housing (94,95) and overcrowding stress (96) have been shown to induce spontaneous GI dysfunction or to increase susceptibility to chemically induced colitis. Though not wholly valid for studies on IBD specifically these models demonstrate the link between stress and GI disturbance and are functional tools in assessing IBS-like symptoms.

CNS disturbances in models of IBD

Anxiety-/depression-like behavioural alterations:

In a recent study, Heydarpour et al. (97) show an increase in immobility in the forced swimming test (FST), a depression-related behaviour in mice 3 days post-TNBS injection. This effect is attenuated using a specific inducible nitric oxide synthase (iNOS) inhibitor (aminoguanidine) administered 30 min before the FST indicating the potential involvement of the nitric oxide pathway in the induction of this behaviour. In the DSS model of colitis, Chen et al. (98) performed anxiety and depression-related behavioural tests in rats following a DSS (5%) colitis induction period and reported that DSS exposure caused a decrease in open arm entries and time spent in the open arm of the elevated plus maze (EPM) indicating anxiety, with an increase in immobility time in the FST indicating learned helplessness. DSS exposure also decreased sucrose preference in the sucrose preference test indicating reduced responsiveness to a rewarding stimulus, anhedonic behaviour symptomatic of depression, and reduced social interaction between animals suggestive of social avoidance and withdrawal. Interestingly, this study also found that the anxious- and depressive-like behaviours were reversed by prolonged desensitisation of transient receptor potential vanilloid 1 (TRPV1)-expressing colonic afferent neurons using a colonic infusion of the potent analogue of

capsaicin and activator of TRPV1, resiniferatoxin. In an earlier study, Messaoudi et al. (99) 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. Painsipp et al. (100) analysed female and male mouse behaviour in the EPM, open field (OF) and FST on days 8, 9 and 11, respectively, of an 11 day DSS (2%) exposure protocol. 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, whereas female rats had increased immobility in the FST indicative of a depressive-like phenotype. Lyte et al. (101) examined anxiety-like behaviour on a hole-board OF apparatus in mice infected with *C. rodentium*, a murine model of IBD. Male mice were tested 7–8h post-infection and results provide evidence for an anxiety-like phenotype: decreased exploration of the inner zone of the OF, decreased number of pokes into the holes as well as a preference for the first corner hole compared with control mice. A more recent study using the same colitis mouse model assessed anxiety-like behaviours in the light/dark box and found no behavioural alterations at 10 days postinfection when inflammation was at its peak (102). However, Emge et al. (103) using a DSS colitis model, reported that during active inflammation (8 days post-DSS) mice exhibited anxiety-like behaviour in the light/dark box, whereas recognition memory was impaired in the novel object recognition test. These behavioural alterations had normalised by 14 days post-DSS when the colitis had resolved. In an investigation by Bercik et al. (104) mice who received 3% DSS in drinking water during three 1-week cycles demonstrated increased anxiety in the step-down test compared with controls.

Sickness behaviour versus depressive-like behavior: Considering the moderate

physiological effects of chemically induced and infection-induced colitis the validity of behavioural tests, particularly those which passively measure depressive-like behaviour during acute sickness should be questioned. In behavioural tests such as OF and FST where lack of activity may be interpreted as anxiety- or depressive-like behaviour one should not discount the impact of abdominal pain being experienced by animals with active colitis. Diarrhoeal symptoms associated with colitis may affect grooming behaviour and may also impact social interaction tests. Few studies, if any, report disease activity scores or take disease activity into account in behavioural tests of colitic animals with many studies also not reporting the timepoint during colitis at which each behavioural test was carried out. In a study of lipopolysaccharide (LPS)-induced alterations in Fos expression in the brain Frenois et al. (105) argue that there is a functional difference in cytokine-induced sickness behaviour observed when LPS-sickness is at its peak (decreased motor activity 6 h post-LPS i.p. injection) and the cytokine-induced depressive-like behaviour observed (increased immobility in FST at 24 h and decreased sucrose preference at 24 and 48 h post-LPS) when LPS-induced sickness was minimal (and motor activity and food/fluid intake had returned to normal). At 24 and 48 h post-LPS increased cellular activity was measured by Fos labelling in brain structures including the amygdala, hippocampus and hypothalamus, however, at 6 h post-LPS no Fos labelling was observed in these regions. This points to an underlying difference in sickness behaviour and depressive-like behaviour induced by a systemic inflammatory insult. It would therefore be of interest to measure behavioural abnormalities in animal models of colitis during the recovery period when IBD-like symptoms have ceased.

Blood brain barrier (BBB) permeability: The BBB is a tightly controlled diffusion barrier which regulates the transport of molecules between the periphery and CNS. Endothelial cells of the BBB are non-fenestrated and have more extensive tight junctions acting as a protective barrier against pathogens and neurotoxic substances, whereas allowing influx of essential nutrients and neurotransmitters (106). Systemic

inflammation can disrupt the BBB and has been linked to syndromes such as sickness behaviour and delirium, and neurological disorders such as Alzheimer's disease and multiple sclerosis (107). The presence of intestinal inflammation in IBD models and the increase in permeability of the gut-blood barrier has been linked to an increase in the permeability of the BBB. Hathaway et al. (108) investigated potential disruption to the BBB in rabbits exposed to TNBS. Barrier disruption was assessed following i.v. administration of low molecular weight fluorescein (MW 376 Da) or a higher molecular weight molecule fluorescein isothiocyanate (FITC)-dextran (MW 71 000 Da)

48 h post-TNBS administration. Results demonstrated a significant increase in the permeability of the BBB to fluorescein, however, no difference in permeability to the higher molecular weight FITC-dextran was found. In a later investigation they confirmed these findings and suggest that free radical damage is not responsible for the BBB disruption (109). More recently, Natah et al. (110) further analysed the BBB disruption in Sprague-Dawley rats exposed to TNBS to determine the anatomical sites of the BBB disruption using sodium fluorescein (MW 376 Da) or IgG (MW 156 000 Da) as a marker of increased permeability. As per Hathaway et al. (62,63) 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, subfornical organ and median eminence during days 1 and 2 following TNBS administration.

Sans et al. (111) measured expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) using a dual radio-labelled antibody technique in four different colitis models: IL-10^{-/-} mice, mice reconstituted with CD45RB^{high} T-cells, mice with DSS-induced colitis and rats with TNBS-induced colitis. VCAM and ICAM are endothelial CAMs of the immunoglobulin super family which are responsible for the adhesion of leucocytes in various inflammatory diseases. This study demonstrated that there is a significant increase in VCAM expression in the brain of all four models of colitis, which corresponded with colonic VCAM expression and colon weight. They also report

that TNBS-induced colitis induces ICAM expression, although this is not the case in the DSS model. These changes were not associated with increased leucocyte 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.

The consequences of BBB disruption in inflammatory conditions such as IBD is that it leaves the CNS vulnerable to inflammatory mediators and gut-derived bacterial or viral antigens. In the case of IBD these inflammatory mediators are likely to be at higher concentrations in the circulation considering the increased permeability of the intestinal barrier in this condition.

Inflammatory mediators in the brain: Cytokines are soluble, regulatory proteins, released by immune cells, which act as intercellular mediators. They also have the ability to interact with the CNS either via the vagus nerve or by directly interacting with the BBB, thus providing a means of communication between the immune system and the brain. Cytokines are implicated in the pathogenesis of IBD, with a pivotal role in regulating intestinal inflammation and the clinical symptoms of IBD (112). The use of TNF- α antagonists as a standard therapy for IBD highlights the crucial role of cytokines in this disease. Following peripheral immune activation cytokines can also 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 (113) as discussed in a previous section.

TNBS model: Riazi et al. (114) investigated the influence of TNBS-induced colitis on hippocampal TNF- α concentrations and microglial activation in male Sprague-Dawley rats. They found an increase in both hippocampal TNF- α 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. (115) confirmed that a single enema of TNBS induces increases in circulating TNF- α concentrations which are paralleled by increased brain TNF- α protein concentrations. However,

unlike the Riazi et al. (114) study the increase was still present at day 15 post-TNBS administration possibly due to differences in the strain of rat used (115). Wang et al. (116) 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 IL-6 messenger RNA (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 were 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 (117). In their study they also report a decrease in the concentrations of the anti-inflammatory cytokine IL-10 at 2 and 7 days post-TNBS. Alhouayek et al. (118) also reported increased central inflammatory cytokine expression following TNBS-induced colitis. Three days post-TNBS administration there was an increase in IL-1 β , TNF- α and monocyte chemoattractant protein 1 mRNA expression in the brains of C57BL6 mice, which was associated with an increase in circulating endotoxin concentrations attributable to extensive histological damage to the colon.

DSS model: Villaran et al. (119) reported a significant increase in TNF- α , IL-6, IL-1 β and iNOS mRNA expression in the substantia nigra of male Wistar rats during acute DSS-induced colonic inflammation. More recently, Reichmann et al. (120) measured levels of IL-1 β , IL-6, IL-17A, IL-18, TNF- α and growth-regulated oncogene (GRO)- α in the circulation and in the hypothalamus, hippocampus and amygdala of mice following 7 days DSS administration (2%) and in combination with water-avoidance stress (WAS). A prolonged immobility in C57BL/6N mice with DSS-induced colitis during WAS was associated with brain region-dependent alterations in the expression genes associated with energy homeostasis [neuropeptide-Y (NPY), NPY receptor Y1], stress pathway activation [corticotropin-releasing factor (CRF), CRF1 receptor and glucocorticoid receptor] and neurogenesis (brain-derived neurotrophic factor). They report increased GRO- α in the hypothalamus as a result of DSS alone. The combination of DSS and WAS induced increases in IL-6 in all three brain regions and in GRO- α in

the hippocampus and hypothalamus. Cytokine concentrations in the brain did not correlate with plasma cytokine levels suggesting that WAS is required to effect a brain inflammatory response in DSS-exposed mice. The authors propose that alterations in gut-brain signalling may be responsible for the observed behavioural changes in response to stress in DSS animals. As well as demonstrating a decrease in hippocampal neurogenesis in DSS (3%)-treated mice Zonis et al. (169) also show an increase in circulating IL-6 and an increase in hippocampal IBA1 and GFAP, markers for activated microglia and astrocytes, respectively, in acute colitis. Following three more rounds of DSS increases were observed in hippocampal TNF- α , IL-1 β and GFAP mRNA expression and IBA1 and IL-6 protein simultaneous to the reductions in neurogenesis.

Effects of cytokines on the brain: Apart from their primary role in the inflammatory immune response cytokines have the ability to interact with the brain. Cytokine receptors located on the BBB allow for non-barrier-disruptive communication between the periphery and brain though in some cases cytokines may actually cross the BBB either via transporters or via a compromised BBB (107). Diapedesis of leucocytes across the BBB may also lead to immune activation and cytokine production in the brain (121). Microglia can respond to these cytokine signals in paracrine and autocrine fashion to facilitate tissue repair, initiate immune responses and recruit immune cells, however, sustained activation of microglia can result in neurotoxicity and production of reactive oxygen species (122). Astrocytes also communicate using the cytokine network to influence immune responses in the CNS and there is also evidence to suggest that activation of astrocytes by inflammatory mediators modulates astrocyte signalling thereby influencing synaptic and neural function and potentially playing a role in the behavioural effects of inflammation such as sickness behaviour and depression (123). Cytokines can directly affect neuronal activity, influencing neuronal excitability neuronal plasticity, neuronal development and synaptogenesis (124–126). Cytokines have been shown to affect neurotransmitter metabolism, specifically glutamate, serotonin and dopamine, in brain regions associated with emotional

regulation namely the nucleus accumbens, amygdala and hippocampus (127). Cytokines can also affect the kynurenine pathway in the brain by stimulating indoleamine 2,3-dioxygenase (IDO) production. As the IDO enzyme is responsible for conversion of tryptophan to kynurenine the amount of tryptophan available for serotonin production is decreased and depressive-like behaviour is observed (128). Pro-inflammatory cytokines may also increase kynurenine-3-mono-oxygenase enzyme activity. This enzyme degrades kynurenine into 3-hydroxykynurenine, shifting the kynurenine pathway from neuroprotection towards neurotoxicity with the production of neurotoxic metabolites and excitotoxicity. Cytokines can also influence the HPA axis impacting glucocorticoid receptor function, HPA feedback regulation and causing activation of the HPA axis (129,130). HPA axis activation results in an increase in glucocorticoids which has been implicated in depression (131). [For a more detailed review of the role of pro-inflammatory cytokines in neuroinflammation and depression see Kim et al. (132).]

Regional patterns of neuronal activation

c-Fos is an immediate-early gene expressed following an action potential which is used to indirectly measure neuronal activity [for reviews see (133,134)]. Original evidence of c-Fos activation in the nervous system following induction of colitis was published by Miampamba and Sharkey (135). Colitis was induced following a per-endoscopic injection of formalin and rats were euthanised 2 h later. Immunohistochemical analysis demonstrated a significant increase in c-Fos in the lumbosacral spinal cord, and in two circumventricular organs: nucleus of the solitary tract (NST) and area postrema. Treatment with the α -2-adrenoceptor agonist xylazine inhibited the colitis-related increase in regional c-Fos expression. A later study by Porcher et al. (136) extensively analysed the expression of c-Fos 2 h post-TNBS administration throughout the brain. They report significant increases in c-Fos immunostaining across a number of brain regions including brain nuclei involved in the autonomic, behavioural and neuroendocrine response to inflammation, in most circumventricular organs and in CRF

pathways particularly the paraventricular nucleus (PVN) of the hypothalamus. At 6 h post-TNBS administration c-Fos mRNA expression in the PVN had completely returned to basal levels. Welch et al. (138,139) focused on TNBS-induced c-Fos activation in a number of brain regions which are abnormal in autism spectrum disorder (ASD); periventricular grey, hypothalamic/visceral thalamic stress axes and cortical domains, and septal/preoptic/amygdalar brain areas. ASD is a complex, multifaceted neurodevelopmental disorder which is often linked to GI disturbance (137). Results from this study support previous evidence of increased c-Fos induction following experimentally induced colitis, however, here the results suggest prolonged neuronal activation (138). In a later study, this group showed that subdiaphragmatic vagotomy did not inhibit the observed increase in c-Fos induction in the PVN of the hypothalamus, basolateral amygdala, central amygdala (CeA), and piriform cortex indicating the unlikely role of the vagus nerve in mediating the brain activation response in these regions (139).

However, a separate study which used *C. jejuni* infection to induce intestinal inflammation in mice showed c-Fos induction in vagal sensory ganglia and in the NST – the primary sensory vagal afferent nucleus indicating that intestinal inflammation signals to the brain via this nerve (140).

The microbiota–gut–brain axis

Clinical IBD, gut microbiota and therapies

In terms of gut–brain axis research, the gut microbiota and its associations with brain and behaviour is currently one of the most promising areas of study. The gut microbiota comprises the largest collection of microorganisms in the body, existing in a symbiotic relationship with the host and in the colon reaching a concentration of 10¹¹ or 10¹² cells/g of luminal contents (141). Collectively, the human gut microbiota is thought to be composed of between 15 000 and 36 000 bacterial species (142). The major bacterial phyla found in the gut are Firmicutes and Bacteroidetes though other phyla including Actinobacteria, and Verrucomicrobia are also

present (143). The gut microbiota is believed to play a role in the pathogenesis of IBD. Dysbiosis of the commensal gut bacteria is commonly observed in IBD, generally as a decrease in diversity of Firmicutes and an increase in Proteobacteria (142), furthermore, many of the susceptibility genes for IBD are related to microbial recognition and processing (144), and antibiotics are known to be effective in reducing IBD symptoms (145). This is also supported by the finding that germ-free (GF) mice do not develop severe colitis (146).

A strong link has been discovered between a healthy gut microbiota and satisfactory CNS functioning. The microbiota has been implicated in several neuropsychiatric disorders including depression, anxiety, autism and schizophrenia [for review see (147)]. The bacterial flora of the gut can communicate with the brain via vagal pathways and immune mediators as previously discussed, as well as by the production of microbial metabolites. In a healthy individual this bidirectional communication maintains host homeostasis, whereas for an IBD patient, for example, this balance is disturbed with potential consequences for the CNS.

The imbalance observed in the gut microbiota of IBD patients presents a potential therapeutic target. Manipulation of the gut microbial flora can be achieved using probiotics, prebiotics or a combination of both (synbiotics). A probiotic is a live microbial food supplement that beneficially affects the host by improving its intestinal microbial balance (148). A prebiotic is a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth, activity, or both of one or a limited number of bacterial species already resident in the colon (149). Faecal microbiota transplantation (FMT), an extremely effective treatment for *Clostridium difficile* infection, is another method of gut microbiota manipulation being explored as a potential therapy for IBD patients. The procedure involves transplant of a faecal preparation from a healthy donor into the colon of the patient via nasoenteric tube, colonic enema or in capsule form. In *C. difficile* infection this works by restoring balance to the gut microbial environment. Although IBD is a more complicated condition with many genetic, environmental and immune factors at play, the restoration of a healthy gut microbiota would

likely be advantageous at least for some patients.

Effects of microbiota modulation on IBD and brain function

To date, clinical studies investigating the effects of probiotics or prebiotics in IBD have focused on the physiological symptoms of IBD itself. Probiotics (a preparation of live, beneficial bacteria) show efficacy in inducing remission and increasing remission times in UC (150), which was not the case in CD (151). Despite promising results in TNBS and DSS colitis, prebiotics (preparations of dietary nutrients which support the growth of beneficial host bacteria) have been tested in few clinical trials. Similar to probiotics, results show efficacy in reducing inflammation and inducing remission in UC (152–154), whereas somewhat controversially reducing disease activity and inflammation in CD (155,156). Although no work has explored the potential therapeutic benefit for probiotics or prebiotics in reducing anxiety/ depression specifically in IBD patients, this therapeutic strategy has been tested in other cohorts. Messaoudi et al. (157) administered a probiotic mixture of *Bifidobacterium longum* and *Lactobacillus helveticus* for 30 days to both rats and healthy human volunteers and showed a decrease in anxiety-like behaviour in rats and a decrease in anxiety and depression scores in humans in the HADS and Hopkins Symptom Checklist (HSCL-90). Similarly Rao et al. (158) administered a strain of *Lactobacillus casei* to chronic fatigue patients resulting in a reduction in anxiety scores. In terms of prebiotics (159) administered a 3 week course of galacto-oligosaccharide (GOS) or fructo-oligosaccharide (FOS) prebiotic to healthy controls. The GOS resulted in a decrease in waking cortisol levels and decreased attentional vigilance to negative versus positive emotional stimuli compared with placebo. FOS had no effects in either test. Considering these encouraging data, the use of microbiota modulation by pre/ probiotics as a simple, non-invasive therapy for the psychological as well as physiological effects of UC and potentially CD is an area which should be further explored in future. In terms of FMT as a treatment for IBD, a systematic review of the literature from case reports and cohort studies showed a modest

increase in remission rates in IBD patients receiving FMT therapy (160). More recently, two randomised, controlled trials have been carried out in UC patients with conflicting results suggesting a beneficial outcome that may be dependent on donor stool, route of administration, dosage, time since diagnosis and whether patients are also receiving immunosuppressive therapy (161,162). Interestingly, Irish researchers have recently postulated that FMT may be of therapeutic benefit in depression after showing that FMT from depressed human patients into microbiota-deficient rats induces a depressive phenotype in the rats with symptoms of anhedonia and anxiety (163).

Animal models of IBD and the gut microbiota

In animal studies the modulation of the microbiota has been used to alter behaviour. GF mice display decreased anxiety-like symptoms in the EPM, OF and light/dark box compared with specific-pathogen- free mice (164). GI microbial infection and inflammation, including exposure to DSS-induced colitis, results in an increased anxiety-like profile (101,165,166). Modulation of the gut microbiota using probiotics can alter the behavioural response. Anxiety and/or depression-related behaviours in the EPM and FST in Balb/c mice (167) and in the FST in maternally separated rats (168) are rescued by probiotics. Probiotics also reduce anxiety-like behaviours induced in rats in response to DSS colitis (165). This points to a potential therapy for neuropsychiatric disturbance in IBD patients. In an aforementioned study by Bercik et al. (104) results demonstrated that increased anxiety measured in the step-down test observed in DSS colitis mice was reversed by the probiotic *B. longum* NCC3001 without affecting gut inflammation (as measured by myeloperoxidase activity and histological scores). They found that the anxiolytic effect of *B. longum* was lost in mice vagotomised before the third cycle of DSS potentially due to the modulatory effects of the fermentation products of *B. longum* on enteric neuron excitability. In another previously mentioned behavioural study Emge et al. (103) reported that the deficits in recognition memory and anxiety-like behaviour during active inflammation on day 8 post-DSS were

ameliorated by administration of a probiotic mixture containing *Lactobacillus rhamnosus* and *Lactobacillus helveticus* (103). This study also observed a significant decrease in c-Fos expression in the CA1 region of the hippocampus in mice at day 8 post-DSS which is similarly rescued by administration of the *L. rhamnosus* and *L. helveticus* probiotic combination. It has recently been observed that intestinal inflammation may impact upon hippocampal neurogenesis (169). Following four cycles of DSS (3%) in mice Zonis et al. (169) observed a downregulation in markers for stem/early progenitor cells, with a concomitant increase in p21, a suppressor of cell proliferation, indicating a reduction in neurogenesis in the hippocampus. As p21 can be stimulated directly by pro-inflammatory cytokines it has been proposed that this decrease in hippocampal neurogenesis may occur as a consequence of the increase in activated microglia and astrocytes observed in the hippocampus at the acute phase of colitis and in chronic colitis, respectively.

Influence of psychological stress on healthy gut function

The HPA axis represents a major axis of the neuroendocrine system that controls reactions to stress. Dysregulation of the HPA axis has been linked to a number of mood disorders including depression, anxiety and bipolar disorder. The HPA axis is regulated by CRF which is released centrally from the hypothalamus or peripherally from the adrenal cortex in response to stress. Central CRF promotes HPA axis activity via the adrenocorticotrophic hormone (ACTH) – glucocorticoid system and peripheral CRF directly influences stress-induced alterations in gut motility.

Effects of stress on the gut

Konturek et al. (170) describes a number of stress- induced disturbances to normal GI physiology: including alterations to GI motility, GI secretion, GI mucosa and mucosal blood flow, intestinal microbiota and also increased visceral perception and intestinal permeability. Deng et al. (171) recently showed that levels of colonic

cytokines (IL-6, IL-1 β and IL-17A) and neutrophil infiltration in DSS- exposed rats are further increased by chronic unpredictable stress. Previous investigations have shown that when chronically stressed, animals can develop spontaneous inflammation in the bowel (94,172). Reber et al. (94) examined the effect of chronic psychosocial 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 mucosal layers of the colon, and an increased secretion of pro- and anti- inflammatory markers by the mesenteric lymph node.

Milde and Murison (173) report decreased time to symptom expression in DSS rats previously exposed to restraint stress, and in a separate study involving electric shock pre-DSS exposure they report a sensitising effect of stress on later vulnerability to intestinal permeability (174). The genotoxic agent azoxymethane predisposes mice to develop CRC when challenged with DSS. Peters et al. (175) report increased risk of inflammation-related CRC when azoxymethane mice were also exposed to chronic subordinate colony housing. Review of maternal separation has also reported disturbances to gut function as a result of early life stress (93). 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 immune dysregulation, increased intestinal permeability and disturbed intestinal microbiota. One caveat when studying the effect of psychological stress or maternal separation on models of 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.

GI effects on the HPA axis

Due to the bi-directionality of the gut-brain axis not only will a disturbance such as stress influence the GI system but a disturbance in the GI system may also influence stress by activation of the HPA axis. In the Reichmann et al. (73) study

DSS treatment increased basal and post-stress (90 min) levels of circulating corticosterone – an index for increased HPA axis activity. Greenwood-Van Meerveld et al. (176) studied the long-term effects of acute colitis on the expression of central CRF in rats. They found a significant increase in CRF mRNA expression in the 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 CeA 3 days post-TNBS administration, however, it had returned to basal levels in this region 30 days post-TNBS. Porcher et al. (136) 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 h of TNBS administration. Kojima et al. (177) report the opposite effect: decreased CRF expression in the PVN 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. (177) study indicating that a higher dose of TNBS may provoke a more severe colitis necessary for increased CRF expression in the brain.

Clinical neuroimaging studies

In the clinical literature, information on the effects of IBD on brain structure and function are limited to neuroimaging studies, of which there are very few. A 2011 neuroimaging study investigating the response of UC sufferers to emotional stimuli strongly indicated that IBD could potentially cause psychological disturbances by altering function of brain regions. Functional MRI, using blood oxygen level detection imaging of UC patients 6 months in remission demonstrated a decreased sensitivity to positive stimuli (178). Due to the subjects being in remission, this study is a strong indicator that IBD can cause persistent psychological alterations in patients. An MRI study conducted by this group in 2013 found that IBD affects grey matter volume and brain size, when CD patients showed decreased grey matter volume in the

frontal cortex and anterior midcingulate cortex. In addition, the study showed that this morphological change occurred in a manner that suggested disease duration was negatively correlated to grey matter volume (179) (Fig. 1). The mechanisms summarised illustrate the bidirectional communication axes between the brain and gut showing how behavioural changes mediated centrally may occur following experimentally induced colitis in rodents and also how stress or alterations in mood states may impact the GI system and result in GI dysfunction and IBD symptoms.

Stress leads to production of CRF from the hypothalamus which acts on the pituitary gland causing it to produce ACTH. This acts on the adrenal gland leading to production of cortisol which then enters into circulation and directly influences the gut. Direct innervation of the gut via the vagus nerve (containing mostly afferent sensory fibres and 10–20% motor and parasympathetic efferent fibres) allows feedback from the gut to the brain and central modulation of the gut. The release of neurotransmitters by the nervous system may influence gut physiology or directly influence the gut microbiota, whereas neurotransmitters produced by enteroendocrine cells in the gut or released as microbial products (short-chain fatty acid, serotonin, gamma amino butyric acid) may in turn signal to the neural network. Increased GI permeability allows microbial products and potentially microbes themselves to infiltrate into the circulatory system, and to interact with the immune system and nervous system. Immune system activation leads to the production of cytokines which may enter the circulation or act upon the nervous system. As the circulation reaches the brain these mediators may impact on BBB permeability or

initiate inflammatory pathways in the brain including activation of microglia and astrocytes.

Future directions

Despite a substantial increase in the number of studies investigating the association between IBD and psychological comorbidities in recent years, there has been a distinct lack of investigation into the cause of increased psychological disturbances in IBD. It is possible that the psychological impact of the GI symptoms could be sufficient to induce depression or anxiety. However, it is also likely that inflammatory mediators themselves could be responsible for altered mood or anxiety. Further attention to specific circulating mediators should be investigated in relation to psychological changes in patients with IBD. This would be beneficial as standard anti-depressants may not be as efficient in minimising the depressive symptoms if inflammatory mediators are responsible for the psychological changes. It is possible that a combination of immunomodulators/anti-inflammatories and conventional anti-depressant therapy, or complimentary psychological management techniques may be the most appropriate treatment approach. Encouraging progress in the area of microbiota–gut–brain axis-based therapies also points to potential treatment options.

As no single animal model can fully reflect the true nature of human IBD, further research using existing models and the development of novel models for the disease will contribute to our understanding of the underlying mechanisms of how IBD interacts with the CNS.

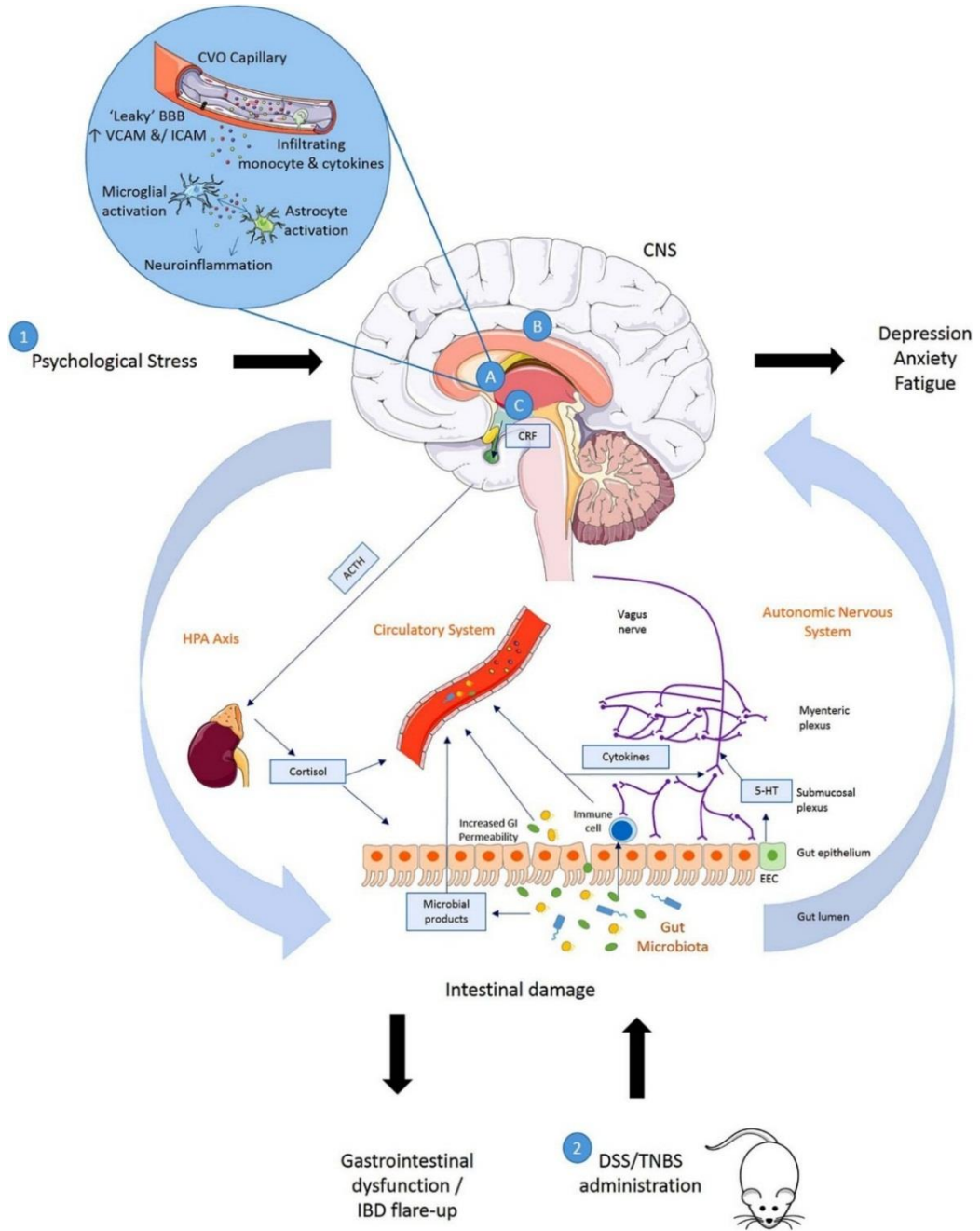


Fig. 1. Summary of disturbances to the gut–brain axis when (1) exposed to psychological stress (brain–gut) or (2) animals exposed to experimentally induced colitis (gut–brain). (1) Exposure to psychological stress, anxiety or depression can result in altered gastrointestinal (GI) motility, increased visceral perception, altered GI secretion, increased intestinal permeability, altered intestinal microbiota and altered GI mucosa and mucosal blood flow. (2) Induction of inflammation in the bowel results in symptoms of inflammatory bowel disease (IBD) accompanied by altered blood brain barrier (BBB) permeability with activation of a central inflammatory response (A), increased regional brain activation (B) and activation of the hypothalamic–pituitary–adrenal (HPA) axis (C) in rats exposed to dextran sulphate sodium (DSS) or 2,4,6 trinitrobenzenesulphonic acid (TNBS). Animals with colitis have been reported to develop behaviour indicative of an anxiety- and/depression-related phenotype when compared with non-colitic animals. 5-HT, serotonin; ACTH, adrenocorticotrophic hormone; CNS, central nervous system; CRF, corticotrophin releasing factor; CVO, circumventricular organ; EEC, enteroendocrine cell; ICAM, intercellular adhesion molecule; SCFA, short-chain fatty acid; VCAM, vascular cell adhesion molecule.

Concluding remarks

Overall, there is a general consensus that IBD is associated with increased vulnerability to depression and anxiety symptoms compared with healthy controls particularly during active disease. Recent evidence has shown that treating psychological symptoms improves HRQOL and reduces the rate of relapse in IBD patients. These results suggest that patients with IBD should be screened for psychological symptoms and where indicated treated accordingly in the overall management of IBD.

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Conflicts of Interest

The authors have no conflicts of interest to declare.

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