ANXIETY BEHAVIOUR AND INFLAMMATORY MARKERS IN C57/BL6J MICE ARE ENHANCED AFTER A CHRONIC DOSE OF DSS COLITIS DURING CONTEXTUAL FEAR CONDITIONING

KAYLEN BEEKMAN

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Department of Neuroscience
University of Lethbridge
LETHBRIDGE, ALBERTA, CANADA

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KAYLEN BEEKMAN

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Dr. A. Gruber Thesis Supervisor	Professor	Ph.D.
Dr. R. J. McDonald Thesis Examination Committee Member	Professor	Ph.D.
Dr. R. Gibb Thesis Examination Committee Member	Professor	Ph.D.
Dr. I. Whishaw Thesis Examination Chair	Professor	Ph.D.

DEDICATION

To my nieces and nephews: thank you for constantly filling my life with joy.

ABSTRACT

This thesis describes how induced gut inflammation induces brain inflammation via the brain-gut axis. It further shows that mice with elevated gut inflammation display post-shock behavioural correlates of anxiety for longer duration than mice that do not have gut inflammation. The gut-inflamed mice also show a reduced ability to recover from fearful experiences and higher relative quantities of inflammatory markers in the nucleus accumbens. Mice receiving both gut inflammation and psilocybin show reduced anxiety behaviour and lower relative quantities of inflammatory markers in the nucleus accumbens. This thesis demonstrates that induced gut inflammation drives increased inflammation in the nucleus accumbens and results in increased measures of anxiety in conditioned and unconditioned behavioural tasks. Some outcomes were ameliorated by the addition of a single dose of psilocybin. Overall, these data improve understanding of potential mechanisms by which anxiety may be produced and treated. Research was completed under protocol number 2018.

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List of Abbreviations

DSS	Dextran Sodium Sulphate
IBD	Inflammatory Bowel Diseases
HPC	Hippocampus
ACC	Anterior Cingulate Cortex
GIDD	Gastrointestinal Diseases and Disorders
CD	Crohn's Disease
Nrf2	Nuclear erythroid 2-Related Factor 2
RNA	Ribonucleic Acid
mRNA	messenger Ribonucleic Acid
RD	Reagent Diluent

CHAPTER 1: GENERAL INTRODUCTION

1.1 ANXIETY

Consider the following. Two students, Leanne and Dina, are approaching exam season and both have five exams back-to-back with less than two weeks to prepare. Whenever these students think about their exams, their heart rates increase, and their breathing becomes faster and shallower. This behavioural response causes Dina to sit down and plan out the next two weeks, carefully selecting blocks of time for each subject as well as food, sleep, and exercise breaks. After this careful planning, Dina can now focus on the task at hand. The same behavioural response causes Leanne to begin imagining a future in which she fails all five exams. In this imaginative future her parents are disappointed, she has to redo all the hard work she put into the semester, or she drops out of university altogether. These thoughts paralyse Leanne's ability to study, as each time she thinks about her exams, her mind is consumed with the potential reality of her future failure.

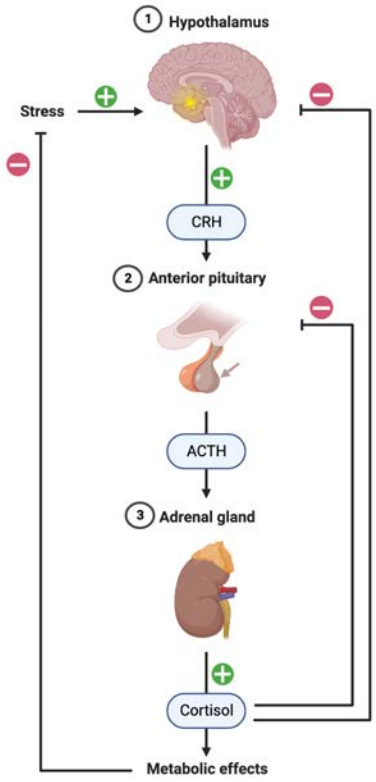

Figure 1.1: HPA Axis
Template from: Camilla Maria Fontana PhD Student, University of Padova - created with BioRender.com

The above responses of both students are labelled "anxiety" responses. For both students the hypothalamus releases corticotropin releasing hormone (CRH). CRH induces the pituitary gland to release adrenocorticotropic hormone (ACTH). ACTH induces their adrenal glands to produce cortisol. The release of cortisol into the bloodstream mobilises glucose, along with many other responses which are interpreted as a sympathetic response.

An important concept to note is that anxiety is different from fear. As defined by Barlow (2000), fear is the response to a "realised threat", while anxiety is a response to a "potential threat". Dina's anxiety response is a normal and beneficial stress response. Anxiety can have many benefits, including the ability to increase attention, enable focus on pressing issues at hand, and inhibit an individual from entering a potentially dangerous situation (Bourin et al., 2007). A therapeutic challenge arises when anxiety does not benefit an individual. This occurs when anxiety is unresolved or is triggered by low level stimulus and becomes a chronic state. This type of anxiety, demonstrated by Leanne, impairs quality of life.

Anxiety as a disorder is described by the Diagnostic and Statistical Manual V as "excessive worry and apprehensive expectations, occurring more days than not for at least 6 months, about a number of events or activities (such as work or school performance)" (5th ed.; DSM–5; American Psychiatric Association, 2013). Once anxiety reaches this point, it has the opposite effect of helpful anxiety, and results in symptoms such as decreased ability to focus and improper judgement of danger.

The human gut harbours 10-100 trillion symbiotic microbial cells and bacteria (Turnbaugh et al, 2007). Foster and Mcvey (2013) argue that the microbiota (or lack of certain microbiota) in the gut have an influence on the process of healthy anxiety responses, as in Leanne's scenario, or the process of unhealthy anxiety responses, as in Dina's scenario. They also show that inflammation in the gut changes the dynamic microbiota population.

There are several different types of anxiety: social anxiety, specific phobias, panic disorder, separation anxiety, and generalised anxiety disorder. This thesis focused on generalised anxiety disorder for the following reasons: comorbidity with inflammatory gut diseases (Matisz & Gruber, 2022), potential for animal behavioural models (Matisz et al., 2022), and lifetime prevalence (6.2%

of the population at some point in their life will experience at least one case of the disorder) (Szuhany & Simon, 2022).

I hypothesise that the comorbidity of generalised anxiety disorder and inflammatory gut disease can occur because there is a connection between the brain and the gut. This connection is able to drive inflammation in the gut to the brain. To test this hypothesis, I will perform several experiments. First, mice will be given a chronic treatment to mimic ulcerative colitis. While receiving this inflammatory disease, mice will be trained on a conditioned fear task. The anxiety behaviours of the mice will then be tested on both conditioned and unconditioned tasks, the remediatory ability of psychedelics will be tested, and finally, their brains will be removed for the analysis of inflammatory markers.

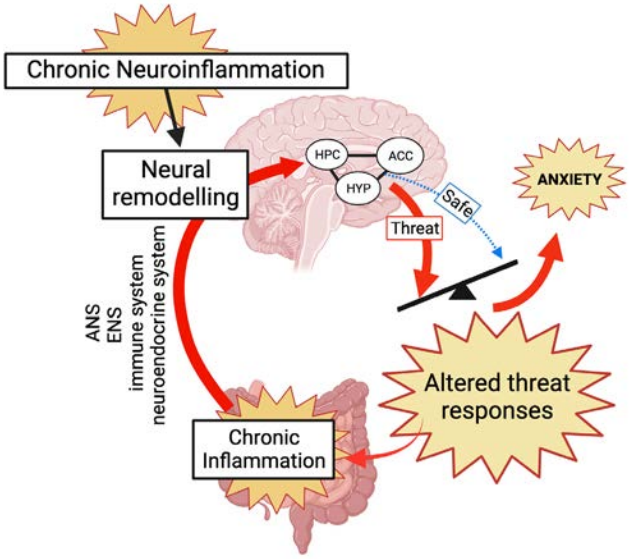

Figure 1.2: Model of Brain-Gut-Axis. Created with BioRender.

1.2 BRAIN REGIONS INVOLVED IN ANXIETY

There are many brain regions that are involved in emotional processing and anxiety. While it is sometimes difficult to understand which regions are more influential in certain disorders, the following section will describe two regions which may be influential in the gut-brain-anxiety process due to their unique connections and roles.

1.2.1 Anterior Cingulate Cortex

Activity in the anterior cingulate cortex (ACC) is shown to be related to diseases such as Inflammatory Bowel Disease (Crohn's Disease and ulcerative colitis), Celiac Disease, Irritable Bowel Syndrome, and Functional Dyspepsia - broadly referred to as gastrointestinal diseases and disorders. This connection is poignantly displayed in a thorough review on the subject by Matisz and Gruber, (2022). The ACC is involved in threat coping and emotional regulation. It also has unique connections with vagal and spinal afferent nerves from the gut, immune system, and endocrine system. This communication gives rise to feedback opportunities between the brain and the gut, enabling the ACC to drive dysfunction in either direction (Matisz & Gruber, 2022).

Along with feedback mechanisms, the ACC also influences pain interpretation and processing. Felger (2018) finds that people with gastrointestinal diseases and disorders are more sensitive to pain and have a lower threshold for pain tolerance. The ACC is also more active in people with gastrointestinal diseases and disorders during bouts of pain, but also when anticipating a painful stimulus (Silverman et al., 1997). One consequence of this is that a heightened activation strongly associates the ACC with anxiety (Modi et al., 2014; Chua et al., 1999). This makes sense, as one of the key features of gastrointestinal diseases and disorders, such as ulcerative colitis, is the potential for sudden, unexpected onsets of disease-related pain. This unpredictability cues the ACC to anticipate pain at unexpected times, which leads to anticipatory anxiety during and between flares (Piche et al., 2010; Bryant et al., 2011)

The ACC is also integral to anxiety due to its wide connections with regions involved in decision making, arousal, and sympathetic activation involving fight or flight responses (Hashemi et al., 2019). These connections allow the ACC's involvement in associations between contexts and stimuli (Rustay et al., 2008), with a preference for negative stimuli in order to best promote

the activation of the sympathetic nervous system (Matisz & Gruber, 2022). It is also shown that the cingulate cortices are involved in acquiring new memories (Pezze & Feldon, 2004), and that lesions of this area will result in deficits to fear conditioning (Rustay et al., 2008). These associations enable the ACC to guide decision making and anticipatory behaviour when in potentially dangerous contexts (Matisz & Gruber, 2022).

Due to involvement in pain processing, anticipation of pain, pain tolerance, associations between context and painful stimuli, and the ability to control the sympathetic response, the ACC has much potential to provide new and interesting information on the mechanism of anxiety production.

1.2.2 Ventral Striatum / Nucleus Accumbens

The striatum has been studied in children at risk for anxiety because of its connection with learning, reward functioning, salience, and goal-directed behaviour (Sollenberger, et al., 2023), all of which are negatively affected by anxiety. The ventral striatum, or nucleus accumbens (NAc), has many different connections with the amygdala, an area of the brain involved in both emotion, and anxiety processes (Britt et al., 2012; Antoniadis & McDonald, 2000). The NAc has also been closely tied to learning, prediction error, and reward valuations (Daw et al., 2011). Studies show that during anticipation of rewards and losses linked to behaviour, youth with behavioural inhibition have an elevated NAc response (Bar-Haim et al., 2009; Guyer et al., 2006). In 2021, Cai et al. summarised over fifteen papers linking abnormal NAc activation to anxiety and depression, while showing in their own study that rats showed dysregulation of proteins in the NAc after receiving chronic mild stress.

The NAc is shown to be involved in both reward-related behaviour (Wise and Bozarth, 1985; Koob,1996; Robbins and Everitt, 1996; Salamone and Correa, 2002), and aversive

reinforcement (see for review Oei and King, 1980; Blackburn et al., 1992; Salamone, 1994; Salamone et al., 1997). But more specifically, it is implicated in specific involvement with contextual fear conditioning, rather than explicit stimulus fear conditioning (Pezze and Feldon, 2004). Contextual fear conditioning involves two differing contexts, one in which a negative stimulus is repeatedly presented and the other in which a negative stimulus is never presented. This differs from an explicit stimulus in that the negative stimulus is preceded by a tone or light prior to its administration. This is demonstrated in a study in which the NAc was temporarily inactivated as rats were undergoing contextual fear conditioning. It is found that in this scenario there is an impairment to fear of context, but not to tone (Westbrook, 1999). Differing from this, Antoniadis & McDonald (2006), show that damage to the nucleus accumbens specifically interferes with conditioned heart rate and ultrasonic vocalisations during contextual fear conditioning, but not with avoidance of the shock-paired context.

The NAc is implicated as having a role in both contextual fear conditioning and anxiety. The NAc's many connections to other anxiety-involved regions, and the fact that the proposed method of studying anxiety specifically is a fear to context experiment, makes the NAc an excellent candidate of study to better understand anxiety.

1.3 ANXIETY AND IRRITABLE BOWEL DISEASE

As referred to above, psychiatric comorbidities, such as anxiety and depression, can be comorbid with gut conditions such as irritable bowel disease (Mikocka-Walus et al., 2016; Mikocka-Walus & Andrews, 2018). This connection between the function of the gut and anxiety were reported as early as 1838, when exceptional circumstances enabled Dr. Beaumont to observe the inside of the stomach while also interacting with the patient. His observations were considered

the “most important practical contributions to the science of physiology.” One such observation was that fear or anger reduced the secretion of “gastric juice”, slowing the rate of digestion (Beaumont W., 1838).

It is currently understood that approximately 50% of irritable bowel disease patients suffer from comorbid anxiety during active disease (Neuendorf et al., 2016). This rate of comorbidity has led to irritable bowel disease and its related psychiatric comorbidities to be considered disorders of the gut-brain axis (Matisz et al., 2022). Bidirectional communication of the brain and the gut is postulated. Communication methods include the autonomic nervous system, the enteric nervous system, the neuroendocrine system, and the immune system (Foster & McVey Neufeld, 2013). This communication forms a gut-brain axis that allows the gut to regulate brain function and vise-versa (Matisz et al., 2022). In a stunning review, Cryan et al. (2019) clearly articulates numerous avenues by which gut microbiota influence health. Initially undertaking the foundational understanding of the gut-brain-axis, he then moves to the consequences of this connection ranging from neuron signalling to Alzheimer's Disease to anxiety (Cryan et al., 2019).

Gut dysbiosis, known as abnormal gut microbiota profiles (Ni et al., 2017), co-occurs with depression and anxiety (Powell et al., 2017), as well as a range of other mental health conditions (Matisz et al., 2022). This relationship has also been demonstrated in several experimental studies (R.D. Heijtz, et al. 2011; K.A. Neufeld, et al. 2011; N. Sudo, et al. 2004; G. Clarke, et al. 2012). In one such study, faecal microbiota are transferred from humans with IBD to healthy mice. This exchange increased the mice's gut motility, gut barrier dysfunction, colon inflammation, and anxiety-like behaviour (De Palma et al., 2017). Another study involved the transfer of healthy gut microbiota from age-matched human patients to patients with irritable bowel disease. This transfer resulted in alleviated disease symptoms such as anxiety severity, depression, and obsession, as

well as a decrease in the irritable bowel disease patients' gastrointestinal symptoms (Kilinçarslan et al., 2020). Not only this, but the use of prebiotics (a source of food for healthy bacteria) and probiotics (food sources containing good bacteria) have been shown to improve both behavioural and neurological abnormalities in mice and humans (Sanders et al., 2019; Barbosa & Vieira-Coelho, 2019).

In summary, there are now many studies that illustrate that there is a close correlation between gut dysfunction and mood disorders such as depression and anxiety. As this proposed bi-directional inflammatory pathway between the brain and the gut has become more evident, it has inspired potential remedial therapies for gut-brain inflammatory dysfunction (Sudeep et al., 2022; Foster & McVey Neufeld, 2013).

1.4 INDUCED INFLAMMATORY BOWEL DISEASE

Inflammatory bowel diseases (IBD), a form of gut inflammation which includes Crohn's disease and ulcerative colitis, have complex and unknown causes. Their complexity is further increased by their tendency to relapse and remit (Podolsky, 1995), often triggered by stress. IBD presents with diarrhoea, abdominal pain, fatigue, and weight loss (Podolsky, 1995) and is often comorbid with anxiety and depression (Hassan et al. 2014; Stuart & Baune, 2014). Ulcerative colitis is included under the umbrella term IBD, but primarily affects the rectum and colon (Kobayashi et al., 2020). Rodent models of gut inflammation have been employed to better understand IBD pathologies. In relevant studies, a chemical model of IBD is commonly introduced by dissolving dextran sulphate sodium (DSS) into drinking water, (Okayasu et al.,1990). DSS colitis exposure has many of the same symptoms as ulcerative colitis, including clinical disease signs such as faecal blood, diarrhoea, and weight loss. These are also accompanied by behavioural

changes (Matisz et al., 2020), such as anxiety and depression (P. Bercik, et al. 2011; Sudeep et al., 2022).

A benefit of the DSS colitis model is that after a 5-7-day cycle of DSS, peak disease lasts for about three days and then subsides on its own (Bento et al., 2012). A common method is to induce an acute model of colitis by giving only one dose for up to ten days. However, by repeating a 5-day cycle of DSS can induce a chronic disease model, better representing the human experience of relapsing and remitting IBD (Neuendorf et al., 2016). DSS colitis also induces an increase in neuroinflammatory markers (Do and Woo, 2018 and Reichmann et al., 2015), neuronal excitation (Barnes et al., 2021), and behavioural alterations (Chen et al., 2015 and Jain et al., 2015 and Nyuyki et al., 2018 and Reichmann et al., 2015), which mirrors the comorbidity of IBD with depression and anxiety (Matisz et al., 2022). For these reasons, DSS is an excellent resource to induce an animal model of the IBD, colitis. Matisz et al. (2022) found that to date, most of the animal research has focused on acute gut inflammation. For example, mice are often given a single 5-10-day dose of DSS in their drinking water. This model does not properly replicate the chronic and relapsing nature of inflammation in IBD experienced by humans. For this reason, Matisz et al. (2022) gave mice 3, 5-day doses of ulcerative colitis to better represent the chronic cycles of inflammation in the human condition of IBD. One limitation of DSS-induced colitis is that we cannot assess abdominal pain apart from posture and mobility. Although these can be monitored, severity of pain is less evident than in a human model. Another consideration is the cells used when the colitis is induced, Denese et al. (2020) mentions that T and B cells are involved in human colitis, but not in DSS-induced colitis. However, apart from these differences, it appears to be one of the most reliable drugs to induce ulcerative colitis in mice.

DSS-induced colon inflammation can be monitored through biomarkers in the faecal matter. It is shown that peripheral inflammation is accurately reflected by the levels of the robust inflammatory marker, Lipocalin-2. Lipocalin-2 is produced by several different cell types such as myeloid and intestinal epithelial cells (Zollner et al., 2021), and is induced in response to proinflammatory stimuli, such as Il-1b, Il-22, and Toll-like receptor activation (Chakraborty et al., 2012; Behnsen et al., 2014). This induced Lipocalin-2 is then secreted into the gut (Nielsen et al., 1996), providing an ideal informant of gut inflammation.

1.5 ANIMAL MODELS OF ANXIETY

If it is true that gut inflammation induces anxiety behaviour, it is important to find accurate methods with which to analyse anxiety in animals. It is easy to ask a human test subject if they are feeling anxious. It is far less simple to determine the anxiety level of a rodent such as a mouse. For this reason, numerous animal models of anxiety have been developed. The aim of these models is to replicate the physiological, pathophysiological, and behavioural features of human anxiety in non-human rodents or primates (Steimer, 2011). By replicating these features of anxiety, the ability to consistently predict anxiety behaviours in rodents is obtained.

Animal anxiety models fall under two broad categories, unconditioned (no training required) and conditioned (training required). In an unconditioned model of anxiety, the rodent has never been exposed to the environment/context/cue, thus the anxiety response is acute anxiety, not connected to memory. Some of these unconditioned tests include, but are not limited to, the elevated plus maze, the light-dark chamber, and the open field task (Rustay et al., 2008). In these contexts, the failure to enter an open space by an animal is indicative of anxiety. In a conditioned model of anxiety, animals are trained to recognize one context as fearful because it is first paired

with an unpleasant stimulus, and the ability to recall this prior pairing is measured. A few conditioned tests include fear potentiated startle, fear to context, or chamber preference (Steimer, 2011). Fear potentiated startle uses an explicit stimulus given prior to a negative stimulus in order to induce anxiety behaviours in rodents. Both fear to context and chamber preference will be described adequately in the methods section of Chapter 2.

A major difference between conditioned and unconditioned tasks is the way in which fear or anxiety is displayed. According to Panksepp (1990), flight or active coping strategies are unconditioned responses to a current threat. Differing from this, freezing or passive coping strategies are a conditioned response to a non-current threat or prediction of danger. This type of passive response was originally described by Engel and Schmale as a conservation-withdrawal strategy (Engel and Schmale, 1972). These two responses are modulated in part by the cognitive apprehension of the environment and in part by the probability of a successful escape. A flight pattern is activated when the danger is near, or current (unconditioned), while a freezing pattern is activated when the threat is far, or non-current (conditioned) (Panksepp et al., 1990; Steimer, 2011; Fanselow, 1980; Fanselow and Helmstetter, 1988; Richmond et al.,1998).

While these threat-coping strategies are different depending on the conditioned or unconditioned model, it is recommended to strengthen the evaluation of anxiety by using more than one anxiety model (Seibenhener & Wooten, 2015). For this reason, as will be described below, the conditioned place preference task, the fear to context task, and the open field task were chosen to appropriately monitor both conditioned, and unconditioned anxiety responses.

1.5.1 Contextual Fear Conditioning

Due to the role of the NAc in contextual fear conditioned anxiety, a fear to context paradigm was used for this study. The definition of contextual fear conditioning in rodents is: a

task involving associative learning, in which an environment which was previously neutral, is paired with one or more foot shocks to produce a learned fear response when re-exposed to the environment without the paired shock (Delgado et al., 2006; Izquierdo et al., 2016). This kind of fear conditioning enables the acquisition of danger associations and subsequent natural fear responses (Antoniadis & McDonald, 1999). Contextual fear conditioning successfully models human anxiety disorders in animals because the learned anxiety can be ameliorated by different anxiolytics, agents that reduce anxiety (Olevska et al., 2021; Rustay et al., 2008). This fear conditioning paradigm can be manipulated in several ways. It can involve a single session, multiple sessions, or discrimination learning, otherwise defined as discrimination contextual fear conditioning (Matisz et al., 2022).

Intriguingly, most studies of the effects of peripheral inflammation, or specifically DSS-induced colitis on fear-related behaviours, examine animal behaviour on singular tasks such as the elevated plus maze, light-dark chamber, or the open field task. These tasks are limited, as the absence of learning eliminates the potential to assess associations involved in fear conditioning having to do with affect (Matisz et al., 2022). Discriminative contextual fear conditioning provides this affect-related response by having two or more unique contexts. This enables mice to discriminate between the context paired with shock (P), and the context not paired with shock (UP). These contexts can differ in shape, scent, size, wall pattern, or sound. Rodents are able to associate these unique contexts with negative affective outcomes, such as one or more foot-shocks. Delivering a negative stimulus in context ‘A’, but not context ‘B’, will typically result in more fear-related behaviours in context A than in context B, thus revealing both anxiety levels, and the discriminative abilities of the rodent (Matisz et al., 2022; Izquierdo et al., 2016).

An exemplar study done by Czerniawski & Guzowski (2014) finds that this type of discriminative contextual fear conditioning can be manipulated by a single dose of peripheral inflammation, and Matisz et al. (2022) showed that a chronic, but not an acute, dose of peripheral inflammation can affect context discrimination, even at a time-point 9-day post peak inflammation. Another study compared rats with and without lesions or inactivation (via muscimol) of the orbitofrontal prefrontal cortex and found that this area is able to prevent overgeneralization during discrimination learning using the discriminative fear conditioning to context task (Zelinski et al., 2010; Trow et al., 2016). These findings suggest that inflammation impacting the function of different frontal cortex areas may be involved in modulating the memory involved in fear discrimination.

1.5.2 Open Field Task

Unlike the contextual fear conditioning, the open field task is a non-conditioned anxiety-like behaviour task. During the open field task, a single mouse or rat is placed in the centre of a novel, (usually) square, open environment with opaque walls, in which they remain for 2-20 minutes depending on the experiment (Seibenhener & Wooten, 2015). The task measures the amount of time they spend in the centre of the context compared to the periphery, with an inverse relationship between centre time and anxiety levels. Anxiety is produced in this environment for two reasons: solitude and agoraphobia (Bourin et al., 2007; Seibenhener & Wooten, 2015), both of which are exaggerated in a high-anxiety mouse and reduced in a low-anxiety mouse. Due to the complexity of the conditioned-place-preference task and fear-to-context tasks, the open field task offers a straight-forward measure of anxiety-like behaviour. Such unconditioned behaviours appear to be modulated by different brain regions, remaining unchanged even with hippocampal and amygdala lesions (Antoniadis & McDonald, 2001). This unlearned anxiety task provides

insight into novel-context anxiety production, compared to the fear to context task and conditioned place preference learned anxiety production.

1.6 PSILOCYBIN AND INFLAMMATION

As there is evidence for a connection between gut inflammation, brain inflammation, and anxiety production, it is important to consider how to remediate this inflammatory cycle. Psychedelics have recently been implicated for their potential to remediate inflammation by acting on the 5-HT2A receptor. The brain 5-HT2A receptor agonist, serotonin, is not only involved in brain inflammation, but also involved in gut inflammation (Ghia et al., 2009) and is implicated in inflammatory bowel diseases (Khan, 2013). Due to the plethora of its roles, dysfunction in the serotonergic system leads to a host of disorders, including depression and anxiety (Thiebot, 1986). Considering the potential damage due to inflammatory processes involved in prevalent diseases such depression, anxiety, and gut diseases, there is a search for anti-inflammatory agents without risky side effects.

One such potential agent has been recently identified in psilocybin, the active component of magic mushrooms. For centuries people have used psilocybin-containing mushrooms for the healing properties they provide. How psilocybin has therapeutic properties is not understood. One proposal is that psilocybin acts as a competitive agonist at the serotonin, 5-HT2A, receptor (Zanicov et al., 2023). When this receptor is activated by serotonin, it has a pro-inflammatory effect, however when activated by different psychedelics, including psilocybin, the action of serotonin is blocked, leading to a decrease in inflammation (Flanagan & Nichols, 2018; Yu et al., 2008). By stimulating a specific receptor formation, psilocybin may trigger anti-inflammatory effects while also blocking the receptor from allowing serotonin to dock and induce inflammation.

The result of this opposing effect is demonstrated by Nau et al. (2013) when they found that psychedelics inhibit TNF-a-induced inflammation.

Considering the connection between inflammation, and depression and anxiety mentioned earlier, it is unsurprising that in a double-blind study in which the 5-HT2A receptor was activated by psilocybin, anxiety and depression are found to have decreased in cancer patients (Griffiths et al., 2016; Ross et al., 2016), again contributing to the theory of the anti-inflammatory effect of psilocybin. It is also found that psilocybin downregulates proinflammatory proteins such as CD80, p65, and TLR4 while upregulating neuroprotective protein, TREM2 (Kozłowska et al., 2021). Psilocybin has also been shown to stimulate neurogenesis (Jones & O'Kelly, 2020).

Due to the overwhelming connection between inflammation, gut diseases, and anxiety, the potent anti-inflammatory effect of psychedelics when activating the 5-HT2A receptor (Yu et al., 2008) is certainly worth exploring in a model of gut and brain inflammation as I will do in the present thesis.

1.7 THE GUT-BRAIN-AXIS THEORY

To explain and expand on the research to date, a theory has been proposed by our lab referred to as The Gut-Brain-Axis Theory. This proposes that the gut and brain can communicate with each other. This communication can benefit the function of both organs, but with respect to the current thesis, increased inflammation in either organ may be related to abnormal function of both organs. As is summarised in the introduction to this thesis, proposed trafficking between the gut and the brain relates the physical symptoms of gut inflammation to the behavioural states of anxiety. The gut-brain axis theory accounts for a range of data including that gut inflammation can induce anxiety. Furthermore, the theory accounts for some of the therapeutic effects treatments of

these conditions. Antianxiety treatments can reduce gut inflammation and anti-gut inflammation treatments can treat anxiety. The theory will be further tested in this thesis by experiments related to the following hypotheses.

1.8 RESEARCH OBJECTIVES AND HYPOTHESIS

The overarching goal of this thesis is to better understand the physiological cause of anxiety. By taking a step in this direction, the door for further discovery of the biological mechanisms of anxiety opens. Based on previous research, induced gut inflammation leads to differences in anxiety-like behaviours in mice. To expand on this question of inflammation and anxiety, it is necessary to discover if induced gut inflammation leads to increased markers of inflammation in the brain. If so, the potential for correlations between increased brain inflammatory markers and higher levels of anxiety must be tested.

1.8.1 Hypothesis I: Mice with chronic gut inflammation exhibit higher levels of conditioned and unconditioned anxiety behaviours

In past studies it was shown that particularly at the remote time-point (19 days post-training and 9-12 days post-DSS), a chronic model of inflammatory bowel disease led to increased anxiety-like behaviour in a conditioned-fear task (Matisz et al. 2022). To further understand anxiety production, it is necessary to reproduce this experiment while adding further analysis and behavioural tasks. The hypothesis is that mice with chronic gut inflammation exhibit higher levels of anxiety in both the conditioned and unconditioned behavioural tasks and improperly differentiate fearful and non-fearful stimuli. Based on past research, specific changes at the remote disease time-point compared to the recent disease time-point are anticipated.

1.8.2 Hypothesis II: Mice with chronic gut inflammation will have increased markers of inflammation in their brains

To understand whether the brain is affected by the chronic gut inflammation, it is necessary to identify specific brain regions for further analysis. Based on studies mentioned above, the NAc region of the striatum, and the ACC region of the cortex distinguished themselves as areas involved in anxiety. For this reason, the NAc and ACC were tissue punched and the mRNA was isolated from these regions for RT qPCR analysis of inflammatory molecules and genes involved in the inflammatory pathway. The prediction is that mice given chronic gut inflammation would have an increase in inflammatory markers, such as Il-1b, in specific brain regions related to anxiety, and that mRNA involved in the inflammatory pathway would be expressed differently in mice given chronic inflammation compared to those without inflammation. It is hypothesised that the increase in anxiety-like behaviours is due to an increase in brain inflammation.

1.8.3 Hypothesis III: Pilot study - A single injection of psilocybin will decrease anxiety-like behaviour in mice with chronic gut inflammation

To further investigate the role of inflammation on anxiety, as well as the potentially remediatory effects of psychedelics such as psilocybin on anxiety, a single high-dose (6 mg/kg) of psilocybin was administered between testing time-points. This will enable comparison of behaviour readouts between the recent and remote testing time-points. In the interest of understanding the potential positive effects psilocybin may have on brain inflammation, a pilot study of 12 mice receiving both three doses DSS and one dose of psilocybin will be run. The results of this study may offer directives for future research on the effect of psilocybin as an anti-inflammatory agent on specific brain regions. The hypothesis is that a single dose (6 mg/kg) of psilocybin administered between the recent and remote testing time-points would decrease

inflammation in the brain. It is further hypothesised that this decreased inflammation would lead to a decrease in anxiety-like behaviour in both the conditioned and unconditioned behavioural tests.

1.8.4 Experimental Design

Three groups of male mice, 1 control, 2 DSS, 3 DSS + Psi, were tested for anxiety-like behaviour at two different time-points after training on a contextual fear task. The reason for only using male mice is explained in the limitations section of this thesis. After experimental endpoint, reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) was performed on the ACC and NAc of these mice to test the relative quantity of RNA of several specific markers involved in the inflammatory pathway. These results will allow understanding for three things, 1. Whether gut inflammation impacts brain inflammation, as described in hypothesis I. 2. How inflammation may affect anxiety behaviour, as described in hypothesis II. 3. If psilocybin has any effect on these outcomes, as described in hypothesis III.

CHAPTER 2: CHRONIC GUT DISEASE CHANGES INFLAMMATORY MARKERS IN THE NUCLEUS ACCUMBENS AND INCREASE ANXIETY-LIKE BEHAVIOURS IN MALE C57/BL6J MICE

2.1 INTRODUCTION

The percentage of the population with mood disorders is progressively rising, with generalised anxiety alone having a life-time prevalence of 6.2%. This means that 6.2% of the population (around 500 million people using a current global population of 8 billion) will suffer from at least one case of generalised anxiety within their lifetime. Unfortunately, suffering from any inflammatory disease dramatically increases one's likelihood of suffering from a comorbid mood disorder such as anxiety (Mikocka-Walus et al., 2016; Mikocka-Walus & Andrews, 2018; Hassan et al. 2014; Stuart & Baune, 2014). It is found that about 50% of patients suffering from inflammatory bowel disease (IBD) also suffer from comorbid anxiety disorders (Neuendorf et al., 2016). This rate of comorbidity has led to IBD and its related psychiatric comorbidities to be considered disorders of the gut-brain axis (Matisz et al., 2022). Communication methods include the autonomic nervous system (ANS), the enteric nervous system (ENS), the neuroendocrine system, and the immune system (Foster & McVey Neufeld, 2013), giving ample opportunity for inflammation to be driven bi-directionally.

To understand the increased risk for anxiety disorders in IBD patients, it is important to find appropriate models for both IBD and mood disorders such as anxiety. IBD presents with diarrhoea, abdominal pain, fatigue, and weight loss (Podolsky, 1995). Ulcerative colitis is included under the umbrella term IBD, but primarily affects the rectum and colon (Kobayashi et al., 2020). An especially effective IBD rodent model is found in dextran sulphate sodium (DSS) (Okayasu et

al.,1990). When added to drinking water, DSS exposure has many of the same symptoms as ulcerative colitis, including clinical disease signs such as faecal blood, diarrhoea, and weight loss. These are also accompanied by behavioural changes such as anxiety and depression (P. Bercik, et al. 2011; Sudeep et al., 2022) and relapsing and remitting disease (Bento et al., 2012; Neuendorf et al., 2016).

While several conditioned and unconditioned anxiety tasks are available for mouse models of anxiety, the fear to context and preference conditioned tasks as well as the open field unconditioned task were chosen. Conditioned tasks such as the fear to context task enable analysis of how fear memory may be modulated by the induction of colitis. Unconditioned fear tasks such as the open field task provide understanding for how inflammatory diseases such as ulcerative colitis impact general, untrained anxiety behaviours. Using more than one model strengthens the overall evaluation of anxiety.

Two brain regions have unique distinct potential to be modulating the inflammatory pathway and increased anxiety behaviour; the anterior cingulate cortex (ACC) and the ventral striatum, also known and referred to in this paper as the nucleus accumbens (NAc). The ACC has extensive connections with vagal and spinal afferent nerves from the gut, immune system, endocrine system, and microbiota. This communication gives rise to feedback opportunities between the brain and the gut, enabling the ACC to drive dysfunction in either direction (Matisz & Gruber, 2022). The ACC also has connections with regions involved in decision making, arousal, and sympathetic activation involving fight or flight responses (Hashemi et al., 2019). These connections allow the ACC to be involved in associations between contexts and stimuli (Rustay et al., 2008).

The nucleus accumbens has been studied in children at risk for anxiety because of its connection with learning, reward functioning, salience, and goal-directed behaviour (Sollenberger, et al., 2023). When suffering with anxiety it is common for these traits to become distorted. The NAc has also been closely tied to learning, prediction error, and reward valuations (Daw et al., 2011). In 2021, Cai et al. cited over fifteen papers linking abnormal NAc activation to anxiety and depression, while showing in their own study that rats showed dysregulation of proteins in the NAc after receiving chronic mild stress. In a study involving its temporary inactivation, the NAc has also been specifically implicated in its involvement with contextual fear conditioning (Westbrook, 1999; Pezze and Feldon, 2004). The ACC and NAc's fundamental connections with the gut and other brain regions enable them to be excellent candidates for the study of associations between anxiety disorders and gut inflammatory disorders such as ulcerative colitis.

The hypothesis is that the comorbidity of generalised anxiety disorder (GAD) and inflammatory gut disease occurs because of a connection between the brain and the gut. This connection enables inflammation to be promoted from the gut to the brain. To test this hypothesis, the study will consist of several steps. First, mice will be given a chronic treatment to mimic ulcerative colitis. While receiving this inflammatory disease, the mice will be trained on a conditioned fear task. Their anxiety behaviours will then be assessed on both conditioned and unconditioned tasks. Their brains will be subsequently analysed for downstream inflammatory markers.

2.2 MATERIALS AND METHODS

Subjects

Twenty-one adult, male, C57BL/6J mice purchased from Jackson Bar Harbour, ME arriving on December 14, 2022, were used in the experiments described in this chapter. The mice were divided into two groups, a control group receiving tap water (n = 9) and a DSS group receiving three doses of dextran sodium sulphate in water (n = 12). The mice were 8 weeks old at the beginning of the experiment and weighed between 21g and 26g. The mice were given ad-libitum access to food and water. They were housed in groups of three and kept on a 12-hour light/12-hour dark cycle with lights on at 7:30 and off at 19:30. After arrival in the housing facility, the mice were handled for ~ten minutes every other day for one week before starting the behavioural experiment. This was done to familiarise them with the experimenter. All animals were cared for in compliance with the Guide for the Care and Use of Laboratory Animals. Ethical approval was obtained from the Animal Experimental Ethics Committee of the University of Lethbridge.

Colitis Induction

For the induction of chronic DSS colitis, mice were exposed to three cycles of DSS (3.0%, 2.75%, 2.75% wt/v) each lasting five days, with 8 days of recovery between the first and the second cycle, and a 15-day recovery period between the second and third cycle. Control mice received regular drinking water for the duration of the experiment. Body weight and disease activity were monitored throughout the experiment. Disease activity index (DAI) was tested for seven days after each 5-day cycle of DSS. The intensity of disease is based on five different measurements: 1.

percentage of body weight lost from the start of the cycle, 2. activity compared to control mice, 3. hydration (tested by giving a skin tent), 4. body conditioning, and 5. Posture.

Contextual Fear Training

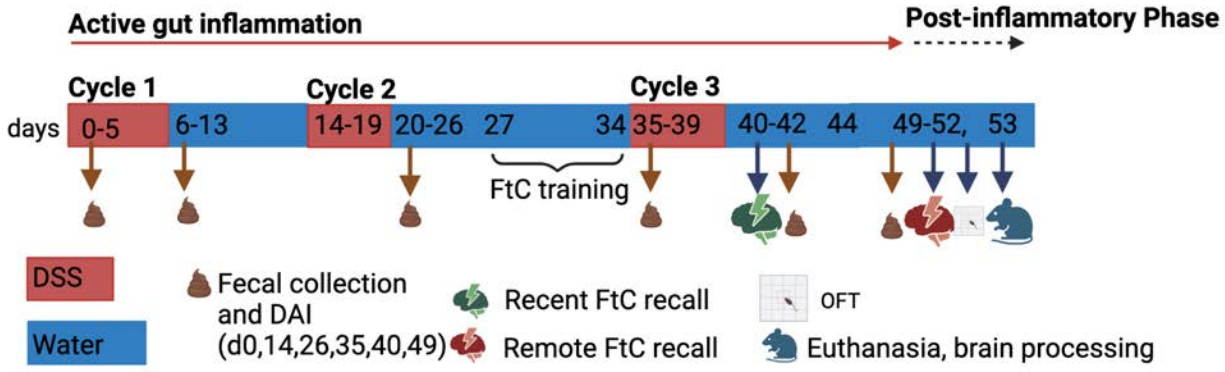

Figure 2.1: Schedule of behavioural experiments, colitis induction, and injections. Created with BioRender.com

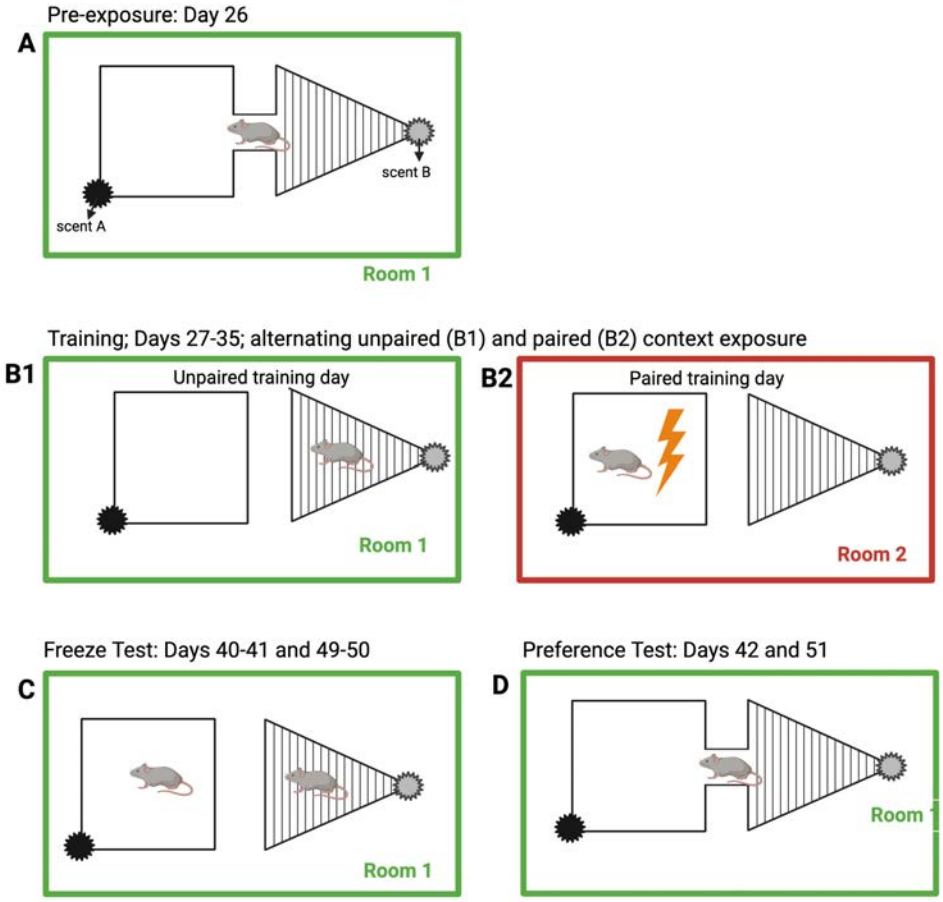

Figure 2.2: Schematic representation of the discriminative fear to context apparatus during training and testing.
Created with BioRender.com. Contexts were cleaned with Virkon prior to the addition of each mouse in each of the training sessions and behavioural experiments.

Table 2.1: Schedule of faecal collection, DSS induction, and behavioural experiments.

Day	Date	Behaviour	DSS Level
0	28-Dec	Baseline	None
26	23-Jan	Pre-Exposure	8-days post-cycle 2
35	01-Feb	End of training	Just prior to cycle 3

40	06-Feb	Recent Test	Peak Inflammation
49	15-Feb	Remote Test	Resolving Inflammation

Day 26: pre-exposure (2.2A)

This procedure took place in the “safe” room. The mouse was placed in the bridge between two contexts. The time spent in each of the contexts was recorded for ten minutes. If a mouse showed a preference for one context over the other by spending at least 10% more time in one of the contexts than the other, that context became the paired context (PC).

Day 27-34: training (Fig. 2.2B)

Each mouse was exposed to both a paired and an unpaired context in an alternating manner. On day one they would be in either the paired or the unpaired, and on day two the mouse would be placed in the context opposite to the day prior. This was repeated for eight days. This procedure took place in two rooms: the “safe” room and the “shock” room. Mice were placed one at a time in their respective unpaired or paired contexts with their back to the sealed door (the bridge was removed and sealed) for five minutes.

Unpaired (Fig. 2.2B1): Mice were placed in their unpaired context for five minutes and then removed and placed in a waiting room until the rest of their cage was complete.

Paired (Fig. 2.2B2): Mice were placed in their paired context and were given two minutes to acclimatise to the context before a 0.5 mA shock was delivered for 2 seconds. This was repeated

three times at the beginning of minutes 2, 3, and 4, and the mouse was removed from the context after minute 5 and then placed in a waiting room until the rest of their cage was complete.

Day 40-41 + 49-50: Freezing Test (Fig. 2.2C)

The freeze test took place over two days and was similar to training days 1 and 2. The testing occurred in the "safe" room. Mice were placed in their respective contexts depending on the training schedule and were recorded with a floor-mounted ELP 2.0 MP 5-50mm USB camera for 5 minutes. No shock was administered during testing. Freeze-time scoring for contextual fear behavioural tasks was scored using a combination of DeepLabCut and a custom Matlab script. However, to ensure accuracy, all videos were also scored by the experimenter. All graphs were created using GraphPad Prism 9.5.0 or GraphPad Prism 10.0.1.

Day 42 and 51: Conditioned Place Preference Task (Fig. 2.2D)

Mice were placed in a bridge connecting both contexts and allowed to roam freely between the contexts for 10 minutes while being recorded with a floor-mounted ELP 2.0 MP 5-50mm USB camera. The total time spent in each context was scored by individuals and compared using a two-way ANOVA. Comparisons between time spent in paired contexts at only recent or remote time points were compared using a t-test. All graphs were created using GraphPad Prism 9.5.0 or GraphPad Prism 10.0.1.

Day 52: Open Field Test

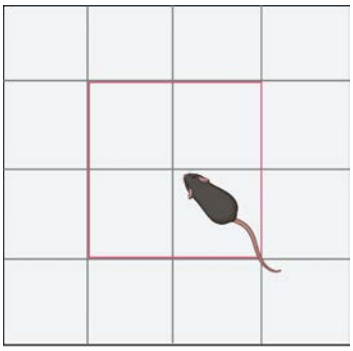

Figure 2.3: Open Field Task Schematic. Created with BioRender.com

Anxiety-like behaviour and locomotor activity were assessed in the open field arena (Fig. 2.3) (w: 50 cm/50 cm, h: 25 cm) under normal lighting conditions (Seibenhener & Wooten, 2015). Mice were placed in the centre of the open field arena (made from white plexiglass) and allowed to roam freely for 10 minutes while being recorded with a ceiling-mounted ELP 2.0 MP 5-50mm USB camera. The OFT was scored using a custom tracking algorithm. The tracking algorithm used OpenCV's implementation of a mixture of Gaussians 2 algorithm (MOG2). The algorithm tracked the centre of mass of the animal as it moved through the arena. The program then created a CSV with the X and Y coordinates of the centre of mass for each frame. This was then used to analyse velocity, centre dwell time, periphery dwell time, total distance travelled, and the time it took to enter the centre. Less time spent in the centre is indicative of more anxiety (Seibenhener & Wooten, 2015). All graphs were created using GraphPad Prism 9.5.0 or GraphPad Prism 10.0.1.

Brain Tissue Extraction and RNA Isolation

Mice were euthanized on day 52 by induction into an isoflurane chamber and subsequent injection with ~1.6mL/kg euthanyl (Bimeda MTC 00141704). The brains were removed and snap-frozen via immersion into pre-chilled isopentane for 60-120 seconds and subsequently stored at -80 ºC.

Section Extraction

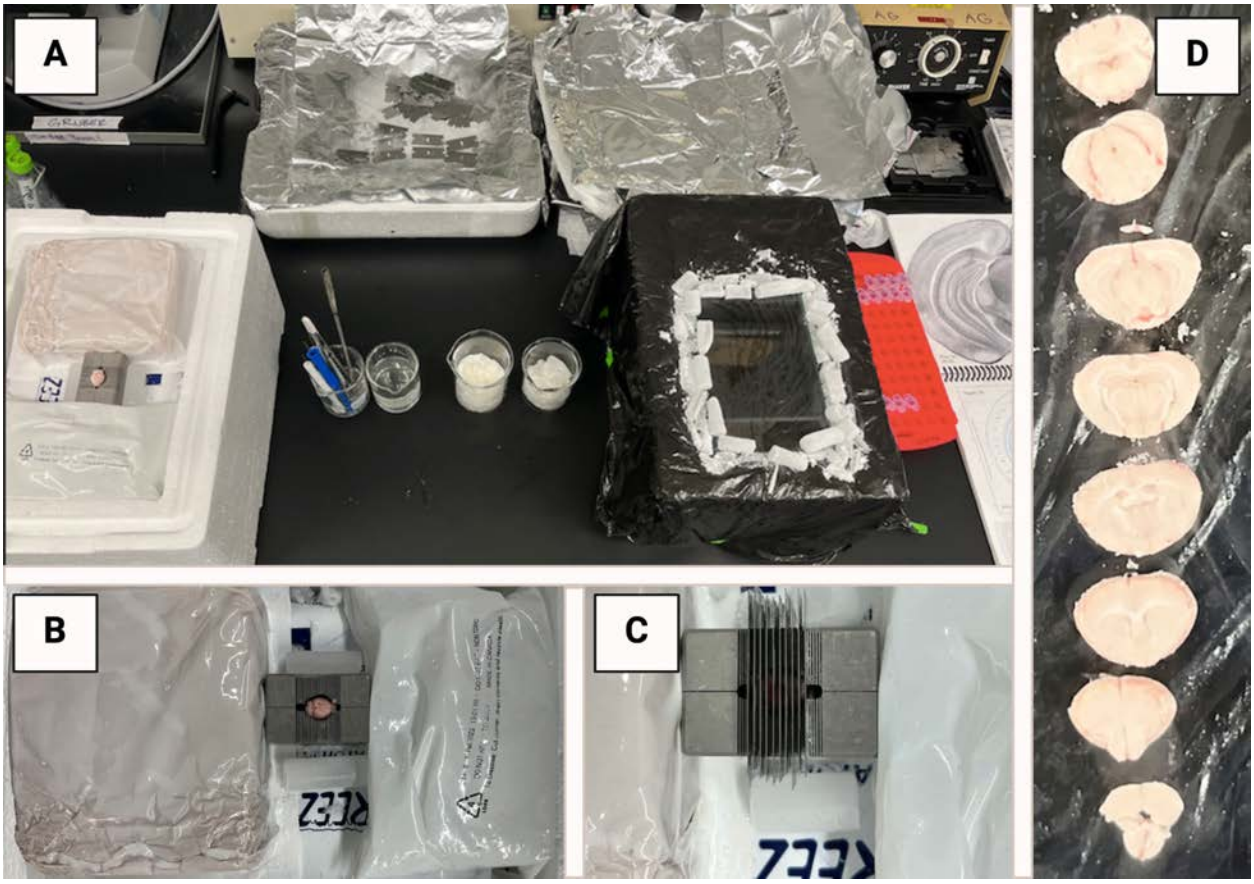

Figure 2.4: Image of brain section extraction process. All procedures were performed in a sterile environment with gloved and masked experimenters. Created with BioRender.com

(A) Setup of wet lab bench prior to slicing. (B) Brain warming from -80ºC to -20ºC in the frozen matrix. (C) Brain within a matrix with frozen blades embedded in the matrix. (D) Frozen brain slices laid out on frozen glass ready for tissue punching.

Six control and six DSS mice were subjected to the following nucleic constituent extraction protocol. These mice were randomly chosen from the group of twelve DSS and nine control mice which underwent the previous experiment.

Protocol acquired from JOVE (Miller et al., 2020): "Collection of Frozen Rodent Brain Regions for Downstream Analyses" was followed apart from a few details. Prior to beginning, all tools and surfaces were cleansed using RNaseZap™ RNase Decontamination Solution and

UltraPure™ DNase/RNase-Free Distilled Water purchased from ThermoFisher Scientific followed by 70% ethanol. Surfaces were not frozen or used until ethanol had fully evaporated.

About 20 hours prior to dissection, an alto 0.5 mm brain matrix was frozen at -20 ºC between two freezer packs. A Styrofoam box was filled with ice and covered with cleaned, black plastic and frozen at -20 ºC and a piece of glass fitting within the Styrofoam box was placed on top of the ice. Using pliers, the metal tops were removed from single-edged razor blades. Blades were then cleaned and frozen at -20 ºC.

Just prior to brain sectioning, dry ice was placed around the glass as pictured in Figure 1.3 (previous protocol suggested placing dry ice under the glass, I found this resulted in the glass being too cold and the brain slices shattering when punched). The brain was placed within the matrix and allowed to come to temperature for ten minutes. Pre-cleaned and frozen razor blades were placed halfway through the brain in the centre, rostral, and caudal positions of the brain, every other slot was filled with 0.23mm thick blades. Once slots were full, blades were pressed down with a flat object until the blades were at the bottom of the matrix. Dry ice was placed beside the blades prior to removal to ensure the brain remained frozen while being removed from the matrix.

All blades were removed in unison, and slices were laid out on chilled glass as shown in Figure 1.3. A pre-cleaned tissue punch was used along with the Allen Brain Atlas to punch the anterior cingulate cortex, and the NAc region of the ventral striatum. These tissues were then dropped into 0.5mL TRIzol (15596026) immediately following the tissue punch and subsequently stored at -80 ºC.

RNA and DNA Extraction

RNA Isolation. All tubes were Thermo Fisher nuclease-free PCR tubes. Samples were thawed to room temperature and homogenised with a homogenizer followed by gentle pipetting with pipettes of decreasing sizes until fully homogenised. Samples were then incubated at room temperature for five minutes to ensure complete membrane lysis. 100µL of chloroform (Sigma Aldrich CAS 67-66-3) was added, and the tubes were gently inverted ten times and left to incubate for three minutes at room temperature. The samples were then centrifuged at 4 ºC and 12000 x g for 15 minutes. The upper aqueous phase was transferred to another tube and 1µL of RNase-free glycogen (Thermo Fisher R0551) and 250µL of isopropanol (Sigma Aldrich CAS 67-63-0) were added to each sample. Samples were then incubated at -20 ºC for 1.5 hours. During this time the DNA isolation protocol was carried out. After 1.5 hours, samples were centrifuged for 10 minutes at 4 ºC and 12000 x g. The supernatant was discarded, with care taken to not disturb the RNA pellet. The RNA pellet was washed with 500µL 75% ethanol and centrifuged for 5 minutes at 4 ºC and 7500 x g. The 75% ethanol supernatant was then discarded. The RNA pellet was air-dried for no longer than 60 seconds. The pellet was then resuspended in 25µL of RNase-free H2O (Thermo Fisher AM9938) and placed on a heat block at 55 ºC for exactly 10 minutes in order to ensure full elution of the RNA. Samples were then immediately placed on ice to ensure RNA quality.

Targets and Primer Selection

Genes of interest were chosen based on their relation to the developed hypothesis, namely, the potential for inflammation to drive changes in the brain which results in anxiety-like behaviours in mice. Primers were chosen based on published literature that used these same targets.

Table 2.2: Genes of interest.

Gene ID	Gene Name	Gene Role	Forward Primer	Reverse Primer	Reference
mt-Co1	cytochrome c oxidase subunit I	Mitochondrial function	TCGCAATTCCTACCGGTGTC	CGTGTAGGGTTGCAAGTCAGC	BLAST ID: MN228597.1
Gpx	glutathione peroxidase 1	Cellular antioxidant	CCTCAAGTACGTCCGACCTG	CAATGTCGTTGCGGCACACC	BLAST ID: BC086649.1
Nrf2	Nuclear factor erythroid 2-related factor 2	Protection against oxidative stress.	ACATTCCCATTTGTAGATGACC	GGTATTAAGACACTGTAATTCGGG	(Kemper, et al., 2013)
Il-1b	Interleukin 1-beta	Inflammatory Cytokine	TGGTGTGTGACGTTCCCATT	CAGCACGAGGCTTTTTTGTTG	(Mona Yasin & Willias, 2020)

cDNA & RT-qPCR

RNA was extracted using Trizol reagent as described above, and reverse transcribed using Superscript IV Buffer (Thermo Fisher 18090200) by the following method: 100 ng total RNA was mixed with 1µL random primers (New England Biolabs (NEU+1F60E S1330S)) and 1µL of 10mM dNTP mix (New England Biolabs (NEU+1F60E N0447S)). The mixture was incubated for 5 min at 65 °C and placed immediately on ice for 1 minute. The mixture was then incubated with 4µL of 5x First strand buffer (Thermo Fisher 18090200), 1µL of 0.1M DTT (Thermo Fisher 18090200), and 1µL Superscript IV (200U/µL) (Thermo Fisher 18090200) for 10 min at 25 °C, 10 min at 55 °C and 10 min at 80 °C. cDNA was analysed by qPCR using 2µL of 1:10 diluted cDNA, 0.5µL of 10µM of each gene-specific primer, 2µL H_2O and 5µL of Luna Universal qPCR Master Mix (M3003E, NEB). Thermocycler conditions are as follows: 3 min at 95 °C (15 s at 95

°C, 30 s at 54 °C, 30 s at 66 °C) × 40 cycles. Fluorometer readings were taken during extension and qPCR was performed using the Bio-Rad CFX384 Real-time detection system. Standard curves were prepared for relative expression and the analysis of PCR efficiency by pooling 2µL of each cDNA sample and standard diluting SD1: 1:2, SD2: 1:4, SD3: 1:8, SD4: 1:16, SD5: 1:32. RT-qPCR analysis was completed using Microsoft Excel where samples were analysed by standard curve relative expression. Student's T-tests were used to study significance as described in the respective figure legends. Primers were ordered from IDT as custom oligos and are listed in Table 1. Three biological replicates were performed for data analysis. Differences among groups were analysed using one-way ANOVA followed by Tukey's post hoc test. $P < 0.05$ was considered as statistical significance.

Faecal Lipocalin

Collection. Faeces were collected at five different time points throughout the experiment:

Table 2.3: Experimental dates of faecal collection

Day	Date	Behaviour	DSS Level
0	28-Dec	Baseline	None
26	23-Jan	Pre-Exposure	8-days post-cycle 2
35	01-Feb	End of training	Just prior to cycle 3
40	06-Feb	Recent Test	Peak Inflammation

49	15-Feb	Remote Test	Resolving Inflammation

All Samples were stored at -20ºC until homogenised.

Homogenization. Samples were defrosted and added to a pre-weighed tube. Once sample weight was determined, 10,000 times the sample weight of 1X phosphate buffered saline (PBS) (DY006) in 0.1% Tween-20 (Sigma-Aldrich P9416) was added to each sample. The samples were then vortexed briefly and incubated in a box vortex for 20 minutes. If samples were not homogenised at this time point, they were individually vortexed and placed back in the box vortex for another 10 minutes. Once samples were homogenised, they were then spun down in the centrifuge at 12000 rpm for 10 minutes at 4ºC. The upper aqueous layer contained the faecal serum and was removed and placed in a labelled tube and stored at -20ºC.

Detection. All components needed to detect levels of Mouse faecal Lipocalin-2 were purchased from R&D Systems as part of the DuoSet Ancillary Reagent Kit 2 (DY008).

For each plate, 83µL of Mouse Lipocalin-2 Capture Antibody (844831) was diluted into 10mL of Sterile filtered PBS (DY006). Using a 96-well microplate (DY990), 100µL of diluted capture antibody was pipetted into each well. The plate was covered in an adhesive sheet (DY992) and incubated on a moving plate overnight at room temperature.

The next day, the plates were drained and washed thoroughly with a Wash Buffer (WA126) by filling each of the wells fully and aspirating fully. This process was repeated three times, and after the third wash and aspiration, the plate was blotted against a towel for complete removal of the diluted capture antibody. The plate was then blocked by the addition of 300µL of Reagent Diluent (RD) (DY995) to each well. The plate was covered in an adhesive sheet (DY992) and incubated on a moving plate for one hour at room temperature.

During the one-hour blocking step, peak disease samples were prepared by diluting 1µL of each faecal serum into 650µL of RD in a separate tube. Standard 1 was prepared by diluting 11.11µL Mouse Lipocalin-2 Standard (842442) into 1000µL RD (DY995) after which it was vortexed thoroughly. A serial dilution was performed by adding 500µL of standard 1 to 500µL of RD to make standard 2. This was repeated by removing 500µL of standard 2 and adding it to 500µL of RD (DY995) to make standard 3 until 7 standards were made. Standard 8 was pure RD.

The 3X wash step was repeated after the one-hour incubation period and was followed by the addition of 100µL of each standard to rows A-H, run in duplicate. The remaining wells were filled with 50µL of RD (DY995) and 50µL of diluted sample, run in duplicate. The plate was covered in an adhesive sheet (DY992) and incubated for two hours at room temperature or overnight at 4ºC.

The 3X wash step was repeated followed by the addition of 100µL of 167µL Mouse Lipocalin-2 Detection Antibody (844832) diluted in 10mL RD (DY995) to each well. The plate was covered in an adhesive sheet (DY992) and incubated for two hours at room temperature or overnight at 4ºC.

The 3X wash step was repeated followed by the addition of 150µL Streptavidin-HRP B (893975) diluted in 10mL RD (DY995) to each well. The plate was covered in an adhesive sheet (DY992) and tin foil to block the light. The plate was then incubated for 20 minutes at room temperature.

The 3X wash step was repeated and a substrate solution was made by combining 5mL Color Reagent A (H2O2) with 5mL Color Reagent B (Tetramethylbenzidine) (DY999). 100µL of substrate solution was added to each well. The plate was covered in an adhesive sheet (DY992) and tin foil to block the light. The plate was then incubated for 15-20 minutes at room temperature.

Once the wells containing the standard reached the desired colour, 50µL of Mouse Lipocalin-2 Stop Solution (DY994) was added to each well (wells were not drained and washed prior to this step). The plate was then gently tapped until the solution was thoroughly combined (all wells were orange, yellow, or clear, not green or blue). Once added, the plates were immediately placed in a Meridian Bioscience Inc. plate reader and optical density was read at 450 nm. Higher optical density was indicative of higher levels of Lipocalin-2. Optical density was represented statistically with a Repeated Measures two-way ANOVA with Tukey's post hoc test. *$p \leq 0.05$ Control vs. DSS.

Statistical analysis

All graphs were created using GraphPad Prism 9.5.0 or GraphPad Prism 10.0.1 (GraphPad Software, San Diego, CA, USA) and are presented as means ± standard deviation (SD). Shapiro-Wilk normality tests were performed where necessary, and Welch's t-test was used to test the means.

Entropy Calculation

Entropy is calculated with the following formula where H is the entropy, n is the number of blocks, and Xi is the number of times the mouse crossed block i in a particular context. The summation is over all blocks (i from 1 to n) in a context with 900 blocks.

$$H = -\sum_{i=1}^{n} \left(\frac{x_i}{\sum_{j=1}^{n} x_j} \right) \cdot \ln \left(\frac{x_i}{\sum_{j=1}^{n} x_j} \right)$$

This formula calculates the entropy for each mouse in each context by summing the product of the ratio of the number of times the mouse crossed each block to the total number of crossings (probability) and the natural logarithm of that ratio (probability) for each block. This calculation deciphers the predictability of the movement and in summary can be expressed as: Shannon Entropy = (p)*ln(p)

2.3 RESULTS

Chronic exposure to DSS produced clinical signs of colitis

Mice given DSS in the present study had reduced body weight (Fig. 2.5A) relative to controls after the DSS treatment and 8-20 days after treatment cessation. Likewise, the faecal lipocalin-2 levels were increased by DSS treatment (Fig. 2.5B). Mice chronically exposed to DSS exhibited greater weight loss and faecal lipocalin levels throughout the study, and at experimental endpoint had heavier spleens and shorter colons compared to control mice (Fig. 2.6A and 2.6B). This suggests that those mice receiving DSS showed significant clinical signs of DSS colitis disease, unlike control mice.

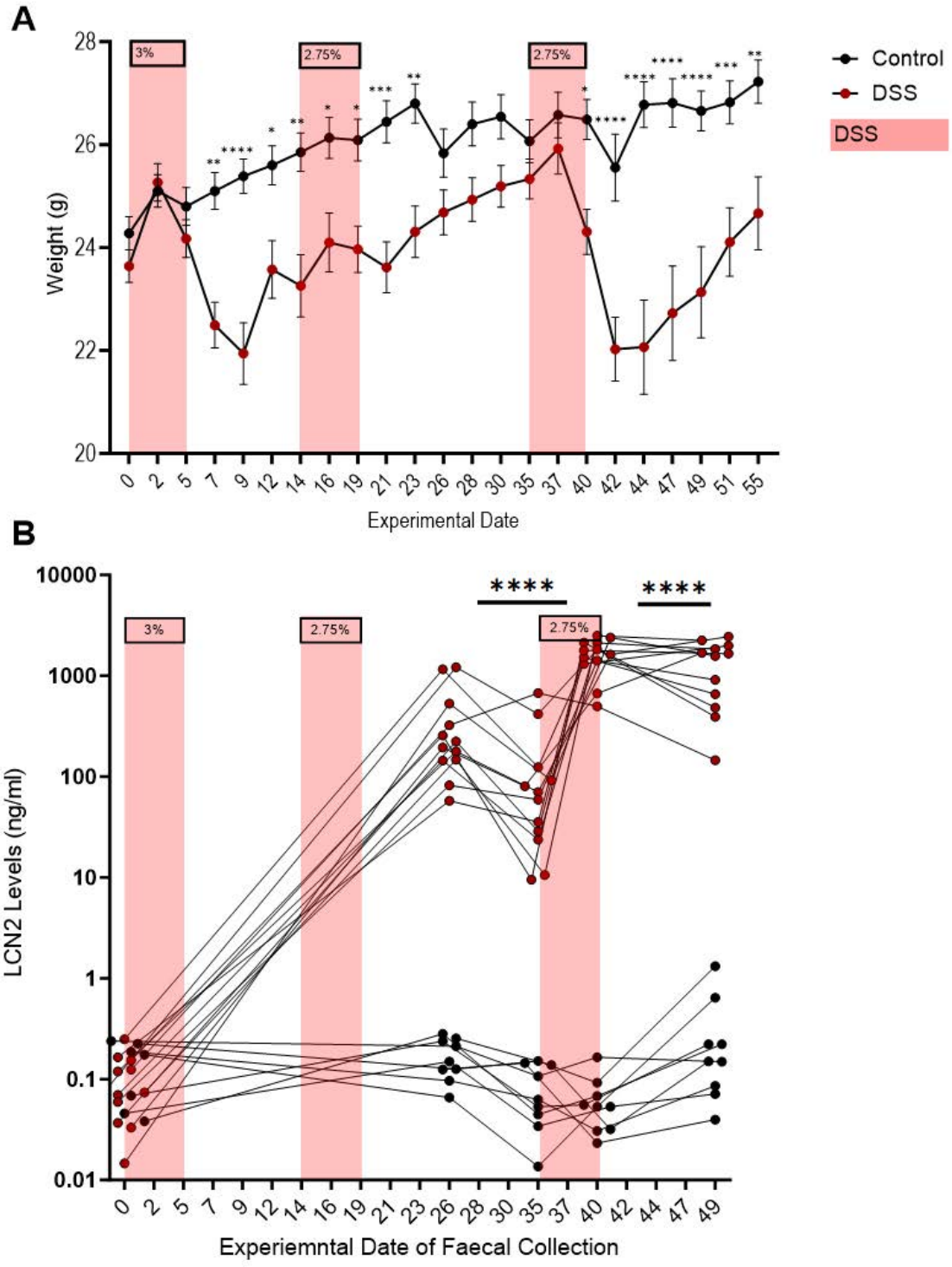

Figure 2.5: Clinical signs of disease.

(A) Body Weight and (B) Lipocalin-2 levels among treatment groups. Mice were given 3 doses of DSS (3%, 2.75%, and 2.75%) in their drinking water to induce colitis. Body weight was observed throughout. Data are shown as mean ± SEM. Lipocalin-2 levels were

discovered from faeces collected at 5-time points throughout 3 doses of colitis (red bars). Control n = 9; DSS n = 12. Repeated Measures two-way ANOVA with Tukey's post-test. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 Control vs. DSS.

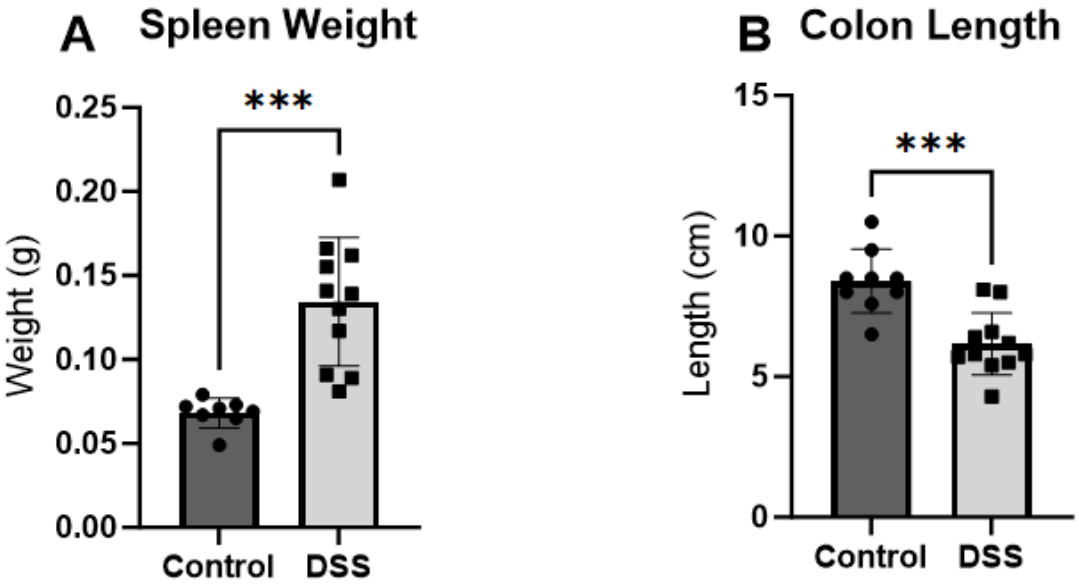

Figure 2.6: Features of organs.

(A) Spleen Weight; control n = 8; DSS n = 11. (B) Colon Length; control n = 9; DSS n = 12. Data are shown as mean ± SD. (A) Passed Welch's test and Shapiro-Wilk Normality test. (B) Unpaired t-test. **p ≤ 0.01; ***p ≤ 0.001.

Mice with Chronic DSS display increased anxiety-like behaviours and seem to have a reduced ability to "recover" from fear conditioning at the remote time point.

The experiment involved pairing one of two chambers (either the square or the triangle) with a mild foot-shock (paired context), and the other with no shock (unpaired context) for eight alternating training days (Fig. 2.2B). After 6-8 days (recent time point), mice were placed in the paired (P) or unpaired (UP) context for 5 minutes on day one, and the opposite context for five minutes the next day in a randomised fashion (Fig. 2.2C). The behavioural readouts of this test are (i) the total distance they travel within each context (Fig.

2.7A), (ii) the entropy of the dwell time in the contexts (Fig. 2.7B), and (iii) total time spent freezing (2.7C). On the third day, during the preference task, mice were given access to both P and UP contexts via a bridge for ten minutes (Fig. 2.2D), the behavioural readout for this experiment is the time spent in each of the contexts (Fig. 2.7D). These tasks are repeated 17-19 days after training (remote time point).

Both groups travelled the same approximate distance within their paired and unpaired contexts (Fig. 2.7A1). The mice receiving DSS colitis exhibited a lower entropy (unpredictability of the area traversed) in their paired context compared to their unpaired context (Fig. 2.7B1). In other words, the area in which the DSS mice traversed in the paired chamber was more predictable than in the unpaired chamber. Both groups spent the same amount of combined time (both contexts) freezing (Fig. 2.7C1). Both groups showed the ability to discriminate between contexts during the preference task and spent significantly more time in their unpaired context (Fig. 2.7D1). This task allowed us to test their threat assessment independent of freezing behaviour because they were able to avoid the fearful environment by moving to the other context rather than to only respond to it, as when the mice spent five minutes in their P context alone. Overall, entropy was the only significant trend apparent between the groups at the recent testing time point.

Nine days after the recent test point, over a four-day period, mice were retested on each of the prior assessments as well as the Open Field Task on day four (2.8E). Similar to the recent time point, both control and DSS mice displayed a preference for the unpaired context in the chamber preference task, where they were able to roam between the contexts (Fig. 2.7D2). And, again aligning with the recent time point, both DSS and Control mice spent equal combined times freezing at the remote time point (Fig. 2.7C2). Differing from

the recent time point, however, at the remote timepoint in the paired context, the DSS group showed decreased distance travelled (Fig. 2.7A2) and entropy (Fig. 2.7B2) relative to the control group. It is also evident that the control mice increase the amount of time they spend in the paired context during the chamber preference task at the remote time point compared to the recent time point, whereas the DSS mice do not show any changes between time points (Fig. 2.7A3). In other words, it appears that the Control mice have diminished their aversion to the previously paired chamber, whereas the DSS mice have not. Moreover, the Control mice spend significantly more time in the centre of the open field than DSS mice (Fig. 2.8D).

In the case of the behavioural readouts of the conditioned anxiety test, the control group does not increase their entropy or distance travelled over time, rather, the DSS group decreases these readouts. Although the DSS group retained their ability to discriminate between contexts as exhibited in the chamber preference task, their fear behaviour either increased, or stayed the same. Differing from this, the control group exhibited either less anxiety behaviours, as in the Open Field Task (Fig. 2.8) and the increased time in the paired context (Fig. 2.7A3), or stayed the same, as in the case of the entropy (Fig. 2.7B2) and distance travelled (2.7A2). Thus, it appears that chronic DSS treatment negatively affects the ability for mice to recover from fear conditioning over time.

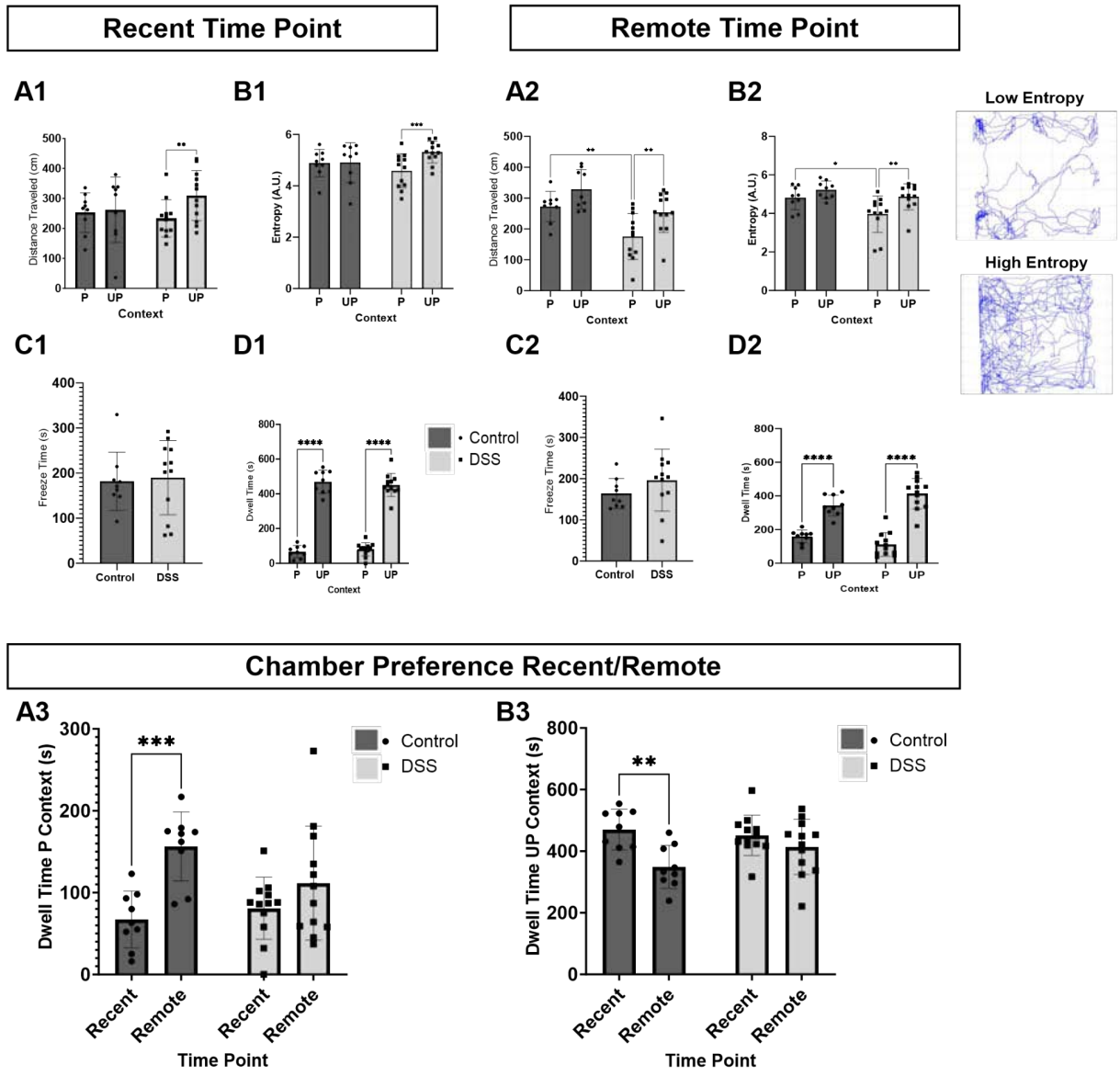

Figure 2.7: Behavioural readouts of the fear to context task at both the recent and remote time-point.

(A1) Distance travelled in paired and unpaired contexts at recent freeze-test time point. (B1) Entropy of dwell time in paired and unpaired contexts at recent freeze-test time point. (C1) Total freeze time in both the paired and the unpaired context combined at the recent freeze-test time point. Unpaired t-Test. *$p < 0.05$. Data are mean ± SD, control n = 9; DSS n = 12. (D1) Dwell time in paired (P) and unpaired (UP) context of chamber preference task at recent testing time points. (A2) Distance travelled in paired and unpaired contexts at remote testing time points. (B2) Entropy of dwell time in paired and unpaired contexts at remote testing time point. (C2) Total freeze time in both the paired and the unpaired context

combined during remote testing intervals. Unpaired t-Test. *p < 0.05. Data are mean ± SD, control n = 9; DSS n = 12. (D2) Dwell time in paired (P) and unpaired (UP) context at remote testing time points. Entropy calculation. (A3) Dwell time in paired context and (B3) unpaired context at both recent and remote testing time points. (Everything apart from the B1 and B2) Multiple comparison two-way ANOVA with Sidak post-test. *p < 0.05; **p < 0.01; ***p < 0.001****p < 0.0001. Data are mean ± SD, control n = 9; DSS n = 12.

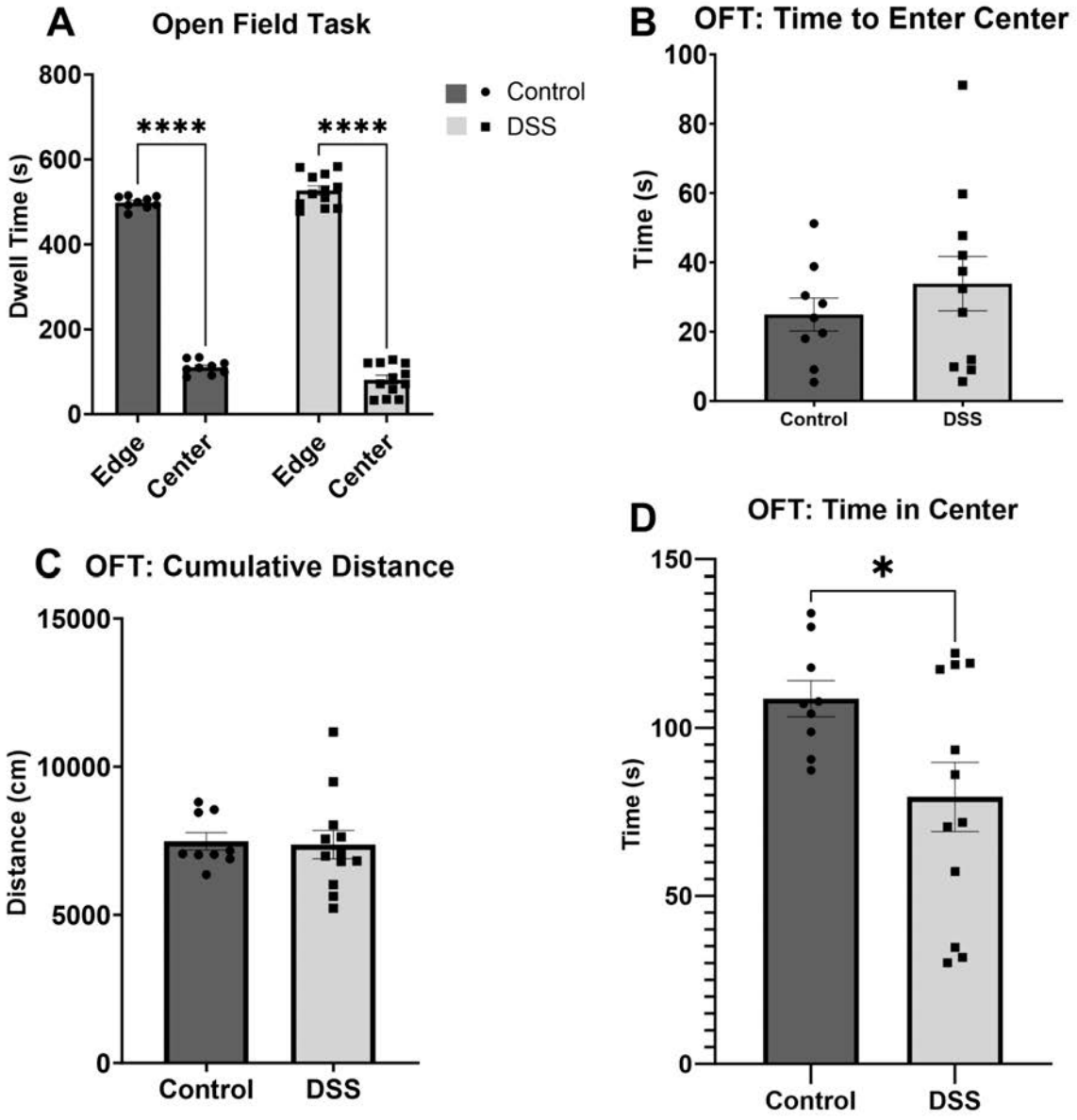

Figure 2.8: Behavioural readouts of the Open Field Task

(A) Open Field task (OFT). Total time on the edge compared to total time in the centre. Multiple comparison two-way ANOVA with Sidak post-test. ****p < 0.0001. Data are

mean ± SD, control n = 9; DSS n = 12. (B) OFT: Time to Enter Center. (C) OFT Cumulative Distance. (D) OFT Time in Center. Data are shown as mean ± SEM; control n = 9; DSS n = 12. Unpaired t-Test. *p < 0.05; ****p < 0.0001.

Correlation between peripheral inflammation and behavioural readout.

When analysing the correlations between faecal inflammation markers (LCN2), and anxiety-like behavioural readouts, differing trends were observed between the control and DSS groups. While there is no significant correlation between the control behaviour readouts and the LCN2 levels, there is a significant negative correlation between the amount of time DSS mice spent in the centre of the open field and the amount of peripheral inflammation they had (Fig. 2.8). This suggests that inflammation beyond physiologically normal levels may lead to increased anxiety and decreased exploratory behaviour.

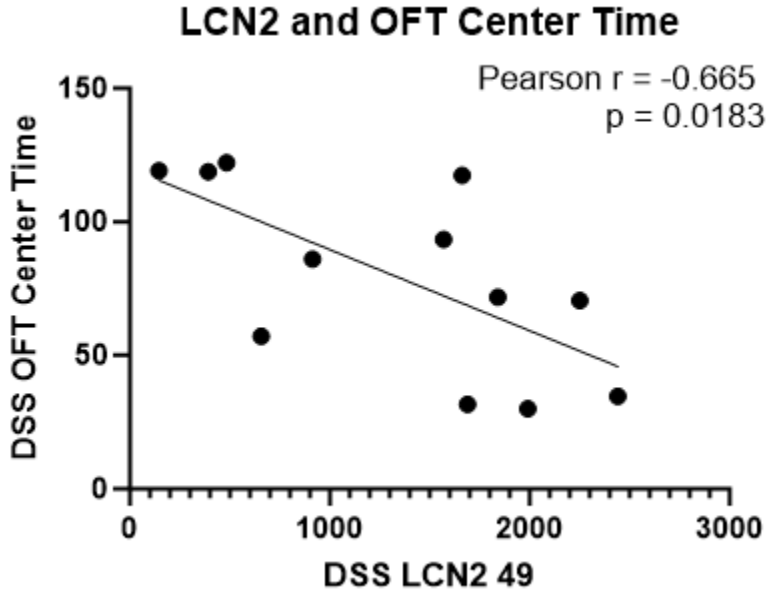

Figure 2.9: Gut inflammation and anxiety-like behaviour correlations.

Faecal LCN2 levels of DSS mice as it relates to the time in the centre of the OFT task. DSS n = 12. Simple Linear Regression.

Chronic exposure to DSS produced changes in inflammatory markers in the brain.

The mRNA of several proteins involved in the inflammatory pathway in the brain were isolated from both the ACC and the NAc of mice given DSS and Control mice. When analysing these genes individually, Il-1b, an inflammatory cytokine, is significantly higher in the NAc of DSS mice than Control mice (Fig. 2.10C).

A Principal Component Analysis (PCA) was run to provide a visual representation of the greatest amount of variance between the different data sets plotted (Fig. 2.11). In both the ACC and the NAc, but especially in the NAc, there are differences in the mRNA content between Control and DSS groups (Fig. 2.11A and 2.11B). This suggests that the induced inflammation in the gut is also impacting inflammatory pathways in the brain of mice given DSS colitis.

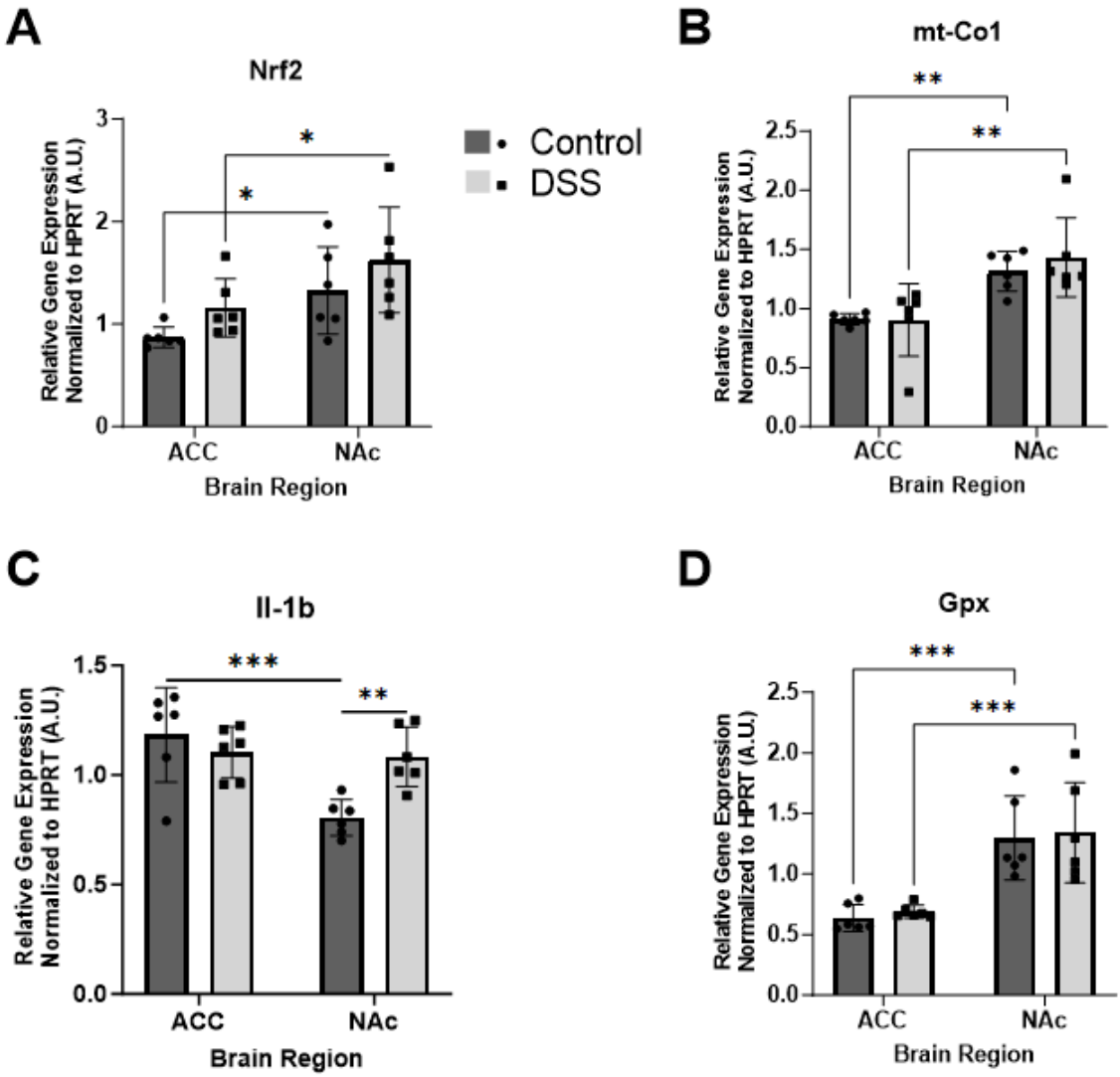

Figure 2.10: Relative mRNA expression in the ACC and NAc of DSS and control mice.

Relative expression of genes (normalised to HPRT) involved in the inflammatory pathway. (A) Nrf2, (B) mt-Co1, (C) Il-1b, and (D) Gpx. Multiple comparisons two-way ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001. Data are mean ± SD, control and DSS n = 6.

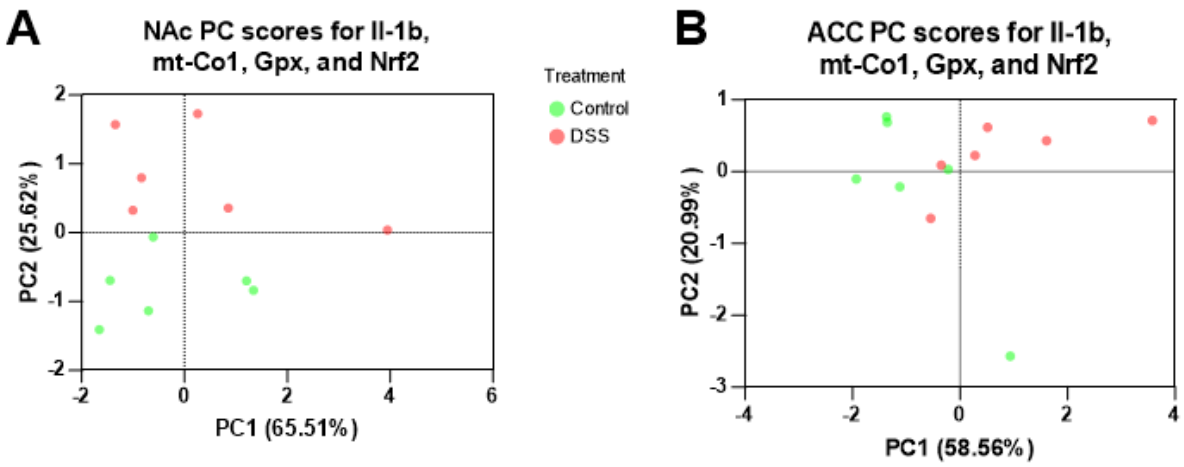

Figure 2.11: Multivariate analysis of inflammation-related gene expression.

(A) Il-1b, mt-Co1, Gpx, and Nrf2 in the NAc of both DSS and Control mice. (B) Il-1b, mt-Co1, Gpx, and Nrf2 in the ACC of both DSS and Control mice. Control n = 6; DSS n = 6 (only 6 controls and 6 DSS were used for the RNA isolation portion of this experiment).

2.4 DISCUSSION

In this experiment, twenty-one mice were trained for eight days on a fear to context task involving two contexts differing in shape, scent, and wall pattern. One group of 12 mice were given three doses of DSS in their drinking water while the other group of 9 mice received normal drinking water. The mice were tested at two different time-points, recent (peak disease for the 12 mice receiving DSS) and remote. At both time-points mice were given the same three tests in their paired, unpaired, and bridged contexts, with the addition of the open field task at the remote time-point. The findings of the experiment were that mice given DSS have a stronger negative stimulus-context association than control mice. This study suggests that chronic gut inflammation drives hyperarousal of ‘dangerous’ contexts in order to maximise survival.

Chronic Colitis Induction

The mice given three doses of DSS show a significant decrease in weight compared to the control group, as well as a significant increase in faecal LCN2 levels. The dramatic upward trend of LCN2 in the DSS mice is indicative of increased inflammation in the colon, also exemplified by an increase in the size of the spleen and shortened colons. The spleen is understood to be involved in immunity processes (Bronte & Pittet, 2013). These results were expected from previous studies using DSS to induce chronic colitis (Zhou et al., 2023) and show that chronic colitis was successfully modelled in the DSS group.

Fear to Context Results

Distance travelled, entropy or uncertainty of movement, and the total time spent freezing in their combined contexts were measured during the fear to context test. Their preference for each context was also measured by placing a bridge between the two contexts and allowing them to freely travel between the unpaired and paired chambers. Each of these tests were repeated at the recent and remote time-point. At the recent time point there is no significant difference between the distance travelled in the paired or unpaired context for either the DSS or Control mice, and there is no difference between the distance the DSS mice travelled in either of their contexts compared to the control group.

Considering both groups had been in each of the contexts several times during training, it is possible that although unafraid in the unpaired context, they had no initiative to explore, as it was a familiar environment. This idea is also represented by Pearson et al. (2010), as mice only showed interest in investigating new social stimuli in a novel environment. This demonstrates the idea that mice are more prone to investigation in an unfamiliar context.

The control group did not show a difference in entropy between the paired and unpaired contexts, but the DSS group did, with a lower entropy in the paired context than the unpaired context. This difference shows that in the paired context the movement is more predictable, demonstrating that the mice spent more time in one specific corner compared to different locations. Both control and DSS groups froze equal amounts of time in the combined contexts at both the recent and remote time-point, and both groups showed a strong preference for the unpaired context during the preference task at both time-points. These results differ from those found by Matisz et al. (2022), in which it was found that all mice froze more in their paired context at the recent testing time-point. One potential cause could be the lower number of mice present in this study's control group which may reduce the test significance. A study done by De Franceschi et al. (2016) suggests another possibility. Their study found that a moving overhead stimulus induces freezing behaviour, while an unmoving overhead stimulus induces flight behaviour. In the present study both contexts were contained within a large white hood, limiting the potential for movement of shadows.

At the remote time-point there is an interesting change. The DSS mice travel less in their paired context at the remote time point than the recent time point. The DSS mice also maintain a lower entropy in their paired context compared to their unpaired context, but even lower at the remote time point than at the recent time-point. Both the distance travelled and the entropy in the paired context are significantly lower for the DSS group than for the control group. The control mice spend significantly more time in the paired context, and significantly less time in the unpaired context at the remote time-point. Meanwhile, the DSS group does not shift their preference between these time-points. These results have several potential explanations: a chronic increase in sympathetic activation leading to hyperarousal, or a stronger association of danger with the paired context. An increased sympathetic activation should result in an increase in overall anxiety

behaviours, such as increased combined freeze time and reduced entropy and travel distance in both contexts. Considering we do not see these results; the more likely explanation is that the DSS group had a stronger association between the negative stimulation and context.

An interesting explanation for this increased association could be a change in gut microbiota composition. Vicentini et al. (2022) find that mice given a faecal transplant from a group given colitis show increased anxiety behaviours. The mice receiving the transplant did not have altered gut or neuroinflammation but did have altered gut microbiota. Although the gut microbiota composition was not considered in this study, in the future it could provide more insight into the observed phenomena.

Open Field Task Results

The open field task was run after the conditioned tests at the remote time-point. Both control and DSS groups show a preference for the periphery compared to the centre of the open field. There is no significant difference in the time it took for the groups to enter the centre of the open field for the first time, nor the cumulative distance that the groups travelled while in the open field. The amount of time that the mice spent in the centre of the open field is significantly higher for the control mice than it is for the DSS mice. This suggests that the anxiety at the remote time-point is higher for the DSS group than for the control group. Together with the previous tests, this suggests that both conditioned and unconditioned anxiety measures were higher in DSS mice than control mice at the remote time-point. This idea is strengthened by the results found in figure 2.9, as the amount of time spent in the centre of the open field is negatively correlated with the amount of LCN2 found in the faeces at the remote time-point. This means that more inflammation in the colon of a mouse leads to less time in the centre of the OFT for that particular mouse. This

phenomenon was also found in rats receiving DSS. During peak disease of an acute dose of colitis less time was spent in the centre, yet after disease resolution their time in the centre returned to control levels (Dempsey et al., 2019). These results provide more understanding of the relative inconsistency of OFT results between studies, as the task appears to be influenced by the levels of inflammation in the rodent.

Tissue Analysis Results

After the mice were euthanized and the brains were snap-frozen, the ACC and the NAc of 6 randomly chosen DSS mice and 6 randomly chosen control mice were removed in order to isolate the RNA for further down-stream analysis. The RNA was analysed via a RT-qPCR in order to find the relative quantity of several genes involved in the inflammatory response, namely Nrf2, mt-CO1, Gpx, and Il-1b. The relative quantities of Nrf2, mt-Co1, and Gpx did not differ between the control and DSS groups in either the ACC or the NAc, but the relative quantities did differ between brain regions in all three of these genes. The relative quantity of Il-1b did not differ between brain regions in the DSS group, but did in the control group, with more Il-1b RNA in the ACC of control mice than the NAc. However, in the NAc there was more Il-1b RNA in the DSS group than in the control group. Il-1b is a cytokine released during an inflammatory response. The increase in Il-1b in the DSS mice suggests that the increased inflammation in the colon also triggered an inflammatory response in the brain, and specifically the NAc, of the DSS group. This effect is supported by a study in which FosB/ΔFosB immunoreactivity was increased in the NAc of rats after an acute dose of DSS colitis (Dempsey et al., 2019), suggesting that gut inflammation is able to alter the cellular activity in the brain. Further evidence of this is found in the unsupervised principal component analysis, in which all four genes were analysed together. In figure 2.11 the

control group is clustered at the bottom, while the DSS group is clustered at the top. This shows that the greatest amount of variance within the data is found between the DSS and control groups. Although each individual gene is not significantly different between groups, when considered together they follow a similar trend of different expression between groups.

Examination of Hypotheses

Hypothesis I predicted that mice with chronic gut inflammation would exhibit higher levels of anxiety in both the conditioned and unconditioned behavioural tasks and improperly differentiate between the contexts. Based on previous research completed by Matisz et al. (2022), changes in anxiety behaviour at the remote disease testing time-point compared to the recent time-point was anticipated. While the mice were able to differentiate between the contexts at both time points, there is an increase in anxiety behaviour at the remote time-point. This is evidenced by significantly lower entropy, less distance travelled, and less time spent in the paired context compared to controls. Hypothesis II predicted that mice given chronic gut inflammation would have an increase in inflammatory markers, such as Il-1b, in specific brain regions related to anxiety, and that mRNA involved in the inflammatory pathway would be expressed differently in mice given chronic inflammation compared to those without inflammation. We are able to reject the null hypothesis as there is an increase in Il-1b in the NAc of DSS mice compared to controls, and as shown in the PCA, all four genes are expressed differently in the NAc of DSS mice than in control mice. These same results are not found in the ACC of DSS compared to control mice.

Conclusion

The findings in this study show that a chronic treatment of DSS is able to model the human condition of colitis. Inflammation induced by DSS induces inflammation in the brain, specifically noticed in the NAc. Mice with chronic inflammation have a stronger stimulus-context association than control mice, showing higher levels of anxiety in both conditioned and unconditioned tasks. Overall, these results add to this field of research in several important ways: 1. They strengthen our understanding of the brain-gut axis, supporting the hypothesis that gut health is able to impact brain health. 2. This study shows that even after the resolution of an inflammatory disease, mood disorders such as anxiety may persist. 3. This study depicts the correlation between peripheral inflammation and anxiety behaviours. 4. This study aids in understanding which brain regions may be involved in the anxiety processes.

CHAPTER 3: EFFECT OF PSILOCYBIN ON INFLAMMATION AND ANXIETY-LIKE BEHAVIOUR IN MALE C57/BL6J MICE

3.1 INTRODUCTION

Inflammatory bowel diseases (IBD), including Crohn's disease and ulcerative colitis, have complex causes and comorbidities (Podolsky, 1995). IBD presents with diarrhoea, abdominal pain, fatigue, and weight loss (Podolsky, 1995) and are often comorbid with anxiety and depression (Hassan et al. 2014; Stuart & Baune, 2014). A chemical model of IBD is commonly introduced by dissolving dextran sulphate sodium (DSS) into drinking water, (Okayasu et al.,1990). DSS colitis exposure has many of the same symptoms as ulcerative colitis, including clinical disease signs such as faecal blood, diarrhoea, and weight loss. These are also accompanied by behavioural changes such as anxiety and depression (P. Bercik, et al. 2011; Sudeep et al., 2022). DSS colitis also induces an increase in neuroinflammatory markers (Do and Woo, 2018 and Reichmann et al., 2015), neuronal excitation, and behavioural alterations (Chen et al., 2015 and Jain et al., 2015 and Nyuyki et al., 2018 and Reichmann et al., 2015), all which mirror the comorbidity of IBD with depression and anxiety (Matisz et al., 2022).

As there is evidence for a connection between gut inflammation, brain inflammation, and anxiety production, it is important to consider how to remediate this inflammatory cycle. Psychedelics have recently been implicated for their potential to remediate inflammation by acting on the 5-HT2A receptor (Robinson et al., 2023). The usual 5-HT2A receptor agonist, serotonin, is not only involved in brain inflammation, but also involved in gut inflammation (Ghia et al., 2009) and is implicated in inflammatory bowel diseases (Khan, 2013). Due to its numerous roles, dysfunction in the serotonergic system contributes to a host of disorders, including depression and anxiety (Thiebot, 1986). Considering the potential damage due to inflammatory processes involved

in prevalent disorders such depression, anxiety, and gut diseases, it is important to find an anti-inflammatory agent with the potential for gut disease and mood disorder treatment efficacy.

One such agent is psilocybin, a hallucinogenic substance found in fungi, including 'magic' mushrooms. For centuries people have used psilocybin-containing mushrooms for the healing properties they provide, however, it is now understood that psilocybin acts as a competitive agonist for the serotonin, 5-HT2A, receptor (Zanicov et al., 2023). When this receptor is activated by serotonin, it has a pro-inflammatory effect, however, when activated by different psychedelics, including psilocybin, it has the opposite effect, leading to a decrease in inflammation (Flanagan & Nichols, 2018; Yu et al., 2008). By stimulating a specific receptor formation, psilocybin triggers anti-inflammatory effects while also blocking the receptor from allowing serotonin to dock and induce inflammation. The result of this opposing effect is demonstrated in that psychedelics inhibit TNF-a-induced inflammation (Nau et al. 2013).

Considering the connection between inflammation and anxiety, it is unsurprising that in a double-blind study in which the 5-HT2A receptor is activated by psilocybin, anxiety and depression are found to have decreased in cancer patients (Griffiths et al., 2016; Ross et al., 2016). It is also found that psilocybin downregulates proinflammatory proteins such as CD80, p65, and TLR4 while upregulating neuroprotective protein, TREM2 (Kozłowska et al., 2021). Psilocybin has also been shown to stimulate neurogenesis (Jones & O'Kelly, 2020).

Due to the overwhelming connection between inflammation, gut diseases, and anxiety, the potent anti-inflammatory effect of psychedelics when activating the 5-HT2A receptor (Yu et al., 2008) is certainly worth exploring in a model of gut and brain inflammation. It is hypothesised that a single dose (6 mg/kg) of psilocybin administered between the recent and remote testing time-points will decrease inflammation in the brain. It is further hypothesised that this decrease in

inflammation will lead to a decrease in anxiety-like behaviour in both the conditioned and unconditioned behavioural tests.

3.2 MATERIALS AND METHODS

Materials and methods are identical to those in chapter 2 apart from details below.

Subjects

Along with the 9 control and 12 DSS mice, an additional 12 adult, male, C57BL/6J mice purchased from Jackson Bar Harbour, ME arriving on December 14, 2022, were used in the experiments described in this chapter. These 12 mice received three doses of DSS in water and a single subcutaneous dose of 6 mg/kg of psilocybin on experimental day 44.

Colitis Induction

For the induction of chronic DSS colitis, mice were exposed to three cycles of DSS (3.0%, 2.75%, 2.75% wt/v) each lasting five days, with 8 days of recovery between the first and the second cycle, and a 15-day recovery period between the second and third cycle, after which psilocybin was injected.

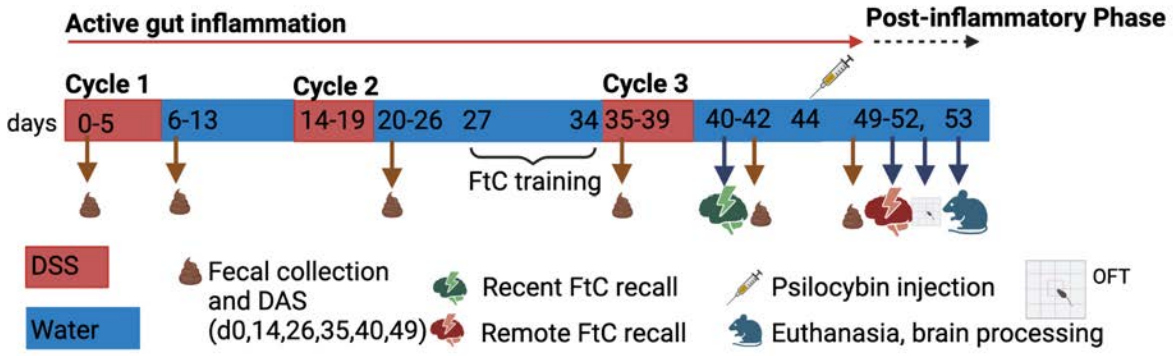

Figure 3.1: Schedule of the behavioural experiments, colitis induction, and injections.

Table 3.1: Schedule of faecal collection, DSS induction, and behavioural experiments.

Day	Date	Behaviour	DSS Level
0	28-Dec	Baseline	None
26	23-Jan	Pre-Exposure	8-days post-cycle 2
35	01-Feb	End of training	Just prior to cycle 3
40	06-Feb	Recent Test	Peak Inflammation
44	10-Feb	Between Test	Resolving Inflammation
49	15-Feb	Remote Test	Resolving Inflammation

RNA Isolation

Four of the twelve DSS+Psi mice were randomly chosen for RNA and DNA extraction and underwent the same protocol as the twelve mice in chapter 2.

Injections. One injection of 6 mg/kg psilocybin purchased from Toronto Research Chemicals was subcutaneously (SC) injected between recent and remote tests on day 44 (2023/02/10).

3.3 RESULTS

Chronic exposure to DSS produced clinical signs of colitis

Mice given DSS in the present study had reduced body weight (Fig. 3.2A) relative to controls during the DSS treatment and 8-20 days after treatment cessation. Likewise, the faecal lipocalin-2 levels were increased by DSS treatment (Fig. 3.2B). Mice chronically exposed to DSS exhibited greater weight loss and lipocalin levels throughout the study, and at experimental endpoint had heavier spleens and shorter colons compared to control mice (Fig. 3.3). This suggests that those mice receiving DSS colitis showed significant clinical signs of DSS colitis compared to control mice. Mice given both DSS and psilocybin (Psi) were injected on experimental day 44 (Table 3.1), this single injection did not affect their weight or faecal inflammation markers after this point. However, DSS + Psi mice did not differ as dramatically from control mice in their colon length or spleen weight compared to DSS-only mice, suggesting some clinical signs of disease may have decreased (Fig. 3.3). Apart from the singular psilocybin injection, the DSS + Psi group was treated exactly as the DSS-only group (methods from Chapter 2 are relevant to this chapter).

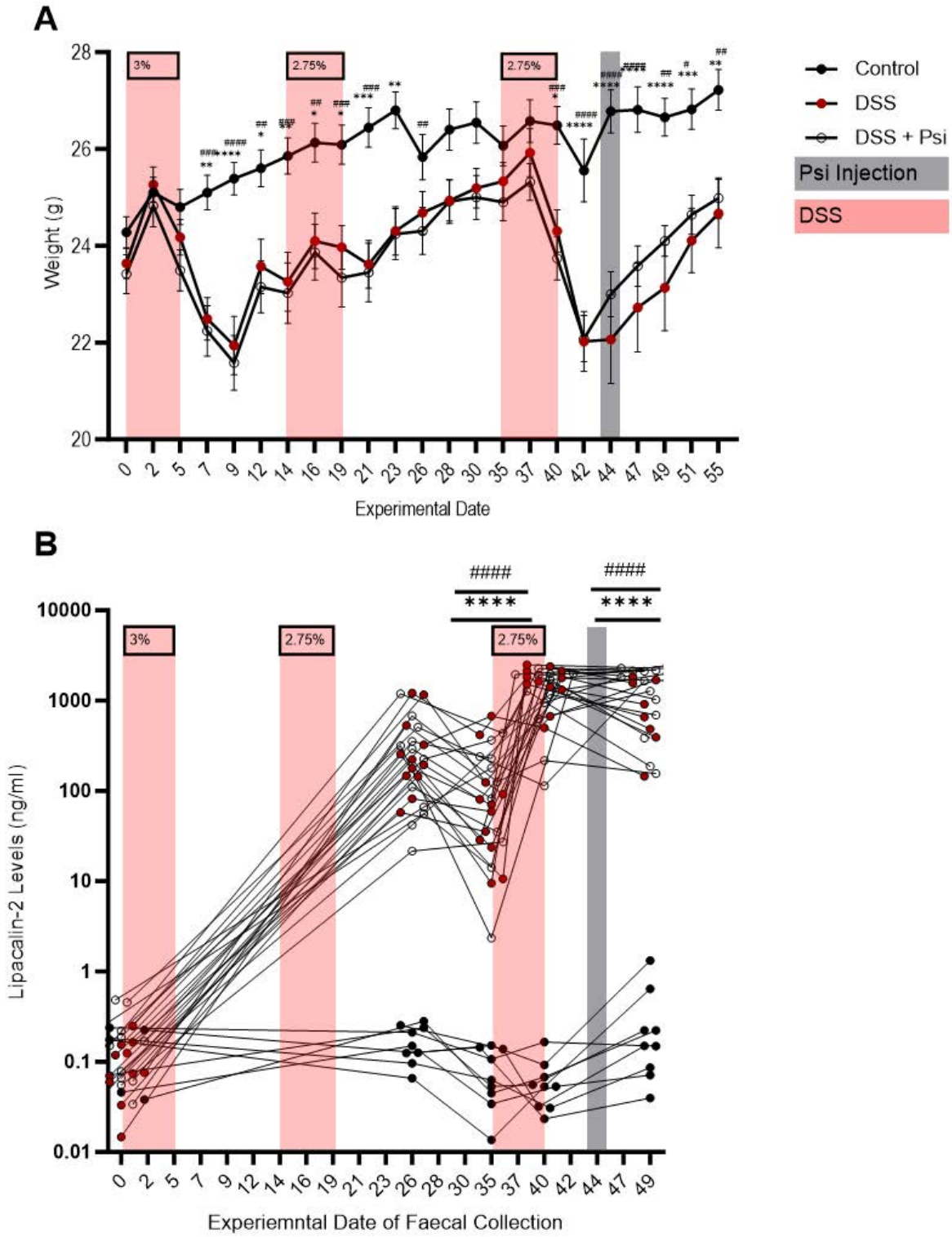

Figure 3.2: Clinical signs of disease.
(A) Body Weight and (B) Lipocalin-2 levels among treatment groups. DSS and DSS+Psi mice were given 3 doses of DSS (3%, 2.75%, and 2.75%) in their drinking water to induce

colitis. Body weight was observed throughout. Data are shown as mean ± SEM. Lipocalin-2 levels were discovered from faeces collected at 5-time points throughout 3 doses of colitis (red bars). Control n = 9; DSS n = 12; DSS + Psi n = 12. Repeated Measures two-way ANOVA with Tukey's post hoc test. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. Control vs. DSS. #p < 0.05; ##p < 0.01; ###p < 0.001; ####p < 0.0001. Control vs. DSS + Psi.

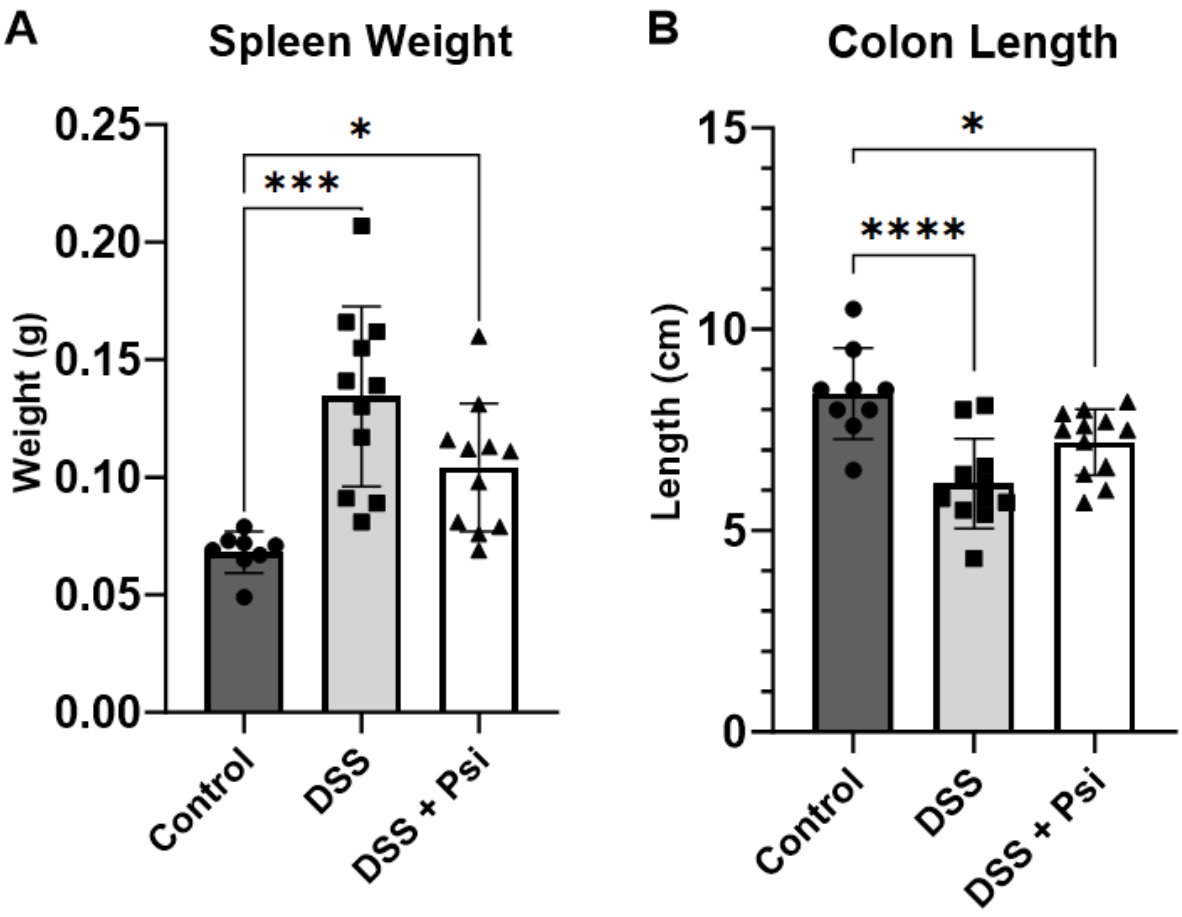

Figure 3.3: Features of organs.

(A) Spleen Weight; control n = 8; DSS n = 11; DSS + Psi n = 11 (one outlier in each group). (B) Colon Length; control n = 9; DSS n = 21; DSS + Psi n = 12. Data are shown as mean ± SD;. Ordinary one-way ANOVA with Tukey's multiple comparisons Post Test. *p < 0.05; **p < 0.01; ****p < 0.0001.

Psilocybin alters anxiety-like behaviours.

Both Control and Psi groups travelled the same approximate distance within their paired and unpaired contexts (Fig. 3.4A1). The mice receiving DSS colitis and the DSS + Psi (identical to DSS-only group at this time-point as they had not yet received psilocybin injection) exhibited a lower entropy in their paired context (higher entropy correlates with less predictability of dwell location and lower entropy correlates with more predictability of dwell location) compared to their unpaired context, but did not exhibit any differences compared to control mice (Fig. 3.4B1). All three groups spent the same amount of combined time (both contexts) freezing (Fig. 3.4C1). All three groups showed the ability to discriminate between contexts during the preference task and spent significantly more time in their unpaired context (Fig. 3.4D1). This preference task allowed us to test their threat assessment independant of freezing behaviour because they were able to avoid the fearful environment by moving to the other context rather than to only respond to it, as when the mice spent five minutes in their P context alone.

Nine days after the recent test point, over a four-day period, mice were retested on the prior two assessments as well as the Open Field Task on day four (fig. 3.5E). Similar to the recent time point, all groups displayed a preference for the unpaired context in the chamber preference task, in which they were able to roam between the contexts (Fig. 3.4D2). And, again aligning with the recent time point, all groups spent equal combined times freezing at the remote time point (Fig. 3.4C2). Differing from the recent time point, however, at the remote timepoint in the paired context, the DSS group showed decreased entropy (Fig. 3.4B2) compared to the control group, and decreased distance travelled (Fig. 3.4A2) relative to the control and psilocybin groups. The control mice increase the amount of time they spent in the paired context during the chamber preference task at the remote time point

compared to the recent time point, whereas the DSS mice do not show any changes between time points (Fig. 3.4A3). Not only this, but during the open field task the Control and DSS + Psi mice spend more time in the centre of the open field than DSS mice (Fig. 3.5D), and DSS + Psi mice are the quickest to enter the centre of the open field (Fig. 3.5B).

In the case of the behavioural readouts of the fear test, the control group does not increase their entropy or distance travelled in the paired context over time, rather, the DSS group decreases these readouts. Although the DSS group retained their ability to discriminate between contexts as exhibited in the Chamber Preference Task (CPT), their fear behaviour either increased, or stayed the same. Differing from this, both the control and DSS + Psi groups exhibited either decreased anxiety behaviours, as in the Open Field Task and increased time in the paired context of the CPT, or stayed the same, as in the case of the entropy and distance travelled. Thus, it appears that chronic DSS treatment negatively affects the ability for mice to recover from fear conditioning over time, and the data suggests that a single dose of psilocybin is able to reverse some of these effects.

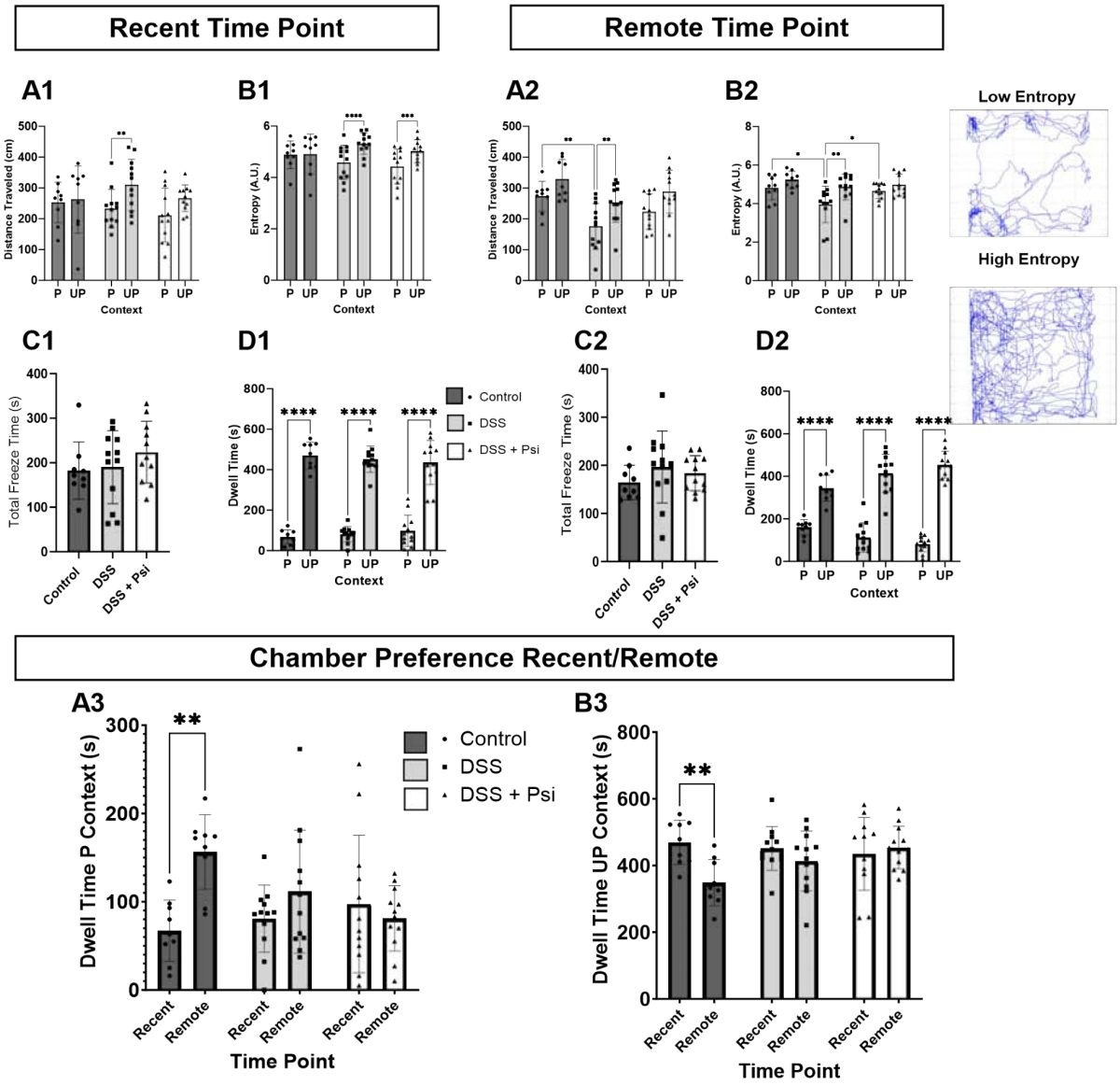

Figure 3.4: Behavioural readouts of the fear to context task at both the recent and remote time-point.

(A1) Distance travelled in paired and unpaired contexts at recent freeze-test time point. (B1) Entropy of dwell time in paired and unpaired contexts at recent freeze-test time point. (C1) Total freeze time in both the paired and the unpaired context combined at the recent freeze-test time point. Unpaired t-Test. *$p < 0.05$. Data are mean ± SD, control n = 9; DSS n = 12. (D1) Dwell time in paired (P) and unpaired (UP) context of chamber preference task at recent testing time points. (A2) Distance travelled in paired and unpaired contexts at remote testing time points. (B2) Entropy of dwell time in paired and unpaired contexts at remote testing time point. (C2) Total freeze time in both the paired and the unpaired context combined during remote testing intervals. Unpaired t-Test. *$p < 0.05$. Data are mean ± SD, control n = 9; DSS n = 12. (D2) Dwell time in paired (P) and unpaired (UP) context at remote

testing time points. Entropy was calculated by summing the number of times the mouse crossed each block (context was divided into 900 equal blocks), dividing each by the summed number, then multiplying each by the natural log of itself and summing that for the entropy of each mouse in each context. (E) Nose-track of a low entropy mouse. (F) Nose-track of a high entropy mouse. (A3) Dwell time in paired context and (B3) unpaired context at both recent and remote testing time points. (Everything apart from the B1 and B2) Multiple comparison two-way ANOVA with Sidak post-test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$****$p < 0.0001$. Data are mean ± SD, control n = 9; DSS n = 12; DSS + Psi n = 12.

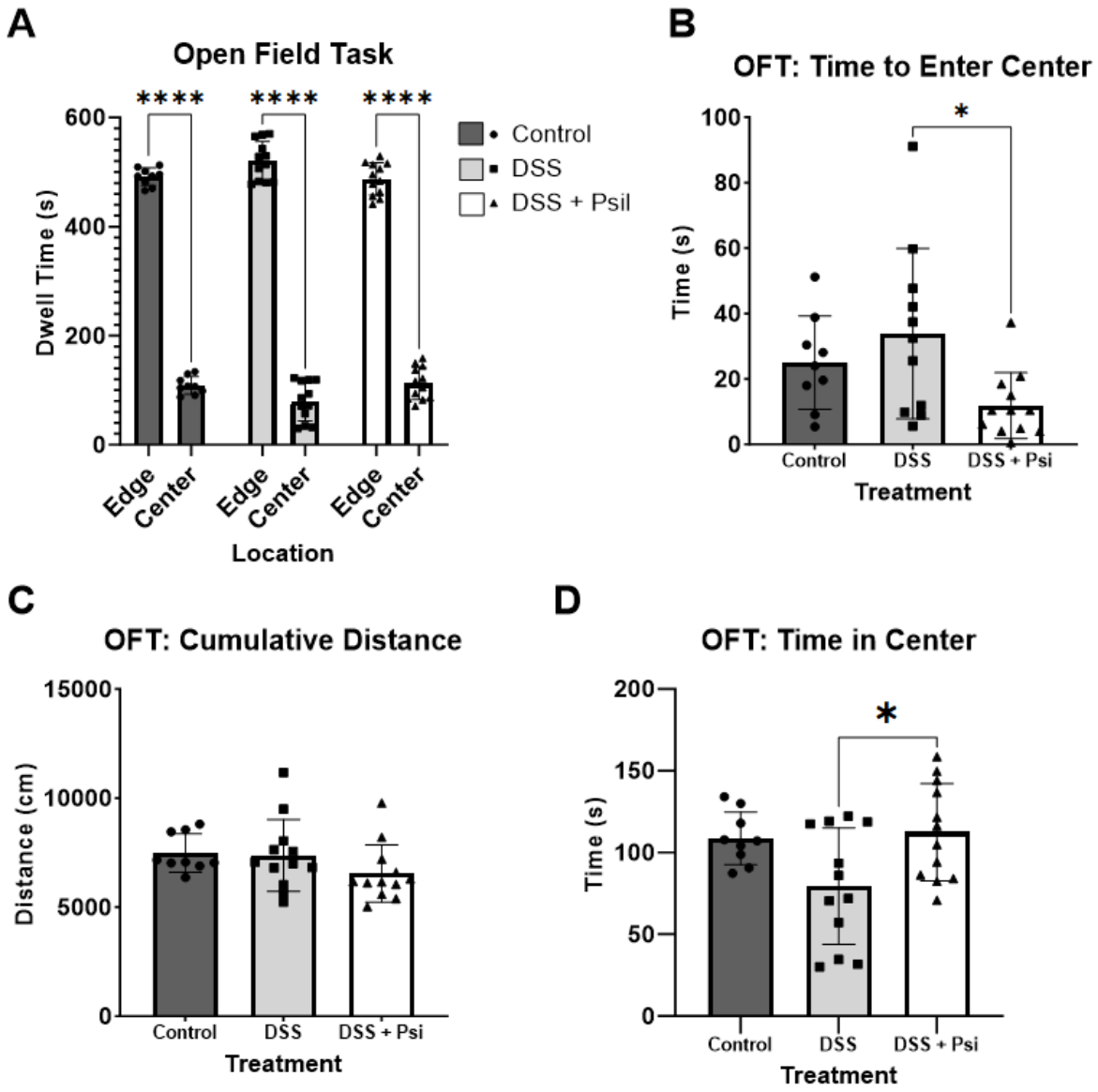

Figure 3.5: Behavioural readouts of the Open Field Task

(A) Open Field task (OFT). Total time on the edge compared to total time in the centre. Multiple comparison two-way ANOVA with Sidak post-test. ****$p < 0.0001$. Data are

mean ± SD, control n = 9; DSS n = 12. (B) OFT: Time to Enter Center. (C) OFT Cumulative Distance. (D) OFT Time in Center. Data are shown as mean ± SD; control n = 9; DSS n = 12; DSS + Psi n = 12. Multiple comparisons one-way ANOVA with Tukey's post-test. *p < 0.05.

Chronic exposure to DSS produced changes in inflammatory markers in the brain which are mitigated by psilocybin injection.

The mRNA of several proteins involved in the inflammatory pathway in the brain were isolated from both the ACC and the NAc of mice given DSS, DSS + Psi, and Control mice. When analysing these genes individually Il-1b, an inflammatory cytokine, is significantly lower in the NAc of both the control and the DSS + Psi groups compared to the DSS-only group (Fig. 3.6C).

Principal Component Analysis (PCA) was run to see how these genes may be working together within the NAc and ACC of each group. Differences are found in both the ACC and the NAc between Control, DSS, and DSS + Psi groups, with DSS + Psi clustering with the control group (Fig. 3.7A). These differences continue with the addition of LCN2 at the remote time-point (Fig. 3.7C).

All this suggests that the induced inflammation in the gut is also impacting inflammatory pathways in the brains of mice given DSS colitis, but it also that a single dose of psilocybin is able to impact these pathways, specifically in the NAc, even while faecal inflammation (LCN2 levels - Fig. 3.2B) remain at the same level as mice given only DSS colitis.

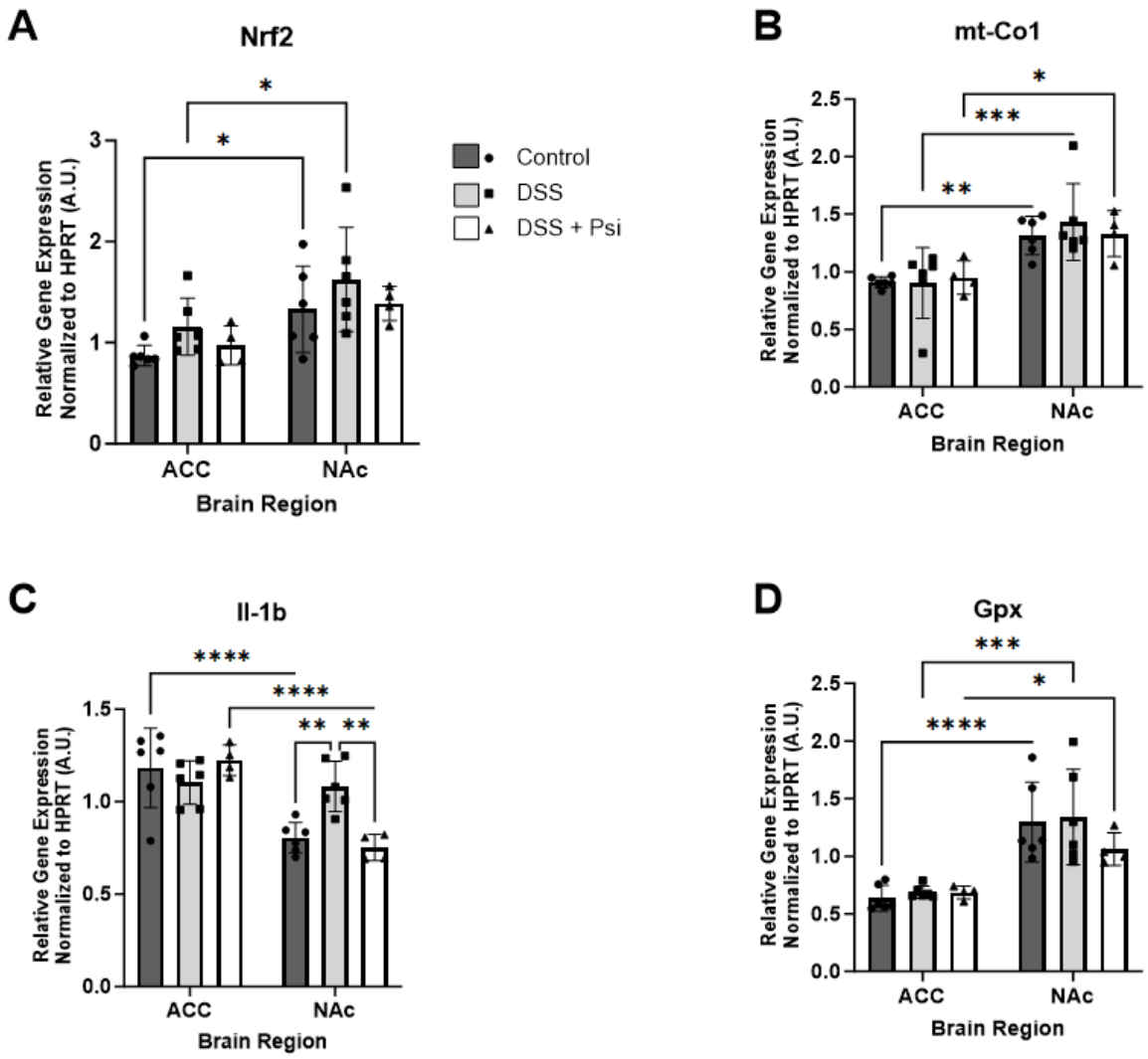

Figure 3.6: Relative mRNA expression in the ACC and NAc of DSS and control mice.

Relative expression of genes (normalised to HPRT) involved in the inflammatory pathway. (A) Nrf2, (B) mt-Co1, (C) Il-1b, and (D) Gpx. Multiple comparisons two-way ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001. Data are mean ± SD, control and DSS n = 6; DSS + Psi n = 4.

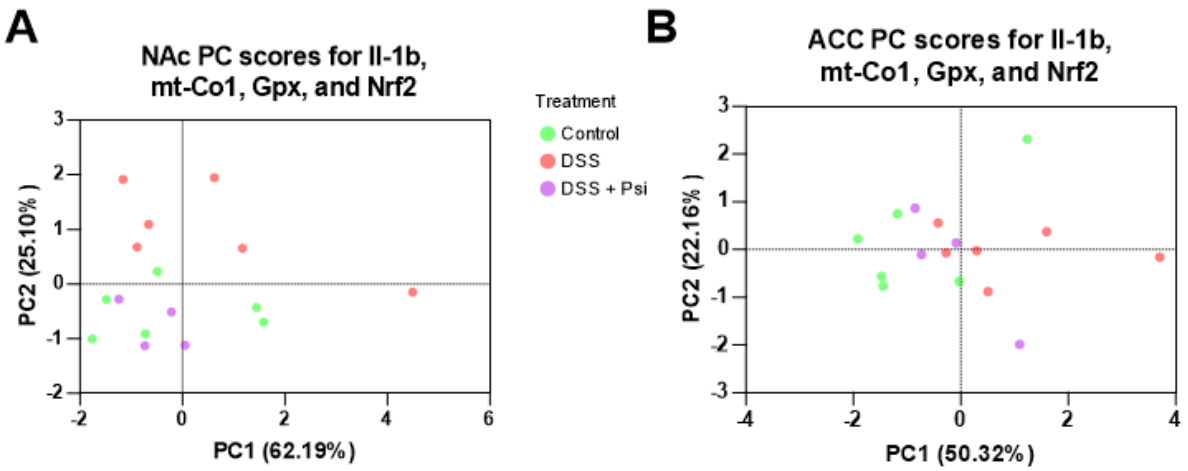

Figure 3.7: Multivariate analysis of inflammation-related gene expression.

(A) Il-1b, mt-Co1, Gpx, and Nrf2 in the NAc of both DSS and Control mice. (B) Il-1b, mt-Co1, Gpx, and Nrf2 in the ACC of both DSS and Control mice. Control n = 6; DSS n = 6; DSS + Psi n = 4 (only 6 controls, 6 DSS, and 4 DSS+Psi were used for the RNA isolation portion of this experiment).

3.4 DISCUSSION

In this experiment, 33 mice were trained for eight days on a fear to context task involving two contexts differing in shape, scent, and wall pattern. Two groups of 12 mice were given three doses of DSS in their drinking water while the other group of 9 mice received normal drinking water. The mice were tested at two different time-points, recent (peak disease for the 12 mice receiving DSS) and remote. One of the two groups receiving DSS also received a single dose of psilocybin between the recent and remote test time-points. At both time-points mice were given the same three tests in their paired, unpaired, and bridged contexts, with the addition of the open field task at the remote time-point. The findings of the experiment are that mice given DSS and one dose of psilocybin show a decrease in inflammatory cytokines in key brain regions related to anxiety, as well as decreased anxiety behaviours. This study suggests that psilocybin is able to remediate negative effects of chronic inflammation on both the brain and behaviour.

Ulcerative Colitis Induction

Similar to Chapter 2, figures 3.2 and 3.3 show that mice given DSS and psilocybin showed evidence of ulcerative colitis through decreased weight, increased LCN2 in faeces, shorter colons and heavier spleens than control mice. Interesting to note, however, is that although the mice receiving both DSS and a single dose of psilocybin had shorter colons and heavier spleens, there is less difference between the control and DSS+Psi group as there is between the control and DSS-only group. This suggests that even in the short 6-day span since the injection, the psilocybin may have decreased the inflammatory and immune response typical of the disease.

Fear to Context Results

At the remote time-point there is a significant increase in entropy in the DSS+Psi group compared to the DSS-only group, with the DSS+Psi group showing entropy values similar to the control group. This suggests that the mice given psilocybin had less predictable movement than the DSS mice, showing a decrease of inhibition in the paired context. While these trends are not consistent in the distance travelled or the chamber preference task, there are interesting results in the OFT.

Open Field Task Results

The open field task was run after the conditioned tests at the remote time-point. All groups showed a preference for the periphery compared to the centre of the open field, and there is no significant difference in the cumulative distance travelled. The DSS+Psi group entered the centre in significantly less time than the DSS group, and spent significantly more time in the centre of

the open field than the DSS group. This suggests that the DSS+Psi group had less general anxiety at the remote time-point than the DSS group, with the decreased time to enter the centre suggesting a decrease in general inhibition. Together, the decreased entropy in the paired context and the increased time in the centre of the OFT suggest that both conditioned and unconditioned anxiety were alleviated by a single dose of psilocybin.

Tissue Analysis Results

After the mice were euthanized and the brains were snap-frozen, the ACC and the NAc of 6 randomly chosen DSS mice, 6 randomly chosen control mice, and 4 randomly chosen DSS+Psi mice were removed in order to isolate the RNA for further down-stream analysis. The RNA was analysed via a RT-qPCR in order to find the relative quantity of several genes involved in the inflammatory response, namely Nrf2, mt-CO1, Gpx, and Il-1b. The relative quantities of Nrf2, mt-Co1, and Gpx did not differ between the control and DSS and DSS+Psi groups in either the ACC or the NAc, but the relative quantities did differ between brain regions in mt-Co1 and Gpx. In the NAc there is a higher relative quantity of Il-1b RNA in the DSS group than in the control or DSS+Psi group. Considering Il-1b is a cytokine released during an inflammatory response, this result suggests that the single dose of psilocybin is enough to decrease the inflammatory response evident in the NAc of the DSS-only mice. Further evidence of this is found in the unsupervised principal component analysis, in which all four genes in the NAc were analysed together. In figure 3.7 the control group is clustered at the bottom with the DSS+Psi group, while the DSS group is clustered at the top. This shows that the greatest amount of variance is not found between the two DSS groups and control, but rather between the DSS-only group and the control and DSS+Psi

groups. This further suggests that the dose of psilocybin had a significant effect on the inflammatory response specifically within the NAc.

Examination of the Hypothesis

Hypothesis 3 predicted that a single dose of psilocybin would decrease the inflammation caused by chronic colitis, and that this decrease in inflammation would decrease the anxiety-like behaviours exhibited in the DSS-only mice. The data were able to reject the null hypothesis, as there is a significant decrease in the expression of inflammatory cytokine in the NAc and a decrease in several anxiety behaviour measures of DSS+Psi mice compared to DSS-only mice.

Conclusion

The findings from this study show that a single dose of psilocybin is able to significantly decrease both brain inflammation and anxiety-like behaviours within a couple days of administration. Not only that, but it shows signs of being able to promote healthy anatomical changes as well. The spleens and colons showed less difference to the control mice than the DSS-only group. Mice given psilocybin had increased entropy in their paired context at the remote time-point, as well as more time in the centre of the open field, and less time to enter the centre of the open field for the first time. These findings add to this field of research by providing evidence of many positive effects of psilocybin, without any recognized negative impacts on the health or function of the mouse. With regards to a disease such as ulcerative colitis, with no known cure, it is remarkable to consider the potential for a simple remediatory molecule such as psilocybin.

CHAPTER 4:

GENERAL DISCUSSION

The goal of this thesis was to conduct experiments to further the understanding of the physiological cause of anxiety. The goal was to test the gut-brain-axis theory by looking at the effects of induced gut inflammation on anxiety-like behaviours and to study inflammatory markers in specific brain regions. Our theory also proposed that if gut inflammation is able to promote brain inflammation, a molecule able to remediate inflammation should also reduce anxiety behaviours. Psilocybin was thus studied as a remediatory option for the inflammatory cycle. Experiments reported in Chapter 2 were designed to investigate hypothesis I and II, the effects of DSS colitis on both the potential for gut inflammation to drive brain inflammation and the potential for this inflammation to induce anxiety behaviours. Experiments reported in Chapter 3 were completed to investigate hypothesis III, the ability of psilocybin to remediate the negative impact of colitis on the promotion of anxiety behaviours.

Hypothesis and summary of Results

Hypothesis I states that mice with chronic gut inflammation would exhibit higher levels of anxiety in both the conditioned and unconditioned behavioural tasks and improperly differentiate fearful and non-fearful stimuli. Based on previous research (Matisz et al, 2022), changes in anxiety behaviour at the remote disease testing time-point compared to the recent disease testing time-point was anticipated. Additionally, hypothesis II states that mice given chronic gut inflammation would have an increase in inflammatory markers, such as Il-1b, in specific brain regions related to anxiety. It additionally states that mRNA of proteins involved in the inflammatory pathway would

be expressed differently in mice given chronic inflammation compared to those without inflammation. Most of these predictions were supported by the results. Mice given chronic colitis were able to differentiate between fearful and non-fearful contexts at both the recent and remote testing time-points. However, mice given chronic ulcerative colitis showed increased anxiety-like behaviours specifically at the remote time-point. Mice given chronic colitis also showed different expression of inflammatory markers, and an increase specifically in the protein Il-1b in the NAc compared to control mice.

Hypothesis III stated that a single 6 mg/kg dose of psilocybin administered between the recent and remote testing time-points would remediate the negative effects of chronic inflammation. This would be evidenced by decreased anxiety-like behaviour and an adjusted inflammatory protein mRNA profile. These predictions were supported by results. Mice given chronic DSS as well as a single dose of psilocybin took less time to enter the centre of the open field task and spent more time in the centre compared to mice receiving only DSS. Decreased anxiety-like behaviour is recognized in one measure of the fear to context task, but not the preference task. These mice also expressed significantly less Il-1b in the NAc than DSS-only mice.

Conditioned anxiety task results

The goal of the experiment in Chapter 2 had two parts. Previous experiments resulted in mice receiving chronic DSS being unable to differentiate between their paired and unpaired contexts at the remote time-point. This result occurred after showing the ability to differentiate between their paired and unpaired contexts at the recent time-point (Matisz et al., 2022). Specifically, the mice spent equal amounts of time freezing in both the paired and unpaired contexts at the remote time-point. These results suggest that there is remodelling of the brain taking

place between the recent and remote testing time points. Thus, one aspect of this thesis is to replicate these results to better understand the mechanisms of this remodelling. In accord with speculations made by Matisz et al. (2022), we hypothesised that the gut is driving dysfunction in specific brain regions which in turn cause anxiety. However, it has also been found that many people experience exacerbated gut dysfunction as a result of an anxious experience (Popa & Dumitrascu, 2015). Together, these results suggest that inflammatory dysfunction can originate in either the gut, or the brain.

The purpose of the experiment in Chapter 3 is to study the potential remediatory effects of psychedelics on the generalised anxiety demonstrated in Matisz et al. 's (2022) paper, as well as to remediate the induced brain inflammation. This study was completed alongside the experiments in Chapter 2. In the Chapter 3 experiment, 12 mice were given both three doses of DSS and a single dose of psilocybin between testing time-points.

The previous experiment completed by Matisz et al. (2022) was thus replicated while also analysing specific brain regions most likely to be involved in the processes of gut inflammation leading to the generalised anxiety at the remote time-point. Prior to conducting the experiments represented in this thesis, two prior replication attempts were completed. Both these experiments were not successful. The results of the first were skewed presumably due to an over-administrated of the scents in each of the contexts, as well as distracting noises during the training period of the task. The second experiment was conducted using different natural sounds in each of the contexts instead of different scents, the shape and wall pattern remained consistent. Unfortunately, the effects were still unreplicated. None of the mice were able to properly learn the task.

Finally, the results present in this thesis were completed using different scents than the Matisz et al. (2022) study, but the same context shape and wall pattern. The chronic ulcerative

colitis was also repeated, with both the DSS and DSS+Psi groups showing evidence of weight loss, increased LCN2, reduced colon length, and increased spleen size. Unfortunately, while the DSS and DSS+Psi groups froze more in their paired contexts than in their unpaired context, the control group did not show any difference in freeze time between the two contexts. Thus, the previous study was not replicated in regard to the generalised anxiety exhibited by the DSS mice at the remote time-point, and the control group did not appear to properly learn the task. This may have had to do with the smaller group size, as the control group had only 9 mice while the DSS group had 12 mice. Further considerations are made below.

Considering the control group did not show a preference for the unpaired context, the freeze-times in both of the contexts were combined in order to better compare overall anxiety behaviour between the groups. As this did not result in any significant differences between the groups, it is assumed that the increased anxiety behaviours in the paired context are due to a more robust association between the negative stimuli and context rather than a more active sympathetic response.

This is supported by further analysis of the fear to context tests specifically at the remote time point. First, it is found that the entropy of the DSS group is lower at the remote time point in the paired context than both the control and DSS+Psi groups. This demonstrates that the control and DSS+Psi groups were more likely to move around in their paired context during the test than the DSS mice were. Second, the amount of distance travelled also differed in the paired context. The DSS mice travel significantly less in their paired context at the remote time point than the control mice. Third, the control mice increase the amount of time they spend in their paired context during the preference task at the remote time point, while the DSS mice do not increase their time in the paired context at the remote time point. Altogether the increased anxiety behaviours

specifically in their paired context suggest that the learned fear of the paired context is more salient for the DSS mice than it is for the control mice. There is also evidence that the DSS+Psi mice retain less of this association than the DSS-only mice.

Considering these results, it is interesting to consider why the freeze time did not also demonstrate this outcome. One reason could be due to the afor-suggested group size, but perhaps a more apparent reason is the difficulty of accurately calculating freeze time. While precise measures were taken to ensure the accuracy of these calculations, it is still difficult for either human scorers or computerised scoring programs to have 100% accuracy when distinguishing between a comfortable, resting mouse and an anxious, freezing mouse. Another aspect of the study worth mentioning is that all the mice involved in this experiment were run as one cohort. While this was chosen both for timeliness and to inhibit cohort and age differences, the difference in the time of day in which the mice were trained may have influenced the freeze-time results. In the future I would advise running half of each group first, and then ordering the second half of each group and running them at the same age as the previous group. It was also found that people use eucalyptus scent as a natural mouse-repellent. While this was unknown prior to the experiment, it may explain the dramatically different freeze time between mice paired in the triangle context and mice paired in the square context. The triangle context was the scent of eucalyptus, while the square was banana scented. In fact, I found that even control mice shocked in the square context on occasion spent more time freezing in the triangle context.

Unconditioned anxiety task results

It was hypothesised that both conditioned and unconditioned anxiety would be greater in mice given DSS compared to controls and decrease in mice given both DSS and Psi. While these

results are partially apparent in the conditioned task at the remote time-point, they are readily apparent in the unconditioned task. The DSS mice spend significantly less time in the centre of the open field than both the control mice and the DSS+Psi mice. Not only that, but the DSS+Psi mice are also more willing to enter the centre than the DSS mice, showing both decreased anxiety-like behaviours and inhibition.

A further support of this analysis is the negative correlation between LCN2 in the faeces and the number of seconds DSS mice spend in the centre of the open field task. To enter the centre of the open field is to risk being vulnerable and visible to predators while increasing the potential for finding food. These results suggest that the level of inflammation in the colon is directly related to the mouse's willingness to risk threat for a potential gain. This demonstrates "sickness behaviour," as the mice with increased inflammation are at a higher risk of predation and are thus less likely to increase their risk by entering the centre of the open field. This agrees with the results of the conditioned task as well, as the mice given DSS-only were less likely to move around and more likely to remain in one position in their paired context. When given the choice, the DSS mice were also less likely to enter their paired context. All of these demonstrate increased vulnerability, or sickness behaviour, each of which are not demonstrated by either the control mice, nor the mice given both chronic DSS and psilocybin.

RNA isolation results

The second aspect of this study was to understand how the brain might be affected by chronic inflammation, and how that affect may be driving the anxiety behaviours witnessed. Initially it was planned to remove and study the hippocampus (HPC), NAc, and ACC. Once removed, the RNA would be isolated and analysed for the relative quantity of several genes

involved in the inflammatory pathway, including but not limited to, Nrf2, Gpx, Il-1b, and mt-Co1. Unfortunately, during the isolation period most of the RNA from the HPC was lost, leading to protocol adjustments prior to the isolation of the NAc and ACC RNA. This resulted in the inability to include the HPC in further downstream gene analysis.

It was also intended that the DNA be isolated from these brain areas in order to calculate the copy number of the mitochondria. This would have provided interesting information about how chronic inflammation may impact the number of mitochondria present in the brain regions and would have enabled correlation with anxiety measures. However, the brain regions were too small to isolate enough DNA for detection levels: by removing and isolating an entire brain section during the necropsy, there may be enough for detection.

Isolation of the RNA from both the NAc and the ACC showed that the relative quantity of Nrf2 and mt-Co1 are significantly different between the ACC and NAc in all three groups, and Gpx is different in the DSS and control groups. While I expected to see differences in the quantities of RNA between the DSS and control groups, this is only found with Il-1b. There is a significantly higher relative quantity of Il-1b RNA in the NAc of DSS mice than either control or DSS mice. Il-1b is an inflammatory cytokine which the body increases during an inflammatory response. Thus, the difference found within the DSS mice compared to the control and DSS+Psi mice provides evidence of induced gut inflammation causing inflammation in the brain. The decrease in Il-1b in the DSS+Psi group shows that a single dose of psilocybin is able to reduce the inflammatory response in the brain and revert it back to control levels. Considering this along with the necropsy results, the spleens, and colons of the DSS+Psi mice also seem to be on a trajectory of returning to control measurements.

Interesting to note is the fact that although there is a significant decrease in the Il-1b found in the brains of DSS+Psi mice compared to DSS mice, the same is not found in the LCN2 levels in their faeces. What this suggests is that while psilocybin reduces inflammation, it is either specifically targeting the inflammatory response in the brain, or the response in the colon is slower to respond. Either explanation makes sense regarding the decreased anxiety behaviour demonstrated by the DSS+Psi group, while still maintaining shorter colons and heavier spleens.

Further evidence of the difference in expression of these genes is found in the unsupervised principal component analysis. In this analysis the groups are separated according to the greatest level of variance. When plotting all four genes together there is a clear separation of the DSS group from the control and DSS+Psi groups in the NAc. This shows that although there are not significant differences on the individual gene level, these genes are cumulatively expressed differently in the NAc of DSS mice than in either control or DSS+Psi mice. Again, this represents the ability of ulcerative colitis to cause an increase in inflammatory processes in the brain and the addition of a single dose of psilocybin to inhibit these inflammatory processes.

These behavioural results support the brain-gut-theory of anxiety, which states that inflammation in the gut increases inflammation in the brain which promotes anxiety behaviours. Our theory that a molecule able to reduce inflammation will also reduce anxiety behaviours is supported by behavioural and physiological results.

Limitations

Throughout the experiments within this thesis there were challenges and limitations, some of which have been forementioned. First and most poignant throughout the study, due to initial miscalculations in group size, the control group is nine subjects instead of twelve. This could be

easily remediated in future studies and would likely add to the efficacy of the study. Not only that, but there were not enough mice for a positive control group receiving psilocybin without the addition of DSS. The addition of a positive control would have enabled a more accurate understanding of the effects of psilocybin. Another limitation was found in the variation in DSS potency between bottles. Due to the large number of mice receiving DSS, we had to use several bottles of the compound, unfortunately it was not predictable how severely sick the mice would get from each bottle, with some bottles leading to far greater weight loss than others. For this reason we decided to change the percentage of DSS in the water to 2.75% each time a new bottle was used, as the more recently purchased DSS seemed to have greater potency.

As mentioned, the inability to reproduce Matisz et al (2022) fear to contex task results may be due to a plethora of aforementioned possibilities. However, another interesting phenomenon is that in this study the mice seemed to prefer the square context compared to the triangle context. Although their preferred context is paired with shock, several mice, specifically in the control group, still froze more in their unpaired context than in their paired. However, it is important to note that this only happened if the paired context was the square context. As in, if the mouse was shocked in the square, it had a tendency to freeze more in the triangle. This is unexpected, as mice are generally more comfortable in dark spaces, and therefore should be more comfortable in the triangle with its striped walls and tighter corners. This confusion led to the consideration that perhaps some of the time scored as “freeze time” is more accurately the mice comfortably “nesting” in the corner. Although this is hard to confirm or deny, it is a possible observation which could make sense both of the confusing freeze-time results, and the fact that these results are not supported by the other anxiety test outcomes.

Again, as mentioned, the lack of training in RNA isolation led to the loss of the HPC RNA, and thus decreased knowledge of how that region may be impacted by a model of ulcerative colitis. Along with this, in order to maintain accuracy, the size of each region was too small to enable accurate copy-number analysis. This completely limited our ability to understand how the mitochondria may be affected by chronic inflammation, and subsequently how mitochondria alterations may have influenced the anxiety behaviours.

This study was also notably only performed on male mice. This was because most of this type of experiment previously reported were performed on male mice. Previous research on this version of contextual fear conditioning had been conducted on male mice, and thus there is no data on how female mice may respond to this type of fear conditioning along with chronic DSS. Adding a group of female mice to the study would have necessitated one or more pilot studies. Although fascinating, this would have increased the time and effort beyond that available for a single thesis.

Future Direction

The results of this thesis show that chronic gut inflammation increases inflammation in the brain (specifically the NAc) and increases anxiety behaviours in mice specifically at a time-period post peak-disease. It is still unknown what kind of alterations are occurring in the brain that may be causing these anxiety behaviours, as well as which other regions may be involved, and whether mitochondria are involved in the dysfunction. Future research involving more brain regions as well as a female cohort would greatly improve our understanding of the gut-brain-axis.

To replicate this experiment, it may be beneficial to change the wall pattern of the square, so the darker chamber also has larger corners and thus equalise the potential “benefits” in the two contexts. It may also be beneficial to remove areas such as the HPC immediately post-necropsy

and thus accurately secure the largest possible amount of each section of interest. This may increase the amount of DNA extractable and allow for further understanding of how the mitochondria may be playing a role in the processes observed in this study. Alternatively, an Ouroboros O2k-FluoRespirometer is able to monitor both oxygen consumption and reactive oxygen species production of live tissue, thus able to provide a plethora of information on mitochondria function of various different tissues. More insight into the effect of DSS treatment, as well as subsequent behavioural alterations may be found if the gut microbiota composition were tracked throughout the study.

It would also be interesting to give psilocybin after three doses of DSS and wait an extra week prior to necropsy. This would enable tests on LCN2 levels and organ tissue length and weight at a time-point further from administration and perhaps show significant changes in these measures. To study the gut microbiota of control and DSS mice at different time-points of the study would also benefit our understanding of how the composition of microbiota may be influencing behaviour.

CONCLUSION

This thesis examined the effects of a chronic treatment of DSS to mimic ulcerative colitis on both conditioned and unconditioned anxiety behaviour as well as the ability of psilocybin to remediate negative inflammatory effects. Several novel contributions were made throughout these experiments. It is observed that chronic DSS increases inflammation in both the colon and the NAc. It is also observed that mice given a chronic treatment of DSS as well as a single dose of psilocybin after peak disease showed a significant decrease in inflammation in the nucleus accumbens, as well as decreased anxiety behaviour. These findings are exciting as they improve

our understanding of both the long-term effects of chronic inflammation, as well as the prompt and remediatory effects of psilocybin on both brain inflammation and anxiety behaviour. Recognizing these key aspects of anxiety take us one step closer to understanding how best to manage this prevalent mood disorder, and how to ensure Leanne does not fail her finals.

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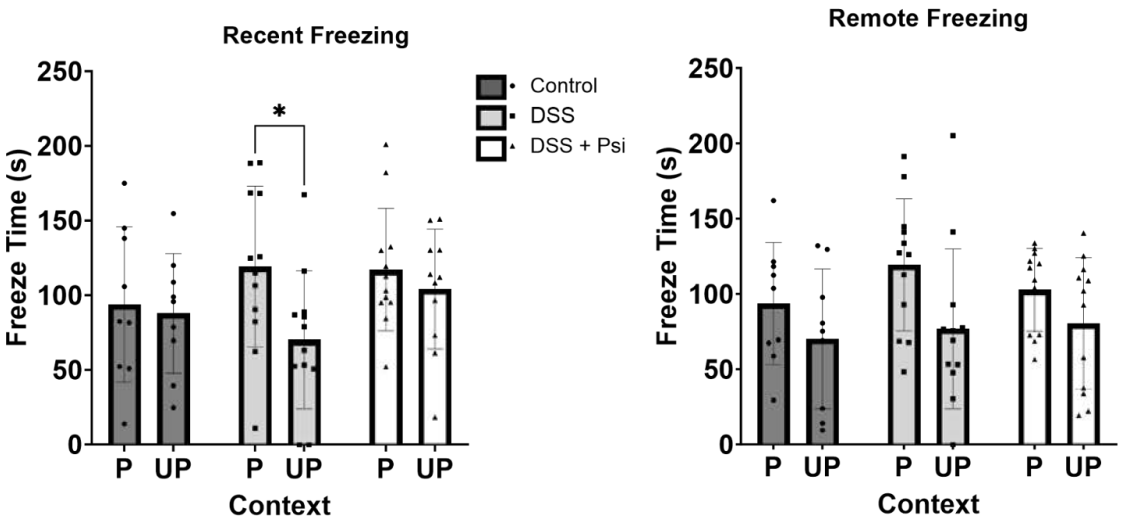
Supplementary Information:



Time Spent Freezing During Recent and Remote Testing. Time freezing in Paired (P) and Unpaired (UP) contexts. Multiple comparison two-way ANOVA with Sidak post-test. *$p < 0.05$. Data are mean ± SD, control n = 9; DSS n = 12; DSS + Psi n = 12.

This figure shows that at the recent timepoint, only the DSS group accurately discriminated between the paired context and the unpaired context. At the remote timepoint, none of the groups were able to discriminate. Prior to fear conditioning the mice preferred the square context. Although the mice with this preference were subsequently shocked in the square context (it became their paired context), it appears as though the original preference was far more robust than anticipated. Some reflections on why this may have occurred are discussed in the limitations section above.