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Novel Models for Understanding Traumatic Stress

Thesis presented by

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism and intellectual property.

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Abstract

Undergoing trauma, be it physical, psychological, or observed, can induce pathological alterations leading to disorders such as Post-Traumatic Stress Disorder (PTSD). PTSD is highlighted by negative cognitive alterations, behavioural changes, and interruptions in arousal and sociability. PTSD is comorbid with disorders such as anxiety and depression, gastrointestinal disturbances, pain, and the gut microbiome is hypothesized to play a role in this trauma-related disorder. Direct experience of traumatic events is the most common method of generating traumatic-stress related pathologies, however indirect exposure through witnessing another endure a traumatic event can also lead to PTSD-like symptoms. This method of traumatic transference is called secondary traumatic stress (STS). While PTSD and STS are clinically relevant, and ever-growing in importance due to the recent COVID-19 pandemic, there is still a lot to be learned about their molecular underpinning, mechanisms, and biomarkers. In order to appropriately investigate these neurobiological features of traumatic stress, valid and effective animal models are absolutely essential. Utilizing the most appropriate animal models for the representation of neuropathologies is essential for extracting critical information in the process of developing novel therapeutic options.

In chapter 2, we aim to develop the knowledge of secondary traumatic stress, we investigated whether a novel observational model, combining visceral pain, a common comorbidity of traumatic stress related disorders, and observed stress could result in a suitable phenotype. Utilizing colo-rectal distension (CRD) to induce visceral pain behaviours, we had rodents observe another rodent undergoing this procedure. These observer rodents then underwent the CRD themselves 24 hours later in order to assess whether they had visceral hypersensitivity. Indeed, it was seen that observer animals had hyperalgesia measured in visceral pain threshold and total behaviours, an impacted

HPA axis, and altered neuronal activation in key brain regions. Our results suggest that this novel model was effective in producing secondary traumatic stress-like phenotypes, and would be well suited for further research into the social transference of pain and developing therapeutic options for traumatic-stress induced disorders and visceral pain comorbidity.

In chapter 3, we look at Single prolonged stress (SPS), which is a well-validated and commonly used model however there are ethical concerns that limit its widespread use. The classical SPS model involves a 2-hour restraint, immediately followed by a 20-minute forced swim, a 15-minute rest and culminates with diethyl ether exposure until loss of consciousness. Recent focus on ethical standards and interests in refining animal models has led to concerns in the usage of diethyl ether, leading us to investigate whether the model would still be effective using isoflurane as a replacement for diethyl ether. Our findings suggest that this model is effective in recapitulating a key PTSD phenotype in the contextual fear conditioning paradigm. Impaired fear learning has been repeatedly found to be a key component of PTSD phenomenology, and our model induced significantly impaired fear learning in stress rats. Further to this, we found that SPS with isoflurane caused significant reduction in learned helplessness in rodents, paired with time specific changes in corticosterone concentration. Anxiety-like behaviours also appear to be implicated by this model, with Isoflurane exposure leading to reduced anxiety-like behaviour, suggesting its potential as an adequate PTSD model.

The encouraging results from these two models of traumatic stress provide a significant interest in further studies using them. With the future intention of developing novel and effective therapeutics for undermedicated sufferers of these disorders, the hope is that these models can help provide valuable insights into the mechanisms of action behind the pathologies, illuminating potential therapeutic avenues.

Chapter 1:

General Introduction

1. What is Trauma

Throughout all of history, humans and our ancestors have been facing existential challenges, threats of injury and death, enduring accidents and disaster, and watching untimely and aversive events occur to loved ones. In other words, humans have been undergoing traumatic events for as long as we can be considered human. From philosophers to poets, Homer to Dickens, recordings of the significant effect of traumatic events on an individual's cognitive ability, emotions, and behaviours have been ever present (Friedman et al., 2011). Since then, a variety of explanations and terms have been used to describe the ensuing maladaptive behaviours, from shell-shock to nostalgia to soldier's heart (Friedman et al., 2011), however, none of these were all-inclusive for the detrimental effects being felt by those suffering. In 1917, Sigmund Freud defined trauma as "an experience which within a short period of time presents the mind with an increase of stimulus too powerful to be dealt with or worked off in the normal way" (Freud, 1963). While, in the last 105 years, our understanding of trauma has developed, the latter half of that sentence stands true; that severe traumatic events lead to deficits in ability to normally process and deal with the stimulus and future stimuli. Further, traumatic experiences can lead to psychopathologies, and reduce to ability of an individual to cope with later life challenges (Richter-Levin & Sandi, 2021). The subjective experience of each individual is crucial to the level of trauma that is experienced and the potential long-term effects of the trauma (Creamer et al., 2005), making categorical claims as to traumatic experiences quite difficult. This encourages continuous research

into trauma and traumatic stressors, in order to more conclusively define trauma by the changes in biomarkers, behaviour, and social activity.

1.1 Stress and Trauma

Stress and trauma are colloquially intertwined terms; however, it is vital to note the distinction between them. While stress and trauma go hand in hand in many cases, it is important to distinguish between them in the context of animal models for research. To preface it plainly, invoking an old cliché, all traumas are stressors, but not all stressors are traumas. The root of this is the understanding that not all stressors are adverse, and stressful experiences nor traumatic experiences do not induce stress-related pathological issues on a uniform basis. In fact, it is considered that resilience to stress is the rule, while stress-related pathology is the exception (Richter-Levin & Sandi, 2021). Similarly, around 70-80% of individuals experience a traumatic event in their lifetime (Watson, 2019), however only somewhere between 10-20% of individuals who endure these traumatic events will develop PTSD (Bromet et al., 2017; Charlson et al., 2016; Kessler et al., 2005). Essentially, according to (Richter-Levin & Sandi, 2021), stress and trauma can be aligned in a stepwise manner, such that stress is a factor of induction for a traumatic experience. For example, stress is implicated in the fear extinction deficits seen in trauma-related impairment (Maeng & Milad, 2017; Maren & Holmes, 2016). For something to be trauma it has to elicit a more significant response than a 'normal' stressor. Critically, the defining difference is the ability for the animal to cope later in life with further stressful scenarios, which is possible after undergoing a stressful experience (Koolhaas et al., 2011), but will be impacted by a traumatic experience and induce

psychopathologies (Richter-Levin & Sandi, 2021). Acute or transient stressors are not inherently damaging, even having the potential for beneficial effects (Averill et al., 2018; Yuen et al., 2009), whereas exposure to a traumatic stressor can induce extended or chronic stress responses which have significant detrimental outcomes to both brain and behaviour (McEwen, 2017; Yuen et al., 2012).

2. Post-Traumatic Stress Disorder

In 1980, the term post-traumatic stress disorder (PTSD) was coined in the third edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-lll) (Crocq & Crocq, 2000). PTSD is a highly prevalent mental health disorder across the globe. While the worldwide prevalence of diagnosed PTSD is between 4-6% (Koenen et al., 2017), which is approximately 300 million individuals, a staggering 70% of individuals will experience at least one traumatic even in their lifetime (Watson, 2019), while 30% of individuals will experience four or more traumatic events (Benjet et al., 2016). These statistics show that while 4% of individuals do go on to develop clinically significant PTSD, there is a gap of approximately 66% of worldwide individuals – over 5 billion people – that experience a traumatic event, and henceforth may suffer from mental health detriments as a result. Potential ailments include increased anxiety, depression, and bipolar disorder which are all commonly comorbid with PTSD (Baldwin, 2013). Further to this, there can be a poor recognition of trauma and PTSD in patients, as many symptoms and presentations of trauma can overlap with other severe mental illness diagnoses, i.e., schizophrenia and major depressive disorder (Mauritz et al., 2013). With this being said, the significance of trauma and PTSD cannot be understated. These conditions involve increased risk and

early onset of conditions such as cardiovascular disease and diabetes (Shalev et al., 2017), as well as serious disability and premature death (Watson, 2019). These risks can be exponentially compounded when the trauma experience occurs in childhood and adolescence, leading to significant detrimental effects on cognitive and emotional development, impaired educational outcomes, and worsened lifelong medical prospects (Magruder et al., 2017).

The current pharmacological treatments for PTSD are anti-depressants, specifically the SSRIs (selective serotonin reuptake inhibitors) fluoxetine and paroxetine. These are slow-acting drugs, and offer mixed therapeutic benefit to PTSD patients, with only 20-30% of patients achieving full remission with these options (Berger et al., 2009; Krystal et al., 2017). This may be due in part to a lack of comprehensive information regarding the molecular underpinnings of PTSD specifically. The current treatments are re-purposed from other neuropsychiatric disorders – namely depression, as opposed to specifically targeting and ameliorating PTSD pathophysiology. Due to this, there is no significant pharmacological relief for the PTSD sufferer. The current COVID-19 pandemic accelerates the need for these tailored therapeutics, as the lockdowns and psychosocial impact are directly leading to increased prevalence of PTSD and many other mental disorders (Dubey et al., 2020). Behavioural therapies are becoming a more common tool for addressing PTSD and other trauma related disorders. The most common options for PTSD include Prolonged Exposure, Cognitive Processing Therapy, and Cognitive Behavioural Therapy (Watkins et al., 2018). In fact, it was found that behavioural therapy treatment was more effective than sertraline, the common pharmacological PTSD

treatment (Storm & Christensen, 2021). However, these behaviour therapies are often not comprehensive in having patients entirely recover from PTSD, with non-response to CBT potentially being as high as 50% (Kar, 2011).

Henceforth, investigating robust animal models is absolutely essential to developing an inclusive understanding of the specific neurobiology and molecular mechanisms of PTSD, with the long-term goal to develop more effective and tailored therapeutic treatment options.

2.1 HPA Axis and PTSD

The HPA axis is the key component in coordinating the response to stress and fear in animals (Felmingham et al., 2014; Stark et al., 2015). Under normal circumstances, the activation of the HPA axis stimulates neurons in the paraventricular nucleus (PVN) of the hypothalamus to secrete corticotropin-releasing hormone (CRH), which then prompt the pituitary gland to produce and secrete of adrenocorticotropic hormone (ACTH). As a result, ACTH induces the secretion of mineralocorticoids and glucocorticoids (specifically, corticosterone in rodents and cortisol in humans) from the adrenal cortex into the bloodstream. Cortisol binds to glucocorticoid receptors (GRs) in the PVN, pituitary gland, and hippocampus, which in turn stimulates an effective negative feedback mechanism that is responding to the high levels of cortisol in the blood which consequently inhibits further release of ACTH and CRF (Cruz-Pereira et al., 2020). In normal circumstances, an effective negative feedback loop is highlighted by fluctuating ACTH and CRH production levels around the circadian rhythm and inhibition by elevated

levels of cortisol in the bloodstream (Guilliams & Edwards, 2010). The continuation of HPA axis dysregulation as a result of repeated stress can lead to hypercortisolism, hypocortisolism, and circadian rhythm dysfunctions (Guilliams & Edwards, 2010). Hypercortisolism can induce volumetric changes in the hippocampus often seen in patients suffering from depression (Cruz-Pereira et al., 2020).

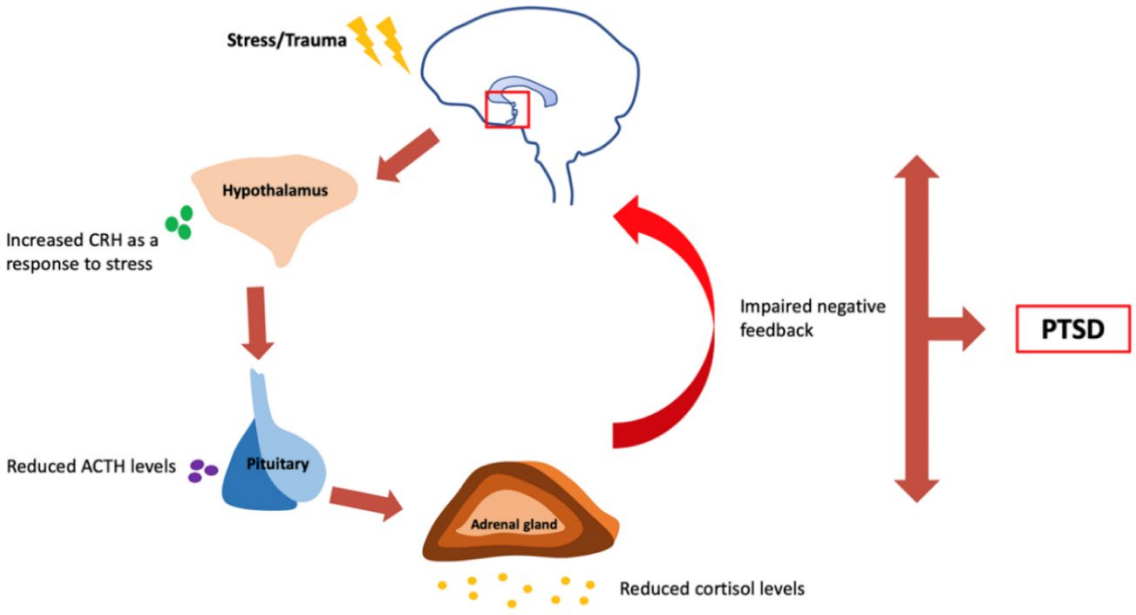

Figure 1: HPA Axis in PTSD. *The HPA axis in PTSD is significantly dysregulated, with reduced levels of cortisol indicating hypoactivation of this stress-response system. While typically, stress induces a hypercortisolism, or hyperactivation of the HPA axis, the opposite is most commonly seen in PTSD. Image (Nisar et al., 2020)*

PTSD, on the other hand, can induce hypocortisolism (Klaassens et al., 2012; Richter-Levin et al., 2019; Yehuda, 2002), involving high sensitivity of the glucocorticoid receptor, negative feedback control inhibition in the form of a blunted response to corticotropin releasing hormone (CRH) leading to reduced levels of adrenocorticotropic hormone (ACTH) from the pituitary gland, and furthermore low level of cortisol/corticosterone

released from the adrenal glands and circulating in the bloodstream (Yehuda, 2002). A forthcoming hormonal imbalance links with negative alterations in cognition and mood, alterations in arousal and activity, and intrusive/avoidance symptoms (D'Elia et al., 2021).

2.2 Central Nervous System and Trauma

Trauma can have a significant effect on all facets of the nervous system, with the brain of course being a focal point. Traumatic events create a situation where the brain is simply hyper-stimulated and overwhelms the ability and capacity of the brain to properly process information (Solomon & Heide, 2005). Specifically, the episodic memory of trauma events is processed in the right limbic system – including the hippocampus, amygdala, and hypothalamus (Rajmohan & Mohandas, 2007) – and this can generate vivid imagery of the traumatic event, leading to terror in the form of body sensations and thoughts and furthermore cause emotional looping among other recurring psychiatric issues due to a lack of effective processing (Solomon & Heide, 2005). In a trauma re-exposure study, hyperactivity in the right limbic system and visual field, couples with hypoactivity of the anterior cingulate cortex – which regulates the limbic system – suggest significant neural changes due to trauma (Rauch et al., 1996). Further to this, hippocampal volumes have been shown to be reduced in human studies of PTSD (Thomason & Marusak, 2017). These hippocampal changes are seen to be bilateral, and in fact there appears to be a correlation between the severity of PTSD and the relative size reduction (Pitman et al., 2012). Further to this, volume deficiency has been noted in the pre-frontal cortex (PFC), most notably in the vmPFC (ventro-medial PFC) (Kasai et al.,

2008) and ACC (Karl & Werner, 2010; Kitayama et al., 2006). Additionally, functionality of the vmPFC has been seen to be relatively decreased in PTSD (Shin et al., 1999).

In animal models, key brain regions seen to be centrally involved in PTSD are the prefrontal cortex, amygdala, hippocampus, and hypothalamus (Bremner, 2006; Fenster et al., 2018). Pathways between the PFC, amygdala, and hippocampus have seen to be implicated in PTSD (Ganon-Elazar & Akirav, 2013; Lisieski et al., 2018), including specifically the prelimbic (PL) and infralimbic (IL) cortices of the PFC (Knox et al., 2016). These regions are also involved in fear and anxiety (Tovote et al., 2015), a common PTSD comorbidity. Circuits from the IL of the PFC to the basolateral amygdala (BLA) are implicated in fear learning as well (Asede et al., 2015). The trauma induced disruptions of these regions crucially contribute to changes in fear expression and response (PL) and reduced fear extinction (IL) (Knox et al., 2016). Further to this, regions such as the insula and striatum have been seen to be involved with PTSD (Felmingham et al., 2014; Stark et al., 2015). The paraventricular nucleus of the hypothalamus (PVN) is at the crux of the stress response, and it has been seen that chronic stress causes significant changes to both its form and function, with it being responsible for both typical behaviour and pathological features in the HPA axis' stress response (Herman & Tasker, 2016). The anterior cingulate cortex (ACC) is known to be involved in empathy, particularly found to be hyperactivated when experiencing pain but also while observing pain (Marsh, 2018). Further to this, both the PFC and ACC are implicated not only in physical pain but also in psychological stress (Meerwijk et al., 2013) as well as visceral pain (Meerveld & Johnson, 2018; Moloney et al., 2016). Clinical studies have shown that similar pain

pathways in the ACC activate when not only experiencing pain, but observing others experiencing a painful stimulus (Hutchison et al., 1999).

2.3 Animal Models of PTSD

Animal models of PTSD tend to produce the phenotypes of PTSD that correspond with the diagnostic criteria of clinical PTSD. The DSM-V (Diagnostic and Statistical Manual of Mental Disorders – 5th Edition) lists 8 criteria [A: Stressor; B: Intrusion Symptoms; C: Avoidance; D: Negative Alterations in cognitions or mood; E: Alterations in arousal and reactivity; F: Duration; G: Functional Significance; H: Exclusion] for the identification and diagnosis of PTSD (*Diagnostic and statistical manual of mental disorders : DSM-5*, 2013). There are many different models used to recapitulate to these specific phenotypes, including electric shock, predator exposure, immobilization/restraint stress, predator-related cues, and single prolonged stress (Cohen et al., 2004; Verbitsky et al., 2020; Yamamoto et al., 2009). The electric foot shock paradigm can be effective for gauging the fear-related outputs of PTSD but clearly differentiating between traumatic and non-traumatic shocks remain a drawback (Aliczki & Haller, 2014). Immobilization stressors can have significant stress-related phenotypes, but lack the efficacy of producing comprehensive PTSD phenotypes (Buynitsky & Mostofsky, 2009). Predator exposure and underwater trauma recapitulate significant anxiety-related responses that can be seen in PTSD (Cohen et al., 2004). Predator stressors include exposure to stimuli such as cats, cat odour, or fox odour and has been validated due to its ability to provoke significant fear and instigate potential long-lasting physiological and/or behavioural deficits (Adamec et al., 2008; Blanchard et al., 1998; Cohen et al., 2000; Diamond et al., 2006).

Adversity in the form of ethical quandaries, full recapitulation of PTSD phenotypes, level of trauma, and co-morbidities makes it hard to research PTSD, but refining and growing knowledge on the existing models will allow for more focused research moving forward (Richter-Levin et al., 2019).

2.3.1 Single Prolonged Stress

Single prolonged stress (SPS) is a well-validated and commonly used animal model that reflects the physiological, molecular, and behavioural alterations that occur in PTSD (Lisieski et al., 2018). In order to be considered a truly viable model of PTSD, the model must reproduce the phenomenology – the impacted behaviours and mechanisms behind these changes – and PTSD therapeutic interventions must also show success in this model; all of which SPS successfully does (Yamamoto et al., 2009). Initially developed by Libzerzon and colleagues (Liberzon et al., 1997; Liberzon et al., 1999), this model involves a 2 hour restraint, 20 minute forced swim, a 15-minute break, and traditionally diethyl ether exposure until loss of consciousness. While the diethyl ether is the most commonly used anaesthetic for the third part of this stress paradigm, other methodological considerations exist, including isoflurane (Ferland-Beckham et al., 2021). SPS is commonly paired with a contextual fear conditioning paradigm which is a beneficial model for observing fear memory relative to PTSD, and interestingly it is only after 7 days that the phenotypes of PTSD manifest (Souza et al., 2017). While this model was initially validated using rats and rats are the most frequently used rodent for this specific model, there has been evidence to support the efficacy of the model in mice as well (Perrine et al., 2016).

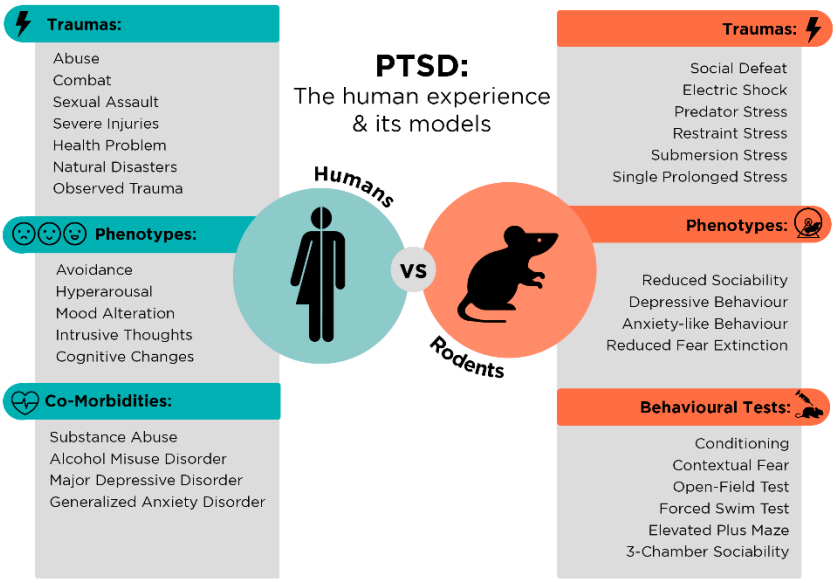

Figure 2: Animal Models of PTSD *– How pre-clinical research aims to recapitulate the human experience of PTSD. Animal models are used in order to provide face validity and relevance to human disorders. The animal models intend to reproduce representative phenotypes seen in human disorders; specifically in PTSD, the diagnostic criteria are intended to be seen by performing behavioural tests for fear-related dysregulation, anxiety and depressive-like behaviours, and social inhibitions.*

3. Secondary Traumatic Stress

There are many studies investigating trauma and PTSD in relation to violence, natural disasters, abuse, or physical injury, however there is a lack of comprehensive mechanistic information regarding indirect exposure to traumatic events - namely secondary traumatic stress (STS). Originally, STS was defined by the adverse behaviours an individual experiences as a result of the learning of a traumatic stress that was

endured by another individual. Indeed, originally attributed to a person who had a desire to help the traumatized person (Figley, 1995). It wasn't until 2014 in the DSM-V that secondary traumatic stress became an independently identified disorder (*Diagnostic and statistical manual of mental disorders : DSM-5*, 2013), however it was still a topic of clinical research for long before (Bride et al., 2004) with a great variety of different titles tied to it (Klarić et al., 2013). Within this clinical realm, a great deal of focus has been in the context of compassion fatigue and observed empathy-related stress (Baird & Kracen, 2006; Beck, 2011; Rauvola et al., 2019), specifically in the health care field, advancing from the focus in the 1980s and 1990s on psychotherapists. More recently, pre-clinical studies have begun to dive into the biological background of STS. Pre-clinically, empathetic relationships and transference of pain and fear between rodents has been seen(Keum & Shin, 2016; Sanders et al., 2013) with observed fear and pain inducing exaggerated responses. These studies have shown that STS related symptoms can manifest in many ways, from development of some characteristics of PTSD, to trauma desensitization and empathy decrements, to significant fatigue and burnout. Indeed, the PTSD symptoms of intrusion, hypervigilance, and avoidance are commonly seen in STS (Figley, 1995). An increase in STS being suffered by nurses and other healthcare staff who have been on the front lines of the COVID-19 pandemic continues to drive a broad understanding of STS (Dubey et al., 2020; Orrù et al., 2021).

3.1 Animal Models of Secondary Traumatic Stress

In order to investigate the biomarkers and mechanisms of secondary traumatic stress, animal models of observation or empathy are required. The transference of hyperalgesia or adverse behaviours as a result of secondary experience are the current ways to assess secondary traumatic stress. Observed foot shocks or observational fear learning, is the most frequently employed model (Fendt et al., 2021; Jeon et al., 2010; Liu et al., 2017) as it provides a rodent the experience of seeing another rodent undergoing a significant stressor, which in itself is used as a PTSD model. As a result of this observation, freezing behaviour is induced in the observing animal (Jeon et al., 2010), representing human PTSD symptoms (Török et al., 2019; Verbitsky et al., 2020). Observation of and cohabitation with stressed animals, without observation of the stressor itself, has been found to affect protein expression related to sensitization (Hawkins et al., 2018), suggesting that familiarity and interaction can be enough to induce mechanistic STS-like effects. Emotional contagion for pain between rodents was comprehensively seen by Langford and colleagues using formalin injections for pain (Langford et al., 2006), and further purported with use of venom injections (Li et al., 2014). The use of the formalin injection method has been reproduced and developed further understanding of this empathetic pain transference (F. Mohammadi et al., 2020; Nazeri et al., 2019), with these showing impacts on anxiety-like behaviour and hyperalgesia, as well as implication of the opioid system.

4. Comorbidities

As mentioned above, PTSD is comorbid with many psychiatric disorders, including depression and anxiety (Baldwin, 2013; Koenen et al., 2017), as well as gastrointestinal disturbances (Moloney, O'Mahony, et al., 2015) which in turn produce visceral pain, and other chronic pain issues (Åkerblom et al., 2017; Beck & Clapp, 2011). Indeed, about 91% of PTSD patients have a comorbid condition (Bryant et al., 2010).

4.1 Psychiatric Comorbidities

Psychiatric comorbidities are exceptionally common in PTSD. Strikingly, approximately 48-55% of PTSD patients present with major depression (Elhai et al., 2008), and between 11-31% present with generalized anxiety disorder (Bryant et al., 2010; Grant et al., 2008). PTSD shares a lot of concurrent symptomologies with these disorders, leading to the high rates of comorbidity. With depression, PTSD shares sleep disturbances, withdrawal and isolation, and difficulty concentrating, while sharing panic attacks and avoidance behaviours with anxiety disorders (Brady et al., 2000). HPA axis disruptions characterize both PTSD and depression, and while they similarly induce increased corticotropin-releasing factor, they differ in that PTSD induces an overactive negative feedback loop while depression produces a weaker negative feedback loop (Cruz-Pereira et al., 2020; Yehuda, 2002). Neural changes, highlighted by specific detriments to the hippocampus, also connect these two disorders (Cruz-Pereira et al., 2020; Thomason & Marusak, 2017). The strong overlap between depression and PTSD suggest the rationale for the anti-depressant SSRIs to be used as a treatment option for PTSD, however this partial

symptom relief is what leaves over 60% of PTSD patients in a state short of remission (Krystal et al., 2017). On top of this, individuals with concomitant disorders such as autism spectrum disorder can be more likely to endure a trauma, even more susceptible to PTSD and furthermore a worse clinical outlook (Haruvi-Lamdan et al., 2020). Deciphering the neurobiological markers of PTSD independently will allow for the development of more successful therapeutics for the great majority who are not receiving the relief that they need. Further, investigating how these disorders overlap will also develop an understanding of how to treat those that have long suffered from a complex blend of significant disorders.

4.2 Pain

Pain is fundamental to the human experience. It can protect us from significant injury, warn us of bodily damage, but also cause significant psychological distress. Pain can be classified into three categories; nociceptive pain such as heat or cold, inflammatory pain activated by the immune system, and pathological pain which can be neuropathic due to nervous system damage or dysfunctional – pain despite no discernible damage (Woolf, 2010). Pain can often outlast its usefulness, leading to cases of allodynia – when a typically innocuous stimulus induces a pain sensation – and hyperalgesia, where normal pain stimuli are dramatically intensified (Basbaum et al., 2009). While pain is exceptionally common and indeed the primary complaint seen in medical settings (Wang & Mullally, 2020), it is exceptionally difficult to comprehensively understand and classify due to the level of subjectivity in regard to pain (Giordano et al., 2010). The nature of this subjectivity heightens the necessity for practical and effective animal models of pain.

As animals are unable to self-report pain symptoms in the way humans can, we rely on behavioural analyses in pre-clinical pain research. Continuously developing appropriate animal models will in turn lead to greater face validity in the context of pain research, amplified translatability from pre-clinical to clinical, and more details as to the biomarkers behind pain (Mogil, 2009). As we continue to understand pain, we can unravel its comorbidity with PTSD. It has been long known that pain and PTSD are interwoven, with PTSD inducing fear anxiety and indeed increasing pain level reporting (Asmundson et al., 2002; Barbano et al., 2021) While PTSD rates normally are around 4-6% (Koenen et al., 2017), the rate in individuals with chronic pain is around 10% (Fishbain et al., 2017; Siqveland et al., 2017), and even as high as 32% in youth (Noel et al., 2016).

4.2.1 Visceral Pain

Visceral pain is a type of pain that originates and radiates from the internal organs. It is a core symptom of functional gastrointestinal disorders, including irritable bowel syndrome, which is frequently found to be concomitant with psychiatric disorders such as PTSD (Moloney, O'Mahony, et al., 2015). While it is highly common, and while it is oft-considered a side effect or symptom of other disorders and diseases, visceral pain exists independently (Cervero & Laird, 1999). Visceral pain related disorders come at significant societal and medical cost, indeed leading to detriments in social, psychological, and work aspects of life (Hungin et al., 2003). Unfortunately, visceral pain has been relatively difficult to study as it is hard to pinpoint the source of pain, and often pain from internal organs present in different areas than they are located (Sikandar & Dickenson, 2012). Further to this, visceral pain can elicit a perception that it is more

threatening and unpleasant in comparison to somatic pain (Boeckxstaens et al., 2016). PTSD has long been linked with visceral pain and disorders inducing visceral pain, such as irritable bowel syndrome (Irwin et al., 1996). Indeed it is very clinically relevant for PTSD sufferers to present with irritable bowel disorders (Cohen et al., 2006; White et al., 2010), with heightened sensitivity to visceral pain being noted (Stam, 2007).

5. PTSD and the Gut

As mentioned above, PTSD is comorbid with gut related disorders such as irritable bowel syndrome, as well as many other psychiatric disorders such as anxiety and depression (Wilmes et al., 2021). With the burgeoning knowledge of the microbiota-gut-brain axis (Cryan et al., 2019), it is to be understood that these psychiatric comorbidities come as a result of a dysregulation of this microbiota-gut-brain axis (Bastiaanssen et al., 2019; Cruz-Pereira et al., 2020; Cryan & Dinan, 2012). The gut microbiome is strongly implicated in stress and stress-related disorders (Foster et al., 2017; Moloney et al., 2014), indicating that the gut microbiota would be relevant in a stress-related disorder like PTSD. However, to date, there is a relative shortage of studies assessing the impact of the gut microbiota on traumatic stress and traumatic-stress induced disorders despite interest in the ameliorative potential (Bersani et al., 2020; Leclercq et al., 2016). Preliminary clinical studies have indicated potential gut microbiome variation in trauma populations (Bajaj et al., 2019; Hemmings et al., 2017; Howard et al., 2017), and a very recent pre-clinical study has suggested that the gut microbiota are relevant to traumatic stress susceptibility (Tanelian et al., 2022), but further focused research studies on the effects of traumatic stress and PTSD in the bidirectional microbiota-gut-brain axis are

certainly necessary. Given that the gut microbiota, via the vagus nerve and concentrations of specific bacteria (Cryan et al., 2019), impacts the HPA axis - which is hypoactivated in the context of PTSD (Herman et al., 2016; Klaassens et al., 2012) - this suggests a linkage between the gut microbiota and stress physiology (O'Mahony et al., 2009). Taken together, the gut microbiome can potentially contribute to the underlying physiological processes in PTSD, suggesting a potential novel therapeutic avenue.

6. Aims and Hypotheses

The overall aim of this thesis is to validate two separate animal models of trauma with a long-term aim of using these to gain a greater understanding of the behavioural, physiological, and neural outcomes of traumatic stress. By validating two different rat models of trauma, one of which being a modified version of the classical Single Prolonged Stress (SPS) paradigm to recapitulate a physically-induced trauma, and the other a novel animal model developed by our lab to investigate secondary traumatic stress in relation to visceral pain which relates to psychologically-related trauma, we aim to shed light on multiple facets of trauma-related symptomology.

For Chapter 2, we hypothesize that

1. A psychological trauma, specifically the observation of a fellow rat undergoing a physical trauma, will induce a secondary traumatic stress-like phenotype.

2. Observation of significant visceral pain will induce hypersensitivity to visceral pain.

For Chapter 3, we hypothesize that,

1. The use of a modified version of the classical SPS paradigm will induce PTSD-like phenotypes

Chapter 2:

A Novel Animal Model for Understanding Secondary Traumatic Stress and Visceral Pain in Male Rats

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Under Review: Neurobiology of Stress

Abstract

Psychological and physical trauma can induce significant pathological alterations, and lead to the manifestation of neuropsychiatric disorders, such as post-traumatic stress disorder (PTSD). Moreover, secondary traumatic stress (STS) develops after witnessing another individual or group undergo a severe or life-altering event. These trauma-related disorders are often comorbid with anxiety, depression, pain, and gastrointestinal disturbances. Visceral pain is a specific type of pain that originates from the internal organs, and despite its widespread prevalence, remains poorly understood. To develop a more comprehensive view of the linkage between visceral pain and STS, we established an observation paradigm that allowed us to assess the impact of STS on visceral pain sensitivity.

Briefly, in male rats, we utilised colorectal distension (CRD) to induce visceral pain behaviours in a stimulus rodent whilst the observer rodent observed. Twenty-four hours post observation, the visceral sensitivity of the observer rodent is assessed using CRD. Intriguingly, and in line with our hypothesis, the observer rats were found to have a significant hyperalgesia as determined by lower visceral pain threshold and a higher number of total pain behaviours compared to control. Moreover, the behaviours of the observer animals during the observation portion were found to be correlated with the behaviours of the stimulus animal employed. The observer animals were seen to have hypoactivity of the hypothalamic-pituitary-adrenal (HPA) axis, highlighted by reduced corticosterone levels at 90 minutes post-CRD. Using

c-Fos immunohistochemistry we showed that observer animals also had increased activation of the anterior cingulate cortex, and decreased activation of the paraventricular nucleus, compared to controls. These results suggest that observing another animal in distress and pain produces a secondary traumatic stress type phenotype at the level of the brain-gut axis. Such a model will aid future development of effective treatments of complex stress disorders especially those co-morbid with functional bowel disorders such as irritable bowel disorder.

Keywords: Pain transmission, trauma, secondary traumatic stress, visceral pain, colorectal distension, empathy

Abbreviations: ACC: Anterior Cingulate Cortex; ARRIVE: Animal Research: Reporting of In Vivo Experiments; BORIS: Behavioral Observation Research Interactive Software; CRD: Colorectal Distension; HPA: Hypothalamic-Pituitary-Adrenal Axis; IL: Infralimbic Cortex; PFC: Prefrontal Cortex; PL: Prelimbic Cortex; PTSD: Post Traumatic Stress Disorder; PVN: Paraventricular Nucleus of the Hypothalamus; STS: Secondary Traumatic Stress.

1. Introduction

Traumatic stressors such as assault, abuse, exposure to severe injury, car accidents and other vicarious traumatic experiences are inducing significantly high levels of trauma-related disorders such as post-traumatic stress disorder (PTSD) (Oakley et al., 2021; van der Kolk, 2000). PTSD can occur in individuals who endure one of these types of traumatic events, or indeed witness an event, with the witnessing of a traumatic event being itself a traumatic event for the observer. The exposure to trauma, long-term stressors, and trauma-related disorders can have significant co-morbidities, including premature aging, hypersensitivity to pain, depression, substance misuse and abuse, anxiety, and chronic pain conditions (Åkerblom et al., 2017; Aliev et al., 2020; Beck & Clapp, 2011; Brady et al., 2000; Debell et al., 2014; Gradus et al., 2015; Morasco et al., 2013; Ng et al., 2019)

While studies investigating trauma and PTSD in relation to combat, violence, or severe physical injury have been prevalent in the literature, there is still a lack of comprehensive understanding regarding indirect exposure to traumatic events - namely secondary traumatic stress (STS). STS was coined in 1995 (Figley, 1995), and typically, but not uniquely, applies to medical personnel, first responders, or other individuals in positions to observe traumatic experiences first-hand (Branson, 2019). Further, it can produce phenotypes similar to PTSD, and it can manifest after just one exposure to a vicarious traumatic event. In preclinical models, empathetic relationships and transference of pain and fear-related behaviours between rodents has been seen which parallel observations

in the clinical community (Ben-Ami Bartal et al., 2011; Langford et al., 2006; Mogil, 2012). Observational paradigms for conditioned fear have shown that the behaviours of observer animals dramatically shift due to the fear experience, and indeed this effect can be exacerbated when the observers are familiar with the stimulus (Keum & Shin, 2016). A relative dearth of information exists regarding social transmission of pain, but research suggests that observation of pain experiences, such as formalin injections and neuropathic pain, induces significant changes in response and behaviour (Baptista-de-Souza et al., 2015; Fatemeh Mohammadi et al., 2020). A very recent study has demonstrated that social pain transmission also induces alterations at the behavioural, neural, and hormonal level (Baptista-de-Souza et al., 2022).

Chronic pain is a particularly common trauma-related comorbidity (Reed et al., 2021). It has been seen that an individual's pain sensitivity and pain prevalence can be significantly increased with pre-existing PTSD symptoms (Brennstuhl et al., 2015; Morasco et al., 2013). The individuals which witness pain experienced by others are themselves more susceptible to pain later on. It is, however, not clear if a similar phenomenon can be observed in the context of visceral pain. Visceral pain originates from internal organs and is a core symptom of functional gastrointestinal disorders such as irritable bowel syndrome which is highly co-morbid with stress-related psychiatric disorders (Moloney, O'Mahony, et al., 2015). We set out to develop a model that would encompass visceral pain and observed trauma, using the most frequently used test for assessing visceral pain, colorectal distension (CRD) (Greenwood-Van Meerveld et al.,

2003; Moloney, Stilling, et al., 2015; O'Mahony et al., 2012; Vaculin et al., 2010). We hypothesize that observation of visceral pain will induce hypersensitivity, and by doing so will provide evidence of social transmission of pain.

2. Materials and Methods

2.1 Animals

Adult male Sprague-Dawley rats (n=36, 250-350 g, age 8 weeks. Envigo, UK) were used in this study. All experiments were conducted in accordance with European Directive 86/609/EEC, Recommendation 2007/526/65/EC, and approved by the Animal Experimentation Ethics Committee of University College Cork, and performed under HPRA project authorization AE19130/P143. All experiments were performed as per the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. Animals were kept under a 12-h light/dark cycle (ON 7:00AM, OFF 7:00PM), with a temperature of 21 ± 1 °C and humidity of 55 ± 10%. Food and water were given ad libitum during the 2-week acclimatization period, and water throughout the entire study. One week prior to the commencement of the study, animals were habituated to handling and marked for easy identification. Rats were randomly divided into three groups (stimulus, observer, control) and housed in groups of 3, caged by group.

2.2 Colorectal Distension (CRD)

Prior to the beginning of CRD, rats were fasted for 16-hours overnight. Rats were then placed under anaesthesia (5% Isoflurane induction, followed by 1.5% maintenance), and

a latex balloon catheter (6 cm) was inserted into the colon via the anal cavity, and held in place using surgical tape firmly wrapped around the tail. Following this, the rat was placed into a clean cage and allowed to recover. Subsequently, the experimental animal was placed in a transparent box and the catheter was connected to the barostat (Distender Series II, G and J Electronics, Toronto, ON, Canada). The distension occurs with the balloon being inflated from 0mmHg to 80mmHg over an 8-minute period. Threshold and total pain behaviours were scored live. Threshold is the pressure (mmHg) at which the rodent first exhibited a visceral pain behaviour. Visceral pain behaviours in the paradigm are identified by abdominal retraction and withdrawal (Mckernan et al., 2010; O'Mahony et al., 2009). Each expression of this abdominal and withdrawal behaviour was counted towards the total pain behaviours. **(Figure 1B, 1D).**

2.3 Experimental design

To evaluate the potential induction of visceral hypersensitivity upon observation of CRD, rats were randomly assigned to three experimental groups: control (n=12), stimulus (n=12) and observer (n=12) and caged separately. To assess the impact of CRD observation, as a stimulus rat underwent the CRD paradigm, an unfamiliar rat from the observer group was placed in an adjacent transparent box to observe the conspecific animal undergoing to the procedure. Then, 24 hours later, the animal that observed the CRD undergoes the paradigm themself as previously described. The control animals, which did not observe the procedure, were habituated to the box 24 hours prior to undergoing the CRD procedure. **(Figure 1)**. All procedures occurred between 9 am and 3

pm. Following completion of CRD, the animals were placed in a clean cage and were euthanized 90 minutes after the start of the CRD.

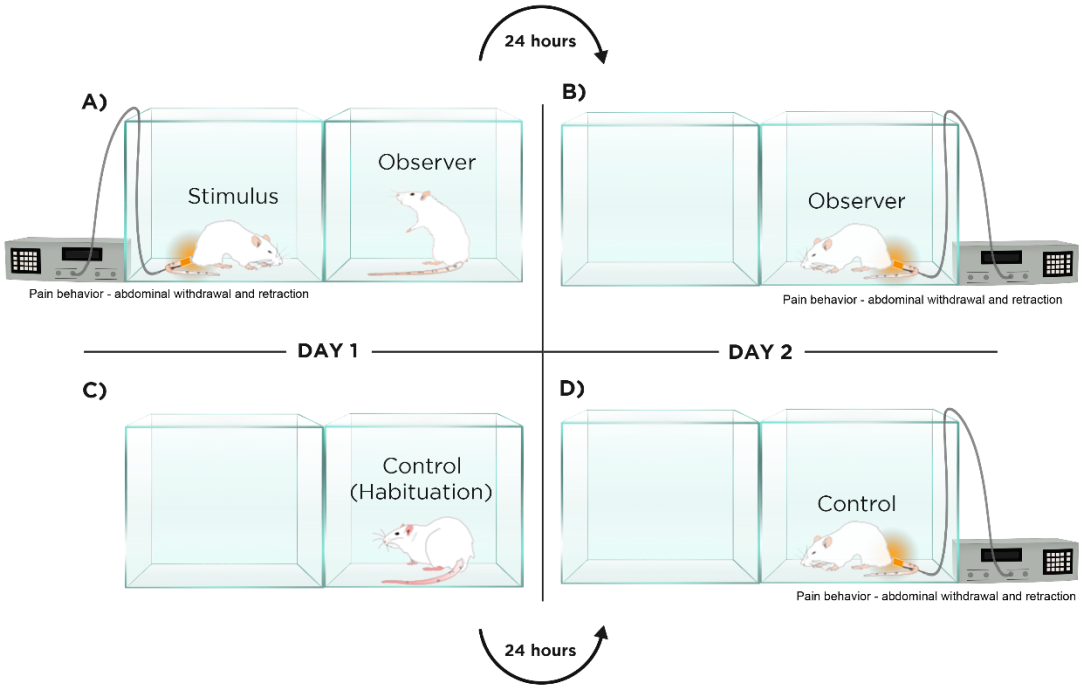

Figure 1:

Experimental Design: To assess the impact of CRD observation, **(A)** as a stimulus rat underwent the CRD paradigm, an observer was placed in an adjacent transparent box to observe the conspecific animal undergoing to the procedure. **(B)** Then, 24 hours later, the rat (observer) that observed CRD on day 1, themself undergoes the test CRD. The control animals, which did not observe the procedure, **(C)** were habituated to the box 24 hours prior to undergoing the CRD procedure to avoid any confounding effects, and **(D)** 24 hours later underwent test CRD. Animals in **(B) (D)** are exhibiting behaviours identified as pain behaviours for scoring and threshold. The control in panel **(C)** is showing normal behaviour.

2.4 Behavioural Analysis

The CRD and observation procedure was recorded live using a mounted video recorder. Videos were saved and uploaded to "Behavioral Observation Research Interactive Software" (BORIS v.12.2, University of Torino, Italy) for scoring. Behaviours scored using this software included immobility (occurrences, total time), grooming (occurrences, total time), rearing (occurrences, total time, assisted/unassisted, facing towards/away from stimulus), and arching/hunching (occurrences, total time). Rodents were considered immobile when paw, body, and head movement had seized. Self-grooming is a frequently performed innate behaviour of rodents, specifically in the manner of cephalocaudal progression, wherein the rodent self-grooms starting at the nose, followed by the face and head, to the rest of the body (Kalueff et al., 2016). Rodents were considered to be arching/hunching when there was a clear and distinct curvature of the back and retraction of the head towards the torso. Rodents were considered rearing when standing upright on the back legs, with supported rearing being against the walls of the chamber, and facing towards the stimulus if against the transparent wall directly adjacent to the stimulus chamber. All data was collected using BORIS, transferred to Excel, and analysed using GraphPad Prism.

2.5 Tissue Collection

In every group (n=12), 90 minutes after undergoing CRD, 6 animals were perfused, and 6 animals were culled for fresh tissue collection. Animals undergoing perfusion were first deeply anaesthetized with sodium pentobarbital, perfused with PBS and 4% PFA, then

rapidly decapitated. Tissue was collected immediately following. Perfused brains and spinal cords were collected and stored in a 30% sucrose solution in a 4°C cold room. Rats not undergoing perfusion were rapidly decapitated using a standard rodent guillotine, and tissue was promptly collected. Trunk blood was collected and centrifuged for 15 minutes at 10,000 g at 4°C. Plasma was then collected and stored at -80°C for subsequent analysis.

2.6 Plasma Corticosterone

Corticosterone concentration analysis of blood plasma samples that were obtained at the time of sacrifice was performed using a corticosterone ELISA kit (Enzo Life Sciences, ADI-901-097), and was carried out according to the manufacturer's guidelines. A multi-mode plate reader (Synergy HT, BioTek Instruments) was used to measure light absorbance.

2.7 Brain Immunohistochemistry

Brains were frozen on dry ice and sectioned on a freezing microtome (Thermo HM 450; ThermoFisher) (section thickness: 35µm). Next, sections were mounted on super-frost slides. Antigen retrieval was performed in citric buffer for 10 min in 90^{o}C. The sections were blocked for 1h in room temperature with 10% donkey serum in 0.3% Triton in PBS. The sections were incubated overnight in 4 ^{o}C in 2.5% donkey serum in 0.3% Triton in PBS with c-Fos anti-rabbit antibody (Cell signaling, REF: 22505, diluted 1:500) and fos-b mouse antibody (Abcam, REF: ab11959, diluted 1:500). Next day sections were washed 3 times for 5min in PBS and incubated for 2h in room temperature with the secondary

antibodies (Alexa 488, Invitrogen, donkey anti-rabbit, REF: a21206; Alexa 555, Invitrogen, donkey anti-mouse, REF: a31570) diluted 1:500 in 2.5% donkey serum in 0.3% Triton in PBS. The slices were washed twice for 5min in PBS and cover-slipped using DABCO (Roth).

2.8 Image Acquisition

The brain images were collected under the fluorescence microscope (Olympus bx51) using 10x. The identification of regions of interest was performed using Paxinos and Watson Rat Brain Atlas, 6th edition. C-Fos and fos-b cell counting was performed in ImageJ using function “Find Maxima”. A minimum of 6 sections per region per animal were quantified, and the area of the region was recorded via the calculated area on ImageJ. Upon completion of the quantification in these areas, they were normalized by dividing the number of cells by the area in which they were quantified. This provided accurate normalized data for the relevant region.

2.9 Statistical Analysis

Statistical analyses were conducted using GraphPad Prism software, version 8.3.0 (GraphPad Software, San Diego, CA). Unpaired samples t-tests were used to assess differences in threshold and total pain behaviours, number of c-Fos and fos-b positive cells, and assess corticosterone concentration between the observer and control groups. Pearson R correlation was used to perform a correlation matrix of rodent behaviours. All data is presented as mean +/- standard error of the mean (S.E.M.). Outliers were excluded using the Grubbs outlier test. Statistical significance was accepted for $P<0.05$.

3. Results

3.1 Observer animals demonstrate more pain behaviours and lower pain threshold than controls

The pain threshold of the rat was the minimum value of the inserted balloon's pressure (mmHg) at which the rat first showed a pain behaviour. The observer rats were found to have a significantly lower threshold for visceral pain behaviours, compared with control rats. **(Figure 2A)** ($t_{1,21}$=2.274, p=.0335). This indicates that witnessing a conspecific exhibiting visceral pain behaviours prior to experiencing the CRD test themselves leads to reduced threshold for pain.

To assess if observation impacted overall visceral pain sensitivity, the total number of pain behaviours was scored. With the threshold being the earliest pain behaviour shown, every exhibition of pain behaviour following that was counted. The observer rats were found to have a significantly greater number of pain behaviours **(Figure 2B)** ($t_{1,16}$=2.811, p=0.0125). This indicates that exposure in the form of observation to the visceral pain behaviour results in heightened hypersensitivity to visceral pain, suggesting STS-like effects.

3.2 Hunching and arching occurrences correlate with total pain behaviours and pain threshold

During the observational portion, rodent behaviours of immobility, arching/hunching, rearing, and grooming were scored, and analysed alongside the aforementioned visceral pain threshold and pain behaviours. A correlation matrix was performed of the data in order to determine whether there existed any correlations between the behaviours of

the rodents while observing and the behaviours they expressed while undergoing CRD or the behaviours of the animals that they observed. A Pearson's R correlation **(Figure 2C)** was run using GraphPad Prism, and results showed that there was a significant correlation between arching/hunching occurrences and observed pain threshold ($p=.040$, $r= -.656$) and observed total pain behaviours ($p=.019$, $r=.718$). Further to this, there was a significant correlation between both the time that the observer spent arching/hunching and the observer's immobility time and the number of occurrences of rearing while facing the stimulus rodent (Arching/Hunching an Rearing: $p=.042$, $r= -.651$; Immobility and Rearing: $p=.010$, $r= -.767$).

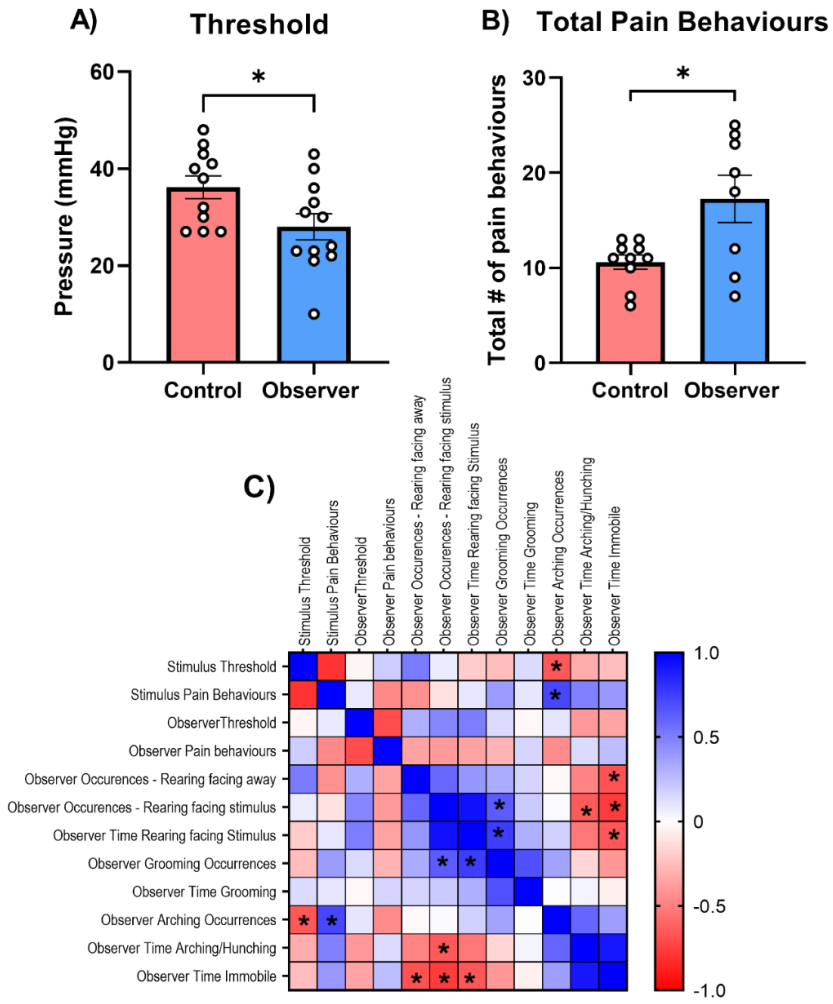

Figure 2:

A) Observation leads to lower threshold for visceral pain. The threshold of pressure (mmHg) at which the animals expressed the first pain behaviour. STS animals had a significantly lower threshold than control. Statistical significance was accepted at p≤.05; (p=.0335). Data represented as mean±SEM. **B) Observation leads to higher number of pain behaviours.** The total number of pain behaviours experienced by each animal. STS animals had a significantly greater amount on average than the control animals. Statistical significance was accepted at p≤.05 (p=.0125). Data represented as mean±SEM. **C) Arching occurrences correlated with observed pain behaviours and threshold.** The number of arching occurrences correlated with the both the total number of pain behaviours and the threshold of the rodents that they observed (p=.019, p=.040). Furthermore, there was a correlation between an observer rodent's (STS) pain behaviours and their threshold (p=.023).

3.3 Corticosterone levels in plasma of observer animals is lower than in controls

Assessment of stress responsivity and HPA-axis reactivity as a result of stress and observation revealed a significant difference between the control and observer groups. An unpaired samples t-test displayed a significant difference between control and observer ($t_{1,9}$=2.293, p=.0475) **(Figure 3A)**. This indicates hypoactivity of the HPA axis as a result of observation.

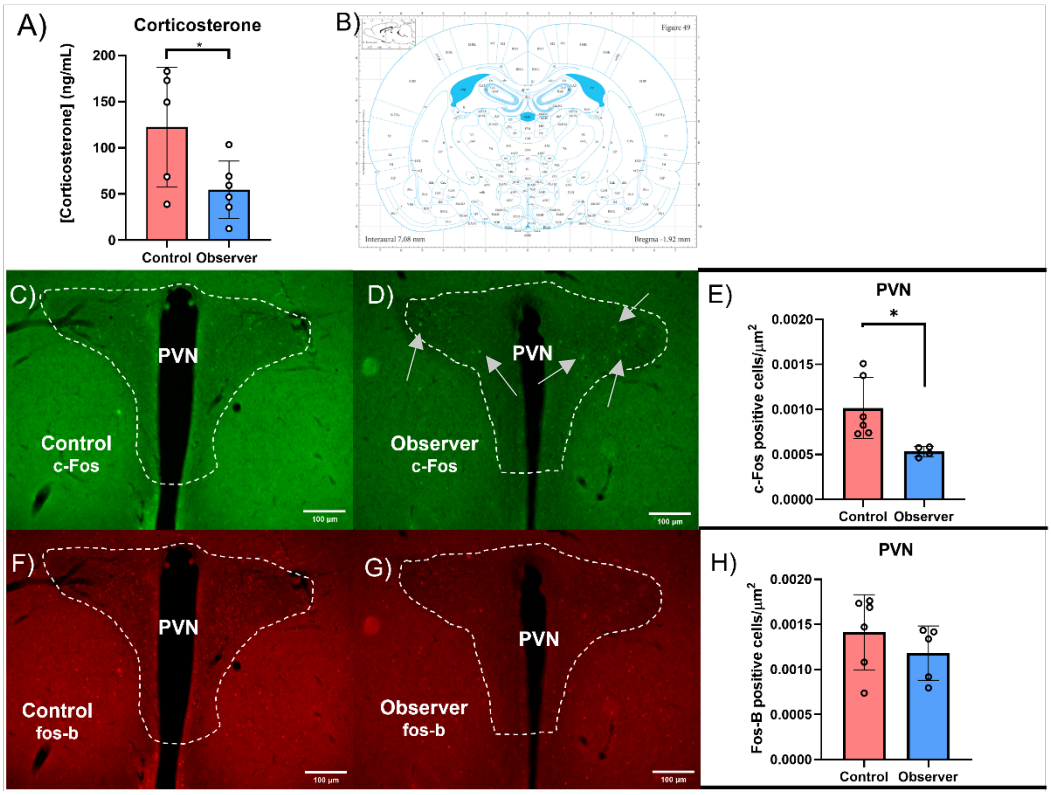

Figure 3:

A) Observation leads to HPA axis hypoactivation. The corticosterone concentration in ng/mL was found to be significant different between control and STS. Statistical difference was accepted at p≤.05 (p=.0475). **B)** Atlas image of representative area for quantification. **C)** Representative image of c-Fos in the PVN for

control. **D)** Representative image of c-Fos in the PVN observer. **E) There was significantly fewer c-Fos positive cells in the PVN in observer animals compared to control (p=.0245). F)** Representative image of fos-b in the PVN for control. **G)** Representative image of fos-b in the PVN for observer. **H)** No significant difference in fos-b was seen in the Insula between control and observer.

3.4 C-Fos expression in anterior cingulate cortex (ACC) is higher in observers than in controls, and lower in the paraventricular nucleus of the hypothalamus (PVN) in observers

To assess changes in neuronal activation patterns in the brain, specific regions were chosen for immunohistochemical staining with c-Fos. Quantification of c-Fos was performed as described above. We analysed the c-Fos for neuronal activation in the ACC, and found there was a significantly greater number of c-Fos positive cells in the brains of observer animals compared to control (p=<.0001, t=7.282, df=9) **(Figure 4C)**. In the PVN, there was a significantly lower amount of c-Fos positive cells in observer animals compared to control (p=0.0245, t=2.764, df=8) **(Figure 3E)**.

In the prelimbic (PL) and infralimbic (IL) cortices of the prefrontal cortex (PFC), there was not a significant difference between observer and control animals **(Figure 4F,** PL: p=0.3524, t=.09753, df=10; **Figure 4I,** IL: p=.0676, t=2.049, df=10**)**. Likewise, in the Insula, there was no significant difference between observer and control (p=0.7237, t=0.3636, df=10) **(Figure 4L)**.

Analysis of fos-b in the aforementioned brain regions showed no significant difference across observer and control animals. (**Figure 5)**

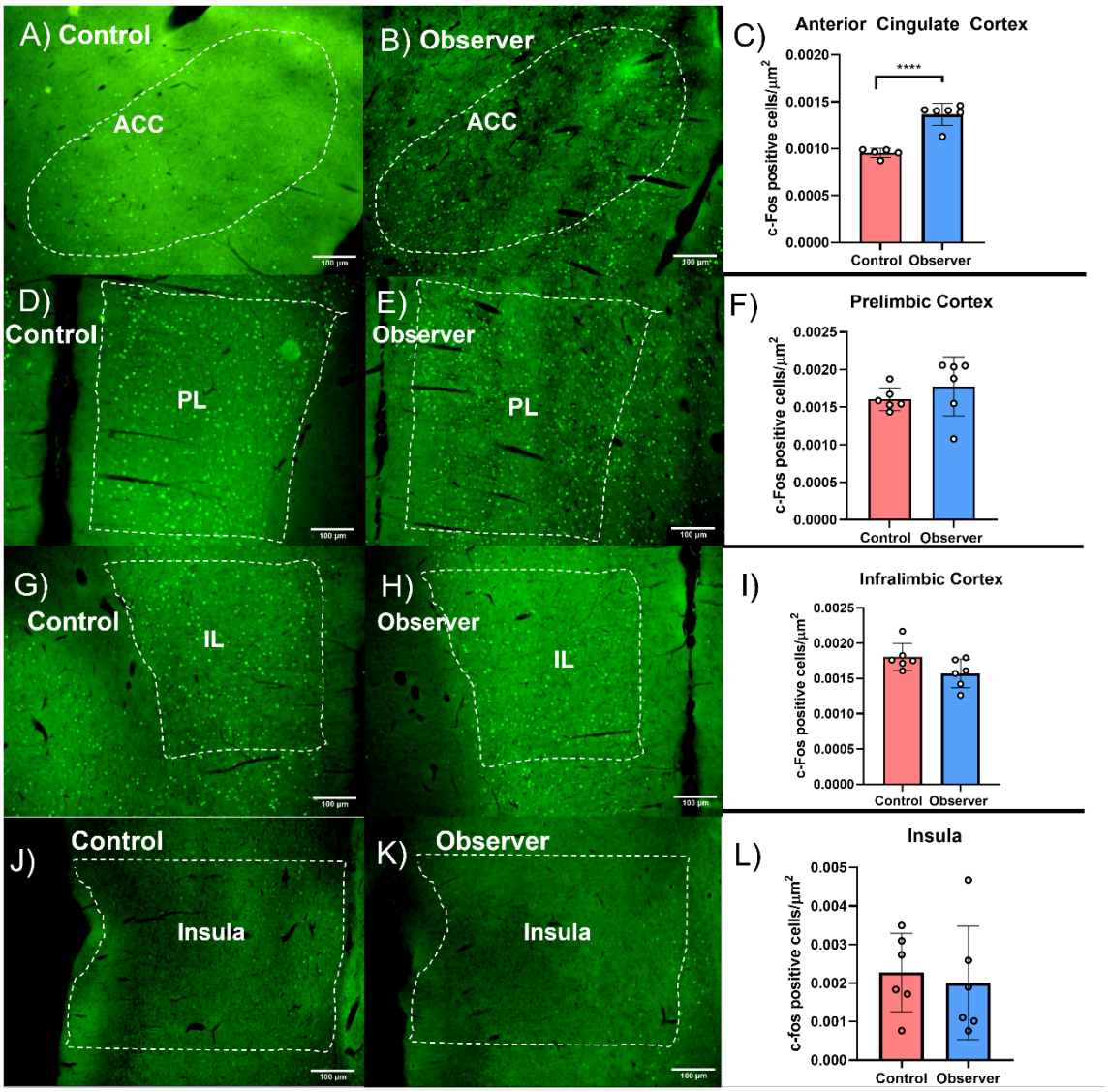

Figure 4:

A) Representative image of c-Fos in the ACC of control. **B)** Representative image of c-Fos in the ACC of observer. **C) Observer animals have greater neuronal activation in the Anterior Cingulate Cortex. There was a significantly higher number of c-Fos positive cells in observer animals compared to control animals (p*<.0001). D)** Representative image of c-Fos in the PL of control. **E)** Representative image of c-Fos in the PL of observer. **F)** No significant difference in c-Fos was seen in the PL between control and observer. **G)** Representative image of c-Fos in the IL of control. **H)** Representative image of c-Fos in the IL of observer. **I)** No significant difference in c-Fos was seen in the IL between control and observer. **J)** Representative image of c-Fos in the Insula of control. **K)** Representative image of c-Fos in the Insula of observer. **L)** No significant difference in c-Fos was seen in the Insula between control and observer.

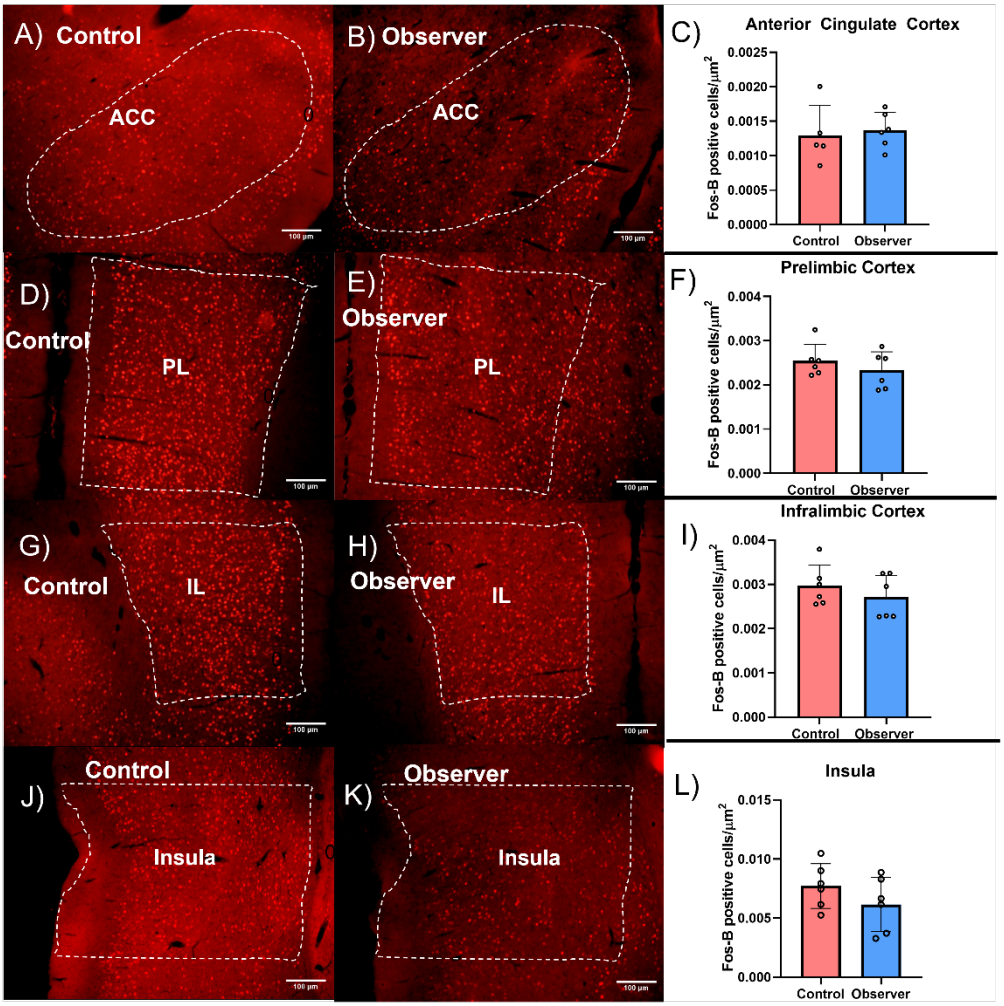

Figure 5:

A) Representative image of fos-b in the ACC of control. **B)** Representative image of fos-b in the ACC of observer. **C)** No significant difference in fos-b was seen in the ACC between control and observer. **D)** Representative image of fos-b in the PL of control. **E)** Representative image of fos-b in the PL of observer. **F)** No significant difference in fos-b was seen in the PL between control and observer. **G**) Representative image of fos-b in the IL of control. **H)** Representative image of fos-b in the IL of observer. **I)** No significant difference in fos-b was seen in the IL between control and observer. **J)** Representative image of fos-b in the Insula of control. **K)** Representative image of fos-b in the Insula of observer. **L)** No significant difference in fos-b was seen in the Insula between control and observer.

4. Discussion

The availability of animal models is key to understanding PTSD-related co-morbidities (Moloney, O'Mahony, et al., 2015). The results from this study suggest that observing a conspecific rodent undergoing CRD and expressing visceral pain behaviours results in hypersensitivity to visceral pain. Thus this model is poised to be an important tool to dissect the neurobiology of visceral pain transference in the context of an observed trauma. This provides further insights into growing evidence that suggest the empathetic transference of visceral pain via sensory modalities.

At a behavioural level we found that the observation of visceral pain behaviours lead to hyperalgesia, in the form of both reduced threshold for experiencing and displaying visceral pain behaviours, and increased total pain behaviours over the eight minute CRD paradigm. This hypersensitivity to visceral pain is in line with our hypothesis, and aligns with other studies regarding rodent empathy and pain transference. As prior studies showed that observation of pain experience induced empathetic response and behavioural changes (Baptista-de-Souza et al., 2015; Fatemeh Mohammadi et al., 2020; Sanders et al., 2013), this study likewise shows the empathetic response extends as far as visceral pain hyperalgesia.

The correlated behaviours that the observer animals showed during the observation task further support the active empathetic processes. The observer animals having increased hunching and arching behaviour positively correlating with increased stimulus animal pain experience indicates an active empathetic relationship between the rodents. While

it is plausible that these hunching behaviours could be stress-induced, it would be the observation of the stimulus animals stress – the CRD – that would be causing this and that would be hand-in-hand with the empathetic relationship between the animals. Further to this, we found that the more times the observer rodent reared while facing the stimulus rodent, the greater amount of time they spent immobile and arching/hunching. This would suggest that the visual stimulus is involved in the empathetic transference of pain, but does not narrow the communication down to this one sensory input. It has been suggested that rodents can pick up on cues beyond visual and auditory cues, such as nuanced environmental cues and even silence; indeed, there are a multitude of factors that influence the transference of fear and pain, including sensory modalities [i.e. vision, vocalization], strength of stimulus, sex, and familiarity (Kim et al., 2019), and future research will dictate which of these are most crucial in the empathetic relationships and communicative nature of pain. For future studies, it would be beneficial to have control animals observe other control, non-stimulated animals. While we controlled for exposure to CRD, be that both observation and undergoing, it would be more comprehensive to include control animals that equally observed other control rats. This would account for a potential effect of exposure to another animal, regardless of their any aversive experience or relevance of empathy.

At the level of the HPA axis the observer animals had significantly lower corticosterone concentration at 90 minutes post-CRD compared to control. In the context of PTSD, the HPA axis has been found to be hypoactive (Richter-Levin et al., 2019), as a result of high sensitivity of the glucocorticoid receptor and negative feedback control inhibition in the

pituitary gland, which causes low level of cortisol circulating in the bloodstream (Yehuda, 2002). A related hormonal imbalance ties to negative alterations in cognition and mood, alterations in arousal and activity, and intrusive/avoidance symptoms (D'Elia et al., 2021). The results from our observer study suggest that witnessing and later experiencing CRD induces similar hypoactivation of the HPA axis. This finding is in contrast to prior work, where observation of stressful events alone did not seem to evoke the same corticosterone response (Li et al., 2014; Patki et al., 2015). While other models were not able to induce this effect, the CRD Observer model developed in our study induced this very interesting HPA hypoactivation effect. Though we cannot definitively say that it was the reduced corticosterone that caused the hyperalgesia, the fact that these results are common among the observer animals suggests a relationship between them. The nature of the effect, be it direct or indirect, requires further intriguing investigation. These results are important in the framework for understanding the experience of witnessing a stressful event, and how visceral pain is involved with traumatic experiences. The acute nature of the HPA axis hypoactivation inspires investigation at other timepoints, including the identification of a typical 120-minute corticosterone curve, but also in days following the observation and CRD to identify if it reflects the long-term effects seen in PTSD. We acknowledge the fact that this is too acute of a timepoint to appropriately state it as a relevant marker for PTSD, as 24 hours post-stress is significantly shorter than necessary to truly assess for a PTSD validity. However, it encourages research using this model in experiments with greater longevity to assess both short-term and long-term effects. Further, an additional naïve control

group that would not undergo CRD would have been interesting as we could identify a true baseline effect of CRD for further comparisons, and we acknowledge this is as a current limitation.

Paired with this hypoactive response of the HPA axis is a reduction in c-Fos positive cells in the PVN in observer animals compared to control animals. The PVN initiates the stress response with the release of CRH and is key in modulating the pathway (Herman et al., 2016). The PVN is at the crux of the stress response, and it has been seen that chronic stress causes significant changes to both its form and function, with it being responsible for both typical behaviour and pathological features in the HPA axis' stress response (Herman & Tasker, 2016). In the case of this study, hypoactivity of the HPA axis in the form of reduced corticosterone at 90 minutes post CRD parallels with hypoactivity of the PVN in the form of c-Fos positive cells at 90 minutes post-CRD. Considering the importance of the PVN in the HPA axis cascade and overall stress response (Herman & Tasker, 2016), it is to be expected their dysregulation is intertwined.

Beyond the PVN, a significant increase in c-Fos positive cells was detected in observers compared to controls in the ACC. It has been previously shown that visceral hypersensitivity leads to increased activity in ACC after colonic distension (Felice et al., 2014; Gao et al., 2006; Gibney et al., 2010). Interestingly, ACC is also implicated in feelings of empathy (Lockwood et al., 2015; Morrison et al., 2004), and particularly found to be hyperactivated when experiencing pain but also while observing pain (Marsh, 2018). Further to this, both the PFC and ACC are implicated not only in physical pain but also in psychological stress (Meerwijk et al., 2013), as well as visceral pain (Meerveld &

Johnson, 2018; Moloney et al., 2016), and show dysfunction in traumatic stress (Fenster et al., 2018). Our findings support the literature, in that the apparent transference of pain, specifically visceral pain behaviours, lead to a hyperactivation of the ACC. The PL and IL of the PFC are key regions in the context of trauma and traumatic stress phenotypes (Giustino & Maren, 2015; Knox et al., 2016; Schreiber et al., 2017) as well as fear and anxiety (Tovote et al., 2015). However, in the case of this study, the PL and IL were not significantly different in activation across control and observer animals. Further studies exploring other key brain areas involved in empathy, pain and pain transference, and stress is critical. We would like to acknowledge that use of a section thickness of 35µm in the immunohistochemistry analysis leads to background staining and identify this is as a limitation. While the specific cells that are expressing c-Fos were not identified in this study, this would be an avenue of interest for future studies. Considering it's relevance to trauma and PTSD, it could be speculated that astrocytes could be playing a significant role during a trauma-related situation and would be the cell-type that would be expressing c-Fos under the conditions. A potential way to investigate this would be by doing a staining for GFAP which is found distinctly in astrocytes. By staining for both c-Fos and GFAP and then assessing whether there is overlap, we could potentially identify astrocytes as the cells in this play in this situation.

There is a common overlap between psychiatric disorders and visceral pain and other irritable bowel disorders. Psychiatric disorders such as depression and anxiety are often presented as comorbid with irritable bowel disorders, as observed in irritable bowel syndrome (Wilmes et al., 2021), and these are extenuating from dysregulation of the

microbiota-gut-brain axis (Bastiaanssen et al., 2019; Cruz-Pereira et al., 2020). Understanding that both physical and psychological stressors induce visceral hypersensitivity (Felice et al., 2015), paired with the knowledge of the bidirectional pathway of the microbiota-gut-brain axis (Cryan et al., 2019), it can be inferred that psychiatric disorders and bowel disorders can exacerbate each other. The nature of these comorbidities urges further research into the relevance within the empathetic transference of pain.

5. Conclusions

Taken together, the evidence of this study suggests that the observation of visceral pain leads to significant neural, hormonal, physiological, and behavioural changes. The experience of observing visceral pain, and later undergoing it, leads to an STS-like phenotype. This intriguing data sparks further interest into unearthing the methods of transference of pain, and identifying how to ameliorate the deficits seen as a result.

Understanding observed trauma and social transference of visceral pain, and its impact on neurocircuitry, HPA axis, and physiology will allow for investigation into STS-related therapeutics and provide invaluable knowledge into the mechanisms of stress and empathy. The establishment and validation of this novel model of STS provides avenues for in depth research into the molecular underpinnings involved in trauma and its comorbidities, as well as the observation of pain.

Chapter 3:

Refining a Stress Model: Is Anaesthesia Exposure a Critical component of the Single Prolonged Stress Model of Post-traumatic Stress Disorder?

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Abstract

Single Prolonged Stress (SPS) is a validated traumatic stress animal model that recapitulates many of the phenotypes associated with post-traumatic stress disorder (PTSD). This model in its original design involves a 2-hour restraint, immediately followed by a 20-minute forced swim, a 15-minute rest and culminates in anaesthesia exposure with diethyl ether until loss of consciousness. However, due to evolving ethical complications and the continued desire to refine animal models, we intended to validate the SPS model using isoflurane as a replacement for diethyl ether. Further, we aimed to investigate the efficacy of the model using only the restraint and group swim parts of the paradigm, leading us to two key groups – SPS with Isoflurane (SPS-Iso), and SPS without Isoflurane (SPS-No Iso).

Intriguingly, we found that both SPS-Iso and SPS-No Iso induced inhibited ability to extinguish fear in the contextual fear conditioning paradigm, which is a hallmark measure of the efficacy of the SPS model and relevance to PTSD. Further, we found that SPS-Iso showed significant change in coping ability and learned helplessness measure in the forced swim test, as well as time and time x group effect in a corticosterone analysis. However measures of gut permeability were not found to be significantly different between groups.

These results suggest that the use of isoflurane as a refinement over diethyl ether in the SPS model is a promising avenue that holds the potential to appropriately represent traumatic stress effects. More in-depth research into the validity of this model in

reproducing the phenotypes of PTSD can provide a path to the development of novel and effective therapeutic options.

1. Introduction

Post-traumatic stress disorder (PTSD) is a mental health disorder characterized by significant detriments to personal well-being and quality of life, sociability inhibition, and significant societal cost (Kessler, 2000; Watson, 2019). As cases continue to rise (Watson, 2019), the necessity for advanced novel therapeutics is rapidly rising. To fulfil these therapeutic needs, accurate representative animal models are essential for developing the most effective therapeutic responses. As with all disorders, animal models of PTSD have limitations individual to each model (Richter-Levin et al., 2019) and choosing an appropriate model depends on the researcher's specific focus. In seeking an approach that effectively recapitulates multiple facets of PTSD, one of the most appealing rodent PTSD model is single prolonged stress (SPS) in rats. SPS induces molecular, behavioural, and physiological alterations, such as hypervigilance and hyperarousal, that mirror PTSD-like pathophysiology (Ferland-Beckham et al., 2021; Lisieski et al., 2018). SPS was first championed by Liberzon and colleagues in the 1990s (Liberzon et al., 1997) and since then has been considered an effective and relevant model of traumatic stress in rodents. This original model of SPS includes a 2-hour restraint, followed by a 20-minute group swim, a 15-minute rest period, and exposure to diethyl ether until loss of consciousness. Vital to the efficacy of this model is the 7-day incubation period starting immediately following completion of the paradigm (Dayan Knox et al., 2012; Liberzon et al., 1999).

Diethyl ether is a hazardous, flammable chemical that is harmful and irritable to both humans and rodents ("2000 Report of the AVMA Panel on Euthanasia," 2001; Carruba et al., 1987), with the possibility of death in rodents (Brandstater et al., 1965) and refinements in the shape of other anesthetic options are being explored (Nagate et al., 2007; Nakatsu et al., 2017). Thus, alternatives to the use of diethyl ether have been discussed and in line with concern about the usage of diethyl ether, moves to find effective refinements are underway. To explore the potential of using an alternative to diethyl ether, we employed isoflurane instead as the third element of the SPS model. Isoflurane is an anesthetic administered via inhalation, and is the most common anesthetic used in rodents (Richardson & Flecknell, 2005). Isoflurane has been utilized in some prior SPS protocols, however inconsistent and incomprehensive results were found (D. Knox et al., 2012; Lee et al., 2018; Mancini, Marchetta, Pignani, et al., 2021; Mancini, Marchetta, Riccardi, et al., 2021), which encourages a focused experiment investigating the efficacy of SPS with Isoflurane.

Fear conditioning is a frequently used paradigm to determine the efficacy of the SPS model; in particular cued and contextual fear conditioning paradigms are important for measuring the fear-related behavioural phenotypes associated with PTSD, such as avoidance and impaired fear extinction/learning (Ferland-Beckham et al., 2021; Rau & Fanselow, 2009; Souza et al., 2017). It provides an etiologically relevant behavioural manifestation (fear behaviour – monitored as freezing during testing) of PTSD, with the initial memory formation and the context of the memory associating closely with the

traumatic stressors that lead to PTSD (Johnson et al., 2012). The context in the fear conditioning paradigm varies; it can be a sound, a shock, the fear conditioning chamber itself, as clinically this translates as the trauma or memory that instigates the fear. Abnormal fear learning, generalization, and fear memory are key components of PTSD and measurables in fear conditioning tests (Liberzon & Abelson, 2016). As such, extinction of fear in a contextual fear conditioning setting is seen to be disrupted as a direct result of SPS in days following fear acquisition (Dayan Knox et al., 2012; Milad et al., 2009).

Beyond fear learning and fear extinction, important behavioural readouts for the context of PTSD include anxiety-like behaviour, social deficits, learned helplessness and depressive-like behaviours. Behavioural tests for these readouts that have been shown to be sensitive to SPS include the open-field test and elevated plus maze for anxiety like behaviours (Chen et al., 2021; Lee et al., 2020; Lee et al., 2018; Zhou et al., 2020) , the forced swim test for depressive-like behaviour and learned helplessness (Chen et al., 2021; Lee et al., 2020), and social interaction tests for sociability deficits (Azevedo et al., 2020; Wang et al., 2019). In the context of PTSD phenotypes, the elevated plus maze and open field test align with DSM-V criterion C: avoidance of trauma related stimuli; the forced swim test and sociability tests aligns with criterion D: negative alterations in cognition and mood(*Diagnostic and statistical manual of mental disorders : DSM-5*, 2013).

Psychiatric disorders are commonly found to be comorbid with irritable bowel and gut-related disorders due to a dysregulation of the bidirectional microbiota-gut-brain axis (Bastiaanssen et al., 2019; Cryan et al., 2019; Wilmes et al., 2021). Stress induces significant changes in the gut (Cryan et al., 2019), with intestinal permeability being a key marker of these changes (Kelly et al., 2015). A recent study suggests that traumatic stress susceptibility is linked to the gut microbiota (Tanelian et al., 2022) indicating the potential for changes in gut functioning, including intestinal permeability.

Thus in this study, using fear condition as one of our major readouts we aimed to assess the efficacy of the SPS paradigm using isoflurane as a replacement for diethyl ether, as well as determining the necessity for an anesthetic portion of the stressor by performing only the restraint and group swim portion.

2. Methods

2.1 Animals

Male Sprague-Dawley rats (Adult – 270-320 g, Envigo) were used in this study. All experiments were conducted in accordance with European Directive 86/609/EEC, Recommendation 2007/526/65/EC, and approved by the Animal Experimentation Ethics Committee of University College Cork. Animals were kept under a 12-h light/dark cycle (ON 7:00AM, OFF 7:00PM), with a temperature of 21 ± 1 °C and humidity of 55 ± 10%. One week prior to the commencement of the study, animals were habituated to

handling and marked for easy identification. Rats were randomly divided into three groups (n=12/group) (Control, SPS-Iso, and SPS-No Iso), and housed in cages of 3 or 4.

2.2 Single Prolonged Stress

SPS was performed in part as per the original protocol laid out by Liberzon (Liberzon et al., 1997), which entails a 2-hour restraint, immediately followed by a 20-minute forced swim. However, unlike the traditional protocol, diethyl ether was replaced with isoflurane. The SPS with Isoflurane group underwent the restraint, swim, and isoflurane exposure until loss of consciousness. The SPS without Isoflurane group underwent only the restraint and swim. Immediately following the procedure comes a 7-day incubation period.

2.3 Experimental Design

Two cohorts of rats were used for this project. Each cohort had the three groups as labelled above (n=36 total; n=12/group) and underwent SPS as described above. We employed three groups: control, SPS with Isoflurane (SPS-Iso), and SPS without Isoflurane (SPS-No Iso). To comprehensively assess the efficacy of the model, animals underwent an array of behavioural tests to assess for the diagnostic criteria for PTSD; anxiety-related behaviours [Criterion C: avoidance of trauma related stimuli] in the open-field test (OFT) and elevated plus-maze (EPM), social behaviours [Criterion D: negative alterations in cognition and mood] in the three-chamber sociability test, learned helplessness and depressive-like behaviours [also Criterion D] via trauma re-

exposure in the forced swim test (FST). Further, the contextual fear conditioning paradigm was performed to identify whether the hallmark characteristic of fear extinction deficits would be produced using SPS with or without Isoflurane.

After the 7-day incubation period, the first cohort underwent the array of behavioural tests (Open-Field Test, Elevated Plus Maze, Three-Chamber Sociability Test, Forced Swim Test with Tail-Tip and Repeated Bleeds), and then were either fresh culled or underwent transcardial perfusion 90 minutes after completing the Forced Swim Test. (**Figure 1**)

The second cohort underwent the Contextual Fear Conditioning paradigm after the 7-day incubation, and 90-minutes after completing the paradigm, underwent transcardial perfusion or fresh cull.

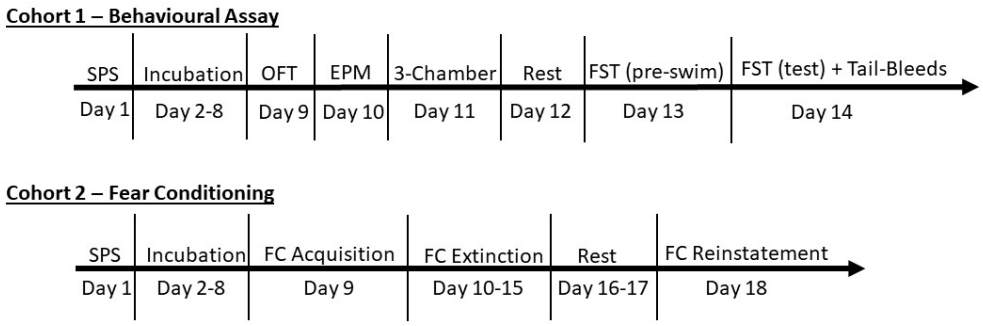

Figure 1: Experimental Timeline

Timeline of events for the experiment, broken into cohort 1 (undergoing the behavioural assay) and cohort 2 (undergoing the contextual fear conditioning paradigm).

2.4 Open Field Test

The open field test was used to assess the impact of SPS with/without Isoflurane on anxiety-like behaviour. The rat is placed in the centre of an open circular brightly lit (~1000 lux) arena, made of grey plastic (90 cm diameter). They can freely explore the arena for 10 minutes, during which the behaviour is recorded by a camera mounted directly above the arena. Immediately following the test, the rat is removed and returned to the home cage. The arena is thoroughly cleaned with 70% ethanol between each test. Time spent in the centre of the arena, entrances to the centre of the arena, and total movement are scored.

2.5 Elevated Plus Maze

The elevated plus maze test was run to assess the impact of SPS with/without Isoflurane on anxiety-like behaviour. The EPM arena is a plus-shaped apparatus elevated above the ground, with two open arms (10cm x 50 cm) and 2 closed arms (10 cm x 50 cm) with a 10 cm x 10 cm centre zone. This test was run using red light (~5 lux). The rat is placed in the centre zone and is allowed to explore the plus maze for 5 minutes. A camera mounted directly above the apparatus records the behaviours. Immediately following the test, the rat is removed from the maze and returned to home cage. The maze is then thoroughly cleaned using 70% ethanol. Time and entrances into the open and closed arms are scored as primary behaviours.

2.6 3-Chamber Sociability Paradigm

The 3-chamber sociability paradigm investigates social preference and sociability, and the potential impacts of traumatic stress on these outcome measures. The arena is made up of 3 separate chambers each measuring 60 cm x 30 cm x 34 cm, with sliding doors on the two dividers which allow for ease of access between chambers. Two identical cylindrical cages are placed in the opposing outer chambers. Phase 1 is habituation, wherein the test rat is placed in the centre chamber. The sliding doors are opened, and the rat can freely explore all 3 chambers for 10 minutes. After 10 minutes, the rat is brought back to the centre chamber, and the sliding doors are closed to restrict movement. Prior to beginning phase 2 (sociability phase), a conspecific rat is placed into one of the cages in the outer chambers. Following this, the doors are opened, and the test rat can freely explore all 3 chambers for a further 10 minutes. At the conclusion of phase 2, the test rat is again brought back to the centre chamber and the doors are shut. A novel conspecific rat is placed in the remaining empty cage. At this point there is a familiar conspecific rat (present for phase 2 and 3), and a novel conspecific rat (present for phase 3). As phase 3 (social novelty) begins, the doors are opened, and the test rat again has 10 minutes to freely explore all 3 chambers. At the conclusion of phase 3, which is the conclusion of the test, the conspecific rats are removed and placed in their respective home cages, and the test rat is also removed and placed in its home cage. The entire arena, sliding doors, and cages are cleaned using 70% ethanol. Time spent in the chamber with the familiar conspecific and novel conspecific rats are scored as primary

behaviours. The entire test is recorded using a camera mounted directly above the arena.

2.7 Forced Swim Test with Tail-Tip and Repeated Bleeds

The forced swim test (FST) was performed as per Slattery & Cryan (Slattery & Cryan, 2012). The rat is carefully placed in a transparent plexiglass cylinder filled with 23-25°C water for a period of 15 minutes. 24 hours later, the rat is once again placed in the plexiglass cylinder (new freshwater 23-25°C) for a 5-minute test swim. The test is recorded by a video camera placed directly above the cylinder. Following all swims, animals are thoroughly hand-dried with a towel and then moved to a recovery cage before being placed back in their home cage. The water is changed between trials. As per Slattery & Cryan (2012), the behaviours of climbing, swimming, and immobility were scored.

Immediately preceding the start of the FST, a tail snip is carried out by removing less than 1mm of the tail at the tip with a sterilized scalpel. The tail is gently massaged, and blood is collected into capillary tube coated with heparin. At 30, 60, and 90 minutes following the FST, the scab is removed and ≤ 0.2ml of blood is collected per timepoint into the capillary tubes.

2.8 Contextual Fear Conditioning

The Contextual Fear Conditioning Paradigm is a 10-day process involving acquisition, 6 days of extinction, 2 days of rest, and reinstatement. A rat is placed into sound-

attenuated chambers (Med Associates, Fairfax VT) with a 33×28×25 cm interior chamber within a 63×45×58 cm sound attenuating outer box and aluminium walls and aluminium rod floor. The fear conditioning paradigm was controlled by Med Associates software. On day one - acquisition, the rodent is placed in the chamber for 8 minutes. At minutes 3, 4, and 5, it receives a 1 second 1mA foot shock. On each of the following 6 [extinction] days, the rodent undergoes extinction whereby the rat is placed into the same chamber for 5 minutes without receiving a foot shock. After the 6 days of extinction, there are 2 days of rest where the animal remains in its home cage. Reinstatement is the final day of the paradigm. The rat is placed into the chamber for 8 minutes, and at minute 3 they receive a 1 second 1mA foot shock. 90 minutes following this procedure, the rat is euthanized and transcardially perfused, or fresh culled.

2.9 Behavioural Scoring

DeepLabCut 2.2 with CUDA Toolkit 10.1 and TensorFlow 1.12.0 was used to perform behavioral analysis (Mathis et al., 2018; Nath et al., 2019) and analyzed using simBA 1.1.3 (simple Behavioural Analysis) (Nilsson et al., 2020).

Video recordings of the behaviours were uploaded to DeepLabCut. Once uploaded, each frame of each video is extracted and saved. These frames are then manually labelled (recommended minimum of 200 labelled frames). Labelling involves identifying a specified number of rodent body parts; in the case of this project, the nose, left ear, right ear, center of body, lateral left of body, lateral right of body, tail base, and tail tip were labelled. These labelled frames are saved and used as a benchmark for the network to

learn the specific body parts. The system was trained using a deep neural network that was trained in 200,000 iterations as the loss relatively flattened (Mathis et al 2018, Nath et al 2019). The trained network could accurately track the position of the rats in the full sets of video segments. The labelled x-axis (i.e. left–right) and y-axis (i.e. bottom-top) positions of the pixels in each frame were stored and exported in CSV format. Once this is complete, the results are evaluated manually, and the researcher determines if the network's ability to identify and locate the specified body parts is as precise and accurate as necessary. Once it is determined to be the case, the behavioural videos can be analyzed.

The output from DeepLabCut is then uploaded to simBA. Using simBA, regions of interest can be added to the videos, specific to each desired behaviour. Additionally, a known width of the arena in centimeters is added, which is compared in pixels creating a pixel-to-centimeter ratio, allowing for movement and velocity to be determined. For the Open Field Test, a center circle zone was drawn to identify center zone vs peripheral zone. In the Elevated Plus Maze, separate regions of interest for open arm 1, open arm 2, closed arm 1, closed arm 2, and center zone were added. For the 3-Chamber Sociability Test, regions for the center chamber, familiar chamber, and novel chamber, as well as interaction zones for the novel and familiar conspecific rodents were drawn. simBA then pairs the regions of interest with the x, y output from DeepLabCut to track the exact location of each animal relative to the region of interest. Videos of animal tracking with visual regions of interest are produced by simBA. Full analysis, including for the project,

movement in the regions of interest, time spent in the regions of interest, and entries into the regions of interest are produced and exported to Excel.

Forced swim test was scored manually as per guidelines laid out in Slattery & Cryan (2012), specifically using the time-sampling technique. The behaviours of climbing, swimming, and immobility were scored and analyzed.

Contextual Fear Conditioning was scored using Med Associates software (Med Associates "Video Freeze" Software; Vermont, USA); the freezing % was scored live with the software. Minute by minute freezing % was gathered using post-video analysis on the same Med Associates software. Freezing % was gathered for each minute of each test day, acquisition, all extinction days, and reinstatement.

2.10 Sample collection

Rats were rapidly decapitated using a standard rodent guillotine, and tissue was promptly collected. Fresh colon and ileum samples were collected, stored in krebs buffer temporarily, and immediately processed for use in Ussing chamber electrophysiology. Plasma was collected as referred to above in the tail-bleeds methods.

2.11 Plasma Corticosterone

Corticosterone quantification of plasma samples obtained at timepoint 0 (immediately preceding the forced swim test), and then 30, 60, and 90 minutes post-forced swim test,

was performed using a corticosterone ELISA (Enzo Life Sciences, ADI-901-097), and was carried out according to the manufacturer's guidelines. Duplicates of each sample were run. A multi-mode plate reader (Synergy HT, BioTek Instruments) was used to quantify light absorbance in the assay, at 405 nm. Only data derived from duplicates with <20% CV were included in the analysis. Concentrations of plasma corticosterone was expressed in pg/mL.

2.12 Assessment of Intestinal Permeability using Ussing Chamber Electrophysiology

In order to assess intestinal permeability, Ussing Chambers were utilized. Distal ileum (1.5cm segment taken 1cm proximal to caecum) samples were mounted into the Ussing chambers with 4mm round aperture (0.126cm2 exposed tissue area) following seromuscular stripping. Krebs buffer (1.2mM NaH_2PO_4, 116mM NaCl, 4.8mM KCl, 1.2mM $MgCl_2$, 25mM $NaHCO_3$, 2.5mM $CaCl_2$) was added to both the mucosal and serosal chambers: krebs buffer contained 10mM D-glucose in the serosal chamber and 10mM mannitol in the mucosal chamber to avoid activation of sodium/glucose co-transporters in the epithelial cells of the small intestine, which is known to increase tight junction permeability (Turner et al., 1997). Chambers were continuously supplied with carbogen (95% O_2 and 5% CO_2). Short-circuit current (Isc) was recorded in a zero-voltage clamp mode; transepithelial electrical resistance (TEER) was measured by discharging a 2 mV pulse. Transepithelial permeability was investigated by measuring mucosal-to-serosal flux of 4kDa fluorescein isothiocyanate (FITC)-dextran (FD4, Sigma-Aldrich, Ireland). FITC was added to the mucosal chamber to a final concentration of 2.5mg/ml and 200µl samples were taken from the serosal chamber every 30 minutes for the

following 120 minutes. Samples taken were measured at 485nm excitation/535nm emission wavelengths. Following the final FITC sample, 10uM carbachol was added to the serosal chamber to confirm tissue viability.

2.13 Statistical Analysis

Statistical analyses was conducted using SPSS v.28 (IBM, USA). All values were calculated as means + standard error of the mean (s.e.m.). Normality was assessed employing the Shapiro-Wilk test. Repeated measure ANOVA was used to test for effects in the contextual fear conditioning paradigm, with an uncorrected Fisher's least significant difference (LSD) *post hoc* test used to check for differences between groups at timepoints. One-way ANOVA was used to assess difference between groups in the OFT, EPM, and FST. 2-way ANOVA was employed in the 3-chamber sociability test. A mixed-effect analysis of a curve was used to analyze the corticosterone concentration over time. Outliers in the behaviour and corticosterone results were excluded when values were greater or less than 2 standard deviations of the mean. Statistical significance was set at $p \leq 0.05$.

3. Results

3.1 SPS with and without anaesthesia exposure induces reduction in fear extinction

The contextual fear conditioning paradigm was run to determine what effects traumatic stress has on the fear memory and fear extinction of rats. Animals that underwent SPS, both with and without isoflurane, had significantly reduced ability to extinguish the fear memory (Figure 2). The level of extinction is measured by freezing behaviour, which is a fear response that is species-specific for these rodents and is considered to be a total lack of movement aside from respiration. Freezing is a direct result of fear that is conditioned to an external or contextual stimuli (Fanselow, 1980). On the Acquisition day, a repeated measures ANOVA revealed significant effect of time ($F=426.1$, $p<.0001$). A repeated measures ANOVA revealed a significant effect of time on each of Extinction Day 1-6 (Ext 1-4: $p<.0001$; Ext 5: $p=.003$; Ext 6: $p=.030$. Extinction Day 4 and 5 also had a significant effect of Group (Ext 4: $p=.040$; Ext 5: $p=.042$). Extinction Day 6 showed an effect of time x group ($p=.027$). Post-hoc analysis of extinction Day 2 found a significant difference between control and SPS-Iso at minute 2 ($p=.0146$), and between control and SPS-No Iso at minute 4 ($p=.029$). Extinction Day 4 post-hoc tests identified a significant difference between control and SPS-No Iso at minute 2 ($p=.029$), and a significant difference between control and both SPS-Iso and SPS-No Iso at minute 4 (SPS-Iso: $p=.031$; SPS-No Iso: $p=.020$). Post-hoc analysis of Extinction Day 5 showed a significant difference between control and SPS-No Iso at minute 2 ($p=.023$). A repeated measure ANOVA of Extinction Total, assessing all days of the extinction process, showed a significant effect of day ($F=52.32$; $p<.0001$) and group ($F=4.89$; $p=.014$). Post-hoc

analysis of Extinction Total expressed a significant difference between control and both SPS-Iso and SPS-No Iso on Day 2 (SPS-Iso: p=.040; SPS-No Iso: p=.040), Day 3 (SPS Iso: p=.036; SPS-No Iso: p=.003), and Day 4 (SPS-Iso: p=.017; SPS-No Iso: p=.045). A repeated measure ANOVA of Reinstatement identified a significant effect of time (F=223.8; p<.0001) and time x group (F=2.743; p=.002). Post-hoc analysis revealed a significant difference between control and SPS-Iso at minute 5 (p=.034). (**Figure 2**).

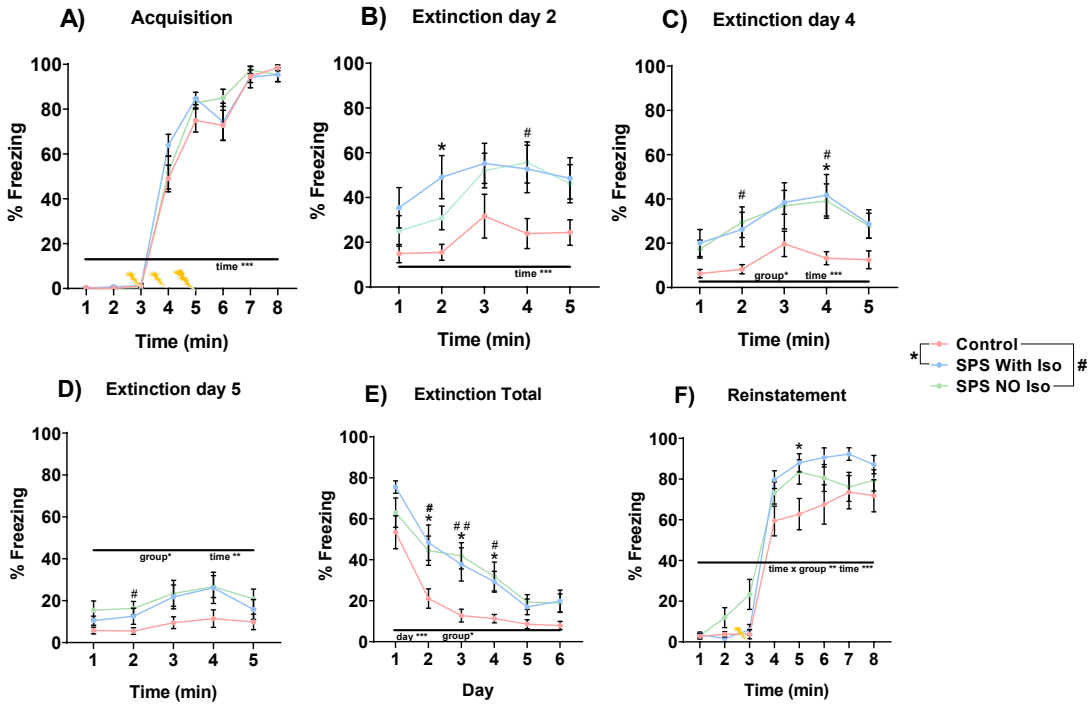

Figure 2: Contextual Fear Conditioning

Freezing % in the Contextual Fear Conditioning Paradigm: **A)** Acquisition Phase: significant effect of time (p<.0001). **B)** Extinction Day 2: Significant effect of time (p<.0001); significant difference between control and SPS with Isoflurane at minute 2 (p=.0146); between control and SPS without Isoflurane at minute 4 (p=.0291). **C)** Extinction Day 4: Significant effect of time (p<.0001), and group (p=.0401). Significant difference between control and SPS-Iso at minute 2 (p=.0287), between control and SPS-Iso (p=.0312) at minute 4, and between control and SPS-No Iso at minute 4 (p=.0201). **D)** Extinction Day 5: Significant effect of time (p=.0029), and group (p=.0419). Significant difference between control and SPS without Isoflurane at minute 2

(p=.0234). **E)** Extinction Total: Significant effect of day (p<.0001), and group (p=.0138). Significant difference between control and SPS-Iso on day 2, 3, and 4 (p=.0398, p=.0355, p=.0172) and between control and SPS-No Iso Day 2, 3, and 4 (p=.0400, p=.0025, p=.0448) **F)** Reinstatement: Significant effect of time (p<.0001), and time x group (p=.0019). Significant difference between control and SPS-Iso at minute 5 (p=.0338).

3.2 SPS with Isoflurane only induced changes in coping behaviour in the forced swim test

The forced swim test was run to assess the level of coping behaviour in the rodents. For the SPS animals, the forced swim test was their third exposure to a swimming scenario; the first being during the initial stressor, the second being the forced swim pre-test, and the third being the forced swim experimental test. A one-way ANOVA showed a significant difference in immobility between the three groups (p<.0001, F=12.65); with a Tukey's multiple comparison test showing a significant difference between SPS-Iso and control (p<.0001), and between SPS-Iso and SPS-No Iso (p=.008). SPS-Iso had significantly reduced immobility time compared to each of the other two groups. Further to this, a one-way ANOVA showed a significant difference in swimming time between the three groups (p<.0001, F=17.71); with a Tukey's multiple comparison test showing a significant difference between SPS-Iso and control (p<.0001), and between SPS-Iso and SPS-No Iso (p=.001). SPS-Iso had a significantly increased amount of swimming compared to each of the other groups. (**Figure 3**)**.**

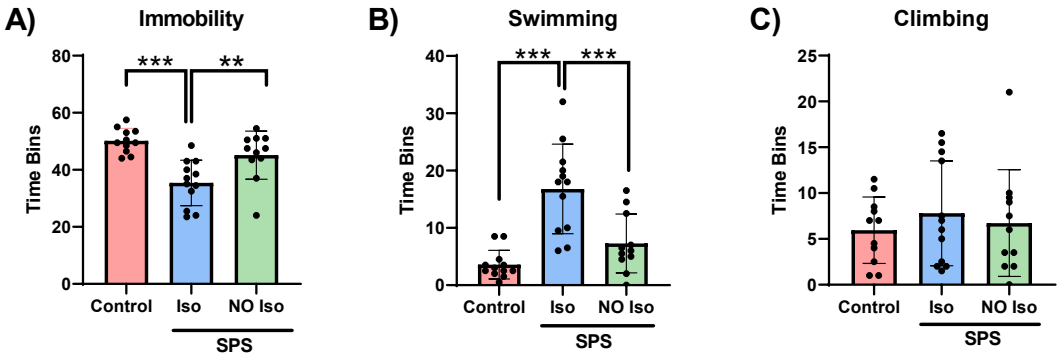

Figure 3: Effect of SPS with or without Isoflurane on Forced Swim Test

A) Immobility was significantly difference across the three groups (p<.0001), with a SPS-Iso being significantly lower than control (p<.0001) and SPS-Iso being significantly lower than SPS-No Iso (p=.0077). **B)** Time spent swimming significantly differed across the three groups (p<.0001), with SPS-Iso being significantly higher than control (p<.0001), and SPS-Iso being significantly higher than SPS-No Iso (p=.0008). **C)** No significant differences were seen in climbing time.

3.3 Effects of SPS on Corticosterone concentration

ELISA analysis of plasma corticosterone concentration was performed to identify potential hyperactivation or hypoactivation of the HPA axis following the FST. A one-way ANOVA showed a significant difference between groups at 90 minutes post-FST (p=.034), with the SPS-Iso group having a higher corticosterone concentration compared to controls (p=.051). Whereas, compared to SPS-No Iso rats the difference does not reach statistical significance (p=.063). A mixed-effects analysis of the curve of plasma corticosterone concentration over time (T0-T90) revealed a significant effect of time (p<.0001) and significant time x group effect (p=.003). (**Figure 4**).

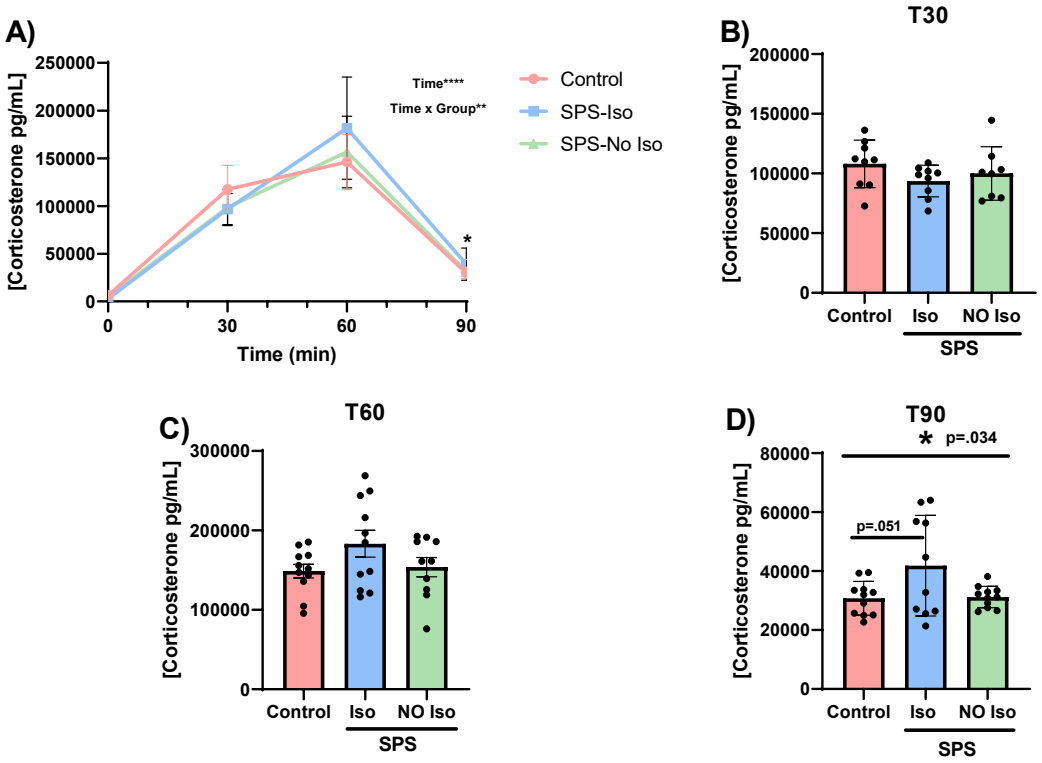

Figure 4: Effects of SPS with or without isoflurane anesthesia on Corticosterone Concentrations

A) A significant effect of time (p<.0001) and a significant effect of time x group (p=.0030) was seen in the concentration of corticosterone between T0-T90. B,C) No significant differences found at these timepoints. However, apparent resilient vs susceptible populations appear to diverge at T60 into T90. D) Timepoint 90 specifically, with ANOVA showing a significant difference across groups of p=.0337, and the difference between Control and SPS-Iso having a p-value of p=.051.

3.4 Effects of SPS on anxiety behaviour in the Elevated Plus Maze

The elevated plus maze was performed to assess anxiety-like behaviour in the rats. SPS-Iso rats showed induced elevated anxiety-like behaviours as shown in the total movement in the open arms of the maze, as a One-way ANOVA indicated a significant difference between groups (p=.026), and post-hoc showing SPS-Iso significantly lower than SPS-No Iso (p=.031) and trending lower than control (p=.081). Further to this, there appeared to be a small reduction in time spent in the open arms, however a one-way ANOVA did not show a significant difference (p=.141). (**Figure 5**).

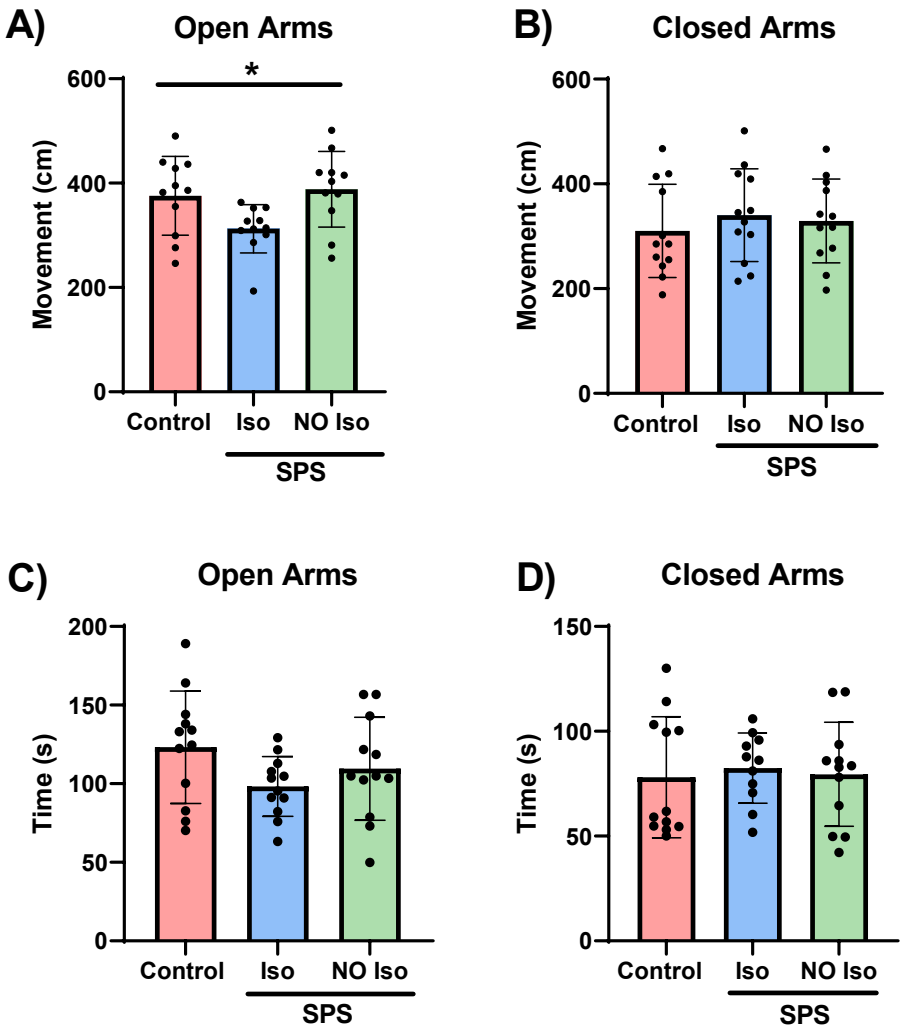

Figure 5: Effect of SPS with or without Isoflurane on Elevated Plus Maze

A) A significant difference across groups was seen in movement in the open arms (p=.0259), with SPS-Iso being significantly lower than SPS-No Iso (p=.0307), and a trending difference between control and SPS-Iso (p=.0805). **B)** No significant difference was found in movement in the closed arms. **C, D)** No significant difference was seen in time spent in the open arms or closed arms.

3.5 SPS did not induce changes in the Open Field Test, 3-Chamber Sociability Test

Analysis of the open field test did not show any significant differences in the major outputs. Time spent in the centre area (p=.203), entries into the centre area (p=.888), and movement in the centre area (p=.712) were all found to not be significant. (Figure 6)

The 3-chamber sociability test was run in order to assess whether SPS induced changes in social preference. Results showed that all rodents showed a normal preference for social novelty, regardless of group. (**Figure 6**).

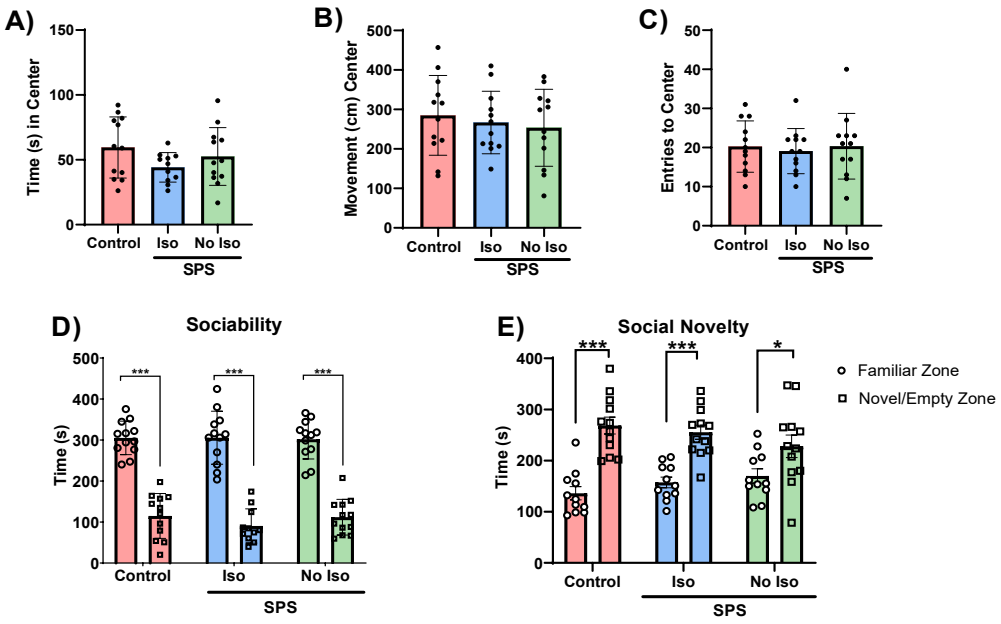

Figure 6: Effect of SPS on Open-Field Test, 3-Chamber Sociability

A) No significant difference was seen between groups in time spent in the center of the arena. **B)** No significant difference was found between groups in movement in the center of the arena. **C)** No significant difference was seen in the number of entries into the center of the arena.

All rats showed a preference for novel social interaction, across all groups and across both tests. **C)** No significant difference was seen across the groups in the sociability phase. **D)** No significant difference was found across groups in the social novelty phase.

3.6 Effects of SPS on intestinal permeability

Given that stress has been shown to affect gut permeability, analysis was done in order to see if the SPS with or without isoflurane model induced any changes in gut permeability. SPS with/without isoflurane exposure did not induce FITC or intestinal TEER alterations compared to control animals. However, rodents undergoing the FST immediately preceding the culls and tissue collection potentially impacted the gut permeability. (**Figure 7**)

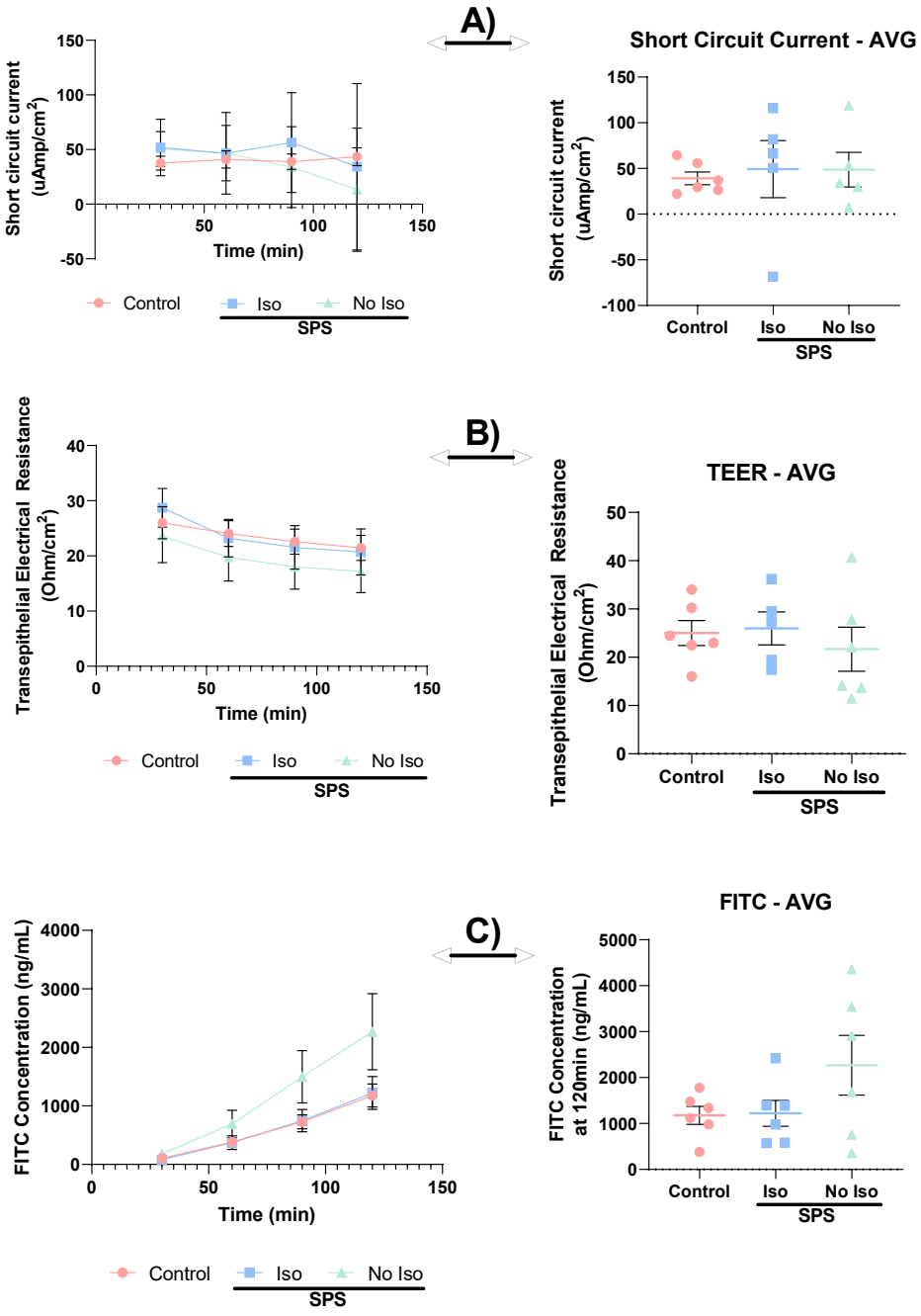

Figure 7: Effect of SPS with or without Isoflurane on intestinal permeability

A) No significant difference was found in short current circuit across the groups. **B)** No significant difference was seen across groups in TEER. **C)** No significant difference was seen across group in FITC concentration.

4. Discussion

The continuous development and refinement of animal models is key to generating a fuller knowledge of the capabilities of each model and understanding of the pathologies or disorders which they represent. While the traditional model of SPS using diethyl ether is well-established, it leaves room for refinement. This avenue for refinement and an alternative point of view inspired the exploration of using SPS with isoflurane, and also investigating the efficacy of SPS without the final gaseous stressor.

The use of fear conditioning paradigms is a commonly used measure for the efficacy of the SPS model (Ferland-Beckham et al., 2021; Lisieski et al., 2018; Souza et al., 2017; Yamamoto et al., 2009), as impaired ability to extinguish fear is a hallmark feature of PTSD and the traditional SPS model (Dayan Knox et al., 2012; Milad et al., 2009; Norrholm et al., 2011). Our results, using SPS with and without isoflurane, indeed show that our traumatic stress model had a significant effect on the ability of a rodent to extinguish fear. This aligns with the existing knowledge of the adverse effect of stress on fear extinction (Maren & Holmes, 2016), characterized by decreased ability to adequately extinguish fear. Similar to prior results in the traditional SPS model which show impairments in fear extinction (Dayan Knox et al., 2012), our SPS with and without Isoflurane model produce results also showing the impaired extinction of fear throughout the paradigm. Throughout the extinction phase of the paradigm, we found that the presence of isoflurane was not a key component of the fear extinction, as both the SPS-Iso and SPS-No Iso groups were seen to have significant reductions in fear

extinguishing capabilities throughout the extinction phase, with both groups overall having significant differences on days 2, 3, and 4. However, on the day of reinstatement, it was only the SPS-Iso group that was found to be significantly different at any timepoint. While SPS-No Iso did trend towards difference from control, it was only SPS-Iso group at minute 5 that resulted in a significant difference in freezing behaviour, representing inhibited fear extinction. Taken together, this model can be considered an effective model of PTSD in the context of impaired fear-related behaviour.

As seen in the forced swim test, the ability of the SPS-Iso rats to cope during the swim appeared to be significantly greater than that of the SPS-No Iso and control rats. SPS-Iso rats had significantly reduced immobility time in the FST, and this was paired with significantly increased time spent swimming, when compared to both controls and SPS-No Iso. Immobility and swimming measures in the FST are representative of relative learned helplessness and depressive-like behaviours (Slattery & Cryan, 2012), and stress generally induced increased immobility and decreased swimming (Bessa et al., 2009; Slattery & Cryan, 2012). The results from our study are of stark contrast to this, as the SPS-Iso group show the inverse effect. Initially, it was considered that this was the SPS group's third exposure to a swim; however, this was the case for both the SPS-Iso group and the SPS-No Iso group. Isoflurane has been seen to have anti-depressant like capabilities (Zhang et al., 2019), and specifically induced an anti-depressant effect in the context of learned helplessness (Antila et al., 2017; Brown et al., 2018) therefore, it is possible that the exposure to isoflurane shortly following the group swim during the SPS

model lead to a resilient effect associated to the swim, manifesting itself in the ability for this group to cope with the swim. Further, isoflurane was seen to induce a reduction in immobility time following a chronic unpredictable mild stress paradigm (Zhang et al., 2019), however the exposure was shortly before the testing, whereas in our study the isoflurane was part of the stressor, 12 days prior to the FST. The hypothesis that the isoflurane was determinant for this effect in our FST can be further purported by the fact that not only was the SPS-Iso group significantly more capable of coping measures compared to control, but also significantly more resilient than the SPS-No Iso group. This suggests that the isoflurane exposure had a significant effect on the outcome of the FST, in the form of a protective coping phenotype. This protective effect has not been seen in the SPS models using diethyl ether (Chen et al., 2021; Nie et al., 2021; Zhang et al., 2021), further suggesting that isoflurane exposure was the critical aspect of this result.

In most stress models, the HPA axis is found to be hyperactive; such that the corticosterone concentration of animals that have undergone stress will be typically greater than that of controls (Guilliams & Edwards, 2010; Herman et al., 2016), as stress induced a dysregulation of the HPA axis. However, in the context of PTSD, the HPA axis has been found to be hypoactive (D'Elia et al., 2021; Richter-Levin et al., 2019), as a result of high sensitivity of the glucocorticoid receptor and negative feedback control inhibition in the pituitary gland, which causes low level of cortisol circulating in the bloodstream (Yehuda, 2002). A related hormonal imbalance ties to negative alterations in cognition and mood, alterations in arousal and activity, and intrusive/avoidance symptoms (D'Elia

et al., 2021). While our contextual fear conditioning results showed that the model was valid in the context of fear extinction deficits, we found that there was not a significant hypoactivation of the HPA axis as seen in prior PTSD literature. However, this hypoactivation of the HPA axis is inconsistently seen (Lisieski et al., 2018; Meewisse et al., 2007; Wheler et al., 2006). While there was a significant effect of time over the 90 minutes, and a significant interaction effect between time and group, there was only one timepoint in which the corticosterone concentration in any group was significantly different, and it was T90. At T30, there was a clustering of SPS-Iso, at T60 and T90, an apparent split of resilient vs susceptible populations became apparent within the SPS-Iso group. The control and SPS-No Iso groups remained relatively consistent and similar in concentration throughout. The experience of prior behaviour testing (OFT, EPM, and 3-chamber), as well as a lengthy time from stressor (12 days), and third exposure to a swim could be contributing factors as to why the response of the HPA axis was muted, but as previously mentioned, this effect is inconsistently seen within PTSD, encouraging further exploration into the effect of SPS with and without isoflurane on corticosterone.

There is a relative absence of literature surrounding the effect of PTSD on gut permeability, specifically in the context of the SPS model. Severe psychological stress has been seen to have a strong impact on gut permeability and function (Baranyi et al., 2021; Rezzi et al., 2009), and changes in the gut microbiota have been recently discovered to be relevant in the SPS model (Tanelian et al., 2022). In our investigation, changes in gut permeability were not found between groups. However, a potential confound for this

could be that all of the animals, regardless of group, underwent the forced swim test (both pre-swim and experimental swim) within approximately 24 hours of tissue collection and gut permeability analysis. This was indicated as control rats in this study had different outputs to naïve control rats from other in-house studies. With the knowledge that stress impacts gut permeability at baseline (Demaude et al., 2006; Wilmes et al., 2021), it is to be assumed that the stress of the forced swim test potentially muted the SPS effects. Future studies with dedicated naïve control groups will be beneficial in assessing the role of SPS in intestinal permeability.

PTSD is characterized by and often comorbid with other psychopathologies, such as anxiety and depressive-like behaviour, social inhibitions, and other mood disorders (Brady et al., 2000; Fond et al., 2014; Gradus et al., 2015; Qassem et al., 2021). As a result, we aimed to identify whether the SPS with/without Isoflurane model would recapitulate phenotypes seen both in PTSD itself, and in comorbidities. SPS is an established PTSD model, but there can be inconsistency in the ability to recapitulate all behavioural phenotypes to a significant level (Eagle, Knox, et al., 2013; Harvey et al., 2006; Lisieski et al., 2018; Lisieski & Perrine, 2017), as the potential for resilience and susceptibility is ever present. This is realistic to the nature of traumatic stress and PTSD, as not each case is identical. Indeed, it is true of PTSD itself that while nearly 70% of individuals experience a traumatic event in their lifetime (Watson, 2019), the lifetime prevalence of PTSD is much lower, near 4% (Koenen et al., 2017). As such, demonstrations of the phenotypes of PTSD in an animal model are certain to be

inconsistent, yet vital to assess. In the elevated plus maze, an indication of anxiety-like behaviour was seen in the SPS-Iso group, where a significant difference across the groups was seen for total movement in the open arms, with SPS-Iso showing the most reduced amount of movement. Further, when individually compared to controls, the SPS-Iso time spent in the open arms was significantly lower, however across all three groups no significant difference was seen. In the Open-field test, however, no indication of anxiety-like behaviour was seen in any measure. The 3-chamber sociability test showed that all animals, irrespective of group, had a strong preference for the novel animal in their environment. Detriments to social preference have been seen following SPS (Eagle, Fitzpatrick, et al., 2013), however this effect was not reproduced in this current study.

5. Conclusion

SPS with and without isoflurane induced significant reduction in fear extinction, further confirming the relationship between trauma and fear memory; moreover, validating the efficacy of the SPS model using isoflurane in the focus of contextual fear conditioning. As well as this, significant changes in the forced swim test were seen, with isoflurane appearing to induce a stress coping resilience to learned helplessness. Anxiety-like behaviour measures trended upwards in the SPS-Iso group.

Overall, the use of isoflurane in the SPS model lead to varying and interesting results. In the context of contextual fear conditioning, our hypothesis was confirmed. Further investigation using this model is needed to validate its relevance to PTSD, including assessment of neuronal circuitry and other biomarkers.

Chapter 4:

General Discussion

1. Summary

Validated and efficient pre-clinical animal models are crucial for developing novel therapeutic avenues in a great variety of psychological and physiological disorders. As the general population continues to boom, prevalence of mental health ailments increases, and focus on mental and physical well-being continues to sharpen, the need for novel, accessible, and effective treatment modalities is rising exponentially. Complex mental health disorders, specifically trauma-related disorders such as PTSD, continue to have insufficient pharmacological treatment options (Berger et al., 2009), encouraging focused research into amending this therapeutic shortcoming. We explore trauma-related disorders in two different avenues, one via observation or secondary traumatic stress [CRD Observer], and one through direct experience by enduring a traumatic stressor with the intent of inducing PTSD-like characteristics [SPS with and without Isoflurane].

Key outputs of animal models are very often identified behavioural changes. Investigating behavioural differences can provide face validity as to the phenotypes that are displayed in the relevant human disorder – in this case trauma-related disorders. The typical models for understanding the transference of pain and fear, or in effect a secondary traumatic stress-like event, incorporate specifically observed fear, commonly in the form of foot shocks (Kim et al., 2019). Pain and fear go hand in hand, such that it is the induction of a painful stimulus (i.e. foot shocks) that generates the fear. Foot shocks, or other pain induction methods such as formalin injections, produce somatic

pain rather than visceral pain. These transferences of somatic or nerve pain have been seen to induce alterations at the neural, behavioural, and hormonal level (Baptista-de-Souza et al., 2022; Mogil, 2012). However, the information about visceral pain transmission remained untapped, until our CRD observer study. We hypothesized that the observation of visceral pain behaviours would induce hyperalgesia. Not only did we confirm this but discovered further neurobiological changes as a result of the observation.

After investigating the role of observation of stress on visceral pain, we identified that the observation of visceral pain behaviour induced significant hyperalgesia. This was seen in both the threshold for showing visceral pain behaviours, and the total number of visceral behaviours elicited during in the CRD paradigm. The visceral pain hypersensitivity we report supports prior studies that showed rodents exhibiting an empathetic transference of pain (Baptista-de-Souza et al., 2015; Fatemeh Mohammadi et al., 2020), but we demonstrate for the first time specifically transference of visceral pain inducing hyperalgesia. Further to this, we saw a correlation between behaviours that the observer expressed during the observation period and the extent to which the stimulus animal – whom the observer watched – expressed pain. This is evidence to an active empathetic relationship between the rodents – the observer witnessing the other rodent undergo a potentially traumatic event induced exaggerated behavioural changes both during the observation and during the testing 24 hours later. While attributing these results entirely to empathy is not possible based solely on this one study, the

evidence we have found combined with the relevant studies previously mentioned would suggest that empathy plays a significant role in the proceedings and outcome. Further work, with emphasis on more control groups that, for example, could be control for the different sensational stimuli, levels of interaction, familiarity with other animals etc., could help identify the relative strength of the empathetic relationship and the specifics of determining factors.

Identifying these behavioural changes is crucial, as it shows distinct and measurable phenotypic changes as a response to a traumatic experience. In the case of the CRD observer study, the significant changes were induced by a secondhand experience, whereas in the SPS with and without Isoflurane study, our behavioural changes came as a result of direct experience and undergoing a traumatic stressor. Likewise, the behavioural changes were significant and were identifiable with phenotypes seen in trauma-related stress disorders.

A primary output of the SPS model, closely related to PTSD phenotypes, is impaired fear extinction and learning (Ferland-Beckham et al., 2021; Dayan Knox et al., 2012). To assess this, contextual fear conditioning paradigms are put to use. Our results show that both SPS with and without isoflurane induce significant deficits in the ability for rats to extinguish fear. This was seen over the course of the six extinction days, as well as the final reinstatement phase of the contextual fear conditioning paradigm. These results support the traditional SPS literature (Giustino & Maren, 2015; Knox et al., 2016; Milad et al., 2009), in that the traumatic stressor induces reduced fear extinction. Interestingly, we found that the presence of the anesthetic portion of the SPS was not a key variable

in the fear extinction deficits throughout the extinction phase of the paradigm, as a significant reduction in ability to extinguish fear was seen in both groups. However, in the reinstatement phase only the SPS with Isoflurane group exhibited the diminished fear extinction. Considering these results, we can conclude that the SPS model using isoflurane instead of diethyl ether would be a valid model within the context of assessing rodent fear learning and extinction.

In the forced swim test, a behavioural test used to identify coping ability and learned helplessness (Slattery & Cryan, 2012) in a re-exposure to trauma, isoflurane appeared to have a protective effect in the context of learned helplessness. Rats that underwent SPS with isoflurane had reduced immobility time and increased swimming time, which can suggest that these animals show a greater resilience to learned helplessness, which was not observed in the other groups. While stress typically increases measures of learned helplessness and depressive-like behaviour, which manifest as exaggerated immobility in the FST (Bessa et al., 2009; Slattery & Cryan, 2012), we found the opposite effect only in SPS with Isoflurane rats. These results suggest a protective capability of isoflurane in this situation. Given the relevance of anxiety and social detriments in PTSD, analysis of behavioural tests that reflect the anxiety-like behaviours and social deficits seen in PTSD were run. However, only in in the EPM was a significant difference found, as SPS with Isoflurane rats exhibited amplified anxiety-like behaviour. Behavioural results in the fashion of recapitulating these extenuating PTSD phenotypes are inconsistent (Eagle, Knox, et al., 2013; Lisieski et al., 2018), reflecting the inconsistency of PTSD as a human disorder.

A common avenue of interest for animal models is through the stress-response system – the HPA axis. The HPA axis is central to stress response, and chronic activation of this system can cause significant dysregulation. Investigation of the HPA axis in the CRD observer study showed that observer animals had a significantly reduced corticosterone concentration 90 minutes following CRD. While some studies have shown that only observation of stressful events did not induce a hypoactivity of the HPA axis (Li et al., 2014; Patki et al., 2015), our results showed one exposure to observation of visceral pain lead to an acute hypoactivation of the HPA axis, similarly to what is seen in traumatic stress models (Yehuda, 2002). Interestingly, this deviation of the HPA axis was paired with a significant reduction in c-Fos positive cells in the PVN. The PVN is at the forefront of the stress response, as it initiates the commencement of the stress response by releasing CRH (Herman et al., 2016). Seeing that this model has produced both hypoactivity of the PVN and the HPA axis and given that the PVN modulates the HPA axis stress response, it is left to be understood that these results go hand in hand. This encourages focused investigation on the neural pathways of the PVN in an observational paradigm, and the connection between visceral pain significance and HPA function. While significant changes were identified in this study, the SPS with and without Isoflurane did not provide an expected output in regard to corticosterone concentration. 90 minutes following the FST, SPS with Isoflurane rats had the higher plasma corticosterone concentration than both other groups, which is contradictory to typical PTSD models (D'Elia et al., 2021; Richter-Levin et al., 2019; Yehuda, 2002), where the HPA axis is found to be hypoactive. It is quite intriguing that the PTSD-related

hypoactivation of the HPA axis was identified in the very acute CRD observer model, while in the traditional PTSD model of SPS, this hypoactivation was not to be found. While there are potential confounds, including exposure to the FST immediately preceding, and the use of isoflurane, it still interesting that this specific change was not seen.

Essential to understanding the neurobiological underpinning of trauma-related disorders is analysis of neurocircuitry. Neuronal activation patterns, measured by analysis of c-fos activation, can provide valuable insight. In the CRD observer study, c-Fos positive cells were found in a greater abundance in the ACC of observer animals compared to control animals. This is intriguing, as the ACC is central to empathy (Lockwood et al., 2015; Morrison et al., 2004), as well as physical, psychological, and visceral pain (Meerwijk et al., 2013; Moloney et al., 2016). Visceral hypersensitivity is also well known to be linked with increased ACC activity, specifically following colonic distension (Felice et al., 2014; Gibney et al., 2010). Hyperactivation of the ACC is also described when observing pain (Marsh, 2018), as the ACC is similarly activated in situations of both experienced and observed pain (Corradi-Dell'Acqua et al., 2011; Yesudas & Lee, 2015). While it would be expected that CRD alone would induce an increased level of activation of the ACC, we nevertheless found that the observer animals still had a greater level of hyperactivation compared to controls. As the control underwent CRD as well, it suggests that it was the observation that lead to the comparative hyperactivation of the ACC. As mentioned above, the PVN also had significant changes in neuronal activation. This paired with the relative HPA axis

hypoactivation, and as such felt appropriate to reference this neurobiological output in that context.

Considering all results, the animal models we employed showed strong validity and inspire further exploration. Changes in behaviour relevant to what is seen in trauma-related disorders, as well as endocrine and neurobiological marker changes were identified. The novel CRD observer study encourages deeper investigation into the empathetic transference of visceral pain and secondary traumatic stress, while the SPS with or without Isoflurane model could be considered an effective model of PTSD within the scope of fear learning and extinction. Inconsistencies in behavioural results lead to further questions as to its efficacy in recapitulating all of the phenotypes of PTSD and its comorbidities. However, these inconsistencies exist also within the classical SPS paradigm that uses diethyl ether. There are shortcomings in the use of the Isoflurane model, for example as seen in the forced swim test, where contrary results were found, evidently due to Isoflurane itself. While this may be a shortcoming for its use in the specific learned helplessness marker, it does not preclude its use overall as it still was effective in recapitulating PTSD phenomenology in the context of fear extinction and learning. It also provides an avenue of research for the potentially protective effect of Isoflurane in the FST and would be worthwhile investigating this effect in a greater array of behavioural tests. As there are many different animal models for trauma and PTSD, there is not one that stands consistently head and shoulders above the rest. This SPS with/without Isoflurane model provides an appropriate option in some contexts, and is worth further investigation as to how far-reaching its effectiveness can be.

2. Future Directions

Our novel model of secondary traumatic stress via visceral pain transmission in the CRD Observer study spurs further investigations using this model. As this was a pilot, it was ethically limited in regard to treatments, overall n number – leading to a reduction in the possible variation of control and experimental groups -, and potential two-hit effects. Now that the model has been validated for its desired effect – the successful transmission of visceral hyperalgesia accompanied with neurobiological changes, further explorations are of exciting prospect.

With there being behavioural, neural, and endocrine changes, the potential avenues for exploration are plentiful. To further understand the behaviour changes, both in regard to visceral hypersensitivity and the active empathetic behaviours, an interesting route of investigation would be to determine the sensory modality that was most critical for the transference. Rodents pick up on visual and auditory cues, as well as minute environmental changes, pheromones, sex differences, and levels of familiarity (Kim et al., 2019), so including controls or pairings to assess each of these would help to identify which is the strongest for this situation. In order to do so, using closed/sound-proofed arenas for auditory and olfactory limitation, fully opaque arenas for visual limitation, and variations to sex and familiarity both in the observer and stimulus would allow investigation into these modalities.

With the corticosterone quantification elucidating an apparent hypoactivity of the HPA axis, an interesting point of investigation would be assessing the HPA axis activation over

time. Identifying a 120-minute corticosterone concentration change, taking multiple timepoints throughout that time period [0, 30, 60, 90, and 120 minutes] would give insight into the time-dependent nature of the HPA axis stress response. Further, assessing corticosterone at timepoints on later dates would provide intriguing evidence in regard to the trauma and its relevance to PTSD. Considering the acute nature of this study, the hypoactivity could not be considered PTSD-related, as it was only 24 hours after the stressor. Analyzing the HPA axis at timepoints perhaps 7-14 days later would be of interest.

Similar to the CRD Observer study, the SPS with or without Isoflurane project was a pilot study, so there were limitations in the exploratory possibilities. With efficacy and significant results seen in some measures, further analysis of tissue taken from this study is highly encouraging, and even inspires further studies using this model.

The most immediate further step will be in exploration of the gut microbiome and brain tissue. Fecal samples were taken throughout the study in order to investigate the effect of traumatic stress on the gut microbiome over time. Given that stress impacts the gut microbiota (Cryan & Dinan, 2012; Dodiya et al., 2020; Foster et al., 2017) and that, in turn, the gut microbiota may impact susceptibility to traumatic stress (Tanelian et al., 2022), analyzing it with samples from this study is very intriguing. Whole brains were taken at the time of cull, allowing for investigation into the changes in neurobiology and neural activation. SPS, and by extension PTSD, induce significant like neural changes in many brain areas, including the PFC, hippocampus, and amygdala (Bremner, 2006; Eagle, Knox, et al., 2013; Kasai et al., 2008; Knox et al., 2016; Thomason & Marusak, 2017). By

digging deeper into specific brain-related PTSD-related targets in these regions, such as glucocorticoid receptors and specifically the PAI-1 protein (Bouarab et al., 2021), brain-derived neurotrophic factor and Pituitary adenylate cyclase-activating polypeptide which are particularly relevant for fear memory and extinction (Notaras & van den Buuse, 2020; Velasco et al., 2022), as well as inflammatory markers including IL-6 and TNF-α that are generally found to be elevated in PTSD (Hori & Kim, 2019), or indeed working in relevant gut-brain axis related targets such as the tryptophan/kynurenine pathway that has been implicated recently in PTSD (Dell'Oste et al., 2023), we can hope to attain a more comprehensive picture of the effects SPS with and without Isoflurane on the greater neural picture. Identifying whether the SPS with and without Isoflurane model reproduces the changes seen in the classical SPS literature and general PTSD literature will be a further step in validating this model.

3. Conclusion

We have now established a novel model of observed trauma and empathetic transmission of visceral pain. This novel model is a significant contribution to the literature, as it is the first of its kind, and is sure to have future greater scientific benefit. This development will allow for a more focused investigation into visceral pain and secondary traumatic stress. Further, we provided a validation for the use of SPS with isoflurane as a refinement, specifically in the context of understanding fear extinction and fear learning, which will be of benefit for research studies under ethical scrutiny. Further, we found very interesting effects of isoflurane in the context of protective capabilities in certain aspects of PTSD-like symptoms, such as learned

helplessness. This novel data will be useful for projects going forward that will use isoflurane as part of a stressor, but also potential side effects of use as anesthetic or otherwise. Overall, the studies in this thesis have produced novel information that expand the current scientific knowledge and literature of animal models of PTSD.

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