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An Investigation into Neuroimmune, Cognitive and Affective Behaviour after Chemotherapy Exposure in a Rat Model: Implications for Chemotherapy-induced Gut Toxicity and 'Chemobrain'

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TABLE OF CONTENTS

ABSTRACT

Patients receiving chemotherapy frequently experience debilitating side-effects. Side-effects such as chemotherapy-induced gut toxicity (CIGT) are acute, however chemotherapy-induced cognitive impairment (CICI) can persist for years following treatment. CIGT and CICI can significantly impact patient quality of life, thus understanding the aetiology and developing therapeutic strategies is critical. Animal models are commonly used in these investigations and are excellent for determining cellular and molecular mechanisms. However, translational validity may be poor if they do not consider actual impacts on the animal's cognitive and affective domains. Although there are a range of well-validated behavioural tests utilised in other research areas, these tests may be insensitive to subtle cognitive impairments observed in CICI and neglect to measure animal emotional experience.

Whilst the underlying mechanisms that contribute to CICI remain unclear, neuroinflammation has been postulated as a key contributor. It is hypothesised that chemotherapy agents can cause direct or indirect CNS damage, negatively impacting cognition. Therefore, peripheral inflammatory events such as CIGT may also play a role in CICI pathogenesis. Our understanding of the effects of chemotherapy agents on gut inflammation in CIGT have been well characterised, however the underlying mechanisms that contribute to CICI and resulting neuroimmune changes occurring in the CNS at acute and chronic time-points remain relatively unclear, as do the effects of palliative treatments such as opioids. Pain mitigation strategies typically involve administration of opioid agents and play a crucial therapeutic role for pain management, yet are largely understudied for their effects on neuroimmune responses and behaviour.

Subsequently, this thesis examined the effects of chemotherapy on neuroimmune outcomes and cognitive and affective behaviour in a rat model of CIGT and CICI. In chapter 2, the judgement bias task was utilised to investigate and validate a reliable test to measure the effect of chemotherapy on affective state in a rat model of 5-FU. Furthermore, the utility of the Rat Grimace Scale (RGS) was investigated for pain assessment in a tumour-bearing rat model of CIGT (chapter 3). A systematic review was undertaken to assess compliance of scientific publications evaluating CICI in rodent models with the 'Animals in Research: Reporting in vivo Experiments' guidelines (chapter 4). Additionally, a systematic scoping review (chapter 5) was conducted to identify and map available literature on neuroimmune reactivity marker expression changes and resulting cognitive changes, in preclinical rodent models of CICI. Chapter 6 comprised an experimental study assessing the effect of opioid co-administration with 5-FU on gut inflammation and architectural changes associated with CIGT, cellular changes in the CNS and associated cognitive changes utilising a tumour-bearing rat model. Additionally, neuroimmune reactivity marker expression changes and resulting cognitive changes were explored in a subacute and chronic rat model of MTX and 5-FU-induced CICI (chapter 7). Importantly, this was achieved through the use of cognitive behavioural tests to assess hippocampal and pre-frontal cortex functioning.

In summary, this thesis describes establishment of a validated and reliable judgement bias task and 5CSRTT as promising cognitive behavioural methods in preclinical models of CIGT and CICI. Furthermore, it identifies neuroimmune reactivity marker changes occurring in tandem with cognitive impairments. This has implications for future study design using animal models in CICI and CIGT to improve translational validity. Furthermore, it paves the way for further investigations of actions at the neuroinflammatory response as a potential therapeutic strategy in CICI.

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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~ This thesis is dedicated to my family, but most of all my mother ~

LIST OF PUBLICATIONS AND PRESENTATIONS BY CANDIDATE

Published Journal Papers

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George, R. P., Howarth, G. S., & Whittaker, A. L. (2019) 'Use of the Rat Grimace Scale to Evaluate Visceral Pain in a Model of Chemotherapy-Induced Mucositis'. *Animals*, *9*(9), 678.

George, R. P., Semendric, I., Hutchinson, M. R., & Whittaker, A. L. (2021). 'Neuroimmune reactivity marker expression in rodent models of chemotherapy-induced cognitive impairment: a systematic scoping review'. *Brain, Behavior, and Immunity.* DOI 10.1016/j.bbi.2021.01.021.

Journal Papers Under Review

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Supporting Journal Papers

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Conference Presentations

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CHAPTER 1

General Introduction

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1.1 Introductory background

1.1.1 Chemotherapy-induced toxicities

Patients receiving chemotherapy frequently experience undesirable and debilitating side-effects termed 'chemotherapy-induced toxicities'. These toxicities occur as a result of chemotherapeutic agents inhibiting proliferation and growth of cancer cells in addition to causing damage to normal functioning cells (Toale et al., 2016). The nature of these toxicities can range in severity and can manifest as short-term or long-term after remission. Often these toxicities cause increasing concern among patients undergoing chemotherapy treatment and can greatly affect patient quality of life. The gastrointestinal (GI) tract and central nervous system (CNS) are particularly vulnerable to undesirable toxicities of chemotherapy exposure (Kayl et al., 2006; Bajic et al., 2018). Side-effects impacting the GI tract are termed chemotherapy-induced gut toxicity (CIGT) and include nausea, vomiting and diarrhoea. These effects are generally short-lived and resolve once treatment has ceased. However, more chronic conditions such as chemotherapy-induced cognitive impairment (CICI) can persist for weeks, months or years following cessation of treatment. The nature of these side effects can lead to poorer adherence to treatment regimens or significantly impair patients' return to normal life post-treatment. The research presented in this thesis focused on both chemotherapy-induced gut toxicity and cognitive impairment with a focus on neuroimmune activation as a potential driving mechanism. Further, this research investigated CIGT and CICI with a focus on assessment of emerging methods to measure affective state and cognition in a rat model, to improve translational validity and improve animal welfare.

The research presented herein addressed the following aims:

1. Evaluate and validate a cognitive bias test to measure affective state in a rat model of 5-FU-induced CIGT.
2. Assess the utility of the rat grimace scale for pain assessment in a tumour-bearing rat model of 5-FU-induced CIGT.
3. Explore the effects of opioid agents that are commonly prescribed analgesics in cancer patients as modulators of neuroimmune expression and cognition in the context of CICI development and/or exacerbation.
4. Determine the extent of chemotherapy-induced cognitive impairment in an acute and chronic time course, as evidenced through impaired performance in cognitive tests assessing hippocampus and prefrontal cortex function, and associated neuroimmune reactivity marker expression changes in a rat model of CICI.

This chapter will firstly discuss the prevalence and pathogenesis of CIGT and CICI, with a focus on neuroimmune activation as a potential key contributor in the development of CICI. I will also discuss quality of life and mitigation strategies used in supportive care in cancer. Lastly, I will discuss animal models of CIGT and CICI, identify barriers associated with translational validity, and highlight potential methodologies to assess affective state and cognition in these animal models.

1.1.2 Chemotherapy-induced gut toxicity (CIGT)

Chemotherapy-induced gut toxicity (CIGT), previously termed mucositis, affects approximately 40-60% of patients receiving standard doses of chemotherapy (Gibson et al., 2013; Rubenstein et al., 2004). The condition can affect the entire GI tract and is characterised

by widespread damage and inflammation to mucosal surfaces (Vanhoecke et al., 2015). Severe abdominal pain is a predominant symptom of CIGT due to inflammation and ulceration of the GI tract. Other accompanying symptoms include diarrhoea, constipation, bloating, dehydration, vomiting, nausea, and diminished oral ingestion which can lead to malnutrition and severe weight loss (Mauger et al., 2007; Sultani et al., 2012). For patients with neutropenia, the ulcerative lesions pose a greater risk of causing secondary systemic infection, which can possibly be life threatening (Sonis, 2004). Duration and severity of CIGT differs depending on chemotherapeutic agent, dose and dosing regimen (Vanhoecke et al., 2015). Consequently, CIGT causes significant burden on the healthcare system and a substantial impact on patients' quality of life during treatment.

1.1.2.1 Mechanisms underlying CIGT

The development of CIGT involves complex cellular and molecular processes that involve several interacting pathways (Keefe, 2007; Sultani et al., 2012). The pathobiology of CIGT has been characterised into five biological stages including 1) initiation, 2) up-regulation and message generation, 3) signaling and amplification, 4) ulceration and inflammation, and 5) healing phase (Sonis, 2004). Initiation phase begins immediately after chemotherapy is performed and involves direct damage to cellular DNA, which causes instant damage or death to cells in the sub mucosa and basal epithelia (Sultani et al., 2012). If tissue damage is extensive reactive oxygen species (ROS) may also be generated, which further damages cells, excites macrophages, and activates numerous inflammatory pathways (Sultani et al., 2012).

During the up-regulation and message generation phase numerous signaling pathways and transcription factors are initiated. The activation of nuclear factor kappa-beta (NFκβ) facilitates gene expression, and the synthesis of pro-inflammatory cytokines such as tumour-necrosis

factor (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), adhesion molecules, and cyclooxygenase-2 (COX-2) from adjoining connective tissue (Sonis, 2004; Sultani et al., 2012). The third phase, signaling and amplification, involves amplification of the inflammation signal by pro-inflammatory cytokines that act in a positive feedback loop to support the NFκβ stimulation. This results in greater production of TNF-α, IL-1β, and IL-6, and increased apoptosis causing further tissue damage (Sultani et al., 2012). The ulceration and inflammation phase occurs when the integrity of the gastrointestinal epithelium is destroyed and leads to CIGT becoming symptomatic. As bacteria colonise the ulceration site, further inflammation occurs as a result of other inflammatory cells and macrophages entering the site of damage (Sonis, 2004; Sultani et al., 2012). CIGT is a self-limiting disease, which involves a natural spontaneous healing process characterised by intense proliferation of the intestinal epithelium once chemotherapy has ceased (Menezes-Garcia et al., 2018).

1.1.3 Chemotherapy-induced cognitive impairment (CICI)

With advances in chemotherapy treatments, the number of cancer survivors continues to increase, and more attention has shifted to focus on neurotoxic symptoms post-treatment as part of survivorship care plans. Chemotherapy-induced cognitive impairment, colloquially referred to as ‘chemobrain’, is a debilitating condition experienced during and after the administration of chemotherapy. The condition causes a variety of cognitive deficits including impairment to memory, reasoning, concentration, learning, executive function, attention, and visuospatial skills (Argyriou et al., 2011). It is estimated that 17-75% of patients will experience CICI, with side effects ranging from subtle and transient, to severe in some cases (Asher & Myers, 2015; Matsos & Johnston, 2019). Furthermore, the duration of CICI can occur 6 months to 10 years post-treatment (Boykoff et al., 2009; Ren et al., 2019). CICI has been reported in patients diagnosed with many different cancers, including breast cancer, leukemia,

ovarian cancer, prostate cancer, lymphoma and colorectal cancer (Lv et al., 2020). As a result of cognitive impairments, activities of daily life become more challenging, with patients' ability to transition back to work and maintain relationships impacted (Boykoff et al., 2009; Hodgson et al., 2013). Thus, CICI is becoming an increasing concern due to increased cancer survivorship, and the profound impact it can have on patient quality of life.

1.1.3.1 Brain regions and cognitive domains implicated in CICI

CICI affects several brain regions, with cognitive impairment occurring across numerous domains of cognition. Affected cognitive domains include attention, concentration, mental flexibility, visual memory, and information processing speed, which are predominantly based in the prefrontal cortex, frontal cortex, parietal and temporal lobes, indicating that damage or interference to these brain regions leads to the presenting symptoms observed in CICI (Figure 1) (Ahles & Saykin, 2007; Foley et al., 2008; Matsos & Johnston, 2019).

Neuroimaging techniques have been used in various studies to assess brain structure and function in patients receiving chemotherapy. These techniques have demonstrated changes in specific brain regions that are likely linked to the cognitive changes apparent (Foley et al., 2008; Inagaki et al., 2007). Studies have shown reduced grey matter density in multiple brain regions immediately post chemotherapy, which only partially recovers one-year post-treatment cessation (Nguyen & Ehrlich, 2020). Positron emission tomography (PET) imaging on breast cancer survivors has shown substantial changes in resting glucose metabolism and cerebral blood flow in the cerebellum and frontal cortex, which was associated with impairment in a short-term memory recall task (Foley et al., 2008; Silverman et al., 2007). Comparably, magnetic resonance imaging (MRI) on breast cancer survivors has shown a decreased volume in numerous brain regions linked to CICI, which were associated with poorer performance in

several working memory tasks, and indices of the Wechsler Memory Scale-Revised, including concentration, attention and visual memory (Foley et al., 2008; Inagaki et al., 2007).

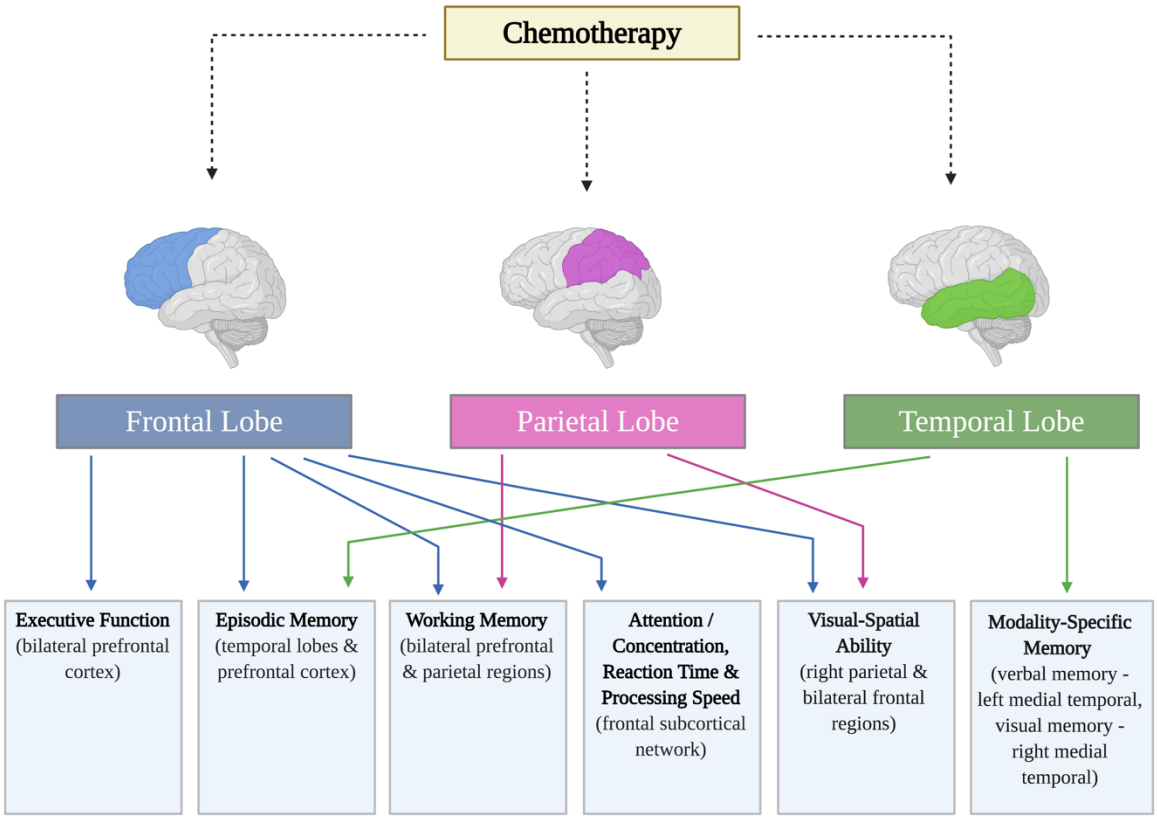

Figure 1. Brain regions implicated in CICI and associated affected cognitive function. Adapted from Ahles and Saykin 2007 (Ahles & Saykin, 2007) and Matsos and Johnston 2019 (Matsos & Johnston, 2019). Created with BioRender.com

1.1.3.2 CICI pathogenesis

Whilst there is considerable evidence on cognitive decline associated with chemotherapy exposure, the pathogenesis involved in the development of CICI remains unclear, although a number of potential mechanisms have been proposed. Neuroinflammation is a potential key contributor that may play an important role in the development of CICI. In particular, previous studies have suggested that some chemotherapy agents are able to cross the blood brain barrier (BBB) and cause direct damage to the CNS, or cause indirect damage via peripheral

mechanisms in the CNS, which in turn negatively impacts cognitive performance. It is postulated that chronic inflammation in the CNS contributes to memory and learning impairments that present in many neurodegenerative diseases, including Alzheimer′s disease (Nguyen & Ehrlich, 2020). Furthermore, elevated cytokine levels have been observed in patients with Parkinson's disease and Alzheimer′s disease, which have been linked to cognitive dysfunction (Nguyen & Ehrlich, 2020). Neuroinflammation occurs when an inflammatory challenge caused by an insult, such as infection or toxic metabolites, activates the innate immune system of the CNS. This activation leads to alterations in homeostasis, irregular protein aggregation, ischemic damage, trauma, aging, and specific disease states and toxins (McLeary et al., 2019).

Microglia and astrocytes are key regulators and contributors to the neuroimmune system within the CNS. Microglia are present in the white and grey matter of the brain and spinal cord, and act as the front line of defence, actively surveying the CNS environment in their ramified (resting) form for damage-associated molecular patterns (DAMPs), pathogen-associated molecular patterns (PAMPs), and to remodel synapses (Land, 2015; Loftis & Janowsky, 2014). Upon activation from either direct CNS damage (infection, disease, insult, drug exposure) or indirect causes via inflammatory peripheral events, microglia can exhibit one of two inflammatory profiles. The classical activation is a pro-inflammatory phenotype that removes unwanted pathogens, responds to tissue damage, clears debris and is involved in neuroinflammation by producing pro-inflammatory cytokines such as TNFα, IL-6, IL-1β, IFNγ and numerous chemokines (Ansar & Ghosh, 2016; Cherry et al., 2014; Salvi et al., 2017). Conversely, the alternate activation is an anti-inflammatory phenotype that acts in the inflammation resolution phase restoring homeostasis to the environment through the production of anti-inflammatory cytokines such as IL-10 and IL-4 (Ansar & Ghosh, 2016; Salvi

et al., 2017). Astrocytes are multifaceted cells that aid in maintaining homeostasis, supporting neurons and synapses, clearing synapses and regulating neuroimmune processes by responding to signals to perpetuate inflammation, or promote immunosuppression and repair (Colombo & Farina, 2016). Additionally, they are involved in the maintenance and permeability of the BBB and modulating the entry of peripheral immune cells into the CNS through the BBB to recruit additional support (Colombo & Farina, 2016; Gimsa et al., 2013). Much like microglia, astrocytes can act to exacerbate or suppress neuroinflammation through signalling pathways. Multiple products such as TGFβ, IFNγ and BDNF have been associated with reparative functions, and others including IL-17, VEGF, and NFkB have been associated with detrimental functions (Colombo & Farina, 2016). As such, when CNS insult occurs via indirect or direct chemotherapy exposure, microglia and astrocytes may become activated and overexpress pro-inflammatory mediators, leading to neuronal damage and a subsequent adverse impact on cognition. The role of neuroinflammation in development of CICI is described in depth in chapter 5.

In addition to neuroinflammation, other proposed mechanisms include: damage associated molecular patterns, disruption to the BBB, increased oxidative stress, decreased hippocampal neurogenesis, cellular metabolism and mitochondrial dysfunction, amongst others (McLeary et al., 2019; Mounier et al., 2020; Vichaya et al., 2015). These are important potential mechanisms however have not been specifically examined as part of this body of work.

1.1.4 Quality of life and mitigation strategies used in supportive care in cancer

A cancer diagnosis is often accompanied by impairment to quality of life (QoL) which begins at diagnosis, continues during treatment, and in some cases persists long-term post-treatment. Chemotherapy treatment can significantly affect patient QoL in both positive and negative

ways. Positive effects during treatment include treatment efficacy and cancer regression, use of a support object/activity, and positive effects on relationships (Sibeoni et al., 2018). While the impact of chemotherapy-induced toxicities caused by adjuvant chemotherapy not only affects patients' physical, but emotional and mental wellbeing, which significantly contribute to reduction of QoL (Sibeoni et al., 2018). CIGT poses a major clinical problem that increases risk of morbidities, with patients experiencing severe disruptions to QoL and negative impact on affective state (Mauger et al., 2007). In CICI, the magnitude of cognitive disturbances in executive function, memory, concentration, amongst others, often impact daily function, social engagement, returning to the workplace and community integration, and have been linked with poor QoL (Hutchinson et al., 2012). In recent years QoL has become a key objective in oncology care with increasing emphasis being placed on the importance of assessing patient QoL to relieve suffering, improve quality of daily life, manage symptoms and evaluate the effectiveness of treatment. Consequently, a number of informative QoL tools such as the Functional Assessment of Cancer Therapy-General (FACT-G) and the Multidimensional Quality of Life Scale-Cancer (MQOLS-CA) have been established for the purpose of assessing health-related QoL in cancer populations (Bell et al., 2018; Dodd et al., 2001). In CICI the Functional Assessment of Cancer Therapy Cognitive Function (FACT-COG, version 3) instrument has been developed to measure perceived self-assessments of cognitive impairments and cognitive abilities, impact on QoL and noticeability by others (Asher & Myers, 2015; Costa et al., 2018; Von Ah & Tallman, 2015). These health-related QoL tools have the ability to provide important therapeutic information that can aid in management of side-effects, advance clinical practice and improve QoL for patients undergoing chemotherapy and survivors without modifying chemotherapeutic efficacy.

The use of opioid analgesics is pivotal in effective pain management for cancer-related pain and side-effects of chemotherapy, including CIGT. Opioids are the most potent drug of choice for treatment of severe pain, however their use is accompanied by side effects such as nausea, respiratory depression, constipation and addiction (Stein et al., 2003). The MASCC/ISOO guidelines recommend use of opioids such as morphine and fentanyl due to their potent analgesic effects (White et al., 2011). Whilst our understanding of the clinical features of CIGT have increased over time, the role of opioids in CIGT and CICI progression and/or modulation remains relatively unknown.

It has recently been postulated that opioids, such as morphine, activate classic opioid receptors or toll-like receptors expressed on neuro-immune cells via central immune signalling events (Grace et al., 2015; Hutchinson et al., 2011; Wang et al., 2012). This opioid immune activation upregulates astrocyte and microglial reactivity markers which triggers the production of pro-inflammatory mediators such as cytokines and chemokines. Considering that the development of CIGT results in debilitating pain, and that supportive treatment includes opioid analgesics, it is not clear whether opioids may contribute to the development of CICI by central immune signalling, mediated by glia. In addition, it has been demonstrated that μ-opioid and κ -opioid agonists are able to exert anti-inflammatory effects via peripheral receptors (Philippe et al., 2003). However, anti-inflammatory properties of opioids remain relatively unrecognised due to a greater understanding of their analgesic effects and side effects they exert (Walker, 2003). Thus, the need to balance analgesic effectiveness and inflammatory properties is paramount to optimise analgesic treatment used in cancer care. As such, the research presented in chapter 6 explored the effects of opioid agents on gut inflammation and architectural changes in CIGT, along with neuroimmune and cognitive changes in the context of CICI development.

1.1.5 Animal models of CIGT and CICI

Animal studies are commonly used to elucidate the mechanisms and development of novel therapeutic strategies for both CIGT and CICI. Animal studies utilising rodent models are particularly valuable as they allow controlled and systematic study of desired research outcomes. Rodent models are commonly used in CIGT research and are well-established, with a variety of chemotherapeutic agents used to induce CIGT, including 5-fluorouracil (5-FU), irinotecan and methotrexate (MTX) (Vanhoecke et al., 2015). Furthermore, the Dark Agouti rat mammary adenocarcinoma model (DAMA) has been established to mimic the clinical setting and investigate gut toxicity and breast cancer tumours simultaneously (Vanhoecke et al., 2015). Similarly, both mouse and rat models are frequently used in CICI research, having been utilised to determine: the extent of chemotherapy-induced learning and memory deficits, the underlying neural mechanisms, and to link impairments with specific chemotherapy agents, combinations and dosages. However, currently there is no standardisation in CICI animal models with studies employing a broad range of chemotherapy agents and behavioural tests to assess impairment (Matsos & Johnston, 2019; Seigers & Fardell, 2011; Winocur et al., 2018). This heterogeneity in animal models mimics the clinical setting, where patient specific treatment is required, resulting in a variety of treatments, treatment regimens and patient experiences. Whilst these animal models have provided valuable information for determining cellular and molecular mechanisms via pathological outcomes, translational validity may be poor if they do not consider actual impacts on the animal's cognitive and affective domains. Additionally, palliative treatments such as opioid analgesic agents are largely understudied for their effects on the neuroimmune response and behaviour. Furthermore, although there are a range of well-validated behavioural tests utilised in other research areas, these tests may be insensitive to subtle cognitive impairments often observed in CICI and neglect to measure animal emotional experience.

1.1.5.1 Assessing affective state

Numerous studies have recognised that animals are able to experience emotional capabilities such as distress and pain. This has led to the theory that animals may also experience a broader range of emotional states, named affective states (Verbeek et al., 2014). As affective states are subjective, observation of these states in animals is challenging. Notwithstanding this, novel behavioural tests that can objectively measure affective states have recently been developed and allow us to better understand animal's emotional experiences and how they may differ from our own (Jirkof et al., 2019; Verbeek et al., 2014).

1.1.5.1.1 Assessing quality of life in animal models

The recognition of animals as sentient beings capable of experiencing senses, consciousness, and emotions has prompted scientists to investigate animal emotions to understand how an animal is 'feeling'. A growing area of interest is the relationship between cognition and emotion. Traditionally, behavioural and physiological measures formed the primary foundation used to assess an animal's emotional state. Whilst these measures offer important insight into the assessment of animal welfare, there are difficulties associated with their interpretation (Paul et al., 2005). These measures generally provide an accurate indication of the activation status of an animal, or level of emotional intensity (emotional arousal), however, they do not necessarily assess whether the animal is in a negative or positive emotional state (emotional valence) (Mendl et al., 2009). As previously mentioned, CIGT is known to be a painful self-limiting condition, with alleviation of symptoms and improvement of patient affective state and quality of life a primary goal. However, previous CIGT investigations often do not include a measure of affective state. Understanding emotional valence can offer insight into affective states and offer an objective way to measure emotions in animals. This will allow for improvement in animal welfare, implementation of humane endpoints, and accelerate

development of therapeutic strategies; ultimately improving translational validity of these animal models.

Cognitive bias testing is a novel approach to measure affective state of animals and establish an objective measure of cognitive performance. The influence of emotion on cognitive function is defined as a cognitive bias. Cognitive biases have the ability to change the strength and nature of emotional output by influencing appraisal processes (Paul et al., 2005). Currently the most commonly used cognitive bias test in animals' measures emotions induced by biases in decision-making in response to ambiguous stimuli (Mendl et al., 2009). The interpretations of ambiguous stimuli are either negative (pessimistic) or positive (optimistic) (Matheson et al., 2008; Mendl et al., 2009). A negative judgement bias predisposes a negative interpretation of ambiguous information induced by a negative emotional state, whilst a positive judgement bias predisposes a positive interpretation of ambiguous information induced by a positive emotional state (Jirkof et al., 2019). The judgement bias task has been well validated in many differenct species, including rats, with particular focus on animal welfare applications. However, the judgement bias task has yet to be validated or incorporated into disease models such as CIGT and provides a valuable tool to assess affective state in these models. This work was performed and reported in chapter 2.

1.1.5.2 Pain assessment in animal models

Pain is postulated as a two-dimensional construct and comprising sensory-discriminative and affective-motivational aspects. Nociceptive stimuli activate a 'pain matrix', consisting of the sensory-discriminative dimension that refers to the intensity, location and duration of pain, and the affective-motivational dimension of the painful experience, which involves the level of unpleasantness or negative affect (Novembre et al., 2015; Talbot et al., 2019). Previous animal

CIGT studies have primarily focused on physiological and clinical measurements for pain assessment. Assessment techniques such as clinical scoring schemes indicate general health parameters and provide retrospective measures of disease severity and physical response to painful stimuli. However, they are not specific to the emotional component of pain (Jirkof et al., 2019). Furthermore, previous gastrointestinal models have mainly examined the sensory-discriminative dimension of pain, utilising nociceptive assays without an affective component, such as the Von Frey filament test (Eijkelkamp et al., 2007; Esquerre et al., 2020). Pain reduction is an essential clinical goal in CIGT, however assessments specific to the affective component of pain are generally not included in rodent models. Recent trends in animal welfare science have seen an increased emphasis on methods of assessment of an animal's affective pain response (Mogil, 2009). Grimace scales are one such method that have been used to identify spontaneous pain response and have been utilised in numerous rodent models of acute pain and impacts of analgesia and anaesthesia (Jirkof et al., 2019; Whittaker & Howarth, 2014). Consequently, the Rat Grimace Scale (RGS) was investigated in chapter 3 of this thesis.

1.1.5.3 Assessment of cognition in CICI

Rodents are commonly used to investigate the role of cytotoxic agents in the disruption of learning and memory processes. To date, previous preclinical research has focused on a variety of cytotoxic agents and dosages including cisplatin, paclitaxel, doxorubicin, cyclophosphamide, 5-FU and MTX (Matsos & Johnston, 2019). Many studies have demonstrated compelling evidence of cognitive impairments in non-tumour bearing CICI animal models, which reflects hippocampal and frontal lobe dysfunction (Matsos & Johnston, 2019). However, some studies have failed to demonstrate cognitive impairment after chemotherapy exposure (Flanigan et al., 2018; Gandal et al., 2008). Currently, there are methodological inconsistencies between studies of CICI in animal models across the board.

These opposing results are likely due to differences in experimental design, treatment protocol, animal models and sensitivity of behavioural tests employed for cognitive assessment. Commonly utilised behavioural tests for examining cognitive impairment in CICI predominantly revolve around hippocampal-dependent facets of cognition such as short and long-term spatial, recognition and contextual memory (Seigers & Fardell, 2011) (Table 1). Specifically, the Morris water maze task has commonly been utilised to evaluate both short and long-term spatial working memory in response to a wide range of chemotherapy agents (Matsos & Johnston, 2019). Furthermore, both the novel location recognition and novel object recognition tests have been frequently employed in CICI preclinical literature to assess recognition memory (Matsos & Johnston, 2019). Importantly, often preclinical CICI studies do not commonly assess executive functioning, dependent on the pre-frontal cortex (Matsos & Johnston, 2019). Reduction in executive functioning and short-term memory are common features of 'chemobrain' in patients (Kovalchuk & Kolb, 2017). Patients who exhibit dysfunction in executive control, often have problems associated with working memory, judgement, planning and decision making emphasising a need to assess this domain (Kovalchuk & Kolb, 2017). More recently, methodologies such as the Five-Choice Serial Reaction Time Task (5CSRTT) and puzzle box have been utilised in CICI research as a measure of executive functioning (Chiang et al., 2019; Huo et al., 2018). The 5CSRTT is designed to test motivation and several paradigms of executive function which includes attention, inhibition and impulsivity in rodents. The advantage of the puzzle box behavioural test is that it can be used to assess both executive function and short-term memory loss and requires less training compared to the 5CSRTT (Ben Abdallah et al., 2011). There is a genuine need for studies to include a range of cognitive tests that assess short and long-term memory and executive functioning. However, tailoring to the model is essential in selection of

appropriate behavioural tests, for example suitability to species and strain, and sensitivity to subtle cognitive changes as it is usually reported by patients.

A large disparity exists between the preclinical research and the clinical reality, in that tumour-bearing models are not regularly being assessed, nor is the potential role of cancer itself on CICI being considered (Seigers & Fardell, 2011). But, the level of feasibility and practicality of cognitive testing presents challenges that can impede use of tumour-bearing rodent models, as employment of humane endpoints at acute timepoints are usually required due to welfare concerns. In addition to choice of behavioural tests with narrow cognitive domain focus, and lack of model standardisation, there is also minimal concurrent assessment of molecular changes and cognitive changes within the literature. Increases in pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6 have been implicated with cognitive impairments of recognition memory and short and long-term memory (Donzis & Tronson, 2014). Furthermore, the onset, duration and prevalence of CICI that are reported in the clinical setting appear to differ considerably, with some patients experiencing memory and learning deficits persisting long-term (Asher & Myers, 2015; Janelsins et al., 2014). However, to date few studies have evaluated cognitive and neuroimmune outcomes, particularly at a chronic time point (Seigers & Fardell, 2011). Therefore, animal models should investigate markers of neuroinflammation with cognitive change ascertained through behavioural assessment in an acute and chronic time course in order to develop intervention strategies and their appropriate timing for prevention or remediation of symptoms. Subsequently, this guided experimental design and was assessed in chapter 7.

Table 1. Common behavioural tests used to examine cognition in CICI (Matsos & Johnston, 2019; Seigers & Fardell, 2011)

Cognitive function assessed	Behavioural test
Short-term memory /spatial working memory	Barnes maze Y-maze Morris water maze
Long-term memory	Morris water maze
Recognition memory (short-and long-term recognition memory)	Novel object recognition Novel object place Object in place / object location Temporal order task
Contextual memory / Stimulus response learning	Contextual and cued fear Fear extinction
Spontaneous exploration	Novelty seeking
Avoidance learning	Passive avoidance Conditioned avoidance
Discrimination learning	Autoshaping task Go/No-Go task Conditional discrimination task Non-matching to sample
Executive control	Social discrimination 5-Choice serial reaction time task Simple reaction time task
Executive control / Short and long-term memory	Puzzle box
Behavioural flexibility	Reversal learning

CHAPTER 2

A Judgement Bias Test to Assess Affective State and Potential Therapeutics in a Rat Model of Chemotherapy-induced Mucositis

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A Judgement Bias Test to Assess Affective State and Potential Therapeutics in a Rat Model of Chemotherapy-induced Mucositis

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Name of Principal Author (Candidate)	Rebecca Peta George		
Contribution to the Paper	Involved in experimental design, carried out experimental work, analysed data, wrote manuscript, acted as corresponding author.		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Involved in experimental design, carried out experimental work and edited the manuscript.		
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Contribution to the Paper	Provided input into experimental design and technical assistance with animal trial.		
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Contribution to the Paper	Conducted experimental work and edited the manuscript.		
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Contribution to the Paper	Contributed to experimental design, data interpretation and preparation of the manuscript.		
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CONTEXTUAL STATEMENT

Recent trends in animal welfare science have seen an increased emphasis on methods of assessment of an animal's affective state. In particular such methods focus on positive as well as negative affective states. Cognitive bias testing is one such strategy. The presumptive basis of this technique is that emotional state alters cognitive processing, such that interpretation of an ambiguous stimulus may be altered. Previous animal models of CIGT typically assess pathological outcomes such as gut histological architecture, however fail to include a measure of affective state; a key clinical therapeutic goal. Therefore, reliable assessment of affective state, integrated with investigation of therapeutic targets, is needed to increase translational validity of the outcomes of these models to the clinical setting.

Chapter 2 examined a cognitive bias technique to validate this test for measurement of affective state in a rat model of CIGT (previously referred to as chemotherapy-induced mucositis). Further validation of the test was performed by assessing response to analgesic, through the addition of a partial mu opioid agonist palliative agent, buprenorphine.

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A Judgement Bias Test to Assess Affective State and Potential Therapeutics in a Rat Model of Chemotherapy-Induced Mucositis

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Chemotherapy-induced mucositis is an extremely painful condition that occurs in 40–60% of patients undergoing chemotherapy. As mucositis currently has no effective treatment, and due to the self-limiting nature of the condition, the major treatment aims are to manage symptoms and limit pain with significance placed on improving patient quality of life. Rodent models are frequently used in mucositis research. These investigations typically assess pathological outcomes, yet fail to include a measure of affective state; the key therapeutic goal. Assessment of cognitive biases is a novel approach to determining the affective state of animals. Consequently, this study aimed to validate a cognitive bias test through a judgement bias paradigm to measure affective state in a rat model of chemotherapy-induced intestinal mucositis. Rats with intestinal mucositis demonstrated a negative affective state, which was partially ameliorated by analgesic administration, whilst healthy rats showed an optimistic response. This study concluded that the judgement bias test was able to evaluate the emotional state of rats with chemotherapy-induced mucositis. These findings provide a foundation for future refinement to the experimental design associated with the animal model that will expedite successful transitioning of novel therapeutics to clinical practice, and also improve humane endpoint implementation.

Cancer is a major cause of morbidity and mortality worldwide, with prevalence expected to increase. Consequently, the need to develop diagnostic tools and novel therapeutics has grown. Chemotherapy treatments have vastly improved cancer survival rates, however they are often accompanied by significant side-effects that severely impact patient quality of life. A common, painful and debilitating side-effect is chemotherapy-induced mucositis, which affects the mucous-membrane lining the digestive tract, primarily in the oral cavity and small intestine[1,2]. Mucositis affects approximately 40% to 60% of patients undergoing standard doses of chemotherapy and occurs in up to 100% of patients undergoing high-dose chemotherapy in combination with radiation or hematopoietic stem cell transplant[3–5]. The condition occurs due to the inability of chemotherapy agents to distinguish between neoplastic and normal cells, consequently causing extensive damage[5,6]. The pathobiology of mucositis is described as a five-phase process involving several complex interacting cellular and molecular processes, including clonogenic cell death, activation of reactive oxygen species, ulceration and healing processes[1,7–9]. Typical symptoms include severe abdominal pain due to ulceration, inflammation and deterioration of the mucosal membrane of the gastrointestinal tract. Other symptoms include vomiting, nausea, diarrhoea, dehydration, constipation, bloating, and diminished oral ingestion that can lead to malnutrition and severe weight loss[5]. For neutropenic cancer patients the ulcerative lesions that occur with mucositis pose a greater risk of causing secondary systemic infection that can be potentially life- threatening[8].

Mucositis is a major dose-limiting factor in cancer treatment, causing possible interruption to treatment regimen by forcing reduction in dose or early termination, rendering it essential to prevent and manage side-effects to the greatest extent possible[8]. As mucositis currently has no effective treatment, and due to the self-limiting nature of the condition, the major treatment aims are to manage symptoms and limit pain with significance placed on

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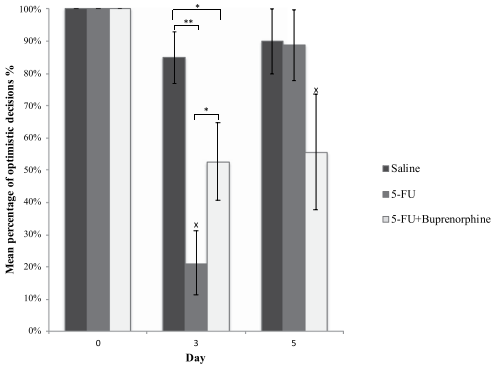

Figure 1. Mean percentage of optimistic decisions on day 0 (baseline prior injection, saline), day 3 (72 hours post injection) and day 5 (120 hours post injection) of rats injected with either saline, 5-FU, and 5-FU + buprenorphine treatments. Data expressed as mean ± estimated SEM. *Indicates significance $p < 0.05$, **indicates significance $p < 0.001$. X indicates significance $p < 0.05$ between time points within the same treatment group.

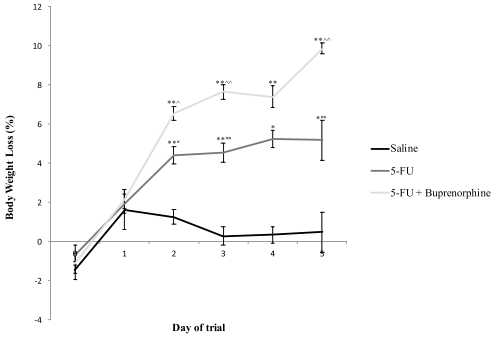

Figure 2. Effects of saline, 5-FU, and 5-FU + buprenorphine on body weight change in SD rats from days 0–5. Bodyweight change calculated from original weight recorded at day 0 prior to saline, 5-FU, buprenorphine injection. Data expressed as mean ± SEM. *$p < 0.05$, **$p < 0.001$ compared to saline, ^$p < 0.05$, ^^$p < 0.001$ compared to 5-FU, ×$p < 0.05$, ××$p < 0.001$ compared to 5-FU + buprenorphine.

improving patient quality of life and the impact on affective state[8,10]. Affective state being a subjective feeling state that encompasses different mood states, such as anxiety, depression, joy or happiness[11].

The majority of preclinical studies in mucositis have used rodents to investigate pathogenesis of the condition and effectiveness of novel therapeutics. These investigations typically assess pathological outcomes such as gut histological architecture or inflammatory response, yet fail to include a measure of affective state; the key therapeutic goal. Reliable assessment of affective state, integrated with investigation of therapeutic targets, is therefore required to improve translational validity of these models to human patients.

Since animals are incapable of verbally reporting their 'feelings', subjective experiences cannot be directly measured. Behavioural and physiological measures have traditionally been used to gauge an animal's affective state. Although these behavioural and physiological measures provide important information on the arousal of an emotion, they are simplistic and do not provide a complex interpretation of the positive and negative valence of an emotion[12–15]. One such method that has been derived from the human psychology field, and has been used as an indicator of affective state, is assessment of cognitive biases[16].

Cognitive bias testing is a novel approach to identify emotional states and establish an objective measure of cognitive performance in animals. Despite the promise of cognitive bias assessment methods as indicators of animal affective state these methods are yet to be employed in an animal disease model. It is pivotal for biomedical studies to find reliable assessment tools to evaluate various emotions experienced by animals to enhance animal model refinement, and improve validity of novel therapeutic assessment. Consequently, this study aimed to validate a cognitive bias test through a judgement bias paradigm to measure affective state in a rat model of

Day of trial	Saline	5-FU	5-FU + Buprenorphine
0	0	0	0
1	1.0	1.1	1.5*,^
2	1.1	1.5*	2.7**,^^
3	1.1	1.5*	3.0**,^^
4	1.2	1.6	2.1**,^^
5	1.1	1.7*	2.6**,^

Table 1. Effects of saline, 5-FU, and 5-FU in combination with buprenorphine on disease activity index from days 0–5. Rats were administered saline, 5-FU or 5-FU + buprenorphine on day 1 by intraperitoneal injection. Buprenorphine administration continued at 12 hourly intervals from days 1–5. Data expressed as mean ± SEM. Data are expressed as the mean disease activity index score. Data expressed on days 0–3 saline n = 20, 5-FU − 5-FU + Buprenorphine n = 19, days 4–5, saline n = 10, 5-FU − 5-FU + Buprenorphine n = 9. *$p < 0.05$, **$p < 0.001$ compared to saline, ^$p < 0.001$, ^^$p < 0.05$ compared to 5-FU.

chemotherapy-induced intestinal mucositis. Further validation of the test was also performed through the addition of an opioid palliative treatment, buprenorphine, which was expected to improve wellbeing.

Results

Judgement Bias Test. The results from the judgement bias data indicated that rats administered 5-FU and buprenorphine exhibited significantly fewer optimistic decisions compared to saline alone treated rats (Fig. 1). There were no differences in optimistic decisions between treatment groups prior to administration of saline, 5-FU and buprenorphine (day 0: 100%, $p > 0.05$). 5-FU-injected rats expressed decreased optimistic decisions compared to animals administered 5-FU + buprenorphine (21%; 53% respectively, $p < 0.05$) and saline control animals (85%, $p < 0.001$) 72 hours post injection. There were no differences between treatment groups in the number of optimistic decisions made at the 120 hour time point (saline 90%, 5-FU 89%, 5-FU + buprenorphine 56%, $p > 0.05$). However, optimistic decisions increased between 72 hour and 120 hour time points for rats administered 5-FU alone ($p < 0.001$). No differences were detected for rats administered 5-FU in combination with buprenorphine or saline alone between the 72 hour and 120-hour time points ($p = 1.000$ respectively).

Daily Bodyweight. There were no differences in bodyweight between groups prior to administration of saline, 5-FU and buprenorphine (day 0: $p = 0.49$; day 1: $p = 0.61$). Bodyweight change differed substantially between the groups post administration of agents (Fig. 2). Intergroup comparisons demonstrated that rats administered 5-FU alone had an increased bodyweight loss on days 2 to 5 compared to saline control (days 2 and 3: $p < 0.001$, days 4 and 5: $p < 0.05$). Furthermore, administration of buprenorphine in conjunction with 5-FU was associated with greater reductions in bodyweight compared to both 5-FU (day 2 $p < 0.05$, days 3, 4, and 5 $p < 0.001$) and saline controls (days 2–5 $p < 0.001$).

Disease Activity Index. There was no significant difference in disease activity index (DAI) prior to saline, 5-FU or buprenorphine injections ($p > 0.05$, Table 1). 5-FU administration increased DAI on days 2, 3 and 5 compared to saline controls ($p = 0.001$, $p = 0.003$, $p = 0.01$ respectively). Buprenorphine administration in 5-FU injected rats increased DAI on days 1–5 compared to both saline (day 1 $p < 0.05$, days 2–5 $p < 0.001$) and 5-FU controls (days 1 and 5 $p < 0.05$, days 2–4 $p < 0.001$).

Histological Severity Score. Histological gut architecture of the proximal jejunum and distal ileum was consistent with previous literature on mucositis on rats. 5-FU injected rats exhibited an increased histological severity score at 72 hours post injection compared to saline injected rats. Features included decreased goblet cells, crypt and enterocyte disruption, and shortening of villi. A decreased histological severity score was recorded at 120 hours post injection, with the occurrence of villi and crypt elongation (Fig. 3, data not shown for distal ileum). Animals administered 5-FU exhibited an increased disease severity score in the proximal jejunum and distal ileum at both 72 hour and 120 hour, compared to saline injected animals ($p < 0.001$, Fig. 4). Buprenorphine in conjunction with 5-FU had no effect on histological severity score at 72 hour and 120 hour time points for both ileum and jejunum compared to 5-FU alone treated rats ($p < 0.05$). Both 5-FU and buprenorphine showed a significant difference from 72 hour and 120 hour time points for jejunum and ileum (jejunum $p = 0.005$, $p < 0.001$; ileum $p = 0.03$, $p < 0.001$ respectively). This difference was not demonstrated in animals administered saline (jejunum $p = 0.90$, ileum $p = 0.71$).

Discussion

Mucositis is a common and serious side-effect following chemotherapy and radiotherapy. Despite the self-limiting nature of the condition and the negative impact on patient quality of life, cognitive parameters such as affective state are rarely studied in animal models. To our knowledge this is the first study to investigate affective state through judgement biases in a disease model. Importantly, this study represents the first to have demonstrated and validated a reliable judgement bias test to assess affective state exhibited by rats with chemotherapy-induced mucositis. The results from this study indicated that the presence of intestinal mucositis caused a negative affective state, which was partially ameliorated by analgesic administration.

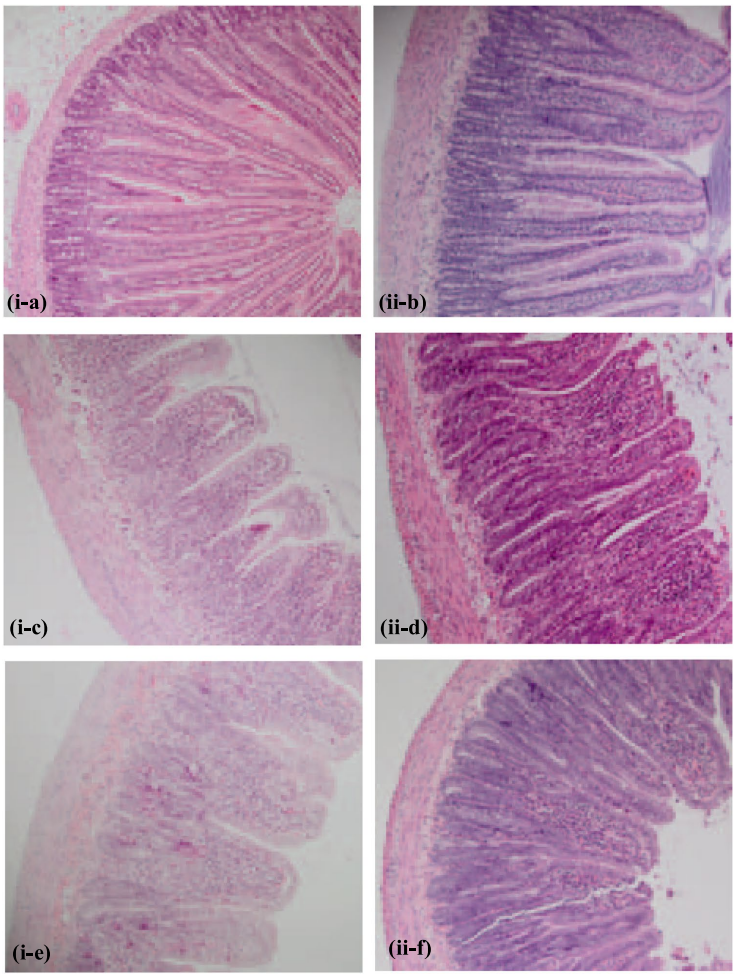

Figure 3. Representative photomicrographs of proximal jejunum at 72 hour (i) and 120 hour (ii) time points stained with haematoxylin and eosin (×40) in rats injected with saline (**a**,**b**), 5-FU (**c**,**d**) and 5-FU + buprenorphine (**e**,**f**).

In the present study, judgement bias results followed a correlation with the pathophysiological progression of mucositis identified from previous studies which have evaluated histology and clinical score[6,17]. Healthy animals, prior to 5-FU and saline injections were in a positive affective state, evidenced by optimistic decision-making by all animals when exposed to the ambiguous probe. Administration of 5-FU significantly decreased optimistic decisions compared to healthy controls 72 hours post injection. This finding of negative affective state showed a correlation with the progression of pathological damage associated with mucositis development, where clinical scoring and histological assessment revealed increased histological severity score, decreased bodyweight and increased disease activity index parameters following 5-FU administration. This finding is consistent with previous literature in rats, in which 5-FU caused mucosal damage in the small intestine, villus blunting and fusion, intestinal inflammation characterized by infiltration of immune cells, increased intestinal wall thickness, and increased bodyweight change and clinical disease score at 72 hours post 5-FU injection[6,18].

Changes in affective state were observed at 120 hours post 5-FU administration. Optimistic decisions increased across time points in animals with mucositis, implying that the healing process of mucositis may have led to an increase in positive affective state. This was evident in the current study since histological severity

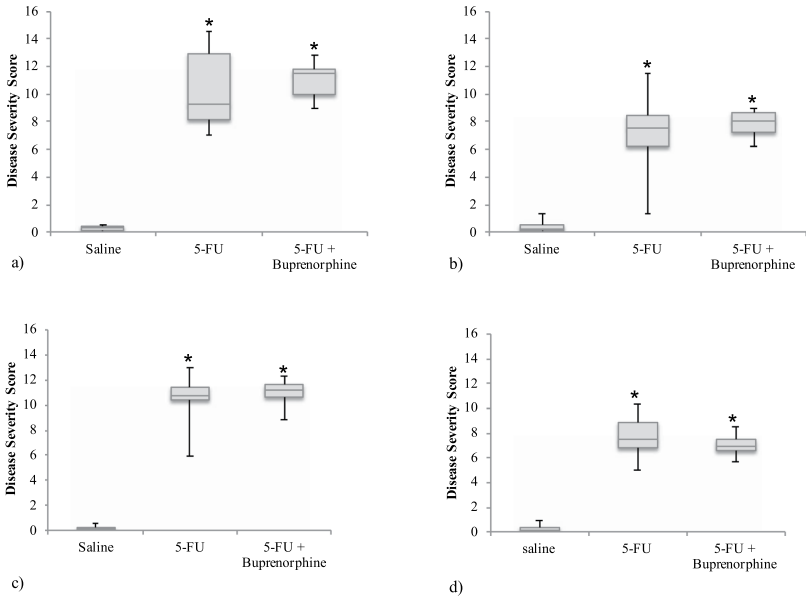

Figure 4. Disease severity score of distal ileum 72 hour (**a**) and 120 hour (**b**) time point and proximal jejunum at 72 hour (**c**) and 120 hour (**d**) time point of rats administered saline, 5-FU and 5-FU + buprenorphine. Data presented as the first and third quartiles, horizontal lines represent the median disease severity score and the whisker ends represent the maximum and minimum score. *Indicates $p < 0.001$ compared to saline.

score decreased with accelerated repair in the damaged intestine 120 hours post 5-FU injection. Histological and disease severity parameters in previous studies have shown evidence of a healing stage 120 hours post 5-FU administration in rats, as gastrointestinal tissue damage subsided, indicated by cell hypertrophy observed via compensatory crypt and villi elongation in the ileum and jejunum, myeloperoxidase activity normalization, and reduction of disease activity index parameters[6,19].

Interestingly, the analgesic agent, buprenorphine, failed to ameliorate the negative impact on affective state across the 72 hour and 120 hour time points. Differences over time were only present in rats treated with 5-FU alone. Whilst this result appears paradoxical given the commonplace use of opioids to treat severe pain, their use is hampered by side-effects including nausea, respiratory depression, constipation, and urinary retention[20,21]. The associated side-effects of buprenorphine exhibited in rodents are likely playing a part in this judgement bias observation. Side-effects of buprenorphine in rats include; appetite suppression, decreased body weight, and pica behaviour[21,22]. In the current study, buprenorphine had a striking effect on the daily disease activity parameters and bodyweight which is consistent with previous literature[17]. Rats administered 5-FU alone or in combination with buprenorphine showed a significant decrease in bodyweight compared to saline control groups. This decrease in bodyweight was further potentiated throughout the duration of the study in animals administered buprenorphine compared to 5-FU alone. The loss of bodyweight, and side-effects of buprenorphine, most likely had a substantial impact on affective state. These clinical signs are a potential cause of the decreased optimistic behaviour observed across time points when the recovery phase of mucositis was underway.

However, there is an alternative theory for the negative affective state recorded across the 72 hour and 120-hour time points. The repeated administration by injection, and the associated physical restraint used to administer buprenorphine may have had a substantial impact on affective state. A study by Stuart and Robinson[23] demonstrated that conventional animal restraint used during intraperitoneal substance administration provoked a negative affective state in male Lister Hooded rats. In the current study the route of administration was subcutaneous and the restraint method used was scruffing. Whilst restraint stress was likely present in our study, the subcutaneous restraint method is expected to have had minimal impact compared to restraint methods used during intraperitoneal administration. Nonetheless, repeated restraint stress has previously been applied to cause depressive-like phenotypes and induce negative affective state in rats[23–25]. This is a limitation of the current study, and further research is required to investigate the effects of analgesics, subcutaneous administration and restraint stress on affective state. Through addition of further groups to include an analgesic alone treatment group, saline injection group, and groups utilising alternative methods of analgesic administration such as oral delivery, it would be possible to tease apart the contribution of restraint and injection technique on affective state. Future

Phase	Description
1	Rats were handled twice daily for a 10-minute duration to become acclimatised to handling. The duration of this phase was five days.
2	Each day rats were tested four times, with test duration of five-minute. Food bowls were located in the testing apparatus, with associated rewards for each individual rat positioned on top of the sand in each bowl. The PVC pipe did not contain any sandpaper. The duration of this phase was five days.
3	Sand paper was positioned in a PVC pipe between the two transparent perspex boxes in the testing apparatus. Each day rats were tested four times, comprised of two cheerio trials and two chocolate trials. The daily order of these trials was determined independently by randomisation. A reward item was positioned in the associated bowl with the associated sandpaper also present. A timer was started once rats were positioned in the start box, and times were recorded for latency to vacate start box, enter goal box, approach a reward bowl, approach the correct reward bowl, and begin to consume reward. Testing ceased once the rat started to consume the reward or when 5 minutes had lapsed. Cleaning of apparatus with seventy percent ethanol solution was performed at the completion of each test. Once rats successfully completed the training on five consecutive days, they were advanced to phase 4.
4	Duplicate protocol to phase 3, except reward items were positioned under the surface of the sand in reward bowls at various depths, and rats were required to unearth reward items. Following successful removal, burial depth of rewards was increased for each succeeding trial, until entirely below the surface. Conditions required for advancement to phase 5 were the same as phase 3.
5	Duplicate protocol to phase 4, except reward items were placed entirely below surface of sand for every trial, and one trial each day selected at random did not contain a reward item. If the first bowl the rat foraged in would normally have contained a reward, the trial was deemed a successful unrewarded trial. Conditions required for advancement to phase 6 were the same as phase 3 and 4.
6	Duplicate protocol to phase 5, except intermediate grade sandpaper was matched with unrewarded trial (P180). The duration of phase 6 was three days.
7	Each day rats were tested once. PVC pipe contained intermediate sandpaper (P180), and food bowls did not contain a reward. The time taken for rats to begin foraging in any bowl was recorded, and record of the first bowl approached and foraged in was taken. The duration of phase 7 was five days.

Table 2. Promotion criteria for each phase of the judgement bias test.

study design would also benefit from inclusion of additional methods of assessing affective state such as the conditioned place preference test.

There is growing recognition of the limitations associated with animal models, and the challenges to translate outcomes from animal research to medical practice. In clinical studies changes in quality of life and affective state have been associated with cancer treatments[26]. Increase in negative affective state caused by chemotherapy has the potential to exacerbate treatment side-effects[11,27]. Whilst animal models in mucositis research are a valuable tool, they are not without limitations and complications and outcomes do not always translate to the clinic. Key limitations include; dose and scheduling issues (dose rates do not translate to humans), the absence of an emetogenic reflux in rats, impact on drug clearance and toxicities due to sex and strain differences in metabolic enzyme profiles, and difficulties assessing emotional states and other affective behaviours[14,28]. In previous studies the most common indicators used to assess disease progression and wellbeing in mucositis models have been non-specific clinical scoring, bodyweight change and histological analysis. These measures have drawbacks of being retrospective, and the possibility of not indicating true emotional experience[29]. Measuring judgement biases provides a clearer understanding of positive and negative emotional valence. Judgement bias assessment could also be utilised to improve humane endpoint implementation, thus improving animal welfare.

Whilst research into psychiatric disease has seen cognitive bias assessment utilised as an important tool, other biomedical areas of research are yet to incorporate this strategy into disease models. Results from this study demonstrate that the judgement bias test utilised was efficacious to evaluate the emotional state of rats with chemotherapy-induced mucositis. Furthermore, these findings provide a foundation for future biomedical research to incorporate cognitive bias methodologies such as a judgement bias test to determine effectiveness of novel therapeutics, and the mechanisms by which emotion can influence cognitive processes in animal models. Refinement to the experimental design associated with use of these animal models will likely expedite successful transitioning of novel therapeutics to clinical practice.

Methods

Animals and Experimental Design. Male Sprague Dawley rats (ArcCrl:CD(SD)IGS, n = 60) were acquired from a specific pathogen-free, barrier-maintained animal facility (Laboratory Animal Services, University of Adelaide, Adelaide, South Australia). Male SD rats were selected due to evidence from previous literature that this strain could be successfully trained using cognitive bias methodologies[12,30,31]. Upon arrival rats were housed in standard open-top cages (415 mm × 260 mm × 145 mm, Tecniplast, Exton, PA, USA) in groups of three, maintained in a room temperature of 21–23 °C with a 12 hour reversed light/dark cycle. All cages were supplied with shredded paper and fibre cycle bedding (Animal Bedding, Fibrecycle Pty Ltd, Queensland, Australia). Food (standard rat chow, Rat and Mouse Cubes, Specialty Feeds, WA, Australia) and RO water was provided at *ad libitum*. All experimental protocols were performed during the dark phase, under red lighting.

Rats were randomly allocated into three experimental groups; saline ip (n = 20); 5-fluorouracil (5-FU) (n = 19) (150 mg/kg 5-FU ip;Mayne Pharma Pty, Ltd, Mulgrave, Vic, Australia); and 5-FU + buprenorphine (n = 19) (150 mg/kg 5-FU ip + 0.05 mg/kg buprenorphine q12hr sc). On day 0 all rats were injected intraperitoneally with 5-FU (150 mg/kg) or saline. Rats in 5-FU + buprenorphine treatment group were injected subcutaneously with 0.05 mg/kg buprenorphine. Buprenorphine was administered at 12-hour intervals for the duration of the study. Buprenorphine was chosen, as it is a commonly used analgesic in rodents. It is favoured due to its simple administration, extended action, partial agonist action at the μ-opioid receptor, and effectiveness in various pain models[22]. Judgement bias response, bodyweight and disease activity index data were collected as described below. Rats were humanely euthanised by CO_2 asphyxiation at two time points; either 72 hours or 120 hours post 5-FU or saline administration in order to assess gut architecture via histology.

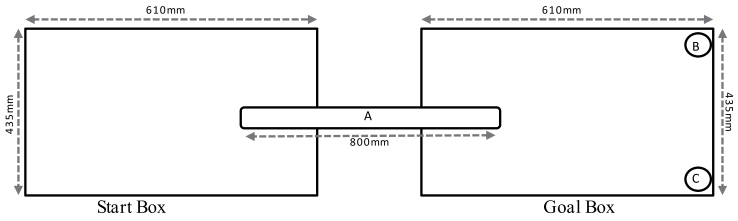

Figure 5. Schematic of the apparatus setup used in the judgement bias test. The apparatus consisted of a start and goal transparent perspex box. The start box was connected to the goal box by a PVC pipe (**a**) that was lined with sandpaper. A blue (**b**) and brown (**c**) reward bowl was placed at the end of the goal box.

Protocols were approved by the University of Adelaide Animal Ethics Committee and conducted in accordance with the Australian code for the care and use of animals for scientific purposes[32].

Judgement Bias Test. Rats were trained using a judgement bias paradigm to distinguish between two tactile stimuli associated with two rewards. The judgement bias paradigm used was based on a previous study by Barker, *et al.*[30], and consisted of seven phases that included a promotion criterion for each phase (Table 2). The training and testing apparatus consisted of two transparent perspex boxes (610 mm × 435 mm × 215 mm). The start box and goal box were connected with a PVC pipe (100 mm diameter). The inner surface of the PVC pipe was lined with either coarse (P80) or fine (P1200) sandpaper depending on the association. A blue and brown bowl was located at the end of the goal box and contained cinnamon and coriander-scented sand (1% by weight of spice) respectively (Fig. 5). The rewards consisted of a high-positive chocolate reward (Cadbury, London, England) or a low-positive cheerio reward (UncleToby's, Victoria, Australia). Each rat was randomly allocated a sandpaper association paired with a reward and bowl. During each trial the reward was placed in the bowl and paired with the associated sandpaper. During the training phase, each rat received two chocolate trials and cheerio trials per day. A trial commenced when the rat was placed in the start box and terminated once the rat started to consume the reward or 5 minutes had lapsed. The daily order of these trials and associations was determined independently by randomisation. During the testing phase rats received one ambiguous test, which consisted of the PVC pipe lined with intermediate sandpaper (P180) and no reward being present. Judgement bias was measured by investigating the foraging behaviour (bowl rat first foraged) in response to the ambiguous probe.

Once each training phase was successfully achieved, rats entered the test phase and were randomly assigned to an experimental group. Judgement bias was measured 24 hours prior to 5-FU and saline injection to obtain a baseline (day 0), 72 hours post injection (day 3; saline n = 20, 5-FU n = 19, 5-FU + buprenorphine n = 19) and 120 hours post injection (day 5; saline n = 10, 5-FU n = 9, 5-FU + buprenorphine n = 9). Animals that were determined to be in a positive emotional state demonstrated foraging behaviours that would correspond with high-positive reward during the ambiguous trial compared to those in a negative emotional state.

Disease Activity Index and Bodyweight. Following 5-FU or saline injection DAI and bodyweight were monitored and measured daily to determine the severity of mucositis. DAI was measured on a scale of 0–3 severity per parameter based on rectal bleeding, stool consistency, bodyweight loss, and overall general condition of the animals described by Mashtoub, *et al.*[6]. General condition was determined based on dull or ruffled coat, hunched, pale or sunken eyes, dehydration, squealing when handled and reluctance to move.

Histological Analysis. Sections (2 cm) of distal ileum and proximal jejunum were collected and fixed in 10% formalin buffer solution. Small intestinal sections were transferred to 70% ethanol 24 h post tissue collection. Tissue samples (4 μm) were processed and embedded in paraffin and stained with haematoxylin and eosin (H&E).

Histological analyses were conducted using a light microscope (Olympus Corporation CX-31, Tokyo, Japan). Histological severity was scored in the jejunal and ileal sections by grading eight histological criteria from zero (normal) to three (maximal damage) in a blinded fashion. This included: enterocyte disruption, reduction in goblet cells numbers, thickening of the submucosa and muscularis externa, villus fusion and stunting, crypt cell disruption, lymphocytic infiltration and crypt disruption[33].

Statistical Analyses. Statistical analyses were performed using Megastat Excel Add-In (version 10.3 Release 3.1.6 Mac, McGraw-Hill Higher Education, New York, NY) and SPSS (SPSS Inc., Chicago, IL, USA). Judgement bias data were analysed using a generalised linear mixed model (binary logistic) with the logit link function, where the implicit residual variance was on the underlying scale $p^2/3$[34]. The fixed effects were day, treatment, and day x treatment interaction taking into account individual animal to allow for repeated measures. Pairwise comparisons of the estimated marginal means were performed with sequential Bonferroni adjustment. Bodyweight and DAI data were analysed using a repeated measures ANOVA with Tukey's post hoc test. Histological disease severity score was analysed using a Kruskal-Wallis test with Mann-Whitney U-test. Data were deemed significant at $p < 0.05$. All data were expressed as means ± standard error of the mean.

Data Availability. All datasets generated and analysed during this study are included in supplementary information files.

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Author Contributions

R.P.G. conducted experimental work, data analysis and prepared the manuscript. T.H.B., K.A.L. and D.A.B. contributed in experimental work and data analysis. G.S.H. assisted in data interpretation and manuscript preparation. A.L.W. was involved in experimental design and work, data interpretation and manuscript preparation.

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CHAPTER 3

Use of the Rat Grimace Scale to Evaluate Visceral Pain in a Model of Chemotherapy-induced Mucositis

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Principal Author

Name of Principal Author (Candidate)	Rebecca Peta George		
Contribution to the Paper	Involved in study design, performed experimental work, analysed data, wrote manuscript, acted as corresponding author.		
Overall percentage (%)	75%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	19/02/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Gordon S. Howarth		
Contribution to the Paper	Provided guidance on content and structure of manuscript, editing of manuscript.		
Signature		Date	24/2/21

Name of Co-Author	Alexandra L. Whittaker		
Contribution to the Paper	Involved in the experimental design, supervision of the development of the work, funding acquisition, data interpretation and manuscript preparation.		
Signature		Date	19/02/2021

CONTEXTUAL STATEMENT

Chapter 2 validated a judgement bias test to assess affective state in a rat model of 5-FU-induced CIGT. The judgement bias test was demonstrated as valuable and efficacious tool to evaluate the emotional states of rats in a disease model of CIGT and has future potential to determine effectiveness of novel therapeutics and improve translational validity. However, this test may not be appropriate for all experimental designs due to the extensive training time required. Additionally, as CIGT is a painful condition and often debilitating, pain mitigation is an important clinical consideration. Similarly, pain evaluation is important in rodent models for the implementation of humane endpoints.

Therefore, the study presented in **Chapter 3** examined the utility of the Rat Grimace Scale (RGS) for pain assessment in a tumour-bearing rat model of chemotherapy-induced mucositis (currently referred to as chemotherapy-induced gut toxicity). A further aim was to assess whether changes in standard general clinical protocol and an established test of anxiety, the open field test reflected the grimace responses recorded. The addition of opioid palliative treatments that are reflective of the clinical setting allowed for further validation of the test to determine if the responses were pain-specific. There is an imperative need to develop methods of effective pain assessment in animal models of CIGT to improve welfare and satisfy regulatory requirements, as well as increasing the translational validity of the model to the human patient.

animals

MDPI

Article

Use of the Rat Grimace Scale to Evaluate Visceral Pain in a Model of Chemotherapy-Induced Mucositis

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Simple Summary: Mucositis is a painful and often debilitating condition associated with cancer treatment. Management of associated symptoms is an important clinical consideration. Animal models are used in mucositis research to model the condition in humans in order to develop novel therapeutic agents to relieve symptoms. Previous animal studies have focused on disease severity and outcomes, but often failed to measure pain. The rat grimace scale (RGS) is a validated observational measure used to gauge pain levels experienced by rats. The aim of this study was to assess the rat grimace scale in a rat model of mucositis, and to examine whether changes in clinical signs and anxiety reflected the grimace responses recorded. We also aimed to determine whether the responses were pain-specific by administering potent opioid painkilling agents. In the present study rat grimace scores did not change significantly between treatments. Development of reliable pain assessment methods in animal models is urgently required to improve model relevance to human clinical practice, in addition to safeguarding animal welfare.

Abstract: The rat grimace scale (RGS) is a measure of spontaneous pain that evaluates pain response. The ability to characterize pain through a non-invasive method has considerable utility for numerous animal models of disease, including mucositis, a painful, self-limiting side-effect of chemotherapy treatment. Preclinical studies investigating novel therapeutics for mucositis often focus on pathological outcomes and disease severity. These investigations fail to measure pain, in spite of reduction of pain being a key clinical therapeutic goal. This study assessed the utility of the RGS for pain assessment in a rat model of mucositis, and whether changes in disease activity index (DAI) and open field test (OFT) reflected the grimace responses recorded. Sixty tumor-bearing female Dark Agouti rats were injected with either saline or 5-Fluourouracil alone, or with co-administration of opioid analgesics. Whilst differences in DAI were observed between treatment groups, no difference in RGS scores or OFT were demonstrated. Significant increases in grimace scores were observed across time. However, whilst a statistically significant change may have been noted, the biological relevance is questionable in terms of practical usage, since an observer is only able to score whole numbers. Development of effective pain assessment methods in animal models is required to improve welfare, satisfy regulatory requirements, and increase translational validity of the model to human patients.

Keywords: rat grimace scale; chemotherapy-induced mucositis; disease activity index; open field test; opioids

1. Introduction

Mucositis is a painful and debilitating condition arising in 40–60% of oncology patients treated with chemotherapeutic agents [1]. The condition results from a series of biological events initiated by the epithelial cell response to cytotoxic damage [2]. Pathological damage manifests as ulceration, inflammation and breakdown of the alimentary mucosal membranes. Mucositis affects the entire length of the gastrointestinal tract from mouth to the rectum [1]. Pain may arise directly due to the activation of nociceptors [3], or in response to other gastrointestinal events such as abdominal bloating and ulceration [4].

The frequency at which mucositis affects chemotherapy patients, and the serious nature of the symptoms it causes, render the mucositic condition to be the major dose-limiting factor in cancer treatment regimens [1]. Consequently, a range of research programs are directed at further elucidating the mechanisms of mucositis pathogenesis, and development of novel therapeutic agents for symptomatic relief of symptoms [5,6]. Many of these programs of research utilize animal models.

Legislation governing the use of animals in research frequently requires severity assessment of procedures performed on animals and amelioration of pain by analgesic administration [7]. However, objective, pain-specific parameters for severity assessment in rodents are lacking, and those that exist have not been widely validated. This has significant impacts on the ability to accurately apply humane endpoints and provide analgesic intervention. Further, since mucositis is a self-limiting condition, with symptoms self-resolving once cancer therapy ends, reduction of patient pain is a key goal of therapy [2]. Similarly, reduction of pain or other clinical signs is the primary aim in rodent models evaluating novel therapeutics. In clinical practice, patients are frequently prescribed potent opioid analgesics such as fentanyl and morphine to manage mucositis pain [8]. By extrapolation it might be assumed that rats with mucositis experience pain, and that this pain can be alleviated with opioid analgesics. Therefore, the use of opioid analgesics in this model may aid in validation of pain specific measures. The identification of a reliable measure of pain would considerably improve the translational validity of rodent models of mucositis.

The rat grimace scale (RGS) is a measure of spontaneous pain that has been considered to evaluate the pain response [9]. The scale comprises four facial action units; orbital tightening, nose/cheek flattening, ear changes and whisker change. The RGS is well-studied, and validated, for the assessment of acute pain, such as that produced post-surgically [10,11]. However, in the face of visceral pain, such as intestinal mucositis, the RGS is less studied and the literature available is generally conflicting as to the validity of this scoring method [12]. Instead, gastrointestinal models tend to employ typical clinical scoring schemes arguably more indicative of general health, rather than specific to pain [5,13]. The disease activity index (DAI) evaluates disease severity based on general clinical observations; overall appearance of the animals, bodyweight loss, rectal bleeding and stool consistency. While an association between clinical signs and pain has been demonstrated in gastrointestinal disorders in human clinical practice, this has not been validated in rodent models of mucositis [3,14]. The open field test is a well validated measure of anxiety in rodent models. Although it does not measure pain, previous studies have shown that visceral pain and anxiety are often interrelated [15]. Therefore, the open field test has the potential to be an adjunctive tool for measuring affective state and associated pain. The aim of the current study was to assess the utility of the RGS for pain assessment in female Dark Agouti tumor-bearing (mammary adenocarcinoma) rats with chemotherapy-induced mucositis. The Dark Agouti rat mammary adenocarcinoma model (DAMA) was specifically chosen as it is extensively used in research investigating mucositis in rats [16]. The Opioid analgesics were administered to determine if the responses were pain-specific. A secondary aim was to examine whether changes in other measures of affective state, such as the DAI and the open field test, reflected the grimace responses recorded.

2. Materials and Methods

2.1. Animals and Experimental Design

Female Dark Agouti rats (DA/Arc; n = 60) were acquired from a Specific-Pathogen Free production facility, Animal Resources Centre (ARC) (Perth, Western Australia). The supply facility undertakes a quarterly health screening program, covering a range of bacterial, viral and parasitic organisms, for which the colony screened negative. Rats were group-housed in groups of 5 in standard open-top polycarbonate rat cages (50 cm × 31 cm; Tecniplast, NSW, Australia) with ad libitum access to potable reverse osmosis treated water, a standard rat chow (Specialty Feeds, Glenn Forest, WA, Australia), and provided shredded paper as enrichment. Lights were set to a 12-h light-dark cycle and animal facility room temperature was maintained between 21 °C and 23 °C. Rats were given a seven-day acclimatization period. During this period rats were handled daily and habituated to the grimace observation chamber and open field arena. Following baseline behavioral analysis, rats, weighing approximately 140 g, were anaesthetized with isoflurane (induction 4%, maintenance 1–2% to effect, in O_2). Isoflurane was selected as it provides rapid induction and recovery. Once anaesthetized, animals were injected with 0.2 mL (2.0×10^7 cells/mL) of breast cancer inoculum into the right flank as described by Gibson et al., 2002; day 0 [17]. Tumors were allowed to grow for seven days. Rats were given wet food and clinical scoring was performed daily. On day 8, a random number generator was used to assign rats to five groups (n = 12) into which the animals were allocated. The experimental groups were: (1) saline injection (ip) + saline (sc q 12 h); (2) 5-Fluourouracil (5-FU) (150 mg/kg; ip) + saline (sc q 12 h); (3) 5-FU (150 mg/kg; ip) + morphine (3.33 mg/kg; q 12 h sc); (4) 5-FU (150 mg/kg; ip) + fentanyl (10 μg/kg; q 12 h sc); and (5) 5-FU (150 mg/kg; ip) + oxycodone (3 mg/kg; q 12 h sc). When determined by group allocation, saline was administered in an equivalent volume to 5-FU. Opioid analgesics and equivalent volume of saline were administered by subcutaneous injection at 12 hourly intervals for the remainder of the study (a total of 6 doses) (Figure 1). Dosages of all agents administered, and dosing schedule, were based on previous literature [18,19]. Sample sizes were chosen using RGS data from a previous publication [20], with an alpha value of 0.05, power 0.8, and mean difference of 0.3, indicating a requirement for 12 animals per treatment group. The study was carried out in 6 balanced replications over a three-month time period. Rats were humanely euthanized via CO_2 asphyxiation 72 h post chemotherapy and saline administration. Rats were placed individually in a CO_2 chamber, with a gradual fill rate of 20% of chamber volume/min. Death was confirmed by lack of corneal reflex and cessation of heartbeat.

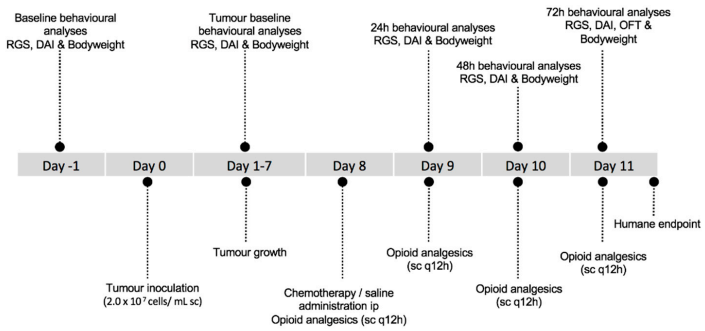

Figure 1. Overview of the experimental timeline for treatment and behavioral analyses. Abbreviations: RGS—rat grimace scale, DAI—disease activity index, OFT—open field test.

Animal housing and experimental protocols were approved by the Animal Ethics Committee of The University of Adelaide and conducted in accordance with the provisions of the Australian Code

for the Care and Use of Animals for Scientific Purposes [21]. This paper was written in accordance with Animal Research: Reporting in vivo experiments: The ARRIVE guidelines [22]. This study was conducted as part of a larger experiment evaluating the influence of chemotherapy-induced mucositis and opioid palliation on the development of chemotherapy-induced cognitive impairment.

2.2. Facial Image Analysis

Grimace scoring was performed at the same time of day for each animal during the light phase of the circadian cycle, at 5 time-points: 24 h prior to injection of tumor inoculum, 24 h prior to 5-FU or saline administration, and 24 h, 48 h and 72 h post-5-FU or saline injection. The latter three time points were selected to include an observation point 2 h following analgesic injection. Rats were removed from the home cage and placed in a 34 cm × 20 cm × 21 cm clear chamber. Two video cameras (Panasonic Video Camera HC-V180, Selangor, Malaysia) were placed perpendicularly on the outside sides of the chamber, with the two remaining sides and base being covered with white paper to enhance the clarity of the recordings. Rats were video recorded for a five minute-duration and then placed back into the home cage. All facial grimace recordings were performed in a quiet environment, in white light (150 lux) with no experimenter present. For image extraction, at each time-point a still image was selected and retrieved every 6 s using a video to JPG converter software (Free Studio v. 5.0.101 build: 201, United Kingdom). This occurred until a total of fifty images was obtained. Ten images of the rat's face were then randomly selected from the still images using a random number generator. Images were only selected when photos showed rats directly facing the camera, and were then cropped to show the face alone. In the scenario where an image did not show the rat directly facing the camera, a new random number was generated. Three final images for scoring were then selected using a random number generator. All images were selected by an operator who was blinded to the group and time-point. The images were placed into a pre-designed excel spreadsheet for scoring and assigned a random number code. Scoring was performed by a treatment-blinded experienced coder using the method described by Sotocinal et al. 2011 [9]. Each image was scored based on four action units: orbital tightening, nose/cheek flattening, ear changes and whisker change. A score from 0–2 (0 = not present, 1 = moderate, 2 = severe) was assigned to each facial unit. The four action unit scores were summed to produce the total score and a mean of the scores for all three images obtained.

2.3. Disease Activity Index

Disease activity index (DAI), [5] was recorded at 5 time-points; 24 h prior to tumor inoculum (baseline), 24 h prior to 5-FU and saline administration (tumor baseline), and 24 h, 48 h and 72 h post-injection. DAI scoring was conducted by a blinded observer, 2 h following analgesic injection prior to facial image analysis. DAI was measured on a scale of 0–3 severity (scored from 0 normal to 3 increasing in morbidity) per parameter based on overall appearance of the animals [23], bodyweight loss, rectal bleeding and stool consistency (Table 1).

Table 1. Disease activity index scoring criteria.

Overall Appearance *	Weight Loss (%)	Stool Consistency	Rectal Bleeding	Score
Normal	No weight loss	Normal Stools	No observable blood	0
Mild	0–5%	Loose	Blood spots in faeces	1
Moderate	5–10%	Mild diarrhoea	Clear evidence of blood in faeces	2
Severe	>10%	Overt diarrhoea	Gross bleeding	3

* Overall appearance was determined based on criteria; dull or ruffled coat, hunched, pale or sunken eyes, change in behavior, reduced food or water intake, dehydration, squealing when handled, reluctant to move.

2.4. Open Field Test

Anxiety level and locomotor activity were measured using the open field test (OFT) 72 h post 5-FU or saline administration. The OFT was conducted 1 h following facial image analysis. The OFT consisted of an enclosed, open field arena (1000 cm × 1000 cm × 1000 cm) in a brightly lit room, with a video camera (Logitech HD Webcam C525, Lausanne, Switzerland) suspended above the arena. The test commenced when a rat was placed in the center of the arena. After five minutes the rat was removed and placed back into the home cage. Each test was video recorded for the five minute period. Between each test the arena was cleaned using 70% ethanol. Analysis was performed retrospectively by a blinded observer using ANY-maze™ software (Stoelting Co.,Wood Dale, IL, USA). Two zones, peripheral and center, were superimposed, and the time spent, distance travelled and number of zone crosses for each zone were analysed.

2.5. Statistical Analysis

Statistical analyses were conducted using Megastat Excel Add-In (version 10.3 Release 3.1.6 Mac, McGraw-Hill Higher Education, New York, NY, USA) and SPSS software (SPSS Inc., Chicago, IL, USA). Data were tested for normality and homogeneity of variance using the Shapiro–Wilk test. One animal was excluded from the study and subsequent analyses due to unrelated health issues, resulting in an n = 11 for the saline control group. RGS and DAI data between groups at each time point were analysed non-parametrically using a Kruskal-Wallis test with a *post-hoc* Mann Whitney U-test. The Friedman test with a *post-hoc* Mann Whitney U-test was applied to determine within group significances across time for both RGS and DAI data. Bonferroni correction was applied where appropriate to account for multiple comparisons. A repeated measures analysis of variance (ANOVA) with Tukey's post hoc test was used to analyze body weight and open field data. Significance was determined at $p < 0.05$.

3. Results

3.1. Facial Image Analysis

There were no significant differences in RGS between treatment groups at any individual time-points (baseline $p = 0.98$; tumor baseline $p = 0.68$; 24 h $p = 0.20$; 48 h $p = 0.57$; 72 h $p = 0.58$) (Figure 2). Friedman analysis determined that the RGS scores were significantly higher post treatment ($\chi^2(4) = 68.7$, $p < 0.001$). Post-hoc Mann Whitney U-test determined that grimace scores were significantly increased for rats in all treatment groups at the 72 h time-point compared to tumor baseline ($p < 0.05$) (Table 2).

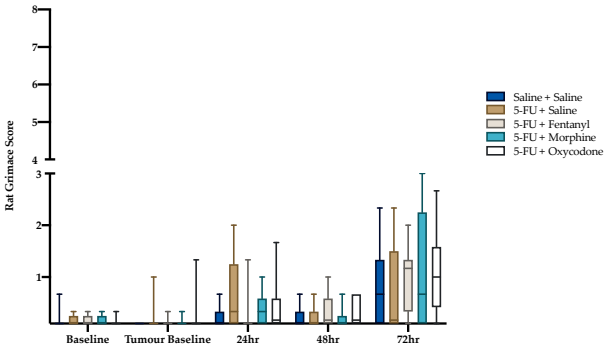

Figure 2. Box plot of rat grimace score. Maximum obtainable score was 8. Statistical comparison within group across time not shown (refer to Table 2). Saline + Saline (n = 11), 5-FU + Saline (n = 12), 5-FU + Fentanyl (n = 12), 5-FU + Morphine (n =12) and 5-FU + Oxycodone (n = 12) at all time-points.

Table 2. Statistical comparison within group across time for RGS and DAI.

	RGS	**DAI**
Saline + Saline	***P* Value**	***P* Value**
Tumour baseline vs. 24hr	0.28	0.54
Tumour baseline vs. 48hr	0.07	0.07
Tumour baseline vs. 72hr	0.03 *	0.06
5-FU + Saline		
Tumour baseline vs. 24hr	0.06	<0.001 *
Tumour baseline vs. 48hr	0.53	<0.001 *
Tumour baseline vs. 72hr	0.03 *	<0.001 *
5-FU + Fentanyl		
Tumour baseline vs. 24hr	0.91	<0.001 *
Tumour baseline vs. 48hr	0.12	<0.001 *
Tumour baseline vs. 72hr	0.001 *	<0.001 *
5-FU +Morphine		
Tumour baseline vs. 24hr	0.03 *	<0.001 *
Tumour baseline vs. 48hr	0.47	<0.001 *
Tumour baseline vs. 72hr	0.01 *	<0.001 *
5-FU + Oxycodone		
Tumour baseline vs. 24hr	0.11	<0.001 *
Tumour baseline vs. 48hr	0.12	<0.001 *
Tumour baseline vs. 72hr	0.001 *	<0.001 *

3.2. Disease Activity Index and Body Weight

Disease activity index scores increased significantly over time ($p < 0.05$). This increase was potentiated at 24 h, 48 h and 72 h compared to tumor baseline ($p < 0.001$, Table 2). There were no significant differences in DAI between treatment groups prior to 5-FU administration ($p > 0.05$). Administration of 5-FU significantly increased DAI within each time-point compared to saline control (Figure 3). The analgesic agents morphine and oxycodone increased DAI compared to 5-FU alone at later time-points (refer to Figure 3).

There were no significant differences in body weight between groups prior to chemotherapy injection. After chemotherapy administration there was a significant reduction in body weight at 24, 48 and 72 h in comparison to tumor baseline ($F(4, 270) = 414$, $p < 0.001$) (Figure 4). Body weight was further decreased for animals that received 5-FU in combination with analgesics compared to saline-injected rats. Morphine and oxycodone potentiated this weight loss compared to the 5-FU control group (24 h oxycodone alone, $p = 0.044$, 48 h; $p = 0.008$, $p < 0.001$, 72 h; $p = 0.01$, $p < 0.001$, respectively).

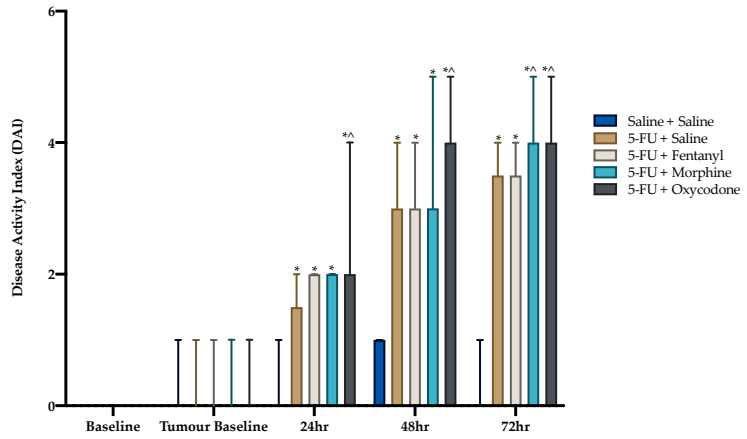

Figure 3. Disease activity index scores. Data expressed as median with range. Symbols denote significance obtained using a Kruskal-Wallis test with a *post-hoc* Mann Whitney U-test, between groups, at each time point. * $p < 0.05$ compared to saline. ^ indicates $p < 0.05$ compared to 5-FU alone. Statistical comparison within group across time not shown (refer to Table 2). Saline + Saline (n = 11), 5-FU + Saline (n = 12), 5-FU + Fentanyl (n = 12), 5-FU + Morphine (n = 12) and 5-FU + Oxycodone (n = 12) at all time-points.

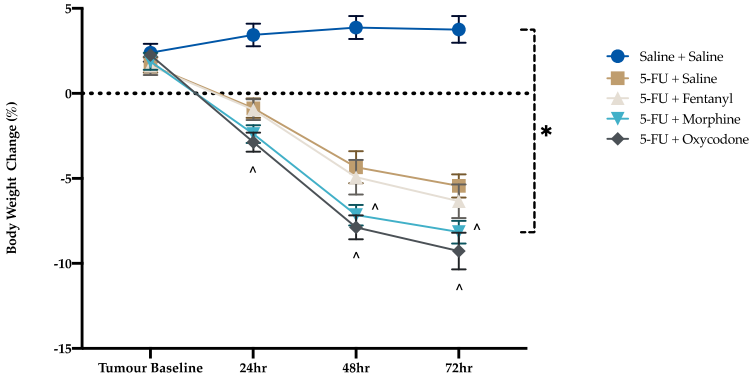

Figure 4. Body weight change. Data presented as mean % change in body weight ± standard error of the mean. * $p < 0.05$ compared to saline, ^ $p < 0.05$ compared to 5-FU. Saline + Saline (n = 11), 5-FU + Saline (n = 12), 5-FU + Fentanyl (n = 12), 5-FU + Morphine (n = 12) and 5-FU + Oxycodone (n = 12) at all time-points.

3.3. Open Field Test

There were no significant differences between treatment groups in the time spent in the centre or peripheral zones ($p = 0.638$, $p = 0.650$; respectively) (Figure 5). However, all rats, irrespective of treatment group, spent more time in the peripheral zone compared to the center zone ($p < 0.05$). No significant difference was detected between treatment groups in the distance travelled (centre, $p = 0.415$; peripheral, $p = 0.494$) and number of zone crosses (entries and exists) (centre, $p = 0468$, $p = 0.470$; peripheral, $p = 0.540$, $p = 0.536$; respectively), for both centre and peripheral zones.

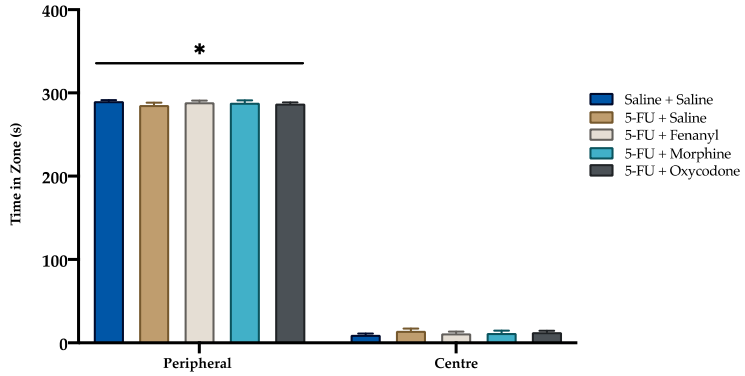

Figure 5. Time spent in center and peripheral zones of the open field test. Data presented as mean ± SEM. * $p < 0.05$ compared to center zone. Saline + Saline (n = 11), 5-FU + Saline (n = 12), 5-FU + Fentanyl (n = 12), 5-FU + Morphine (n = 12) and 5-FU + Oxycodone (n = 12).

4. Discussion

In the current study we aimed to determine, firstly, whether rats with implanted tumors and experiencing mucositis displayed pain as determined through expression of a 'pain face', and secondly, whether this pain could be ameliorated through the use of potent opioid analgesics, hence validating the RGS as a pain measure in this model. A further aim was to examine whether the condition of the rats and accompanying analgesic treatment led to a change in affective state as evaluated through a standard general clinical scoring protocol and an established test of anxiety. No difference in pain scores was demonstrated between groups at any individual time points. An important observation was that, while a significant increase in RGS was observed from baseline to 72 h post 5-FU and saline administration for all groups, the scores were consistently low, and frequently below the threshold able to be scored by an unbiased observer using a point system.

Our study findings imply that either: (1) rats with tumors/mucositis experienced minimal pain; or (2) the RGS lacked the sensitivity to successfully discriminate pain in this model. In consideration of the DAI scores, it becomes clear, however, that animals were experiencing some adverse welfare impact as evidenced by the increased scores, which reached approximately 50% of the total score possible at the later time-point. This scoring method is not unique to assessment of pain. Given the current findings of weight loss and changes in appearance in these rats, and previous studies demonstrating that other behavioral indicators of pain were exhibited at the time points evaluated [12], it is probable that the RGS is not a valid indicator of pain in this model. These results may have more general application to other gastrointestinal models of disease such as IBD and colitis where an acute visceral pain insult is anticipated, although there is some evidence to the contrary [24].

It is feasible that in the current study there was a confounding effect of the analgesic agent on assessment of the RGS score. For example, opioid agents may have produced a sedative effect thus influencing the facial action units. This may explain the significant RGS score exhibited in the morphine group at the 24 h time-point compared to tumor baseline, although this was not observed with other opioid agents. However, no change in spontaneous locomotor activity was measured in the open field apparatus, and based on previous literature this renders a sedative effect unlikely [25]. Furthermore, previous studies have disputed an influence of opioids on grimace tests [11,26]. The selected dosing regimens and time period for behavioral analysis may be factors in the minimal RGS response observed to the opioid treatments. Additionally, a significant increase in RGS score was observed from tumor baseline to 72 h in the saline control group. This elevation in RGS score may have been influenced by the pain caused by tumor burden, since this group had the largest tumors, being untreated. At 72 h

there was also a trend towards significance in the DAI scores for these animals. However, we again urge caution in interpretation of these findings given the low scores, and the lack of sensitivity of the RGS over small changes, due to ordinal scoring.

Alternatively, the minimal RGS change exhibited may have arisen as a result of the time points chosen for data analysis. The peak of histological damage in 5FU-induced mucositis typically occurs at 48–72 h following injection [5,27], and thus pain was expected to be maximal at this point. However, it is feasible that histological damage and maximal pain experience may not be coincident. Alternatively, the 'pain face' may be inhibited by animals in longer term pain, due to the need to avoid predator attention [9]. The majority of studies to date have demonstrated declines in RGS following an initial peak in response to a painful insult by 6–8 h following the noxious stimulation [28–30]. However, previous studies involved a surgical stimulus [28–30], or inflammatory pain model [28], hence pain duration in the current study may have been reduced in comparison to the visceral pain sensation of mucositis. Contrarily, an assumed chronic pain insult caused by tooth movement caused a grimace response in rats for several days post-pain insult [31], as did the presence of colitis [14]. It is noteworthy that in the current study, grimace values were a small fraction of the maximum obtainable score (i.e., 8); often scoring under one. Therefore, while a statistically significant change may have been noted, the biological relevance is questionable in terms of practical usage, since an observer is only able to score whole numbers.

It is plausible that the chosen observation period for grimace analysis and strain of rat used in the current study may have contributed to the RGS results obtained. Animals in this study were video recorded for a shorter duration compared to previous studies [9,14]. Exploration of the relatively new environment may have masked grimace response, with animals not having time to habituate to the chamber. However, changes in rat grimace score following recovery from exposure to either isoflurane or air have been reported using an identical scoring period to that used in the current study (five minutes) [32]. Furthermore, the rat strain used may have impacted on the ability to accurately score facial features. Previous research has implied that detection of grimace facial features in dark-colored animals is impaired [33,34]. This may however be as a result of poor achievement of background contrast, rather than the pigmentation of the animal per se. We tried to minimize this factor by using a white background when performing grimace scoring. Nonetheless, this could be a limitation of the study. Furthermore, our findings concur with previous results in a 5-FU induced mucositis model in white Sprague-Dawley rats, where mucositis did not elevate RGS in a longitudinal study design in which rats acted as their own controls [12].

The DAI has been widely used in models of mucositis to assess presence of the mucositic condition, in addition to severity, and is generally the sole assessment method utilized for making decisions on humane endpoint implementation, as prescribed by Animal Ethics Committees. The DAI findings in this study mirror those described in other mucositis studies with a progression in clinical signs until 72 h post-chemotherapy injection. However, the DAI is not specific to pain and therefore may not be useful in guiding when analgesic intervention is required. Further, it is apparent that the analgesic agents, morphine and oxycodone, actually increased the DAI score, thus challenging its interpretation. Closer inspection of the individual scores contributing to total DAI reveals that score is heavily influenced by weight loss, which increased over time in concert with histological disease progression, as described in previous studies. Since morphine and oxycodone potentiated weight loss, the DAI scores for these groups were relatively higher. If we assume that oxycodone is controlling pain, it is assumed that this exacerbation of bodyweight loss was brought about either through reduced appetite as a result of nausea, or as a result of a sedative effect rendering a larger proportion of the time budget being spent engaged in sleep. These are well recognized side effects of opioid administration [35,36]. This observation is consistent with previous studies, which have shown that the opioid analgesic, buprenorphine, similarly exacerbated bodyweight loss [27,35,37]. This lack of sensitivity of the DAI poses an issue when implementing humane endpoints. If strict point-score cut-offs are applied without the input of clinical judgement, animals may be withdrawn from a study as a result of analgesic

side-effects such as weight loss, which influenced DAI. These side effects may not in themselves negatively impact on affective state. This poses an ethical issue in terms of animal wastage.

5. Conclusions

There is a genuine need to develop and validate objective pain assessment tools in animal models of gastrointestinal disease such as the mucositis model described here. This will not only improve animal welfare and satisfy legislative requirements, but improve the translational validity of the models. Our data imply that the RGS fails to meet this need in a rat model of chemotherapy-induced mucositis and alternative pain assessment strategies are required. Alternative methods of emotional affect should be investigated, to include activities of daily living, such as burrowing and nest building, measures of affective biasing, or the use of running wheel activity. The conditioned place preference test might also be employed to examine preference for a substance assumed to provide an ameliorating effect [38]. Taking our findings, and those of others, into consideration, we also urge caution in conflating statistical significance with clinical significance when it comes to validating tools for practical implementation. Furthermore, given an apparent lack of sensitivity of traditional DAI scoring to side-effects of opioid analgesics, the comparative efficacy of opioid analgesics in these models cannot be established.

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CHAPTER 4

Reporting in Rodent Models of 'Chemobrain': A Systematic Review Assessing Compliance with the ARRIVE Guidelines

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Reporting in Rodent Models of 'Chemobrain': A Systematic Review Assessing Compliance with the ARRIVE Guidelines

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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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CONTEXTUAL STATEMENT

Rodent models are commonly used to investigate pathogenesis and potential therapeutic strategies in CICI. However, concerns have been raised regarding inadequacies in reporting of animal studies rendering them unreliable and irreproducible. The ARRIVE (Animals in Research: Reporting in vivo Experiments) Guidelines were introduced to improve critical appraisal of experimental design, interpretation of results and advance animal welfare and ethical standards. Currently, there is considerable variability in experimental design and measured research parameters in animal models of CICI. Due to this heterogeneity, accurate and transparent reporting is of even greater concern in this research area, in order to allow for replication and translation of research findings.

In **Chapter 4** a comprehensive systematic search of the literature was conducted to identify relevant peer reviewed publications evaluating CICI in rodent models, to assess compliance with the ARRIVE guidelines. A further aim was to determine if the introduction of the ARRIVE guidelines has made an impact on quality of reporting.

Reporting in Rodent Models of 'Chemobrain': A Systematic Review Assessing Compliance with the ARRIVE Guidelines

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Abstract

Patients diagnosed with cancer are often plagued with debilitating side effects post chemotherapy treatment. One such side effect is chemotherapy-induced cognitive impairment or 'chemobrain'. Rodent models are commonly used to investigate pathogenesis and potential therapeutic strategies. However, concerns have been raised regarding inadequacies in reporting of animal studies rendering them unreliable and irreproducible. The aim of this systematic review was to assess compliance with the ARRIVE reporting guidelines in peer-reviewed publications evaluating chemotherapy-induced cognitive changes in rodent models, and to determine if the introduction of the ARRIVE guidelines has improved quality of reporting. A comprehensive search was conducted to identify relevant peer reviewed publications. Ninety-seven studies met the eligibility criteria and publication compliance with the ARRIVE guidelines reporting was assessed. No studies achieved full adherence with the ARRIVE guidelines. Furthermore, no significant improvement was demonstrated in the overall compliance score post-ARRIVE. Given the lack of standardisation of animal models in this research area, these results pose particular threat to future progress and translation of findings in this area of research. These results highlight the need for stricter adherence to the ARRIVE

guidelines by journal editors and reviewers. Animal Ethics Committees also have an important educative role in improving knowledge and awareness of the guidelines amongst researchers.

Keywords chemobrain; CICI; cognitive impairment; ARRIVE, reporting guidelines

Introduction

The development of new, diverse chemotherapeutic agents for cancer therapy has led to a considerably reduced reoccurrence and higher survival rates for numerous types of cancer [36]. Despite this relative success, patients are often plagued with undesirable, debilitating side effects arising from treatment, resulting in forced dose reduction or even cessation of treatment [35]. Chemotherapy agents act to prevent cancer cell multiplication in surrounding tissues whilst limiting overall cell proliferation. However, this process is non-specific and toxic effects on normal functioning cells are common. The CNS is particularly vulnerable to toxicity resulting in a condition colloquially termed 'chemobrain' [30]. Chemotherapy-induced cognitive impairment (CICI) or 'chemobrain' may affect up to 75% of patients for around 2 years following treatment courses, with around 35% of those patients experiencing deficits lasting some decades [1, 30]. Common side effects include deficiencies in attention, language, memory and executive function, as well as fatigue, psychomotor function and motivational deficits [15, 23, 35, 37]. These symptoms have been consistently observed across all cancer types, including those not of CNS origin [14, 15]. Cognitive impairments can have a significant impact on activities of daily life, employment, leisure and maintenance of relationships [10, 14]. Chemobrain thus presents a significant personal and societal burden, especially as cancer survivorship increases. In recognition of this multiple survivorship frameworks have identified the need for greater understanding of the pathogenesis of chemobrain, as well as the development of treatment and mitigation options, as priorities for research [22].

Patient research plays a key role in understanding the prevalence, severity and lived experience of this condition [3, 7, 20, 21, 25, 29]. However, interpretation of patient data can be challenging, due to the number of confounding factors inherent in observational study designs, including patient age, cancer type, treatment administered, presence of comorbidities, disease progression, pre-treatment cognitive status, as well as experiences of anxiety or depression [8, 17, 27, 32]. These factors hamper our progress in gaining mechanistic understanding and developing effective therapeutics. Animal studies are therefore commonly used since they allow control of extraneous variables, such as genetics and tumour status. In the most part these studies utilise a range of rodent models of the condition [28].

However, in recent years, concerns have been raised about the extent of inadequate reporting of animal studies rendering them unreliable and irreproducible [2, 19, 33]. Incomplete descriptions of methodological items, as well as inappropriate or incomplete reporting of data raise scientific, ethical and economic concerns. Therefore, transparent reporting of methods and results is a vital component in increasing reproducibility of findings [5]. In an attempt to address these concerns, The ARRIVE (Animals in Research: Reporting in vivo Experiments) guidelines were released in June 2010 [11]. These guidelines aimed to universally improve the quality of animal reporting, increase transparency of findings and ultimately allow for greater reproducibility and translation of results of animal studies [5, 11]. Landis and colleagues in 2012 further refined the list to four key attributes that should be reported on at a minimum. Randomisation, blinding, sample-size estimation and data handling were deemed to be universally accepted as core issues impacting on study evaluation [13]. The ARRIVE guidelines were updated in 2020 by reorganizing items to facilitate their use [24]. The current guidelines are organized into two sets, comprising an essential ten that should be included as a minimum in any publication where animal research was performed, and a recommended set to

complement these. Reporting of the items in both sets represents best practice, and should be the ultimate goal. The guidelines also consist of a simple checklist summarizing the minimum information required. This document is designed to aid authors when preparing manuscripts, and allow journal reviewers and editors to simply confirm compliance with guidelines [24].

In comparison to other more established pre-clinical research areas, there is greater variability in animal models for chemobrain, with no established 'standard' model [4, 17]. Furthermore, outcomes assessed are heterogeneous and commonly include results of a range of behavioural tests which are known to be highly variable both within and between animals [6]. This variability makes accurate and transparent reporting, especially of animal-related characteristics, even more important in this area of research to enhance opportunities for translation of findings to clinical settings. Therefore, the goal of this systematic review was to assess compliance with the ARRIVE guidelines in scientific publications evaluating chemotherapy-induced cognitive changes in rodent models. The secondary aim was to determine if the introduction of the ARRIVE guidelines has made an impact on quality of reporting.

Methods

Search Strategy

A comprehensive search was conducted using Medline via PubMed and Scopus to identify relevant peer reviewed publications. The search strategy consisted of terms such as "chemotherapy", "anti-cancer agents", "animal models", "cognitive impairment", "cancer induced cognition changes" and related synonyms (refer to supplementary material S1). The search was conducted in August 2020. Independent reviewers (CC, AW, and EB) screened

articles based on pre-defined inclusion and exclusion criteria and any conflicts were resolved by discussion. The third reviewer was consulted if a consensus could not be met.

Inclusion and Exclusion Criteria

In order to be considered for review, the articles were required to meet the following criteria: [1] original full text articles available from database; [2] use of a rat/mouse model regardless of strain, sex or age; [3] administration of cancer chemotherapy agent regardless of route of administration or dosage, to naïve, cancer-inoculated, or tumour-inoculated animals; [4] studies that evaluated chemotherapy-induced cognitive impairment via either behavioural or tissue based measures or combination thereof; [5] English language publications.

The exclusion criteria for articles were as follows: abstracts and conference proceedings, review articles, use of non-rat/mouse animal models or human clinical studies, studies that did not assess chemotherapy-induced cognitive impairment, and non-English publications.

Search Results

The database search identified a total of 638 studies. All citations were uploaded to EndNote (EndNote ™ X9) and then imported into Covidence (Covidence systematic review software, Veritas Health Innovation, Melbourne, Australia) where duplicates were automatically identified, removed and manually cross referenced. The remaining studies were screened based on title and abstract against the pre-defined inclusion and exclusion criteria by three independent reviewers (CC, AW, and EB). A total of 399 studies were excluded based on title and abstract screening, with 117 studies remaining for full text assessment.

Full texts were manually retrieved and imported into Covidence. Three independent reviewers thoroughly assessed the studies by full text for their eligibility. During the eligibility process, 20 studies were excluded due to not being an original full text article, not assessing chemotherapy agents, not utilising a rodent model and full text being unavailable**.** Details of the identification process are described in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA).

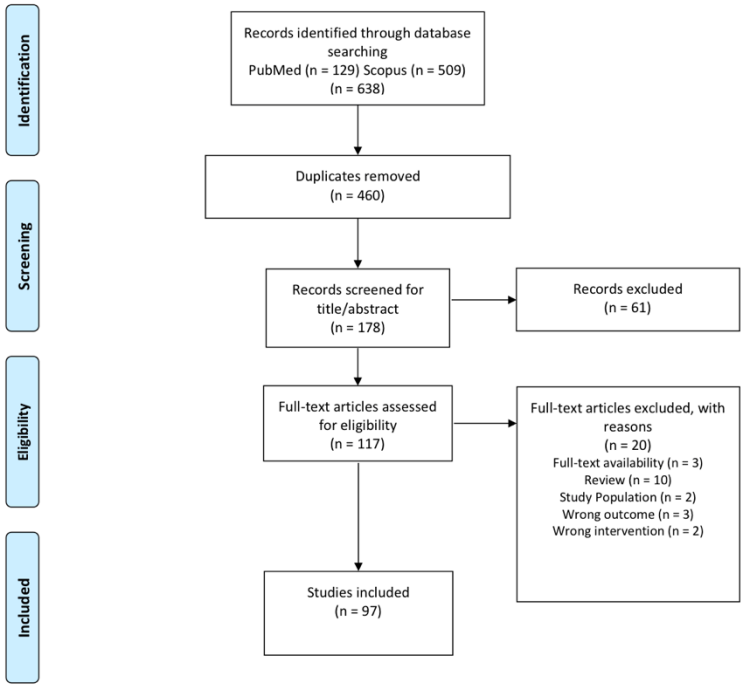

Figure 1. PRISMA flow diagram for the review process [18].

Data Extraction

Data were extracted from 97 included studies by two independent reviewers (RG and IS) with a third independent reviewer consulted if necessary (A.L.W). Prior to data extraction two reviewers independently extracted data from two studies to ensure uniformity in the reviewing process. To assess publication compliance with the ARRIVE guidelines reporting was assessed at an item and sub-item level (table 1), namely ethical statement (item 5), study design (item 6a and 6b), experimental procedures (item 7a, 7b, 7c and 7d), experimental animals (item 8), housing and husbandry (item 9a and 9b), sample size (item 10a and 10b), allocating animals to

experimental groups (item 11a), statistical methods (item 13a), baseline data (item 14), numbers analysed (item 15a), outcomes and estimations (item 16) and adverse events (item 17a and 17b). Each included study was critically appraised for compliance with the guidelines for the items with a rating score from 0-2 assigned (0 = not reported; 1 = partially reported, 2 = reported) (table 1). In addition, item 6b (blinding) was differentiated to item 11a (randomisation of animal allocation to treatment group) as blinding may include randomisation as a method of reducing subjective bias. Since ethical statement (item 5) and adverse events (item 17a and 17b) can only be scored in a binary fashion (yes or no), these items were either allocated a 0 or a 1 by the independent reviewers and this is reflected in the total score. For visualisation of data these items were classified as '0 = not reported' and '2 = reported'. The rating score for each ARRIVE guideline item was summed to produce a total compliance score out of 36. Information regarding citation information (authors, year of publication, titles, country of corresponding author) was also extracted. Additionally, in order to determine if the introduction of the ARRIVE guidelines has made an impact on quality of reporting, included studies were divided into two groups; pre-introduction of the ARRIVE guidelines (pre-ARRIVE) and post- introduction of the ARRIVE guidelines (post-ARRIVE). Extracted data were cross-checked and verified by two independent reviewers.

Statistical Analysis

The statistical analysis was performed utilising IBM SPSS (SPSS Inc., Chicago, IL, USA) statistical software. Data were tested for normality and homogeneity of variance utilising the Shapiro-Wilk test. A repeated measures ANOVA was performed to determine changes in reporting across years. Due to unequal variance between groups compliance scores pre- and post- ARRIVE guidelines were compared using a Welch's t-test. Statistical significance was determined at $p < 0.05$.

Table 1. Rating score used to evaluate the quality of reporting in rodent models of chemobrain based on the ARRIVE Guidelines

Category	Item	Recommendation Description	Rating score
Methods			
Ethical statement	5	Ethical statement	0 – not reported 1 – reported
Study Design	6a	The number of experimental and control groups.	0 – not reported 1 – partially reported 2 – reported
	6b	Blinding - minimise the effects of subjective bias when allocating animals to treatment (e.g. randomisation procedure) and when assessing results (e.g. blinded observer).	0 – not reported 1 – partially reported 2 – reported
Experimental procedures	7a	How (e.g. drug formulation and dose, site and route of administration, anaesthesia and analgesia used). Details of any specialist equipment used, including supplier(s).	0 – not reported 1 – partially reported 2 – reported
	7b	When (e.g. time of day).	0 – not reported 1 – partially reported 2 – reported
	7c	Where (e.g. home cage, laboratory, water maze).	0 – not reported 1 – partially reported 2 – reported
	7d	Why (e.g. rationale for choice of procedures)	0 – not reported 1 – partially reported 2 – reported
Experimental animals	8a	Animal characteristics (including species, strain, sex, developmental stage and weight	0 – not reported 1 – partially reported 2 – reported
Housing and husbandry	9a	Housing (type of facility, type of cage or housing; bedding material; number of cage companions)	0 – not reported 1 – partially reported 2 – reported
	9b	Husbandry conditions (e.g. light/dark cycle, temperature, access to food and water, environmental enrichment).	0 – not reported 1 – partially reported 2 – reported
	9c	Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment.	0 – not reported 1 – partially reported 2 – reported
Sample size	10a	Total number of animals used in each experiment, and the number of animals in each experimental group.	0 – not reported 1 – partially reported 2 – reported
	10b	Explain how the number of animals was arrived at. Details of any sample size calculation used.	0 – not reported 1 – partially reported 2 – reported
Allocating animals to experimental groups	11a	Randomisation - Details of how animals were allocated to experimental groups, including randomisation or matching if done.	0 – not reported 1 – partially reported 2 – reported
Statistical methods	13a	Details of the statistical methods used for each analysis.	0 – not reported 1 – partially reported 2 – reported
	13c	Methods used to assess whether the data met the assumptions of the statistical approach.	
Results			
Baseline data	14	For each experimental group, relevant characteristics and health status of animals (e.g. weight, microbiological status, and drug or test naïve) prior to treatment or testing.	0 – not reported 1 – partially reported 2 – reported
Numbers analysed	15a	Number of animals in each group included in each analysis. Report absolute numbers.	0 – not reported 1 – partially reported 2 – reported
Outcomes and estimation	16	Results for each analysis carried out, with a measure of precision (e.g. standard error or confidence interval).	0 – not reported 1 – partially reported 2 – reported
Adverse events	17a	Details of all important adverse events in each experimental group.	0 – not reported 1 – reported

Results

Descriptive characteristics of included studies

A total of 97 studies met the eligibility criteria and were included in this review. The publication dates ranged from 2008 to 2020, with 88.7% (86 studies) of the included studies being published after the ARRIVE Guidelines were published in June 2010. Data were provided from 19 countries; Canada (n=7), United States (n=43), China (n=12), Brazil (n=2), The Netherlands (n=2), United Kingdom (n=3), Australia (n=7), Egypt (n=1), Republic of Korea (n=7), France (n=2), Japan (n=1), Ireland (n=1), Amsterdam (n=1), Chile (n=1), Germany (n=1), India (n=2), Saudi Arabia (n=1), Thailand (n=2) and Slovakia (n=1) (Figure 2).

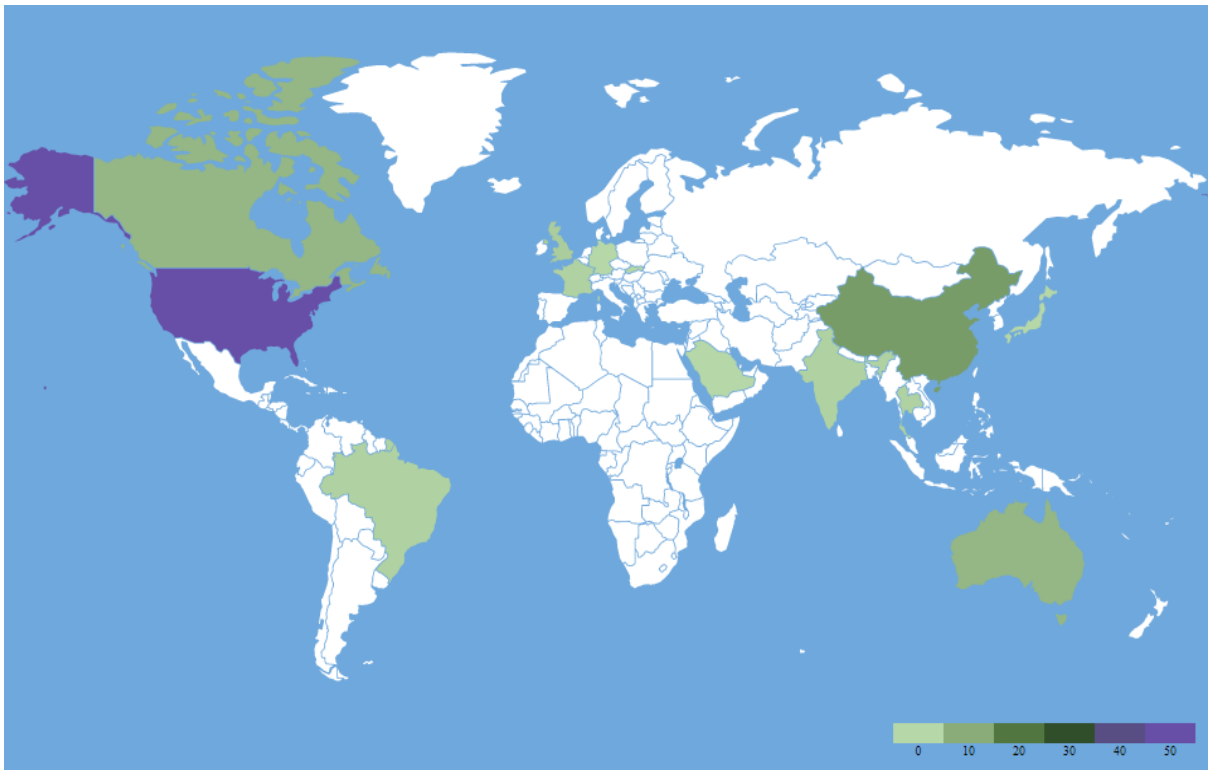

Figure 2. World heat map depicting geographical location of corresponding author of included studies.

Overall compliance score

To achieve 100% compliance, the included studies had to fully report each item and sub-item included in the evaluation (Table 1). A total of 12 items comprising 20 sub-items concerning reporting in the Methods and Results sections were evaluated in this study. None of the included studies achieved full adherence with the ARRIVE guidelines. Further, no significant improvement was demonstrated in the overall compliance score post-ARRIVE ($t_{13.593} = 2.021$, $p = 0.063$). The mean compliance score rated out of 36 for pre-ARRIVE and post-ARRIVE was less than 70% (pre-ARRIVE, mean compliance score 22.36; post-ARRIVE, mean compliance score 24.58) (Figure 3A). Out of the items evaluated only the outcomes and estimations (item 16) were reported in 100% of the included studies, both pre-ARRIVE and post-ARRIVE (Figure 3B). Overall compliance rating for each sub-item for Pre-ARRIVE and Post-ARRIVE guidelines are displayed in Figure 5 and Figure 6, respectively. There were no statistically significant differences observed between the overall reporting compliance across the years published ($F (14, 82) = 0.7200$, $p = p = 0.7485$) (Figure 4).

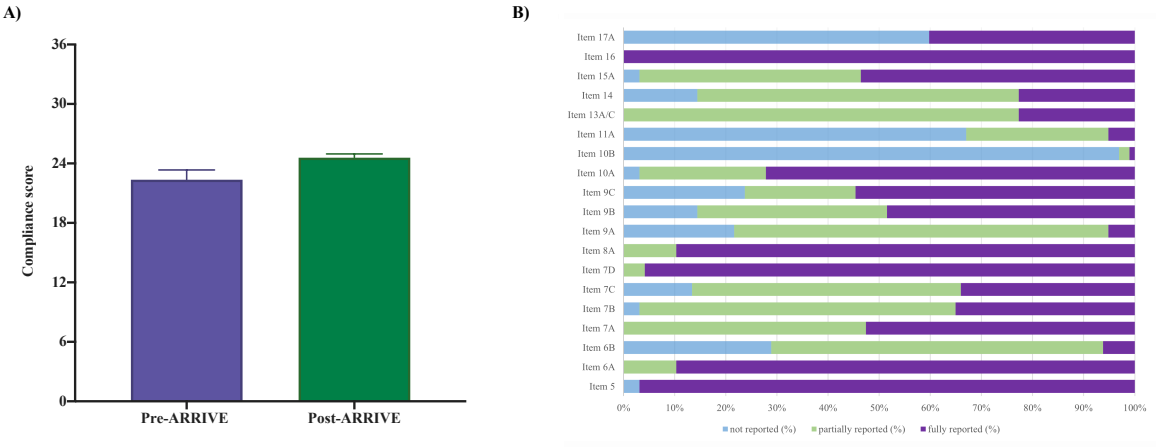

Figure 3. A) Mean compliance score of studies pre-ARRIVE and post-ARRIVE. *N = 97*, B) Percentage (%) of items not reported, partially reported, and fully reported for each ARRIVE item analysed.

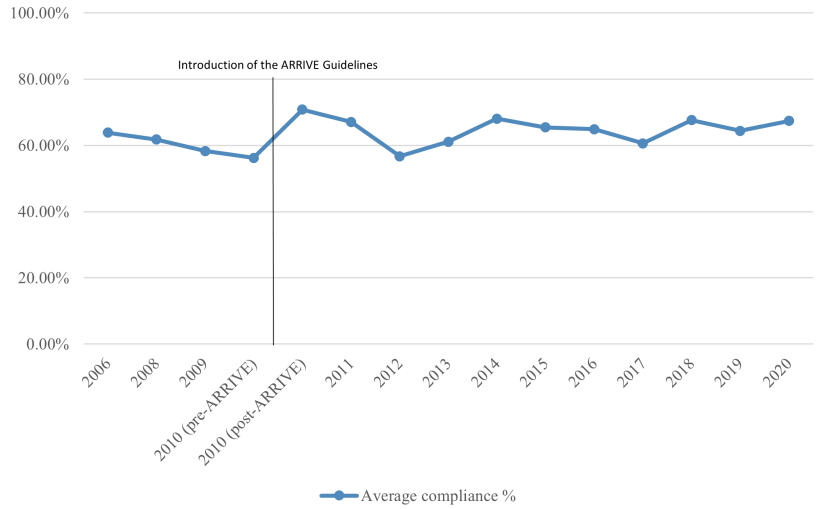

Figure 4. Mean adherence (%) with the ARRIVE guidelines of included studies ranging from 2006-2020 visualising the introduction of the ARRIVE Guidelines in July 2010.

Per-item analysis

Item 5 – ethical statement

Overall, 3.1% of studies reported no ethical statement and 96.9% of studies fully reported an ethical statement. In studies published pre-ARRIVE, 18.2% reported no ethical statement and 81.8% reported an ethical statement. In contrast, in studies published post-ARRIVE 1.2% reported no ethical statement and 98.8% reported an ethical statement.

Item 6a and 6b – study design

It was found that 10.3% of studies partially reported the number of experimental and control groups (item 6a), and 89.7% of studies fully reported. In studies published pre-ARRIVE, 27.3% partially reported and 72.7% fully reported this item, while in studies published post-ARRIVE 8.1% partially reported and 91.9% fully reported. Overall, 28.9% of studies did not report blinding procedures, 65% partially reported, and 6.2% fully reported. Of these studies, those

published pre-ARRIVE, 54.6% did not report and 45.5% partially reported. In contrast, in post-ARRIVE studies 25.6% did not report, 67.4% partially reported, and 7% fully reported.

Item 7a, 7b, 7c, and 7d – experimental procedures

Overall, 47.4% of studies partially reported full experimental procedures (item 7a), and 52.6% fully reported. Of these, 63.6% of pre-ARRIVE studies partially reported and 36.4% fully reported. 45.4% of post-ARRIVE studies partially reported and 54.7% fully reported. In general, 3.1% of studies did not report when procedures were conducted (item 7b), 61.9% partially reported, and 35.1% fully reported. Of these, 72.73% of pre-ARRIVE studies partially reported and 27.3% fully reported. 3.5% of post-ARRIVE studies did not report, 60.5% partially reported, and 36.1% fully reported. Overall, 13.4% of studies did not report where procedures were conducted (item 7c), 52.6% partially reported, and 34% fully reported. Of these, 54.6% of pre-ARRIVE studies partially reported, and 45.5% fully reported. It was discovered that 15.1% of post-ARRIVE studies did not report, 52.3% partially reported, and 32.6% fully reported. Study rationale (item 7d) was partially reported in 4.1% of studies and 95.9% fully reported. Of the pre-ARRIVE studies 9.1% partially reported, and 90.9% fully reported, while 3.5% of post-ARRIVE studies partially reported, and 96.5% fully reported.

Item 8a – experimental animals

Animal characteristics (item 8a) were fully reported in 95.9% of studies and partially reported in 4.1% of overall studies. Pre-ARRIVE studies fully reported in 100% of studies, while 11.6% of post-ARRIVE studies partially reported, and 88.4% fully reported.

Item 9a, 9b, and 9c – housing and husbandry

Overall, 21.7% of studies did not report housing conditions (item 9a), 73.2% partially reported, and 5.2% fully reported. It was found that 18.2% of pre-ARRIVE studies did not report, 72.7% partially reported, and 9.1% fully reported. Also, 22.1% of post-ARRIVE studies did not report, 73.3% partially reported, and 4.7% fully reported. Overall, 14.4% of studies did not report husbandry conditions (item 9b), 37.1% partially reported, and 48.5% fully reported. Of these 63.6% of pre-ARRIVE studies partially reported, and 36.4% fully reported, while 16.3% of post-ARRIVE studies did not report, 33.7% partially reported, and 50% fully reported. Overall, 23.7% of studies did not report welfare related assessments and interventions (item 9c), 21.7% partially reported, and 54.6% fully reported. Of the pre-ARRIVE studies, 54.6% did not report, 9.1% partially reported, and 36.4% fully reported. Alternatively, 19.8% of post-ARRIVE studies did not report, 23.3% partially reported, and 57% fully reported.

Item 10a and 10b – sample size

It was found that 3.1% of studies did not report total number of animals used in each experiment and in each experimental group (item 10a), 24.7% partially reported, and 72.2% fully reported. Of the pre-ARRIVE studies, 45.5% partially reported and 54.6% fully reported, while 3.5% of post-ARRIVE studies did not report, 22.1% partially reported, 74.4% fully reported. Overall, 96.9% of studies did not report sample size calculation (item 10b), 2.1% partially reported, and 1% fully reported. Furthermore, 100% of pre-ARRIVE studies did not report. 96.5% of post-ARRIVE studies did not report, 2.3% partially reported, and 1.2% fully reported.

Item 11a – allocation of animals to experimental groups

Overall, 67% of studies did not report randomisation details for group allocation, 27.8% partially reported, and 5.2% fully reported. It was found that 72.7% of pre-ARRIVE studies

did not report, 18.2% partially reported, and 9.1% fully reported, while 66.3% of post-ARRIVE studies did not report, 29.1% partially reported, and 4.7% fully reported.

Item 13a/c – statistical methods

The vast majority of studies (77.3%) partially reported details of statistical methods (item 13a) and methods used to assess if data approached statistical significance (item 13c), 22.7% fully reported. Overall, 81.8% of pre-ARRIVE studies partially reported, and 18.2% fully reported. Similarly, 76.7% of post-ARRIVE studies partially reported, and 23.3% fully reported.

Item 14 – baseline data

It was found that 14.4% of studies did not report relevant characteristics or health status prior to testing/treatment (item 14), 62.9% partially reported, and 22.7% fully reported. In total 18.2% of pre-ARRIVE studies did not report, 72.7% partially reported, and 9.1% fully reported. Comparably, 14% of post-ARRIVE studies did not report, 61.6% partially reported, and 24.4% fully reported.

Item 15a – numbers analysed

Overall, 3.1% of studies did not report number of animals from each group included in analysis (item 15a), 43.3% partially reported, and 53.6% fully reported. It was also determined that 9.1% of pre-ARRIVE studies did not report, 63.6% partially reported, and 27.3% fully reported, while 2.3% of post-ARRIVE studies did not report, 40.7% partially reported, and 57% fully reported.

Item 16 – outcomes and estimation

It was shown that 100% of pre-ARRIVE and post-ARRIVE studies reported results for each analysis carried out reported with precision (item 16).

Item 17a – adverse events

Overall, 59.8% of studies did not report adverse events (item 17a), and 40.2% fully reported. Furthermore, 72.7% of pre-ARRIVE studies did not report, and 27.3% fully reported. Comparably, a total of 58.1% of post-ARRIVE studies did not report, while 41.9% fully reported.

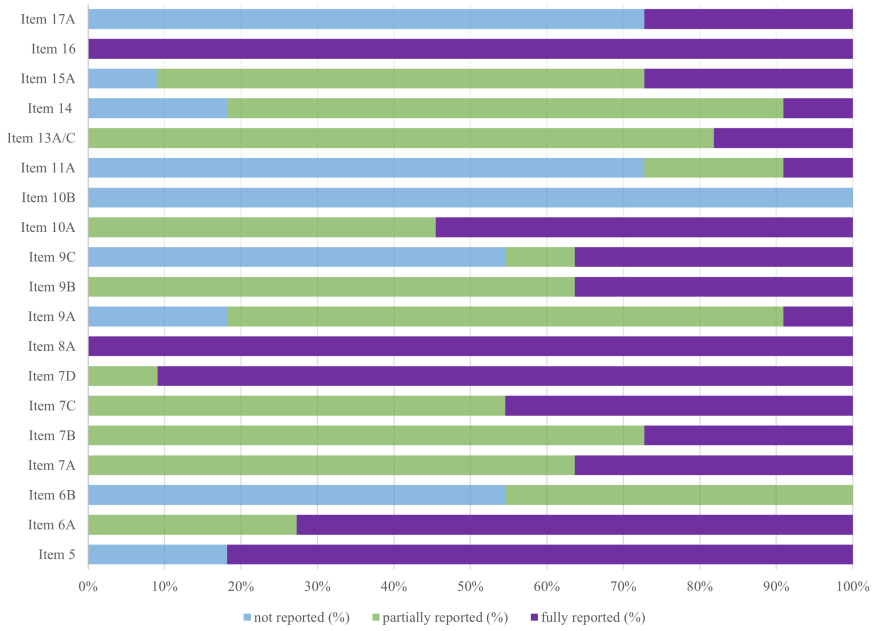

Figure 5. Percentage (%) of items not reported, partially reported, and fully reported for each ARRIVE item analysed pre-ARRIVE. *N = 11*.

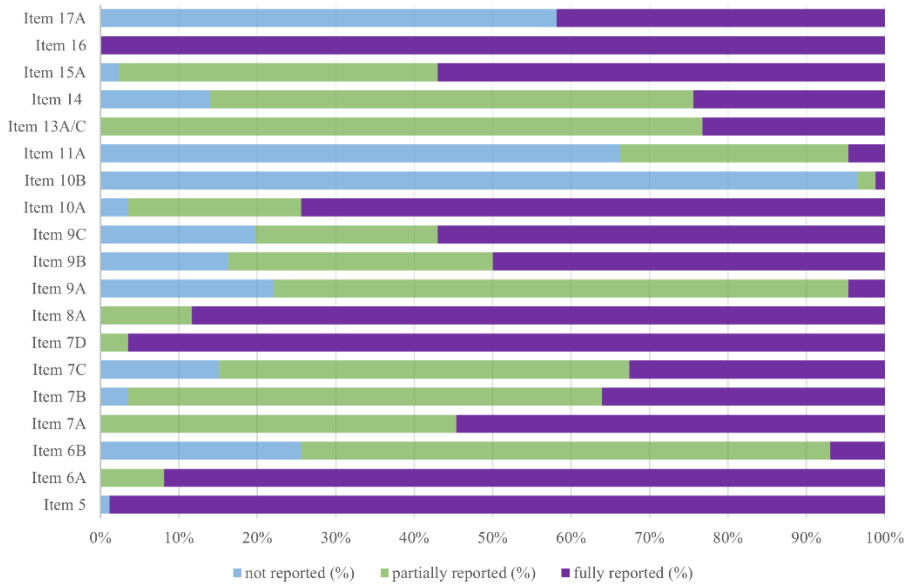

Figure 6. Percentage (%) of items not reported, partially reported, and fully reported for each ARRIVE item analysed post-ARRIVE. *N = 86.*

Discussion

Quality and consistency of reporting in animal-based studies is vital for the replication of results, translation of findings to the clinic, and to ensure resource investment in this type of research is not wasted. This is especially so in research areas such as CICI, where animal models are less well-defined and there is considerable heterogeneity of approach. The ARRIVE guidelines are generally regarded as the benchmark for this reporting, being the most widely accepted by a range of journals publishing pre-clinical animal studies [5]. It is therefore surprising that in spite of the widespread purported journal adherence to these standards no articles achieved full adherence with these guidelines.

Our results illustrate that out of the ARRIVE subitems evaluated few were reported well and in complete adherence with the ARRIVE guidelines, indicating considerable room for improvement. Outcomes and estimations (item 16), ethical statement (item 5), study rationale (item 7d) and animal characteristics (item 8a) were reported in more than 90% of the articles.

It is suggested that these items may be well reported due to being recognised and well taught elements of study design and reporting, which most researchers are au fait with, or are enforced during journal submission processes (ethical statement). For example, researchers often have a good grounding in statistics and presentation of data through formal training, and are aware of the importance of making clear the 'knowledge gap' and study rationale through their experiences in grant writing and acquiring ethical approvals. However, other critical elements in reducing bias were less well reported. In consideration of only some of the key reporting attributes which make up the Landis 4 and the ARRIVE essential list, that of randomization, blinding, and sample-size estimation, in spite of the weight placed on their reporting by these sets of guidelines, adherence was poor.

Although an essential element in reducing bias, randomisation (item 11a) was not mentioned in 67% of articles. Even when randomisation was documented, there was rarely a description of how the randomisation was conducted in order to produce truly comparable groups at selection, or for detection of outcomes. This lack of randomisation can lead to significant risk of introduction of bias. Complete reporting of the nature of randomisation is a key element of appraisal when using the SYRCLE Risk of Bias Tool for quality assessment of animal studies [9]. Other articles have reported similar findings, with Gulin et al., 2015 finding poorer reporting than shown here with only 7 out of 44 (16%) publications reporting on randomization in animal models for Chagas disease [5]. Randomization should also extend further than animal allocation to groups, considering cage placement within rooms, and order of experimental treatments or performance of assays.

Blinding similarly is crucial in terms of reducing bias, however a considerable percentage of studies did not include, or only partially reported this criterion (item 6b; 28.9%, 65%

respectively). Blinding limits bias particularly when qualitative or subjective scoring of experimental observations is performed. This is likely to be especially important in studies in CICI which commonly include behavioural tests or assessment of clinical score where subjectivity can be introduced. Furthermore, true blinding should be feasible in CICI animal models, since the procedures involved tend not to lead to overt signs betraying the nature of group allocation. Our findings reflect previous literature with a survey undertaken across a range of pre-clinical research areas finding that blinding was not reported in 87% of cases [12].

Finally, whilst most authors reported the total number of animals used in each experimental group, the least presented criterion in our investigation was a sample size calculation on how this group size was derived. This went unreported in 96.9% of articles. This is perhaps unusual given the clinical linkages inherent in studies in this area; reporting of a power analysis is strictly enforced in human clinical trials and it might be assumed that researchers are engaged in both human and animal studies making power calculations second nature to them. Furthermore, applications for animal ethical approval often require the demonstration of a power analysis hence this non-compliance is likely to be a true case of non-reporting rather than non-consideration. Power calculations are essential to evaluation, statistical interpretation and replication of findings [12]. They also serve an important ethical role, ensuring prevention of unnecessary animal use, yet also ensuring that studies are not underpowered, hence animal lives being wasted. There is considerable heterogeneity in preclinical CICI studies resulting from aspects of experimental design, animal model choice, treatment procedures and nature of behavioural testing [40]. This is in addition to the variability that arises ordinarily in the use of preclinical animal models due to aspects such as husbandry, innate behaviour and the environment [39]. Improving reporting on methods would be beneficial in reducing the heterogeneity observed. Additionally, in relation to study attrition, differences in samples sizes

were commonly observed between the Methods section and Results. This would suggest the occurrence of adverse events, yet these were infrequently reported (59.8% of articles). Adverse event reporting is probably even more important in CICI studies compared to other pre-clinical animal studies given the lack of standardisation of models. Full and accurate reporting is imperative to avoid future use of animal models that have a high welfare cost or that are not truly representative of the human condition.

The ARRIVE guidelines were introduced in 2010 in an attempt to address poor reporting, especially of critical items [11]. However, our findings suggest that their introduction has had little to no effect on the overall compliance in study reporting. Whilst, impacts of their implementation are expected to take some time to become apparent, over the 10-year passage of time since the guideline introduction this compliance trend has remained static. The ARRIVE guidelines 2.0 were introduced in an attempt to counteract poor compliance rates [24]. In the revised document, rewording to improve clarity and prioritization of certain criteria into the 'Essential 10' has been performed. It remains to be seen what impact these amended guidelines will have on compliance.

By its nature, we have only been able to make an assessment of reporting in this review. Reporting guidelines can only go so far in addressing issues of reproducibility and translatability, since many of the criteria require consideration in experimental planning and conduct. It is the need for pre-consideration of these items that is considered in planning guidelines such as the PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines [31]. It is of course quite possible that researchers were using ARRIVE criteria but failing to report on these. In such cases, internal validity of the study would be preserved but the incomplete reporting is still a considerable

threat to external validity or generalisation of the study findings to inform future work or allow translation. Furthermore, previous study has suggested that inadequate reporting correlates with overstatement of study outcomes [16, 34]. Use of both sets of guidelines will greatly increase the likelihood of translation success and adherence to the 3R's principles [31].

Lack of compliance with the guidelines may arise due to a lack of awareness of their existence, in which case there is a clear need for expanding the education of researchers, probably at the experiment planning stage. Animal Ethics Committees may be best placed to drive this education since they evaluate all protocols and have the ability to only allow protocols to proceed that meet their criteria. Referral to ARRIVE criteria in ethics application forms will not only ensure the criteria are addressed, but will serve to disseminate awareness of the concepts. At the other end of the research process, stricter adherence to the ARRIVE guidelines by journal editors and reviewers, along with the instigation of clear and unambiguous processes associated with them, such as submission of an ARRIVE checklist, will assist in improving reporting and provide a further educative function. This is especially appropriate given the establishment of the international cancer and cognition task force (ICCTF) which aims to identify future research directions and provide recommendations to help standardise experimental design and procedures in animal models of CICI [38]. Improving the quality of reporting will also aid the production of systematic reviews, the means through which the weight of evidence in a particular area can be assessed [26].

Conclusion

The results from this systematic literature review reveal reporting of rodent models in relation to the ARRIVE guidelines rarely met the full set of essential criteria. Given the lack of standardisation of animal models in this research area, this is a particular threat to future

progress and translation of findings. Furthermore, the lack of improvement since the guidelines introduction implies there is still a lack awareness or disbelief of the importance of this reporting. In the short term, this finding may be best remedied through the actions of journal editors and reviewers. In the longer term, the role that animal ethics committees play as the enablers of animal-based research should be considered.

Supplementary Information

Table S1; search strategy, citation details of included studies available on further request.

Declarations

Author contribution

RPG designed the study, assisted in performing the literature search, data extraction, analysis and interpretation and writing the manuscript; IS assisted in study design, data extraction, analysis and interpretation and writing of the manuscript; CC, AW, EBS performed literature search and screened articles by title and abstract; AW involved in the study design, supervision of the development of the work, assisted in performing the literature search, data analysis and interpretation and writing of the manuscript.

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Conflict of interest

The authors declare no competing interests.

Ethics approval

Not applicable.

Consent to participate

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Consent for publication

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Code availability

Not applicable.

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CHAPTER 5

Neuroimmune Reactivity Marker Expression in Rodent Models of Chemotherapy-Induced Cognitive Impairment: A Systematic Scoping Review

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Neuroimmune Reactivity Marker Expression in Rodent Models of Chemotherapy-induced Cognitive Impairment: A Systematic Scoping Review

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Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Assisted in research design, interpretation of results and writing of the manuscript		
Signature		Date	10 Feb 2021

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Contribution to the Paper	Designed study, supervision of the development of the work, assisted in performing the literature search, data analysis and interpretation and writing of the manuscript		
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CONTEXTUAL STATEMENT

Whilst the underlying mechanisms that contribute to CICI remain relatively unknown, neuroinflammation has been suggested as one key contributor. Given the established link between neuroinflammation and cognitive decline in other neurodegenerative diseases such as Alzheimer's disease, similar neuroinflammatory mechanisms would be expected in CICI. Therefore, it is important to determine if there is a link between neuroinflammation and cognitive decline in preclinical rodent models of CICI. This will provide a strong base for future targeted investigation of neuroinflammation as a driver of CICI and assist in developing therapeutic strategies. The systematic scoping review in **Chapter 5** systematically identified and mapped the current evidence on neuroimmune reactivity marker expression changes and resulting cognitive changes in preclinical rodent models of CICI.

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Review Article

Neuroimmune reactivity marker expression in rodent models of chemotherapy-induced cognitive impairment: A systematic scoping review

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ABSTRACT

Background: Chemotherapy-induced cognitive impairment (CICI) is a debilitating side effect arising from chemotherapy treatments. The condition is characterised by a range of cognitive deficits including impairment to memory, attention, and concentration. Whilst the underlying mechanisms that contribute to CICI remain unclear, neuroinflammation has been suggested as one key contributor.
Method: A comprehensive systematic search of EMBASE and Medline via PubMed was conducted to identify studies on neuroimmune reactivity marker expression changes and resulting cognitive changes in preclinical rodent models of CICI.
Results: A total of twenty studies met the eligibility criteria and were included in the scoping review. There was significant heterogeneity in the methodology employed in the included studies. Our findings demonstrate that widespread changes in cytokines, chemokines, microglia reactivity, and astrocyte reactivity are observed in CICI in the brain regions expected to be affected, given the nature of the cognitive impairment observed in CICI.
Conclusions: Although there was considerable heterogeneity in study design that made comparisons between studies difficult, our findings suggest that neuroinflammation commonly occurs in CICI preclinical rodent models and shows an association with cognitive impairment.

1. Introduction

Chemotherapy-induced cognitive impairment (CICI), or colloquially 'chemobrain', is a debilitating sequela resulting from the administration of antineoplastic agents. The condition is characterised by a range of cognitive deficits occurring during and after cessation of cancer chemotherapy treatment. The most common neuropsychological effects include impairment to visual processing, visual motor function, attention and executive functioning (Wigmore et al., 2013). The estimated prevalence of CICI differs widely amongst the literature, from 17 to 75% (El-Agamy et al., 2019; Myers, 2009; Wefel et al., 2004). Those affected by CICI report side effects ranging in severity, from subtle yet notable manifestation, to more severe and sustained levels of impairment, with the duration of side effects persisting for weeks, months, or years post treatment (Cheung et al., 2013; Vardy, 2009). Consequently, cognitive disability caused by chemotherapy treatment has a significant negative impact on patients' quality of life, including: ability to work, care for family, difficulty in multitasking, and maintenance of a social life (Hodgson et al., 2013; Boykoff et al., 2009; Sleight, 2016). Chemotherapy treatments have vastly improved remission rates and long-term survival, creating a "silver tsunami" of cancer survivors. CICI may have a profound impact on the healthcare system due to patients requiring long-term cognitive therapy and rehabilitation (Sleight, 2016; Wertheimer et al., 2010; Janelsins et al., 2011). Thus, understanding the aetiology of CICI and developing therapeutic strategies is critically important, both to minimise the burden on the healthcare system and improve patient quality of life.

Currently, the underlying mechanisms that contribute to CICI remain unclear. However, several mechanisms have been postulated to contribute. These include: blood–brain barrier (BBB) disruption, cellular metabolism and mitochondrial dysfunction, damage-associated molecular patterns (DAMPs) and neuroinflammation (McLeary et al., 2019; Vichaya et al., 2015). There is growing evidence from other central nervous system (CNS) diseases such as Alzheimer's disease and Multiple Sclerosis, that chronic reactivity of glial cells and chronic neuroinflammation can lead to neurodegeneration resulting in long-term

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cognitive deficits (Cherry et al., 2014). Likewise, evidence from both clinical and preclinical research demonstrates that neuroinflammatory mechanisms play a significant role in the sequence of events leading to cognitive decline in CICI (Fig. 1). In particular, it has been recognised that chemotherapy agents exert CNS damage via both direct and indirect effects, having a negative impact on cognition (Wigmore et al., 2013; El-Agamy et al., 2019; Dietrich et al., 2015).

2. The neuroimmune system and CICI

The neuroimmune system has a number of important roles in maintaining homeostasis, maintenance of the blood–brain barrier (BBB), mediating inflammation, healing, and protecting against foreign pathogens (Gimsa et al., 2013; Loftis and Janowsky, 2014; Daneman and Prat, 2015). The system comprises glial cells, which include microglia, astrocytes, and oligodendrocytes. These cell types function to protect the CNS, maintain homeostasis, support neurons and drive neuro-inflammation (Gimsa et al., 2013; Loftis and Janowsky, 2014).

Neuroinflammation refers to the inflammation of nervous tissue caused by a stimulus such as infection, traumatic brain injury or toxic metabolites (Ebert et al., 2019). Acute inflammation typically presents immediately after an injury with rapid glial cell activation and resolution (Fleit et al., 2014; Ansar and Ghosh, 2016). Alternately, chronic inflammation is characterised by slower onset time, greater cellular infiltration and cell recruitment leading to increased duration and injury severity (Fleit et al., 2014; Ansar and Ghosh, 2016). Whilst, previously it was thought that inflammatory mediators could not pass the BBB to enter the CNS, it has now been shown that elevated levels of cytokines alter the integrity and permeability of the BBB, and allow the transport of peripheral immune cells into the CNS further contributing to neuro-inflammation (Lyman et al., 2014). Therefore, peripheral inflammatory events may also play a role in the pathogenesis of CICI (Wardill et al., 2016). A challenge in investigations of neurodegenerative disorders, such as in CICI, is in determining when levels of inflammatory mediators go beyond that required for a homeostatic maintenance function and can be referred to as neuroinflammation (Bilbo et al., 2012).

As a sequelae of amplified or chronic glial activation, inhibition of neuronal regeneration and consequent neuronal death may occur. This leads to functional and structural changes in the CNS. These changes have implications for cognition, for example impairment to learning and memory (McLeary et al., 2019). The release of neurotoxic products post chemotherapeutic treatment, including reactive oxygen species (ROS) and damaging enzymes, can destroy brain tissue (McLeary et al., 2019; Sartori et al., 2012; Raz and Rodrigue, 2006; Seigers and Fardell, 2011). Brain structures that play important roles in cognition, including the limbic system, basal ganglia, and hippocampus, contain more enzymes involved in the inflammatory response compared to sensory or motor related brain structures. Therefore, these areas may be more susceptible to cumulative damage, leading to knock-on detrimental effects to cognitive process such as attention, memory, and perception as commonly observed in CICI (Sartori et al., 2012). In addition, neuro-inflammation may lead to complete loss of synaptic function or impairment of synaptic plasticity, which is associated with memory formation and consolidation. Over expression of pro-inflammatory mediators such as IL-6, IL-1β and TNF-α, which are strongly expressed in the hippocampus post-chemotherapy insult, have been implicated in: inhibition of neurogenesis, prevention of long-term potentiation (LTP) induction and maintenance, amplification of sickness behaviour and memory and learning impairment (McLeary et al., 2019; Donzis and Tronson, 2014).

Additionally, in the clinical setting, neuroimaging studies have provided evidence of structural and functional brain changes related to chemotherapy. Neurostructural deterioration induced by chemotherapy has been shown in breast cancer patients through diffusion tensor

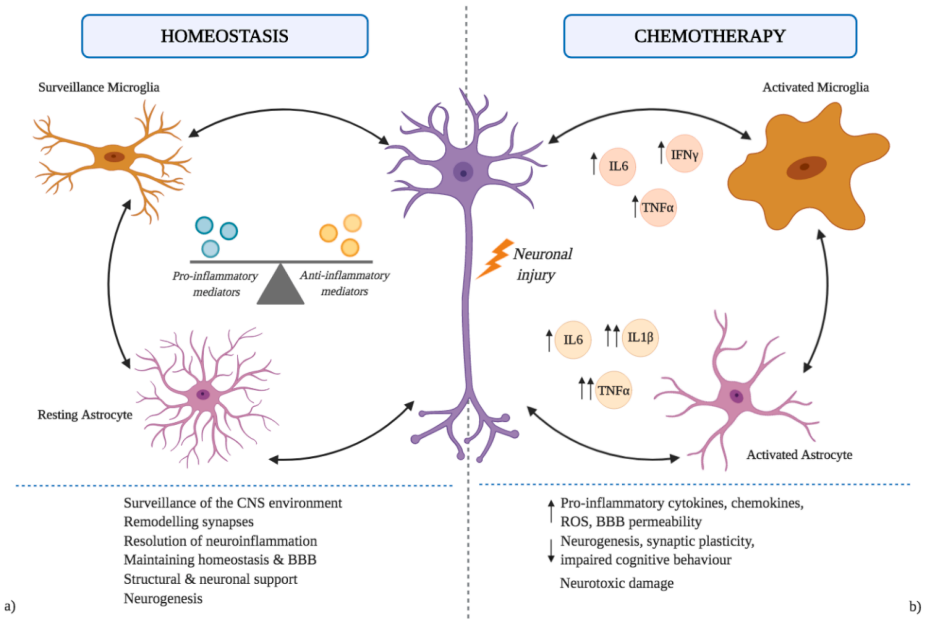

Fig. 1. Neuroinflammation in CICI. (a) Resting microglia and astrocytes maintain homeostasis in a healthy CNS environment (structural support, surveillance, maintenance of BBB). (b) When neuronal insult occurs via chemotherapy, microglia and astrocytes become activated and overexpress pro-inflammatory mediators (ROS, cytokines, chemokines) leading to neuronal damage (neurogenesis, synaptic plasticity, impaired cognition). Created with BioRender.com.

neuroimaging and has been linked to reduced cognitive performance (Santos and Pyter, 2018; Bergouignan et al., 2011). Specifically, chemotherapeutic agents have been shown to change brain structure, with reduced grey and white matter in the cortex and corpus callosum and decreased hippocampal volume (Vichaya et al., 2015; Dietrich et al., 2008; Kesler et al., 2013; McDonald et al., 2010; Deprez et al., 2011). These structural changes have been detected up to twenty years post cessation of chemotherapy and have the potential to be progressive (Santos and Pyter, 2018). As such, this lends support to the idea that neuroinflammation is a potential cause of cognitive impairment, with peripheral inflammation, such as elevated cytokine release as a consequence of chemotherapy-induced gut toxicity, also being linked to cognitive symptoms in cancer patients (Wardill et al., 2016; Santos and Pyter, 2018; Seigers et al., 2016; Ganz et al., 2013; Pomykala et al., 2013; Janelsins et al., 2012). Although neuroimaging cannot be used to directly link neuroinflammation to cognitive impairment, circulating levels of inflammatory markers have been positively associated with symptoms of cognitive dysfunction (Vichaya et al., 2015; Santos and Pyter, 2018).

3. Animal models of CICI

Whilst clinical studies allow us to have a better understanding of symptoms associated with CICI, as well as patient lived experience, they are unable to confirm the neurological mechanisms that cause CICI as they primarily utilise self-report surveys and MRI scans. Additionally, these studies have a number of significant limitations including the study of small patient populations, the inability to exclude confounding factors such as disease-related co-morbidities that could affect cognitive performance, and the use of cross-sectional study designs preventing determination of trajectory of cognitive change or baseline cognitive ability (Seigers and Fardell, 2011; Taillibert et al., 2016).

Given the difficulty in designing the ideal study employing CICI patients, pre-clinical animal studies play a pivotal role. Compared to clinical research, these studies provide controlled experiments to investigate the extent of learning and memory deficits, access CNS tissue to ascertain direct measures of neuroinflammation, and link effects with specific chemotherapy agents (Vichaya et al., 2015). Many of these studies utilise rodent models. Unfortunately, preclinical rodent models of CICI are diverse, and a gold-standard, off the shelf model does not exist. Prominent discrepancies exist in intervention characteristics (chemotherapeutic agents and/or combination, route of administration, dose frequency, treatment regime), cancer presence, outcome measures used e.g. behavioural test types and neurobiological outcomes (measures of immune system functionality, CNS function, inflammation) and the timepoints utilised for outcome measures (Matsos and Johnston, 2019). To some extent, these discrepancies likely reflect the clinical scenario with a wide range of chemotherapy regimens being utilised depending on cancer type. However, non-standardisation of models does present a challenge when assimilating the pre-clinical evidence to guide future research directions.

Given the established link between neuroinflammation and cognitive decline in other neurodegenerative diseases, similar neuroinflammatory mechanisms would be expected in CICI. Several review papers in this area also imply that there is a neuroinflammatory component to CICI pathogenesis (McLeary et al., 2019; Vichaya et al., 2015; Wardill et al., 2016; Santos and Pyter, 2018; Seigers et al., 2016). However, to date there has been no systematic identification of all the preclinical literature investigating this link. Such a review is needed to guide directions in research, allowing for future targeted investigation of neuroinflammation as a driver of CICI, which may assist in the development of therapeutic strategies. Thus, the specific aim of this scoping review was to identify and map the available literature on neuroimmune reactivity marker expression changes and resulting cognitive changes, assessed through behavioural tests, in preclinical rodent models of CICI.

4. Method

4.1. Search strategy

A comprehensive search using EMBASE and Medline via PubMed was conducted to identify eligible peer reviewed publications. The search consisted of a pre-conceived list of synonyms for "chemobrain", "rodent", "cognitive dysfunction" and "neuroimmunity''. An initial search was conducted in August 2019 and was updated in May 2020. The search strategy (refer to Supplementary material S1) was developed in consultation with an information specialist to ensure all identified index terms and keywords were suitable and results rendered from the initial search were reproducible. Additionally, hand searching of reference lists was performed. Two independent reviewers (RG and IS) screened articles based on inclusion and exclusion criteria and conflicts were resolved by discussion. In the event that conflicts were unable to be resolved via discussion a third reviewer was consulted.

4.2. Neuroimmune definition

Based on our understanding of the underlying mechanisms of well-researched neurodegenerative disease, such as Alzheimer's disease, we hypothesise that similar pathways might contribute to the pathogenesis of CICI. As such, there is a need to focus on the expression of reactivity markers of microglia and astrocytes, and the quantification of cytokines, and chemokines in the CNS. Neuroimmune was defined as changes in the components of the central nervous system that modulate immune responses. This included glial cells (astrocytes and microglia), cytokines (pro-inflammatory and anti-inflammatory) and chemokines.

4.3. Inclusion and exclusion criteria

In order to be included for review, articles were required to meet the following criteria: [1] original full text articles available from database inception; [2] use of a rodent model regardless of age, sex or strain (P); [3] administration of a cancer chemotherapeutic agent regardless of route of administration or dosage to either naive or cancer-incoulated animals (I); [4] studies that included an approriate control group; [5] studies that assessed chemotherapy agent effects on the neuroimmune response, as defined previously, alone or in conjunction with one or more behavioural tests of cognition (O); [6] English language publications.

The exclusion criteria for articles were as follows: studies that gave additional supportive medications/treatments in conjunction with chemotherapy without inclusion of the appropriate control group; studies investigating mechanisms that precede or result from neuroimmune reactivity marker expression, such as production of reactive oxygen species if neuroimmune reactivity marker expression itself was not determined; studies investigating peripheral neuropathy, papers looking at behavioural outcomes alone.

4.4. Search results

The database search identified a total of 592 studies. An additional 10 studies were sourced from reference lists of peer review publications (Fig. 2) Following the database search, all citations were initially uploaded to EndNote (EndNote™ X9) and imported into Covidence (Covidence systematic review software, Veritas Health Innovation, Melbourne, Australia) where duplicates were identified and removed. Each of the identified studies were screened based on title and abstract against the pre-defined eligibility criteria by two independent reviewers (RG and IS). Disagreements were discussed and settled by consensus, with a third reviewer (AW) being consulted if consensus was not reached. A total of 547 studies were excluded based on title and abstract, rendering 39 potentially relevant studies for full text assessment.

Full text and Supplementary material were retrieved for the

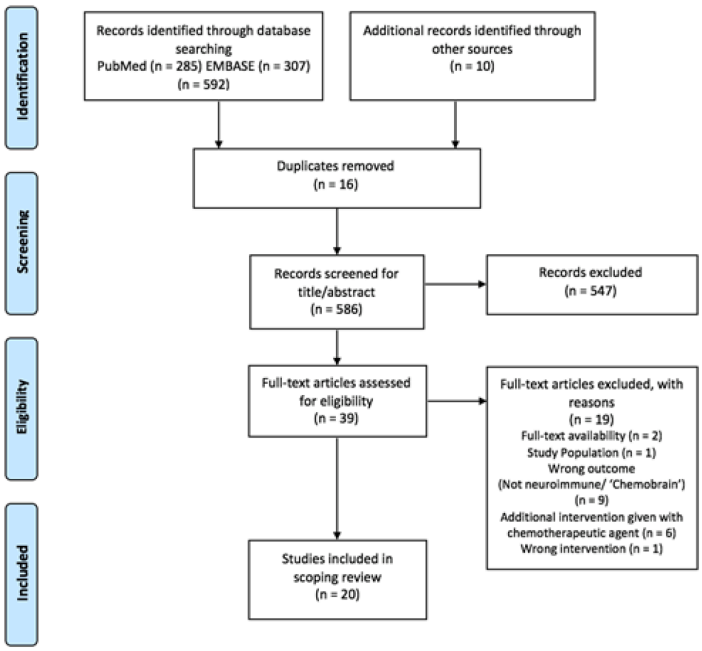

Fig. 2. PRISMA flow diagram for the scoping review process (Moher et al., 2009).

remaining studies and imported in Covidence. Two independent reviewers thoroughly assessed the studies by full text for their eligibility for inclusion. Discrepancies were resolved through an initial discussion between the two reviewers and consultation with a third reviewer when further disagreements arose. During the eligibility process, 19 studies were excluded from the scoping review. Studies were excluded due to not including a neuroimmune outcome measure, not assessing chemotherapy-induced cognitive impairment, not utilising a chemotherapeutic agent, studies in humans or in vitro, the inclusion of additional treatment in conjunction with chemotherapy agents without the presence of a control group and full-text not available. Details of the identification process are described in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (Fig. 2).

4.5. Data extraction

Data were extracted from included studies by two independent reviewers using an extraction template developed in Microsoft Excel (Microsoft Excel for Mac, Version 16.16.19) (refer to Supplementary material). Prior to data extraction both reviewers independently extracted data from two studies and compared the results to ensure consistency in the process. Relevant information regarding citation information (authors, year of publication, title), study objective, population (species, strain, sex, age, weight), study design, intervention characteristics (chemotherapeutic agents and/or combination, route of administration, dose frequency, treatment regime), outcome measurements (tissue analysed, glial cells, cytokines and chemokines, behavioural tests), and time-point (0–7 days acute, 8 days-12 weeks sub-acute, and 12 weeks and over chronic) related to the review question were extracted. Where the study investigated the effects of a treatment in conjunction with chemotherapy, only data from control groups were extracted as relevant to the review question. Extracted data were collated into a master copy and cross-checked and verified. Where required, Supplementary material was utilised for additional data and authors were contacted to request additional information.

5. Results

5.1. Descriptive characteristics of included papers

A total of 20 studies met the eligibility criteria and were included in the scoping review. A detailed summary of the descriptive characteristics of included studies is presented in Table 1. The publication dates ranged between 2006 and 2020, with 30% of the included papers published in 2018. Data was provided from ten countries (USA, South Korea, China, Netherlands, Canada, Australia, Thailand, India, Saudi Arabia and Egypt). The majority of the studies (75%) utilised a mouse model, including nine different strains. Of these studies, five assessed study outcomes utilising multiple strains. Conversely, the use of a rat model was reported in 25% of the studies with the utilization of three strains (Athymic nude, Sprague Dawley, Albino). Eleven studies (55%) investigated study outcomes in females. Males were represented in seven studies (35%). One study assessed both sexes and one study failed to report on sex. Information regarding samples sizes (animals per group) varied amongst the included studies ranging between 8 and 20 per treatment group. The selected time-point to assess neurobiological outcomes varied greatly across studies ranging between 24 h – 168 days post chemotherapy treatment. In the following presentation of results, changes presented are in comparison to the relevant control group.

5.2. Intervention characteristics (chemotherapy agents and dosage schedule)

Within the past decade, administration of a range of

Table 1
Summary characteristics of included papers.

Author, Year, Country	Objective	Species/ Strain	Sex	Age/ Weight	Animals per Group	Intervention	Comparator	Accompanying Conditions	Humane Endpoint	Outcome Neuroimmune engagement
(Acharya et al., 2015), USA	Assess benefits of transplantation of human neural stem cells (hNSC) in ameliorating chemobrain, in part through suppression of neuroinflammation	Rat Athymic nude	NR	Four months	n = 8	Cyclophosphamide (CYP)	Saline	Intrahippocampal transplantation of hNSCs. Control animals received sham surgery (hibernation buffer at the same stereotaxic coordinates)	8 weeks	CD68β, ED1+
(Alexander et al., 2019), USA	Assess how a relatively low dose of thioTEPA would affect cognition and motor coordination, dendritic complexity, and cytokine production levels	Mouse C57BL/6J	M	Two months	n = 10 control; n = 8 treatment	ThioTEPA	Saline	Naive	5 weeks	IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-10, IL-12p70, TNF-α RANTES (CCL5), MCP-1, GM-CSF
(Allen et al., 2019), USA	Assess neurocognitive and anti-inflammatory effects of two distinct strategies including a dietary treatment with the CSF-1R inhibitor PLX5622 and intravenous injections of EV isolated from human induced pluripotent stem cell (iPSC)-derived microglia (iMG)	Mouse Wild type C57BL/6J	M	Six months	CSF1R inhibition study: n = 10–12	Adriamycin (ADR)	Saline	Naive	7 weeks	IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, IFNγ, CD68+, Iba1, RANTES (CCL5), MCP-1, MIP-1α, GM-CSF, *mRNA RANTES (CCL5) & CCL20 mRNA IL-6, IL-4, & IFNα1*
		Mouse Wild type C57BL/6J	M	Six months	IMG-EV study: n = 8	Adriamycin (ADR)	PBS	Naive	8 weeks	CD68+, Iba1
(Bagnall-Moreau et al., 2019), USA	Examine the effects of chemotherapy on markers in inflammation, oxidative stress, and the antioxidant defence in the hippocampus, as a potential mechanism for chemotherapy-induced cognitive impairment	Rat Sprague Dawley	F	8–10 weeks	NR	Doxorubicin & Cyclophosphamide	Saline	Naive	1 week	IFNγ, IL-1α, IL-1β, IL-1RA, IL-2, IL-3, IL-4, IL-6, IL-10, IL-13, IL-17, TNF-α, TIMP-1, VEGF, CINC-1, CINC-2, CINC-3, CNTF, FRACTAL-KINE, GM-CSF, SICAM-1, IP-10 (CXCL10), LIX (CXCL5), L-SELECTIN, MIG (CXCL9), MIP-1α (CCL3), MIP-3α (CCL20), RANTES, Thymus Chemokine, *mRNA TNF-α*
(Briones and Woods, 2014), USA	Assess the role of neuroinflammation and myelination in chemotherapy-related cognitive impairment	Rat Sprague Dawley	F	12 months	n = 13	Cyclophosphamide, Methotrexate & 5-Fluo-rouracil (CMF)	Saline	Cyclooxygenase-2 (COX-2) inhibitor. Control animals received saline (vehicle)	4 weeks	IL-1β, IL-10, TNF-α
(El-Agamy et al., 2018), Egypt	Determine whether AST could confer a neuroprotective effect against DOX-induced chemobrain and elucidate its underlying molecular mechanisms in terms of oxidative stress, inflammatory, apoptotic and acetylcholinesterase modulatory arbiters	Rat Albino	M	150–200 g	n = 10	Doxorubicin hydrochloride (DOX)	Saline + Olive oil	Naive	9 days	TNF-α, GFAP
(Fardell et al., 2014), Canada, Australia	Assess impact of systemic intermittent and sustained DTX on neural morphology and cognition in healthy mice	Mouse CD-1	M	27–33 g	n = 9–12	Docetaxel (DTX)	Saline	Naive	1 hr, 6 hr, 24 hr, 48 hr, 9 days & 29 days	GFAP
(Feiock et al., 2016), USA	Evaluate the cognitive impact of radiation in a rodent model system and its potential relationship with cancer/chemotherapy induced cognitive impairment	Mouse BALB/c	F	16–18 weeks	n = 9	Methotrexate (MTX)	Saline	Naive	72 hr, 30 days	TNF-α, Iba1, GFAP

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Table 1 (*continued*)

Author, Year, Country	Objective	Species/ Strain	Sex	Age/ Weight	Animals per Group	Intervention	Comparator	Accompanying Conditions	Humane Endpoint	Outcome Neuroimmune engagement
(Flanigan et al., 2018), USA	Understand post-chemotherapy cognitive impairment deficits in ovariectomized female mice treated with DOX, CYP, or DOX and CYP, assessing a wide range of behaviours, hippocampal spine densities, brain antioxidant activity, and hippocampal cytokine levels	Mouse C57BL/6J (B6)	F	Approx. postnatal day 76	n = 19–20	Cyclophosphamide (CYP); Doxorubicin hydrochloride (DOX); Doxorubicin & Cyclophosphamide	Saline	Ovariectomized approximately postnatal day 56	73 days	IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17, TNF-α, IFNγ RANTES (CCL5), MCP-1, MIP-1α, GM-CSF
(Gibson et al., 2019), USA	Assess underlying mechanism of neurological dysfunction resulting from methotrexate treatment	Mouse CB57BL/6J bred with CD-1	F M	NR	NR	Methotrexate (MTX)	PBS	Naive	4 weeks & 6 months	CD11b+, Glast+
(Ramalingayya et al., 2019), India, Saudi Arabia	Investigate if Rutin can protect against DICD in N-methyl-N-nitrosourea (MNU) –induced mammary carcinoma in rats without interfering with Dox anticancer potential	Rat Sprague Dawley	F	30–35 days	n = 9	Doxorubicin hydrochloride (DOX)	0.3% carboxy methyl cellulose in distilled water	MNU-induced mammary carcinoma	12 days	TNF-α
(Seigers et al., 2016), Netherlands	Assess 6 chemotherapeutics on cognition and immunohistochemical analysis short-term and long-term after treatment	Mouse CB57BL/6J	M	11 weeks 26.6 g average	NR	Cyclophosphamide, Docetaxel, Doxorubicin, 5-Fluorouracil, Topotecan	Saline	Naive	3 weeks & 16 weeks	Iba1
		Mouse FVB wild type	M	11 weeks 26.6 g average	NR	Topotecan	Saline	Naive	3 weeks	Iba1
		Mouse Abcg2; Abcb1a/b (KO)	M	11 weeks 26.6 g average	NR	Topotecan	Saline	Naive	3 weeks	Iba1
(Shi et al., 2018), China	Examine whether resveratrol, a natural polyphenol that has nootropic effects, could prevent chemobrain and its underlying mechanisms	Mouse C57BL/6J	F	18–20 g	n = 10	Docetaxel, Adriamycin & Cyclophosphamide (DAC)	Saline	Naive	18 days	IL-4, IL-6, IL-10, TNF-α
(Shi et al., 2019), China	Assess effect of Rg1 (anti-inflammatory) on cytokines in a mouse model of chemotherapy-induced cognitive impairment	Mouse C57BL/6J	F	18–20 g	n = 10	Docetaxel, Adriamycin & Cyclophosphamide (DAC)	Saline	Naive	18 days	IL-4, IL-6, IL-10, TNF-α, Iba1, GFAP
		Mouse Thy1-YFP H-line transgenic	NR	NR	n = 10	Docetaxel, Adriamycin & Cyclophosphamide (DAC)	Saline	Naive	13 days	IL-4, IL-6, IL-10, TNF-α
(Shi et al., 2019), China	To test the hypothesis that chemotherapy-induced cytokine dysregulation and disrupted neuroplasticity in related brain regions may lead to the development of chemobrain in a DAC mouse model of chemobrain	Mouse C57BL/6J	F	18–20 g	n = 10	Docetaxel, Adriamycin & Cyclophosphamide (DAC)	Saline	Naive	18 days	IL-4, IL-6, IL-10, TNF-α
		Mouse Thy1-YFP H-line transgenic	F	18–20 g	n = 10	Docetaxel, Adriamycin & Cyclophosphamide (DAC)	Saline	Naive	13 days	IL-6, TNF-α
(Smith et al., 2014), USA	Develop a mouse model of cancer treatment related symptoms to assess IL-1β and TNF-α signalling and its role in these symptoms	Mouse NR	F	NR	n = 12	Cyclophosphamide, Doxorubicin & 5-Fluoro-uracil (CAF)	Saline	Naive	24 hr	IL1-β, TNF-α
	Evaluate the relationship between ADR-induced TNF production,	Mouse B6C3	M	8 weeks 25–30 g	NR	Doxorubicin hydrochloride (DOX)	Saline	Anti-TNF antibody or preimmune IgG Control	3 hr	TNF

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Table 1 (*continued*)

Author, Year, Country	Objective	Species/ Strain	Sex	Age/ Weight	Animals per Group	Intervention	Comparator	Accompanying Conditions	Humane Endpoint	Outcome Neuroimmune engagement
(Tangpong et al., 2006), USA, Thailand	mitochondrial dysfunction, and CNS injury							animals received saline (vehicle)		
(Yang et al., 2012), South Korea	Evaluate MTX in a breast cancer model through hippocampal cognitive tests, neurogenesis and neuroinflammation	Mouse C3H/HeN	F	6 weeks	n = 9	Methotrexate (MTX)	Saline	FM3A mammary carcinoma Control animals received sham injection of PBS	24 hr	Iba1
(Yi et al., 2020), China	Assess the cognitive changes after curcumin administration in a mouse model of cisplatin-induced cognitive impairment	Mouse C57BL/6	M	8 weeks 24 ± 2 g	n = 15	Cisplatin	PBS	Naive	NR	Iba1, *mRNA IL-1ß, IL-6 & TNF-α*
(Zhou et al., 2016), USA	Establish a mouse model of cisplatin-induced cognitive impairment and determine potential prevention effects of metformin	Mouse C57BL/6J	F	8–10 weeks	n = 10–14	Cisplatin	Saline	Naive	4 weeks	CD11β, GFAP

chemotherapeutic agents has been associated with development of neuropsychological deficits. The choice of chemotherapy agents varied substantially across studies including regimens involving solo and combination drugs. A total of fourteen studies utilised a sole agent with eight different chemotherapy agents identified. These chemotherapy regimens included three alkylating agents, three mitotic inhibitors and two antimetabolites; Cyclophosphamide (CYP; n = 3), ThioTEPA (n = 1), Doxorubicin (DOX; n = 7), Cisplatin (n = 2), Docetaxel (DTX; n = 2), Topotecan (n = 2), 5-Fluorouracil (5-FU; n = 1) and Methotrexate (MTX; n = 3). Chemotherapy agents administered in combination were employed by seven studies utilising four different combinations; DAC (n = 3), CMF (n = 1), CAF (n = 1) and DOX/CYP (n = 2). DOX was the most commonly used chemotherapy agent, either as a sole agent or in combination with other chemotherapy agents. Likewise, CYP was also frequently used in combination or alone. The intraperitoneal route was most commonly used for administration. Dose and dosing frequency varied both between chemotherapy agents but also for the same agent across studies. Utilising a repeated dose cycle is generally considered ideal to mimic the clinical setting. Repeated dose cycle was employed in the majority of studies and observed in the majority of chemotherapy agents reported, except Topotecan and 5-FU. A single dose was reported for MTX, 5-FU, Topotecan, DTX, DOX and CYP. One study assessed multiple chemotherapy agents, in combination and as a sole agent. Another study specifically assessed five different chemotherapy agents. Characteristics of chemotherapy agents and dosage schedule are provided in Table 2.

5.3. Cognitive assessment through behavioural tests and neuroimmune linkage

The effects of chemotherapy agents on cognitive function as assessed by behavioural tests of cognition was investigated in fifteen studies (75%) (refer to Supplementary material S2 for further detail). Cognitive domains assessed were predominantly short-term memory, long-term memory and executive function. A total of eleven cognitive tests were employed across the included studies, with cognitive impairment observed in 83% of the tests, and no difference observed in 17% of tests (Fig. 3). Of these studies, a total of two studies assessed cognition at an acute time-point, thirteen studies assessed cognition at a sub-acute time-point, including one study that additionally assessed a chronic time-point. No differences were observed at a sub-acute time-point across three studies.

Across the fifteen studies that assessed cognitive function the following molecular changes were also assessed in tandem, including: seven pro-inflammatory cytokines, three anti-inflammatory cytokines, two chemokines, seven microglia reactivity markers and two astrocytic reactivity markers (Table 3). Short-term and long-term memory were significantly impaired ($p < 0.05$), with increases found in TNF-α (57.14%, n = 7), IL-6 (37.5%, n = 5), GFAP (100%, n = 2), and IL-6 + Iba1 (100%, n = 1) and decreases found in IL-6 (12.5%, n = 5), IL-2, IL-3, IL-5, GM-CSF, IL-12 and IL-10 (50%, n = 2 respectively), IL-1ß (33%, n = 3), IL-4 (60%, n = 5) and Arg-1 (100%, n = 1). Recognition memory was significantly impaired ($p < 0.05$), with increases found in IL-1ß (66.67%, n = 3), ED-1+, FACS, Glast + and IFNα (100%, n = 1 respectively), IL-3, IL-5, IL-12, GM-CSF (50%, n = 2 respectively), RANTES, GFAP, CD68+/Iba1 (50%, n = 1 respectively), CD68+ (66.67%, n = 2), and TNF-α (50%, n = 4) and decreases found in IL-1ß, IL-6, IL-10 (33%, n = 3 respectively), and IL-12 (50%, n = 2). Contextual memory/stimulus response learning was significantly impaired, with increases found in ED-1+, IL-6, RANTES, IL-1ß, IL-3, IL-5, IL-12, IFNα1, GM-CSF and CD68+ (50%, n = 1 respectively), no decreases were found (n = 2). Avoidance learning was significantly impaired, with increases found in TNF-α (100%, n = 1), GFAP, and Iba1 (50%, n = 1 respectively), no decreases were found (n = 2). Executive control was significantly impaired, however no increases or decreases in molecular analysis was found (n = 1).

Table 2
Overview of Chemotherapy dosage schedule used in included papers.

Species	Chemotherapy	Dosage	Dosing Schedule	Route of Administration	References
Mouse	Cyclophosphamide (CYP)	50 mg/kg	4 doses (once weekly/4 weeks)	Intravenous	(Flanigan et al., 2018)
		150 mg/kg	1 dose	Intravenous	(Seigers et al., 2016)
	ThioTEPA	10 mg/kg	3 doses (once weekly/3 weeks)	Intraperitoneal	(Alexander et al., 2019)
	Adriamycin/Doxorubicin hydrochloride (DOX)	2 mg/kg	4 doses (once weekly/4 weeks)	Intraperitoneal, Intravenous,	(Allen et al., 2019; Flanigan et al., 2018)
		5 mg/kg	1 dose	Intravenous	(Seigers et al., 2016)
		10 mg/kg	1 dose	Intravenous	(Seigers et al., 2016)
		20 mg/kg	1 dose	Intraperitoneal	(Tangpong et al., 2006)
	Cisplatin	2.3 mg/kg	15 doses (5 daily injections followed by 5 days rest for 3 cycles)	Intraperitoneal	(Yi et al., 2020; Zhou et al., 2016)
	Docetaxel (DTX)	8 mg/kg	1 dose (sustained release/DTX-PoLigel once weekly/4 weeks); 4 doses (once weekly/4 weeks)	Intraperitoneal	(Fardell et al., 2014)
		33 mg/kg	1 dose	Intravenous	(Seigers et al., 2016)
	Topotecan	15 mg/kg	1 dose	Intraperitoneal	(Seigers et al., 2016)
		25 mg/kg	1 dose	Intraperitoneal	(Seigers et al., 2016)
	5-Fluorouracil (5-FU)	75 mg/kg	1 dose	Intraperitoneal	(Seigers et al., 2016)
	Methotrexate (MTX)	100 mg/kg	3 doses (once weekly/3 weeks)	Intraperitoneal	(Gibson et al., 2019)
		40 mg/kg	1 dose	Intraperitoneal	(Yang et al., 2012)
		0.75 mg/kg	3 doses (once weekly/3 weeks)	Intraperitoneal	(Feiock et al., 2016)
	Doxorubicin and Cyclophosphamide	DOX 2 mg/kg CYP 50 mg/kg	4 doses (once weekly/4 weeks)	Intravenous	(Flanigan et al., 2018)
	Docetaxel, Adriamycin, and Cyclophosphamide (DAC)	DTX 10 mg/kg ADR 10 mg/kg CYP 40 mg/kg	3 doses (2-day intervals for 7 days)	Intraperitoneal	(Shi et al., 2018; Shi et al., 2019; Shi et al., 2019)
	Cyclophosphamide, Doxorubicin, and 5-Fluorouracil (CAF)	CYP 167 mg/kg DOX 4 mg/kg 5-FU 167 mg/kg	1 dose; 4 doses (at 10–20-day intervals)	Intraperitoneal	(Smith et al., 2014)
Rat	Cyclophosphamide (CYP)	100 mg/kg	4 doses (once weekly/4 weeks)	Intraperitoneal	(Acharya et al., 2015)
	Adriamycin/Doxorubicin hydrochloride (DOX)	2 mg/kg	4 doses (once weekly/4 weeks)	Intraperitoneal	(El-Agamy et al., 2018)
		2.5 mg/kg	10 doses (every 5 days/50 days)	Intraperitoneal	(Ramalingayya et al., 2019)
	Cyclophosphamide, Methotrexate, and 5-Fluorouracil (CMF)	CYP 40 mg/kg MTX 37.5 mg/kg 5-FU 75 mg/kg	4 doses (once weekly/4 weeks)	Intraperitoneal	(Briones and Woods, 2014)
	Doxorubicin and Cyclophosphamide	DOX 4 mg/kg CYP 40 mg/kg	3 doses (once weekly/3 weeks)	Intravenous	(Bagnall-Moreau et al., 2019)

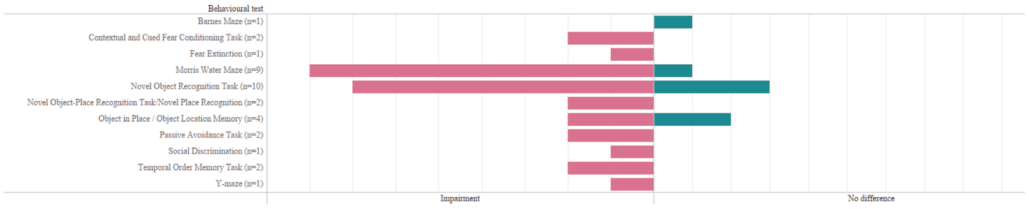

Fig. 3. Effect of chemotherapy on cognition measured using behavioural tests of cognition. Note that some papers assessed behavioural outcomes utilising the same test for multiple experiments in a single study. *n* represents the number of tests.

5.4. Outcome measures

5.4.1. Microglia

A total of nine studies (45%) assessed markers for microglial reactivity marker expression (Table 4). Ionized calcium-binding adapter molecule 1 (Iba1) was the most frequently investigated marker of microglial reactivity marker expression across the studies. Additionally, microglial reactivity marker expression was evaluated in fifteen brain regions, with the hippocampus most commonly investigated (n = 6). There were statistically significant ($p < 0.05$) increases found in microglial reactivity marker expression in the dentate hilus (n = 1), dentate gyrus (n = 1), CA1/C3 subfields (n = 1), hippocampus (n = 2),

Table 3
Neuroimmune reactivity expression and implicated cognitive domains (%) represents the percentage of studies that examined this cognitive domain that found cognitive impairment and associated neuroimmune expression marker changes.

Cognitive domains impaired	Behavioural assessment	Neuroimmune engagement	No. of studies that assessed behavioural outcomes	References
Short-term memory/ spatial working memory (80%)	Barnes maze Y maze Morris water maze	↑ TNF-α, IL-6, GFAP, IL-6 + Iba1 ↓ IL-2, IL-3, IL-5, GM-CSF, IL-1ß, IL-6, IL-12, IL-4, IL-10, Arg-1	n = 8	(Alexander et al., 2019; Flanigan et al., 2018; Ramalingayya et al., 2019; Shi et al., 2018; Shi et al., 2019; Shi et al., 2019; Yi et al., 2020; Fardell et al., 2014)
Long-term memory 80%)	Morris water maze	↑ TNF-α, IL-6, GFAP, IL-6 + Iba1 ↓ IL-2, IL-3, IL-5, GM-CSF, IL-1ß, IL-6, IL-12, IL-4, IL-10, Arg-1	n = 8	(Alexander et al., 2019; Flanigan et al., 2018; Ramalingayya et al., 2019; Shi et al., 2018; Shi et al., 2019; Shi et al., 2019; Yi et al., 2020; Fardell et al., 2014)
Recognition memory (78.6%)	Novel object recognition Novel object place recognition/ novel place recognition Object in place/object location Temporal order	↑ ED-1+, IL-1ß, IL-3, IL-5, IL-12, IL-6, IFNα1, RANTES, GM-CFS, CD68+, TNF-α, GFAP, Glast+, CD68+/ Iba1, FACS (microglia activation) ↓ IL-10, IL-1ß, IL-6, IL-12	n = 9	(Acharya et al., 2015; Allen et al., 2019; Briones and Woods, 2014; Flanigan et al., 2018; Ramalingayya et al., 2019; Yi et al., 2020; Fardell et al., 2014; Gibson et al., 2019; Zhou et al., 2016)
Contextual memory/ Stimulus response learning (100%)	Contextual and cued fear Fear extinction	↑ ED-1+, IL-1ß, IL-3, IL-5, IL-12, IL-6, IFNα1, RANTES, GM-CSF, CD68+	n = 2	(Acharya et al., 2015; Allen et al., 2019)
Avoidance learning (100%)	Passive avoidance	↑ TNF-α, GFAP, Iba1	n = 2	(El-Agamy et al., 2018; Yang et al., 2012)
Executive control (0%)	Social discrimination	No differences	n = 1	(Zhou et al., 2016)

↑ increase; ↓ decrease.

corpus callosum (n = 1), isolated microglia exposed to chemotherapy (n = 1), rostral cortex (n = 1), striatum (n = 1), medulla (n = 1) and cerebellum (n = 1). There were statistically significant ($p < 0.05$) decreases found in microglial reactivity marker expression in the prefrontal cortex (n = 1). No differences were found in microglial reactivity marker expression in the hippocampus (n = 5), prefrontal cortex (n = 1), superficial gray matter (n = 1), coronal brain (n = 1), rostral cortex (n = 1), striatum (n = 1), midbrain (n = 2) and caudal cortex (n = 2).

Increases in classical microglial reactivity (defined as IL6 + Iba1) were found in the dentate gyrus (n = 1), CA1/CA3 subfields, (n = 1), hippocampus (n = 1) and prefrontal cortex (n = 1) whilst a marked decrease in alternate microglial reactivity (defined as Arg-1) was found in the hippocampus (n = 1) and prefrontal cortex (n = 1). No differences were found in classical microglial reactivity in the CA3 region (n = 1), or alternate microglial reactivity in the dentate gyrus (n = 1), CA1/CA3 subfields (n = 1) and CA3 region (n = 1).

5.4.2. Astrocytes

Glial fibrillary acidic protein (GFAP) expression was quantified in multiple brain regions, represented in five studies. One study assessed the astrocytic marker, glutamate transporter GLAST + cells in the frontal deep cortex and corpus callosum. A summary of astrocytic reactivity marker expression can be found in Table 5. There were statistically significant ($p < 0.05$) increases found in GFAP expression in the hippocampus (n = 1), whole-brain (n = 1), prefrontal cortex (n = 1), dentate gyrus (n = 1), CA1 subfield (n = 1), CA3 subfield (n = 1), striatum (n = 1), rostral cortex (n = 1), caudal cortex (n = 1), cerebellum (n = 1) and Glast + cells in the frontal deep cortex (n = 1) and corpus callosum (n = 1). No difference was found in GFAP expression in the hippocampus (n = 1), coronal brain (n = 1), midbrain (n = 1) and medulla (n = 1).

5.4.3. Cytokines

A total of 15 cytokines were analysed in 70% of studies (n = 14), including 11 pro-inflammatory cytokines and 4 anti-inflammatory cytokines. The hippocampus was the most analysed brain region for both pro-inflammatory and anti-inflammatory cytokines.

5.4.4. Pro-inflammatory cytokines

TNF-α was the most frequently analysed pro-inflammatory cytokine across the studies (n = 11). A total of seven brain regions were analysed. Pro-inflammatory cytokine-related findings are displayed in Table 6. There were statistically significant ($p < 0.05$) increases found in 9 cytokines and decreases were seen in 5 cytokines.

5.4.5. Anti-inflammatory cytokines

Anti-inflammatory cytokines were analysed in eight of the fourteen studies that assessed cytokine markers (Table 7). Interleukin 10 (IL-10) was the most common anti-inflammatory cytokine assessed across four different brain regions. The hippocampus was the most analysed brain region with four anti-inflammatory cytokines assessed. There were statistically significant ($p < 0.05$) increases found in 4 cytokines, decreases were found in 3 cytokines and no differences were found in 3.

5.4.6. Chemokines

Chemokine markers were evaluated in 20% (n = 4) of the included studies, with a total of 9 chemokines analysed. Across the four studies, the hippocampus was the only brain region analysed. Summary of chemokine-related findings is provided in Table 8. There were statistically significant ($p < 0.05$) increases found in 9 chemokines and decreases were found in 1 chemokine and no differences were found in 10 chemokines in the hippocampus.

5.4.7. Chemotherapy agents and neuroimmune expression in hippocampus

Magnitude of neuroimmune expression in the hippocampus was assessed in 9 of the 12 included chemotherapy regimens administered, including 8 solo agents and 4 combination regimens. Greater magnitude of fold change was found in DOX + CYP compared to DOX alone (Table 9).

6. Discussion

CICI is a debilitating side effect resulting from the administration of antineoplastic agents. The condition impacts on memory, attention, concentration and ultimately on patient quality of life. Neuroinflammation has been suggested as a key mechanism underlying CICI development. To our knowledge, this is the first scoping review to provide a comprehensive synthesis of the literature on neuroimmune

Table 4
Microglial reactivity marker expression.

Brain region	Marker for microglial reactivity	*Outcome*			No. of studies	References
		Acute	*Subacute*	*Chronic*		
Dentate hilus	CD68+/ED-1+		↑		n = 1	(Acharya et al., 2015)
Dentate gyrus	CD68+/ED-1+		~		n = 1	(Acharya et al., 2015)
	IL6 + Iba1		↑		n = 1	(Shi et al., 2019)
	Arg-1		~		n = 1	(Shi et al., 2019)
CA1/CA3 subfields	CD68+/ED-1+		↑		n = 1	(Acharya et al., 2015)
	IL6 + Iba1		↑		n = 1	(Shi et al., 2019)
	Arg-1		~		n = 1	(Shi et al., 2019)
CA3	IL6 + Iba1		~		n = 1	(Shi et al., 2019)
	Arg-1		~		n = 1	(Shi et al., 2019)
Hippocampus	Iba1	↑	~(2)	~(6)	n = 5	*(Allen et al., 2019)
			~			*(Feiock et al., 2016)
			~(8)			*(Seigers et al., 2016)
		~↑	~			*(Yang et al., 2012)
						(Yi et al., 2020)
	CD68+/ED-1+		↑		n = 1	(Allen et al., 2019)
	IL6 + Iba1		↑		n = 1	(Shi et al., 2019)
	Arg-1		↓		n = 1	(Shi et al., 2019)
Prefrontal cortex	Iba1		~(4) ↓(4)	~(6)	n = 1	*(Seigers et al., 2016)
	IL6 + Iba1		↑		n = 1	(Shi et al., 2019)
	Arg-1		↓		n = 1	(Shi et al., 2019)
Superficial gray matter	CD68+/Iba1+		~		n = 1	(Gibson et al., 2019)
Corpus callosum	CD68+/Iba1+		↑	↑	n = 1	*(Gibson et al., 2019)
Isolated from animal	Microglia	↑			n = 1	(Gibson et al., 2019)
Coronal brain	CD11b		~		n = 1	(Zhou et al., 2016)
Rostral cortex	Iba1	↑	~		n = 1	*(Feiock et al., 2016)
Striatum	Iba1	↑	~		n = 1	*(Feiock et al., 2016)
Midbrain	Iba1	~	~		n = 1	*(Feiock et al., 2016)
Caudal cortex	Iba1	~	~		n = 1	*(Feiock et al., 2016)
Medulla	Iba1	↑	↑		n = 1	*(Feiock et al., 2016)
Cerebellum	Iba1	↑	↑		n = 1	*(Feiock et al., 2016)

~ no difference; ↑ increase; ↓ decrease; *denotes findings corroborated using more than one analysis method from one study; () denotes number of findings associated with the direction of effect from one study.

Table 5
Astrocytic reactivity marker expression.

Brain region	Marker for astrocytic reactivity expression	Outcome			No. of studies	References
		Acute	*Subacute*	*Chronic*		
Hippocampus	GFAP	~	↑		n = 2	(El-Agamy et al., 2019)
			~			*(Feiock et al., 2016)
Whole brain	GFAP	↑(9)	↑		n = 1	*(Fardell et al., 2014)
Prefrontal cortex	GFAP		↑		n = 1	(Shi et al., 2019)
Frontal deep cortex	Glast + cells		↑		n = 1	(Gibson et al., 2019)
Corpus callosum	Glast + cells		↑		n = 1	(Gibson et al., 2019)
Dentate gyrus	GFAP		↑		n = 1	(Shi et al., 2019)
CA1	GFAP		↑		n = 1	(Shi et al., 2019)
CA3	GFAP		↑		n = 1	(Shi et al., 2019)
Coronal brain	GFAP		~		n = 1	(Zhou et al., 2016)
Striatum	GFAP	↑	↑		n = 1	*(Feiock et al., 2016)
Midbrain	GFAP	~	~		n = 1	*(Feiock et al., 2016)
Rostral cortex	GFAP	↑	↑		n = 1	*(Feiock et al., 2016)
Caudal cortex	GFAP	↑	↑		n = 1	*(Feiock et al., 2016)
Medulla	GFAP	~	~		n = 1	*(Feiock et al., 2016)
Cerebellum	GFAP	↑	~		n = 1	*(Feiock et al., 2016)

~ no difference; ↑ increase; ↓ decrease; *denotes findings corroborated using more than one analysis method from one study; () denotes number of findings associated with the direction of effect from one study.

reactivity marker expression changes in rodent models of CICI.

6.1. Animal models to assess neuroinflammation in CICI

Animal models play an important role in understanding the underlying mechanisms of CICI. Unfortunately, there was significant heterogeneity in the methodology employed in the included studies. This makes it challenging to draw firm conclusions on some aspects reviewed. Significant experimental differences (rodent strain, chemotherapeutic agents and/or combination, route of administration, dose frequency, treatment regime) were observed across the board. Notably, selected chemotherapy agents differed substantially between the studies. Assessing single chemotherapeutic agents alone is important to understand potential mechanisms and effects on cognition. Tumour recurrence and drug resistance commonly occur with single-drug based chemotherapy, primarily due to a neutralising response, cross-talk and pathway overlapping (Hu et al., 2016). Currently combination therapy is well-established in oncologic practice and provides effective solutions for cancer treatment (Hu et al., 2016). However, combination treatment has the potential to increase cytotoxicity, thus exacerbating the neuroimmune response. Due to the limited study replication of experimental design factors, such as chemotherapy agent use and outcomes measured,

Table 6
Pro-inflammatory cytokine-related findings.

Brain region	Cytokine	Outcome			No. of studies	References
		Acute	*Subacute*	*Chronic*		
Hippocampus	TNF-α	[↑]	~		n = 11	(Alexander et al., 2019)
						(Bagnall-Moreau et al., 2019)
						(El-Agamy et al., 2019)
		↑	↑			(Feiock et al., 2016)
						(Flanigan et al., 2018)
			~			(Ramalingayya et al., 2019)
			↑			(Shi et al., 2018)
			↑			*(Shi et al., 2019)
			↑(2)			*(Shi et al., 2019)
		↑	↑(2)			(Tangpong et al., 2006)
						(Yi et al., 2020)
			~			
	IL-1α		~		n = 4	(Alexander et al., 2019)
						(Allen et al., 2019)
		~	~			(Bagnall-Moreau et al., 2019)
						(Flanigan et al., 2018)
			~			
	IL-1β	~	~		n = 5	(Alexander et al., 2019)
						(Allen et al., 2019)
			↑			(Bagnall-Moreau et al., 2019)
						(Flanigan et al., 2018)
			↓			(Yi et al., 2020)
			[~]			
	IL-2		↓		n = 4	(Alexander et al., 2019)
						(Allen et al., 2019)
		↑	~			(Bagnall-Moreau et al., 2019)
						(Flanigan et al., 2018)
			~			
	IL-3		↓		n = 4	(Alexander et al., 2019)
						(Allen et al., 2019)
		↑	↑			(Bagnall-Moreau et al., 2019)
						(Flanigan et al., 2018)
			~			
	IL-6		~ [↑]		n = 7	(Allen et al., 2019)
						(Flanigan et al., 2018)
			↓			(Shi et al., 2018)
			↑			*(Shi et al., 2019)
			↑(2)			*(Shi et al., 2019)
			↑(2)			(Yi et al., 2020)
			~			(Bagnall-Moreau et al., 2019)
		↑				
	IL-12/IL-12p70		~		n = 3	(Alexander et al., 2019)
						(Allen et al., 2019)
			↑			(Flanigan et al., 2018)
			↓			
	IL-13	~			n = 1	(Bagnall-Moreau et al., 2019)
	IL-17	↑			n = 1	(Bagnall-Moreau et al., 2019)
	IFNγ	↑	~		n = 3	(Allen et al., 2019)
			~			(Bagnall-Moreau et al., 2019)
						(Flanigan et al., 2018)
	IFNα1		[↑]		n = 1	(Allen et al., 2019)
Corpus callosum	TNF- α		↑		n = 1	(Briones and Woods, 2014)
	IL-1β		↑		n = 1	(Briones and Woods, 2014)
Frontal cortex	TNF- α		↑		n = 1	(Ramalingayya et al., 2019)
Hypothalamus	IL-1β	[~]			n = 1	(Smith et al., 2014)
	TNF- α	[~]			n = 1	(Smith et al., 2014)
Cerebellum	IL-1β	[↑]			n = 1	(Smith et al., 2014)
	TNF-α	[↑]			n = 1	(Smith et al., 2014)
Prefrontal cortex	TNF- α		↑		n = 3	(Shi et al., 2018)
						*(Shi et al., 2019)
			↑(2)			*(Shi et al., 2019)
			↑(2)			
	IL-6		~		n = 3	(Shi et al., 2018)
						*(Shi et al., 2019)
			~ ↑			*(Shi et al., 2019)
			~(2)			
Whole brain	TNF- α		↑		n = 3	(Shi et al., 2018)
						*(Shi et al., 2019)
			↑			*(Shi et al., 2019)
			↑			
	IL-6		↑		n = 3	(Shi et al., 2018)
						*(Shi et al., 2019)
			↑			*(Shi et al., 2019)
			↑			

~ no difference; ↑ increase; ↓ decrease; *denotes findings corroborated using more than one analysis method from one study; () denotes number of findings associated with the direction of effect from one study; [ ] denotes mRNA finding.

Table 7
Anti-inflammatory cytokine-related findings.

Brain region	Anti-inflammatory Cytokine	Outcome			No. of studies	References
		Acute	*Subacute*	*Chronic*		
Hippocampus	IL-4	↑	~		n = 7	(Alexander et al., 2019)
						(Allen et al., 2019)
			[~]			(Bagnall-Moreau et al., 2019)
						(Flanigan et al., 2018)
			~			(Shi et al., 2018)
			↓			*(Shi et al., 2019)
			↓(2)			(Shi et al., 2019)
			↓			
	IL-5		↓		n = 3	(Alexander et al., 2019)
						(Allen et al., 2019)
			↑			(Flanigan et al., 2018)
			~			
	IL-10	↑	~		n = 7	(Alexander et al., 2019)
						(Allen et al., 2019)
			~			(Bagnall-Moreau et al., 2019)
						(Flanigan et al., 2018)
			~			(Shi et al., 2018)
			↓			*(Shi et al., 2019)
			↓(2)			(Shi et al., 2019)
			↓			
	IL-1RA	↑			n = 1	(Bagnall-Moreau et al., 2019)
Corpus callosum	IL-10		↓		n = 1	(Briones and Woods, 2014)
Prefrontal cortex	IL-4		↓		n = 3	(Shi et al., 2018)
						*(Shi et al., 2019)
			↓(2)			(Shi et al., 2019)
			↓			
	IL-10		↓		n = 3	(Shi et al., 2018)
			↓(2)			*(Shi et al., 2019)
			~			(Shi et al., 2019)
Whole brain	IL-4		↓		n = 3	(Shi et al., 2018)
			↓			(Shi et al., 2019)
			↓			(Shi et al., 2019)
	IL-10		↓		n = 3	(Shi et al., 2018)
			↓			(Shi et al., 2019)
			↓			(Shi et al., 2019)

~ no difference; ↑ increase; ↓ decrease; *denotes findings corroborated using more than one analysis method from one study; () denotes number of findings associated with the direction of effect from one study; [ ] denotes mRNA finding.

it is not possible to suggest agents that cause greater neuroimmune reactivity marker expression change with high confidence. However, based on our quantitative representation of the data available from the studies (Table 9), there is suggestion that the combination of DOX and CYP leads to greater magnitude of fold-change than DOX alone. This summary of magnitude of neuroimmune reactivity marker expression change is not without limitation. Unfortunately, due to the heterogeneous nature of the studies it was not appropriate to use meta-analytical techniques and the table should be interpreted in light of this. The implications of the increased expression of neuroimmune markers are not well characterised in CICI. However, they are perhaps best interpreted through consideration of the relative cognitive changes, this being the phenotypic expression of pathology. Increases in cytokine expression were found with impairments of short-term/long-term memory and recognition memory linked to increases of TNF-α, IL-6 and IL-1β. Ultimately, the magnitude of expression is relevant if it crosses a threshold from protective to pathological effect and manifests in a detrimental manner, such as cognitive impairment (Bilbo et al., 2012; Goshen et al., 2007). The majority of studies found concurrent cognitive impairment and molecular changes (Table 3) suggesting that there is a linkage between the neuroimmune reactivity expression demonstrated and pathological cognitive outcomes.

Thus, further research is warranted to explore the neurotoxic effect of quantifiable neuroimmune reactivity marker expression change post administration of chemotherapy agents across a variety of brain regions employing similar study designs to published work to allow more robust evidence synthesis.

Importantly, the majority of the studies included in this review assessed outcomes in naïve animals. Whilst these studies provide valuable information, it is important to acknowledge the potential effects of cancer itself. It has been estimated, through longitudinal neuropsychological research, that cancer-related cognitive impairment (CRCI) occurs in 30% of cancer patients prior to treatment (Janelsins et al., 2014). This has been further elucidated in rodent cancer models, where an increase in circulating inflammatory mediators (cytokines and chemokines) by tumour and non-tumour cells in the tumour microenvironment are released to attract additional immune cells and promote tumour growth and metastasis (Santos and Pyter, 2018). Furthermore, patients may experience cognitive dysfunction due to other common side-effects. Cancer-related anemia, fatigue and emotional states (anxiety and depression) are common co-morbidities reported in patients undergoing chemotherapy and have been shown to contribute to cognitive decline (Kayl et al., 2006).

There is currently no 'gold standard' animal model for assessing CICI.

Table 8
Chemokine-related findings.

Brain region	Chemokine	Outcome			No. of studies	References
		Acute	*Subacute*	*Chronic*		
Hippocampus	RANTES (CCL5)		~		n = 3	(Alexander et al., 2019) *(Allen et al., 2019)
			[~] ↑			(Bagnall-Moreau et al., 2019)
		~				
	GM-CSF		↓		n = 4	(Alexander et al., 2019) (Allen et al., 2019)
		~	↑			(Bagnall-Moreau et al., 2019) (Flanigan et al., 2018)
			~			
	MCP-1		~		n = 3	(Alexander et al., 2019) (Allen et al., 2019)
			~			(Flanigan et al., 2018)
			~			
	MIP-1α		~		n = 2	(Allen et al., 2019) (Bagnall-Moreau et al., 2019)
		↑				
	MIP-3α (CCL20)		[↑]		n = 2	(Allen et al., 2019) (Bagnall-Moreau et al., 2019)
		↑				
	CINC-1	~			n = 1	(Bagnall-Moreau et al., 2019)
	CINC-2	~			n = 1	(Bagnall-Moreau et al., 2019)
	CIN-3	~			n = 1	(Bagnall-Moreau et al., 2019)
	FRACTAL-KINE	~			n = 1	(Bagnall-Moreau et al., 2019)
	CNTF	~			n = 1	(Bagnall-Moreau et al., 2019)
	SICAM-1	↑			n = 1	(Bagnall-Moreau et al., 2019)
	IP-10 (CXCL10)	↑			n = 1	(Bagnall-Moreau et al., 2019)
	LIX (CXCL5)	↑			n = 1	(Bagnall-Moreau et al., 2019)
	L-SELECTIN	↑			n = 1	(Bagnall-Moreau et al., 2019)
	MIG (CXCL9)	↑			n = 1	(Bagnall-Moreau et al., 2019)
	Thymus cytokine	~			n = 1	(Bagnall-Moreau et al., 2019)

~ no difference; ↑ increase; ↓ decrease; *denotes findings corroborated using more than one analysis method from one study; () denotes number of findings associated with the direction of effect from one study; [ ] denotes mRNA finding.

Having an established animal model for CICI, which allows for the teasing apart of cancer-related impacts, would aid in our mechanistic understanding, and improve the translational validity of CICI models. Furthermore, it would be beneficial for future reviews to concentrate on methodological quality of animal models of CICI to reduce heterogeneity as observed in this scoping review. There is increasing demand for improving reporting standards in preclinical animal research, in accordance with guidelines such as Animal Research: Reporting of In Vivo Experiments (ARRIVE) (Kilkenny et al., 2011; Percie Du Sert et al., 2020).

6.2. Markers of neuroimmune engagement

The pro-inflammatory cytokines which received the most focus were TNF-α and IL-6. TNF-α is a powerful pro-inflammatory cytokine and cytotoxic factor released by macrophages. Whilst it is released by macrophages, it also has the ability to regulate and encourage macrophage activity (Parameswaran and Patial, 2010). It is known to be the main driver and regulator of the pro-inflammatory cytokine cascade and plays a key role in a number of diseases which feature chronic inflammation as an underlying mechanism, and with cognitive impairment as an outcome, such as rheumatoid arthritis, Alzheimer's and Parkinson's (Parameswaran and Patial, 2010; Misiak et al., 2018). Unlike TNF-α and other traditional pro-inflammatory cytokines, IL-6 is capable of modulating homeostasis by exerting either pro- or anti-inflammatory effects. The capacity of IL-6 to act as an immune stimulant and respond to injury being of most importance within this context (Luo and Zheng, 2016; Tanaka et al., 2014). During acute inflammation IL-6 responds aptly to acute stressors to fight off infections, regulate the level of pro-inflammatory cytokines in both local and systemic regions of inflammation and ensure balance is restored after inflammation is resolved (Gabay, 2006). However, chronically, IL-6 is shown to exhibit pro-inflammatory properties for example in models of schizophrenia, depression and Alzheimer's (Misiak et al., 2018; Zheng et al., 2016). Specifically, regarding CICI, IL-6 is often assumed to act as a pro-inflammatory cytokine (Allen et al., 2019; Bagnall-Moreau et al., 2019; Flanigan et al., 2018; Shi et al., 2018, 2019). Given the duality of action of IL-6 there is an emphasis on the need to resolve the time course and disease progression of CICI to interpret findings regarding the properties of IL-6 expression. Understanding the mechanisms that drive differences between classical and trans-signaling pathways in IL-6 will assist in elucidating the nature of the inflammatory responses as either pro or anti-inflammatory (Narazaki and Kishimoto, 2018; Reeh et al., 2019). Application of an iterative systems biology approach should be considered in CICI models to definitively characterise the effects of IL-6.

Our findings demonstrate that TNF-α is predominantly increased in the hippocampus, whole brain, and prefrontal cortex. In contrast, IL-6 expression changes were inconsistent across brain regions. Taken together, these data imply that acute inflammation in CICI may be driven by TNF-α. Given the lack of study at a chronic time-point no conclusions on the contribution of TNF-α can be drawn. There was however synergy between expression patterns and the regions expected to be affected given the nature of the cognitive impairment observed in CICI. For example, increases in pro-inflammatory markers in the hippocampus and prefrontal cortex result in impairment to executive functioning, memory, and learning.

In tandem with the findings of increased TNF-α, it follows that levels of IL-4 and IL-10 would decrease, as was evidenced in our synthesis. IL-4 has been shown to be critical for memory and learning, adult neurogenesis, and in counteracting levels of pro-inflammatory cytokines, specifically TNF-α (Gadani et al., 2012). A decrease or absence of IL-4 results in cognitive impairment, reduced neurogenesis, increases of pro-inflammatory cytokines and has been implicated in models of Alzheimer's and multiple sclerosis (Gadani et al., 2012). Additionally, IL-10 also acts to inhibit pro-inflammatory cytokines produced by microglia thus protecting astrocytes from excessive inflammation, preventing

Table 9
Heatmap demonstrating magnitude of neuroimmune reactivity marker changes in hippocampal tissue following administration of chemotherapy agents. Neuroimmune reactivity marker expression (represented by colour change) presented as fold change compared to relevant control.

Marker(s) of neuroimmune engagement	Chemotherapy Agent(s)											
	CYP	ThioTEPA	DOX	CMF	DOX + CYP	DAC	Cisplatin	DTX	Topotecan	5-FU	MTX	CAF
CD68+/ED-1+			1									
Iba1	1		2					1	1	1	2	
CD11b												
IL6 + Iba1						1						
Arg-1						1						
GFAP											2	
IL-4			1		2	3						
IL-5		1	1		1							
IL-10			1		2	3						
IL-1RA					1							
TNF-α			3		2	3						
IL-1α			1		2							
IL-1β			1		2							
IL-2		1	1		2							
IL-3		1	1		2							
IL-6			1		2	3						
IL-12/IL-12p70			1		1							
IL-13					1							
IL-17					1							
IFNγ			1		2							
IFNα1												
RANTES (CCL5)			1		1							
GM-CSF			1		2							
MCP-1			1		1							
MIP-1α			1		1							
MIP-3α (CCL20)												
CINC-1					1							
CINC-2					1							
CIN-3					1							
FRACTAL-KINE					1							
CNTF					1							
SICAM-1					1							
IP-10 (CXCL10)					1							
LIX (CXCL5)					1							
L-SELECTIN					1							
MIG (CXCL9)					1							
Thymus cytokine					1							

CYP = Cyclophosphamide; DOX = Adriamycin/Doxorubicin hydrochloride; CMF = Cyclophosphamide, Methotrexate, and 5-Fluorouracil; DAC = Doxorubicin, Adriamycin, and Cyclophosphamide; DTX = Docetaxel; 5-FU = 5-Fluorouracil; MTX = Methotrexate; CAF = Cyclophosphamide, Doxorubicin, and 5-Fluorouracil. Note fold change was extracted directly from papers if stated or obtained utilising WebPlotDigitizer (Rohatgi 2020, Pacifica, California, USA) when accessible. Grey represents no assessment or quantitative data not available. 'number within coloured box' signifies the number of papers.

apoptosis and regulating adult neurogenesis (Salvi et al., 2017). Similarly, decreases have been reported in Alzheimer's, multiple sclerosis and Parkinson's (Salvi et al., 2017).

Microglial reactivity marker expression was predominantly analysed utilising Iba1, a protein specific to microglia and macrophages. Outcomes were relatively heterogenous across the studies. The majority of studies found no difference in microglial reactivity marker expression across a range of brain regions, including the hippocampus, at an acute and sub-acute time-point. Methodological issues may however be at play. Although often employed in studies investigating microglial reactivity marker expression, Iba1 is considered a marker of all counts of microglia regardless of their phenotypic expression (Hopperton et al.,

2018). In Alzheimer's, the use of Iba1 has not produced consistent findings. However, markers specific for cell activation, such as major histocompatibility complex-II (MHCII) and CD68, demonstrated consistent increases in diseased brain tissue potentially making it a more telling marker (Hopperton et al., 2018; Hoogland et al., 2015). Comparably, findings from our review indicate that studies that utilised CD68 demonstrated significant increases, supporting the use of this marker as an indicator of microglial reactivity. In a similar vein, astrocyte reactivity was predominantly assessed using GFAP. However, this is considered to be a more reliable and highly specific marker for reactive astrocytes making it an ideal marker (Hol et al., 2003; Preston et al., 2019).

6.3. Brain regions assessed & cognitive domains explored

The hippocampus was the primary analysed brain region for all markers of neuroimmune reactivity expression (cytokines, chemokines, microglia reactivity marker expression, and astrocyte reactivity marker expression). CICI is characterised by impairments to working, episodic, remote, verbal, and visual memory, executive function, processing speed, attention, concentration, and visual-spatial ability during and after the cessation of chemotherapy treatment (Argyriou et al., 2011; van Asselen et al., 2006). Many of these cognitive skills are linked to intact hippocampal functioning and, as such, this fits with a majority of the studies incorporating hippocampal analysis. Given the range of cognitive abilities affected by CICI, simply assessing the hippocampus is not sufficient. For example, the prefrontal cortex is also implicated in facets of cognition such as working and episodic memory, executive function, attention, and concentration and, although present in the reviewed studies, is underrepresented. Thus, assessment of markers of neuroimmune reactivity marker expression in different brain regions, such as the prefrontal cortex, is needed.

Molecular or immunohistochemistry (IHC) analysis allows insight into the nature of changes occurring at a cellular level. However, behavioural analysis is imperative in assessing whether these changes cause a cognitive effect that causes a measurable deficit. Cognitive tests utilised in the included studies assessed the animals' ability to temporarily store and retrieve information, remember different locations and spatial relations between objects, and recognize previously encountered contexts in both the short and long-term.

These tests are reliant on intact hippocampal and pre-frontal cortex functioning (Matsos and Johnston, 2019). It is important to note that whilst the pre-frontal cortex can support the hippocampus in short-term memory, it is particularly associated with long-term memory consolidation (Funahashi, 2017). Impairment was noted in the majority of the cognitive tests employed, however, not all papers found impairments in these assessments. It is possible that the lack of impairment observed in some studies may have resulted from the lack of available cognitive tests that can reliably assess mild cognitive impairment, rather than an actual absence of impairment. This may explain why cognitive impairment was not evident in some behavioural tests despite the presence of changes upon molecular biology analysis. In addition, transient cognitive impairment may occur and resolve before long-term cognitive impairment is evident, and therefore choice of time-point for behavioural testing is important. Further investigation of cognitive testing in conjunction with in vitro analyses is needed. In addition to hippocampal based impairment, patients also report experiencing impairments in attention and executive control, thus a focus on additional tests of these characteristics driven by the prefrontal cortex may offer additional insight(Wigmore et al., 2013).

6.4. Neuroimmune reactivity marker expression and cognitive outcomes

Analysing molecular outcomes and cognitive outcomes in tandem allows exploration of the relationship between a potential mechanism and CICI. Short and long-term memory and recognition memory were the most examined cognitive domains. Impairments in short and long-term memory (80%) and recognition memory (78.6%) were associated with consistent increases in pro-inflammatory cytokines and decreases in anti-inflammatory cytokines. Impairment in context/stimulus response learning (100%) was associated with increases in microglial expression, chemokines, and primarily pro-inflammatory cytokines. Impairment in avoidance learning (100%) was associated with an increase in TNF-α and GFAP. Executive control was not found to be significantly impaired, and no molecular changes were found in CD11b or GFAP, however executive function is a difficult cognitive domain to assess in rodents and requires further research as this was only assessed in one paper. Time-point is important to consider when interpreting these results; the included studies assessed acute and sub-acute time-points. Unfortunately, few studies assessed cognitive and molecular outcomes at a chronic time-point where we would expect neuroinflammation to be of greatest concern.

Neuroimmune reactivity marker expression is not inherently detrimental and is, in fact, necessary for neural and cognitive function. However, this expression becomes problematic if it becomes chronic, with persistent activation of microglia and astrocytes and the production of cytokines and chemokines resulting in neural and/or cognitive changes (Tchessalova et al., 2018). Cytokine analyses predominated in the included studies, presumably due to their role in persistent activation. In contrast, chemokine analysis was underrepresented. It has been demonstrated in other conditions, such as Alzheimer's and traumatic brain injury, that low-grade chronic inflammation drives negative neural and cognitive alterations, therefore it was important to synthesise behavioural outcomes in this review as it provides a tangible representation of cognitive changes (Akiyama et al., 2000; Schimmel et al., 2017; Chen et al., 2017). Therefore, an important consideration in CICI pathology is whether acute reactivity marker expression occurs with consequent resolution, or whether chronic reactivity marker expression persists. Neuroinflammation has been linked to cognitive impairment in rodents and humans at both acute and chronic time-points in neurodegenerative conditions (Simen et al., 2011; Lee et al., 2010). The chemobrain clinical literature suggests that significant impairment may occur between 6-months and 20-years post-treatment indicating a chronic component to the mechanism (Koppelmans et al., 2012). A large subset of the included papers assessed acute or sub-acute time-points, with very few focusing on chronic time-points. Therefore, further research focus needs to be directed towards evaluation of chronic expression changes.

7. Conclusion and future directions

This is the first systematic scoping review to comprehensively map the evidence of the literature on neuroimmune reactivity marker expression changes in rodent models of CICI. Although the included studies demonstrated significant heterogeneity in design which precludes the making of firm conclusions in some aspects, our findings suggest a close association between neuroimmune engagement and CICI in preclinical rodent models. There is a need to establish causation between neuroimmune engagement and cognitive impairment observed in CICI, potentially through blocking inflammatory pathways which are implicated in cognitive changes. In order to establish a link, future study designs also need to consider analysing molecular changes and cognitive changes in tandem rather than independently. It would also be worth revisiting the possibility of performing a meta-analysis in the future, should more studies be published providing greater consistency in outcome measures and methods used. Whilst, glial cells (astrocytes and microglia), cytokines (pro-inflammatory and anti-inflammatory) and chemokines are principally responsible for modulating immune responses, there are other mediators of inflammation including nitric oxide synthase, reactive oxygen species and oligodendrocytes. These mediators play a crucial role in enhancing inflammation and facilitating neuronal death. As such, future reviews should examine the role, and

interlinking, of the inflammatory mediators in CICI development.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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CHAPTER 6

Influence of Chemotherapy-induced Gut Toxicity and Opioid Palliation in the Development of Chemotherapy-induced Cognitive Impairment in a Tumour Bearing Rat Model

CONTEXTUAL STATEMENT

Pain mitigation strategies play an important role in symptom management in CIGT due to the painful nature of the condition. Commonly, this involves administration of potent opioid analgesics such as morphine, oxycodone and fentanyl to relieve moderate to severe pain. In spite of their widespread clinical usage, the effect of different opioid agents in the gastrointestinal inflammatory environment created in CIGT is yet to be investigated. Additionally, considerable inconsistencies exist between preclinical animal models and patients, in that tumour-bearing models are not regularly being assessed, nor is the potential role of cancer itself on CICI being considered. In particular the use of tumour bearing animal models has been recommended by the international cancer and cognition task force (ICCTF) (Winocur et al. 2018).

In the current chapter a tumour-bearing rat model of CIGT was utilised with assessment of cognitive outcomes as marker for cognitive decline. As such, **Chapter 6** examines the effect of 5-FU in conjunction with clinically relevant analgesic opioids on gut inflammation and architecture changes associated with CIGT and cognitive markers in a female Dark Agouti tumour-bearing (mammary adenocarcinoma) rat model.

CHAPTER 6

Influence of Chemotherapy-induced Gut Toxicity and Opioid Palliation in the Development of Chemotherapy-induced Cognitive Impairment in a Tumour Bearing Rat Model

6.1 Introduction

Rates of cancer remission and long-term survival of patients diagnosed with cancer has improved greatly due to modern chemotherapy treatments. As a result, a larger population of cancer survivors is predicted. Despite improved survivorship, patients often experience debilitating side effects associated with chemotherapy treatments, which can persist for weeks, months or years after patients have recovered. Side effects such as chemotherapy-induced gut toxicity (CIGT), previously termed mucositis, are short-lived, however chemotherapy-induced cognitive impairment (CICI) can affect patients years following cessation of treatment [1]. It is estimated that CICI can affect up to 75% of patients with many patients self-reporting problems associated with memory and concentration [2,3]. The most common side effects of CICI include defects to visual processing, visual motor function, attention and executive function [4]. Furthermore, CICI can greatly impact patient quality of life, and also place additional burden on the healthcare system as these patients have poorer adherence to treatment plans [5,6].

Despite the high prevalence of CICI, little is known regarding the underlying causes of these deficits in the CNS [7]. Furthermore, it is postulated that CICI may be triggered by peripheral inflammatory events through a cytokine-mediated neuroimmune mechanism [8]. One such peripherally driven inflammatory event that may trigger this cognitive change is CIGT. CIGT affects approximately 40% to 60% of patients undergoing standard doses of chemotherapy [9,10]. Symptoms include vomiting, nausea, constipation, diarrhoea, weight loss and abdominal

pain due to ulceration of the gastrointestinal tract [11,12]. The mucositic condition is a biologically complex process characterised by both inflammation and damage to the epithelial barrier of the gastrointestinal tract [13]. During the development of CIGT, pro-inflammatory cytokines play an important role in the inflammatory processes [14].

In recent years, it has been postulated that the gastrointestinal tract and central nervous system (CNS) are intimately connected and have a complex bidirectional communication pathway which link the gastrointestinal tract to the CNS (gut-brain axis) [15,16]. It has been proposed that during chemotherapy exposure, the gut-brain axis becomes dysregulated and may play a pivotal role in the development of both CIGT and CICI [16]. Whilst our understanding of the effects of chemotherapy agents on gut inflammation in CIGT have been well characterised, the underlying mechanisms that contribute to CICI and resulting neuroimmune changes occurring in the CNS remain relatively unclear. These neuroimmune changes have the potential to contribute to, and exacerbate, cognitive decline in people treated with chemotherapy.

Currently, there is no wholly effective preventative or treatment agent for CIGT. CIGT is an important dose-limiting factor in chemotherapy treatment since severe symptoms can necessitate dose reduction or early termination of chemotherapy, rendering amelioration of symptoms critical [11]. Analgesic treatments play a crucial symptom management role due to the painful, yet self-limiting nature of CIGT. Typically, this involves administration of potent opioid agents for control of moderate to severe pain. The MASCC/ISOO guidelines recommend potent μ -opioid receptor agonists such as morphine and fentanyl as analgesia of choice, due to their potent analgesic nature [17]. Whilst opioids are highly effective for pain management, it has been proposed that opioid agents such as morphine upregulate astrocyte and microglial reactivity markers resulting in overproduction of pro-inflammatory cytokines

and chemokines via opioid-induced central immune signalling events. Although not fully understood, the mechanism for this opioid immune activation is likely through classic opioid receptors or via toll-like receptors expressed on neuro-immune cells [18,19]. Therefore, it is not clear whether opioids themselves may affect central immune signalling, mediated by glia, and thus contribute to development of CICI. Furthermore, Walker *et al.* [20] demonstrated that the nonsteroidal anti-inflammatory drug (NSAID), aspirin, blocked tumour-induced memory impairment in tumour bearing mice suggesting a link between inflammation and development of CICI [21]. Thus, it is plausible that opioid agents such morphine, oxycodone and fentanyl act similarly and may impact cognition.

As such, the aim of this study was to investigate the effect of opioid co-administration with chemotherapy on gut inflammation and architecture changes associated with CIGT, cellular changes in the central nervous system, and associated cognitive changes in a female Dark Agouti tumour-bearing (mammary adenocarcinoma) rat model.

6.2 Methods

6.2.1 Animals

Female Dark Agouti rats (DA/Arc; n=67) approximately 6-8 weeks of age were sourced from a certified laboratory animal producer, Animal Resources Centre (ARC) (Perth, Western Australia). The ARC is Specific-Pathogen Free Facility which carries out quarterly health screening to ensure the colony is free of bacterial, viral and parasitic organisms. Upon arrival rats were housed in standard polycarbonate open-top cages (415 mm x 260 mm x 145 mm, Tecniplast, Exton, PA, USA) in groups of five and given shredded paper for nesting material. Food (standard rat chow; Speciality Feeds, Glenn Forest, WA) and acidified RO water were accessible *ad libitum*. The animal facility was temperature (21°C – 23°C) controlled with a

12 h light-dark cycle (0700 h – 1900 h light). All animals were given an extended seven-day acclimatisation period prior to the commencement of experimental procedures. During this time all animals were handled daily.

All experimental procedures were approved by the University of Adelaide Animal Ethics Committee and conducted in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes, 8th Edition, 2013. The study was conducted in six balanced replications and in conjunction with an additional study evaluating the utility of the rat grimace scale for pain assessment in rats with CIGT [22]. This study is reported in accordance with Animal Research: Reporting in vivo experiments: The ARRIVE guidelines [23].

6.2.2 Preparation of tumour inoculum

The mammary adenocarcinoma used in this model arose spontaneously in the 1970s. The methods used in this study were previously established and described in Gibson *et al.* 2002 [24]. Seven donor female Dark Agouti rats, weighing approximately 140g, were anaesthetised with isoflurane (induction 4%, maintenance 1-2% to effect, in O_2) and subcutaneously injected with 0.2 ml (2.0×10^7 cells/mL) of tumour inoculum into both flanks. After eight days of tumour growth, rats were humanely euthanised via CO_2 asphyxiation. Tumours were removed, placed in sterile phosphate buffer solution (PBS), diced, homogenised and filtered through sterile gauze. The resultant cell suspension was spun at 1200 rpm, the supernatant removed and re-suspended in fresh PBS. This step was repeated three times. A viable tumour cell count was performed using 0.4% w/v trypan blue.

6.2.3 Experimental design

Experimental animals (n=60) weighing approximately 140 g, were anaesthetised and subcutaneously injected with tumour cells ($2.0x10^7$cells in 0.2 ml PBS) into the right flank as described above. Tumours were allowed to grow for seven days. During tumour growth phase all rats were monitored daily using clinical record sheets and given wet food. Using a random number generator, rats were allocated to five experimental groups (n=12) with one animal from each treatment group represented per cage: saline (ip) / saline (sc q 12 h), 5- fluorouracil (5-FU) (150 mg/kg 5-FU ip) / saline (sc q 12h); 5- FU (150 mg/kg 5-FU ip) / Oxycodone (3 mg/kg sc q 12h), 5- FU (150 mg/kg 5-FU ip) / Morphine (3 mg/kg sc q 12 h) and 5- FU (150 mg/kg 5-FU ip) / Fentanyl (10 µg/kg sc q 12 h). Sample sizes were chosen based on previous data from our laboratory for outcomes in the novel object recognition test. The number of animals required was derived using a standard deviation of 3.5s, with an effect size of 0.74, providing a statistical power of 82.6%. Cages were assigned to racks randomly and moved regularly throughout the duration of the study. Investigators were blinded to all treatments by allocation concealment. Opioid agents and doses were selected based on previous literature [25,26] and were designed to represent clinical practice (with appropriate allometric scaling for species), where opioid analgesics are prescribed to treat pain associated with cancer and CIGT. Rats received subcutaneous injections of analgesics or equivalent dose of saline every 12 hours from the initial injection of chemotherapy until 72 hours post injection (total of 6 doses).

Behavioural assessments as described below were taken 72 h post chemotherapy/saline injection. This timepoint was specifically chosen to assess cognitive deficits during peak severity of CIGT which typically occurs at 48-72 h following treatment [27,28]. Following behavioural assessment, rats were humanely euthanised using CO_2 asphyxiation in a CO_2 chamber, with a gradual fill rate of 20% of chamber volume/minute. Gastrointestinal tissue

samples were taken for histological and cytokine analysis. Hippocampal samples were taken for western blot and cytokine analysis.

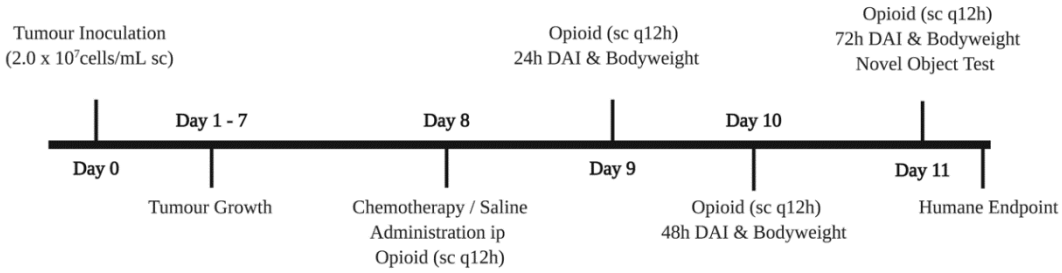

Figure 1. Summary of experimental timeline. Created with BioRender.com

6.2.4 Tumour burden, clinical assessment & bodyweight

All animals were monitored daily for disease activity index and tumour burden with humane endpoints implemented if any animal exceeded the maximum acceptable total score, as prescribed by the Animal Ethics Committee. This scenario did not eventuate. Tumour progression, bodyweight change and clinical assessment (overall appearance) were analysed at 24 h prior to treatment (baseline), 24 h, 48 h and 72 h post chemotherapy and saline administration. Tumour size as a percentage of bodyweight was measured using a digital vernier calliper (Craftright, I/N: 5660742). Tumour burden was calculated utilising the following formulae; (tumour size/bodyweight (g)) × 100. Clinical assessment of the animals was based on eight criteria. These criteria included dull or ruffled coat, hunched, pale or sunken eyes, change in behaviour, reduced food or water intake, dehydration, squealing when handled and reluctant to move. Clinical assessment was recorded using a disease severity score from zero (normal) to three (maximal damage).

6.2.5 Novel-object recognition test (NOR)

The novel object recognition test (NOR) is a well-validated measure of hippocampal-dependent spatial and recognition memory. The NOR was performed according to the protocol published by Bevins & Besheer (2006) [29]. The test consisted of a clear acrylic rectangular arena (610 mm x 435 mm) with a video camera (Panasonic Video Camera HC-V180, Selangor, Malaysia) suspended above the arena in a brightly lit room. Rats were habituated to the NOR arena for a five-minute period prior to the commencement of the test. Rats were then placed into the NOR arena twice, separated by a 1-hour break 72 h post 5-FU and saline administration. Animals were video recorded in the testing arena for both phases of the testing session. In the first phase, object familiarisation, two identical objects were placed on equal sides of the arena 15 cm apart. Rats were placed nose facing away at the midpoint of the wall opposite from the 'familiar' objects. Rat were allowed to freely explore and manipulate (e.g. drag and roll) the objects for 5 minutes. The arena and objects were cleaned with 70 % ethanol after each trial. In the second phase, novel object recognition, one 'familiar' object was replaced by a novel object, with a different colour, shape and texture and rats were again allowed to freely explore the objects for a 5-minute period (Figure 1). Analysis was performed retrospectively by a blinded observer using CowLog software (3.0.2) [30]. Interactions were scored for duration when a rat approached, and nose poked the object or was directed within 2 cm of the object. An interaction was deemed 'nonscorable' when the rat leaned or stood on the object to observe the arena. The percentage preference for the novel object was determined by dividing the amount of time spent with the novel object by the total time the rat spent investigating both objects during phase two and multiplied by 100. A preference index greater than 50% indicated novel object preference, and preference index value of less than 50% indicated familiar object preference.

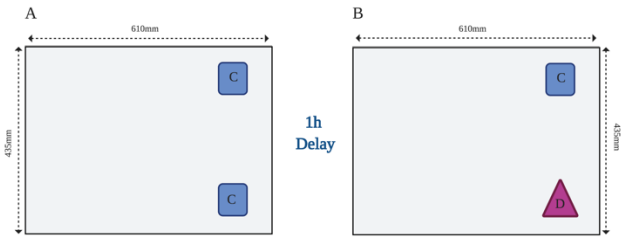

Figure 2. Schematic of the apparatus setup used in the novel-object recognition test. Phase 1: object familiarisation (A). Phase 2: novel object recognition (B). Rats were allowed to explore an identical pair of objects (C) during phase 1. After 1h break rats were presented with a familiar object (C) and a novel object (D) during phase 2.

6.2.6 Histological analysis

Histological analysis of jejunal samples was conducted to assess severity of 5-FU-induced CIGT 72 hours post chemotherapy. Following humane euthanasia, the jejunum was removed, and contents emptied. Jejunum segments (2 cm) were dissected and fixed in 10% neutral buffered formalin solution. Following 24 h incubation, sections were then transferred to 70% ethanol. Preparation and embedding of tissue samples (4 µm) was performed following standard procedure. Samples were stained with haematoxylin and eosin (H&E). All histological analysis was conducted by a blinded observer using a light microscope (Olympus CX-41, Olympus, Tokyo, Japan). The blinded observer scored each jejunal sample for histological severity from zero (normal) to three (maximal damage) on eight independent criteria (villus fusion and stunting, enterocyte disruption, reduction in goblet cells numbers, crypt disruption, crypt cell disruption, lymphocytic infiltration, thickening of the submucosa and thickening of the muscularis externa) [28].

6.2.7 Luminex microbead assay

Cytokine analysis was performed by a commercial assay facility (Adelaide Research Assay Facility Robinson Research Institute, School of Medicine, University of Adelaide). Sections (2 cm) of jejunum were collected and snap frozen in liquid nitrogen and stored at -80^{o}C for subsequent cytokine analysis of tumour-necrosis factor (TNFα), interleukin-6 (IL-6), interleukin-1β (IL-1β) and interleukin-10 (IL-10).

Total protein was extracted from rat jejunum tissue samples using a Powerlyzer® 24 Bead Based Homogenizer (Mo Bio Laboratories, Carlsbad, CA). Briefly, tissue samples were placed in individual 2 ml tubes with 500 ul of RIPA buffer (Sigma Aldrich, Australia) containing protease inhibitor (complete™, Mini, EDTA-free Protease Inhibitor Cocktail; Roche, Switzerland) and 0.6 g of 1.4 mm diameter ceramic beads and tissue disrupted by two cycles of homogenisation at 3500 rpm for 10 seconds at 4^{o}C. Following homogenisation, samples were transferred to new 1.7 ml tubes and centrifuged at 11000 g for 20 minutes at 4^{o}C to remove cellular debris. The resultant supernatants were then transferred to new tubes and stored at -80^{o}C until assayed for cytokine content.

The abundance of IL-1β, IL-6, IL-10 and TNF-α in rat jejunum tissue extracts was quantified by Milliplex MAP Magnetic Bead Luminex Assay (Catalog No. RECYTMAG-65K, Merck Millipore; Darmstadt, Germany) according to the manufacturers' instructions with all samples tested without further dilution. Microbead data were analysed using a MAGPIX® Luminex instrument and data acquired using xPONENT 4.2 software (Luminex Corporation; Texas, USA).

6.2.8 Enzyme-linked immunosorbent assay (ELISA)

Hippocampal tissue was collected, snap frozen in liquid nitrogen and stored at -80°C. Samples (2 cm) were homogenised, and the supernatant was collected for determining protein concentration using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Victoria, Australia). Assay procedure was conducted in accordance to manufacture's instruction, TNF-α ELISA kit (cat. no. ab46070; Abcam, Cambridge, UK). Samples were assayed in duplicate with an average value taken for analysis using a spectrophotometer (Victor X4 Multilabel Reader, Perkin Elmer, Singapore)

6.2.9 Western blot analysis

The hippocampal region was removed, snap frozen in liquid nitrogen and stored at -80 °C. Sections (1-2 cm) of hippocampus were extracted and homogenised in standard RIPA buffer. Protein concentration was determined using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Victoria, Australia). Gel electrophoresis was performed using Bolt 4–12% Bis-Tris Plus gels (Thermo Fisher Scientific Inc) with 25 μg of protein loaded per well. Gels were run at 150 V for approximately 1.5 h or until the dye reached the bottom of the gel. In accordance with the manufacturer's protocol, gels were transferred to a polyvinylidene fluoride (PVDF) membrane using the iBlot 2 Dry Blotting System (Thermo Fisher Scientific Inc). To ensure each well had equal protein loading, membranes were stained with Ponceau S red solution (Fluka Analytical) to allow for visual inspection. Following visual inspection membranes were washed three times using 1X tris-buffered saline with tween (TBST) and left to incubate on agitator for five-minutes between each wash. Membranes were blocked in 5% skim milk solution (5 g skim milk powder, 100 mL TBST) for 2 h at room temperature. Membranes were then washed three times as previously described and then incubated in 2% skim milk solution (1 g skim milk powder, 50 mL TBST) at 4°C overnight with primary

antibodies (GFAP; #ab7260, Abcam, Cambridge, UK) and housekeeper protein (GAPDH; #ab8245 Abcam, Cambridge, UK) on a rotating device. Following the overnight incubation period, membranes were washed three times in TBST and then incubated with secondary antibodies (LICOR 800CW donkey/anti-rabbit,1:10,000; LICOR 800CW donkey/anti-mouse, 1:10,000) in 2% skim milk solution for 2 h at room temperature on a rotating device covered with aluminium foil. Western blots were visualised using the Odyssey Infrared Imaging System (model 9120; software version 3.0.21). Western blot quantification was performed by a blinded observer using ImageJ software (Wayne Rashband, National Institutes of Health, USA).

6.2.10 Statistical analyses

Statistical analysis was conducted using Megastat Excel Add-In (version 10.3 Release 3.1.6 Mac, McGraw-Hill Higher Education, New York, NY, USA) and SPSS (SPSS Inc., Chicago, IL, USA) software. Data were tested for normality using the Shapiro–Wilk test. One rat was excluded from the study due to unrelated health issues rendering an n=11 for the saline control group. A second rat was excluded from the novel object analysis due to an error during phase two of the NOR for the saline control group (n=10). Histological severity, gastrointestinal cytokine**,** tumour burden, clinical assessment and bodyweight data between groups were analysed non-parametrically using a Kruskal-Wallis U-test with *post-hoc* Mann Whitney U-test. Within group differences across time were analysed using a Friedman test with *post-hoc* Wilcoxon signed-rank test. A one-way analysis of variance (ANOVA) with Tukey's *post-hoc* test was used to analyse NOR, TNFα expression and western blot data. Data are presented as mean ± SEM for parametric data and median with range for non-parametric data. Significance was determined at $p < 0.05$.

6.3 Results

6.3.1 Tumour burden, clinical assessment & bodyweight

A Kruskal-Wallis test revealed intergroup significance for tumour burden at each timepoint (24 h $H(4) = 10.13$, $p = 0.03$; 48 h $H(4) = 11.59$, $p=0.02$; 72 h $H(4) = 9.80$, $p = 0.04$ (Figure 3). Post-hoc analysis showed that tumour burden as a percentage of bodyweight was significantly decreased for rats injected with 5-FU alone and in conjunction with opioid analgesics, morphine and oxycodone at 48 h and 72 h compared to rats treated with saline ($p<0.05$). Treatment consisting of 5-FU in conjunction with fentanyl showed no significant differences with saline control animals at 72 h post saline and chemotherapy injection ($p = 0.06$). There were however significant differences observed at later time-points. Friedman analysis determined significant differences across time ($X^2(2) = 70.33$, $p<0.001$). Post-hoc Wilcoxon signed-rank test determined that tumour burden was significantly increased at 72 h compared to 24 h for rats in all treatment groups except 5-FU in combination with oxycodone (saline $p = 0.005$; 5-FU $p = 0.005$; Fentanyl $p = 0.003$; Morphine $p = 0.002$; Oxycodone $p = 0.06$).

Clinical assessment scores increased significantly over time for animals treated with 5-FU compared to saline alone ($X^2(3) = 123.33$, $p<0.001$). There were no differences between groups prior to administration of saline and 5-FU. Clinical assessment scores differed substantially between groups 24 h, 48 h and 72 h for animals treated with 5-FU and analgesics compared to saline control animals (Table 1). No differences in bodyweight scores were observed between treatment groups at baseline (prior to chemotherapy or saline treatment). Bodyweight was significantly decreased across all time points post chemotherapy in comparison to saline (Table 1). Administration of opioid analgesics, fentanyl, morphine and oxycodone was associated with greater reduction in bodyweight compared to saline at 24 h, 48 h and 72 h post chemotherapy. Furthermore, administration of morphine and oxycodone further decreased bodyweight

compared to 5-FU alone (48 h; morphine p = 0.01, oxycodone p = 0.02; 72 h oxycodone p = 0.02).

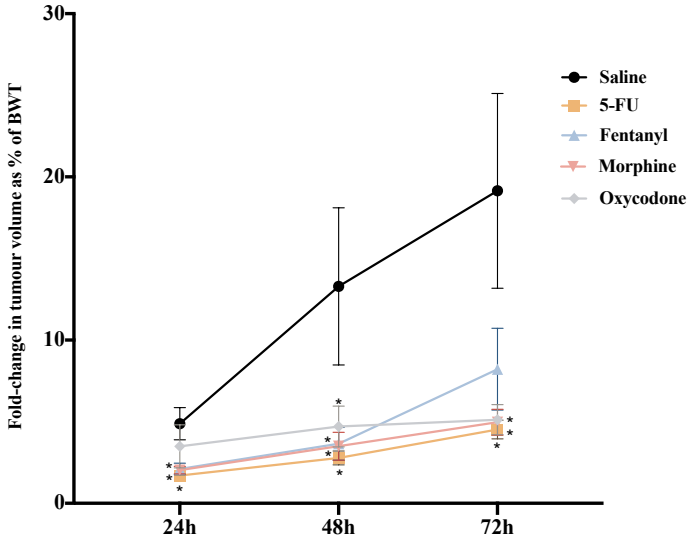

Figure 3. Tumour burden in female Dark Agouti rats 72 h following saline and 5-FU administration and daily injections of saline, fentanyl, morphine and oxycodone. Data are expressed as median with range. Saline (n = 11), 5-FU (n = 12), Fentanyl (n = 12), Morphine (n = 12) and Oxycodone (n = 12). * indicates significance p<0.05 compared to saline.

Table 1. Clinical assessment and bodyweight change of tumour-bearing rats.

Treatment	Baseline		24h		48h		72h	
	Clinical assessment score	Bodyweight change (%)	Clinical assessment score	Bodyweight change (%)	Clinical assessment score	Bodyweight change (%)	Clinical assessment score	Bodyweight change (%)
Saline	0 ± 0	2.39 ± 0.53	0.09 ± 0.09	3.44 ± 0.66	0.45 ± 0.16	3.87 ± 0.67	0.36 ± 0.15	3.76 ± 0.78
5-FU	0 ± 0	1.66 ± 0.44	0.75 ± 0.13*	-0.87 ± 0.57*	1.42 ± 0.19*	-4.34 ± 0.94*	1.75 ± 0.13*	-5.44 ± 0.68*
Fentanyl	0 ± 0	1.62 ± 0.54	1.08 ± 0.15*	-0.96 ± 0.61*	1.33 ± 0.14*	-4.93 ± 1.02*	1.75 ± 0.13*	-6.34 ± 0.99*
Morphine	0 ± 0	1.88 ± 0.50	0.83 ± 0.11*	-2.39 ± 0.52*	1.42 ± 0.15*	-7.16 ± 0.60*^	2.00 ± 0*	-8.12 ± 0.66*^
Oxycodone	0 ± 0	2.26 ± 0.37	1.17 ± 0.17*	-2.87 ± 0.56*	1.75 ± 0.13*	-7.88 ± 0.70*^	1.83 ± 0.11*	-9.27 ± 1.08*^

Symbols denote significance compared with saline * p<0.05 and ^ p<0.05 compared to 5-FU. Saline (n = 11), 5-FU (n = 12), Fentanyl (n = 12), Morphine (n = 12) and Oxycodone (n = 12).

6.3.2 Novel-object recognition test (NOR)

There were no significant differences in the preference index towards investigating the novel object between treatment groups 72 h post 5-FU and saline administration in the second phase of the NOR ($F(4,53) = 1.428$, $p = 0.238$) (Figure 3).

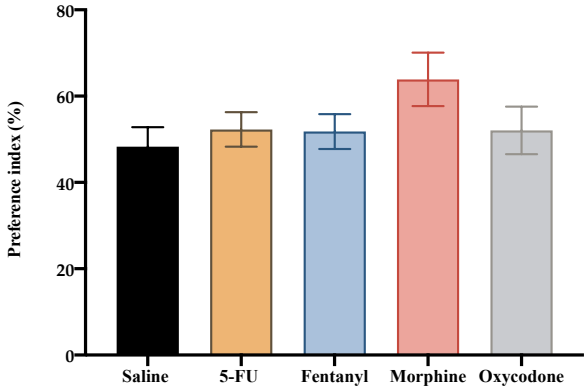

Figure 3. Preference index for the novel object of tumour bearing female Dark Agouti rats treated with 5-FU or saline in combination with opioid analgesics or saline. Data expressed as mean ± standard error of the mean. 5-Fluorouracil; 5-FU. Saline (n = 10), 5-FU (n = 12), Fentanyl (n = 12), Morphine (n = 12) and Oxycodone (n = 12).

6.3.3 Histological Analysis

Histological severity score was significantly increased in rats treated with 5-FU alone and 5-FU in conjunction with opioid analgesics, fentanyl, morphine and oxycodone compared to saline controls ($H(4) = 30.36$, $p = 0.0001$) (Figure 6). Post-hoc Mann Whitney U-test determined that the analgesic agent oxycodone decreased histological severity compared to 5-FU alone treated animals ($p = 0.03$).

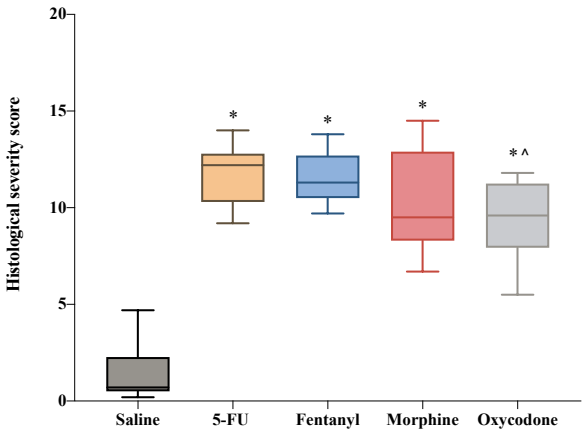

Figure 6. Histological severity score. Data represented as median with IQR. 5-Fluorouracil; 5-FU. Saline (n = 11), 5-FU (n = 12), Fentanyl (n = 12), Morphine (n = 12) and Oxycodone (n = 12). Symbols denote significance compared with saline * $p<0.001$, and 5-FU *^ $p = 0.03$.

6.3.4 Luminex microbead assay & Enzyme-linked immunosorbent assay (ELISA)

Following 5-FU administration, levels of TNFα in the jejunum were significantly elevated in the 5-FU control group compared to saline controls ($H(4)=9.975$, $p=0.041$; $p=0.007$) (Figure 7a). Administration of 5-FU alone and in conjunction with morphine showed significantly elevated levels of TNFα in jejunal tissue compared to saline controls ($p=0.014$). However, this was not observed for opioid analgesic groups oxycodone and fentanyl ($p=0.074$; $p=0.056$, respectively). Animals treated with 5-FU alone and in combination with opioid analgesics, morphine and oxycodone, had significantly decreased expression of IL-1β in the jejunum compared to saline control animals ($H(4) = 10.019$, $p=0.04$; $p=0.012$, $p=0.042$, $p=0.003$, respectively) (Figure 7b). No significant difference was observed in the expression of IL-1β between animals treated with 5-FU in conjunction with fentanyl ($p=0.124$) (Figure 7b). With respect to IL-6 and IL-10 levels, no significant differences between treatment groups were demonstrated ($H(4)=0.867$, $p=0.929$; $H(4)=3.695$, $p=0.449$, respectively) (Figure 7c and 7d).

There was no significant difference between treatment groups ($F(4, 25) = 0.418$, $p = 0.794$) in the expression of TNFα in the hippocampus, 72 hours post saline and 5-FU administration (Figure 8).

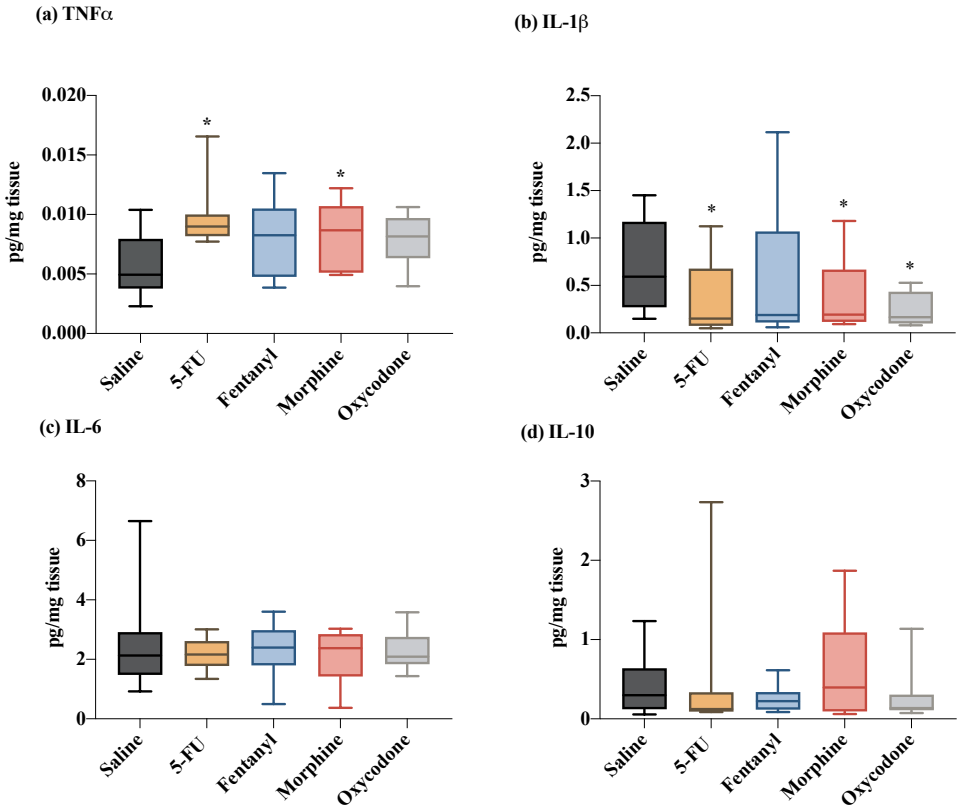

Figure 7. Multiplex analysis of TNFα (a), IL-1ß (b), IL-6 (c) and IL-10 (d) in the jejunum at 72 hours post-chemotherapy and saline injection. Data expressed as median with range. Saline (n = 11), 5-FU (n = 12), Fentanyl (n = 12), Morphine (n = 12) and Oxycodone (n = 12). Symbols denote significance compared with saline * $p<0.05$. Cytokine data presented as pg/mg of tissue.

6.3.5 Western Blot Analysis

Western blot analysis indicated 5-FU had no effect on hippocampal GFAP expression when compared with saline controls ($F(4,25)=0.2661$, $p=0.897$). No statistical significance was

observed in hippocampal GFAP expression on 5-FU in combination with opioid analgesics, fentanyl, morphine and oxycodone (Figure 9).

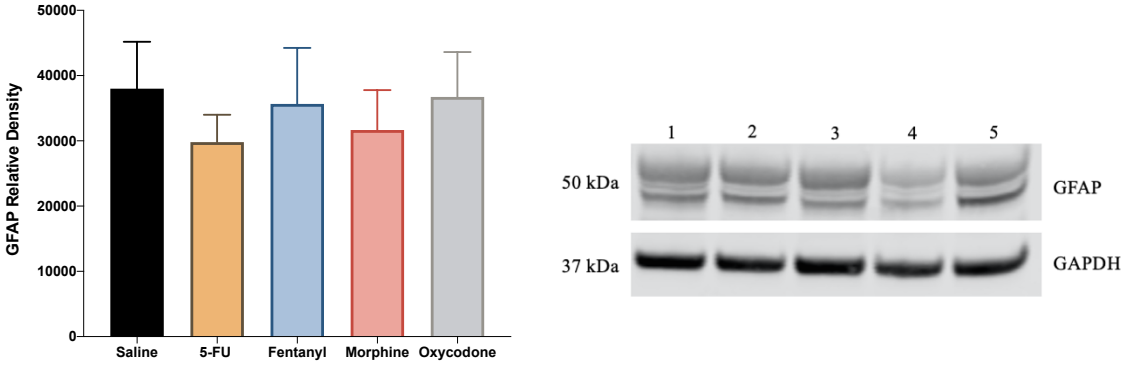

Figure 9. Western blot analysis of the expression of GFAP in the hippocampus in saline and 5-FU treated rats injected daily with saline, fentanyl, morphine and oxycodone. Data expressed as mean ± SEM. 5-Fluorouracil; 5-FU. n = 6 per treatment group

6.4 Discussion

This study aimed to determine the role of CIGT, and its opioid palliation, on central neuroimmune mechanisms underlying CICI in a tumour-bearing rat model. To our knowledge this study represents the first to investigate 5-FU in conjunction with potent μ opioid receptor agonists, fentanyl, morphine and oxycodone in a gastrointestinal inflammatory environment created by CIGT and cognitive markers, simultaneously. The methodology employed in the current study was unique in that it investigated a combination of histological and molecular changes along with alterations in cognitive function through behavioural assessment in rats with tumours. The results indicate that a single high dose of 5-FU caused deleterious effects in the jejunum however did not modulate a hippocampal neuroinflammatory response based on the chosen neuroimmune reactivity markers or induce cognitive dysfunction at this acute

timepoint (72 h post chemotherapy). Furthermore, opioid analgesics fentanyl, morphine and oxycodone did not potentiate CICI in this scenario by astrocytic reactivity marker expression.

The results indicate that CIGT was present as evidenced by increased intestinal histological scores and clinical assessment scores post chemotherapy administration. Observations in previous CIGT studies demonstrated that 5-FU constitutively causes small intestinal damage consisting of villus blunting, thickening of the submucosa and muscularis externa, crypt cell distortion and reduction of goblet cells during the peak injury phase of 5FU-induced CIGT typically occurring at 48–72 h following injection [27,28,31]. These results correspond with the current results.

Changes in clinical assessment and bodyweight were also observed 24-72 h post chemotherapy administration which is also consistent with previous literature. Both clinical assessment and bodyweight loss were elevated across time-points in rats with CIGT, implying systemic toxicity. Similar to previous studies that utilised the partial μ opiate agonist buprenorphine, opioid analgesics herein had a striking effect on bodyweight loss and change in appearance compared saline alone controls [27,28]. Additionally, morphine and oxycodone potentiated this effect on bodyweight compared to 5-FU alone treated animals, most likely due to associated opioid-induced side-effects such as appetite suppression and constipation. Common side effects associated with analgesic use in rodents include respiratory depression, constipation, urinary retention, nausea, increased sensitivity to pain rather than relief, namely opioid induced hyperalgesia, and tolerance that can result in limited response to the medication [32]. In the clinical setting similar adverse effects can be seen in patients following opioid use, including respiratory depression, skin itchiness, and CNS toxicities such as cognitive impairment, confusion, drowsiness, and more infrequently hyperalgesia. Gastrointestinal dysfunction

including bloating, nausea, vomiting, constipation or reduced frequency of bowel movements, and gastroesophageal reflux are also commonly observed [33].

The pathobiological development of CIGT is described as five interlinked phases: initiation, primary damage response (upregulation of inflammation via generation of messenger signals), signal amplification, ulceration and healing [6]. During the upregulation and message generation phase numerous signalling pathways and transcription factors are initiated. The activation of nuclear factor kappa-beta (NFκB) is of greatest importance as it facilitates gene expression, and the synthesis of pro-inflammatory cytokines, in particular IL-6, IL-1β and TNFα [6,11]. Chemotherapy agents have been found to considerably increase production of NFκB, causing pro-inflammatory cytokines to be produced [11]. In this study 5-FU and 5-FU in conjunction with morphine increased TNFα expression compared to saline control group. This observation was expected as previous studies have shown increased expression of TNFα involved in the development of CIGT and the inflammatory response [13,34].

Interestingly, 5-FU and 5-FU in combination with morphine and oxycodone had decreased IL-1β expression compared to the saline alone control group. This finding was unexpected as previous studies have found increased production of IL-1β during 5-FU-inudced CIGT, as IL-1β has been implemented in promotion of inflammation and enhanced development of the condition [13]. It has been postulated that microbiological and non-microbiological factors can stimulate IL-1β production. IL-1β may act on a wide variety of cell types and cause various biological effects, including systemic reactions and involvement in the activation of NFκB pathway [34]. A possible contributing factor could be that morphine and oxycodone may exert an anti-inflammatory mechanism. There has been considerable research on the role of peripheral opioid mechanisms underlying inflammatory conditions, however very few studies

have evaluated peripheral opioid anti-inflammatory effects, especially in gastrointestinal disorders [27,35]. Whittaker *et al.* [27] demonstrated rats with 5-FU-induced CIGT administered with opioid analgesics, buprenorphine and tramadol, demonstrated reduced acute inflammation in jejunal tissue via the myeloperoxidase (MPO) pathway compared with healthy controls [27]. Nonsteroidal anti-inflammatory agents such as benzydamine have been shown to inhibit the production of IL-1β and TNFα and have been recommended to prevent and reduce the severity of oral mucositis [36]. This notion further supports the finding herein that oxycodone attenuated the histological damage caused by 5-FU administration. However, it is important to note that this theory does not account for the decrease in IL-1β expression observed in the 5-FU control group.

Alternatively, the presence of untreated tumours could potentially have played a role in the changes in circulating pro-inflammatory cytokines such as IL-1β levels encountered in this current study. The saline control animals had an increased tumour burden compared to the rest of the groups. Previous studies have demonstrated increased IL-1β expression in various cancers and has been connected to 'metastatic behaviour of breast cancer cells in vitro' [37]. Furthermore, it has been postulated that secretion of inflammatory mediators (i.e. cytokines and chemokines) arise from the tumour microenvironment and promote recruitment of additional immune cells [38]. This could have been measured by having cytokine analysis conducted at various points along the tumour growth trajectory.

In the present study, we used the NOR to assess hippocampal mediated memory [20]. The NOR is a well-validated and robust behavioural assay to assess non-spatial memory in rodents which is reliant on the hippocampus and perirhinal cortex [39]. The test utilises the innate proclivity of rodents to explore novel items, and determinations on memory can be established by

calculating the latency of rodents to explore the objects during the test phase [39]. The results herein showed that there were no significant effects of 5-FU treatment on cognition. Previous studies that have employed the NOR have demonstrated conflicting results. Similar to our findings, other studies have reported no detrimental effects on cognition and improved cognitive performance post chemotherapy [40-43]. Contradictory to our results, others have demonstrated various chemotherapy agents, including 5-FU, causing impairments in cognition utilising the NOR task [44-46].

One possible explanation for the lack of cognitive impairment demonstrated is the dosing frequency that was employed. In the current study a single high-dose of 5-FU was utilised. It is plausible that a single dose of 5-FU was not enough to elicit impaired novel object recognition. Repeated treatment regimens and combination treatment has the potential to increase cytotoxicity, thus exacerbating cognitive impairment. For example, Fardell *et al.* [47] demonstrated that rats treated with chemotherapy agents 5-FU and oxaliplatin alone impaired object recognition, however when given in combination had a significant detrimental effect on cognition in hippocampal-dependent tasks. However, previous studies have reported impairment following single injection of chemotherapeutic agents including 5-FU [48,49]. It is noteworthy to mention that the methodological differences within the literature regarding chemotherapeutic agents, treatment regimen, animal model and NOR experimental setup has vast heterogeneity, making comparisons between studies difficult. It is also feasible that cognitive impairment might not coincide with toxic effects occurring in the gut observed at the 72 h timepoint. Chemotherapy effects on the gastrointestinal system may precede those on the CNS. Thus, it may be too early to detect cognitive changes that could be forthcoming.

Clinical literature indicates that the side effects of CICI can range in severity, from subtle to more severe and persistent. It is plausible that cognitive impairment may have occurred but may have been mild and the cognitive test employed was not sensitive enough to detect subtle cognitive changes. Furthermore, CICI can result in impairment to memory, processing speed, executive function, concentration, attention, and visuospatial ability [4]. Whilst most of these cognitive abilities are related to hippocampal function, which fits in with numerous studies that analyse the hippocampus [20,50,51], CICI affects such a wide range of cognitive skills that hippocampal analysis alone may be insufficient. Thus, the need to assess multiple brain regions and cognitive domains implicated by CICI is paramount. The findings presented in this study, and others, pose a question regarding the effectiveness of current behavioural methods in assessing subtle cognitive deficits. Thus, performing assessment tasks measuring outcomes related to different brain regions, such as the prefrontal cortex, would be beneficial. Alternatively, a battery of cognitive tests could aid characterisation of potential cognitive deficits across multiple domains. However, challenges regarding practicality and feasibility of cognitive testing pose a challenge when investigating acute timepoints such as 72 h.

An alternative interpretation is that the saline control group experienced cognitive impairment due to tumour presence. Clinical literature suggests up to 30% of patients report cognitive dysfunction in facets of executive functioning and memory prior to chemotherapy treatment [52]. Likewise, previous studies have identified cancer associated memory and learning impairments in tumour-bearing rodents [21,38]. Preclinical studies that have utilised standardised behavioural tests to assess cognitive function in tumour-bearing models have reported impairment of components of memory and learning. For example, in tumour-bearing rats and mice performance in the NOR have been considerably impaired. A limitation of the current study is that a saline control group without tumours was not utilised. Inclusion of

additional treatment groups poses an ethical issue in terms of subsequent use of additional animals and this not being directly relevant to clinical practice. Nonetheless, future studies should build on the current results by including a saline control group without tumours to distinguish cancer related cognitive impairment from 5-FU-inudced cognitive impairment.

Continuing the pattern observed from the behavioural analyses, 5-FU did not modulate a hippocampal neuroinflammatory response. Furthermore, opioid analgesics did not have any direct effect on GFAP and TNFα expression in the hippocampus. Chemotherapy agents such as 5-FU are able to penetrate the blood brain barrier potentially resulting in CNS damage via a direct mechanism, leading to cognitive decline in CICI [44,50]. It was expected in the current study that GFAP and TNFα expression would increase in the hippocampus in 5-FU treated animals and opioid analgesics would potentiate this effect. The gastrointestinal tract has the ability to modulate central nervous system function through the peripheral immune system [1], and the peak of mucosal damage characteristically occurs 48-72 h following 5-FU treatment. It is feasible that the selected time-point was too early to observe a neuroinflammatory change with an increase in astrocytic activation in the hippocampus. Thus, hippocampal damage may be occurring at a later time-point through an indirect effect via peripheral cytokines possibly leading to neuroinflammation. Future studies should employ a comprehensive analysis on neuroimmune reactivity marker expression changes such as microglial reactivity across a range of brain regions and time course implicated in CICI. Alternatively, the tumour microenvironment may have had an influence. Peripheral tumour-induced immune changes such as pro-inflammatory cytokines (IL-1β, IL-6 and TNFα) and inflammatory enzymes (nitric oxide synthesis) in the brain and periphery have been reported and associated with cognitive impairments [38]. Thus, an increase in TNFα and GFAP expression observed in the saline control may be attributed to a neuroinflammatory response.

6.5 References

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CHAPTER 7

Investigation of Onset and Duration of Cognitive Decline in a Juvenile Rat Model of Chemotherapy-induced Cognitive Impairment

CONTEXTUAL STATEMENT

In chapter 6 it was posed that the selected acute time-point was too early to detect cognitive changes following peripheral (gut-related) inflammation. Furthermore, the results highlighted the necessity to include a battery of behavioural tests that can assess multiple brain regions and cognitive domains implicated in CICI. As a result, it was proposed that future studies should incorporate a comprehensive analysis of molecular outcomes and behavioural assessments that measure both memory and executive function, with a focus on later time-points in CICI pathogenesis. Furthermore, results from chapter 5 demonstrated that few preclinical rodent models of CICI have assessed molecular and cognitive effects at a chronic time point, where neuroinflammation would be expected to be of great concern. Subsequently, the aim of **Chapter 7** was to investigate 5-FU- and MTX-induced cognitive changes and associated CNS cellular changes in a CICI rat model at a subacute time-point (31 days post treatment) and chronic time-point (93 days post treatment), utilising behavioural paradigms that assess both hippocampal and prefrontal cortex function.

CHAPTER 7

Investigation of Onset and Duration of Cognitive Decline in a Juvenile Rat Model of Chemotherapy-induced Cognitive Impairment

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7.1 Introduction

Patients that have received chemotherapy treatment often experience debilitating side-effects, in the short and long-term, that adversely affect patient quality of life. In particular, a considerable number of patients experience cognitive dysfunction associated with chemotherapy that affect memory, thinking, and ability to concentrate and multitask [1]. This condition is termed chemotherapy-induced cognitive impairment (CICI); often referred to as "Chemobrain". The cognitive domains affected are diverse with the most common neuropsychological effects including deficits to episodic, verbal, visual memory, and visual-spatial ability, alongside impairments in facets of executive function such as working memory, attention, and impulsivity [2-4]. These wide-ranging cognitive deficiencies result from changes to multiple brain regions, including frontal, prefrontal, parietal and temporal regions [2].

Currently, the time of onset and duration of CICI reported in the literature is unclear and varies widely [1]. Clinical studies report cognitive impairment in approximately 17% - 75% of patients, with the severity of cognitive decline ranging from mild to severe [1,5]. The duration of persistent impairments has been described to last for months to years following cessation of treatment in 15% - 35% of patients [6]. Additionally, some studies have reported long-term cognitive impairment for up to 20 years post chemotherapy treatment [7]. Multiple challenges hamper accurate assessment of the prevalence of cognitive impairment including: the absence of standardised cognitive assessment tools and batteries of neuropsychological tests, cross-sectional study designs with lack of cognitive assessment prior to chemotherapy treatment and

heterogeneity in observed populations [1]. As reported prevalence, onset, and duration of CICI appear to vary greatly in the clinical setting, specific guidance on interventional strategies is limited. Thus, investigation of the onset and trajectory of CICI is required, with a focus on the chronic impact of chemotherapy, in order to develop reliable treatment and management strategies for patients, and to ultimately improve patient quality of life post treatment.

The mechanisms of CICI pathogenesis have not been fully elucidated. However, candidate mechanisms identified include neuroinflammation, the action of damage associated molecular patterns (DAMPs), oxidative stress, blood-brain barrier (BBB) degradation, impaired neurogenesis, and myelin degradation [8-10]. Neuroinflammation is one such mechanism that has been proposed to play a pivotal role in the development of CICI and associated cognitive impairment. Specifically, neuroinflammation may result from both direct and indirect mechanisms that cause a cascade of neuroinflammatory events and neurotoxicity, resulting in reduced cognitive performance [8,10,11]. Whilst clinical research in CICI is crucial to improve understanding of the human experience and associated symptoms, it is difficult to design an ideal study to confirm underlying neurobiological mechanisms, hence preclinical animal studies are needed to improve mechanistic understanding.

Chemotherapeutic agents 5-Fluorouracil (5-FU) and Methotrexate (MTX) are frequently used cytotoxic drugs to treat a variety of cancers including acute lymphoblastic leukaemia, colorectal and breast cancer, and are regularly administered alone or in conjunction [12-14]. Studies have demonstrated that both MTX and 5-FU are associated with a decline in cognitive function [5,15-17]. Furthermore, preclinical studies have shown changes in neuroimmune reactivity marker expression in rodents treated with MTX alone, and MTX in conjunction with

5-FU [18-21]. Previously it was thought that chemotherapy drugs could not readily cross the BBB. However, it is now proposed that some cytotoxic agents, such as 5-FU, are able to penetrate the BBB and cause direct neurotoxic damage to the CNS resulting in cognitive dysfunction [3]. Additionally, it has been suggested that MTX can damage blood vessel walls and disrupt BBB permeability resulting in further damage to CNS [5].

Previous animal studies have particularly focused on acute and subacute time-points for CICI progression, with very few studies assessing cognitive outcomes at a chronic time-point [19,22-24]. Furthermore, studies that assess cognitive and molecular outcomes simultaneously at a chronic time-point are vastly under-represented [25]. In other neurodegenerative conditions, neuroinflammation has been linked to cognitive impairment in rodents and humans at both acute and chronic time-points [26,27]. Thus, considering the protracted nature and trajectory of cognitive deficits experienced by patients, it is imperative to assess the onset and time course of CICI in tandem with potential cellular changes in the CNS in order to develop intervention strategies, and their appropriate timing for prevention or remediation of symptoms. Additionally, previous studies in CICI that have employed behavioural tests of cognition have utilised traditional tests that are sensitive to deficits and lesions in the hippocampus. Whilst these tests are well-validated measures utilised in cognitive research, CICI affects a diverse range of cognitive domains such that hippocampal analysis alone may be insufficient. The current study therefore employed behavioural paradigms to assess both hippocampal-dependent and prefrontal cortex dependent tasks, which may be more sensitive to the subtle changes often observed in CICI.

Subsequently, the aim of this study was to determine the time course of chemotherapy-induced cognitive changes and associated CNS cellular changes in a CICI rat model at a subacute time-

point (31 days post treatment) and chronic time-point (93 days post treatment), utilising behavioural paradigms that assess both hippocampal and prefrontal cortex function.

7.2 Methods

7.2.1 Animals

Female Sprague Dawley (SD) rats (Hsd: SD; n=72) aged approximately 4 weeks were sourced from a certified Specific Pathogen Free facility (Laboratory Animal Services, the University of Adelaide). Laboratory Animal Services undertakes regular comprehensive quality assurance health checks to ensure the rodent populations are free of viral, bacterial and parasitological infections. Female SD rats are a commonly used outbred strain and were selected to mimic the heterogeneity existing in the human population. Animals were housed in groups of six in standard polycarbonate open-top cages (415 mm x 260 mm x 145 mm, Tecniplast, Exton, PA, USA). Rats were given cardboard structures and shredded paper as nesting material and enrichment. Acidified RO water and a standard diet of chow (Teklad Irradiated Global SoyProtein-Free Extrduded Rodent Diet, Envigo, Madison, WI) was available *ad libitum* throughout acclimation and treatment regimen. Thereafter, food was restricted to 4g/100gbwt throughout behavioural testing to maintain motivation for the sugar pellet reward. Animals were weighed and monitored daily with additional food provided if bodyweight dropped below 15% of pre-feeding weight. Rats were acclimatised to the animal facility for 5 days prior to the commencement of experimental procedures. During this period animals were handled and given wet food daily. The animal facility was maintained under a 12-hour light / dark cycle (0700 h – 1900 h light) and room temperature at 21-23°C.

All experimental procedures carried out were approved by the University of Adelaide Animal Ethics Committee and in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes, 8th Edition, 2013 guidelines. This paper was written in accordance with Animal Research: Reporting in vivo experiments (ARRIVE) guidelines.

7.2.3 Experimental design and treatment administration

Animals weighing >140 g were randomly allocated, using a random number generator, to either 5-Fluorouracil (5-FU) (75mg/kg ip; Hospira Australia Pty Ltd, Mulgrave, Victoria, Australia), Methotrexate (MTX) (37.5mg/kg ip; Accord Healthcare Pty Ltd, Melbourne, Victoria, Australia) or saline (0.9% NaCI). Animals were then further allocated to two time-points to detect both subacute and chronic effects; 31 days post treatment (MTX n=12, 5-FU n=12; Saline n=12) (Figure 1) and 93 days post treatment (MTX n=12, 5-FU n=12; Saline n=12) (Figure 2). Rats received a single intraperitoneal (i.p) injection of 5-FU, MTX or saline once weekly for two consecutive weeks. Rats in the saline control group received an equivalent dose. Animals were monitored and weighed daily throughout the duration of treatment. To minimise adverse systemic effects of chemotherapy agents and increase nutrient intake rats were given Nutrigel (High-energy dietary supplement, *Troy Laboratories Pty Ltd*, NSW, Australia) throughout the duration of treatment and 5 days post last chemotherapy administration. Chemotherapy agents and dosages were selected based on clinical relevance and previous literature that had demonstrated significant cognitive impairment following treatment. The derived sample size per treatment group was calculated to achieve 80% power based on previous behavioural data.

Following behavioural assessment, rats were humanely euthanized via CO_2 asphyxiation (individually placed in a CO_2 chamber with gradual fill rate of 20% of chamber volume/minute)

at two time points; either 31 days or 93 days post chemotherapy or saline administration. Following humane euthanasia, the prefrontal cortex was dissected and snap frozen in liquid nitrogen and stored at -80°C for cytokine and western blot analysis.

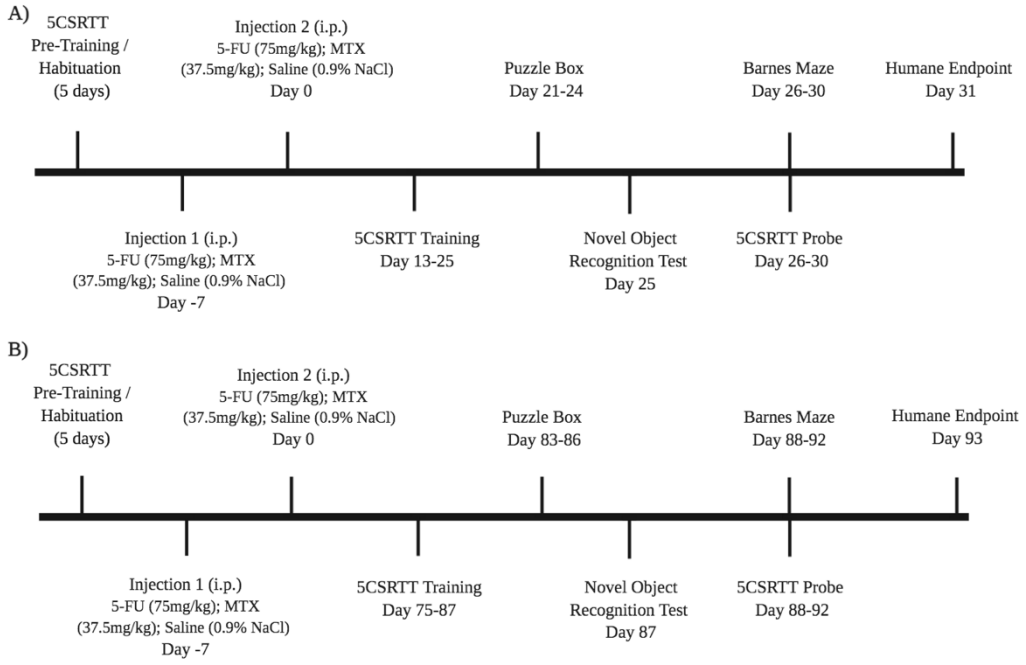

Figure 1. Experimental timeline including chemotherapy administration and behavioural testing at (A) sub-acute time-point (31-days post treatment) and (B) chronic time-point (93-days post treatment) (n=12 per treatment group).

7.2.4 Cognitive tests

Animals were assessed for cognitive function using the novel object recognition test, puzzle box, Barnes maze and five-choice serial reaction time task (5CSRTT) (Figure 1). All animals underwent habituation to behavioural tests and commenced pre-training for the 5CSRTT prior to commencement of treatment. All behavioural testing was conducted between 0700h – 1900h and was performed in an order of least to most aversive with appropriate breaks between

behavioural tests. The testing order within each test trial was randomised across animals utilising a random number generator.

7.2.4.1 Novel Object Recognition test (NOR)

The novel object recognition test (NOR) was performed on day 25 and day 87 following last chemotherapy and saline injection. The NOR is a well validated test to assess non-spatial memory and is dependent on the perirhinal cortex and hippocampus [28]. The testing arena consisted of an enclosed open field arena (50 cm x 50 cm x 50 cm). All procedures were conducted in a brightly lit room with a video camera (Panasonic Video Camera HC-V180, Selangor, Malaysia) suspended above the arena. Animals were first habituated to the testing arena without any objects for a 5-minute period. Post habituation rats were placed into the NOR arena twice, separated by a 1 hr break. During object familiarisation (phase one) two identical sample objects were placed on equal sides of the arena. The animal was placed in the middle of the arena, facing away from the objects. The investigator immediately left the testing room, and the animal was allowed to freely explore for 5 minutes, before being removed and returned to the home cage for 1 hr. The object recognition phase (phase two) was conducted identically to phase one however one of the sample objects was replaced with a novel object. The novel object was distinctive from the sample object (e.g. shape and colour). The arena and objects were cleaned with 70% ethanol between tests to avoid odour cues. Retrospective video analysis was conducted using ANY-maze™ software (Stoeltingco, Wood Dale, IL). The preference index for the novel object was determined by blinded observer utilising the following formulae; (time spent with the novel object / time spent with both familiar and novel objects) x 100. The ethological basis for this test is that rats with intact recognition memory will choose to preferentially explore the novel object.

7.2.4.2 Puzzle box

The puzzle box was utilised to measure short and long-term memory and executive function. The test was adapted from previous studies, with modifications made to the testing apparatus and obstacles to ensure suitability for a rat model [29,30]. The puzzle box was conducted on days 21-24 and 83-86. The test consisted of a rectangular acrylic white box (973 mm x 309 mm x 291 mm) divided into compartments; a large start zone exposed to bright overhead light (start box), and a small enclosed dark zone (goal box) connected by a removal barrier with a small door (5 cm x 5 cm) that allowed the animal to move between the compartments. A video camera (Panasonic Video Camera, HC-V180) was suspended above the puzzle box apparatus for each trial for retrospective analysis. Prior to puzzle box testing all animals were first habituated to the apparatus in two separate trials. The barrier was removed during the first trial (H1), and then contained an unobstructed open door during the second trial (H2) allowing the animal to freely explore the apparatus and learn to move from the starting zone to the goal zone. The testing phase was performed over three consecutive days, with three trials per day during which the door was obstructed, necessitating the animal to develop strategies to reach the goal box. Briefly, during the first trial on day one of the test, the barrier had an unobstructed open door (T1). During trial 2 (T2) and 3 (T3) the door was covered with clean corn cob bedding (Corncobology Pty Ltd, NSW, Australia), requiring the rats to burrow through to the goal zone (burrowing puzzle). On day two of testing, the first trial (T4) was identical to T2-T3 (burrowing puzzle). During trial 2 (T5) and 3 (T6) a soft rectangular plug consisting of a medium density foam obstructed the door, requiring the rats to remove the plug to enter the goal zone (soft plug puzzle). On the third day, the first trial (T7) was again the same as the last two trials from the previous day (T5-T6). The final two trials consisted of a hard-wooden plug positioned in the door to obstruct the door requiring rats to push or pull the plug (T8-T9) (hard plug puzzle). Each trial was considered completed once the rat reached the goal zone or once

5-minutes had elapsed. Between each trial the apparatus was cleaned with 70% ethanol. Latency to reach the goal box was recorded for each trial by a blinded observer using CowLog software (3.0.2). The arrangement of trials allows for assessment of executive functioning (problem-solving ability) (T1,T2,T5,T8), short-term memory (T3,T6,T9) and long-term memory functioning (T4, T7).

7.2.4.3 Barnes maze

The Barnes maze is used to assess spatial memory and learning [31] and was performed over five consecutive days 26-30 and 88-92 (Figure 1). The apparatus consisted of a large round platform (120 cm diameter) with eighteen holes (5 cm diameter) evenly positioned around the circumference of the maze. An escape box was located beneath one of the holes, whilst the remaining holes were false bottomed. All trials were performed in a dim room, with a bright light used to illuminate the surface of the maze to act as a mild negative stimulus to motivate the animal to find the escape box. During the training phase all animals underwent two trials per day with a 15-minute break, over a 4-day period. The animal was placed in the centre of the platform and free to explore the apparatus. If the rat located the escape box during the test timeframe, the light was turned off and it was subsequently returned to the home cage. The rat was gently guided to the escape box by the experimenter if it did not enter the box after 3 minutes had lapsed. The light was immediately turned off and the rat left for 20 seconds and then returned to the home cage. This was repeated over the subsequent training days. On day 5 (probe trial) the location of the escape box was rotated by 90º from the original position. The trial was conducted in the same manner as the training trial with a 30-minute break between probe trials. Location of the escape hole was randomised and counterbalanced across treatment groups and time-point. The escape box and maze were cleaned with 70% ethanol between each trial. All trials were video recorded and retrospectively analysed by a blinded observer. Latency

to find the escape box during training trials, latency to old and new location of the escape box, and number of revisits to the old escape box location during both probe trials was recorded.

7.2.4.4 Five-Choice Serial Reaction Time Task (5CSRTT)

The 5CSRTT is designed to evaluate several paradigms addressing executive function, including attention, inhibition and impulsivity in rodents [32]. The 5CSRTT was performed in the Bussey-Saksida Touchscreen operant chamber (Campden Instruments Ltd., England). Briefly, the touchscreen cognitive chambers (model 80604-20) consisted of four identical fully validated chambers fixed in a 2 x 2 grid. Each operant chamber consisted of a touchscreen monitor fixed to one side (15-inch touchscreen, screen resolution of 1024 x 768), a food dispenser fixed to the other side, house light and a tone generator attached to the ceiling of the apparatus. A Perspex 'mask' window (3 cm x 3 cm) was positioned in front of the touchscreen to allow rats to nose-poke responses on the touchscreen through the designated window locations without accidentally triggering unwanted areas of the touchscreen. Sugar pellets (Dustless Precision Pellet-Sugar Formulation 45mg, ASF0042, Able Scientific, Australia) that were utilised as a reward were dispensed when the animal selected the correct response during the 5CSRTT. The touchscreen operant system was linked to a computer that used the 'Whisker Server' software to simultaneously operate and control each chamber. ABETII software with pre-set programs was used to measure and record training sessions and probe trials during the 5CSRTT task in each chamber. The ABETII software automatically controlled outcome measurements, presentation of the stimulus, house light, tone generation and dispensing of the sugar pellets.

The 5CSRTT was performed over four phases included habituation, pre-training, training and probe trials (Figure 1). Each animal received one 5CSRTT session per day. Prior to the

commencement of chemotherapy and saline treatment rats were habituated to testing apparatus and reward system and underwent pre-training for 5 days. During habituation animals were placed in the operant chambers with 10 sugar pellets in the food dispenser for a 30-minute duration. During pre-training animals underwent 'initial touch' and 'must touch' training where they were trained to nose poke the correct touchscreen in response to the stimulus presented. Rats were required to complete 20 trials in 30 minutes to pass criteria for 'initial touch' and 20 trials in 30 minutes for two consecutive days to pass criteria for 'must touch'.

On completion of pre-training animals were moved to training phase, where they were taught to respond to the correct stimulus in decreasing stimulus duration (60, 30, 20, 10 and 5 seconds) over 13 days. During each session a sugar pellet was released, and the food dispenser switched on. Once the animal retrieved the pellet, the dispenser light switched off. Then a stimulus was presented within any one of the 5 windows. The animal was required to nose poke the correct window within the specified time. If the correct window was nose poked, a tone was generated, a sugar pellet was released and the food dispenser light lit. Each session was completed at 100 trials or when 30 minutes has elapsed. Criteria to pass each training session required rats to achieve at least 80% accuracy and less than 20% omissions. Animals that achieved training criteria early received retention training.

Upon completion of training, animals underwent five days of probe trials which did not have a specific pass criterion. During this phase the stimulus duration was reduced between each trial (4, 2.5, 1.5, 1, 0.5 seconds). The number of trials, accuracy, omissions, premature responses and correct response latency were analysed (Table 1).

Table 1. Definitions of 5CSRTT outcomes

5CSRTT Variables	Definition
Number of trials	One trial is defined a stimulus presenting in one of the five windows and the animal responding/not responding in the allocated stimulus duration
Accuracy	Number of correct responses divided by the sum of correct and incorrect responses, expressed as a percentage
Omissions	The total number of no responses to stimuli, divided by total trials, expressed as a percentage
Premature Responses	The total number of responses that occur before the light stimulus divided by the total number of trials
Correct Response Latency	The average time from onset of the light stimulus to correct nose poke

7.2.5 Enzyme-linked immunosorbent assay (ELISA)

Expression levels of pro-inflammatory tumour necrosis factor alpha (TNF-α) in prefrontal cortex tissue were detected using a commercial Rat TNF-α ELISA Kit (TNF-α cat. no. ab46070, Abcam, Cambridge, UK) performed in accordance with the manufacturer's instructions. Prior to running the ELISA, the concentration of protein was detected using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Victoria, Australia) with an average value taken of each tissue sample for analysis using a spectrophotometer (Victor X4 Multilabel Reader, Perkin Elmer, Singapore).

7.2.6 Western blot analysis

Glial fibrillary acidic protein (GFAP) marker expression was used to assess astrocytic reactivity marker expression changes in prefrontal cortex tissue samples. Each prefrontal cortex tissue sample was weighed, and 1-2 cm sections of the prefrontal cortex were extracted and

homogenised in standard RIPA buffer (Tris-base saline, pH 7.5-8; 50mM, 5M NaCl 150mM, 1% NP-40, 0.5% 10% sodium deoxycholate, 0.1% 10% SDS) with a pestle. Samples were sonicated in 10 second bursts followed by 1 minute on ice for a total of 30 seconds then centrifuged at 15,000rpm for 40 minutes at 4°C. The protein concentration was determined by a BCA protein assay (Pierce® BCA Protein Assay Kit; 23225, Thermo Fisher Scientific Inc., Victoria, Australia). Gel electrophoresis was performed using Bolt 4–12% Bis-Tris Plus gels (Life Technologies) with 25 μg of protein loaded per well. Gels were run at 150 V for approximately 1.5 h or until the dye reached the bottom of the gel. Gels were transferred to a polyvinylidene fluoride (PVDF) membrane using the iBlot 2 Dry Blotting System (Thermo Fisher Scientific Inc). Membranes were stained with Ponceau S red solution (Fluka Analytical) to allow for visual inspection and then washed three times using 1X tris-buffered saline with tween (TBST). Between each wash membranes were left to incubate on an agitator for 5-minutes. Membranes were then blocked in 5% skim milk solution (5g skim milk powder, 100mL TBST) for 2 h at room temperature. Following this, membranes were then washed three times and then incubated in 2% skim milk solution (1g skim milk powder, 50mL TBST) at 4°C overnight with primary antibodies (GFAP; #ab7260, Abcam, Cambridge, UK) and housekeeper protein (GAPDH; #ab8245 Abcam, Cambridge, UK) on a rotating device. The following day membranes were washed three times in TBST as previously described. Membranes were then incubated with secondary antibodies (LICOR 800CW donkey/anti-rabbit,1:10,000; LICOR 800CW donkey/anti-mouse, 1:10,000) in 2% skim milk solution for 2 hours at room temperature on a rotating device covered with aluminium foil. Western blot visualisation was conducted using the Odyssey Infrared Imaging System (model 9120; software version 3.0.21) (LI-COR, Inc.) and analysis was performed by a blinded observer using ImageJ software (Wayne Rashband, National Institutes of Health, USA).

7.2.7 Statistical analyses

Data were analysed using IBM SPSS (SPSS Inc., Chicago, IL, USA) and Megastat Excel Add-In (version 10.3 Release 3.1.6 Mac, McGraw-Hill Higher Education, New York, NY, USA) statistics software. Four animals were excluded from the study due to humane endpoint implementation, rendering the following sample size per treatment group 31-days post treatment; Saline (n = 11), MTX (n = 11), 5-FU (n = 11), 93-days post treatment; Saline (n = 12), MTX (n = 11), 5-FU (n = 12). Data were checked for normality using a Shapiro–Wilk test. Novel object, Western Blot (GFAP), and ELISA (TNFα) data were analysed using a one-way analysis of variance (ANOVA) with Tukey's *post-hoc* test. A two-way repeated measures ANOVA was performed to assess performance in the puzzle box with treatment as a between-subjects factor and obstruction type/trial as a within-subjects factor with a Bonferroni correction applied. The Barnes maze and 5CSRTT training data between groups at each time-point were analysed non-parametrically using a Kruskal-Wallis U-test with *post-hoc* Mann Whitney U-test for task acquisition, latency to old and new escape box on probe day, and the number of sessions required to pass criteria during training phase of the 5CSRTT. Within group differences across time were analysed using a Friedman test with *post-hoc* Wilcoxon signed-rank test. A two-way repeated measures ANOVA with treatment as a between-subjects factor and time as a within-subjects factor with a Bonferroni correction was performed for outcomes assessed on probe day of the 5CSRTT. Data are presented as mean ± SEM for parametric data and median with interquartile range for non-parametric data. A $p<0.05$ was regarded as statistically significant.

7.3 Results

Effect of MTX and 5-FU on object recognition memory in the NOR

Object recognition memory was assessed utilising the NOR. There was no significant effect of treatment on object recognition memory, as assessed by the preference index towards investigating the novel object during phase two, at either 31-days or 93-days post treatment ($F(2,29) = 0.412$, $p = 0.67$; $F(2,29) = 1.246$, $p = 0.30$, respectively) (Figure 2).

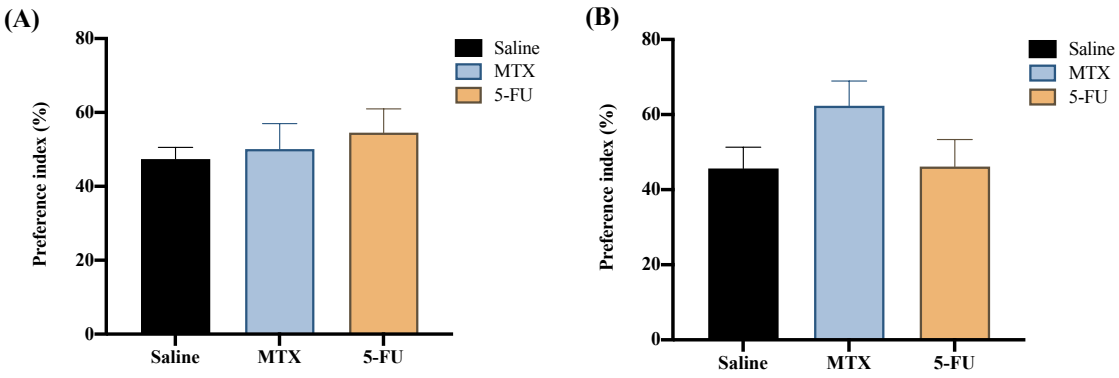

Figure 2. Preference index towards investigating the novel object in the novel-object recognition test 31-days and 93-days post treatment. Data presented as Mean ± SEM. 31-days post treatment; 31-days post treatment; Saline (n = 11), MTX (n = 11), 5-FU (n = 11), 93-days post treatment; Saline (n = 12), MTX (n = 11), 5-FU (n = 12).

Effect of MTX and 5-FU on performance in the puzzle box test

Executive function, short-term and long-term memory were assessed through the puzzle box test. A repeated-measures ANOVA showed a significant difference across trial time points for subacute time-point ($F(2,29) = 1.778$, $p = 0.035$). Within group analysis at each individual trial point for the subacute time-point demonstrated no significant differences at any of the trials

except trial 8 (T8). Animals treated with 5-FU and saline showed significantly reduced puzzle box performance compared to MTX treated animals in T8 ($p = 0.009$; $p = 0.039$, respectively). No differences were observed between treatment groups at any individual trial point for the chronic time-point ($F(2,29) = 1.473$, $p = 0.246$)

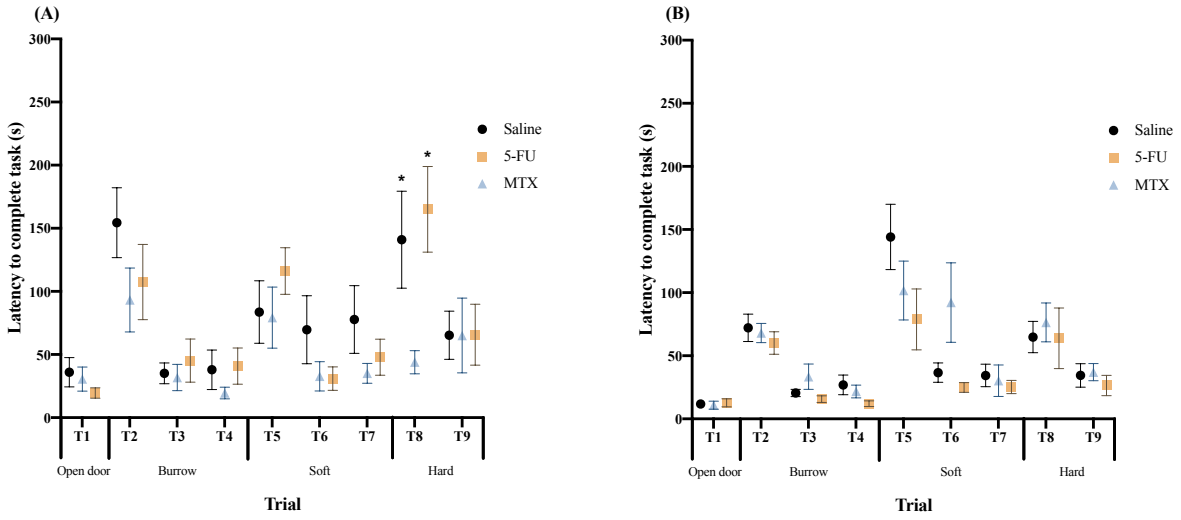

Figure 3. Puzzle Box task performance of female SD rats at 31-days (A) and 93-days (B) post treatment with either Saline, MTX and 5-FU. Data presented as Mean ± SEM. 5-Fluorouracil; 5-FU, MTX; Methotrexate. 31-days post treatment; Saline (n = 11), MTX (n = 11), 5-FU (n = 11), 93-days post treatment; Saline (n = 12), MTX (n = 11), 5-FU (n = 12).

Effects of MTX and 5-FU on memory and learning in the Barnes maze test

During the learning acquisition phase of the Barnes maze test animals demonstrated no significant difference between treatment groups in daily average escape latency for any individual days of trial acquisition for the subacute time-point ($p > 0.05$) (Figure 4A) and the chronic time-point ($p > 0.05$) (Figure 4B). However, a Friedman analysis determined significant difference across time for latency to escape box during task acquisition for both subacute time-point (χ^2 (3) = 52.313, $p < 0.001$) and the chronic time-point (χ^2 (3) = 56.863, p

< 0.001). Post-hoc Mann Whitney U-test determined a significant improvement in daily average escape latency from acquisition day 1 compared to day 2 for rats in all treatment groups at both the subacute and chronic time-point ($p < 0.05$).

During the probe trial at the subacute time-point, no significant differences were observed between treatment groups on latency to find the old escape box and new escape box for probe trial 1 ($H(2) = 1.085$, $p = 0.581$; $H(2) = 1.356$, $p = 0.508$, respectively). However, in trial 2 on probe day there was a significant effect of treatment on latency to find the location of the old box ($H(2) = 6.155$, $p = 0.046$). Animals treated with MTX or 5-FU had a significantly longer latency to find the old escape box location compared to saline control animals (MTX, $p = 0.050$; 5-FU, 0.026). Likewise, this also occurred for latency to find the location of the new escape box in trial 2 on probe day ($H(2) = 10.947$, $p = 0.004$). Compared to saline treated animals, animals treated with MTX and 5-FU took significantly longer to find the new escape box location (MTX, $p = 0.011$; 5-FU, $p = 0.004$). No significant treatment effects were demonstrated in latency to find the old and new escape box during both the probe trials at the chronic time-point ($p > 0.05$). There was no significant effect between treatment group and preservative errors made (number of revisits to old escape box) during probe trials at either subacute time-point ($H(2) = 1.221$, $p = 0.543$) or chronic time-point ($H(2) = 0.257$, $p = 0.879$) (data not graphically represented).

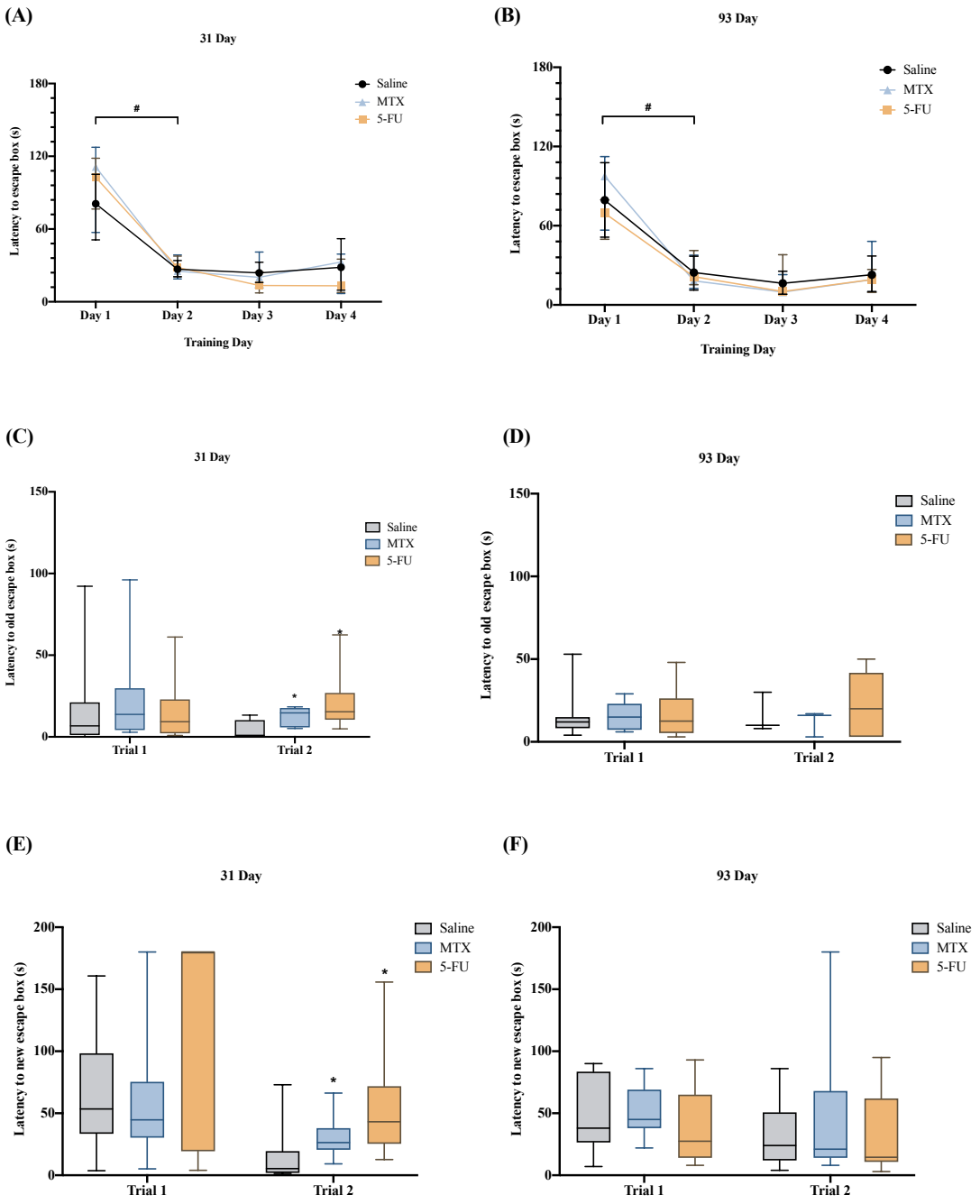

Figure 4. Latency to escape box on acquisition days 1-4 (training) at (A) 31-days and (B) 93-days post treatment. Latency to old escape box at (C) 31-days and (D) 93-days post treatment, latency to new escape box at (E) 31-days and (F) 93-days post treatment on probe day. Data presented as median with interquartile range. 31-days post treatment; Saline (n = 11), MTX (n = 11), 5-FU (n = 11), 93-days post treatment; Saline (n = 12), MTX (n = 11), 5-FU (n = 12). * $p < 0.05$ compared to saline within the same trial. # $p < 0.05$ statistical comparison within group across time.

Effect of MTX and 5-FU on performance in the 5CSRTT

Learning was assessed during the training phase of the 5CSRTT. A significant treatment difference was observed in the number of sessions required to pass training criteria at the subacute time-point ($H(4) = 136.127$, $p < 0.001$). Post-hoc Mann Whitney U-test determined that animals treated with the chemotherapy agent 5-FU required significantly greater number of sessions to reach 20 s stimulus duration ($p = 0.019$), 10 s stimulus duration ($p = 0.018$) and 5 s stimulus duration ($p = 0.020$) compared to saline control animals. 5-FU treated animals also took significantly longer to reach 10 s stimulus duration compared to MTX treated animals ($p = 0.041$). No treatment differences were noted at the chronic time-point for any of the training criteria assessed ($p > 0.05$).

During probe testing of 5CSRTT the number of trials, omissions, accuracy, correct response latency and premature responses were analysed (Table 1). No significant treatment differences between groups were found for the outcome measures omission percentage or accuracy percentage at either subacute or chronic time-point ($p > 0.05$) post treatment (Figure 6). No significant differences between treatment groups were noted for the number of trials completed for each probe test at either 31-days or at 93-days post treatment (data not graphically represented). Treatment of MTX or 5-FU did not affect the percentage of premature responses or the correct response latency at either time-point assessed ($p > 0.05$) (Figure 6).

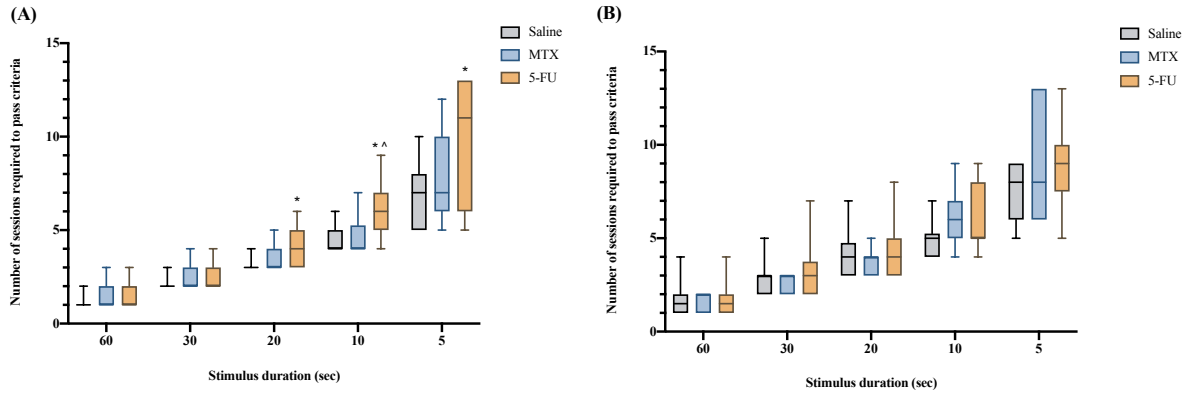

Figure 5. Number of sessions required to pass criteria during training phase of the 5CSRTT at (A) 31-days and (B) 93-days post treatment. Data represented as median with interquartile range. * $p<0.05$ compared with saline, ^ $p<0.05$ compared with 5-FU. 31-days post treatment; Saline (n = 11), MTX (n = 11), 5-FU (n = 11), 93-days post treatment; Saline (n = 12), MTX (n = 11), 5-FU (n = 12).

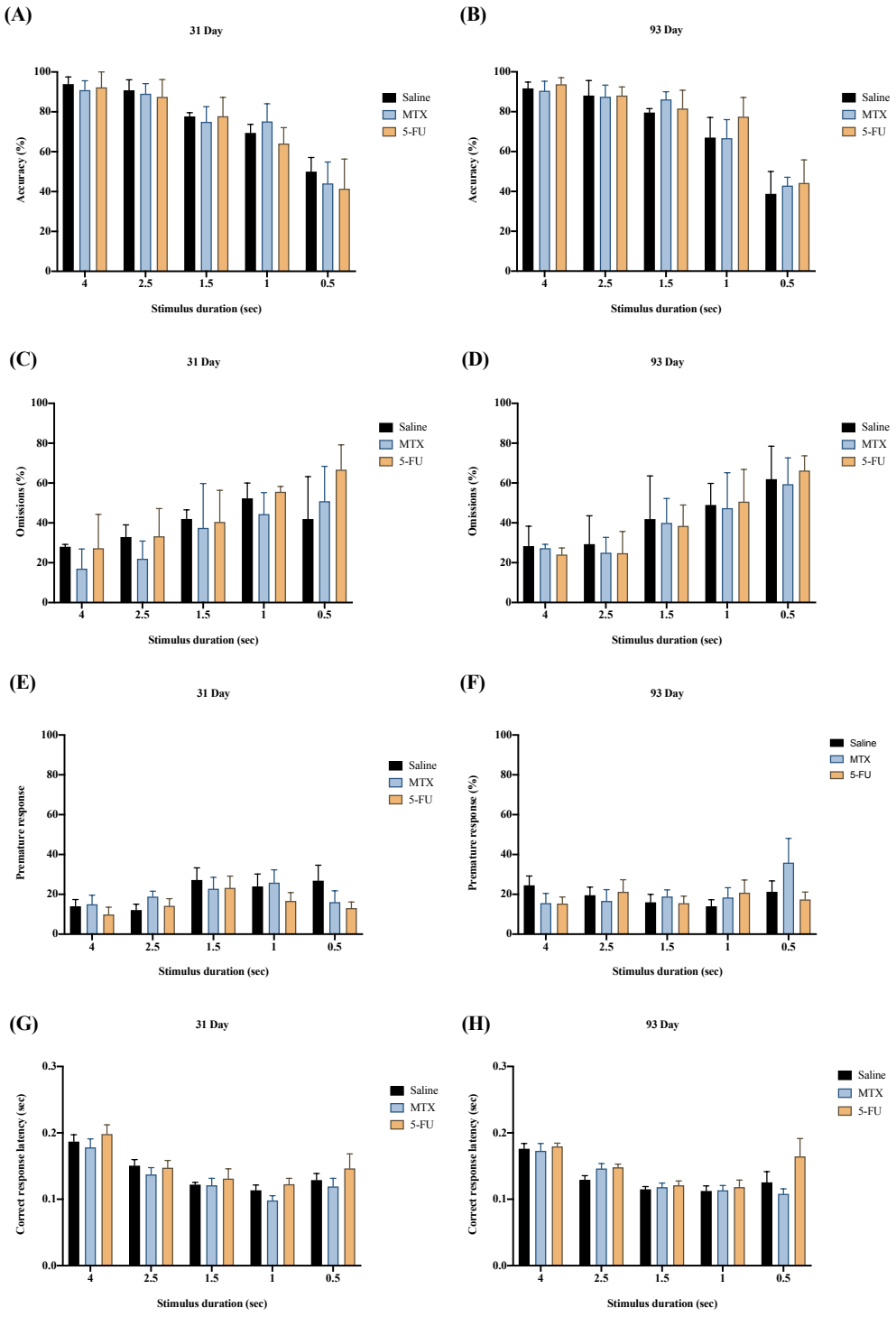

Figure 6. Effects of chemotherapy and saline on performance in the 5CSRTT assessed by accuracy (A-B), omissions (C-D), premature response (E-F) and correct response latency (G-H), at 31-days and 93-days post treatment. Data presented as mean ± SEM. 31-days post treatment; Saline (n = 11), MTX (n = 11), 5-FU (n = 11), 93-days post treatment; Saline (n = 12), MTX (n = 11), 5-FU (n = 12).

Effect of MTX and 5-FU on TNFα expression in the Prefrontal Cortex

The pro-inflammatory cytokine TNFα was analysed in the prefrontal cortex 31-days and 93-days post treatment. There was no significant effect of treatment on the expression of TNFα in the prefrontal cortex at either subacute ($F(2,15) = 0.3702$, $p = 0.697$) or chronic time-points ($F(2,15) = 0.8946$, $p = 0.4295$).

Effect of MTX and 5-FU on astrocytic reactivity marker expression changes in the prefrontal cortex

Glial fibrillary acidic protein (GFAP) marker expression was used to assess astrocytic reactivity marker expression changes in the prefrontal cortex at 31-days and 93-days post last chemotherapy and saline treatment (Figure 7). Western blot analysis demonstrated a significant treatment effect in the expression of GFAP at the 31-day time-point ($F(2,15 = 3.952$, $p = 0.04$). Post-hoc analysis determined that there was increased prefrontal cortex GFAP expression in the MTX group compared to saline ($p = 0.04$) (Figure 7A). No significant difference was observed in GFAP expression in 5-FU-treated rats compared to saline control rats ($p = 0.89$). No significant differences in GFAP expression in the prefrontal cortex was noted between treatment groups at the chronic time-point ($F(2,15) = 1.540$, $p = 0.247$) (Figure 7B).

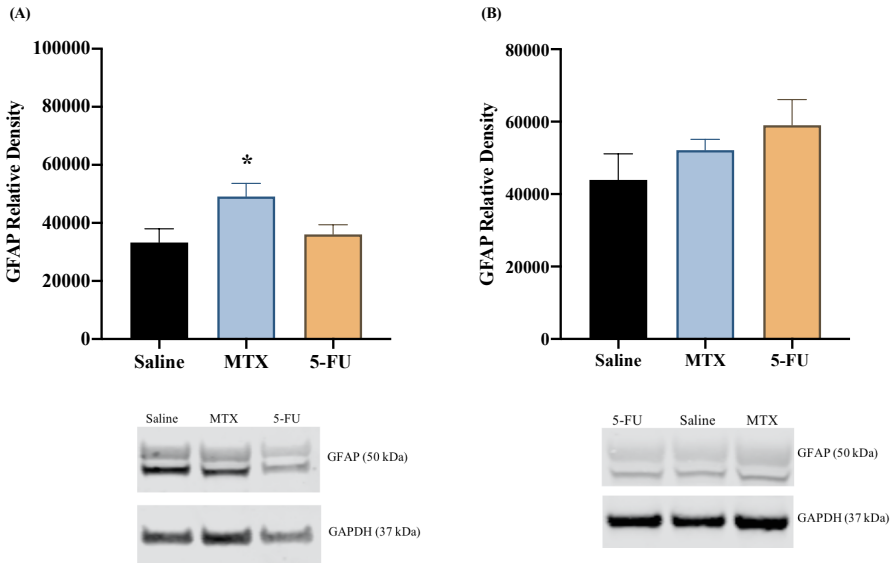

Figure 7. Western blot analysis of the expression of GFAP in the Prefrontal Cortex (PFC) in saline, 5-FU and MTX treated female SD rats at 31-days (A) and 93-days (B) post treatment. Data expressed as mean ± SEM. Representative n = 6 per treatment group. * $p<0.05$ compared with saline.

7.4 Discussion

CICI manifests as cognitive deficits presenting during or post-chemotherapy treatment, however symptoms often persist over years, negatively impacting patients' return to former social, personal and occupational functions. Preclinical animal studies have employed a wide range of methodologies to explore the effects of chemotherapy agents on a range of cognitive functions. Whilst this has provided valuable insight into CICI mechanisms, there remains no consensus on the timeline of the neuroinflammatory onset, progression and/or regression in the condition. In particular, few studies have examined events occurring at a chronic time-point. Subsequently, the current study aimed to elucidate the cognitive changes induced by MTX and 5-FU and its association with neuroimmune reactivity marker expression changes at a subacute and chronic time-point (defined as 31 and 93 days respectively). An additional aim was to

evaluate which cognitive domains and functions were impaired through use of a battery of behavioural paradigms that assess both hippocampal and prefrontal cortex functioning.

In brief, the results in the present study suggest that chemotherapy agents MTX and 5-FU predominantly impair cognitive function at the subacute time-point (31 days post treatment), with impairment ameliorated at the chronic time-point (93 days post treatment). Furthermore, the results imply that impairment is not limited to a single cognitive domain as demonstrated by impairment of memory and cognitive adaptability in the Barnes maze, and impairment of task acquisition in the 5CSRTT. In tandem with the behavioural results MTX caused astrocytic reactivity marker expression changes in the prefrontal cortex at the subacute time-point, but not at the chronic time-point.

Cognitive impairment occurs subacutely with later resolution.

A notable finding in the current study was that administration of MTX and 5-FU led to cognitive impairment at the subacute time-point, however no impairment was observed at the chronic time-point. Specifically, animals treated with MTX and 5-FU exhibited chemotherapy-induced cognitive impairments in the Barnes maze test and 5CSRTT at the subacute time-point. A number of studies have found similar effects on cognition within days, and up to five weeks following MTX and 5-FU administration [12,17,33,34]. Our findings that cognitive impairment was ameliorated may suggest transient effects or activation of a repair mechanism occurring at the selected chronic time-point (93 days).

Further in line with observations in this study, previous studies have reported impairment at the acute and subacute time-point, with no impairment at a chronic time-point post treatment cessation [22,23,35]. Seigers et al. 2015 demonstrated that animals treated with frequently used

cytotoxic agents docetaxel, doxorubicin, cyclophosphamide, 5-FU, topotecan and MTX, suffered short-term cognitive impairments after treatment but did not have any long-term detrimental effects [22]. Additionally, Borbélyová et al. 2020 reported cognitive disturbances immediately after treatment with multi-agent chemotherapy, which were not present at the chronic time-point (3 months) [23]. In contrast to our results, cognitive changes have been reported in the literature at chronic time-points following chemotherapy administration [19,24]. Both Fardell et al. 2010 and Gibson et al. 2019 reported continuation of cognitive impairment at 95 days and 8 months, and 6 months post chemotherapy exposure respectively [19,24]. It should be considered that inter-study comparison is difficult due to the paucity of animal studies that utilise chronic time-points, and heterogeneity between study designs. Gibson et al. 2019 assessed CB57BL/6J female and male mice bred with CD-1 with MTX (100mg/kg) and Fardell et al. 2010 assessed male Wistar rats with a single, high dose of MTX (250mg/kg) in contrast to our study that assessed female Sprague Dawley rats with (75mg/kg MTX, 37.5mg/kg 5-FU). Discrepancies in observed outcomes may be due to variations in gender due to physiological differences and inter-species (mice vs rat model) differences in drug metabolism and sensitivity [22,36,37]. Patient reports of cognitive deficits persisting long-term in the clinical setting in conjunction with the few animal studies that have found cognitive impairment at chronic time-points, suggest components of long-term cognitive impairment following chemotherapy can likely be expected. Currently, there is no standardisation regarding what is defined as ‘chronic’ within CICI. Future research is warranted to further assess a series of chronic time-points with standardised models to determine consistent patterns of cognitive impairment.

Multiple cognitive domains are impacted by chemotherapy administration

Impairments to episodic, verbal, visual memory and visual-spatial ability are regularly reported alongside impairments in facets of executive function such as working memory, attention, and impulsivity by patients during and after cessation of cancer treatment [2]. The methodology employed in the current study expressly investigated alterations in cognition through a battery of behavioural paradigms that assessed various aspects of learning and memory which can be attributed to different brain regions. Specifically, the Barnes maze test measures spatial learning and memory and is dependent on intact hippocampal functioning [31]. The NOR test assesses non-spatial memory and is dependent on the hippocampus and perirhinal cortex [28]. The Puzzle Box tests cognition in terms of aspects of executive function, short- and long-term memory, and is linked to the prefrontal cortex as well as the hippocampus [38]. Lastly, the 5CSRTT evaluates several facets of executive function which includes attention, inhibition and impulsivity and was utilised as a novel assessment of prefrontal cortex-based tasks [39,40].

The present study demonstrates that cognitive impairment was not limited to a single cognitive domain. In the Barnes maze test, rats treated with both chemotherapy agents had significantly longer latency to navigate the original escape box location and to find the new escape box location compared to healthy saline control animals. These findings imply impairment in spatial memory and cognitive adaptability, impaired ability to recognise contextual changes and adapt behaviours to suit this change and are consistent with previous studies utilising the Barnes maze test [22]. Previous animal studies in CICI have primarily assessed spatial memory utilising the Morris water maze, however demonstrated similar results to those herein, that animals treated with chemotherapy exhibit impairment in spatial memory consolidation, spatial recall and decreased hippocampal functioning as indicated by reduced duration and less entries to the target quadrant in the probe trial [41-44].

In the 5CSRTT cognitive impairment of task acquisition and attentional performance was observed. Animals treated with MTX and 5-FU required significantly longer sessions to pass training phase criterion. When attentional demands of the task were increased by decreasing the stimulus duration, animals treated with MTX and 5-FU demonstrated difficulty in attaining the pass criterion (<20% omissions and >80% accuracy), suggesting impairment in task acquisition and attentional performance. During the probe trials, no differences in omissions, accuracy, premature responses, and correct response latency were observed, signifying that impulsivity and attention were spared. Previous studies in CICI have demonstrated differing results in visual attentional processing with the 5CSRTT [45,46]. For example, Boyette-Davis and Fuchs 2009 reported normal attention and information processing speed in rats treated with paclitaxel [45]. In CICI, patients often report deficits in learning and task acquisition which was corroborated by our findings [47,48]. Clinical research has demonstrated that engaging in mentally stimulating tasks can improve symptoms of cognitive impairment, including executive function, through repetitive tasks with adaptive difficulty level [47]. As the 5CSRTT requires a time intensive repetitive training period with hierarchical difficulty, it is plausible that the animals may have improved or restored attentional performance through engagement in the 5CSRTT, which has been reflected in the probe trials. In contrast, Huo et al. found that cisplatin-treated mice displayed attention deficits [46]. In the preclinical literature cisplatin has been shown to cause impairment in spatial learning and recall, recognition memory and executive functioning, however executive function has not been readily studied in animal models of CICI [5]. Furthermore, the results should be interpreted in light of species and strain differences as patterns of impairment differ between species due to heterogeneity of behavioural models [5]. It is noteworthy to mention that these studies trained animals prior to chemotherapy administration. To our knowledge the current study is the first to have

administered treatment prior to completion of 5CSRTT training in order to measure task acquisition.

Challenges in using rodent behaviour tests in assessment of the subtle cognitive changes expected in CICI

In the clinical setting there is no gold-standard for subjective or objective assessment of chemotherapy-induced cognitive changes [1]. Likewise, in preclinical models a range of methodologies have been employed across studies, and a gold-standard CICI model is yet to be established. Furthermore, many established behavioural tests fail to measure subtle cognitive changes as would be expected in CICI. The puzzle box task was designed as a simple test to measure aspects of executive function, short- and long-term memory, and has an ethological basis on an animal's innate problem-solving ability. Puzzle box methodologies have been successfully applied in mouse models of schizophrenia, stroke and CICI [29,49-51]. In the current study, differences were only observed in the trial testing for executive function for the hard plug obstacle (T8) with 5-FU and saline-treated animals, showing reduced puzzle box performance compared to MTX-treated animals. Previous studies have shown that mice treated with cisplatin had significantly reduced performance in the puzzle box [49-51]. It is possible that the impairment observed in previous literature may be attributed to different classes of cytotoxic agents being used. For example, cisplatin is an alkylating agent and can target cells during any phase of the cell cycle, compared to both MTX and 5-FU which are antimetabolite agents and are cell-cycle specific class [52]. Thus, cisplatin may cause greater cytotoxicity, consequently exacerbating impairment in this assessment. However, this is most likely not the case and conclusions made from results in the current study should be interpreted with caution for the following reasons. In this study, several animals failed to complete trials in the designated time and the data did not follow the expected trend as obstacle difficulty

increased. The puzzle box has been validated in a mouse model however, to our knowledge, it has yet to be evaluated in a rat model of CICI. Therefore, the puzzle box methodology utilised may not translate to rats due to inherent behavioural differences, for instance in reward – motivation paradigms. Further validation is required in a rat model utilising prior preference testing to ensure the methodology and obstacles chosen are suitable.

In addition, cognitive impairment was not observed in the NOR at either time-point. Results from the NOR in CICI models are inconsistent in the literature. It is plausible that cognitive impairment may have been mild and the NOR was not sensitive enough to detect subtle cognitive changes. Furthermore, there is considerable debate over the role that the hippocampus plays in the NOR [24]. It has been found that hippocampal damage impairs MWM performance but spares the NOR, while perirhinal cortex damage results in impairment in the NOR but spares MWM performance [24]. This suggests that perirhinal cortex was spared in the current study. However, additional research is required to elucidate this.

Neuroinflammation occurs subacutely but is dependent on agent administered

In addition to the cognitive outcomes measured, astrocytic reactivity marker and TNF-α expression changes in the prefrontal cortex were also evaluated to explore possible MTX and 5-FU-induced damage to the CNS. Astrocyte reactivity marker expression changes were assessed using GFAP. Our results demonstrate an increase in GFAP expression in the prefrontal cortex in animals treated with MTX at the subacute time-point. No differences were observed in the 5-FU treated animals at the subacute time-point, or in either treatment group at the chronic time-point. The prefrontal cortex is important in facets of cognition such as working memory, executive function, attention, and concentration. In response to acute neurotoxic injury, astrocytic reactivity provides a protective cellular response. However, when astrocytic

reactivity is prolonged inflammation becomes excessive, resulting in impairments in neuronal regeneration, increases in neuronal death, and reduction in neuronal survival negatively impacting CNS integrity and function [9]. Therefore, an increase in GFAP expression may be connected with the cognitive impairment observed in the MTX-treated animals implying that damage has occurred in these brain regions.

Increases in GFAP were not observed in the 5-FU treatment despite the presence of cognitive impairment. In contrast to MTX, 5-FU can penetrate the BBB and has the potential to cause direct insult to the CNS [16,53]. It is plausible that the study may have missed the window of opportunity in identifying the peak time of astrocytic reactivity. Thus, molecular changes may have occurred prior to the selected time-point (31 days) and were resolved by the time of assessment. Cognitive impairment was still evident suggesting that impairment may persist after injury resolution. Additionally, astrocyte reactivity expression has been found to be dependent on the brain region assessed, proximity to injury and type of injury [54]. Therefore, there it is possible that 5-FU causes more damage to the hippocampus, in comparison to the PFC. Indeed, 5-FU alone and in combination has been associated with impairments in memory-based tasks in rodents [5,55]. However, our data infers that this is unlikely, and does not explain findings regarding impairment in executive function in animals treated with 5-FU. It is important to note that expression of neuroimmune markers are not well characterised in CICI and the definition between homeostatic expression and pathological effect requires defining within this context. Although GFAP is a commonly utilised marker of astrocytic reactivity and is useful to assess this in animal models of injury and disease, when assessed alone it is not sufficient to inform whether neuroprotective or neurodegenerative processes are occurring [56]. Markers of microglial activation such as Iba1, MHCII, and CD-68 would prove useful to aid in this differentiation. As such, future studies utilising measures of glial activation across a

trajectory of time-points would provide a more comprehensive picture between neuroimmune engagement and CICI and the significance of these findings.

Changes in memory, learning and executive function have been associated with increases in pro-inflammatory markers in the prefrontal cortex and hippocampus [25]. In the current study no differences in TNF-α expression were observed at either subacute or chronic time-points. Previous findings have reported an increase in TNF-α in the hippocampus, whole brain, and prefrontal cortex at an acute and subacute time-point [25]. Furthermore, in neurodegenerative conditions such as Alzheimer's disease TNF-α has been reported to be a driver of the pro-inflammatory cytokine cascade and plays an important role in chronic inflammation associated with cognitive impairment [57]. Thus, it was expected that an increase in TNF-α expression would be present in animals treated with MTX and 5-FU in this study due to impairment observed. As cognitive impairments were primarily observed in changes in facets of memory and task acquisition the analysis of additional cytokines that are strongly linked, such as IL-4, IL-6 and IL-1β, are necessitated in future research [58].

Conclusion

With the increasing survival rate of cancer, there is an imperative need to explore the effects, onset and trajectory of impairment in CICI with additional assessment at a chronic time-point. This study has identified that treatment with chemotherapy agents MTX and 5-FU impairs cognitive function at a subacute time-point with impairment not limited to a single cognitive domain. However, this impairment was ameliorated at the chronic time-point suggesting transient effects or activation of a repair mechanism. Future research is required to further elucidate the trajectory of severity and persistence of impairment occurring at a chronic time-point. Furthermore, this study has demonstrated that astrocyte reactivity marker expression

changes assessed using GFAP occur in tandem with cognitive impairments observed over the time course. These findings also demonstrate that a lack of consistency in outcomes may arise between behavioural tests and there needs to be greater consideration to validating tests which are sensitive to mild cognitive deficits. This has implications for future study design using animal models in CICI to improve translational validity and provide valuable insight into potential time course of CICI in tandem with potential cellular changes in the CNS. In future, consideration should be taken to move beyond the analysis of isolating principal factors of inflammation. Further investigation regarding additional cell types, cytokines, chemokines, and other mediators of inflammation such as reactive oxygen species is warranted, specifically across a time course.

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CHAPTER 8

General Discussion

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8.1 Research summary

Cancer survival rates have significantly improved due to advances in screening, diagnosis and treatment strategies. However debilitating side-effects, particularly CICI and CIGT, frequently accompany chemotherapy treatment and significantly impact patient quality of life. CICI affects various cognitive domains, causing executive function impairment and memory deficits that can impact daily activities. Likewise, the severity and distressing nature of symptoms associated with CIGT, combined with a lack of effective treatment strategies, results in CIGT being a major dose-limiting factor in chemotherapeutic regimens, as well as substantially affecting patient quality of life. In addition to the personal burden that these conditions bring, they also place additional strain on the healthcare system. It is therefore imperative to understand the aetiology of CIGT and CICI and develop therapeutic strategies to mitigate these impacts. This research aimed to determine the effects of chemotherapy agents 5-FU and MTX on neuroimmune outcomes, cognition, and affective behaviour, in a preclinical rodent model of CIGT and CICI.

Development of effective supportive care protocols requires understanding of CIGT and CICI pathogenesis. Animal models provide control over study design which assists in determining underlying molecular and cellular mechanisms of these conditions. To date, preclinical research that has utilised rodent models in CIGT studies have focused primarily on measurements of physiology or pathology, with less regard for measures of affective state or how the animal ‘feels’. This focus leads to reduced translational validity of these models to human patients. Considering the painful side effects of CIGT, a key therapeutic goal in the

clinical setting is to manage symptoms, limit pain, and reduce negative impacts on patient quality of life. To improve translational validity of preclinical animal studies, investigation of therapeutic agents must be integrated with reliable assessment of affective state. The initial two studies in this thesis focused on establishing a measure of affective state in a rodent model of CIGT (Chapter 2 and Chapter 3).

In Chapter 2, a cognitive bias test through a judgement bias paradigm was utilised as a novel approach to identify emotional states and establish an objective measure of cognitive performance in a rat model of CIGT. Results indicated that the presence of 5-FU-induced CIGT caused negative affective states, demonstrating a correlation between pathological damage and disease progression. Further validation of the test was also performed through the addition of an opioid palliative treatment, buprenorphine, which partially ameliorated the negative affective response. To our knowledge, this is the first study to investigate and validate a reliable judgement bias test to assess affective state in a disease model such as CIGT. These results support the notion that cognitive bias methodologies, such as the judgement bias test employed here, can be a valuable addition to CIGT investigations to help provide an understanding of the means by which emotion affects cognition in animal models, improve effectiveness of novel therapeutics and aid in the transitioning of animal models to clinic.

In order to assess pain, a method of assessment of spontaneous pain was employed; the Rat Grimace Scale (RGS) (Chapter 3). Furthermore, a standard general clinical scoring protocol and an established test of anxiety were also utilised to examine whether administration of clinically relevant opioid analgesic treatments ameliorated the pain response, hence validating the RGS as a pain measure in this model. No difference in pain scores were demonstrated between groups. These results indicate that the RGS lacked the sensitivity to successfully

discriminate pain in a tumour-bearing rat CIGT model. Pain-specific parameters for severity assessment in CIGT models are required, however measuring pain in rodent models is challenging (Turner et al., 2019). The RGS has been well researched and validated in numerous acute pain models (De Rantere et al., 2016; Leach et al., 2012; Whittaker et al., 2015). However, in gastrointestinal disease models the utility of the RGS continues to have varied results, especially in regard to CIGT models (Leung et al., 2019; Whittaker et al., 2016). Future investigations into the RGS are required to determine whether it might be a reliable tool in this particular model. This will increase translational validity, as well as improve animal welfare and humane endpoint implementation.

In recent years an emphasis has been placed on transparent reporting of animal research to improve reproducibility of research. The introduction of the ARRIVE (Animals in Research: Reporting in vivo Experiments) guidelines was developed to improve critical appraisal of experimental design, interpretation of results and animal welfare and ethical standards (Kilkenny et al., 2010). There is variability in experimental design and research outcomes in animal models of CICI, emphasising the importance of accurate and transparent reporting for the replication of results and translation of research findings. Therefore, the systematic review described in Chapter 4 aimed to assess compliance with the ARRIVE reporting guidelines in scientific publications evaluating CICI and determine whether the introduction of the ARRIVE guidelines has improved quality of reporting. No studies achieved full adherence with the ARRIVE guidelines, nor was there any improvement in quality of reporting post introduction of the guidelines. Importantly, these results provided valuable insight into the need for stricter adherence in reporting standards to improve rigour of experimental design and reproducibility of research outcomes, especially considering the heterogeneity of animal models employed in CICI research.

To date, the underlying mechanisms that contribute to CICI are relatively unclear, although direct and indirect neuroinflammatory mechanisms have been suggested as a key contributor to the development of CICI. In order to determine if there was a link between neuroinflammation and cognitive decline within the preclinical literature, a systematic scoping review was conducted to identify and map the available literature in preclinical rodent models of CICI on neuroimmune reactivity marker expression changes and resulting cognitive changes (Chapter 5). The findings illustrated that neuroimmune reactivity marker expression changes (cytokines, chemokines, microglia reactivity, and astrocyte reactivity) were common, and were linked with cognitive impairment commonly observed in CICI. These results suggest a close association between neuroimmune engagement and CICI in preclinical rodent models. However, the presence of a causative link between neuroimmune engagement and cognitive impairment needs to be ascertained in future research.

Currently there is little consensus on effective strategies to prevent or treat CIGT or CICI. Furthermore, due to the painful nature of CIGT, opioid agents are often employed as part of pain mitigation strategies. However, the effects of opioid agents and peripheral inflammatory events that occur during CIGT, and neuroimmune and cognitive changes have been understudied simultaneously. In addition, the onset and trajectory of observed cognitive impairment varies widely in patients suffering CICI. To date, many preclinical CICI studies have focused on the early time-course of CICI, with few studies including a chronic time-point and cognitive assessments that measure both memory and executive function. Consequently, the effects of 5-FU and opioid co-administration on gut inflammation and architectural changes in CIGT, along with neuroimmune and cognitive changes in an acute tumour-bearing rat model was assessed in Chapter 6, and neuroimmune reactivity marker expression changes and

resulting cognitive changes following 5-FU and MTX administration in a subacute and chronic time-course in a rat model of CICI was assessed in Chapter 7.

The findings demonstrated that a single high dose of 5-FU caused deleterious CIGT effects however did not have any direct effect on GFAP and TNFα expression in the hippocampus or cognitive changes at an acute time-point (72 h post chemotherapy). Furthermore, the opioid analgesics assessed did not modify CIGT or potentiate cognitive markers in this scenario (Chapter 6). It was postulated that the selected time-point was too early to observe subsequent cognitive changes occurring after peripheral inflammation. Therefore, future studies should address this by incorporating a comprehensive analysis of early markers for brain changes across a range of brain regions, and over the trajectory of early time-course, to ascertain subsequent central changes indictive of CICI occurring after CIGT. This did however provide a basis for the investigations in Chapter 7 which focused on later time-points in CICI pathogenesis.

Following chemotherapy treatment cognitive impairment and associated expression changes were observed predominantly at a subacute time-point, with impairment not limited to a singular cognitive domain, however this was ameliorated at the chronic time-point (Chapter 7). Importantly, this was achieved through the use of behavioural paradigms that assessed hippocampal and prefrontal cortex functioning. However, few previous studies have included an assessment of cognitive and molecular outcomes at a chronic time-point (Chapter 5), which precludes making firm conclusions in some respects regarding the results observed at the chronic time-point. Thus, further assessment at both an acute and chronic time-point coupled with changes in cognitive markers is important to continue to improve our understanding of CICI.

8.2 Recommendations and future directions

8.2.1. Animal models of CIGT and CICI

This thesis describes and validates a judgement bias task and 5CSRTT as promising behavioural assessment methods in preclinical models of CIGT and CICI. This has implications for future study design using animal models in CICI and CIGT to improve translational validity. It is pivotal for biomedical studies to find reliable assessment tools to evaluate various emotional and cognitive experiences to enhance animal model refinement and improve validity of novel therapeutic assessment.

Unfortunately, the RGS employed here was not efficacious in evaluating the pain response in a rat model of CIGT. To improve animal welfare and increase translational validity, there is a necessity to develop and validate assessment methods that accurately measure the emotional component of pain in animal models of CIGT. Whilst not necessarily specific to pain, two generalised measures of affective state that are of particular interest are measures of activities of daily living, such as burrowing and running wheel activity, and assessment of analgesic self-administration preference using the conditioned place preference test (Turner et al., 2019). Additionally, the use of automated methods for assessment of affective states via spontaneous behaviours would have considerable utility in animal models of CIGT.

The investigations in Chapter 6 employed a tumour-bearing rodent model, which was a valuable approach as it reflected the clinical scenario, however a limitation was that a healthy control without tumours was not utilised. There is increasing evidence that patients experience cancer-related cognitive impairment (Janelsins et al., 2014; Pendergrass et al., 2018). Hence there is a need to differentiate between chemotherapy-related and cancer-related cognitive

impairment, and also to consider the potential effects of cancer in animal models. This consideration should feed into future study design when tumour-bearing animals are used.

Currently there is no 'gold standard' preclinical animal model of CICI, with a lack of standardisation regarding study design (chemotherapeutic agents, treatment regimen, animal species and molecular and cognitive outcome measurements). As such it is imperative moving forward that we improve transparent reporting of experimental design and research outcomes, and develop standardised experimental practices to help reduce the heterogeneity between studies and improve replication of results and translation of research findings. This is especially appropriate given the recognition of the variability in animal models of this condition, and the development of the international cancer and cognition task force (ICCTF) to address this (Winocur et al., 2018). Moving forward, in the short term, journal editors and reviewers can assist in improving stricter adherence to the ARRIVE guidelines through encouraging the use of the ARRIVE checklist during journal submission. In the longer-term, education and referral to ARRIVE guidelines should be implemented during the ethics application process by Animal Ethics Committees.

Whilst other neurodegenerative research areas have used a variety of well-validated behavioural tests, they may not be appropriate to measure animal emotion or the subtle cognitive impairments that can occur with CICI. Furthermore, the findings within this thesis pave the way for further investigations of actions at the neuroinflammatory response as a potential therapeutic strategy in CICI. The findings summarised above warrant further exploration into more comprehensive assessment of neuroimmune engagement and behavioural measures at different time-points. This should focus on reliable behavioural tests assessing hippocampal and prefrontal cortex functioning, concurrently with molecular

outcomes across numerous chronic time-points to create a more complete picture in preclinical models of CICI. For example, assessment of spatial memory and executive functioning such as Morris water maze, Barnes maze and non-matching to sample and conditional associated learning tasks across an extended trajectory of time-points (between acute, subacute and chronic) with associated molecular analysis (glial cells, pro- and anti-inflammatory cytokines and chemokines).

8.2.2 Neuroimmune reactivity marker expression in CICI

Findings from this research identified neuroimmune reactivity marker changes occurred in tandem with cognitive impairments (Chapter 5 & Chapter 7). In order to explore damage to the CNS, TNF-α expression and astrocytic reactivity marker expression using GFAP was utilised. Following chemotherapy administration, an increase in GFAP expression was observed, however there was no difference in TNF-α expression in prefrontal cortex compared to saline control. Whilst astrocytes account for approximately one third of the cell population in the CNS and play an important role in the immune response (McLeary et al., 2019), a limitation of the current body of work was that additional mediators of inflammation were not explored. Therefore, future research should build on these research findings and investigate additional mediators of inflammation including nitric oxide synthase, ROS and oligodendrocytes, which play a key role in the inflammatory cascade and aid in neuronal death (McLeary et al., 2019). Additionally, microglial activation markers including Iba1, MHCII, and CD-68 would help elucidate these findings. As cognitive impairments were predominantly detected through differences in facets of memory and task acquisition in Chapter 7, it is necessary to further analyse cytokines that are related to these cognitive changes occurring, including IL-4, IL-6 and IL-1β.

Furthermore, in view of neuroimmune reactivity marker changes occurring in CICI, assessment on the role of inflammasome-mediated inflammatory pathways in the CNS would be highly beneficial. Inflammasome protein complexes are important due to involvement in activation of pro-inflammatory caspases, which cleave inflammatory cytokine precursors including IL-1β, IL-18, and IL-33 into active forms. Findings in previous literature have linked inflammasomes to the pathophysiology of neuroinflammation during neuronal ageing and related neurodegenerative diseases, resulting in cognitive impairment and memory loss (Singhal et al., 2014). Specifically, it has been shown that the NOD-like receptor pyrin (NLRP) inflammasome in microglia play a role in the development of numerous neurological diseases including Alzheimer's disease and Parkinson's disease (Haque et al., 2020; Singhal et al., 2014; Tan et al., 2013). The role of inflammasome-mediated inflammatory pathways is yet to be explored in CICI. Given the involvement in other neurodegenerative diseases, it is plausible that NLRP inflammasomes may play a role in neuroinflammation and subsequent cognitive changes occurring in CICI, thus warrants investigation. Exploring potential inhibitors of NLRP3 inflammasome would be valuable as a therapeutic target to prevent CICI, and thus improve quality of life for cancer survivors. As such, ongoing research from our laboratory group Animal Welfare and Cognitive Affective Neuroscience (AWCAN) is currently looking at the role of the NLRP3 inflammasome in the development of CICI over an acute and chronic time-course.

CHAPTER 9

Consolidated List of References

CHAPTER 9

Consolidated List of References

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APPENDICES

Assessment of Housing Density, Space Allocation and Social Hierarchy of Laboratory Rats on Behavioural Measures of Welfare

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Assessment of Housing Density, Space Allocation and Social Hierarchy of Laboratory Rats on Behavioural Measures of Welfare

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RESEARCH ARTICLE

Assessment of housing density, space allocation and social hierarchy of laboratory rats on behavioural measures of welfare

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Abstract

Minimum space allowances for laboratory rats are legislated based on weight and stocking rates, with the understanding that increased housing density encourages crowding stress. However, there is little evidence for these recommendations, especially when considering positive welfare outcomes. This study consisted of two experiments which investigated the effects of housing density (rats per cage), space allocation (surface area per rat) and social rank (dominance hierarchy) on the ability to perform simple behavioural tests. Male Sprague Dawley (SD) rats (n = 64) were allocated to either high-density (n = 8) or low-density (n = 8) cages. The second experiment investigated the effects of surface area. SD rats (n = 40) were housed in dyads in either the large (n = 10) or small (n = 10) cage. In both experiments, animals were tested on a judgment bias paradigm, with their responses to an ambiguous stimulus being ascribed as optimistic or pessimistic. Animals were also tested on open-field, novel-object recognition and social-interaction tests. Recordings were taken from 1700-2100h daily for rat observation and social rank establishment. Dominant animals responded with significantly more optimistic decisions compared to subordinates for both the housing density ($p<0.001$) and space allocation ($p = 0.0015$) experiment. Dominant animals responded with increased social affiliative behaviours in the social-interaction test, and spent more time in the centre of the open-field test for both experiments. No significance was detected between housing density or space allocation treatments. These findings suggest that social rank is a significantly greater modifier of affective state than either housing density or space allocation. This finding has not yet been reported and suggests that future drafts of housing guidelines should consider animal social status in addition to floor space requirements.

Introduction

International standards for the care and housing of Lab Animals provide relatively uniform guidelines regarding stocking rates and surface area allowance afforded to all rodents used for scientific purposes. The *Guide for the Care and Use of Lab Animals* [1] (United States) and the

EC Directive 2010/63/EU [2] (Europe) are among a few of the regulatory bodies that provide guidelines for rodent housing. These guidelines usually state specific measurements regarding the weight of the animal and the floor area allocated per animal to reduce the effects of crowding-related stressors. However, it has been recently noted that these guidelines rarely cite scientific literature to support these space requirements [3]. It has also been noted in the *Resolution on Accommodation and Care of Lab Animals* [4] that evidence-based data is lacking on this specific subject. While the scientific community have identified this knowledge gap, recent literature has focussed primarily on mice [5]. Few publications have identified the need to establish the effects of housing density and space allocation in rats (*Rattus norvegicus)*.

Investigations of space use by rats have been historically well-researched, however the primary focus was conducted in wild rats [6, 7]. Cage floor area and space allocation provide logistical limitations to the types of research activities that can occur [5] which perhaps prompted early research methods to study the effects that crowding can elicit [8]. It was first noted by Calhoun [8] that when a population of laboratory rats was allowed to increase in a confined space, abnormal behavioural patterns began to occur. It was argued that these behaviours could lead to the extinction of the entire caged population. This crowding effect has led to the prominence of guidelines and legislative documentation that encourage strict space allowances for each caged animal. However, this may not be an accurate portrayal of the multitude of factors that interact. Housing density is defined hereafter as the number of animals that occupy the same caged floor area while space allocation is defined as the surface area allocated to each animal within a shared cage. Housing density and space allocation are two separate, yet closely linked factors that interact to produce 'crowding'. Crowding is the operational word used by these regulatory bodies that defines the motivational state that occurs when spatial and social factors interact [9]. Many studies that have previously discussed the effects of housing density or space allocation on rodent behaviour have been confounded by their inability to successfully separate these two factors [10, 11]. Studies in which cage size is kept constant and animals are added or subtracted are not designed to investigate either housing density or space allocation, instead they report the effects of crowding.

We sought to assess the effects of both housing density and space allocation on the performance of rats in an array of simple behavioural tests. We also aimed to determine if the social class (as determined through a dominance hierarchy) of the animals would interact with these factors, as this interaction had been significantly underreported in the literature, despite the knowledge of social composition contributing to crowding stress [9]. The behavioural tests utilised included the open-field test (OFT), the novel-object recognition test (NORT), the social-interaction test (SIT) and cognitive bias detection through a judgment bias paradigm (JBP). These tests were chosen due to their repeatability and their reliability at providing evidence of anxiety-like behaviours. The JBP has the added advantage of being able to identify positive welfare outcomes.

Materials and methods

Animals and housing

This study was separated into two distinct experiments. The first studied the effects of housing density and used 64 male Hsd: Sprague Dawley (SD) rats. The second study studied the effects of space allocation and used 40 male SD rats. All animals were sourced from a barrier-maintained, specific pathogen free production facility (University of Adelaide, Laboratory Animal Services, Adelaide, Australia). Upon arrival at the testing facility, animals were housed in their treatment groups (discussed later) in commercially available cages (Tecniplast, Exton, PA, USA). Cage design and specifications are discussed below. Each cage was provided with a

paper-based bedding substrate (Animal Bedding, Fibrecycle Pty Ltd, Yatala, Queensland, Australia). Standard rat chow (Rat and Mouse Cubes, Speciality Feeds, Western Australia, Australia) and reverse-osmosis water were provided *ad libitum*. Room temperature was maintained between 21˚C and 23˚C and a reversed 12-hour light/dark cycle (lights on at 1800h, off at 0600h) was used. The animals were acclimatized to the facility environment for 5 days before behavioural training on the judgment bias paradigm commenced. All animal use and housing protocols were approved by the Animal Ethics Committee of the University of Adelaide and conducted in accordance with the provisions of the *Australian Code for the Care and Use of Animals for Scientific Purposes [12]*.

Housing density experiment

Upon arrival at the testing facility, rats (n = 64) were housed in groups of either 6 (high-density), or 2 (low-density) rats per cage. The high-density cages (n = 8, total of 48 rats) were the Eurostandard type IV (Tecniplast, Exton, PA, USA) with dimensions of 59.8cm by 38cm with 26cm of vertical space at the lowest point (Fig 1A). These cages had an area of 2,280cm^2 and were appropriate to house 6 rats of approximately 450 grams, per the guidelines as prescribed in the eighth edition of the *Guide for the Care and Use of Lab Animals* [1]. Low-density cages (n = 8, total of 16 rats) were the Eurostandard type IIL (Tecniplast, Exton, PA, USA) with dimensions of 36.5cm by 20.7cm with 22cm of vertical space at the lowest point (Fig 1B). These cages had an area of 755.55cm^2 and were appropriate to house 2 rats up to 450 grams.

Space allocation experiment

The second experiment used 40 male SD rats and occurred immediately following the completion of all behavioural testing of the housing density experiment. Animals were randomly housed in either the large cage (Eurostandard type IV) or the small cage (Eurostandard type IIL) with one other conspecific and allowed to acclimatize to these conditions for 3 weeks. Animals housed in the large cages were the large surface area treatment group (n = 10, total of 20 rats) and those housed in the small cages were the small surface area treatment group (n = 10, total of 20 rats).

Cognitive bias test

Cognitive bias detection has been used as an indicator of animal affective state (emotional state) [13]. These biases were measured using a judgment bias paradigm (JBP), that was based on an earlier JBP design [14]. Commonly used terminology of the JBP has been defined in

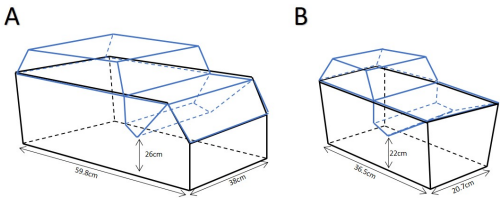

Fig 1. Diagram of cage sizes used. A) The Eurostandard type IV cage drawn to approximate 1:10 scale. This cage is appropriate to house up to 6 rats of approximately 450 grams. B) The Eurostandard type IIL cage drawn to approximate1:10 scale. This cage is appropriate to house up to 2 rats of approximately 450 grams. The black lines represent the cage bottom while the blue lines represent the cage lid.

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Table 1. This JBP makes use of a single testing/training apparatus that comprises two Perspex boxes (610mm x 435mm x 500mm) connected via a PVC pipe with a 100mm diameter. During certain training phases, the pipe was lined with one of two-grades of sandpaper, one being coarse (P80) the other being a fine sandpaper (P1200). Inside one box (henceforth referred as the 'goal box') were two reward bowls positioned in the two far corners. These reward bowls were filled with either a coriander or cinnamon scented sand (1% by weight of spice to sifted sand). The coriander scented reward bowl remained in the right-hand corner for each trial, while the cinnamon scented reward bowl remained in the left-hand corner (Fig 2). Milk chocolate baking chips (Cadbury, London, England) were used as the high-positive reward items whilst Cheerios (Uncle Toby's, Victoria, Australia) were considered a low-positive reward items. Every animal was randomly assigned a sandpaper association to the reward items and rewarded location for both the housing density experiment (Table 2) and the space allocation experiment (Table 3). This paradigm has been divided into phases where different experimental outcomes are expected. A summary of these phases is included in Table 4. The animals would learn to associate the different type of sandpaper with the type of reward item and where that reward item was located. During the testing phase, the sandpaper in the PVC pipe was replaced with sandpaper of an intermediate grade (P180) and no reward items were present in the reward bowls. This sandpaper type behaved as the intermediate, ambiguous probe and the responses to this probe could be considered either optimistic or pessimistic. An optimistic decision was defined when the rat displayed foraging behaviours for these intermediate ambiguous trials in the bowl that would normally contain the chocolate reward. A pessimistic behaviour was defined when the rat displayed foraging behaviour in the bowl that would normally contain the cheerio reward [15]. Testing in the JBP occurred once a day, for 5 days (Table 4).

Open-field test

On day 2 of the five-day testing period, each animal was subjected to the open-field test (OFT) after recording a cognitive bias decision for that day. Testing was performed as described by the methods of Wallace [16] and utilised a square testing arena (100cm by 100cm by 100cm) made from black corflute in a homogenously illuminated arena (150 lux) away from where the animals were normally housed. Animals were placed individually into the centre of the arena upon which a video camera (Logitech HD Webcam C525, Lausanne, Switzerland) suspended

Table 1. Definitions of commonly used terminology for the judgment bias paradigm.

Term	Definition
Approach	When the rat actively and intentionally placed its forelimbs and face into a reward bowl to extract the reward.
Forage	When the rat continuously and deliberately displaced the sand in the food bowl to obtain the reward.
Consumption	When the rat actively and intentionally interacted with the food by bringing it to its mouth.
Success	Successful trial was determined after the animal had approached and foraged in the correct (reward containing) food bowl before approaching or foraging in the incorrect food bowl.
Promotion	Animals were promoted to the succeeding trial (where appropriate) after achieving successful trails per day, for 5 consecutive days.
Failure	If the rat failed to consume the reward within 10 minutes of being placed into the testing chamber.

First described by Barker et al. (15)

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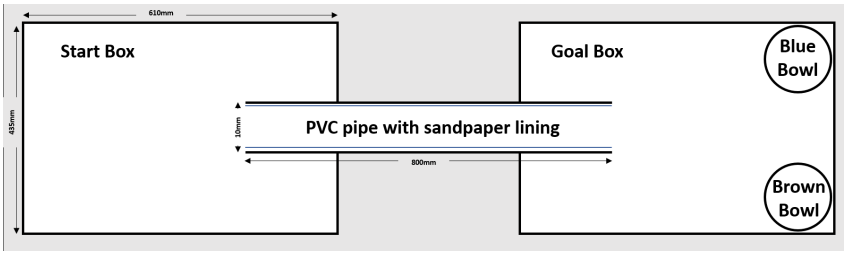

Fig 2. Diagram and size of judgment bias testing apparatus utilized. The judgment bias test was comprised of two Perspex boxes connected via a PVC pipe. Two reward bowls were placed in either corner of the goal box containing scented sand.

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over the centre of the arena began recording. The experimenter then immediately left the room and the animals stayed in the arena for 10 minutes. After 10 minutes the animal was removed from the arena and placed back into its home cage. The arena was then cleaned with a 70% ethanol solution. OFT video analysis was conducted manually utilising the CowLog open source software. Three zones were superimposed to the open field test video files (Fig 3) and the time spent in each zone was recorded, as was the number of transitions into each zone. Observers also recorded the time spent inactive during the test, time spent rearing, and number of defecation or urination incidents. A rat was considered to have entered a 'zone' when its centre of gravity had crossed into the new zone.

Novel-object recognition test

On the third day of testing, after recording a cognitive bias result, each animal was subjected to the novel-object recognition test (NORT). Testing was performed according to the methods presented by Bevins and Besheer [17], the arena utilised was a barren (no bedding), 'high-density' home cage, with accompanying wire-lid. Testing involved two behavioural phases, during phase 1 (Fig 4A) an individual animal was placed into this testing arena with two identical objects (stainless steel, water bottle tops) in either corner. The experimenter than left the room after starting the video recording from the suspended camera. After 10 minutes, the animal was removed from the arena and placed back into the home cage and the arena was cleaned with a 70% ethanol solution. After one hour, the animal was ready to be tested again in the

Table 2. Associations of reward items and locations for treatments of the housing density experiment.

	Cage Density	Chocolate Stimulus	Chocolate Location	Cheerio Stimulus	Cheerio Location
Association 1 (n = 12)	High	Coarse Sandpaper	Brown Bowl / Right	Fine Sandpaper	Blue Bowl / Left
Association 2 (n = 12)	High	Coarse Sandpaper	Blue Bowl / Left	Fine Sandpaper	Brown Bowl / Right
Association 3 (n = 12)	High	Fine Sandpaper	Brown Bowl / Right	Coarse Sandpaper	Blue Bowl / Left
Association 4 (n = 12)	High	Fine Sandpaper	Blue Bowl / Left	Coarse Sandpaper	Brown Bowl / Right
Association 5 (n = 4)	Low	Coarse Sandpaper	Brown Bowl / Right	Fine Sandpaper	Blue Bowl / Left
Association 6 (n = 4)	Low	Coarse Sandpaper	Blue Bowl / Left	Fine Sandpaper	Brown Bowl / Right
Association 7 (n = 4)	Low	Fine Sandpaper	Brown Bowl / Right	Coarse Sandpaper	Blue Bowl / Left
Association 8 (n = 4)	Low	Fine Sandpaper	Blue Bowl / Left	Coarse Sandpaper	Brown Bowl / Right

Each association was randomly assigned and counter-balanced between treatments.

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Table 3. Associations of reward items and locations for treatments of the space allocation experiment.

	Cage Size	Chocolate Stimulus	Chocolate Location	Cheerio Stimulus	Cheerio Location
Association 1 (n = 5)	Large	Coarse Sandpaper	Brown Bowl / Right	Fine Sandpaper	Blue Bowl / Left
Association 2 (n = 5)	Large	Coarse Sandpaper	Blue Bowl / Left	Fine Sandpaper	Brown Bowl / Right
Association 3 (n = 5)	Large	Fine Sandpaper	Brown Bowl / Right	Coarse Sandpaper	Blue Bowl / Left
Association 4 (n = 5)	Large	Fine Sandpaper	Blue Bowl / Left	Coarse Sandpaper	Brown Bowl / Right
Association 5 (n = 5)	Small	Coarse Sandpaper	Brown Bowl / Right	Fine Sandpaper	Blue Bowl / Left
Association 6 (n = 5)	Small	Coarse Sandpaper	Blue Bowl / Left	Fine Sandpaper	Brown Bowl / Right
Association 7 (n = 5)	Small	Fine Sandpaper	Brown Bowl / Right	Coarse Sandpaper	Blue Bowl / Left
Association 8 (n = 5)	Small	Fine Sandpaper	Blue Bowl / Left	Coarse Sandpaper	Brown Bowl / Right

Each association was randomly assigned and counter-balanced between treatments.

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same arena, however during phase 2 (Fig 4B) one of the two familiar items from phase 1, was randomly replaced with a novel item (red, large die), of approximate equal mass to the familiar item. After the animal was placed into the testing arena for phase 2, the video recording was again started and the experimenter left the room for 5 minutes. After 5 minutes, the animal was placed back into the home cage and the arena was cleaned with a 70% ethanol solution. Video footage from phase 2 was used to analyse the NORT data. Using the CowLog open source software, the observer documented the time each rat spent interacting with both the familiar and the novel object, as well as the number of interactions that occurred for each object. Other measures taken include the time the animal spent exploring the confines of the arena, time spent rearing and inactive, and the number of defecation and urination incidents.

Social-interaction test

On day 4 of the five-day testing period, each animal was subjected to the social-interaction test (SIT) after they had recorded a cognitive bias decision for that day. Testing was slightly modified from the methods of Sams-Dodd [18], and was performed in a circular arena with a diameter of 100cm made from hessian supported by flexible plastic sheeting. The arena was in a homogenously illuminated (150 lux) area in a different room to where the animals were normally housed. Test animals was marked with a nontoxic black marker immediately prior to being placed in the arena. The test animal was placed simultaneously into the arena with an unfamiliar SD rat (non-cage mate) that was not used as part of this study. This animal will henceforth be referred as the unfamiliar rat. Both test and unfamiliar rats were placed approximately 40cm apart in the arena. The video camera suspended over the arena began recording and the experimenter then left the room. Testing lasted for 10 minutes after which time the experimenter re-entered the room and returned each animal to its appropriate home cage before cleaning the arena with a 70% ethanol solution. Video analysis was performed manually with the CowLog open source software, animal behaviour was scored using a continuous sampling method. Behavioural expression was categorised using the ethogram as prescribed in Sams-Dodd [18] (Table 5). The time spent exhibiting each behaviour was totalled and a time-budget was generated. Each behavioural expression is presented as a percentage of the total time exhibited from the time-budget.

Social classification

Classification of the rats into their social classes was achieved through observing video footage recorded with CCTV cameras (OzSpy, Brisbane, Australia). Rats were recorded from 1500-

Table 4. Descriptions and promotion criteria of each training and testing phase involved in the JBP.

Phase	Description	Promotion Condition
A	Rats handled for two 10-minute periods. The first period between 0900 and 1200 hours, the second period between 1400 and 1700 hours.	Phase lasted for 5 days.
B	Rats placed into the testing apparatus, four times a day for 5-minute intervals. The food bowls contained the reward items appropriate to the individual rat, these rewards were placed on the surface of the sand in the reward bowls. No sandpaper was present within the PCV pipe.	Phase lasted for 5 days.
C	The testing apparatus now contained the appropriate sandpaper stimuli. Animals had two training trials between 0900-1200h and two between 1400–1700 hours. Each period had one chocolate trial and one cheerio trial that occurred in a random order. For each trial, a single reward item was placed on the surface of the appropriate reward bowl, to the appropriate sandpaper that was present in the apparatus. Rats were placed in the start box, upon which a timer was started. Latency for the rat to leave the start box, enter the goal box, approach any reward bowl, approach the correct reward bowl and start to consume the reward was recorded. The rat was immediately removed from the apparatus once it had consumed the reward or if it failed the test. The whole apparatus was then cleaned with 70% ethanol solution.	Promotion to phase D was achieved after the animals achieved success on 3 of their 4 daily trials for once a day, for 5 days in a row.
D	Identical to phase C, however during phase D the reward items were buried in the sand of the reward bowls. Each rat was required to forage for the reward item and extract it from the sand. Following the successful extraction of the reward, the depth at which the reward was buried for the next trial increased. Burial depth continued to increase with each successive trial until the reward was always completely buried in the sand.	Promotion to phase E was identical to the promotion conditions of phase C
E	Identical to phase D, but the reward items were always completely buried in the sand and one randomly selected trial per day contained no reward item. A successful, unrewarded trial was defined when the first bowl that the rat foraged in would normally contain a reward item.	Promotion to phase E was identical to the promotion conditions of phase C
F	Identical to phase E, except the unrewarded trial was now paired with a sandpaper of intermediate grade (P180).	Phase lasted for 3 days
G	Testing Phase. The rats received one test per day that involved the intermediate sandpaper (P180) being present in the pipe and no reward being present in the food bowl. During testing, the time was recorded for the rat to forage in any bowl, and the bowl the rat approached, and foraged in first was documented.	Phase lasted for 5 days

Adapted from Brydges *et al.* (14)

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2300h each day over the five day testing period. Each animal was observed for 10 minutes with the start time of each sample being randomly selected between 1500-1750h. Behaviours of each

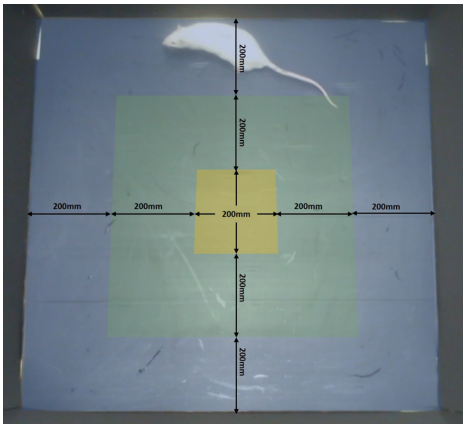

Fig 3. Diagram of the open-field test. Image of the video recording of the open-field test detailing the location and size of the zones used. Blue: Peripheral Zone, Green: Inner Zone, Yellow: Centre Zone. Arrows have been superimposed to indicate size.

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animal were scored per the ethogram as originally devised by Hurst et al. (1996) (Table 6.). Interactions between each individual with respect to each of its caged conspecifics were examined to determine the dominance relationship. Total numbers of aggressive encounters initiated were summed and compared against the number of aggressive encounters received, to assign the animal an agonistic score. As per the experimental design of Hurst et al. [19], dominance within a dyad was assigned if the animal initiated greater numbers of aggressive encounters than it received over each of the five 10-minute videos. Social class of rats for the housing density experiment is included in Table 7, and social class of rats for the space allocation experiment is included in Table 8.

Statistical analysis

All data were analysed using the IBM SPSS Statistics 22 (IBM, NY, USA) software package. Levene's test was used in all cases to test for normality of the data set, all data were found to be

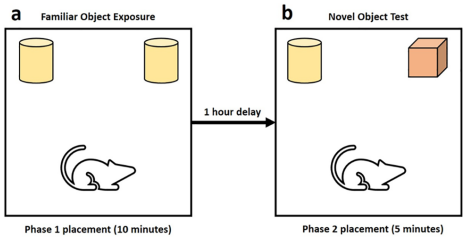

Fig 4. Novel object recognition test design. (A) Phase 1: Familiar object exposure. (B) Phase 2, Novel-object recognition test. From the methods of Bevins and Besheer [17]

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Table 5. Ethogram used for behavioural analysis during the social interaction test.

Behaviour	Description
Exploration	Movement in the arena, sniffing at floor, walls and inspecting arena.
Rearing	Raised on the hind legs, sniffing into the air.
Investigation	Sniffs at and investigates the unfamiliar rat.
Follow	Follows the unfamiliar rat.
Grooming	Cleaning the fur and/or scratching.
Inactive	No discernible action.
Stationary Stereotyped Behaviour	Stationary and performs circular head movements and/or head weaving.
Lateral Threat	Body is arched in a sideward posture towards the unfamiliar rat.
Upright	Standing on its hind legs and is facing the unfamiliar rat.
Stand Over	Standing on top of the unfamiliar rat.
Lie Under	Lying on its back beneath the unfamiliar rat.
Clinch	Active fighting with the unfamiliar rat.
Pursue	Runs after the unfamiliar rat during "Clinch"
Escape	Runs away from the unfamiliar rat during "Clinch"

Adapted from Sams-Dodd (18)

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parametric unless otherwise stated. Numerical data have been presented as mean ± standard error of the mean. Differences between means were considered significant when p was less than 0.05. All normally distributed data was analysed using a two-way multivariate analysis of variance (MANOVA) test, fitting housing density and social class on the test variable. Where the data were not normally distributed, they were analysed using the Kruskal-Wallis H test and/or the Mann-Whitney U test. Further details of statistical analysis are found in the results section.

A complete flowchart of the experimental procedure is illustrated in Fig 5.

Table 6. Ethogram used for behavioural analysis during social classification.

Behavioural category	Behavioural elements of the viewed rat
Sleeping	Lying or sitting unalert, eyes closed
Feeding/drinking	Eating food or faeces; drinking
Non-intake maintenance	Grooming; yawning; stretching; sneezing; urinating; defecating
Exploration	Sniffing air, floor, wall, water bottle, faeces, urine or bedding
Stationary	Alert (eyes open) but no directed attention while lying, sitting or leaning
Movement	Alert but no directed attention while walking, stretching, climbing or running
Other non-social behaviour	Chewing bedding; digging/scrabbling; jumping
Aggressive action	Bite; chase; aggressive over (pinning rat on its back); aggressive groom; aggressive sideways; upright; mounting; pull tail, pursuit of fleeing rat
Defensive action	Defensive over (on back, being pinned), defensive sideways, flight (with and without pursuit)
Social investigation	Sniffing nose, mouth, head, shoulders, back, flank, anogenital area, belly, tail
Other social behaviour	Attend; allogroom

Originally designed by Hurst *et al.* (19) This ethogram was used to assess social hierarchy within cages and to assign dominance to animals within dyads.

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Table 7. Social classes of caged rats for the housing density experiment.

Social Class	Definition	No.
(D) Dominant	Dominant over all cage mates–dominant in every dyad	n = 16
(DS) Dominant subdominant	Mostly dominant–dominant in most dyads but not all	n = 14
(SS) Subordinate subdominant	Mostly subordinate–subordinate in most dyads but not all	n = 22
(S) Subordinate	Subordinate to all cage mates–subordinate in every dyad	n = 12

Definitions of each class and criteria for assignment into a social class for the housing density experiment. As designed by Hurst *et al.*(19).

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Results

Effects of housing density and social class on the cognitive bias test

The Shapiro-Wilk test determined that the data were not normally distributed. A Kruskal-Wallis H test showed that there was no significant effect or interaction involving housing density on the number of days featuring an optimistic decision $\chi 2(3) = 0.082$, $p = 0.521$. However, there was a statistically significant difference observed in social class on number of optimistic decisions made, $\chi^2(3) = 26.95$, $p<0.001$. The Mann-Whitney U test was used to investigate the nature of this effect. Dominant (D) animals responded with significantly greater number of optimistic decisions (4.94 ± 0.25) compared to both Subordinate Subdominant (SS) animals (3.68 ± 0.22; $p<0.001$) and Subordinate (S) animals (2.77 ± 0.28; $p<0.001$). Likewise, Dominant Subdominant (DS) animals (4.5 ± 0.28) responded with significantly greater numbers of optimistic decisions compared to the SS (3.68 ± 0.22; $p = 0.044$) and S animals (2.77 ± 0.28; $p = 0.002$) (Fig 6A).

Effects of space allocation and social class on the cognitive bias test

The Shapiro-Wilk test determined that these data were not normally distributed. A Kruskal-Wallis H test showed that there was no significant effect or interaction between cage size on the number of days featuring an optimistic decision $\chi^2(1) = 0.044$, $p = 0.725$. However, there was a statistically significant difference observed between social class on number of optimistic decisions made, $\chi^2(1) = 5.865$, $p = 0.015$. D animals responded with significantly greater number of optimistic decisions (453 ± 0.23) compared to S animals (3.63 ± 0.28; $p = 0.015$). (Fig 6B).

Effects of housing density and social class on the social-interaction test

A two-way MANOVA was performed fitting housing density and social class against the percentage of time spent exhibiting each of the scored behaviours. There was a statistically significant interaction between housing density and social class on the percentage of time the animal spent 'investigating' the unfamiliar, $F(1, 57) = 6.878$, $p = 0.011$. Simple main effects analysis

Table 8. Social classes of caged rats for the space allocation experiment.

Social Class	Definition	No.
(D) Dominant	Dominant over conspecific	n = 20
(S) Subordinate	Subordinate to conspecific	n = 20

Definitions of each class and criteria for assignment into a social class for the space allocation experiment. As designed by Hurst *et al.*(19).

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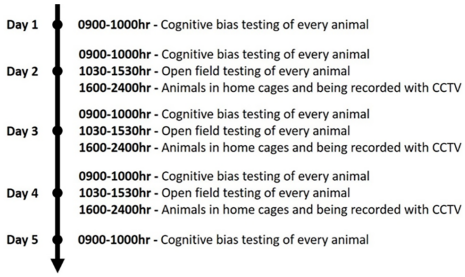

Fig 5. Flowchart of the experimental procedure and the days and times tests are performed. This flowchart details the steps used for both the housing density and the space allocation experiment. Despite the protocol remaining the same, it is important to note that these did not occur at the same time. The space allocation experiment occurred after the animals in the housing density experiment finished testing.

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showed that S rats housed in the high-density cages responded with a significantly reduced percentage of time investigating the unfamiliar rat (7.22% ± 1.91) compared to the S rats housed in the low-density cages (15.92% ± 1.85; p = 0.004) (Fig 7).

There was also a significant effect of social class on the percentage of time spent exploring the confines of the social-interaction test F (3,57) = 5.370, p = 0.003. D animals (49.98% ± 1.62) spent a significantly reduced percentage of time exploring the apparatus of the test when compared with both SS animals (56.81% ± 1.78; p = 0.004) and S animals (57.6% ± 1.44; p = 0.002) (Fig 8A). There was no significance detected for housing density on percentage time exploring, F (1,57) = 0.001, p = 0.992.

Finally, significance was also detected between social class and the percentage of time spent following the unfamiliar rat F (3,57) = 3.684, p = 0.017. D animals (3.27% ± 0.42) spent a significantly greater percentage of time following the unfamiliar rat then both SS animals (1.96% ± 0.43; p = 0.03) and S animals (1.37% ± 0.35; p = 0.002) (Fig 8B). There was no significance detected for housing density on percentage time following the unfamiliar rat, F (1,57) = 0.108, p = 0.744.

Effects of space allocation and social class on the social-interaction test

A two-way MANOVA was performed fitting space allocation and social class against the percentage of time spent exhibiting each of the scored behaviours. There were no statistically significant interactions between space allocation and social class on the percentage of time spent exhibiting any scored behaviour. However, there was a significant effect of social class on the

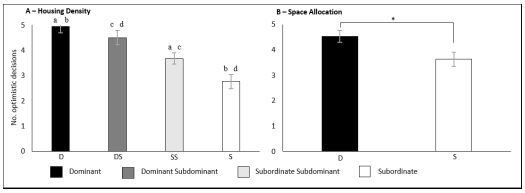

Fig 6. The effects of social class on the number of optimistic responses made in the JBP. A) For the housing density experiment. B) For the space allocation experiment. Significance is denoted at p< 0.05.

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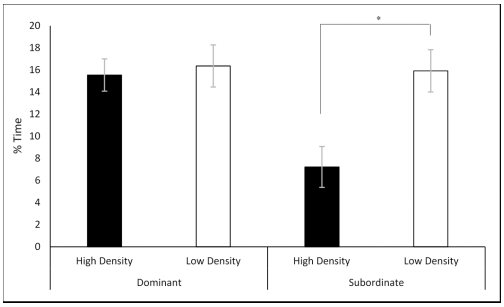

Fig 7. Interaction between density and class on the percentage time spent investigating the unfamiliar rat. Significance is denoted at p< 0.05.

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percentage of time a rat spent investigating the unfamiliar rat. Dominant animals (12.64% ± 1.03) spent significantly greater percentages of time investigating the unfamiliar animals than the subordinate animals (9.79% ± 0.93) F (1, 22) = 4.301, p = 0.049 (Fig 9). No significance was detected between space allocation on the percentage of time investigating F (1, 22) = 0.572, p = 0.891.

There was also a significant effect of space allocation on the percentage of time spent following the unfamiliar rat. Animals in the small cages (5.98% ± 1.45) spent a significantly greater percentage of time following the unfamiliar rat than compared to animals in the large cages (0.77% ± 1.23) F (1, 22) = 4.863, p = 0.038 (Fig 10). No significance was detected between social class on the percentage of time investigating F (1, 22) = 0.321, p = 0.613.

Effects of housing density and social class on the novel-object recognition test

A two-way MANOVA was performed fitting housing density and social class against the percentage of time spent interacting with the familiar and the novel objects as well as the time spent exploring the cage parameters. There was a statistically significant interaction between housing density and social class on the percentage of time the animal spent interacting with the novel object F (1, 57) = 4.798, p = 0.033. Simple main effects analysis identified that S animals in the high-density cages responded with a significantly reduced percentage of time (4.673% ± 3.26) interacting with the novel object compared to S animals in the low-density cages (17.9% ± 3.36) (p = 0.012) (Fig 11).

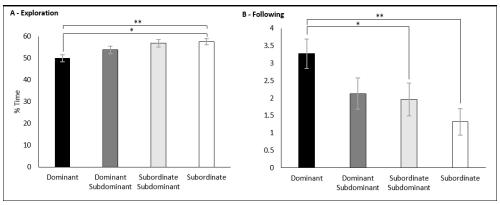

Fig 8. Effects of social class on the percentage of time spent exploring in the SIT. A) For the housing density experiment. B) For the space allocation experiment. Significance is denoted at p<0.05.

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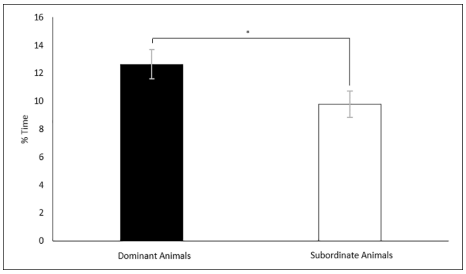

Fig 9. Effects of social class on the percentage of time spent exploring in the SIT. Results of the space allocation experiment. Significance is denoted at $p < 0.05$.

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Effects of space allocation and social class on the novel-object recognition test

A two-way MANOVA was performed fitting space allocation and social class against the percentage of time spent interacting with the familiar and the novel objects as well as the time spent exploring the cage parameters. There was no statistically significant interaction or effect between these parameters on the test variables and therefore the data has been omitted.

Effects of housing density and social class on the open-field test

A two-way MANOVA was performed fitting housing density and social class against the percentage of time the animal spent in each of the open field testing 'zones' as well as the percentage of time spent defecating/urinating, rearing and being inactive. There was a significant interaction between housing density and social class on the percentage of time the animal spent in the peripheral zone of the OFT $F(1,57) = 10.396$, $p = 0.002$. Simple main effects analysis showed that S rats (81.99% ± 3.81) in the high-density cages responded with a significantly increased percentage of time in the peripheral zone compared to D rats (62.08% ± 3.01; $p < 0.001$), DS rats (64.1% ± 2.33; $p < 0.001$) and SS rats (69.43 ± 1.97; $p = 0.003$) also housed in the high-density cages. Likewise, S animals in the high-density cages also responded with increased percentage of time compared to S rats (65.82% ± 3.92; $p = 0.009$) housed in the low-density cages. SS rats of the high-density cages also responded with a significantly increased

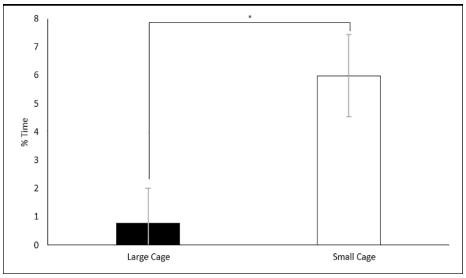

Fig 10. Effects of space allocation on the percentage of time following the unfamiliar in the SIT. Significance is denoted at $p<0.05$.

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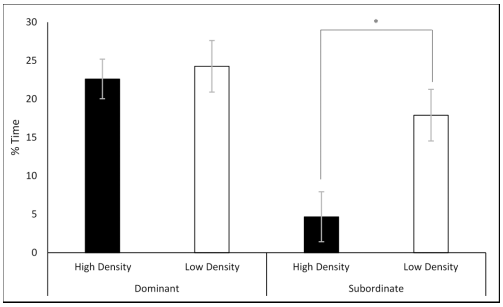

Fig 11. Interaction between housing density and class on percentage of time interacting with the novel object. Significance is denoted at p<0.05.

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percentage of time in the peripheral zone compared to the D rats (p = 0.034) in the same cages. (Fig 12A). No significance was detected between social class for the low-density cages.

There was also a significant interaction between social class and housing density on the percentage of time the animals spent in the centre zone of the OFT, F (1,57) = 5.123, p = 0.027. S rats in the high-density cages (2.09% ± 1.6) responded with statistically significant decreases in the percentage of time spent in the centre compared to D rats (10.18% ± 1.27; p<0.001) and DS rats (7.34% ± 0.98; p = 0.005) of the high-density cages. Similarly, SS rats in the high-density cages (4.88% ± 0.83) also responded with a significantly reduced percentage of time in the centre zone compared to both D rats (p<0.001) and DS rats (p = 0.042) also in the high-density cages. Once again, there was no significance detected between social classes for the rats in the low-density cages (Fig 12B).

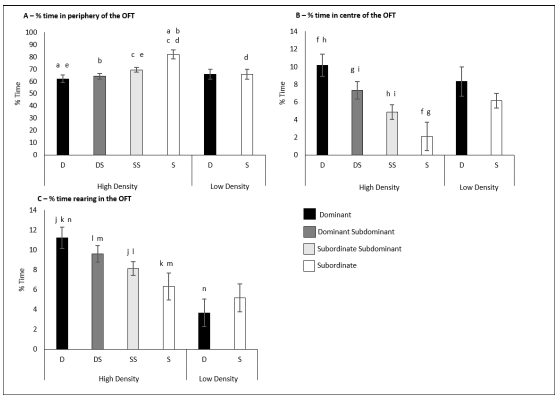

Fig 12. Results of the open field test for the housing density experiment. A) The interaction of housing density and social class on the percentage of time spent in the peripheral zone of the OFT. B) The interaction of housing density and social class on the percentage of time spent in the centre zone of the OFT. C) The interaction of housing density and social class on the percentage of time spent rearing in the OFT. Significance is denoted at p<0.05.

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The final significant interaction between social class and housing density was between the percentage of time the animals spent rearing in the OFT, $F(1,57) = 8.478$, $p = 0.005$. S rats in the high-density cages (6.292% ± 1.35) once again responded with statistically significant decreases in the percentage of time spent rearing in the OFT compared to both the D rats (11.19% ± 1.07) ($p<0.001$) and the DS rats (9.57% ± 0.83) ($p<0.001$) also housed in high-density cages. SS rats in the high-density cages also responded with decreased percentage of time rearing compared to both D rats ($p<0.001$) and DS rat ($p = 0.042$). Finally, D rats housed in the low-density cages (3.66% ± 1.39) responded with a statistically significant decrease in the percentage of time rearing compared to D rats in the high-density cages ($p<0.001$). (Fig 12C).

Effects of space allocation and social class on the open-field test

A two-way MANOVA was performed fitting space allocation and social class against the percentage of time the animal spent in each of the open field testing 'zones' as well as the percentage of time spent defecating/urinating, rearing and being inactive. The data for the percentage of time spent defecating/urinating was found to be non-parametric. Consequently, a Kruskal-Wallis H test was performed which revealed no significant effects or interactions between space allocation $\chi^2(1) = 0.042$, $p = 0.838$, and social class $\chi^2(1) = 3.101$, $p = 0.078$, on the percentage of time spent defecating/urinating in the open field test, and therefore this data has been omitted.

There was a significant interaction as observed from the two-way MANOVA of space allocation and social class on the percentage of time the rats spent in the peripheral zone, $F(1,33) = 7.725$, $p = 0.009$. Simple main effects analysis was employed to investigate this interaction. D rats in the large cages (56.74% ± 4.03) responded with a significantly reduced percentage of time in the peripheral zone compared to the S rats in the large cages (75.93% ± 4.03) ($p<0.001$) (Fig 13A).

There was another significant interaction between space allocation and social class on the percentage of time the rats spent in the inner zone, $F(1,33) = 9.410$, $p = 0.004$. Simple main effects analysis identified that S rats in the large cages (10.74% ± 2.42) responded with a significantly reduced percentage of time in the inner zone compared to D rats in the large cages (20.53% ± 2.41) ($p = 0.001$). S rats in the large cages also responded with a significantly reduced percentage compared to S rats in the small cages (22.53% ± 2.61) ($p = 0.009$) (Fig 13B).

The final significant interaction between space allocation and social class was on the percentage of time the animal spent in the centre zone, $F(1,33) = 8.907$, $p = 0.005$. Simple main effects analysis was used to investigate this interaction and showed that the S rats in the large cages (2.73% ± 1.5) responded with a significantly reduced percentage of time in the centre zone compared to the D rats in the large cages (11.36% ± 1.5) ($p<0.001$) (Fig 13C).

Discussion

The study aimed to investigate the effects of housing density and space allocation on the behavioural performance of rats. No significant effects of housing density, and only one significant effect of space allocation was observed. There were several interactions between these factors and social status. Animal performance in the open-field [20], novel-object recognition [21] and social-interaction tests [18] are all highly repeated methods to observe anxiety-like behaviours in the rat. As hypothesised, subordinate animals regularly responded in these behavioural tests with significantly higher numbers of behaviours considered anxiety-like. Subordinate animals responded with significantly fewer optimistic decisions compared to their more highly ranked conspecifics. These significant effects and interactions indicated that cage size and

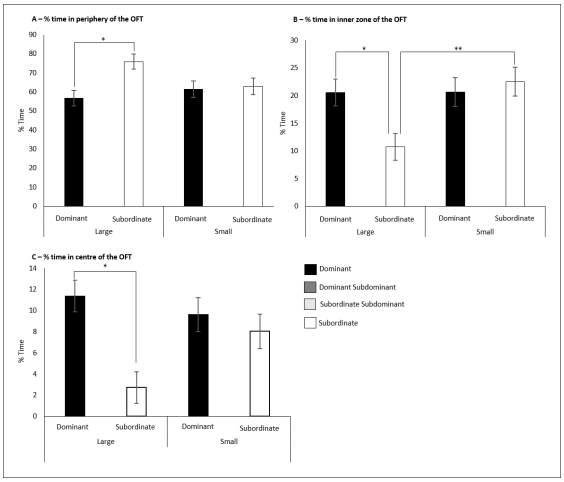

Fig 13. Results of the open field test for the space allocation experiment. A) The interaction of space allocation and social class on the percentage of time spent in the peripheral zone of the OFT. B) The interaction of space allocation and social class on the percentage of time spent in the inner zone of the OFT. C) The interaction of space allocation and social class on the percentage of time spent in the centre zone of the OFT. Significance is denoted at $p < 0.05$.

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social structure could potentially play a significantly greater role in the minimisation of social stressors than previously identified.

Subordinate status is a source of anxiety in group-housed male rats

For both the housing density and space allocation experiments subordinate and subordinate-subdominant animals routinely responded with greater numbers of behaviours associated with an anxiogenic state. A reduced expression of optimistic cognitive biases (Fig 6A and 6B) has been associated with anxiety-like states in multiple animal species [13]. Subordinate animals demonstrated a decrease in the percentage of time spent following the unfamiliar rat in the SIT (Fig 8B) in both experiments, and a decreased percentage of time investigating the unfamiliar rat (Fig 9). A decrease in social investigatory behaviours has been reviewed and is associated with conditions known to cause anxiogenic responses [22]. Finally, a decrease in the amount of time an animal spent in the centre zones of the OFT (Fig 12B and 12C) was associated with anxiety and/or depression like conditions [20].

Subordination has been correlated with significant physiological changes, including decreases in bodyweight [23], reductions in dopamine activity of the brain [24], and teste weight, and decreased plasma testosterone and corticosterone levels [25]; changes consistent with chronic stress responses. Behavioural adaptions have also been associated with social class, with dominant animals spending significantly more time in the open arms of an elevated plus maze compared to subordinates [26]. Subordinate rats housed with a dominant conspecific also responded with an increase in the number of ultrasonic vocalisations (response to aversive stimuli) being emitted and displayed greater freezing responses than dominant conspecifics [27].

The results of the current study suggest that social status of rats can be a reliable cause of anxiety. Whilst there were several interactions of either housing density or space allocation with social status, there was no significant effect of housing density alone on any of the observed parameters and only one significant effect of space allocation (Fig 10). These interactions are discussed in more detail below. Investigations into housing density and space allocation on animal welfare are often confounded. The current study provides evidence that social status needs to be considered as an independent variable when studying the effects of either housing density, space allocation or the effects of crowding.

Subordinate stress varies with housing density

The results suggested that the social stressors associated with subordination were not as severe in the low-density cages compared to the high-density cages. Subordinate animals of the high-density cages responded with a significantly decreased percentage of time investigating the unfamiliar rat in the SIT (Fig 7) and a decreased percentage of time interacting with the novel-object in the NORT (Fig 11). Reduced novel-object recognition is a sign of cognitive impairment in animals suffering from chronic stress [21]. In addition, subordinate animals of the high-density cages responded with significantly greater anxiety-like responses to the OFT (Fig 12A–12C) Previous study findings conducted in mice [28–30] and rats [10] support this observation in which aggression was intensified by increasing group size. In the current study, subordinate animals in the high-density cages experienced greater numbers of aggressive acts initiated upon them compared to the subordinates of the low-density cages. However, these discussed studies did not separate space allocation (surface area per animal) and housing density (animals per cage) this is discussed in greater detail later.

Studies that successfully decoupled space allocation from housing density have reported that aggressive encounters increased in larger groups of caged mice [31]. Another study reported that the number of aggressive encounters between the dominant and subordinate of highly dense cages (8 animals per cage) were significantly greater than animals housed in low density cages (3 animals per cage) [32]. A larger population size encourages the dominant animal to display greater levels of aggressive behaviour to sustain its dominant status. Meanwhile, subordinate animals of the high-density cages showed increased aggressive behaviours to possibly earn a higher social status within the hierarchy [32]. As summarised by Poole and Morgan [29] the greater the population size per cage, the more unstable the hierarchy, increasing the likelihood that dominance status would change between individuals.

Most previous studies have investigated mice, and given inherent species differences, it could be argued that these studies have limited relevance to the current study. However, many common behavioural tests of anxiety [33], learned helplessness [34] and general cognitive ability [35] report mouse and rat behaviour as being 'equivalent'. This suggests that comparisons between the two species are valid when using tests to identify an anxiety or depressive like state. Therefore, while we cannot state that the subordinate stress experienced by the rats in the current study was equivalent to mice, or that the stress was caused in the same mechanistic manner, we can confidently state that the behavioural tests we employed to detect subordinate stress were appropriate.

The current study has shown that larger group sizes of rats lead to increases in the number of anxiety-like behaviours expressed by subordinate rats, and an overall increase in social stressors. This finding has been reported in mice, but has yet to be reported in rats. This encourages future research to focus on understanding the mechanisms underlying subordination stress in rats. Housing recommendations for lab animals are currently based on weight, with few guidelines based on experimental observations of appropriate group size. The

findings of the current study should therefore be considered in future guidelines and legislative drafting.

Subordinate stress varies with space allocation

As illustrated in Figs 6B and 13A–13C, subordinate animals in the large cages responded with significantly more anxiety-like behaviours compared to subordinate animals in the small cages. Furthermore, there were no significant differences observed at all, between dominant and subordinate animals in the small cages compared to those in the large cages. This suggested that subordinate stress was amplified with a larger area which the subordinate shared with the dominant. Guidelines and legislation tend to promote larger cages on the belief that an increased space allocation reduces crowding stress [3]. However, as discussed previously, this may stem from a failing of much peer-reviewed literature to successfully separate housing density and space allocation.

When provided with different cage sizes, rats would preferentially choose the larger cage (1620cm^2 of usable floor space) that housed four other rats over a small cage (540cm^2 of useable floor space), despite the larger cage providing less physical space for the rat to occupy than the small cage [36]. This suggested that rats preferentially chose conditions with greater crowding stressors than lone housing with a greater surface area allowance. Monogamous breeding pairs of Dahl salt-sensitive rats housed in small cages (922.6cm^2 of useable floor space) showed no significant differences in breeding parameters compared to similar pairs housed in larger cages (1355cm^2 of useable floor space) [37]. The 3rd edition of the *Guide for the Care and Use of Agricultural Animals in Research and Testing* [38] states that reproductive parameters are an important indicator of animal welfare, suggesting that the use of smaller rat cages for breeding purposes is acceptable, despite it being considered 'over-crowded'.

Crowding is often confounded with housing density, as an increase in housing density invariably leads to a crowding effect. Density has been defined as the number of animals occupying the same floor area. Crowding is defined as the motivational state that occurs when spatial and social factors interact, which influences behaviour to mitigate the effects of the restricted space [9]. The current study demonstrated that the larger the space allocation per animal, the greater the effect of the stressors associated with subordination. Van Loo, Mol [32] found both dominant and subordinate male mice housed in a small cage responded with fewer acts of aggression compared to those in larger cages. These authors concluded that a small cage was associated with dominant animals having a smaller defendable territory that reduced the number of aggressive acts needed to maintain control over this territory [32]. Therefore, it was hypothesised for the current study that the subordinate rats in the larger cages were subjected to more acts of aggression from their dominant cage-mates compared to the subordinate rats of the small cages.

This discussion highlights the need to consider the effects of crowding stress versus subordination stress, and future research should identify if the stressors from crowding produce more anxiety-like responses than subordination stress. Whilst increasing the surface area per animal will reduce the stressors associated with crowding, it may in turn increase the stressors associated with subordination.

Social class and the effects of motivation in the judgment bias paradigm

Subordinate-subdominant and subordinate rats responded with fewer optimistic decisions then both the dominant and dominant-subdominant rats, when exposed to the JBP for both the housing density (Fig 6A) and space allocation experiments (Fig 6B). Likewise, these rats responded with increased time exploring the confines of the SIT (Fig 8A) and spent less time

following the unfamiliar rat (Fig 8B) during the housing density experiment, signs previously associated with compromised sociability [39]. This suggested that an individual animal having a lower social rank not only encouraged pessimistic cognitive biases, but also encouraged social dissonance. A similar finding of sociability on cognitive bias expression reported that bottlenose dolphins that displayed more social affiliative behaviours (synchronous swimming) responded with greater numbers of optimistic decisions to a JBP [40]. This may provide an explanation as to why subordinate animals, which are the subjects of more acts of aggression and therefore fewer social affiliative behaviours, responded with significantly fewer optimistic decisions. We hypothesised that dominant animals experience a more harmonious social standing and therefore experience fewer anxiety-like effects associated with group housing. As discussed previously, large group sizes encourage animals of lower social standing to challenge other low ranked animals in order to gain a higher social status [32]. This finding is important as it suggests that animal status in a social hierarchy is an important covariate that needs to be taken into consideration when assessing animals on their cognitive bias expression using a JBP.

Dominance has also been correlated with an increased motivation for food reward, when housed in the visible burrow system (VBS), a model in which unfamiliar rats form dominance hierarchies. Dominant rats have been reported to respond to a palatable food reward with an increase in operant responses [26]. Therefore, it could be argued that a dominant social status in rats can significantly augment their ability to respond to palatable food rewards. Rats experiencing chronic social-stress have also shown reduced motivation-related behaviours. Using an indirect marker of dopamine activity (dopamine transporter binding density), non-responsive subordinate rats displayed long-lasting (3-week) changes in their mesolimbic dopaminergic system after experiencing a chronic-social stressor (VBS housing) [24]. This is significant when discussing the JBP as used in the current study, which relied on the motivating factor of food that encouraged the expression of a cognitive bias. Dominant animals have an innate increased motivation to attain a food reward, whilst subordinates experience physiological changes that decrease their ability to be 'rewarded'. This implies that animals experiencing chronic-social stressors are less-motivated to perform reward-motivated behaviours, as they no longer receive "pleasure" by doing so.

If dominance status and subordination stress significantly alter behaviour [24] and food-motivation [26], then a food rewarded JBP to assess affective state of group-housed rats was a significant limitation of the study. Food-motivation has been established as a confounding variable when discussing animal behaviour in general [41, 42]. The possibility that coupling of food-motivation and social status acts as a confounding factor in the JBP is a novel theory. This suggestion renders many studies involving group-housed animals and a food-rewarded JBP confounded unless social status is included as a covariate in design. Logically, future work should then avoid a JBP that relies on animal motivation to food. This may prove difficult as the majority of previous JBP designs have utilised the presence or absence of food as the motivating factor [43]. Few JBP studies have reported success using location or social based rewards as the motivator to express a cognitive bias [44]. These tests may prove superior and should receive attention in future research.

Conclusion

This study used an array of behavioural tests to explore the effects of housing density and space allocation on common laboratory rats. We were unable to confirm the hypothesis that increased housing density or a decreased space allocation would result in increased numbers of anxiety-like behaviours. However, we confirmed the hypothesis that subordinate rats would

respond with greater anxiety-like behavioural traits compared to dominant rats. Furthermore, it was concluded that subordination stress in rats could be exacerbated by housing a greater number of rats in the same cage and by providing a greater surface area per animal. These findings are novel, being the first to successfully dissociate the commonly confused factors of space allocation and housing density in rats. Future work should include treatment groups of variable densities than those utilised in the current study, and a greater range of differently sized, commercially available cages.

Furthermore, the continued use of a reward-based JBP to assess the affective state in at least a group-housed rat model is discouraged. The combined factors of motivation and status within the social hierarchy can significantly augment behavioural expression of the rat. Future studies using a JBP in group-housed animal models should consider controlling for the social status of the animals.

This study has challenged the notion that rats have a greater standard of welfare when housed in larger cages, with more surface area per animal, a common presumption of rodent housing guidelines. An increased surface area does lead to a decrease in the negative effects associated with crowding. However, increasing the surface area also encourages the prevalence of anxiety-like behaviours associated with subordination. Therefore, simply increasing the surface area per rat may not lead to increased animal wellbeing. Furthermore, even when rats are housed with an approximately equivalent floor area per animal, those housed with more conspecifics experience greater levels of social stressors than those housed with a single cage-mate. Therefore, the data encourages the drafting of guidelines and legislative documents that do not simply increase the surface area of cages in which animals can be legally housed. Consideration of other factors such as cage complexity, housing density and social status will provide a higher standard of welfare for caged laboratory rats.

Supporting information

S1 File. Raw behavioural data used in the analysis. Data is presented as one excel spreadsheet. data has been separated into sheets by experiment (housing density or space allocation) and by behavioural test.
(XLSX)

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