NOX2-mediated regulation of NLRP3 inflammasome activation in traumatic brain injury.

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B.A. (Mod.) Physiology

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Declaration of Authorship

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____________________________
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Abstract

Traumatic brain injury (TBI) is described as damage to the brain due to a mechanical impact to the head and is the leading cause of death and disability in developed countries. Following the primary mechanical injury, secondary injury evolves and activates molecular, biochemical, and cellular events such as oxidative stress, cell death and glial cell activation contributing to increased neuroinflammation and neurological impairments. NADPH oxidase 2 (NOX2) is an enzyme complex responsible for reactive oxygen species (ROS) production in phagocytes, including microglia. Chronic NOX2 expression induces oxidative stress, drives neuroinflammation, and leads to progressive cortical and hippocampal degeneration. NOX2 also acts as a priming signal for NLRP3 inflammasome activation, which has been implicated in many neurodegenerative diseases and plays a pivotal role in secondary TBI. GSK2795039 is a small molecule brain penetrable drug that inhibits NOX2 in an NADPH competitive manner. The hypothesis of this study states that pharmacological inhibition of NOX2 by GSK2795039 can attenuate pro-inflammatory mediators, including NLRP3 inflammasome activation, in brain-resident microglia and infiltrating myeloid cells, and improve neurological outcomes following TBI.

*In vitro*, immortalised Microglial (IMG) cells or primary microglia from p1 Wistar rat pups were used to assess microglial activation. Cells were pre-treated with GSK2795039, diphenyleneiodonium (antioxidant) or MCC950 (NLRP3 inhibitor) and stimulated with lipopolysaccharide (LPS) and ATP/nigericin to induce NOX2/ROS and NLRP3 inflammasome activation. *In vivo* studies, using controlled cortical impact (CCI), multi-dimensional flow cytometry, histology and neuro-behavioural tasks aimed to translate *in vitro* findings to an experimental TBI model in C57BL6/J mice.

Results from the *in vitro* studies revealed that GSK2795039 attenuated LPS/nigericin-induced microglial NOX2 activity, ROS production in the cells and reduced nitrite, TNFα, LDH, IL-1β and IL-18 release, as well as the major components of the NLRP3 inflammasome; ASC, cleaved caspase-1 and cleaved IL-1β in the conditioned media. *In vivo* studies demonstrated increased infiltration of NOX2/ROS/Caspase-1/IL-1β+ inflammatory neutrophils and monocytes over a time-course analysis with peak
microglial and monocytic NOX2/ROS production at 3 days post-injury (DPI). Pharmacokinetic results indicated that GSK2795039 entered the brain following CCI. Systemic administration of GSK2795039 (100mg/kg; IP) starting 2h post-injury attenuated NOX2⁺/IL-1β⁺ microglia/macrophage activation at 3 DPI. In addition, GSK2795039 reduced numbers of IL-1R⁺CD4⁺ and IL-1R⁺CD8⁺ T cells in the brains of CCI mice, indicating that microglial-T cell crosstalk was altered by treatment. These neuroimmune changes were associated with minor improvements in motor function post-TBI. At 28 DPI, GSK2795039 treatment showed modest improvements in neuro-behavioural and neuropathological deficits.

In conclusion, TBI is a highly complex disease resulting from a multitude of secondary injury cascades. Addressing the many research gaps of the innate and adaptive immune responses is essential for future TBI therapies. The findings from this thesis imply the NOX2-NLRP3 inflammatory axis along with microglial-T cell crosstalk as effective targets in the neuroinflammatory response following TBI. Our translational studies indicate that GSK2795039 may be a promising therapeutic drug for mitigating NOX2-mediated neuroinflammation in microglia, and peripheral immune cells, following experimental TBI in mice.
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First and foremost, I would like to thank my supervisor, Dr. David Loane, for all his guidance and motivation throughout my PhD. Your door was always open, and I really appreciate your enthusiasm for TBI research and encouragement for my studies. I feel very lucky to be the first PhD student in your lab, I have learnt so many skills during the challenging obstacles we faced setting up the lab. I cannot express how grateful I am for your support and kindness during the most difficult times inside and outside of the lab.

Professor Marina Lynch, without my time in your lab I may not have even learnt what a PhD was, let alone pursue one in David’s lab! I have so many wonderful memories of your lectures, my research project and the terrifying weekly lab meetings that have given me the confidence in presenting today. You have been an incredible mentor who has made a lasting impression on me.

Isabella, the Loane Lab OG, and my amazing lab partner! I am so grateful we started our research projects at the same time, you have become such an important friend to me. We had so many laughs in the lab which kept us sane when experiments were not going to plan, maybe because the ROS machine software was unticked (ooops)! Thank you, not so much for introducing me to Bailey’s “hug in a mug”, David is even a fan now! I would like to say a huge thanks to Marie for your help in the early days of my project. I appreciate your generous guidance with the primary microglia studies and for teaching me the CCI model!

To the amazing members of the Loane lab, I could not have asked for a better group of people to share my PhD experience with. My fellow mice whisperers, Carly and Gloria, I can’t express how thankful I am for your support throughout the behavioural studies, you guys are amazing! Carly, thank you for always being up for anything whether it’s dancing in Munich at ENO, Ryan’s on a Tuesday, or a chilly sea swim - thank you for the laughs, or the roosters I should say. Gloria, thank you for teaching me histology in the last 6 months of my PhD and for showing me the best ways to illustrate data! You are a fantastic teacher (posso avere uno spritz per favore?!?) and a wonderful addition to the lab. To Nathan, the stats man and formatting guru. Thank you very much for always
being so patient with my statistical questions. Also, none of these GSK experiments could have been done without your i.p injections and overnight stints! I really appreciate your kindness and all you do for the lab. Sahil, thank you for your willingness to drop everything and help, for generously offering the only available FlowJo dongle to me when you were using it. Your project is truly fascinating, and I can’t wait to read your PhD thesis!

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Curriculum Vitae

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- 2022, A 3 slides in 3-minutes talk for the Tipton Prize in the School of Biochemistry & Immunology, Trinity College Dublin, Ireland.
- 2021, Neuroscience Ireland (NSI) 2021 Virtual Conference poster competition.
- 2021, Winner’s Spotlight Talk with Ulysses Neuroscien, Virtual webinar.

MANUSCRIPTS IN PREPARATION


## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AD</td>
<td>Alzheimer’s Disease</td>
</tr>
<tr>
<td>AIF</td>
<td>Apoptosis-inducing factor</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>ASC</td>
<td>Apoptosis-associated speck-like protein containing a CARD</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-triphosphate</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid beta</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood Brain Barrier</td>
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<tr>
<td>CARD</td>
<td>Caspase recruitment domain</td>
</tr>
<tr>
<td>CMU</td>
<td>Comparative Medicines Unit</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>Contra</td>
<td>Contralateral to injury</td>
</tr>
<tr>
<td>CTE</td>
<td>Chronic Traumatic Encephalopathy</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage-associated molecular patterns</td>
</tr>
<tr>
<td>DI</td>
<td>Discrimination index</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>DPI</td>
<td>Days post-injury</td>
</tr>
<tr>
<td>DPI*</td>
<td>Diphenyleneiodonium chloride</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental Autoimmune Encephalomyelitis</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunoabsorbant assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FMO</td>
<td>Fluorescence minus one</td>
</tr>
<tr>
<td>GPX1</td>
<td>Glutathione peroxidase 1</td>
</tr>
<tr>
<td>GSDMD</td>
<td>Gasdermin D</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen Peroxide</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSPs</td>
<td>Heat-shock proteins</td>
</tr>
<tr>
<td>i.p</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>Iba1</td>
<td>Ionised calcium-binding adapter molecule 1</td>
</tr>
<tr>
<td>ICP</td>
<td>Intracranial pressure</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IMG</td>
<td>Immortalised microglial cell line</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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Chapter 1: General Introduction
1.1 Traumatic brain injury

Traumatic brain injury (TBI) is described as damage to the brain due to a mechanical impact to the head that results in an “alteration in brain function” [1]. Worldwide, TBI is estimated to affect 50-60 million individuals each year, has the highest incidence rate of all neurological disorders, and more than 90% of all TBI patients have mild TBI [2]. Furthermore, the annual economic cost of TBI exceeds €60 billion per year in Europe [3]. The first *Lancet Neurology* Commission on TBI published in 2017 estimated that TBI would remain one of the top three injury-related deaths or disabilities up to 2030 [1], particularly in young people below the age of 45 years in developed countries [4]. Classification of clinical injury severity of TBI is based on the Glasgow Coma Scale (GCS) which sum scores from responses in three domains (eye, motor, verbal) to indicate mild (GCS 13-15), moderate (GCS 9-12) and severe (GCS 3-8) TBI [1]. Approximately 78% of severe brain injury patients in a vegetative state 2 weeks following injury, recover consciousness 12 months thereafter, with just 25% regaining orientation [2]. Moreover, it is predicted that 50% of mild TBI patients do not return to pre-TBI health levels within 6-months of injury, and females tend to have worse outcomes than males, making this an overall global health problem [2].

TBI can result from a single severe blow to the head such as in road traffic accidents or from mild repetitive head impacts in contact sports, such as rugby union/rugby league or National Football League (NFL) American Football. The incidence of TBI in the elderly is steadily increasing by unintentional fall-related injury [5]. It is important to note the differences between a mild TBI and a concussion, which is often used interchangeably, particularly in contact sports. A concussion is a sub-category of a mild TBI caused by rapid acceleration and deceleration of the brain which gives rise to a “traumatically induced transient disturbances of brain function” [6]. Concussions are not thought to be life-threatening and often individuals recover. However, multiple concussions influence the severity of the brain injury. TBIs are more serious and brain atrophy can be detected by CT and MRI neuroimaging years after severe injury, which is not the case following concussion [6].
Repetitive head impacts (RHI) include both concussions and subconcussions, meaning the individual does not obtain symptoms following hits to the head, for example, headering the ball in soccer [7]. Evidence suggests that soccer players (mean age 14.9 ± 1.1 years) that were exposed to RHI showed impaired cognitive performance in both cognitive and sensorimotor tasks, compared with their aged-matched controls [8]. Currently, the REIMPACT Consortium study which incorporates six research groups in six countries, aims to address the questions regarding the effects of RHI on cumulative structural and functional brain alterations leading to cognitive impairments [7]. In severe incidences, repetitive exposures to head injuries may give rise to a disease known as chronic traumatic encephalopathy (CTE), which has been described in former NFL players [9]. In this deteriorating disease, the structure of the brain shrinks, there is build-up of unique tau filaments and ventricular enlargements which is associated with extreme behavioural alterations, such as sleeping disorders, increased aggression, depression, and anxiety [9]. CTE can only be diagnosed post-mortem and initial symptoms of CTE are subtle, including loss of attention or headaches. As the disease progresses, severe cases consist of complete cognitive dysfunction and early forms of dementia. Indeed, repetitive head impacts do not always result in CTE. In fact, CTE represents just one consequence of several TBI which is mostly observed in NFL players but rarely seen in the general population [10]. Moreover, it has been extensively documented that exposure to a single moderate-severe TBI or repetitive mild-TBI is related to an increased risk of developing neurodegenerative diseases such as dementia [11, 12].

Undoubtedly, life after TBI poses a dramatic impact on patients and their families. Many TBI survivors, ranging from mild-severe cases, experience long-lasting consequences including physical and mental disabilities such as cognitive impairment and psychiatric disorders, which may require endless rehabilitation [13]. Therefore, therapy, counselling and support services are vital in promoting rest and wellness to TBI survivors. Indeed, there is a necessity to address long-term neuropsychiatric problems and related issues, such as anxiety, depression, and sleep disorders [1].
1.2 Primary and secondary injury
There is emerging evidence that TBI is a chronic disease comprised of a primary and secondary injury with an increased risk of neurodegeneration [11]. The primary injury, caused by the initial physical impact, results in immediate cell loss at the site of injury, along with vascular damage and neuronal death [14]. During the primary injury, damage-associate molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) are released from dying cells which triggers the secondary injury that evolves over minutes to days and even years after the initial insult [14, 15]. During the secondary injury, multiple pathophysiological cascades become activated and are thought to cause central nervous system (CNS) homeostatic dysfunction which overtime lead to the development of many neurological diseases after TBI. Secondary injury events include increased oxidative stress, due to increased free radical production, increased excitotoxicity through enhanced glutamate release, mitochondrial dysfunction, and caspase-dependent and independent cell death [14]. Moreover, DAMPs and PAMPs trigger glial cell activation which results in the release of a plethora of pro-inflammatory mediators, which leads to blood brain barrier (BBB) disruption and consequently the influx of peripheral cells into the brain promoting chronic oxidative stress. In summary, all secondary injury mechanisms are highly interactive and contribute to upregulating neuroinflammation and thus worsens long-term TBI outcomes [15, 16].

1.3 Lack of TBI therapeutics
Although there are approaches to treat patients that present to the emergency department with a TBI such as craniotomy, which removes part of the skull to release the build-up of intracranial pressure (ICP), fluid management, cerebral microdialysis for local monitoring [1], there are no therapeutic cures for TBI patients. Clinical research showed that steroid treatment could attenuate intracranial pressure in TBI patients [17]. However, a multitude of carefully designed clinical trials revealed that glucocorticoid treatment actually worsened TBI outcomes and in some cases increases mortality [18], which may be due to a blunted immune response. Steroids are now contraindicated in TBI, and there is an emphasis on more focused and targeted approaches in alleviating
brain inflammation in TBI patients. Considerable research has focused on targeting secondary injury mechanisms to reverse molecular and cellular pathophysiology and develop neuroprotective treatments [19]. It has been demonstrated by $[^{11}C]$PK11195 positron emission tomography (PET) imaging that glial activation persists from 11 months to 17 years post injury in TBI patients [20]. This sustained microglial or astrocyte activation coincides with increased pathology and cognitive dysfunction. The authors then sought to target this chronic evolving neuroinflammation and therefore investigated the effect of the antibiotic minocycline on microglial activation in patients at least six months after moderate to severe TBI. The results of this small intervention study in severe TBI patients showed minocycline reduced microglial activation but elevated levels of plasma neurofilament light protein, which were associated with faster rates of brain atrophy, and therefore was not the desired result [21]. To date, there are no effective neuroprotective treatments for TBI. However, there are many treatment options being develop and considered for TBI neuroprotection. For example, researchers are currently evaluating mesenchymal stem cell (MSC) transplantation for severe TBI patients [22] as MSC stimulate neurogenesis, promote regenerative microglia, and elicit an immunomodulatory effect [23]. Further research is needed to identify a specific target molecule, with little off-target effects to regulate TBI-elicited sustained neuroinflammation.

1.4 Controlled Cortical Impact as a mouse model of TBI

Animal models of TBI are an essential preclinical tool to study the pathophysiology of the disease and identify novel targets to improve therapeutic interventions. But how well do animal models reflect brain injury in humans? There are numerous preclinical challenges faced by *in vivo* researchers including species, sex, and strain, as even the same experimental TBI model can produce different injury severity and neurobehavioral outcomes depending on the strain of mice used [24]. Moreover, translation of preclinical findings of neuroprotective strategies to human TBI has resulted in more than thirty failed clinical trials [14, 25]. For these reasons there is a strong need in the field to improve preclinical research to resemble clinical pathophysiology and outcomes more closely. In this present study the objective was to use an animal model that will mimic
the complexity of human TBI to better understand injury-induced mechanisms and achieve therapeutic breakthroughs for TBI.

Developed in the 1980s, controlled cortical impact (CCI) is one of the most widely used murine mechanical models of TBI [26]. It consists of a pneumatic device that has a high degree of control over experimental factors including speed, dwell-time, and depth. Following craniotomy, the CCI device rapidly extends a rod to penetrate the exposed intact dura mater in the anaesthetised mouse, resulting in a focal contusion. One of the strengths of this model is that it produces cortical tissue loss, cerebrovascular damage, BBB disruption, acute hippocampal degeneration, oedema, neuroimmune dysfunction and inflammation, which mimics critical features of human TBI [27]. Moreover, due to rupture of the dura mater, CCI is considered a moderate-level injury in this project.

1.5 The immune system

The immune system consists of a network of immune cells and signalling molecules and is comprised of both the innate and adaptive system [28]. The innate immune system is the first line of defence and responds immediately to infectious agents. It is comprised of physical defences, such as skin, epithelia and cellular defences including neutrophils and macrophages. These cells express pattern recognition receptors (PRR) such as toll-like receptors (TLR) and nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) which detect and phagocytose harmful pathogens, releasing pro-inflammatory stimuli, chemokines and cytokines, to recruit and activate nearby cells with the ultimate aim of producing an immune response [28]. Macrophages, dendritic cells, and microglia in the CNS are antigen presenting cells (APCs), which present antigens on their surface to T cells. APCs and cytokine-mediated inflammation of the innate immune cells initiates an adaptive immune response [29]. The adaptive immune system is composed of T and B lymphocytes and its main function is to respond and recognise highly specific antigens to prevent re-infection. T cells comprise of CD8+ cytotoxic T cells and CD4+ T helper cells which are further subdivided into CD4+ Th1, Th2 and Th17 cells and T regulatory (Tregs) cells. In homeostatic conditions, the immune system is tightly regulated by the release of anti-inflammatory mediators from myeloid cells and interleukin-10 (IL-10)-producing Tregs which dampen the pro-inflammatory
response [29]. However, overreacting inflammatory responses leads to immune system dysregulation which has been implicated in many chronic neuroinflammatory diseases, including TBI [30, 31].

1.6 Microglia

1.6.1 Microglia are the immune cells of the brain

Microglia are the resident immune macrophages of the CNS [32]. In physiological conditions, microglia function in surveillance, scanning the brain for potential threats. In their surveillant state, microglia adopt a ramified morphology possessing a small cell body (soma) and elongated processes that extend and retract to monitor the brain. Microglia are plastic cells that change morphology and activation states depending on the environment in the brain. Microglia are continuously monitoring the brain so labelling them as “resting” when they are not activated by stimuli is likely inaccurate [33]. One of the ways microglia maintain brain homeostasis is by phagocytosis. Upon detecting a threat, microglia retract their processes and adopt a more ameboid shape where they become more motile [32]. Microglia then migrate to the site of injury, by means of chemotactic signaling [34], with the aim of controlling the insult before it becomes detrimental to neurons. Microglia engulf the apoptotic cells, repair the damaged tissue, and ultimately return to their quiescent state. The neuroprotective microglia also interact with astrocytes, which are the most abundant cells in the brain and are crucial for BBB development, and neurons to maintain CNS homeostasis [33]. Microglia also function in brain circuit development by synaptic pruning and the removal of excess excitatory synapses [32] to ensure a healthy microenvironment. Therefore, the surveillant, responsive and repairing states of microglia provide a neuroprotective microenvironment in the brain.

1.6.2 Microglia become reactive in acute and chronic pathology

Intrinsic factors including inflammation, brain injury and ischemia as well as extrinsic factors like inflammatory cytokines or lipopolysaccharide (LPS), a component of gram-negative bacteria, induce activation of microglia. This activation may be triggered by the release of DAMPs from stressed neurons. When a DAMP such as ATP binds to P2Y12
purinergic receptors on microglia, the stimulated microglia promote the release of pro-inflammatory mediators including cytokines and inducible nitric oxide synthase (iNOS) [35] promoting an acute inflammatory response, which is beneficial to normal brain function. These inflammatory factors signal to peripheral monocyte-derived macrophages to influx into the CNS and triggers an innate immune response. Activated microglia leads to the prolonged release of pro-inflammatory cytokines, such as TNFα, IL-1β, IL-18, which gives rise to a pro-inflammatory cascade. Sustained microglia activation plays a pivotal role in neuroinflammation and acts as a key indicator of neurodegenerative disease, such as in Alzheimer’s Disease (AD) [36]. Microglia respond to a multitude of factors including misfolded protein amyloid beta (Aβ) in mouse models of AD, leading to chronic activation whereby the microglia adopt a more pro-inflammatory state and become less phagocytic [37, 38].

1.6.3 Microglia contribute to secondary injury in chronic TBI

Chronic microglia activation and dysfunction plays a crucial role in long-term neurodegeneration and the evolution of TBI pathology [15, 39]. Alterations in microglial morphological and phenotypic abilities have also been reported following TBI [40]. Dystrophic microglia appear small and de-ramified with beaded tortured processes, and a reduced ability to phagocytose apoptotic cells. Differentiating the morphology of microglia in various activation states will allow for better understanding of microglia function in disease. Mouse models of TBI demonstrated chronic microglial activation up to 1 year post injury which is associated with increased pro-inflammatory release, cell dysfunction and tissue damage [39]. Non-invasive PET imaging studies in the thalamus of humans illustrated that chronic microglial reactivity persists for up to 17 years following moderate to severe TBI, which coincided with increased pathology and dysfunction [20]. Furthermore, chronic glial activation was observed in the brains of young active and former NFL players as demonstrated by PET imaging and increased binding of [11C] DPA-713 to translocator protein (TSPO), a proposed biomarker of neuroinflammation indicating chronic inflammation and injury [41, 42].

Indeed, uncontrolled and sustained microglial activation is detrimental and can contribute to neurodegeneration and neuro-behavioural dysfunction, thus aggravating
the disease in the CNS. Microglia express colony stimulating factor 1 receptor (CSF1R) which is essential for their survival [43]. Recent advances in pharmacology have identified CSF1R inhibitors which enable > 95% microglial elimination in the CNS [44]. Short-term microglial depletion during the chronic phase of TBI showed reduced cortical lesions and long-term improvements in neurological function [45]. Briefly, 4 weeks post-injury, Plexxikon 5622 (PLX5622), a CSF1R inhibitor was administered to the mice followed by removal after 1 week to allow for microglial repopulation. In vehicle-treated TBI mice, microglia in the injured cortex had a chronic hypertrophic cellular morphology and had increased expression of pro-inflammatory mediators, NLRP3 inflammasome, IL-1β and NADPH oxidase (NOX2) that correlated with chronic neurodegeneration in the cortex and hippocampus. In contrast, delayed PLX5622 treated TBI mice had a reduced expression of pro-inflammatory mediators and microglia displayed ramified morphological features like that of the control mice. PLX5622 treated TBI mice also had significantly reduced neurodegeneration compared to their vehicle treated TBI counterparts. This preclinical study, along with others using PLX5622 to deplete microglia following experimental TBI [46, 47], indicate that chronic microglial activation contributes to neurodegeneration and neurological dysfunction after TBI.

The effect of microglial depletion has also shown promise in a mouse model of post-traumatic stress disorder (PTSD) [48]. Following administration of electric foot shocks, there was increased microglia activation and upregulated pro-inflammatory gene expressions including IL-1β. Mice treated with PLX3397 had depleted microglia and showed reduced PTSD symptoms. Microglial depletion altered microglial phenotype as shown by an increased ratio of ki67+ microglia and reduced iNOS gene expression, indicating the repopulating microglia, due to proliferation, exhibit reduced inflammatory gene expression. This study also treated the mice with minocycline which suppressed microglial activation and PTSD-related behaviours were attenuated [48].

Thus, inhibiting chronic microglial activation may act as a potential strategy in attenuating neuroinflammatory responses and ultimately preventing neurodegeneration. However, prolonged inhibition of microglia would unlikely be therapeutic as acute microglial activation is critical to maintain normal brain function. Therefore, more nuanced therapeutic strategies that specifically target and attenuate
the damaging effects of microglia (pro-inflammation and oxidative stress) and promote potential benefits such as anti-inflammation and tissue repair are needed to manage chronic TBI.

1.7 NADPH oxidase (NOX2)

NADPH oxidase (NOX) is a critical multi-subunit enzyme system responsible for the generation of intracellular and extracellular reactive oxygen species (ROS) [49]. Many cellular processes produce ROS as a by-product such as in mitochondrial respiration at the electron transport chain [50]. However, NOX enzymes are the only complex with the sole purpose to produce ROS [49]. Oxidative stress arises from the imbalance and overproduction of ROS implying NOX complexes have become promising therapeutic targets [51]. The NOX family is comprised of seven isoforms: NOX1-5 and two dual oxidases (DUOX 1/2). NOX2 was first discovered because of a missing protein, gp91phox (now known as NOX2), associated with chronic granulomatous disease (CGD) [52]. This is an inherited disease whereby non-functional NOX2 leads to immunodeficiency and recurrent infections such as pneumonia, septicaemia, and gastrointestinal infections [53].

NOX2 is the prototypical enzyme that has been the most widely studied in TBI because it is highly expressed in microglia and other immune cells. As indicated in Figure 1.1, NOX2 becomes activated when cytosolic subunits p67phox, p40phox and p47phox translocate from the cytosol to the membrane and bind to the membrane bound subunits, gp91phox and p22phox. Once activated, the complex catalyses the conversion of NADPH to its reduced NADP+ form and transfers an electron from NADPH to an oxygen molecule to produce a superoxide anion (O2·). NOX2 activation initiates the release of pro-inflammatory cytokines, ultimately leading to a pro-inflammatory cascade of events and thus a pro-inflammatory microglial phenotype.
Figure 1.1: NADPH oxidase (NOX2) assembly and activation.

NOX2 activation results in superoxide and ROS production and can give rise to microglial-related neuroinflammation. NOX2 becomes activated with cytosolic subunits p67\textsuperscript{phox}, p40\textsuperscript{phox} and p47\textsuperscript{phox} phosphorylate and translocate from the cytosol to the membrane and bind to the membrane bound subunits gp91\textsuperscript{phox} and p22\textsuperscript{phox}. Once assembled the activated complex transfers electrons from NADPH to \(\text{O}_2\) to produce superoxide (\(\text{O}_2^-\)). This initiates a pro-inflammatory microglial phenotype with the release of inflammatory mediators.

ROS generation occurs as a normal product of cellular metabolism, contributes to cellular signalling, and can be overproduced in disease [54]. Superoxide radicals (\(\text{O}_2^-\)) are a prominent ROS produced by the reduction of oxygen (\(\text{O}_2\)) by superoxide dismutases, which can then convert into hydrogen peroxide (\(\text{H}_2\text{O}_2\)) and ultimately lead to endoplasmic reticulum (ER) and oxidative stress. It is the formation of these free radicals which contribute to inflammatory changes.

Indeed, NOX2-driven ROS production is a critical feature of the innate immune response and is necessary for host defence [49]. The importance of this is highlighted in CGD patients who have increased susceptibility to infection due to defects in ROS production. However, researchers found that patients deficient of NOX2 could still produce ROS, indicating multiple NOX enzymes, whose discoveries arrived thereafter [49]. NOX1 has been described as the closest homologue to NOX2. Moreover, the membrane bound p22\textsuperscript{phox} is necessary for the activation of NOX3, which differs to NOX5 whose activation is independent of p22\textsuperscript{phox} and cytosolic subunits [51]. NOX4 has been detected in many cell types, including neurons [55], macrophages [56], and endothelial cells [57]. Researchers have demonstrated NOX4 is upregulated following intracerebral haemorrhage and is related to increased pyroptosis and BBB permeability mediated through mitochondrial oxidative stress [58]. Although NOX4 has structurally evolved...
from NOX2, both enzyme complexes induce a neuroinflammatory response through ROS production [49]. Evidently, chronic NOX2 activity promotes oxidative stress, drives neuroinflammation and leads to progressive cortical and hippocampal degeneration [59]. Therefore, it is no surprise that NOX2 has been implicated in many neuroinflammatory and neurodegenerative disorders such as TBI [59] and AD [60].

1.7.1 NOX2 activation drives TBI pathogenesis

The rationale for this project focusing on NOX2 is due to the detrimental effects it has in TBI pathology. NOX2 has been shown to be upregulated in reactive microglia following CCI and contributes to chronic neurodegeneration [39, 61]. Furthermore, NOX2 plays a critical role in neuronal damage and brain oedema [62]. NOX2 is robustly increased in CD68+ microglia as well as F4/80+ monocytes in the injured brain, indicating accumulation of peripheral macrophages [16]. This was associated with ameboid morphological features, cortical neurodegeneration and long-term neurological dysfunction following CCI, which is not observed in uninjured mice [16]. Moreover, unpublished data from the Loane lab using Image stream, a technique incorporating morphology and functionality of confocal microscopy coupled with flow cytometry, demonstrated high NOX2 expression in infiltrating brain macrophages and resident microglia 2 days post-TBI. The results revealed increased NOX2/ROS activation and more ameboid morphology in CD45hi macrophages compared to resident CD45int microglia. The data surrounding the relative NOX2 expression between resident microglia and infiltrating macrophages is limited but understanding the source and function of NOX2 is critical for therapeutic intervention for TBI and other neuroinflammatory disorders.

NOX2 inhibition, as demonstrated by genetically altered NOX2 deficient (NOX2−/−) mice alleviates ROS production, decreases microglial-mediated neurotoxicity, and provides neuroprotection after TBI [16, 61, 63]. Specifically, NOX2−/− mice had reduced lesion volume, upregulated neuronal density in the injured cortex and hippocampus, and had improved fine motor coordination on the beam walk test after TBI, when compared to injured wild type (WT) mice. Along with reduced pro-inflammatory mediators, NOX2 inhibition conferred neuroprotective activity by upregulating anti-inflammatory properties, Arginase 1, YM-1, and IL-4 in microglia/macrophages when compared to
injured WT mice [16]. A separate study showed that BMDMs from NOX2−/− mice stimulated with LPS/IL-4 decreased pro-inflammatory cytokines TNFα, IL-1β and IL-6 and nitrite production compared to WT mice following TBI [63]. Moreover, there were increased levels of IL-10 and phosphorylated STAT3 (pSTAT3) expression in NOX2−/− compared to WT LPS/IL-4 stimulated BMDMs. Co-incubation with neutralising anti-IL-10 reduced pSTAT3 and Arginase 1 expression in BMDMs. Additionally, anti-inflammatory markers were also upregulated in the ipsilateral cortex of NOX2−/− TBI mice [63]. These results indicate NOX2 deficiency increases anti-inflammatory activation that is mediated by IL-10 signalling through the STAT3 pathway and suggests a NOX2-dependent shift towards an anti-inflammatory phenotype that may contribute to neuroprotection. Combined, these findings indicate NOX2 as a major contributor in regulating microglial transitions. In the CNS microglia can alter their activation states depending on the NOX2 redox state [64]. Inhibiting NOX2 activation switched microglia from a classically activated state to an alternative state associated with anti-inflammatory properties [65]. In experimental TBI studies to date NOX2-dependent transitions between pro- and anti-inflammatory activation states have been carefully described [16, 63, 65].

In addition to TBI, the neuroprotective effects of NOX2 depletion have been studied in AD. Mice genetically deficient in the NOX2 cytosolic subunit p47phox, showed higher levels of YM1 mRNA in Aβ-induced microglial activation compared to WT mice [65]. Furthermore, NOX2−/− mice over expressing amyloid precursor protein, showed reduced oxidative stress and improved behavioural outcomes [66]. In a separate study investigating the effect of fasting-mimicking diet (FMD) in AD mice crossed with NOX2KO (3xTgNOX2KO), the protective effects of FMD resulted in reduced microglial activation, NOX2 expression, and superoxide toxicity [67]. NOX2 deletion also improved cognitive function and mitigated AD pathology progression. In a mouse model of spinal cord injury (SCI), inhibiting NOX2 using apocynin in monocyte-derived macrophages reduced ROS, improved functional and histological recovery in 14 but not 4-month-old mice, suggesting an age-dependent treatment effect with NOX2 inhibition [68].

Hence in TBI, and many other neuroinflammatory diseases, NOX2 inhibition is neuroprotective by suppressing sustained neuroinflammation and promoting an
alternative microglial state that upregulates the release of anti-inflammatory cytokines and restores tissue integrity in the injured brain [69]. Homeostatic microglia can enhance functional recovery after TBI and participate in CNS remodelling, such as promoting neurogenesis and the proliferation of neuroblasts in the injured hippocampus, which may be beneficial for recovery post-injury [70].

1.7.2 NOX2 as a double-edged sword

It is worth noting that in a mouse model of cerebral ischaemia, NOX2 has been reported to act as a “double-edged sword”, exaggerating brain injury acutely but promoting functional recovery in the chronic phase [71]. Yingze and colleagues demonstrated NOX2-mediated ROS worsened brain injury acutely as demonstrated by increased infarct volume, reduced neurological score and motor function at 3 days post-injury (DPI), but promoted neuroprotection through autophagy in the chronic phase of recovery. Gene Ontology analysis revealed NOX2 inhibition reduced angiogenesis, which is vital for vascular growth and improving functional recovery, and this was associated with increased NLRP3 inflammasome activation at 7 and 14 DPI. In humans, studies in CGD patients with defective ROS production showed upregulation of NLRP3 inflammasome activation due to mitochondrial damage [72]. NOX2 CRSIPRKO macrophages had enhanced nuclear factor kappa B (NFκB) transcriptional priming of NLRP3 along with increased mitochondrial damage resulting in the activation of the NLRP3 inflammasome. These findings, contrary to what was initially understood, suggest the timing of injury is crucial, and that NOX2 has dual roles in both the brain and in the periphery. Inhibiting NOX2 elicits an inflammatory response in the chronic phase while loss-of-function mutations in the genes encoding NOX2 subunits may trigger inflammatory cascades [71, 72]. These conflicting results consolidate the fact that further research into this complicated ROS-producing enzyme is warranted.

1.7.3 NOX2 inhibitors

Aside from the on-going debate of NOX2 function in disease, it is evident from the studies explained previously that chronic NOX2 activation plays a critical role in neuroinflammation and worsens long-term neurological outcomes, particularly in
neurodegenerative diseases (NDD). Therefore, identifying specific NOX2 inhibitors to attenuate this proinflammatory cascade is of utmost importance. Diphenyleneiodonium chloride (DPI\textsuperscript{*}) is a broad antioxidant and non-specific NOX inhibitor that prevents the generation of ROS by accepting an electron from flavin adenine dinucleotide (FAD), thus hindering the transfer to oxygen to form superoxide [73, 74]. Consequently, DPI\textsuperscript{*} inhibits other flavoenzymes and NOX isoforms which may have negative side effects for patients’ host defence [75].

In the late 2000s, apocynin was said to be the most selective NOX2 inhibitor [76], and was predicted to function by preventing the translocation of p47\textsuperscript{phox} from the cytosol to the membrane [77]. Researchers found that treating mice with apocynin post-CCI revealed improved sensorimotor function on the beam walk at 14 and 21-DPI, reduced lesion volume [78], and in another study reduced superoxide levels in the injured cortex and hippocampus [62]. In a mouse model of cerebral ischaemia, apocynin was administered 0.5h prior to ischaemia. Mice showed reduced infarct volumes and improved neurological scores in WT but not in NOX2\textsuperscript{-/-} ischemic mice, suggesting apocynin is targeting NOX2. Importantly, mice administered apocynin 1h post ischaemia did not have reduced superoxide levels or show any signs of neuroprotection by 24h [76]. The lack of NOX2 inhibition post-injury implies apocynin as a poor translatable NOX2 inhibitor, as pre-treatments or prophylaxis regimens are unlikely to be used in the clinical setting. This highlights the importance of developing highly specific NOX2 inhibitors for CNS injury.

GSK2795039 is a novel brain penetrant and specific NOX2 inhibitor shown to reduce NOX2-dependent ROS in vitro and in vivo [74]. GSK2795039, discovered by Hirano and colleagues, was synthesised at the National Center for Advancing Translational Sciences (NCATS) whereby the compound showed > 95% purity [79]. To understand the selectivity of GSK2795039 for NOX2, the competition for NADPH binding site of NOX2 was determined. Increasing concentrations of NADPH decreased the potency of GSK2795039 which indicates a competitive mode of action [74]. In contrast, the inhibitory potency of DPI\textsuperscript{*} was independent of NADPH concentrations. Hirano et al., demonstrated, for the first time, that systemic administration of GSK2795039 in vivo reduced ROS production by activated NOX2 enzyme following a paw inflammation
model. GSK2795039 was compared against other reported NOX2 inhibitors, including apocynin, which showed no reduction in NOX2 enzyme activity. Levels of NOX2-dependent ROS following GSK2795039 inhibition were similar to that in the genetically deficient NOX2 mice, indicating the specificity of GSK2795039 for NOX2.

In the TBI field, there is emerging preclinical evidence that GSK2795039 attenuates NOX2 dependent ROS production which is associated with reduced BBB damage, and neurological dysfunction including improved hippocampal dependent spatial memory assessed by the Morris Water Maze (MWM) [80, 81]. Recently, this compound was also tested in a mouse model of spared-nerve injury induced neuropathic pain. GSK2795039 treatment reduced microglial activation at 2 days post-spared-nerve injury and anxiety-like behaviour in female mice only, assessed by the marble burying test [82]. This study, and others [74, 80, 81], administered GSK2795039 pre-TBI to show acute NOX2 inhibition and improved neurological function. While these studies act as proof-of-concept data they are not clinically relevant given the prophylactic administration. Indeed, the effect of GSK2795039 as a post-TBI treatment on cell-specific NOX2-ROS signalling and related pro-inflammatory activation is unknown.

1.8 Inflammasomes

Immune cells express an array of receptors on their surface to respond to PAMPs/DAMPs and to initiate an innate immune response [28]. Of these innate immune receptors, NLRs sense pathogens in the cytoplasm. The NLR group of inflammasomes comprise of a central nucleotide-binding and oligomerisation (NACHT) domain, a C-terminal leucine rich repeat (LRR), and a N-terminal protein-protein interaction domain of either caspase-recruitment (CARD), a feature of the NLRC subfamily, or a pyrin domain (PYD), as is the case for NLRP family [83]. NLRs assemble with other proteins to form a multi-protein complex called inflammasomes, which activate caspase-1 to ultimately cleave IL-1β and IL-18 into their active form. Notably, inflammasomes do not exclusively require NLR and can be comprised of an absent in melanoma 2 (AIM 2), which is activated by double stranded DNA [84] and gives rise to casaspe-1 dependent IL-1β release. The inflammasome that was the major focus in this project is the NLRP3 inflammasome.
1.9 NLRP3 Inflammasome assembly and activation

NLRP3 inflammasome is a caspase-1 activating multi-protein complex which gives rise to a major inflammatory pathway [85]. NLRP3 is the most widely studied inflammasome due to its major contributions in the immune system in health and disease. It provides critical host defence against bacterial and viral infections. In the canonical NLRP3 inflammasome pathway, two signals are required for inflammasome initiation, priming followed by activation. As shown in Figure 1.2, DAMPs such as LPS can prime the cells through binding to TLRs and stimulate the NFκB pathway. IKK degrades IκB, the inhibitor molecule binding to NFκB, allowing translocation of NFκB to the nucleus. This priming step leads to the inactive form of NLRP3 and the upregulation of IL-1β and IL-18 genes. The second or activation step is induced by a broad range of PAMPs and DAMPs, including ROS, extracellular ATP/nigericin, and misfolded proteins such as Aβ. Upon activation, the inactive NLRP3 binds to the adaptor molecule apoptosis-associated speck-like protein containing a CARD (ASC) via the pyrin domain (shown in green in Figure 1.2). ASC interacts with pro-caspase-1 via the CARD domain (shown in blue in Figure 1.2) which form the cyclic assembly of the NLRP3 inflammasome complex [85]. This assembly promotes the cleavage of pro-caspase-1 into active caspase-1, which manifests into downstream inflammatory products including pyroptosis, IL-1β and IL-18 release. In non-canonical NLRP3 inflammasome activation, various gram-negative bacteria activate TLR4-MyD88 signalling, which promotes NFκB translocation and ultimately drives caspase-11 activation resulting in inflammasome activation and pro-inflammatory cytokine release [86].

1.9.1 Caspase-1 a key mediator of inflammatory processes

Active caspase-1 cleaves the cytosolic protein Gasdermin D (GSDMD) to create pores in the plasma membrane triggering pyroptotic cell death and subsequent release of mature IL-1β and IL-18 outside of the cell resulting in a robust inflammatory cascade [87]. IL-1β can also be released in the absence of cell lysis, but importantly GSDMD is required for pore formation and is crucial for caspase-11-dependent pyroptosis and subsequent IL-1β release [88]. Caspase-1 activity is a major output of inflammasome activation and has been shown to be upregulated in the CSF of moderate-severe TBI patients (male and female; GCS score ≤ 13; age 22-80 years), 96 hours after hospital
admission [89]. This increased caspase-1 activity positively correlated with increased ICP and poorer outcomes which gives further reason to study this pathway. Preclinically, caspase-1-mediated pyroptosis, including GSDMD cleavage and pro-inflammatory cytokines, IL-1β and IL-18, were attenuated with VX765, a caspase-1 inhibitor following TBI [90]. This was associated with increased anti-inflammatory cytokines IL-10 and TGF-β1 expression, improved BBB integrity and long-term neurological function following TBI.

On the other hand, a caspase-1-independent proapoptotic protein known as apoptosis-inducing factor (AIF) has been shown to be required for neuronal cell death in vitro [91]. siRNA downregulation of AIF, reduced neuronal cell death induced by glutamate toxicity and oxygen-glucose deprivation. Moreover, harlequin mutant mice that express 80% less AIF protein had reduced infarct volumes with little cell loss compared to WT ischemic mice; these molecular changes were associated with preserved motor function after ischemic injury [91]. Together, these findings indicate both pyroptotic- and apoptotic-mediated cell death that are caspase-1-dependent and independent mechanisms respectively, as targets to mitigate cell loss and downstream neuroinflammatory cascades.

1.9.2 NLRP3 inflammasome activation in TBI

NLRP3 inflammasome activation plays a vital role in the secondary injury of TBI [92]. The expression levels of NLRP3, ASC and caspase-1 were upregulated in microglia of the peri-contusion cortex acutely post-injury, and this was related to brain oedema and cortical lesion development [93]. Furthermore, genes related to NLRP3 inflammasome and caspase-1 protein activity were increased in the ipsilateral cortical tissue at 2-3 months post-injury [45].

The rationale for investigating NLRP3 inflammasome activation in the present study is because NOX2 activation has been shown to be upstream of the NLRP3 inflammasome. TBI showed increased immunoreactivity of NLRP3, ASC and IL-1β in the injured cortex and upregulates NLRP3-ASC complex formation which is significantly reduced in NOX2−/− mice compared to WT [94]. Combined, upregulation of NOX2 leads to oxidative stress dysregulation and damage, which primes and activates the NLRP3 inflammasome.
leading to pro-inflammatory cytokine-mediated neuroinflammation as depicted in the schematic below (Figure 1.2). This therefore highlights the NOX2-NLRP3 inflammatory axis as a potential target in neurological diseases.

**Figure 1.2: NLRP3 inflammasome assembly and activation**

The NLRP3 inflammasome is a caspase-1 activating multi-protein complex which gives rise to a major inflammatory pathway. There are two signals, priming and activation, required to activate the NLRP3. LPS primes the cells and promotes translocation of NFkB to the nucleus which gives rise to the inactive form of NLRP3 inflammasome and pro IL-1β and IL-18. DAMPS including ATP, ROS and Aβ stimulate the assembly and activation of the NLRP3 inflammasome which cleaves pro-caspase-1 into active caspase-1 and this stimulates the cleavage of active IL-1β and IL-18 from the pro form. Image created using BioRender.

However, because NOX2 may act as a double-edged sword, researchers indicate NLRP3 plays a dual and time-dependent role in brain injury. Following closed head injury in NLRP3<sup>-/-</sup> mice, the inflammatory response was exaggerated, there was increased BBB permeability and neurological damage compared to WT mice 24h [95]. However, NLRP3 inflammasome inhibition by MCC950 treatment 1h after TBI, reduced BBB damage and neurological impairments suggesting an important window in NLRP3 time-dependent activation along with an opportunity for treatment by targeting this pathway.
1.9.3 MCC950 is a potent NLRP3 inflammasome inhibitor

MCC950 is a potent and specific NLRP3 inhibitor which directly targets the ATPase site on the NACHT domain of NLRP3, blocking ATP hydrolysis responsible for assembly and activation of the NLRP3 inflammasome [96]. MCC950 blocks NLRP3-dependent ASC oligomerisation, a pivotal step in the NLRP3 inflammasome activation, but has no effect on non-NLR AIM2 inflammasomes, indicating the selectivity of MCC950 for NLRP3 [97]. MCC950 has been studied extensively in many inflammatory diseases, including cryopyrin associated periodic syndromes (CAPS), type-2 diabetes and atherosclerosis. Moreover, MCC950 shows promising therapeutic potential in several neuroinflammatory and neurodegenerative diseases in vivo. In EAE, a mouse model of multiple sclerosis, MCC950 attenuated NLRP3 inflammasome-mediated inflammation and simultaneously reduced EAE clinical score severity [97]. MCC950 inhibited NLRP3 inflammasome activation, microglial activation, and improved cognitive function in APP/PS1 mouse model of AD by increasing phagocytosis of Aβ in LPS/Aβ stimulated microglia [98]. Furthermore, MCC950 demonstrated reduced NLRP3-elicited neuroinflammation, specifically reduced counts of NLRP3-expressing microglia [93] and reduced brain oedema [99] following TBI.

Indeed, there is heterogeneity within neurological diseases such as TBI, AD, Parkinson’s Disease, Amyloid lateral sclerosis, and stroke but a common feature is chronic NLRP3 inflammasome activation [100]. Inhibitors of NLRP3 may therefore act as a powerful neurotherapeutic to relieve these chronic neuroinflammatory processes that negatively impact on neurological function.

1.10 The role of the immune system in neuroinflammation

It was previously thought that the CNS was an immune privileged organ, however in the past 15 years there is emerging evidence between the central-peripheral neuroimmune crosstalk [101]. As previously mentioned, microglia are key players in the response to trauma, but they do not act alone, and these neuroprotective glial cells also communicate with cells in the circulation. A study using bone marrow chimeric mice (C57BL/6J-GFP mice) elegantly demonstrated microglia (GFP⁺) activation precedes peripheral cell infiltration (GFP⁺) in cerebral ischemia [102]. The BBB participates in
microenvironment homeostasis by limiting the trafficking of cells and substances between the blood and brain, and so tightly regulates the brain’s immune response. However, dysregulation and damage to the BBB leads to increased permeability, allowing the influx of peripheral immune cells, therefore accelerating the inflammatory process [37]. Along with microglia, another type of innate immune cells in the brain are the perivascular macrophages (PVMs) which originate in the embryonic yolk sac and undergo several crucial activities at the interface between the tissue and the blood [103]. Located at the abluminal surface of blood vessels, PVMs maintain tight junctions and regulate vascular permeability, phagocytose harmful pathogens before they cross the vasculature into tissues, such as the brain [104]. In neurovascular disease, PVMs can employ a pro-inflammatory phenotype which is beneficial in limiting the effects of injury, but when uncontrolled can lead to chronic inflammation [104].

In TBI, there is increasing interest in the role the immune system plays in the progression of neuroinflammation. There is evidence of a temporal progression of the immune response to TBI [15, 105]. Immediately, the danger signals including ATP, HMGB1, and heat-shock proteins (HSPs) released by the damaged parenchyma and meninges bind to and activate resident myeloid cells such as by microglia as illustrated in Figure 1.3. Activated microglia signal to cells in the circulation by means of inflammatory mediators such as chemokines and cytokines. Within minutes to hours, neutrophils are recruited to the meninges and perivascular space with the aim of producing a respiratory oxidative burst to prevent secondary infection. NOX2-derived ROS is essential to the phagocytic activity of leucocytes, primarily neutrophils. [30]. The timing and cellular source of these immune responses is crucial in developing novel therapeutic strategies to mitigate neuroinflammation. For example, the infiltration of neutrophils is short lived as numbers start to decline within a few days [105] as illustrated in the time-course (Figure 1.4). Neutrophil recruitment is typically followed by infiltration of peripheral monocytes-derived macrophages, mainly CCR2-expressing monocytes, within the first 2-3 days, which phagocytose cell debris and aid in tissue repair and remodelling, alongside the microglia. However, macrophages can cause further damage by promoting sustained pro-inflammatory release, which can also further increase microglia activation in a cyclic manner resulting in a perpetuation of pro-inflammatory
cascades. This highlights the complex neuroimmune response to TBI. In a mouse model of TBI, a CCR2 antagonist, CCX872 significantly reduced inflammatory macrophage accumulation which was associated with improved TBI-induced hippocampal cognitive dysfunction [106].

The role of the adaptive immunity in TBI is not entirely clear, but what is known is that inflammation can continue days, weeks and even months post-injury [15]. As shown in Figure 1.3, both microglia and macrophages act as APCs, presenting antigens on their surface to signal T cell recruitment and direct an adaptive immune response. In the absence of resolution of the inflammatory response by the adaptive immune system, chronic inflammation can persist following TBI.

Figure 1.3: Temporal progression of the Immune Response to contusion TBI
Following TBI, DAMPs and PAMPs are released from damaged meninges and perivascular spaces which activate resident microglia cells. Microglia release pro-inflammatory cytokines and chemokines to signal to cells in the circulation which then infiltrate into the CNS through damaged and increased BBB permeability. Neutrophils are the first cells to enter the CNS and upregulate ROS production which is followed by monocyte-derived macrophage infiltration.
Both microglia and macrophages phagocytose cell debris and act as APCs presenting antigens to T cells and directing an adaptive immune response with the aim of resolving inflammation following TBI. Image adapted from Jassam et al., Neuron, 2017 [15].

As illustrated in the time-course (Figure 1.4) outlining the kinetics of the immune response to injury, by 2-weeks post-injury the brain is largely devoid of infiltrating myeloid cells, but levels of microglia and astrocytes remain elevated [105]. Although the relative T and B cell responses are lower than microglia and astrocytes, it is now widely accepted that adaptive immune cells accumulate and persist in the CNS for months following TBI (Daglas, 2019 #2083). Recently, it has been shown that cytotoxic T cells are linked to cognitive dysfunction and worsened outcomes following TBI [107], while IL-2-producing Tregs have anti-inflammatory properties and reduced TBI-elicited neuroinflammation in mice [108]. These findings indicate the dual roles of cells of the adaptive immune system in TBI and highlight the importance of the neuroinflammatory – neuroimmune crosstalk.

![Figure 1.4: Time-course of the immune response to TBI.](image)
Immediately following TBI, cellular damage results in the release of DAMPs, which prompt resident glial cells, microglia, and astrocytes, to release cytokines and chemokines to recruit neutrophils which aid in the containment of the injury site. As neutrophil numbers diminish there is influx of peripheral monocytes which, along with activate microglia, accumulate around the site of injury and aid in tissue repair. T and B cells are recruited into the CNS at later timepoints in response to injury and facilitate an adaptive immune response. Image adapted from McKee et al., Frontiers Immunology, 2016 [105] and created using BioRender.

Not only does the immune system play a role in driving neuroinflammation, TBI has been reported to negatively impact organs in the peripheral system. Human TBI studies have reported increased neutrophilia and oxidative burst in the blood with concomitant decreased phagocytic ability [109]. In mice, TBI showed chronic immune system dysfunction demonstrated by neutrophil and monocyte phagocytic impairments and impaired respiratory burst activity in the blood and spleen [30]. Moreover, TBI induced acute suppression and chronic augmentation of pro-inflammatory cytokines in peripheral myeloid cells along with exacerbated Th1 cytokine production in the chronic phase. Combined, these results indicate altered immune function following TBI. In a separate preclinical study, intestinal inflammation (acute colitis) during chronic TBI was found to exacerbate systemic immune responses, deficits in fine motor coordination, hippocampal neurodegeneration, and cognitive dysfunction compared to mice subjected to TBI only. The authors conclude that systemic inflammation drives a prolonged and altered immune response that concurrently caused neuro-behavioural deficits [31].

Taken together, upregulated innate and adaptive immune responses, inflammatory mediators, oxidative bursts and immune dysregulation may lead to systemic damage further enhancing secondary injury. Targeting these pathophysiological cascades of events could potentially alleviate chronic neuroinflammation and prevent multiple organ dysfunction/failure following TBI [110].
1.11 Aims of the project

The rationale for this project stems from the detrimental effects of NOX2 activation in TBI and how inhibiting NOX2 promotes an anti-inflammatory transition that is neuroprotective [16, 63]. Moreover, the data surrounding pharmacological inhibition of NOX2 is more limited. Therefore, the hypothesis of this PhD project states that pharmacological inhibition of NOX2 by GSK2795039 can attenuate pro-inflammatory mediators, including NLRP3 inflammasome activation, in resident microglia and infiltrating myeloid cells, and improve neurological outcomes following TBI. This is schematically illustrated in Figure 1.5 below.

To address this hypothesis, the specific aims of the project were:

- To investigate the role of NOX2-mediated neuroinflammation in microglia
- To characterize GSK2795039, a novel specific NOX2 inhibitor, in neuroinflammatory models of microglial activation and identify the specific mechanism of inhibition
- To translate in vitro findings in vivo and establish cell-specific functional outputs and neuro-behavioural tasks in an experimental model of TBI
- To identify the source of NOX2/ROS and NLRP3 inflammasome activation in vivo over the temporal progression of TBI
- To evaluate the efficacy of GSK2795039 treatment post-TBI in vivo in regulating NOX2-mediated NLRP3 inflammasome activation and neurological impairments
Figure 1.5: Working hypothesis
The hypothesis of this PhD project states that pharmacological inhibition of NOX2 by GSK2795039 can attenuate pro-inflammatory mediators, including NLRP3 inflammasome activation, in resident microglia and infiltrating myeloid cells to improve neurological outcomes following TBI.
Chapter 2: Materials and Methods
2.1 Materials

2.1.1 Media, buffers and solutions

**Complete Dulbecco’s Modified Eagle’s-Medium (cDMEM) for IMGs**

- 500 ml DMEM (Sigma-Aldrich/Gibco)
- 10% Fetal bovine serum (FBS; Sigma-Aldrich)
- 1% Penicillin/Streptomycin (Sigma-Aldrich)
- 1% L-Glutamine (Sigma-Aldrich)

**Complete Roswell Park Memorial Institute (RPMI) medium for mononuclear cells**

- 500 ml RPMI
- 10% Fetal bovine serum (FBS)
- 1% Penicillin/Streptomycin
- 1% L-Glutamine

**Complete DMEM/F-12 for Primary Microglia**

- 500 ml DMEM/F12 containing L-Glutamine
- 10% FBS
- 1% Penicillin/Streptomycin

**Phosphate buffered saline (PBS) 10X**

- 400 g Sodium chloride (NaCl; 1.4 M; Sigma-Aldrich)
- 58 g Sodium hydrogen phosphate (Na$_2$HPO$_4$; 0.08 M)
- 10 g Potassium di-hydrogen phosphate (KH$_2$PO$_4$; 0.01 M)
- 10 g Potassium chloride (KCl; 0.03 M)

**ELISA wash buffer (PBST)**

- 500 ml 10X PBS
- 2.5 ml Tween 20 (Sigma-Aldrich)
- 4.5 L dH$_2$O

**ELISA blocking buffer (1% BSA)**
5 g Bovine serum albumin (BSA; Sigma-Aldrich)
500 ml PBS (1X; Sigma-Aldrich)

**ELISA stop solution**
28.05 ml Hydrogen sulfate (H$_2$SO$_4$; 2N; Sigma-Aldrich)
471.95 ml dH$_2$O

**Western blot lysis buffer**
10 µl Phosphatase inhibitor cocktail 1 (Sigma-Aldrich)
10 µl Phosphatase inhibitor cocktail 2 (Sigma-Aldrich)
10 µl Protease Inhibitor cocktail (Sigma-Aldrich)
100 µl Pierce™ RIPA buffer (Thermo Scientific)

**Tris-buffered saline (TBS) 10X**
63.04 g Tris HCL (Trizma; Sigma-Aldrich)
175.32 g Sodium Chloride
2 L dH$_2$O
pH to 7.5

**Western blot wash buffer TBS-Tween 20 (TBST)**
100ml TBS
900ml dH$_2$O
0.5 ml Tween 20

**Western blot blocking buffer 5% Marvel in TBST**
5 g Skim milk powder (Sigma)
100 ml TBST

**Separating gel buffer (1.5M)**
90.8 g Tris base
1 g Sodium dodecyl sulfate (SDS)
450 ml dH$_2$O
pH to 8.8

**Stacking gel buffer (1M)**
15.13 g Tris base
1 g SDS
200 ml dH₂O
pH to 6.8

**Stripping buffer**
15 g Glycine (Sigma-Aldrich)
1 g SDS
10 ml Tween 20
pH to 2.2

**FACS buffer for flow cytometry**
500 ml PBS (Sigma-Aldrich)
5 ml FBS (Sigma-Aldrich)
10 ml EDTA (Sigma-Aldrich)

### 2.1.2 General reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Product code</th>
<th>Supplier</th>
</tr>
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<tbody>
<tr>
<td>Bovine Serum Albumin (BSA)</td>
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<td>Sigma-Aldrich</td>
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<tr>
<td>DMEM</td>
<td>41965062</td>
<td>Gibco</td>
</tr>
<tr>
<td>DMEM/ F12</td>
<td>31330-038</td>
<td>Gibco</td>
</tr>
<tr>
<td>DMSO</td>
<td>472301</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>DNase Type 1</td>
<td>D4263</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Ethanol, Absolute, Molecular Biology Grade</td>
<td>16606002</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>Fetal Bovine Serum (FBS)</td>
<td>F9665</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Penicillin-Streptomycin</td>
<td>P4333</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Phosphate buffered saline (PBS)</td>
<td>D8537</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>Streptavidin HRP</td>
<td>Part of ELISA kit</td>
<td>R&amp;D Systems</td>
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<tr>
<td>Reagent</td>
<td>Working concentration</td>
<td>Treatment time</td>
</tr>
<tr>
<td>--------------------------------------</td>
<td>------------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>ATP</td>
<td>1mM</td>
<td>10 min</td>
</tr>
<tr>
<td>Diphenyleneiodonium (DPI⁺)</td>
<td>0.1-0.5 μM</td>
<td>1h</td>
</tr>
<tr>
<td>GSK2795039</td>
<td>5-25 μM</td>
<td>1h</td>
</tr>
<tr>
<td>Lipopolysaccharide (LPS)</td>
<td>100ng/ml</td>
<td>8h or 24h</td>
</tr>
<tr>
<td>MCC950</td>
<td>0.01-0.1 μM</td>
<td>1h</td>
</tr>
<tr>
<td>Nigericin</td>
<td>10μM</td>
<td>30 min</td>
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### 2.1.4 In vivo drug administration

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<th>Supplier</th>
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</thead>
<tbody>
<tr>
<td>GSK2795039</td>
<td>100 mg/kg</td>
<td>Intraperitoneal</td>
<td>HY-18950</td>
<td>MedChem Express</td>
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<tr>
<td>Vehicle</td>
<td>10% DMSO, 90% Corn oil</td>
<td>Intraperitoneal</td>
<td>472301</td>
<td>Sigma-Aldrich</td>
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</table>

### 2.1.5 ELISA antibodies

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Block</th>
<th>Species</th>
<th>Top Working (pg/ml)</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>1 % BSA</td>
<td>Mouse</td>
<td>1000</td>
<td>R&amp;D</td>
</tr>
<tr>
<td>TNFα</td>
<td>1 % BSA</td>
<td>Mouse</td>
<td>2000</td>
<td>R&amp;D</td>
</tr>
</tbody>
</table>
IL-10 | 1 % BSA | Mouse | 2000 | R&D
IL-6 | 1 % BSA | Mouse | 1000 | R&D
IL-1ra | 1 % BSA | Mouse | 10,000 | R&D
IL-18 | 1 % BSA | Mouse | 3,000 | R&D
IL-1β | 1 % BSA | Rat | 4,000 | R&D
TNFα | 1 % BSA | Rat | 4,000 | R&D

2.1.6 Other ELISA reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Product code</th>
<th>Supplier</th>
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<tr>
<td>1x TMB Substrate Solutions</td>
<td>002023</td>
<td>Life Technologies</td>
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2.1.7 Percentage of separating gels for Western Immunoblot

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<tr>
<th>Separating gel</th>
<th>8%</th>
<th>10%</th>
<th>12%</th>
<th>15%</th>
<th>Stacking gel</th>
<th>4%</th>
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</thead>
<tbody>
<tr>
<td>30% Acrylamide</td>
<td>2.7 ml</td>
<td>3.3 ml</td>
<td>4 ml</td>
<td>5 ml</td>
<td>30% Acrylamide</td>
<td>660 µl</td>
</tr>
<tr>
<td>dH₂O</td>
<td>4.6 ml</td>
<td>4.0 ml</td>
<td>3.3 ml</td>
<td>2.3 ml</td>
<td>dH₂O</td>
<td>2.8 ml</td>
</tr>
<tr>
<td>Buffer pH 8.8</td>
<td>2.5 ml</td>
<td>2.5 ml</td>
<td>2.5 ml</td>
<td>2.5 ml</td>
<td>Buffer pH 6.8</td>
<td>500 µl</td>
</tr>
<tr>
<td>10% Ammonium sulfate</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>10% Ammonium sulfate</td>
<td>40 µl</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>10% SDS</td>
<td>40 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>6 µl</td>
<td>4 µl</td>
<td>4 µl</td>
<td>4 µl</td>
<td>TEMED</td>
<td>4 µl</td>
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2.1.8 Primary Western blot antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Molecular Weight (kDa)</th>
<th>Species used</th>
<th>Host of antibody</th>
<th>Working dilution</th>
<th>Product code</th>
<th>Supplier</th>
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<tr>
<td>ASC</td>
<td>22</td>
<td>Mouse</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>AG-25B-0006</td>
<td>Adipogen Life Sciences</td>
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<tr>
<td>β-actin</td>
<td>42</td>
<td>Mouse</td>
<td>Mouse</td>
<td>1:50,000</td>
<td>A1978</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>Caspase-1</td>
<td>Pro: 40 Cleaved: 20</td>
<td>Mouse</td>
<td>Mouse</td>
<td>1:1000</td>
<td>AG-20B-0042</td>
<td>Adipogen Life Sciences</td>
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### 2.1.9 Secondary Western Immunoblot antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Working dilution</th>
<th>Product code</th>
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</thead>
<tbody>
<tr>
<td>Rabbit Anti-Goat</td>
<td>1:5000</td>
<td>305-035-003</td>
<td>Jackson ImmunoResearch</td>
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<tr>
<td>Goat Anti-Mouse</td>
<td>1:5000</td>
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<td>Goat Anti-Rabbit</td>
<td>1:5000</td>
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### 2.1.10 Other Western Immunoblot reagents

<table>
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<tr>
<th>Reagent</th>
<th>Product code</th>
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</thead>
<tbody>
<tr>
<td>30 % Acrylamide/Bis solution 37.5:1</td>
<td>1610158</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>4x Lamelli Sample Buffer</td>
<td>1610747</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>10x Tris/Glycine/SDS Buffer</td>
<td>1610772</td>
<td>Bio-Rad</td>
</tr>
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<td>10x Tris/Glycine Buffer</td>
<td>1610771</td>
<td>Bio-Rad</td>
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<tr>
<td>Trans-Blot Turbo 5x Transfer Buffer</td>
<td>10026938</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>WesternBright™ Quantum Chemiluminescent HRP Substrate kit</td>
<td>K-12042-D20</td>
<td>Advansta</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>M6250</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>WesternBright™ ECL-spray</td>
<td>K-12049-D50</td>
<td>Advansta</td>
</tr>
<tr>
<td>Western blot detection system</td>
<td>T7024</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>TEMED</td>
<td></td>
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### 2.1.11 Flow Cytometry antibodies, dyes

<table>
<thead>
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<th>Antibody</th>
<th>Fluorochrome</th>
<th>Working dilution</th>
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<tr>
<td>Fc block</td>
<td></td>
<td>1:200</td>
<td>553141</td>
<td>BD Biosciences</td>
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<tr>
<td>NOX2/gp91phox</td>
<td>Alexa Fluor 647</td>
<td>1:100</td>
<td>bs-3889R-A647-BSS</td>
<td>Bios</td>
</tr>
<tr>
<td>CD45</td>
<td>BV785</td>
<td>1:400</td>
<td>103149</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD11b</td>
<td>APCeF780</td>
<td>1:400</td>
<td>47-0112-82</td>
<td>Invitrogen</td>
</tr>
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</table>

---

**Life Sciences**

**NLRP3** 110 Mouse Mouse 1:1000 AG-20B-0014-C100 Adipogen Life Sciences

**NOX2/gp91** 58 Mouse Rabbit 1:1000 Ab129068 Abcam

**IL-1β** Pro: 37 Cleaved: 17 Mouse Rabbit 1:1000 Ab254360 Abcam
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Product code</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOXP3/ Transcription Factor Staining Buffer Set</td>
<td>00-5523-00</td>
<td></td>
<td>Invitrogen</td>
</tr>
<tr>
<td>CountBright™ Absolute Counting Beads</td>
<td>C36950</td>
<td></td>
<td>Invitrogen</td>
</tr>
<tr>
<td>ARC TM Amine Reactive Compensation Bead kit</td>
<td>A10346</td>
<td></td>
<td>Invitrogen</td>
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<tr>
<td>OneCompeBeads</td>
<td>01-1111-42</td>
<td></td>
<td>Invitrogen</td>
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<tr>
<td>Brefeldin A</td>
<td>5µg/ml</td>
<td>B7651</td>
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<tr>
<td>Monensin (GolgiStop)</td>
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<td>554724</td>
<td>BD Biosciences</td>
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<td>Collagenase-D</td>
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<td>11088866001</td>
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</tr>
<tr>
<td>Deoxyribonuclease I (DNase I)</td>
<td>20 U/ml</td>
<td>D4263</td>
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### 2.1.12 Other Flow Cytometry reagents

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<td>TNFa</td>
<td>Mm00443258_m1</td>
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<td>IL1b</td>
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<td>Cybb</td>
<td>Mm01287743_m1</td>
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<td>Cyba</td>
<td>Mm00514478_m1</td>
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<td>IL6</td>
<td>Mm00446190_m1</td>
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<td>Cd68</td>
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<td>Applied BioSystems</td>
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<tr>
<td>gapdh</td>
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<td>Applied BioSystems</td>
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2.1.14 Other RT-PCR reagents

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<td>11832113</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>RNeasy Mini kit (50)</td>
<td>74104</td>
<td>Qiagen</td>
</tr>
<tr>
<td>CellPath™ Cryospray Freezer Spray</td>
<td>12705308</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>RNase AWAY™ Surface Decontaminant</td>
<td>10666421</td>
<td>Thermo Scientific</td>
</tr>
</tbody>
</table>

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Immortalised microglia cell line culture

IMG cells were obtained from Millipore and cultured in complete Dulbecco’s Modified Eagle’s Medium (cDMEM) containing 4.5 g/L glutamine (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich), 1% (v/v) penicillin/streptomycin (Sigma-Aldrich) at 37°C with 5% CO₂. The IMG cells were cultured in T75 flasks for 3-4 days until fully confluent and cells between passage 5-15 were used for experimentation.

2.2.1.2 Cell counting and seeding

Once confluent, cells were trypsinised using Trypsin-EDTA (Sigma-Aldrich; 5 min; 37°C), centrifuged (1350 RPM; 4°C; 5 min) and the pelleted cells were resuspended in cDMEM. Cell solution was diluted in Trypan Blue (Sigma-Aldrich) and 10 µl was pipetted into a haemocytometer for counting. Viable cells were stained white and differentiated from dead cells in blue. The concentration of cells/ml was determined by multiplying the average number of viable cells by the trypan blue dilution factor and by 10⁴ to calculate the number of cells per ml. Depending on the experiment, IMG cells were seeded into either 96- or 12-well plates at the densities shown in Table 2.2.1.

<table>
<thead>
<tr>
<th>Well size of plates</th>
<th>Dimensions</th>
<th>Density (Cells/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>96-well</td>
<td>Area of 1 well= 0.32 cm²</td>
<td>7.5 x10⁴</td>
</tr>
<tr>
<td>12-well</td>
<td>area of 1 well =3.8cm²</td>
<td>1x10⁶</td>
</tr>
</tbody>
</table>

Table 2.2.1: Densities of cells seeded into different well sizes.
2.2.1.3 IMG treatments

There are two models of stimulation used in these IMG cells. Firstly, stimulating the IMG cells with LPS, a TLR4 agonist which activates the microglia, upregulates NFκB pathway and induces inflammation. In this model, IMG cells were pre-treated with GSK2795039 (Sigma; 1-25 µM; 1h; 37°C) or diphenyleneiodonium chloride (DPI*; Sigma-Aldrich; 0.01-0.5 µM; 1h; 37°C) and then stimulated with LPS (Sigma-Aldrich; 100 ng/ml; 24h). The second model of stimulation involved activating the NLRP3 inflammasome through priming and activating microglia cells. The IMG cells were pre-treated with GSK2795039 (1-25 µM; 1h; 37°C) or MCC950 (Millipore; 0.001-0.5 µM; 1h), stimulated with LPS (100 ng/ml; 8h), and subsequently activated with ATP (Sigma-Aldrich; 1 mM; 10 min) or nigericin (Invivogen; 10 µM; 30 min). Following stimulation, the supernatant was collected to measure nitrite levels (2.2.2.2), cytokines (2.2.3) and lactate dehydrogenase (2.2.2.4). ROS (2.2.2.1), cell viability (2.2.2.3), NOX activity assay (2.2.2.5), Western blot (2.2.4) or Flow cytometry (2.2.5) was carried out on the cells.

2.2.1.4 Primary microglia cell cultures

Primary microglia were cultured from cerebral cortices of postnatal day (p) 1 male and female Wistar Rat pups. A total of 31 p1 Wistar rat pups were used in experiments. Decapitation was used as the method of euthanasia. The brains were isolated by cutting through the skull along the mid-line of the brain. The skull was peeled back from the brain using forceps and the cortices were removed using a spatula. The cerebral cortices were placed in a petri-dish containing DMEM/F12 (Gibco) and freed from meninges. The cortices were washed once in DMEM/F12 and trypsinized (0.25% Trypsin/EDTA, 1X; Invitrogen) for 10 min at 37°C. This enzymatic digestion was stopped by placing the cortices into DNase/Trypsinase solution (DNase Type I; Sigma/ Trypsin Inhibitor from Egg whites; Sigma) for 10 min at 37°C. This enzymatic digestion was stopped by placing the cortices into DNase/Trypsinase solution (DNase Type I; Sigma/ Trypsin Inhibitor from Egg whites; Sigma) for 10 min at 37°C. The tissue is then washed three times with DMEM/F12. Growing media (DMEM/F12 supplemented with 10% FBS and 1% (v/v) penicillin-streptomycin; 3-5 ml) was added to the tissue and used to titrate 10 times using a sterile transfer pipette. This was repeated twice to ensure sufficient homogenisation of the tissue. Cells were collected by centrifugation (1,000 RPM; 10 min;
RT), resuspended in DMEM/F12 (10 ml) and this mixed glial suspension were plated in Poly-D-Lysine (Sigma-Aldrich) coated T75 culture flasks at 37°C with 5% CO₂.

After 7-10 days in culture, the microglia were detached from the mixed glia (astrocyte-microglia) cultures using an orbital shaker (Incu-Shaker mini, Benchmark Scientific; 100 RPM; 1h; 37°C), centrifuged (1000 RPM; 10 min; RT) and cells were counted using Trypan blue. Microglia were plated in 96-well plates at a density of 1x10⁵ cells/well in DMEM/F12 medium (Gibco) supplemented with 10% FBS and 1% (v/v) penicillin-streptomycin. The next day the media was changed to remove non-adherent cells and one day later the cells were ready for experimentation where they were stimulated with LPS (100 ng/ml) for 8h or 24h. Following this, ROS production and cell viability was measured in the cells and the supernatants were collected to assess nitrite production, the pro-inflammatory cytokine release of TNFα and IL-1β (R&D systems) and lactate dehydrogenase (LDH) as a measure of pyroptosis.

**Figure 2.1:** Primary microglia cultured from cortices of neonatal rat pups. This image was created using BioRender.

### 2.2.2 Plate based assays

#### 2.2.2.1 Measurement of intracellular ROS

Intracellular reactive oxygen species (ROS) levels were measured using 5-(and-6)-chloromethyl-2’,7’-dichlorodihydrofluoresceindiacetate (CM-H2DCFDA; Invitrogen). Following cell stimulation, supernatants were collected, and the cells were incubated with CM-H2DCFDA (6 µM; 200 ml/well) diluted in PBS for 45 min at 37°C in 5% CO₂. Hydrogen peroxide (100 µM; 20 µl/well) was added to control wells for a further 5 min to act as a positive control for ROS production. Fluorescence was measured immediately at excitation and emission wavelengths of 485 and 528nm respectively using Spectra

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Max Gemini Microplate Reader (Molecular Devices, USA). Data are presented as a percentage of controls.

2.2.2.2 Nitric oxide assay

The nitrite released into the supernatant was measured as an indicator of nitric oxide production using the Griess reagent assay (Invitrogen). Sodium nitrite (NaNO₂) standard solution (20 µM) was serially diluted three times (1:2) in serum free media and added to the plate in triplicate (150 µl/well). Cell supernatants (150 µl/well) were added to the plate. Deionised water (130 µl/well) and Griess Reagent (20 µl/well; 1:1 N-(1-naphthyl) ethylenediamine dihydrochloride and sulfanilic acid) were added into each well. The plate was incubated for 30 min at room temperature. The absorbance was read at 548 nm using Versa MAX tunable Microplate Reader with SoftMax Pro software (Molecular Devices, USA). A standard curve was generated using NaNO₂ and absorbance values of unknown samples were converted into micromoles in reference to this standard curve.

2.2.2.3 Cell Viability Assay

Cell viability was assessed using a microculture (3-(4,5- dimethylthiazol-2-yl)- 2,5- diphenyltetrazolium bromide (MTT, EMD Millipore)- based colorimetric assay. Immediately following the ROS assay, the cells are washed once with PBS (100 µl/well) and incubated with MTT dissolved in media containing 10% FBS for 4h at 37°C in 5% CO₂. The viable cells produced MTT formazan crystals which was solubilised with 0.04N HCL and isopropanol. The absorbance was read at 570nm using Versa MAX tunable Microplate Reader with SoftMax Pro software (Molecular Devices, USA). Cell viability was expressed as a percentage of the viable cells in the control group.

2.2.2.4 Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) was measured in the supernatant as an indicator of pyroptosis using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega; G1780). As LDH is an enzyme that converts NAD into NADH which interacts with a probe to elicit colour, this can be quantified on a colorimetric plate reader. LDH assay buffer and LDH substrate were thawed at room temperature before adding the buffer (12 ml) into the
substrate to form the LDH reagent, which was protected from light. 1h prior to collecting supernatants, 1X lysis buffer was added to a separate group of cells to mimic cell death and predict an LDH response. Following stimulation, the conditioned media (40 µl) was added to a 96-well flat bottom plate and the LDH reagent (40 µl) was added on top. The positive control (1 µl) and stimulation media (39 µl) was also added to the plate combined with LDH reagent (40 µl). The plates were covered with foil, left at room temperature for 30-60 min and checked regularly to ensure the reaction was not saturated. Stop solution (40 µl) was then added to the plate to stop the reaction and the plate was read in a spectrophotometer at 490nm. Data are presented as a percentage of controls.

2.2.2.5 NOX activity assay
Microglial NADPH oxidase activity was measured using lucigenin-enhanced chemiluminescence [111]. IMG cells seeded in 12-well plates were harvested in ice-cold PBS (400 µL), lifted using a sterile cell scraper, and homogenised using a 1 mL micropipette. Total superoxide production was assessed in 100 µL of cell suspension, transferred to white 96 well microplates containing NADPH (100 µM; TCI Europe, #T0521) and lucigenin (100 µM; TCI Europe, #2315971). Additional wells containing superoxide dismutase (SOD; 15 IU) for each sample were used to subtract the SOD-uninhibitable portion from the total superoxide production. An additional well containing general NOX inhibitor apocynin (2mM; Sigma, #W508454) was added as a negative control. The plate was incubated while relative luminescence was measured at 37°C for 1h (Luminoskan™ Ascent, ThermoFischer). Relative NOX-driven superoxide production was calculated as the fold of control of the integrated area under the curve of SOD-inhibitable lucigenin chemiluminescence.

2.2.3 Enzyme-Linked Immunosorbent Assay (ELISA)
ELISA kits (R&D Systems) were used to measure an array of cytokines from cell supernatant (Table 2.1.5). Medium binding 96-well microtiter ELISA plates (Griener Bio-one) were coated in cytokine specific capture antibody diluted in PBS (50 µl/well) and incubated overnight at room temperature. Plates were then washed three times with
ELISA wash buffer, PBS containing 0.05% Tween-20 (PBS-T) and blocked in 1% BSA in PBS (150 µl/well) for 1h at room temperature. The plates were washed (PBS-T x 3) and cytokine specific standards were serially diluted (1:2) in 1% BSA from a top working standard and added to the plate in triplicate (50 µl/well). Cell supernatants were added undiluted or diluted in 1% BSA (50 µl/well) and each plate contained three blank wells of 1% BSA (50 µl/well). Plates were incubated for 2h at room temperature or overnight at 4°C. Plates were washed (PBS-T x 3) and cytokine specific detection antibody diluted in 1% BSA was added to each well (50 µl/well) and incubated for 2h at room temperature. Plates were washed (PBS-T x 3) and horse-radish peroxidase (HRP)-conjugated streptavidin diluted in 1% BSA was added to each well and incubated for a further 20 min in the dark at room temperature. Plates were washed (PBST x 5) and substrate solution (TMB; Life Technologies) was added to the wells (50 µl/well) and the plates were incubated in the dark at room temperature. Once the standard wells turned blue, which indicates the standard curve had developed, the enzyme reaction was stopped with sulphuric acid (1M H₂SO₄; 25 µl/well). The absorbance was read immediately at 450nm using Versa MAX tunable Microplate Reader with SoftMax Pro software (Molecular Devices, USA). A standard curve was generated, and unknown cytokine levels produced were determined in reference to this standard curve.

2.2.4 Western blot

2.2.4.1 Protein isolation and quantification

IMG Cells were cultured in 12-well plates, rinsed once with PBS and protein was isolated using lysis buffer containing protease and phosphatase inhibitor cocktails 1 and 2 (1:100 in RIPA buffer; Sigma-Aldrich) for 20 min on ice. Cells were scraped into eppendorfs, sonicated (6 pulses on ice at a power setting of 2-continuos) and centrifuged (15,000 RPM; 15 min; 4°C) to pellet the insoluble protein. The cell lysates (supernatant) were collected and a sample of this was used to determine the protein concentration using Pierce™ BCA Protein Assay kit (Thermo Scientific). The cell lysate (10 µl) was diluted in RIPA (90 µl) and added to the plate in duplicate (25 µl/well). BCA Standards were serially diluted in RIPA at a working concentration of 1 µg/µl and added to the plate in duplicate. BCA reagent (50:1, Reagent A:B) was added into each well (200 µl/well) and the plate
was incubated (30 min; 37°C) before the absorbance was read at 562nm using VersaMAX tunable Microplate Reader with SoftMax Pro software (Molecular Devices, USA). A standard curve was generated, and the protein concentrations of unknown samples were determined from the interpolated values obtained from this standard curve. Samples were normalised to 1 ug/ul using RIPA, 4x Lamelli buffer (Bio-Rad) and 2-mercaptoethanol (Sigma-Aldrich) to reduce disulfide bonds. The samples were heated (90°C; 10 min) before loading onto gels.

2.2.4.2 Immunoblotting and protein analysis

Cell lysates of equal protein were electrophoresed through 8-15% SDS-polyacrylamide gels (Table 2.1.7) depending on the molecular weights of the proteins, with the higher molecular weight proteins running at a lower % of acrylamide. The gels were then transferred to 0.2 µM pore-sized nitrocellulose membranes using Trans-Blot® Turbo™ Transfer Starter System (Bio-Rad) followed by blocking in 5% milk in Tris-buffered saline-Tween-20 (TBST; 2.1.1). After blocking, the membranes were incubated with respective primary antibodies in 5% milk in TBST as described in Table 2.1.8, overnight at 4°C. The membranes were washed in TBST (3 x 5 min) and incubated in HRP-conjugated secondary antibodies (Table 2.1.9) in 5% milk in TBST for 2h at room temperature. Lastly, the membranes were washed (3 x 5 min) and detection was carried out using enhanced chemiluminescence (WesternBright™ ECL-spray, Western blot detection system; Advansta). Proteins were normalised to β-actin, a house keeping gene and quantified using Image Lab software v6.0.1 build 34.

2.2.5 Flow cytometry

2.2.5.1 Flow cytometry in IMG and Isolated mononuclear cells from CNS

IMG cells, blood or isolated mononuclear cells from the ipsilateral cortex of the CNS were processed into a single-cell suspension in 96-well V-plates and immediately stained with the ROS indicator DHR123 (Invitrogen; 1:500; 37°C; 30 min) or FLICA (BioRad; 1:60; 37°C; 30 min). Cells were washed with PBS and stained with Live/Dead (Invitrogen; 50 µl/tube diluted 1:600) in PBS and incubated (15 min; dark; RT). Isolated mononuclear cells were then washed with FACS buffer (150 µl) and resuspended in a master mix (15 min; dark; RT on orbital shaker) of surface fluor-conjugated antibodies (CD16/32 flow...
cytometry block (Fc block), CD11b, CD45, Ly6G, Ly6C, CD3, CD4, CD8, IL-1R) described in detail in Table 2.1.11. Following a wash step, cells underwent intracellular staining whereby cells were fixed by adding Fixation/Permeabilization working solution (1:4 ratio; 50 µl/well), pulse vortexed and incubated (30 min; dark; RT on orbital shaker). Cells were washed (2 x 150 µl) in 1X Permeabilization buffer (diluted in dH2O), centrifuged (1350 RPM; 5 min; 4°C). The pelleted cells were re-suspended in NOX2/gp91^phox (1:100) and IL-1β (1:200) in 1X Permeabilization buffer, and incubated (overnight; 4°C; dark). The following day, cells were washed in 1X Permeabilization buffer (2 x 150 µl) and finally washed in FACS buffer (150 µl/well). Cells were re-suspended in FACS buffer (200 µl/well), transferred into facs tubes, and stored at 4°C in the dark until analysed by the flow cytometer. In the case of IMG cells, following FLICA stimulation, cells were washed with FACS buffer (150 µl) and either directly fixed (4% PFA) or continued with intracellular staining.

2.2.5.2 Flow cytometry analysis

Flow cytometric data was acquired using Cytek Aurora Spectral flow cytometer with SpectroFlo® software. Compensation was calculated using single stained cells. Cell-specific fluorescence minus one (FMO) controls were used as a guide for gating strategies. Live, single, CD45^+ cell populations were identified using the gating strategy illustrated in 2.2.6. FloJo software (TreeStar Inc.) was used to analyse flow cytometric data. Mean Fluorescence Intensity (MFI) of the cells was measured on live, single cells. The absolute number of cells were calculated based on the frequency of the total parent population.
2.2.6 Flow cytometry gating strategies

Figure 2.2: Flow cytometry gating strategy for brain.

CD45+ live cells consisting of resident microglia (CD11b−CD45int) and infiltrating peripheral cells (CD11b+CD45hi) consisting of CD11b+ neutrophils, monocytes and CD11b+ CD3+ T cells in CCI mice brain at 3 DPI.
Figure 2.3: Flow cytometry gating strategy for blood.
CD45+ live cells consisting of infiltrating peripheral cells (CD45\textsuperscript{hi}) consisting of CD11b\textsuperscript{+} neutrophils, monocytes and CD11b\textsuperscript{-} CD3\textsuperscript{+} T cells in CCI mice blood at 3 DPI.

2.2.7 Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

2.2.7.1 RNA extraction
Hippocampal tissue punches from ipsilateral cortex of mice were flash frozen using CellPath\textsuperscript{TM} Cryospray Freezer Spray and stored at -80°C until RNA extraction using RNeasy Mini kit (Qiagen). Tissue was thawed, weighed and placed into a 2 ml RNase free Microcentrifuge tube containing 350 µl of Buffer RLT with β-mercaptoethanol (1:100) and 1 stainless steel bead (5mm; Qiagen). Tissue was lysed using a TissueLyserII (Qiagen) for 5 min at 27.5 Hz with the adaptor rotated half-way through. Once homogenised, the bead was removed from the tube and the tissue was centrifuged at full speed for 3 min.
The supernatant was collected and carefully placed into a new 1.5 ml RNase free
microcentrifuge tube. 1 volume (350 µl) of 70% ethanol was added to the lysates and mixed by pipetting. Up to 700 µl of sample was then transferred in a spin column placed in 2 ml collection tube and centrifuged (8,000g x 15s). The flow-through was discarded and Buffer RW1 (700 µl) was added to the spin column and centrifuged (8,000g x 15s). The flow-through was discarded and Buffer RPE (500 µl) was added to the column and centrifuged (8,000g x 15s). A second addition of Buffer RPE (500 µl) was added and centrifuged (8,000g x 2 min) to further wash the membrane. The longer spin dries the column to ensure no ethanol is carried over during RNA elution. Once all ethanol was evaporated, the spin column was placed in a new 1.5 ml collection tube and the RNA pellet was resuspended in RNase-free H₂O (40 µl). This was centrifuged (8,000g x 1 min) to elute RNA and repeated twice to increase the yield of RNA eluted. The RNA concentration in each sample was determined using NanoDrop software (Applied Biosystems) and the concentrations (ng/ul) were normalised to the lowest sample of RNA. The pure RNA was stored in -80°C until conversion to complementary DNA (cDNA).

2.2.7.2 cDNA synthesis

RNA samples were reverse transcribed into cDNA using Verso cDNA Synthesis kit according to manufacturer’s instructions. The cDNA was synthesised using a DNA engine Dyas Cycler under the conditions described in Table 2.2.7. The cDNA produced was diluted to 10 ng/µl reaction with RNase-free water and stored at -80°C before Real-Time PCR analysis.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA synthesis</td>
<td>42°C</td>
<td>30 min</td>
<td>1</td>
</tr>
<tr>
<td>Inactivation</td>
<td>95°C</td>
<td>2 min</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2.2.7 cDNA Reverse Transcription Cycle

2.2.7.3 Real-time qPCR and analysis

Real-time qPCR for the detection of mRNA was performed using SensiFAST Probe Lo-ROX 2x master mix (Bioline; BIO-84020). Briefly, 4.5 µl of cDNA was mixed with 5.5 µl of master mix consisting of 4.5 µl master mix reagent, 0.5 µl endogenous control, and 0.5 µl reaction primers, as described in table 2.1.13. RT-qPCR amplification was carried out
using 7500 Fast Real-Time PCR system (Applied Biosystems). Relative mRNA expression was calculated using the comparative cycle threshold ($2^{-\Delta\Delta C_{t}}$) method and GAPDH served as endogenous control. Relative amount of mRNA was presented as fold change over sham control.

2.2.8 Mice

In vivo studies were performed using adult male; 24-27g, and female; 20-24g, 10–12-week-old C57BL/6J mice bred by the Comparative Medicine Unit (CMU) in Trinity College Dublin. All mice were housed in groups of five per cage in specific pathogen-free conditions (12h light/dark cycle, 24 ± 1°C and 55 ± 5% humidity) during the experimental period, with ad libitum access to food and water. Mice were maintained under guidelines and regulations of the Health Products Regulatory Authority. All surgical procedures were performed in accordance with protocols approved by the Trinity College Dublin Animal Research Ethics Committee (Protocol Number: AE19136/P138). All animal experiments in this study were designed in accordance with the Animals in Research: Reporting In vivo Experiments (ARRIVE) guidelines which focused on the 3R’s; Refinement, Reduction and Replacement [112]. The recently updated ARRIVE guidelines 2.0 consists of a 21-item checklist which enables good quality reporting of animal research and ensures reproducibility of the experiments [113]. A copy of the ARRIVE guidelines is included in supplemental table 2 of the Appendix (Chapter 7).

2.2.9 Study design

Mice were randomly assigned to the different experimental groups prior to the onset of the study ensuring there was no age, weight or cage effect. If behavioural tasks required training prior to CCI, as is the case of beam walk and rotarod, any differences were accounted for in the assigning of mice to groups. All assessments in the present study were carried out by researchers blinded to the experimental groups. GSK2795039 interventions were administered by an independent researcher not involved in behavioural or cellular assessments.
2.2.10 Controlled Cortical Impact (CCI)

TBI in mice was induced using a controlled cortical (CCI) impact device (RWD; 6809II) (Figure 2.4). C57BL/6J mice were anaesthetised in a chamber containing a mixture of 0.75% O₂ flow rate along with isoflurane (induction at 3% and maintenance at 1.5%). Depth of anaesthesia was assessed before initiation of the surgical procedure by monitoring respiration rate, and pedal withdrawal reflexes. Body temperature was maintained at 37°C. Prior to mounting the head of the mouse in a stereotaxic frame, the site of the incision was shaved and bupivacaine (0.083%) was administered subcutaneously near the site of incision. The researcher then became sterile with the help of a non-sterile assistant. This involved the use of sterile gloves and tools. A sterile sheet was placed over the mouse and the researcher remained sterile for the rest of the procedure. The surgical site was cleaned with ethanol and chlorohexidine and a 10 mm midline incision was made over the skull. After reflecting the skin and fascia using bulldog clamps (#18050-50; Fine Science Tools), a 5 mm craniotomy was made on the central aspect of the left parietal bone, using a 5 mm drill bit (Figure 2.4A). The stage holding the mouse was then moved and rotated so that the impounder tip of the injury device, after extending the impactor to its full stroke distance (44mm), was positioned to the surface of the exposed dura, ensuring it was as flat as possible. The device was then reset to impact the cortical surface. Moderate-severe level injury was induced using an impactor velocity of 3 m/sec, deformation depth of 1.5 mm and dwell time of 0.18 m/sec.

Figure 2.4: Controlled Cortical Impact (CCI) delivers a focal contusion to the left parietal lobe. This image was created using BioRender.
After CCI, the time of impact and notes on the swelling/bleeding were taken. Bupivacaine (0.083%; 100 µl) was administered on top of the injury site, the incision was closed with interrupted 5-0 silk sutures and saline (1 ml) was administered subcutaneously on the dorsal side of the mouse. Following anaesthesia withdrawal, the animal was placed into a heated cage for 60 min post-injury to maintain the normal body temperature and then returned to a clean home cage. During the 60 min, the mouse was monitored and the righting reflex was noted. Sham animals underwent the same procedure as injured mice except for craniotomy and cortical impact. The sutures were cleaned daily for three consecutive days with saline and chlorohexidine and animals were monitored throughout the study using HPRA approved score sheets. Weight, incision check or re-suturing requirement, pain assessment scoring, whether the mouse is bright, alert, responsive, and any other additional comments were documented on these recovery sheets as shown in Figure 2.5. If the weight loss was more than 11-19%, 1 ml saline was provided subcutaneously, and the designated veterinary was contacted.

Figure 2.5: Animal welfare monitoring form for the CCI procedure.

Example of recovery sheet for a 3 DPI study. Weight loss, incision site, pain assessment scoring and any additional comments such as hydration level of the animal is all monitored daily until the completion of the study.

2.2.11 GSK2795039 intervention

GSK2795039 (MedChem Express) was prepared in 10% DMSO, 90% Corn Oil on the day of the injections. Randomly assigned mice were administered GSK2795039
intraperitoneally (i.p) post CCI, with repeated injections delivered by an independent researcher over the course of the study.

2.2.12 Tissue collection and preparation

2.2.12.1 Blood processing
At sacrifice, mice were anaesthetised (3% isoflurane) followed by blood collection from cardiac puncture in EDTA-coated tubes. The blood was kept on ice prior to red blood cell lysis. Briefly, 2 ml of 1X Lysing buffer (BD Bioscience; #555899) was added to 200 µl of blood and incubated (15 min; RT). The blood was centrifuged (1,300 RPM; 4°C; 5 min) and the supernatant was decanted. The lysing step was repeated once more, the pellet was resuspended in cRPMI, plated in a 96-well V-plate and stained for flow cytometry as described in section 2.2.5.

2.2.12.2 Isolation of mononuclear cells from the CNS for flow cytometry
Following blood collected by cardiac puncture, mice were perfused through the left ventricle with 30ml cold PBS with a hand-held syringe before the removal of the brain. The ipsilateral cortex, excluding olfactory bulb and cerebellum, were sectioned and collected into 1 ml cRPMI. DNase and Collagenase (1 mg/ml) were added into the brain tubes and incubated at 37°C; 1h; on a shaker (Incu-Shaker mini, Benchmark Scientific). The homogenate was then passed through 70 µM cell strainer, topped up with cRPMI, centrifuged and resuspended in 40% Percoll before being layered over 70% percoll and spun (1600 rpm; RT; 20 min). The upper layer of myelin was removed and discarded. The isolated mononuclear cells were collected from the interface of the Percoll gradient. Cells were washed with cRPMI and plated on a 96-well V-plate and immediately stained for flow cytometry as described in section 2.2.5.

2.2.12.3 Brains collected for Immunohistochemistry
In the experiments where brains were collected for immunohistochemistry, mice were perfused using a pump (Watson-Marlow 120S) with a constant flow rate (10 ml/min) to prevent bursting of blood vessels which may interfere with antibody staining and increase background. The perfusion consisted of 60 ml saline over 6 min, followed by
60ml of 4% PFA over 6 min. The brain was then isolated from the skull and placed in 4% PFA for a 3h post fix.

**2.2.12.4 Cryopreservation and cryosectioning of brains**

Following 4% PFA post-fix, brains were transferred into 30% sucrose for cryopreservation until they sank (2 days), frozen using isopentane and liquid nitrogen and stored in foil in -80°C until sectioning.

Brains were sectioned at 40 µm thickness using the Cryostat (Leica CM3050 S). Slices were serially collected at from bregma +1.2 mm to -3.6 mm at 200 µm apart, directly onto glass slides (Superfrost™ Plus Adhesion Microscope Slides; epredia; J1800AMNZ) or into 24-well plates containing 0.01M PBS. This 5-series cutting scheme (the first two slices placed on each of the two glass slides and the next three slices in the wells) allowed for a wide range of brain regions across the anterior-posterior axis and is illustrated in Figure 2.6. There was 5 slices/well and these were stored in freezing solution (250 ml PBS; 75 g Sucrose; 75 ml ethylene glycol) in -80°C until further analysis.

Slices were stained with Cresyl violet for lesion volume quantification. Briefly, slices were first de-hydrated following incubation in 70% - 90% - 100% ethanol prior to incubation with Xylene to de-lipidise the tissue. Next the tissue was incubated in 100%-90%-70% ethanol and finally H₂O to fully hydrate the tissue prior to staining with Cresyl violet. Lastly, the slices were dehydrated again with an additional incubation of Acetic acid (3%) to de-stain and clean up parenchyma before mounting with DPX mounting medium.

Cresyl violet images were acquired by Leica DM4 B microscope and LAS X software. Lesion volume quantification was analysed by Fiji software. The total volume (mm³) of the lesion on the ipsilateral side was calculated as the tissue loss compared to the contralateral side. Briefly, starting at +0.6 mm bregma, the volume of the ipsilateral side was subtracted from the volume of the contralateral side. This was then added to the difference observed between contra-ipsilateral at 0 mm from bregma and multiplied by 0.6 (distance between the anterior posterior; AP) and divided by 2. This was continued
through to -3.6 mm and the sum of the volume loss at each AP gives rise to the total lesion volume (mm$^3$).

Figure 2.6: Sectioning scheme of serial coronal brain sections for lesion volume quantification.

2.2.13 Neuro-behavioural Tests

2.2.13.1 General information and experimental outline

Researchers handled mice for 5 min/day for 14 days prior to starting experiments. This prevented stress and anxiety for the mice as they became familiar with handling. Prior to starting all tests, the mice were allowed to acclimatise for 30-60 min in the behaviour room. The lids were changed with one holding a water bottle so the mice could access
water during long periods of testing and habituation. A separate holding cage (with food and water) was used per experimental cage to avoid placing mice already tested back in with the untested mice, because those tested were stressed and may stress the next to be tested. All apparatus was cleaned with water and 70% ethanol prior to testing, in between animals, and after testing. The appropriate lamps were switched on to give an even illumination in the room (35 lux, measured using a luminometer (Extech LT300 Light Meter, Amazon) and to ensure clear images for the video recordings where necessary. The white noise machine was also switched on. By keeping the lighting and noise constant (45dB, measured using a decibel meter (Extech 407730 Digital Sound Level Meter) throughout all tests and for all animals throughout the experiments, these factors should not affect analysis. Mice were video-tracked throughout all behavioural tests using ANY-maze software (Stoelting Company, Europe). The neuro-behavioural tests consisted of Beam walk, 2-trial Y-maze, Novel Object Recognition (NOR), Morris Water Maze (MWM), Rotarod and Simple Neuroassessment of Asymmetric impairment (SNAP). The order of these behavioural tasks is outlined below (Figure 2.7). See individual chapters for precise behavioural outline according to the experiment.

![Diagram](image.png)

**Figure 2.7:** General outline of neuro-behavioural tests on the days they were carried out.

### 2.2.13.2 Beam Walk

The Beam walk test evaluated the fine motor coordination and balance of mice [114]. The apparatus involved a narrow beam of 5-6mm wide and 1300mm in length which was suspended 30-40cm above foam padding and there was an escape house at the end of the beam. This test required 3 consecutive training days prior to testing. On day -3, the mouse was placed on the beam close to the escape house and guided towards it. The mouse was allowed to remain in the house for a few minutes to understand that this is its safe place. Gradually, the mouse was placed further away to learn that it must travel across the beam towards the house. Ultimately, the mouse started at the other
end of the beam and learnt to go from the start of the beam to the house, as demonstrated in Figure 2.8. At this point, the number of foot-faults in a total of 50 steps was determined. A foot-fault occurred when the mouse’s right hind limb slipped off the beam or when the lower body was not lifted, and the mouse dragged itself to the house. This indicates inability to use the right hind limb and therefore was a foot-fault. By day -1, all experimental mice had less than 10 foot-faults. If not, they were excluded from the study. All tests were recorded for later analysis and by an independent researcher blinded to any treatment groups.

Figure 2.8: The beam walk.
The beam walk apparatus comprised of a narrow beam with an escape house at the end, suspended over foam padding which assessed fine motor coordination longitudinally.

Below is a representative image from a mouse of a “hit” and “miss” score on the beam walk. The red circle indicates the right hind limb either stepping on the beam, in the upper panel, or slipping off the beam as shown in the lower panel (Figure 2.9).
Figure 2.9: Visual representation of a “hit” and “miss” score on the beam walk.

2.2.13.3 2-Trials Y-Maze

The 2-trials Y-maze test evaluated spatial memory [115]. The apparatus involved a Y-maze arena of 5cm width, 36cm length, and 10cm height placed on a folding table with transparent Plexiglas placed on top to avoid mice jumping out of the arena during the test. Visual cues (different shapes) were placed in the Y-maze so the mice can identify each of the arms. Bedding was placed on the ground of the maze to reduce stress and provide contrast for the tracking videos. This test required two trials. In the first, the randomised selected arm was blocked with a guillotine. The mouse was placed in a randomly selected starting arm, facing the wall. Both the blocked and starting arms were randomised between all mice before the start of the experiment. The mouse was allowed to freely explore the maze for 5 min. The time that the test ended was recorded, and the mouse was placed in the holding cage for an inter-trial-interval of 1h before starting trial 2. In this second trial, the guillotine was removed from the blocked arm, the mouse was placed in the same starting arm as trial 1 and allowed to freely explore all 3 arms for 5 min, as shown below (Figure 2.10). The main outcome of this test was calculating the amount of time the mice spent in the novel arm as follows: (time in arm (novel or familiar) / (time in novel + time in familiar). The hypothesis of this test was that mice with an intact cognitive function can discriminate between novel and familiar arms and should spend more time in the novel arm, indicating a better performance.
2.10: 2-trials Y-Maze.
2-trials Y-Maze consisted of blocked arm trial and 1h later, unblocked trial and assessed spatial memory. This image was created using BioRender.

2.2.13.4 Novel Object Recognition

The Novel Object Recognition (NOR) test evaluated recognition memory [31]. The apparatus involved a square open-top black plexiglass box testing arena, and an array of objects such as Rubik’s cubes, heart/stars, and intertwined-circles, examples of which are shown in Figure 2.11, and. The objects are chosen based on the fact that naive mice have no preference between objects at baseline testing. On the first day of testing (habituation), the mouse was placed into the empty arena, facing the wall furthest away from where the objects will be in future tests, for 10 min. The researcher left the room so as not to disturb the test. The first day was also used for an Open Field test to determine the locomotor activity (in terms of distance moved) and anxiety-like behaviour (in terms of distance from the border of the arena, known as thigmotaxis). Mice are curious species and have a natural desire to exploring new areas. Therefore, the closer the mouse was to the border indicated higher anxiety-like behaviour. The second day also consisted of an identical 10 min habituation. The third day was the familiarisation test whereby two identical objects were placed in the arena, such as the intertwined circles shown in Figure 2.11, and mice were allowed to freely explore the
objects for 5 min. The amount of time the mice spent exploring the objects was recorded manually using timers. Sniffing and interacting with the object directly or pointing towards the object but just millimetres away were all considered “exploring the object” and the time was accounted for. In contrast, if the mouse was climbing on top of or just walked past the object or sat next to but did not interact with the object, then this was not included in the time recorded. The time spent exploring the object was graphed after the familiarisation step to ensure there was no preference for the left or right side of the arena. Mice with a cumulative exploration time (both objects) of less than 6 seconds were excluded from the study. The fourth day was the novel object test. One of the objects was replaced with a new object (novel), such as the Rubik’s cube as shown in Figure 2.11, and mice were allowed to freely explore the objects for 5 min. The time the mice spent with both the familiar and the novel object was recorded with timers. The novel object was equally distributed across experimental groups and also the side the novel object was placed was randomised and balanced across groups to avoid object and side bias. The main outcome of the NOR was the discrimination index (DI) which was calculated by; (time exploring novel – time exploring familiar)/ (total time novel + familiar object). Mice with a DI of > 0.2 are considered to have an intact cognitive function. The higher the DI, the better the performance.

Figure 2.11: The Novel Object Recognition test
The Novel Object Recognition was a 4-day test which evaluated recognition memory. The first 2 days allowed the mouse to freely explore and habituate the empty arena. On day 3 familiarisation, two identical objects were placed in the arena and the time the mice spent interacting with the objects was recorded. On day 4, the novel test consisted of one object being replaced by a novel object and the interaction time was recorded.
2.2.13.5 Morris Water Maze

The Morris Water Maze (MWM) test evaluated spatial learning and hippocampal-dependent memory [31]. The MWM circular tank (100cm in diameter) was filled with water (21± 1°C), until a transparent platform (5cm in diameter) was submerged 1cm below the surface of the water. Visual cues such as a cross, square, circle and wires already present on the wall were pinned onto the curtain / wall surrounding the MWM as shown in Figure 2.12. These cues were kept in the same position throughout the test. The first four consecutive days was the hidden platform acquisition phase which consisted of four trials per day. Both the position of the platform (SE, NE, SW) and the position the mouse was placed into the tank (N, W, S, E) was randomised across groups. The position of the platform remained the same for each mouse throughout the experiment, but the quadrant that the mouse was placed into was different at each trial per day. Once the mouse was placed into the tank at the pre-assigned randomised quadrant, the researcher exited the testing arena (on the opposite side to where the platform was to ensure the mouse did not follow the operator) and the mouse was allowed 90 seconds to find the platform. If the mouse found the platform before the 90 seconds, they were made to wait on the platform for 10 seconds before being dried with a towel and placed into the holding cage under a heated lamp to dry between trials. If the mouse did not find the platform they were guided towards the platform and made to wait for 10 seconds. On day 5, the probe test was carried out whereby the platform was removed; the mouse was placed into the tank and the time spent in each quadrant was recorded for 60 seconds. The mouse was then removed, dried, and placed back into the holding cage. Once all the mice completed the probe test, the visual acuity test took place to determine if the mice had visual impairments and therefore resulted in an inability to locate the platform. For the visual acuity test, the platform was placed in the SE quadrant with a flag on top and mice were placed into the NW quadrant. The time it took the mice to locate the platform was recorded and this test was repeated twice more changing the platform position each trial (entry platform remains the same) so that the mice did not learn where the platform was, and it was purely a visual test. Mice that did not find the platform within 20 seconds were excluded from the experiments. The main outcome of the MWM was the latency to reach the platform on the training days (1-4) and the time the mouse spent in the platform quadrant when it was removed.
for the probe test (day 5). The mice were expected to experience a learning curve as they learnt where the platform was and during the probe test, the mice with intact spatial learning and memory were expected to spend more time in the quadrant that the platform was in.

Figure 2.12: The Morris Water Maze test.
Morris Water Maze test consisted of a 4-day training and learning phase to locate the missing platform. This was followed by a probe test on day 5 which evaluated hippocampal-dependent memory. Visual acuity was assessed after the probe test using a flag placed on the platform.

2.2.13.6 Accelerating rotarod
The accelerating rotarod evaluated gross motor function in mice [116]. The apparatus consisted of an accelerating rotarod device (Ugo Basile; Italy) with an initial speed of 4RPM to a final speed of 60 RPM over 180 seconds, as demonstrated by “ramp” in the set up in Figure 2.13. This test required 3 consecutive training days to obtain a baseline reading prior to CCI. Mice were then tested each day following injury. The mice were placed on the rod facing the wall (away from the researcher) and three mice were tested at any one time to ensure accuracy. Once all the mice were on the rod, “run” was pressed and the rod began moving at a constant 4RPM. If the mice tried to turn around on the rod they were corrected to always face the wall. Once all the mice were facing the wall “start” was pressed and the speed increased. The latency (seconds) for the mice to remain on the rod was recorded. Mice underwent three trials per day with a 5-10 min rest with access to food and water between each trial. The scores were averaged from the three trials to give a single score per mouse per day. If the mouse falls off or clings to the rod for two consecutive complete rotations, the time is stopped.
Figure 2.13: The accelerating rotarod
The accelerating rotarod measured gross motor function. This image was created using BioRender.

2.2.13.7 Simple neuroassessment of asymmetric impairment (SNAP)

The simple neuroassessment of asymmetric impairment (SNAP) test evaluated neurological deficits in mice following CCI [117]. SNAP is comprised of a series of eight neurological tests assessing vision, coordination and proprioception, motor strength, and posture as demonstrated visually in the schematic below (Figure 2.14). Each of the eight tests were scored ranging from 0 (neurologically intact) to 5 (severely impaired). The scores of each test were summed for a final overall score. The higher the score the greater the neurological impairment. The tests and guidelines for scoring were carried out using the SNAP testing protocol [117].
Figure 2.14: Eight individual SNAP tests.
SNAP measured neurological deficits. This image was created using BioRender.
2.2.14 Statistical analysis

Power analysis calculations were based on previous data from the Loane lab to predict effect size and variability. Statistical analysis and the generation of graphs were performed using Prism v10.2.1 software (GraphPad software, San Diego, CA, USA; RRID:SCR_002798). Normality testing was performed and where data sets passed normality in the Shapiro Willks test, parametric statistical analysis was used. Outliers were identified using Grubb’s test to detect an outlier and were removed prior to analysis. Data from these experiments was analysed by unpaired student’s t test (two groups), One-way ANOVA (more than two groups) with post hoc Dunnett’s (compared to one group of interest) or Tukey’s (compare all groups to each other) multiple comparisons test. For more than two groups and two factors, a Two-Way ANOVA, with or without repeated measures with uncorrected Fisher’s LSD test was performed. All quantitative data are represented as mean ± standard error of the mean (SEM). p values < 0.05 were considered statistically significant. In vitro work demonstrates data represented of at least three independent experiments. See supplemental table 1 in the appendix (Chapter 8) for statistical breakdown on parametric and non-parametric in vitro data.
Chapter 3: NOX2-mediated regulation of NLRP3 inflammasome in models of microglial activation \textit{in vitro}
3.1 Introduction

The mechanisms of which NOX2/ROS signalling pathway induced oxidative stress and disease pathogenesis was of interest in this present study due to the detrimental effects it has in TBI pathology. NOX2 has been shown to be robustly increased in microglia after TBI and contributes to chronic neurodegeneration which is associated with microglial activation [39]. Specifically, NOX2 knock out (KO) mice, subjected to TBI, had reduced cortical neurodegeneration as shown by a decreased lesion volume, upregulated neuronal density and had improved fine motor coordination in a beam walk test compared to wild type (WT) TBI mice [16]. In this present study, ROS, nitrite, and TNFα levels were indicators of NOX2 activation and inducible nitric oxide synthase production which are upregulated following inflammation in the brain. NOX2/ROS also acts a priming signal for NLRP3 inflammasome activation, which is implicated in microglial-mediated neuroinflammation during chronic neurodegeneration. Studies have shown that mice lacking NOX2 had reduced cleaved-caspase 1 activity and NLRP3 activation following TBI [94], therefore confirming the role of NOX2 in NLRP3 inflammasome activation.

Therefore, in knowing that microglia are chronically active following TBI and that NOX2/ROS and NLRP3 inflammasome activation play an important role in sustained neuroinflammation, the aim of this chapter was to mimic TBI in vitro and understand mechanisms of microglial responses under three models of activation. The assessment of a variety of microglial responses was determined in both an immortalised microglial (IMG) cell line and primary microglia. IMGs are derived from adult murine brain and express markers specific to primary adult microglial cells. IMGs respond to pro and anti-inflammatory signals and the changes induced by LPS are greater than those in BV-2 cells, another common microglial cell line [118]. Experiments were also carried out in primary microglia as these cultures have similar properties to those of in vivo cells, including secretory products that occur endogenously [119].

In the first model of microglial activation the cells were stimulated with LPS, the bacterial cell wall component and a TLR4 agonist. Although not physiologically relevant in the brain, LPS is a potent activator of microglia by upregulating MAP kinase (MAPK) and
inducing NFκB activation and promoting inflammation. Accordingly, this mimicked the long-lived pro-inflammatory microglial responses observed following a TBI in vivo [39]. In fact, TLR4 KO mice demonstrated smaller brain lesions and decreased expression of inflammatory markers following controlled cortical impact [120]. These findings provide a rationale and relevance of using LPS to activate TLR4 signalling, stimulate microglia and mimic TBI effects in this present study.

Secondly, the role of LPS/ATP stimulation on microglia cells to induce NLRP3 inflammasome was investigated to study the mechanistic link between NOX2 activation and NLRP3 inflammasome. The additional stimulation with ATP was a more relevant neurotrauma model as ATP is found in abundance in the brain following TBI [121]. The third model of microglial activation focused on LPS/nigericin stimulation. Nigericin induces potassium (K⁺) efflux resulting in pore formation of the plasma membrane to induce NLRP3 inflammasome activation [122, 123] and this model was hypothesised to potently induce pyroptotic dependent IL-1β release.

Once these three models of microglial activation were optimised to closely mimic TBI in vitro, this present study examined if GSK2795039, a novel small molecule NOX2 inhibitor, could attenuate NOX2-mediated pro-inflammation in microglia in vitro. Previous work from the Loane lab showed BMDMs from NOX2 KO mice stimulated with LPS/IL-4 had reduced TNFα, IL-1β, IL-6 and nitrite compared to WT mice following injury [63]. Furthermore, inhibition of NOX2, using dp91ds-tat, altered the phenotype similar to the NOX2 KO TBI studies including improved spatial working memory [16]. Given these rationales, this present study questioned whether pharmacological inhibition of NOX2 by GSK2795039, as a more specific approach, could have similar neuroprotective properties. Studies showed mice pre-treated with GSK2795039 significantly reduced NOX2-dependent ROS production compared to vehicle, and levels were similar to that of the positive control gp91⁻/⁻ [74]. In a mouse model of neuropathic pain, GSK2795039 administration reduced microglial activation along with an attenuation of anxiety-like behaviour [82].

Therefore, the first objective of this study was to characterise models of microglial activation in vitro that depict NOX2/ROS and NLRP3 inflammasome activation in IMGs
and primary microglia. Once the model was optimised, the next objective was to determine the effect of GSK2795039 on NOX2-mediated regulation of NLRP3 inflammasome in microglia, comparing it to either DPI*, a broad antioxidant or MCC950, a potent NLRP3 inhibitor.

### 3.2 Results

#### 3.2.1 LPS stimulated IMG cells and primary microglia to produce reactive oxygen species, nitrite, and TNFα.

Several microglial cell lines such as BV2 and HAPI cells have been utilised to mimic microglial function in vitro. However, these cell lines are neonatal-derived and do not depict accurate representations of microglial responses in adult-associated pathogenesis. Therefore, this present study utilised an established immortalised microglial (IMG) cell line derived from adult murine brain made immortalised by infecting with v-raf and v-myc retrovirus [118]. IMGs were stimulated with LPS to mimic the long-lived pro-inflammatory microglial responses that occur following a TBI. IMG cells were stimulated with LPS at increasing concentrations (10, 50 and 100 ng/ml) diluted in serum-free media (cDMEM) for 24h in a 96-well plate at 7.5 x10⁴ cells/well. As NADPH oxidase (NOX2) activation is responsible for the generation of reactive oxygen species (ROS), cells were incubated with a CM-H2DCFDA dye to measure ROS production in cells by fluorescence. Furthermore, ROS has been shown to induce NFkB which promotes the activation of inducible nitric oxide synthase and ultimately the release of nitric oxide (NO) [124]. NO is a key intra- and extracellular messenger that mediates diverse signalling pathways in the brain. NO can be neurotoxic under neuroinflammatory conditions, including after TBI [125]. Therefore, NO was assessed in stimulated IMGs to investigate important mechanisms that could potentially translate in vivo.

In this study, nitrite was measured as an indication of nitric oxide from the supernatants, using Griess reagent. TNFα, a potent pro-inflammatory cytokine, was also measured from the supernatants using enzyme-linked immunosorbent assay (ELISA). LPS (100 ng/ml) increased ROS (Figure 3.1A) nitrite (Figure 3.1B) and TNFα ml (Figure 3.1C) but
levels failed to reach statistical significance. Hydrogen peroxide (H$_2$O$_2$) was used as a positive control for ROS production.

As IMGs are a cell line, it was necessary to repeat key experiments in primary microglia as they offer more of an insight and relevance into the responses and functions seen in vivo. Primary microglia were isolated from the cerebral cortices of P1 Wistar rats as described in section 2.2.1.4. Once detached from the mixed glial culture, the microglia were seeded in a 96-well plate at $1 \times 10^5$ cells/well overnight, followed by LPS (10, 50, 100 ng/ml) stimulation for 24h. Similar to what was observed in the IMG cells, LPS increased ROS, nitrite and TNF$\alpha$ (Figure 3.1D-F; $p<0.05$; $p<0.01$; $p<0.001$ vs control), in primary microglia. ROS was measured as a % of control. Levels of nitrite and TNF$\alpha$ were determined from a standard curve.

![Image](Immortalised Microglial (IMG) Cell Line)

![Image](Primary Microglia)

**Figure 3.1:** LPS increased ROS, Nitrite and TNF$\alpha$ in IMGs and primary microglia. Immortalised microglial (IMG) cell line (A-C) and primary microglia (D-F) were stimulated with LPS (10-100 ng/ml; 24h) in a 96-well plate. ROS was measured in the cells using CM-H2DCFDA. Nitrite and TNF$\alpha$ levels were measured in the conditioned media using Griess reagent and ELISA respectfully. LPS (100 ng/ml) increased ROS (A, D; "$p<0.05$), nitrite (B, E; **"$p<0.01$; ***"$p<0.001$) and TNF$\alpha$ (C, F; ***"$p<0.01$) compared to controls in both IMG and primary microglia. Data are mean ± SEM (n=3, averages from three-independent experiments). "$p<0.05$; **"$p<0.01$; ***"$p<0.001$ versus control; one-way ANOVA with post hoc Tukey’s multiple comparisons test.
3.2.2 GSK2795039 attenuated LPS induced ROS, Nitrite, TNFα in IMGs and primary microglia.

The previous experiment determined LPS (100 ng/ml) as the optimal concentration for the remaining experiments based on the ROS, nitrite, and TNFα production in both IMG and primary microglia (Figure 3.1). The following aim was to investigate the effect of NOX2 inhibition using GSK2795039, a novel NOX2 inhibitor on LPS stimulated cells and compare with DPI⁺, a broad antioxidant. DPI⁺ inhibits ROS by accepting electrons from flavin-dependent enzyme complexes such as nitric oxidase synthase and so is not specific to NOX. Hirano et al, illustrated that DPI⁺ inhibited NOX4-mediated oxygen consumption. In contrast, GSK2795039 did not. GSK2795039 inhibits NOX2 in a NADPH competitive manner [74]. The objective was to determine an optimal dose of GSK2795039 and identify downstream pathways following NOX2 inhibition.

To assess this, IMG and primary microglia cells were pre-treated with either GSK2795039 (10-50 µM) or DPI⁺ (0.1 µM) for 1h prior to stimulation with LPS (100 ng/ml; 24h) in a 96-well plate. ROS and cell viability were carried out in the cells using CM-H2DCFDA and MTT respectfully. It was important to measure changes in cell viability to avoid results being due to drug toxicity. Nitrite and TNFα were determined in the conditioned media using Griess reagent and ELISA respectfully.

In IMGs, LPS increased ROS production, nitrite, and TNFα (Figure 3.2A-C; p<0.0001 vs control) and had no effect on cell viability (Figure 3.2D). GSK2795039 significantly reduced ROS and nitrite, (Figure 3.2A, B; p<0.0001 vs LPS). DPI⁺ also attenuated ROS and nitrite (Figure 3.2A, B; p<0.0001 vs LPS). GSK2795039 (50 µM) reduced TNFα (Figure 3.2C; p<0.01 vs LPS), which was not altered with DPI⁺. GSK2795039 (25 µM) reduced cell viability (Figure 3.2D; p<0.05 vs LPS) and DPI⁺ had no effect on cell viability.

The following experiments carried out in primary microglia show similar results to that of the IMGs. LPS increased ROS production, nitrite, and TNFα (Figure 3.2A-C; p<0.01; p<0.0001 vs control). GSK2795039 (10, 25 µM) attenuated LPS-induced ROS, nitrite, and TNFα (Figure 3.2E-G; p<0.05; p<0.01: p<0.001; p<0.0001 vs LPS). DPI⁺ (0.1 µM) attenuated ROS and nitrite levels (Figure 3.2E, F; p<0.0001 vs LPS) but no effect was observed in levels of TNFα (G). GSK2795039 (10µM) and DPI⁺ (0.1 µM) did not have any
effect on cell viability, whereas GSK2795039 (25 µM) reduced cell viability (Figure 3.2H; p<0.05 vs LPS). It was important to address that the primary microglia were more sensitive to cell death than the IMGs and therefore a lower dose of 10 µM was used and found to show significant attenuation of LPS induced ROS, nitrite, and TNFα.

Figure 3.2: GSK2795039 attenuated LPS induced ROS, Nitrite, TNFα in IMGs and primary microglia. IMGs and primary microglia were pre-treated with GSK2795039 (10-50 µM) or DPI.
(0.1 µM) for 1h prior to stimulation with LPS (100 ng/ml; 24h) in a 96-well plate. GSK2795039 (10 µM) was used as the drug control. ROS and cell viability were measured in the cells using CM-H2DCFDA and MTT respectfully. Nitrite and TNFα levels were measured in the conditioned media using Griess reagent and ELISA respectfully. LPS increased ROS, nitrite, and TNFα in IMG and primary microglia (A-C, E-G; “p<0.01; ****p<0.0001) and had no effect on cell viability (D, H) versus controls. GSK2795039 (10-50µM) reduced ROS, nitrite, and TNFα in IMGs and primary microglia (A-C, E-G; ”p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 versus LPS. DPI* (0.1µM) attenuated ROS and nitrite (A, B, F; ****p<0.0001) but had no effect on TNFα (C, G) compared to LPS. GSK2795039, DPI* or LPS had no effect on cell viability (D, H). Data are mean ± SEM. For IMGs, n=4-6 per group as a representative from independent experiments. For primary microglia, n=3 averages from three-independent experiments). **p<0.01; ***p<0.001; ****p<0.0001 versus control, ”p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 versus LPS; one-way ANOVA with post hoc Dunnett’s multiple comparisons test versus LPS, unless stated otherwise in supplemental table 1.

### 3.2.3 LPS/ATP induced ROS, IL-1β and cell death but not nitrite release 8h post LPS in IMGs.

It is known that NOX2 activation has been demonstrated to promote NLRP3 inflammasome activation which has been shown to contribute to TBI pathology [94]. Previous studies in NOX2 knockout mice have shown decreased NLRP3 inflammasome activation after TBI and this is associated with decreased cleaved-IL-1β in the injured cortex [94]. This gave rationale to study the NLRP3 inflammasome in IMGs and primary microglia and ultimately test the effect of GSK2795039 on this model.

Two signals were required to activate the NLRP3 inflammasome. Similar to the first experiments, cells were primed with LPS (100ng/ml) but a further activation with ATP (1mM) stimulation for 10 min was required for NLRP3 inflammasome activation. The second stimulation with ATP was more physiologically relevant, than LPS alone, in mimicking TBI in vitro because ATP is a potent DAMP and high levels are released in the brain following TBI [121]. It was necessary to optimise and understand the temporal course of the LPS/ATP stimulation model. To assess this, IMGs were stimulated with LPS (100 ng/ml) in 96-well plates for 4h, 8h or 24h along with a second stimulation of ATP (1mM; 10 min). ROS and cell viability were carried out in the cells using CM-H2DCFDA and MTT respectfully. Nitrite and IL-1β were determined in the conditioned media using Griess reagent and ELISA respectfully.
LPS/ATP induced ROS at 8 and 24h compared to controls (Figure 3.3B, C; p<0.001, p<0.0001 vs control), but not at 4h (Figure 3.3A; p<0.05 vs control). LPS alone further increased ROS at 8h (Figure 3.3B; p<0.0001 vs LPS/ATP). LPS/ATP increased IL-1β at 4h and 8h (Figure 3.3D, E; p<0.0001 vs controls; p<0.0001 vs LPS) and levels of IL-1β were lower by 24h (Figure 3.3F; p<0.05 vs control). LPS/ATP reduced cell viability following stimulation at 4h and 8h by approximately 40% (Figure 3.3G, H; p<0.01, p<0.001 vs control), while there was no significant difference in cell viability at 24h (Figure 3.3I). LPS alone showed higher cell viability at 4h and 8h (Figure 3.3G, H; p<0.0001 vs LPS/ATP), which suggests the additional ATP stimulation is causing cell death and not the LPS priming alone. However, the further ATP stimulation following LPS at 24h was not sufficient to reduce cell viability (Figure 3.3I). Lastly, LPS/ATP significantly increased nitrite at 24h (Figure 3.3L; p<0.0001 vs control), which was not observed at 4h and 8h (Figure 3.3J, K). There was no difference in nitrite between the LPS/ATP and LPS only groups, suggesting the nitrite production was because of LPS priming.
Figure 3.3: LPS priming (4h, 8h, 24h) followed by ATP induced ROS, IL-1β, cell death and nitrite in IMGs. IMG cells were stimulated with LPS (100 ng/ml; 4h, 8h and 24h) with separate groups receiving an additional stimulation of ATP (1mM; 10 min) in a 96-well plate. ROS and cell viability were measured in the cells using CM-H2DCFDA and MTT respectfully. Nitrite and IL-1β levels were measured in the conditioned media using Griess reagent and ELISA respectfully. LPS/ATP
induced ROS production at 8h and 24h (B, C; ****P<0.0001) but not at 4h (A) versus controls. LPS/ATP induced IL-1β at 4h and 8h (D, E; ****P<0.0001; ****P<0.0001) which was greater than at 24h (F; *p<0.05), compared to controls and LPS. LPS/ATP reduced cell viability following 4h (G; **P<0.01) and 8h (H; ***P<0.001) and had no effect at 24h (I). LPS/ATP did not induce nitrite at 4h or 8h (J, K), but increased levels at 24h (L; ****P<0.0001) compared with controls. Data are mean ± SEM (n=6-12 per group) and are a representative of model characterisation. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 versus control, ++++p<0.0001 versus LPS/ATP; one-way ANOVA with post hoc Dunnett’s multiple comparisons test versus LPS/ATP, unless stated otherwise in supplemental table 1.

3.2.4 LPS/ATP induced NADPH oxidase (NOX2) and NLRP3 inflammasome activation in IMGs.

Priming with LPS (100ng/ml) for 8h and activating with ATP (1mM; 10 min) was considered the optimal timing for inducing ROS production, the major output of NOX2 activation, along with IL-1β release, the major output of NLRP3 inflammasome activation (Figure 3.3). The next study investigated whether this model could directly upregulate the intracellular protein expression of NOX2 and NLRP3 protein expression, as both pathways play a vital role in the pathogenesis of TBI [93, 94].

To assess this, IMGs were stimulated with LPS/ATP in 12-well plates and cells were processed into a single cell suspension for measurement of NADPH oxidase (NOX2) by flow cytometry or cell lysates were processed and prepared for measurement of NLRP3 by Western immunoblot.

LPS/ATP increased NOX2 mean florescence intensity (MFI) as shown by the histogram of the fluorescence and the corresponding quantification (Figure 3.4A; p<0.05 vs control). LPS/ATP also increased NLRP3 protein expression (Figure 3.4B; p<0.01 vs control). These findings suggest LPS/ATP induced NOX2/ROS signalling as well as NLRP3 inflammasome activation.
3.4 LPS/ATP increased NOX2 activity and NLRP3 inflammasome activation in IMGs.

IMG cells were stimulated with LPS (100 ng/ml; 8h) followed by ATP (1mM; 10 min) in a 12-well plate. Intracellular expression of NOX2 was measured by flow cytometry and NLRP3 protein expression was measured by Western immunoblot in the cells. LPS/ATP increased NOX2 mean fluorescent intensity (MFI) by flow cytometry as shown by the histogram and corresponding quantification (A; ‘p<0.05) versus control. LPS/ATP increased NLRP3 protein expression (B; “p<0.01) versus control. Data are mean ± SEM (n=3 per group) and are a representative of model characterisation. ‘p<0.05; “p<0.01 versus control; one-way ANOVA with post hoc Dunnett’s multiple comparisons test versus LPS/ATP (A), or unpaired students t-test (B).
3.2.5 GSK2795039 ameliorates LPS/ATP induced ROS production, IL-1β and TNFα in IMGs.

Having shown the microglial response to LPS/ATP in IMGs (Figure 3.3; 3.4), it was next examined if GSK2795039 pre-treatment could reduce indicators of NLRP3 inflammasome activation, and other pro-inflammatory mediators that could potentially lead to a downstream pro-inflammatory cascade.

In the current study, IMGs were pre-treated with GSK2795039 (25 µM) for 1h prior to stimulation with LPS (100 ng/ml; 8h) and ATP (1mM; 10 min) in a 96-well plate. ROS and cell viability were carried out in the cells using CM-H2DCFDA and MTT respectfully and an array of cytokines (IL-1β, IL-18, TNFα and IL-1ra) were measured in the conditioned media using ELISA. IL-1β and IL-18 are both released by the cleavage of active Caspase-1, once the NLRP3 inflammasome is activated. When IL-1β is processed and released, there is also release of IL-1 receptor antagonist (IL-1ra) which can modulate the inflammatory effects of IL-1β [126].

As previously shown, LPS/ATP increased ROS, IL-1β, IL-18, TNFα and IL-1ra (Figure 3.5A-C, E, F; p<0.0001 vs control) and reduced cell viability (Figure 3.5D; p<0.0001 vs control). Pre-treatment with GSK2795039 significantly reduced LPS/ATP induced ROS, IL-1β and TNFα (Figure 3.5A, B, E; p<0.0001 vs LPS/ATP). GSK2795039 reduced IL-18 and IL-1ra but levels failed to reach statistical significance (Figure 3.5C, F). The loss of cell viability was not reversed by pre-treatment with GSK2795039 (Figure 3.5D). These findings suggest GSK2795039 reduced LPS/ATP induced NLRP3 inflammasome activation in IMGs independent of inhibiting cell death but possibly through an upstream mechanism, as demonstrated by ROS inhibition.
Figure 3.5: GSK2795039 attenuates LPS/ATP-induced ROS, IL-1β, TNFα and IL-1ra in IMGs.

Immortalised Microglial (IMG) Cell Line

IMG cells were pre-treated with GSK2795039 (25 µM; 1h), stimulated with LPS (100 ng/ml; 8h) and activated with ATP (1 mM; 10 min) in a 96-well plate. GSK2795039 (25 µM) was used as the drug control. ROS and cell viability were measured in the cells using CM-H2DCFDA and MTT respectively. Nitrite was assessed by Griess reagent and IL-1β, IL-18, TNFα, IL-1ra levels were measured by ELISA in the conditioned media. GSK2795039 (25 µM) was used as the drug control. LPS/ATP increased ROS, IL-1β, IL-18, TNFα, IL-1ra and reduced cell viability (A-F; ***p<0.001; ****p<0.0001) compared with controls. GSK2795039 attenuated ROS, IL-1β, TNF-α (A, B, E; ++++p<0.0001) and IL-18 and IL-1ra but failed to reach statistical significance, compared to LPS/ATP. Data are mean ± SEM (n=6-12 per group) and are a representative of model characterisation. **p<0.01; ****p<0.0001 versus control, ++++p<0.0001 versus LPS/ATP; one-way ANOVA with post hoc Dunnett’s multiple comparisons test versus LPS/ATP, unless stated otherwise in supplemental table 1.
3.2.6 GSK2795039 attenuated LPS/ATP induced NOX2 and NLRP3 inflammasome in primary microglia.

Having shown the effect of GSK2795039, a NOX2 inhibitor, in reducing NLRP3 inflammasome activation in IMGs (Figure 5.5), the role of GSK2795039 inhibition on NOX2-NLRP3 inflammasome in primary microglia was determined and compared to MCC950 a potent NLRP3 inhibitor [96].

Primary microglia were pre-treated with GSK2795039 (5, 10, or 20 µM) or MCC950 (0.01 µM), for 1h prior to LPS stimulation (100 ng/ml; 8h) and ATP (1mM; 10 min) activation in a 96-well plate. Three concentrations of GSK2795039 were investigated as the primary microglia were more sensitive to cell death and it was important to find the correct concentration in reducing NOX2-NLRP3 activation without further inducing cell death due to drug toxicity. ROS and cell viability were carried out in the cells using CM-H2DCFDA and MTT respectfully. Lactate dehydrogenase (LDH) was measured as an indicator of pyroptotic cell death in the conditioned media by CytoTox 96® Non-Radioactive Cytotoxicity Assay. Pyroptosis is a type of cell death caused by pro-inflammatory signals and increased neuroinflammation following a traumatic brain injury [127]. Physiologically, LDH enzyme is too large to pass through Gasdermin D pore and so LDH release is an indication of pore formation, cell lysis and cytokine release such as IL-1β.

Like the IMG experiments, LPS/ATP (L/A) increased ROS and IL-1β (Figure 3.6A, B; p<0.01; p<0.0001 vs control) and reduced cell viability (Figure 3.6D; p<0.01 vs control). GSK2795039 attenuated LPS/ATP induced ROS and IL-1β (Figure 3.6A, B; p<0.05; p<0.01; p<0.001; p<0.0001 vs LPS/ATP) and demonstrated a reduction in cell viability at the higher concentration of (20µM) but failed to reach statistical significance. MCC950 attenuated IL-1β (Figure 3.6B; p<0.05 versus LPS/ATP) and had no effect on the rescue of cell viability (Figure 3.6D). These results demonstrated an inhibitory effect of GSK2795039 on NOX2-NLRP3 activation. In contrast, MCC950 inhibition was more specifically on NLRP3 inflammasome activation as no reduction in ROS production was observed (Figure 3.6A).
Figure 3.6: GSK2795039 attenuated LPS/ATP increased ROS, IL-1β, LDH in primary microglia.

Primary microglia were pre-treated with GSK2795039 (5, 10, or 20 µM) or MCC950 (0.01 µM) for 1h prior to stimulation with LPS/ATP (L/A; 1mM; 10 min) in a 96-well plate. GSK2795039 (10 µM) and MCC950 (0.01 µM) were used as the drug controls. ROS and cell viability were measured in the cells using CM-H2DCFDA and MTT respectfully. Lactate dehydrogenase (LDH), used to assess pyroptosis, and TNFα were measured in the conditioned media by CytoTox 96® Non-Radioactive Cytotoxicity Assay and ELISA respectfully. LPS/ATP increased ROS and IL-1β (A, B; **p<0.01; ****p<0.0001) and reduced cell viability D; **p<0.01 vs control. GSK2795039 (5-20µM) reduced LPS/ATP induced ROS and IL-1β (A, B; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001). GSK2795039 (20µM) augmented, levels of LDH compared to LPS/ATP but levels failed to reach statistical significance. Data are mean ± SEM (n=3, averages from three independent experiments). *p<0.01; ****p<0.0001 versus control, *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 versus LPS/ATP; one-way ANOVA with post hoc Dunnett’s multiple comparisons test versus LPS/ATP.
3.2.7 Nigericin acts as a more potent activator of the NLRP3 inflammasome compared to ATP.

The results so far have demonstrated that LPS/ATP activated IMGs and primary microglia to induce NOX2-ROS production and the products of an activated NLRP3 inflammasome in an array of plate-based assays. However, in these studies, LPS/ATP activation was not potent enough to stimulate the cleaved forms of caspase-1 and IL-1β, which are vital downstream components of the NLRP3 inflammasome. A more potent stimulus was required to demonstrate the effect of GSK2795039 inhibition on an activated NLRP3 inflammasome in more complex biochemical analysis. Nigericin is a pore-forming toxin which binds directly to K⁺ ions and initiates efflux along with sodium (Na⁺) influx on the plasma membrane, both necessary for NLRP3 inflammasome activation. [122, 123].

The aim of this study was to investigate differences between ATP and nigericin stimulation, as well as identifying an optimal concentration of nigericin. Primary microglia or IMGs were stimulated with LPS (100ng/ml; 8h) and ATP (1mM; 10 min) or nigericin (1, 5 or 10µM; 30 min) in a 96-well plate. ROS and cell viability were carried out in the cells using CM-H2DCFDA and MTT respectfully. Lactate dehydrogenase (LDH) was measured as an indicator of pyroptotic cell death in the conditioned media by CytoTox 96® Non-Radioactive Cytotoxicity Assay. IL-1β was measured in the supernatant by ELISA.

Firstly, primary microglia stimulated with LPS/ATP or LPS/nigericin (1µM) induced ROS production, IL-1β and reduced cell viability (Figure 3.7A, B, D; p<0001, p<0.05 p<0.0001 vs control) and had no effect on LDH release (Figure 3.7C). However, the higher concentrations of LPS/nigericin (5 and 10 µM) had no effect on ROS production compared to controls (A) but significantly increased IL-1β, LDH and reduced cell death when compared to both control and LPS/ATP stimulation (Figure 3.7B, C, D; p<0.0001, p<0.05, p<0.0001 vs control; B, D; p<0.0001 vs LPS/ATP). It was important to note that LPS/nigericin (1-10 µM) had significantly lower ROS compared to LPS/ATP (Figure 3.7A; p<0.0001 vs LPS/ATP). These findings suggest a higher concentration of nigericin drives IL-1β and LDH release with simultaneous cell death, more potently than LPS/ATP stimulation.
Figure 3.7: LPS/nigericin increased IL-1β, LDH and cell death compared to LPS/ATP in primary microglia.

Primary microglia were stimulated with LPS (100 ng/ml; 8h) followed by activation with either ATP (1mM; 10 min) or nigericin (1, 5, 10 µM; 30 min) in a 96-well plate. ROS and cell viability were measured in the cells using CM-H2DCFDA and MTT respectfully. Lactate dehydrogenase (LDH), used to assess pyroptosis, and IL-1β were measured in the conditioned media by CytoTox 96® Non-Radioactive Cytotoxicity Assay and ELISA respectfully. LPS/ATP and LPS/nigericin (1 µM) increased ROS, modest levels of IL-1β and cell death (A, B, D (∗p<0.05; ****p<0.0001) but had no effect on LDH (C) versus control. LPS/nigericin (5-10 µM) did not induce ROS production, increased LDH (C; ∗p<0.05) compared to controls, but increased levels of IL-1β and promoted greater cell loss (A,D; ****p<0.0001) when compared to LPS/ATP. Data are mean ± SEM (n=3-6 per group) and are a representative of model characterisation. ∗p<0.05; ****p<0.0001 versus control, ****p<0.0001 versus LPS/ATP; one-way ANOVA with post hoc Tukey’s multiple comparisons, unless stated otherwise in supplemental table 1.
Secondly, to further characterise the potent model of LPS/nigericin in triggering NLRP3 inflammasome, IMGs were stimulated with LPS (100ng/ml; 8h) and nigericin (10µM; 30 min) in either 96-well (Figure 3.8B, F) and 12-well (Figure 3.8C-E) plates. Microscopic images were taken to qualitatively identify morphologically changes in the IMGs from 1) being cultured in growth media (cDMEM), 2) following LPS priming and 3) with the additional stimulation of nigericin. Similar plate-based outputs as the primary microglia described above (Figure 3.7), were also measured.

IMGs cultured in cDMEM appeared sparse with few processes (Figure 3.8A). Following LPS stimulation, IMGs seemed to proliferate and extend their processes in search of the insult. An additional stimulation with nigericin resulted in morphological change whereby the cells adopted a round ameboid shape. Although these observations were qualitative as there was no quantification attached, nigericin stimulation revealed more activated IMGs with possible signs of vesicle rupture and cell death (Figure 3.8A).

In terms of activity within the cells and supernatant, LPS/nigericin failed to increased ROS production (Figure 3.8B), which was similar to what was observed in the primary microglia (Figure 3.7; A). LPS/nigericin increased IL-1β and LDH (Figure 3.8C, E; p<0.01, p<0.001 vs control) and IL-18 but levels failed to reach statistical significance. LPS/nigericin also decreased cell viability (Figure 3.8F; p<0.05 vs control) which was also observed in the primary microglia (Figure 3.7; D).

These results from both primary microglia and IMGs demonstrate LPS/nigericin as a more potent inducer of the NLRP3 inflammasome compared to the LPS/ATP model and will therefore be used for the remainder of the figures in this results chapter.
Figure 3.8: LPS/nigericin induced ameboid morphology and increased IL-1β, IL-18, LDH and cell death in IMGs.

Microscopic images of IMG cells cultured in serum free media (cDMEM), LPS (100 ng/ml; 8h) and LPS/nigericin (10 µM; 30 min) in a 12-well plate (A). LPS/nigericin induced morphological change whereby the cells adopt a round ameboid shape and become more activated with possibly signs of vesicle rupture and cell death. ROS and cell viability were measured in the cells using CM-H2DCFDA and MTT respectfully. Lactate dehydrogenase (LDH), used to assess pyroptosis, and IL-1β, IL-18 were measured in the conditioned media by CytoTox 96® Non-Radioactive Cytotoxicity Assay and ELISA respectfully. LPS/nigericin had no effect on ROS production (A), increased IL-1β, LDH and reduced cell viability (B, E, F; *p<0.05; **p<0.01; ***p<0.001) and increased IL-18 but failed to reach statistical significance (D) compared to control. Data are mean ± SEM (n=3-6 per group) and are a representative of model characterisation. *p<0.05; **p<0.01; ***p<0.0001 versus control; unpaired students t-test, unless stated otherwise in supplemental table 1.
3.2.8 LPS/nigericin promoted NLRP3 inflammasome and caspase-1 activity in IMGs.

To further prove the potency of nigericin, compared to ATP, in promoting NLRP3 inflammasome activation, the activity of cleaved caspase-1 in IMGs was determined. The cleavage of caspase-1 is an indicator of NLRP3 activation as this is upstream of the release of pro-inflammatory cytokines IL-1β and IL-18. Previous studies demonstrated that mice that have been ablated of caspase-1 prior to TBI have reduced pro-inflammatory cytokines and improved neurological effects thus proving the importance of caspase-1 in TBI-induced pyroptosis [128].

To investigate this, IMGs were stimulated with LPS (100 ng/ml; 8h) and ATP (1mM; 10 min) or nigericin (10 µM; 30 min) in a 12-well plate. Cells were processed into a single cell suspension for measurement of Caspase-1 activity by flow cytometry. In this present study, a cell-permeable Fluorochrome Inhibitor of CAspases (FLICA) 660 Caspase-1 kit (BioRad (Catalogue #: ICT9122) was the probe used to detect active caspase-1 activity. This kit uses a Caspase-1 inhibitor (YVAD) coupled to a far-red fluorescent dye (660) and a fluoromethylketone (FMK) to specifically bind to active caspase-1 which can be measured fluorescently. Furthermore, the supernatant from these cells were analysed for products of an activated NLRP3 inflammasome; IL-1β, IL-18 by ELISA and LDH by CytoTox 96® Non-Radioactive Cytotoxicity Assay.

IMGs stimulated with LPS/nigericin revealed an increased percentage of FLICA+ population within single cells when compared to control and LPS/ATP stimulation as shown by the individual data plots, including the fluorescence minus one (FMO) for gating purposes, and corresponding quantification (Figure 3.9A; p<0.01 vs control; p<0.01 vs LPS/ATP). The mean fluorescence intensity (MFI) of FLICA was also upregulated following LPS/nigericin stimulation as shown by the histogram of FLICA eF660 fluorescence intensity and corresponding quantification (Figure 3.9B; p<0.05 vs control). LPS/nigericin increased IL-1β, IL-18 and LDH in the corresponding supernatants (Figure 3.9C-E; p<0.0001, p<0.01, p<0.0001 vs control; C-E; p<0.0001, p<0.01, p<0.0001 vs LPS/ATP). These findings along with the previous results (Figure 3.7 and 3.8) indicate that LPS/nigericin acts as a more potent inducer of the NLRP3 inflammasome compared to the LPS/ATP model demonstrating increased caspase-1 activity, IL-1β, IL-18, LDH released with reduced cell viability and qualitatively more cell lysis.
Figure 3.9: LPS/nigericin increased cleaved caspase-1, IL-1β, IL-18 and LDH in IMGs. IMGs were cultured with LPS (100 ng/ml; 8h) and ATP (1mM; 10 min) or nigericin (10 µM; 30 min) in a 12-well plate. Active caspase-1 was measured in the cells using FLICA 660 Caspase-1 kit and analysed by flow cytometry. LDH and IL-1β, IL-18 were measured in the conditioned media by CytoTox 96® Non-Radioactive Cytotoxicity Assay and ELISA respectfully. LPS/nigericin increased the frequency of FLICA+ single cells as shown by the individual cells in the dot plot and the corresponding quantification (A; "p<0.01; "p<0.01) compared to control and LPS/ATP. LPS/nigericin increased the mean fluorescence intensity (MFI) of FLICA as shown by the
histogram and corresponding quantification (B; ‘p<0.05) compared to LPS/ATP. LPS/nigericin increased IL-1β, IL-18 and LDH (C-E; ‘p<0.01; ****p<0.0001 versus control, **p<0.01; ****p<0.0001 versus LPS/ATP. Data are mean ± SEM (n=3-6 per group) and are a representative of model characterisation. **p<0.01; ****p<0.0001 versus control, *p<0.05; **p<0.01; ****p<0.0001 versus LPS/ATP; one-way ANOVA with post hoc Dunnett’s multiple comparisons test versus LPS/nigericin.

3.2.9 GSK2795039 attenuated LPS/nigericin induced NOX2 and NLRP3 inflammasome activation in primary microglia.

Given that LPS/nigericin was found to be a more potent inducer of the NLRP3 inflammasome in IMGs and primary microglia, we further investigated the effect of GSK2795039 on NOX2-NLRP3 inflammasome inhibition in this more potent model in primary microglia.

To evaluate this, primary microglia were pre-treated with GSK2795039 (5, 10, or 20 µM) or MCC950 (0.01 µM), for 1h prior to LPS stimulation (100 ng/ml; 8h) and nigericin (10 µM; 30 min) activation in a 96-well plate. ROS and cell viability were carried out in the cells using CM-H2DCFDA and MTT respectfully. Lactate dehydrogenase (LDH) was measured as an indicator of pyroptotic cell death in the conditioned media by CytoTox 96® Non-Radioactive Cytotoxicity Assay. IL-1β was measured in the supernatant by ELISA.

LPS/nigericin (L/N) stimulation increased ROS, IL-1β, LDH and reduced cell viability (Figure 3.10A-D; p<0.001, p<0.01, p<0.0001, p<0.001 versus control). GSK2795039 attenuated ROS, IL-1β and LDH (A-C; p<0.0001, p<0.05, p<0.05; p<0.01 versus LPS/nigericin) and there was no effect on cell viability (Figure 3.10D). MCC950 had no effect on ROS and IL-1β (Figure 3.10A, B), albeit mainly due to the scatter of the data. MCC950 reduced LDH (Figure 3.10C; p<0.05 versus LPS/nigericin) and had no effect on rescuing LPS/nigericin induced-cell death (Figure 3.10D). These findings suggest that GSK2795039 inhibits LPS/nigericin elicited ROS production and NLRP3 inflammasome activation whereas MCC950, although expected not to have an effect against ROS production, did not attenuate NLPR3 inflammasome activation or improve cell viability as much as expected, which may be due to the low dose of (0.01µM).
Figure 3.10: GSK2795039 attenuated LPS/nigericin increased ROS, IL-1β, LDH in primary microglia. Primary microglia were pre-treated with GSK2795039 (5, 10, or 20 µM) or MCC950 (0.01 µM) for 1h prior to stimulation with LPS/nigericin (L/N; 10 µM; 30 min) in a 96-well plate. GSK2795039 (10 µM) and MCC950 (0.01 µM) were used as drug controls. ROS and cell viability were measured in the cells using CM-H2DCFDA and MTT respectfultly. Lactate dehydrogenase (LDH), used to assess pyroptosis, and IL-1β were measured in the conditioned media by CytoTox 96® Non-Radioactive Cytotoxicity Assay and ELISA respectfully. LPS/nigericin increased ROS, IL-1β, LDH and reduced cell viability (A-D; **p<0.01; ***p<0.001; ****p<0.0001) compared to control. GSK2795039 reduced ROS, IL-1β and LDH (A-C; *p<0.05; **p<0.01; ***p<0.0001) versus LPS/nigericin. MCC950 reduced LDH (D; *p<0.05) compared to LPS/nigericin. GSK2795049 or MCC950 did not rescue cell viability loss (D). Data are mean ± SEM (n=3-6 per group) and are a representative of two independent experiments. **p<0.01; ****p<0.0001 versus control, *p<0.05, **p<0.01, ****p<0.0001 versus LPS/nigericin; one-way ANOVA with post hoc Dunnett’s multiple comparisons test versus LPS/nigericin.
3.2.10 GSK2795039 attenuated NOX2 intracellular expression and activity in IMGs.

As cell lines provide a higher yield of cells and allow for more biochemical analysis, IMGs were used for the remaining studies in this results chapter. Given that GSK2795039 reduced LPS/nigericin induced NLRP3 inflammasome activation in primary microglia, the effect of GSK2795039 on NOX2 inhibition in a model of NLRP3 inflammasome activation was next determined.

To investigate this, IMGs were pre-treated with GSK2795039 (10, or 20 µM) or MCC950 (0.5 µM), for 1h prior to LPS stimulation (100 ng/ml; 8h) and nigericin (10 µM; 30 min) activation in a 12-well plate. Cells were processed into a single cell suspension for either the measurement of intracellular NOX2 expression by flow cytometry or for measurement of NOX-driven superoxide production using lucigenin-enhanced chemiluminescence.

LPS/nigericin (L/N) increased the mean florescent intensity (MFI) of NOX2 protein as shown by the histogram of NOX2 fluorescence intensity and corresponding quantification (Figure 3.11A; p<0.0001 vs control). LPS/nigericin increased NOX-driven superoxide production, calculated as fold of control and measured by relative luminescence units (RLUs) (Figure 3.11B; p<0.01 vs control). GSK2795039 attenuated NOX2 MFI (Figure 3.11A; p<0.001 vs LPS/nigericin) whereas MCC950 pre-treatment had no effect on NOX2 MFI (Figure 3.11A). GSK2795039 (10µM) reduced NOX activity which was also reduced with MCC950 (Figure 3.11B; p<0.05; p<0.01 vs LPS/nigericin).

These findings suggest GSK2795039 inhibits NOX2 protein expression and activity in a model of NLRP3 inflammasome activation. Although MCC950, a potent NLRP3 inhibitor, fails to reduce NOX2 expression it inhibits NOX-driven superoxide activity, perhaps because the activity assay is not specific to NOX2 and therefore it is inhibiting NOX through a different means compared to GSK2795039. These findings provide evidence of GSK2795039 inhibition upstream of the inflammasome and MCC950 inhibition at the assembly of the NLRP3 inflammasome activation.
Figure 3.11: GSK2795039 attenuated NOX2 expression and activity in IMGs.

IMGs were pre-treated with GSK2795039 (10 or 20 µM) or MCC950 (0.5 µM) for 1h prior to stimulation with LPS/nigericin (L/N; 10 µM; 30 min) in a 12-well plate. GSK2795039 (10 µM) and MCC950 (0.01 µM) were used as drug controls. Intracellular expression of NOX2 was measured by flow cytometry and NOX-driven superoxide activity was measured by NADPH oxidase activity assay in the cells. LPS/nigericin increased NOX2 mean florescent intensity (MFI) by flow cytometry as shown by the histogram and corresponding quantification (A; ****p<0.0001) compared to control. LPS/nigericin increased NOX2 activity (B; **p<0.01) compared to control. GSK2795039 reduced NOX2 MFI (A; ++++p<0.0001) and NOX activity (B; ++p<0.01) versus LPS/nigericin. MCC950 had no effect on NOX2 MFI (A) but reduced superoxide activity (B; +p<0.05) versus LPS/nigericin. Data are mean ± SEM (n=3-6 per group) and are a single (A) or pooled (B) NOX2 outcome. **p<0.01; ****p<0.0001 versus control, +p<0.05; +++p<0.01; ++++p<0.0001 versus LPS/nigericin; one-way ANOVA with post hoc Dunnett’s multiple comparisons test versus LPS/nigericin.

3.2.11 GSK2795039 attenuated nitrite release, and TNFα in IMGs.

Given that the previous result illustrated the inhibition of NOX2 protein expression and activation (Figure 3.11) by GSK2795039, it was next investigated what effect this inhibition had on the pro-inflammatory mediators downstream of the NOX2/ROS signalling pathway. ROS production induces NFkB translocation and ultimately iNOS activation, followed by the chronic release of nitric oxide [124, 129]. Moreover, ROS and IL-1β can induce an overproduction of TNFα which has been shown to promote caspase-1 activity and drive neuroinflammation [130]. For these reasons, the role of GSK2795039 and MCC950 on nitrite and TNFα levels were analysed in the supernatants.

To evaluate this, IMGs were pre-treated with GSK2795039 (10, or 20 µM) or MCC950 (0.5 µM), for 1h prior to LPS stimulation (100 ng/ml; 8h) and nigericin (10 µM; 30 min)
activation in a 12-well plate. Nitrite and TNFα were determined in the conditioned media using Griess reagent and ELISA respectively.

LPS/nigericin increased nitrite and TNFα (Figure 3.12A, B; p<0.01 versus control). GSK2795039 attenuated nitrite and TNFα (Figure 3.12A, B; p<0.05; p<0.01 versus LPS/nigericin). MCC950 had no effect on nitrite or TNFα levels (Figure 3.12A, B). These findings suggest that GSK2795039 reduced NOX2 activation and downstream signalling of ROS, nitrite, and TNFα which are key mediators of microglial inflammatory response.

3.2.12 GSK2795039 attenuates NLRP3 inflammasome activation in cell lysates and supernatants in IMGs.

To further elucidate the role of GSK2795039 inhibition on the NOX2-NLRP3 inflammatory axis, components of the NLRP3 inflammasome were analysed in cell...
lysates and corresponding supernatants by Western blot. This allowed for the identification of apoptosis-associated speck-like protein containing a CARD (ASC). The oligomerisation of ASC into ASC speck is required for the formation of the NLRP3 inflammasome and binding to pro-caspase 1. Cleaved capsase-1 and cleaved IL-1β were also determined to investigate if GSK2795039 inhibition targets these active components because it is important to note that the ELISA technique does not differentiate if the IL-1β released is the pro- or active form of the molecule.

To study this, IMGs were pre-treated with GSK2795039 (10, or 20 µM) or MCC950 (0.5 µM), for 1h prior to LPS stimulation (100 ng/ml; 8h) and nigericin (10 µM; 30 min) activation in a 12-well plate. Cell lysates were processed for Western blot, and supernatants were collected for either Western blot or for analysis of cytokines using ELISA or LDH by CytoTox 96® Non-Radioactive Cytotoxicity Assay.

LPS/nigericin showed modest NLRP3 increase in cell lysates (CL) which were further upregulated in the secreted supernatant (SN) (Figure 3.13A) as demonstrated by the immunoblots. Although no difference in pro-caspase 1 expression, LPS/nigericin increased cleaved-caspase-1 in the cell lysates (Figure 3.13A; p<0.05 versus control). LPS/nigericin upregulated both pro- and cleaved-caspase 1 in the SN as shown by the immunoblots and further increased pro- and cleaved – IL-1β in both the CL and SN as shown by immunoblots and corresponding quantification for the CL (Figure 3.13A; p<0.0001 vs control). There was no induction of ASC in the CL with LPS/nigericin stimulation but increased ASC expression in the supernatant, compared to control. GSK2795039 had no effect on NLRP3 protein expression in CL but qualitatively showed attenuation in the corresponding supernatants as shown by immunoblot (Figure 3.13A). MCC950 did not show reduction of NLRP3 in CL but illustrated complete abrogation in the corresponding supernatants. GSK2795039 had no effect on pro-caspase 1 expression in the CL but demonstrated an attenuation of cleaved-caspase-1 in the CL but levels failed to reach statistical significance. Subsequently, in the corresponding supernatants, GSK2795039 reduced pro- and cleaved- caspase 1 expression. MCC950 had no effect on pro-caspase 1 expression in the CL but demonstrated complete reduction of cleaved- caspase-1 in the CL (Figure 3.13A; p<0.05 vs LPS/nigericin). Again, in the corresponding supernatants, MCC950 showed complete inhibition of
LPS/nigericin induced pro- and cleaved- caspase 1 expression. GSK2795039 reduced pro-IL-1β (Figure 3.13A; p<0.05; p<0.01 vs LPS/nigericin) and cleaved IL-1β but levels failed to reach statistical significance in the CL. In the corresponding supernatants, GSK2795039 reduced pro- and cleaved- IL-1β expression. MCC950 had no effect on either pro- or cleaved- IL-1β in the CL, but levels of both appeared reduced in the supernatants. Despite no effect of GSK2795039 or MCC950 on ASC protein expression in the CL, GSK2795039 showed reduced ASC expression in the supernatants, which was also observed with MCC950.

In separate analysis of the supernatants, LPS/nigericin increased IL-1β and IL-18 (Figure 3.13B, C; p<0.05; p<0.001 versus control). GSK2795039 attenuated IL-1β and IL-18 in a dose-dependent manner (Figure 3.13B, C; p<0.05; p<0.01 versus LPS/nigericin). MCC950 also completely suppressed IL-1β and IL-18 (Figure 3.13B, C; p<0.05; p<0.001 versus LPS/nigericin). After analysis of the NLRP3 inflammasome pathway and downstream products, the next step was to examine if the GSK2795039 attenuation of IL-1β and IL-18 is pyroptosis dependent. LPS/nigericin increased LDH (Figure 3.13D; p<0.0001 vs control) and GSK2795039 attenuated LDH (Figure 3.13D; p<0.05; p<0.0001 vs LPS/nigericin). MCC950 treated IMGs caused LDH to return to control levels (Figure 3.13D; p<0.0001 vs LPS/nigericin).

These findings indicate that the inhibition of ASC and cleaved caspase 1, by GSK2795039, prevents the activation of the NLRP3 inflammasome which is confirmed by the reduction in the downstream products; IL-1β, IL-18 and LDH and therefore preventing a pro-inflammatory cascade.
Figure 3.13: GSK2795039 attenuated LPS/nigericin induced NLRP3 inflammasome activation in supernatants of IMGs.

IMGs pre-treated with GSK2795039 (10 or 20 µM) or MCC950 (0.5 µM) for 1h prior to stimulation with LPS/nigericin (L/N; 10 µM; 30 min) in a 12-well plate. GSK2795039 (10 µM) and MCC950 (0.01 µM) were used as drug controls. Cell lysates and supernatants were processed and NLRP3, Caspase-1, IL-1β and ASC protein expression were measured by Western blot. IL-1β, IL-18 and lactate dehydrogenase (LDH), used to assess pyroptosis, were measured in the conditioned media by ELISA and CytoTox 96® Non-Radioactive Cytotoxicity Assay respectfully. In the cell lysates, LPS/nigericin increased NLRP3, cleaved-caspase-1, pro- and cleaved-IL-1β protein expression lysates as shown by immunoblot and corresponding quantification (A; *p<0.05; ****p<0.0001) compared to controls. GSK2795039 reduced pro-IL-1β (A; **p<0.01; +++p<0.0001) and cleaved-caspase-1 and cleaved-IL-1β but failed to reach significance, versus
LPS/nigericin. MCC950 reduced cleaved-caspase-1 (A; *p<0.05) versus LPS/nigericin. In the corresponding supernatants, GSK2795039 reduced LPS/nigericin induced NLRP3, pro- and cleaved-caspase-1, pro- and cleaved-IL-1β and ASC (A), which were also reduced with MCC950. LPS/nigericin increased IL-1β, IL-18, and LDH (B-D; *p<0.05; ***p<0.001; ****p<0.0001 versus control. GSK2795039 reduced IL-1β, IL-18, and LDH (B-D; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001). MCC950 also reduced IL-1β, IL-18, and LDH (B-D; *p<0.05; **p<0.01; ****p<0.0001). Data are mean ± SEM (n=3, averages of three independent experiments. *p<0.05; **p<0.01; ****p<0.0001 versus control, **p<0.01 versus LPS/nigericin; one-way ANOVA with post hoc Dunnett’s multiple comparisons test versus LPS/nigericin, unless stated otherwise in supplemental table 1.

3.3 Discussion

NOX2 activation is a major contributor to neuroinflammation and is associated with persistent microglial activation up to 1 year post TBI [39]. Mice deficient in NOX2 have reduced lesion volume and neuronal loss along with attenuated NLRP3 inflammasome activation, as depicted by reduced ASC and cleaved IL-1β protein, in the injured cortex post TBI [94]. Thus, NOX2 plays a pivotal role in oxidative stress, due to an imbalance of high levels of ROS and neurotoxicity, and therefore is an important therapeutic target of neuroinflammation in TBI.

The studies in this chapter investigated the mechanisms that underlie the link between NOX2/ROS signalling and NLRP3 inflammasome activation. For this reason, three models of microglial activation and pro-inflammatory responses were utilised. LPS primed the cells and upregulated NOX2 activation along with ROS production and downstream release of nitrite (Figures 3.1-4) in immortalised microglial cell lines (IMGs) and primary microglia. Nitrite is an indication of nitric oxide (NO) which is a key intra- and extracellular messenger that mediates diverse signalling pathways in the brain. NO can be neurotoxic under neuroinflammatory conditions, including after TBI [125]. Indeed, a longer priming with LPS (24h) was required for enhanced ROS and subsequent nitrite production compared with acute priming (4h or 8h) whereby no nitrite was observed (Figure 3.3) thus proving ROS signalling is upstream of nitrite release. This has been shown by others to be because ROS promotes NFκB, leading to iNOS activation and NO release [124, 129].
Notably, in contrast to the above, IL-1β was produced at 4 and 8 h but not at 24 h post LPS/ATP stimulation. This temporal aspect became hugely relevant when linking both LPS and LPS/ATP models of neuroinflammation. Previous studies have shown that NO induced by long-term LPS priming inhibits NLRP3 inflammasome activation and supresses IL-1β levels [131, 132]. Furthermore, NO inhibits NLRP3-mediated formation of ASC in LPS/ATP stimulated macrophages. In iNOS KO mice, ASC pyroptosome is efficiently assembled at both 6h and 24h, which is not the case in WT mice at 24h, as the capacity to assemble ASC diminished following long-term LPS priming [133]. A separate study indicated that iNOS works synergistically with itaconate, an anti-inflammatory molecule, to tolerate the NLRP3 inflammasome. Long-term priming of LPS promotes endotoxin-induced tolerance to inflammasome activation via NO production [132]. In agreement with the literature, this study showed that there was an important temporal aspect for regulating the inflammasome and at the longer LPS priming of 24h, significant release of nitrite suppressed levels of IL-1β. Moreover, acute LPS priming of 4 and 8h was too early to activate iNOS and subsequent nitrite release, therefore no inhibition in IL-1β was observed (Figure 3.3). This provided the rationale for priming with LPS for 8h followed by ATP to induce both ROS production and NLRP3 inflammasome activation in the remaining studies.

NOX2 production initiates NLRP3 inflammasome activation and drives neuropathogenesis in TBI. NOX2 KO mice have reduced NLRP3 inflammasome activation after TBI associated with decreased cleaved IL-1β in the injured cortex [94]. To mimic effects of NOX2-NLRP3 in vitro, LPS/ATP, and more potently, LPS/nigericin acted as inducers of the NLRP3 inflammasome in both IMGs and primary microglia and therefore ultimately enabled the assessment of GSK2795039 on this activated model. Extracellular ATP acts as an agonist for P2X7R and allows for the balanced exchange of potassium (K⁺) out of the cell and influx of sodium (Na⁺). Nigericin is a free membrane-impermeant ionophore that directly binds to hydrogen (H⁺) outside the cell transporting it into the cell leading to acidification of the cytosol along with binding to K⁺ inside the cell and transporting it out [123]. As well as promoting K⁺ efflux, nigericin initiates the translocation of ASC into the cytoplasm and induces oligomerisation which is required for NLRP3 inflammasome activation and may be why nigericin appeared to act more
potently in this present study. LPS/nigericin demonstrated augmented potency and activation in the cells compared to LPS/ATP by increased caspase-1 activity and downstream IL-1β and LDH release. However, the higher concentrations of LPS/nigericin stimulation showed reduced ROS production compared to LPS/ATP or the less potent LPS/nigericin (1 µM). This indicated that a weaker stimulus was needed to induce ROS and more potent stimulations leading to cell death and IL-1β release failed to enhance ROS production, perhaps due to a decrease in the viability of cells as shown in Figure 3.7.

Once the model of microglial pro-inflammatory activation was optimised, the aim was to identify a therapeutic approach using a specific NOX2 inhibitor, GSK2795039, which was hypothesised to downregulate NOX2 expression and activity such as ROS production and promote neuroprotective properties. GSK2795039 is a small molecule, specific NOX2 inhibitor that has been demonstrated in preclinical models to cross the blood brain barrier [74]. Mice pre-treated with GSK2795039 significantly reduced NOX2-dependent ROS production compared to vehicle, and levels were similar to that of the positive control gp91<sup>−</sup>−. However, there was no reduction in NOX2 driven ROS by apocynin, a broad antioxidant.

In this project, GSK2795039 has been characterised as a NOX2 inhibitor, reducing LPS, LPS/ATP and LPS/nigericin induced NOX2 protein expression and activity (Figure 3.11) and NLRP3 inflammasome activation in immortalised microglial cell lines (IMGs) and primary microglia. It was observed that GSK2795039 but not DPI<sup>+</sup>, a broad antioxidant and non-specific NOX2 inhibitor, did not reduce TNFα (Figure 3.2) which indicates GSK2795039 inhibited a general pro-inflammatory cytokine which may potentially be because of NOX2-ROS driven production. Moreover, GSK2795039 significantly decreased LPS/ATP and LPS/nigericin induced ROS, IL-1β, IL-18, LDH (Figure 3.5, 3.6, 3.10, 3.13) demonstrating its effects against NOX2/NLRP3 inflammasome activation. Indeed, GSK2795039 was hypothesised to act on NOX2 inhibition upstream of the NLRP3 inflammasome and not directly acting on the cleavage of IL-1β. This was further supported by the fact that GSK2795039 attenuated IL-1ra (Figure 3.5) which acts as a modulatory factor, released in conjunction with IL-1β release and dampens down the inflammatory effects of IL-1β [126]. Both IL-1β and IL-1ra bind to IL-1 receptor 1 (IL-1R1).
IL-1ra actively blocks IL-1R1 and therefore the activation of IL-1β [134]. As GSK2795039 reduced IL-1β it concomitantly reduced IL-1ra release, proving that this drug does not work on IL-1R1, and hence must be acting on upstream pathways of the NLRP3 inflammasome, such as NOX2.

In further elucidating the mechanism of GSK2795039 inhibition, it was shown that by reducing ASC protein expression and further preventing the interaction with cleaved caspase-1 (Figure 3.13), GSK2795039 attenuated the release of IL-1β and IL-18 cytokines and thus a pro-inflammatory cascade. This was compared to MCC950, a potent NLRP3 inhibitor, which acted as a positive control for the NLRP3 inflammasome experiments. MCC950 has been used therapeutically to inhibit the NLRP3 inflammasome activation and prevent IL-1β release [93, 98] and in this present study demonstrated reduced cleaved caspase-1, IL-1β, IL-18 and LDH.

Ultimately, the key finding of the present study is that GSK2795039 inhibited NLRP3 inflammasome activation, which may be due to reduced NOX2/ROS signalling. It was observed that GSK2795039 elicited a reduction in NOX2 protein in cells, and this was positively correlated with a reduction in IL-1β release into the supernatant as shown by the Pearson correlation and linear regression analysis generating a $R^2$ value= 0.6818 (p<0.0001) in Figure 3.14.

![Figure 3.14: Positive correlation of GSK2795039 inhibition on NOX2 and IL-1β in IMGs.](image)

GSK2795039 inhibited LPS/nigericin induced NOX2 mean fluorescent intensity (MFI) in the cells, which positively correlated with a reduction in IL-1β released in the corresponding supernatant in IMGs. Data are mean ± SEM (n=4 per group) and are a representative of three independent experiments. P<0.0001; by Pearson Correlation and linear regression analysis.
In summary of this results chapter, GSK2795039 inhibited NOX2 activation, and pro-inflammatory microglial responses such as ROS and TNFα production, therefore inhibiting potential second signals required for inflammasome activation. NOX2 inhibition led to attenuation of pro-inflammatory downstream signalling as illustrated by the schematic in Figure 3.15 below. These *in vitro* findings indicate that GSK2795039 may be a promising therapeutic drug for mitigating the damaging effects of NOX2-mediated neuroinflammation in microglia.

**Figure 3.15:** Summary schematic of GSK2795039 inhibition of NOX2/NLRP3 inflammatory axis. GSK2795039 reduced NOX/ROS activation upstream of the NLRP3 inflammasome leading to the reduction in downstream inflammatory products such as Caspase-1, IL-1β, IL-18 and LDH. Image created using BioRender.
Chapter 4: Optimisation of Controlled Cortical Impact in mice to characterise the cellular and temporal dynamics of NOX2-NLRP3 activation after TBI
4.1 Introduction

The previous in vitro chapter presented mechanisms involved in NOX2-mediated NLRP3 inflammasome activation in stimulated microglia from a murine-derived cell line; immortalised microglia (IMGs) and primary microglia. In addition to the in vitro work, preclinical studies have shown NOX2 acts upstream of the NLRP3 inflammasome and contributes to TBI pathology. NOX2 deficient mice had decreased NLRP3 inflammasome activation associated with reduced cleaved IL-1β [94]. Furthermore, inhibiting microglial-mediated NLRP3 inflammasome activation using MCC950 alleviated neuroinflammation post CCI [93], indicating NOX2 and NLRP3 inflammasome activation involvement in progressive neuroinflammation following TBI.

However, it is known that brain injury is a much more complicated immune milieu in the CNS. Not only have microglia been shown to be chronically activated in mice [39] and humans [20, 135] following TBI, the pro-inflammatory infiltrating peripheral cells play a pivotal role in the progression of TBI-elicited neuroinflammation. Kumar et al., illustrated influx and accumulation of NOX2⁺ CCR2⁺ macrophages into the ipsilateral cortex of CCI mice which peaked at 7 days post injury (DPI) and had higher expression than NOX2⁺ microglia suggesting a greater pro-inflammatory profile than the resident cells. [16]. A separate study demonstrated pro-inflammatory CCR2⁺ macrophage accumulation was reduced with a CCR2 antagonist which led to attenuated NOX2 activation and ultimately reduced neuroinflammation [106]. Recent studies have suggested a potential mechanism whereby microglia-mediated T cell influx through chemotactic signalling, promotes neuroinflammation in radiation-induced brain injury patients [107]. This emerging evidence not merely provides the rationale for this results chapter but suggests the inflammatory resident microglia and infiltrating cells as potential targets in attenuating neuroinflammation following TBI.

In order to translate in vitro findings into the in vivo setting, optimisation of the TBI model was first required. Controlled cortical compact (CCI) delivers a unilateral focal contusion to the left parietal lobe which induces cortical tissue loss, subdural hematoma and blood brain barrier (BBB) disruption [27]. CCI mimics pathologies observed in human TBI patients [26, 136] such as reduced motor and cognitive function and increased
microglial activation [135]. As the first PhD student in Dr. David Loane’s lab in Trinity College Dublin, it was imperative to optimise the in vivo model of TBI. Preclinical researchers in the TBI field using the CCI model demonstrate both mild and moderate-severe injury with a variety of impact parameters such as speed, depth and dwell-time. Therefore, the aim in this project was to optimise the parameters which induce a moderate level CCI under sterile conditions, with low mortality and minimal variability between experiments.

In refining this model of TBI, this project focused on the importance of cell-specific functions over the acute temporal course of injury. In conjunction with TBI inducing microglial activation and upregulated neuroinflammation, ongoing research proves a strong link between the CNS and the immune system following TBI [30]. A comprehensive review by McKee et al., outlined the kinetics of the immune response to TBI highlighting neutrophils as the immediate peripheral cells to infiltrate the brain following injury. This was followed by activated monocytes trafficking into the brain with concomitant microglial activation and in time, the infiltration of T cells promoting an adaptive immune response [105]. Physiologically, in this phase the apoptotic cells are present on antigen presenting cells (APC) such as microglia or macrophages, to induce T cell activation and resolve the inflammatory response [15]. However, following chronic TBI pathology, this may lead to an over-reactive T cell response and increased inflammation [137]. In knowing the important function the immune system plays in TBI pathogenesis, this present study aimed to identify the role of resident microglia, infiltrating myeloid cells; neutrophils and monocytes and lymphocytes; T cells in NOX2/ROS and NLRP3 inflammasome activation in a time-course study of 1-, 3- and 7 DPI.

In addition to characterising the effect of CCI on neuroimmunology at a cellular level, this project aimed to optimise cognitive and motor behavioural tasks in male and female mice at a more chronic time-point post CCI. The majority of experimental TBI studies focusing on neuroinflammation have been carried out in males [138]. Given that hormonal differences may impact immune response, more interest has been taken into investigating sex differences in preclinical studies [139]. Mechanistically, research in the TBI field has shown that sex differences are mediated by infiltrating myeloid cells. CCI
males showed greater numbers of infiltrating cells into the brain at 1 DPI which preceded an exaggerated microglial response at 3 DPI in comparison to females, shown by flow cytometry [116]. An independent study, using histology, also found rapid microglial activation and infiltration of cells in male but not females at 1- and 3 DPI which was associated with neuronal loss and greater lesion volume [140]. These findings would suggest females are more protected than males acutely post injury and would coincide with the finding that females showed improved motor function on the rotarod at 1 DPI compared to males [116]. However these results presented acute and transient protection in females which was not sustained overtime as shown by similar rotarod performance between males and females by 3 DPI [116] and equal brain volume loss at 30 DPI [140]. Thus, there is a need for future research aimed at targeting sex differences at more chronic timepoints post TBI.

Overall in this results chapter, the first objective was to optimise the CCI model along with the flow cytometry and neuro-behavioural protocols. The next objective was to determine temporal CCI-induced cell-specific functions. Finally, sex differences in cognitive and motor function over a 28d CCI study was examined.
4.2 Results

4.2.1 Qualitative analysis revealed increased gross pathology in CCI mice.
This present study showed, qualitatively, that controlled cortical impact (CCI) induced gross pathology and lesion development in the ipsilateral cortex over time as shown in Figure 4.1. Acutely, there was evidence of bleeding and oedema of the brain at 1 DPI. 14 DPI revealed a deeper lesion on the left parietal lobe of the ipsilateral cortex which preceded an enlarged lesion development at 28 DPI.

![Figure 4.1: Gross pathology and lesion volume development following CCI in adult male mice.](image)

Given that previous studies have indicated that enlarged lesion volume is associated with cognitive dysfunction and worsened outcomes following TBI [16, 78] the next experiments investigated the effect of CCI at different timepoints and identified the cell populations that play a role in progressive neuroinflammation following CCI.

4.2.2 CCI induced a greater righting reflex compared to sham mice after surgery.
To investigate the role of resident microglia and infiltrating cells following CCI, a time-course study was carried out in adult male C57BL6/J mice and revealed the number and function of these cell population at 1-, 3- and 7 DPI. The surgeries took place on different
days so that all mice in the study were euthanised, brains were processed, and samples were analysed by flow cytometry on the same day. Sham mice surgery took place at 7 DPI.

To ensure rigor and reproducibility of these experiments, a multitude of parameters including the duration of the surgery, the time it took the mice to right themselves and the weights were recorded. As the mice were anaesthetised with isoflurane for the duration of the surgery it was important that sham and CCI mice spent equal amounts of time under anaesthetic and therefore this factor could not impact experimental results. Isoflurane has been shown to alter stress related physiological responses in rodents through increased levels of plasma corticosterone which can alter behaviour [141, 142]. Given this was a time-course study, we ensured the surgery duration was equal for all CCI groups.

Although no difference in surgery duration between groups (Figure 4.2A), CCI induced increased time it took the mice to right themselves after injury (Figure 4.2B) across all groups compared to sham (Figure 4.2B; p<0.05; p<0.001). The weights of mice did not differ significantly throughout the experiment and only the 3 DPI mice had significantly reduced weight at 2 DPI (C; p<0.01 vs 0 DPI) with levels returned to baseline by 3 DPI.

Figure 4.2: CCI induced a greater righting reflex compared to sham mice after surgery.
Adult male C57BL6/J mice were subjected to sham or CCI surgery and euthanised at 1-, 3- or 7 DPI. The surgery duration and righting reflex time was recorded on the day of the surgery and weight was recorded throughout the study. There was no difference in surgery duration (A). CCI mice had increased righting reflex time (B; *p<0.05; ***p<0.001 vs sham) and reduced weight at 2 DPI compared to baseline (C; **p<0.01 vs 0 DPI) in 3 DPI mice. The weight was consistent between groups after 3 DPI. Data are mean ±SEM (n=4-5 per group). *p<0.05; ***p<0.001 vs sham; by One-Way ANOVA with Dunnett’s multiple comparisons test (A, B); Two-way Mixed effect analysis with repeated measures (C).
4.2.3 NOX2 and NLRP3 inflammasome gene expression peaked at 3 DPI in CCI mice.

To determine the effect of CCI at the tissue level, hippocampal tissue punches were taken from the ipsilateral cortex of sham or CCI mice and Real-Time quantitative polymerase chain reaction (RT-qPCR) was carried out. The following Taqman mRNA probes were measured; Cyba and Cybb which are NOX2 membrane bound subunits, Tnf which is a general pro-inflammatory marker, Cd68 a marker of microglial activation and Nlrp3 and Il1b which indicate NLRP3 inflammasome activation at the gene level. The graphs are illustrated by relative expression (RE), which was calculated as fold difference from the ΔΔCT method.

The results revealed a significant increase in Cyba, Cybb, Cd68 and Nlrp3 (RE) at 3 DPI (Figure 4.3A-D; p<0.05 vs sham). Cd68 was significantly reduced at 7 DPI (Figure 4.3C; p<0.05 vs 3 DPI), along with a reduction in Cyba, Cybb and Nlrp3 (Figure 4.3A, B, D) at 7 DPI, but levels failed to reach statistical significance. In terms of pro-inflammatory cytokine gene expression, CCI induced a significant upregulation of Il1b and Tnf at 1 DPI (Figure 4.3E, F; p<0.001 vs sham). Il1b and Tnf peaked at 1 DPI as demonstrated by a reduced expression at 3-, and 7 DPI (Figure 4.3E, F; p<0.05; p<0.01; p<0.001 vs 1 DPI).

These results suggest cytokine gene expression peaks early, followed by the protein release at a later timepoint. Furthermore, given the inflammatory environment within the tissue by 3 DPI, it was next sought to determine the function at the cellular level by flow cytometry.
Figure 4.3: NOX2 and NLRP3 inflammasome gene expression peaked at 3 DPI in CCI mice.

Adult male C57BL6/J mice were subjected to sham or CCI surgery and euthanised at 1-3 or 7 DPI. Hippocampal tissue punches were taken from the ipsilateral cortex of sham or CCI mice. RT-qPCR amplification was carried out which measured Cyba, Cybb, Cd68, Nlrp3, Il1b and Tnf. CCI induced increased Cyba, Cybb, Cd68 and Nlrp3 at 3 DPI (A-D; 'p<0.05 vs sham) and there was significantly lower Cd68 at 7 d (C; 'p<0.05 vs 3 DPI). CCI increased Il1b and Tnf gene expression at 1 DPI (E, F; ***p<0.001 vs sham) which was significantly lower at 3 DPI (E, F; *p<0.05 vs 1 DPI) and at 7 DPI (E, F; **p<0.01; ***p<0.001 vs 1 DPI). Data are mean ± SEM (n=4-5 per group). *p<0.05; ***p<0.001 vs sham; 'p<0.05; **p<0.01; ***p<0.001 vs 1 DPI; *p<0.05 vs 3 DPI; by One-Way ANOVA with Tukey’s multiple comparisons test.

4.2.4 CCI induced increased numbers of infiltrating neutrophils and monocytes at 1-3 DPI.

Given the previous figure demonstrated the inflammatory profile at the tissue level, the next experiments evaluated the number of resident microglia and infiltrating cells at 1-, 3- and 7 DPI, and ultimately determined cellular function by multi-dimensional flow cytometry.

To assess this, mononuclear cells isolated from the ipsilateral cortex, using a Percoll® gradient, were stained with CD11b, CD45, Ly6G and Ly6C to label resident microglia (CD11b⁺CD45<sup>int</sup>), total infiltrating myeloid cells (CD11b⁺CD45<sup>hi</sup>), neutrophils
(CD11b^CD45^{hi}Ly6G^+) and monocytes (CD11b^CD45^{hi}Ly6C^+) and analysed by flow cytometry. The flow plots below are gated on live single cells, with the full gating strategy illustrated in detail in Chapter 2: Materials and Methods, section 2.2.6. The absolute number of cells was calculated based on the frequency of the total parent population.

Although number of microglia was not significantly different between groups (Figure 4.4C), CCI induced increased infiltration of cells, which express CD45^{hi}, into the brain at 1- and 3 DPI as shown in the representative dot plots (Figure 4.4A) and corresponding quantification (Figure 4.4D; p<0.001; p<0.0001 vs sham). The infiltrating myeloid cells consisted of Ly6G^+ neutrophils and Ly6C^+ monocytes as shown in the lower panel (Figure 4.4B). Neutrophils are the first responders following an infection/insult in the brain [105] in response to pro-inflammatory cytokines released by the activated microglia [15]. Neutrophils were shown here to infiltrate early after injury, by a significant increase in absolute numbers at 1 DPI as demonstrated in the representative dot plots (Figure 4.4B) and corresponding quantification (Figure 4.4E; p<0.0001 vs sham). The number of neutrophils peaked at 1 DPI and there was significant reduction in neutrophils at 3- and 7 DPI (Figure 4.4E; p<0.01; p<0.001 vs 1 DPI). The number of monocytes was significantly upregulated in CCI mice at 1 DPI (Figure 4.4F; p<0.01 vs sham) which peaked at 3 DPI (Figure 4.4F; p<0.05 vs 1 DPI. Similar to the neutrophils, the number of monocytes was diminished at 7 DPI which is evident from the flow plots (B) and quantification (Figure 4.4F; p<0.0001 vs 3 DPI). These data provide an insight into the number of cells within the different cell populations and demonstrate the dominant cell type across the acute time-points following CCI.
Figure 4.4: CCI induced increased numbers of infiltrating neutrophils and monocytes at 1- and 3 DPI.

Adult male C57BL6/J mice were subjected to sham or CCI surgery and euthanised at 1-, 3- or 7 DPI. Mononuclear cells isolated from the ipsilateral cortex were stained for CD11b, CD45, Ly6G and Ly6C, and analysed by flow cytometry. CCI induced increased number of infiltrating myeloid cells (CD11b^hi^CD45^hi^) at 1- and 3 DPI as shown in the representative dot plots (A) and quantification (D; ***p<0.001; ****p<0.0001 vs sham). Of these infiltrating cells, the number of neutrophils (Ly6G^hi^) were increased and peaked at 1 DPI (E; ****p<0.0001 vs sham) and the number of monocytes (Ly6C^hi^) increased at 1 DPI (F; **p<0.01 vs sham) and peaked at 3 DPI.
Data are mean ± SEM (n=4-5 per group). *p<0.05; **p<0.01; ***p<0.001 vs sham; *p<0.05; **p<0.01; ***p<0.001 vs 1 DPI; *p<0.05 vs 3 DPI; by One-Way ANOVA with Tukey’s multiple comparisons test.

4.2.5 Microglial NOX2/ROS and IL-1β production peaked at 3 DPI in CCI mice.

Assessment of microglial number revealed a modest increase after injury which failed to reach statistical significance (Figure 4.4). Along with identifying the absolute number of cells, determining the function of these cells is necessary in identifying the population of cells responsible for promoting neuroinflammation at specific time-points following CCI. Firstly, the microglial phenotypic function across the temporal CCI study was investigated.

To assess cell-specific functional output, cells were stained with surface markers, DHR123, an indicator of ROS production, followed by intracellular staining of NOX2 and IL-1β and analysed by flow cytometry. The number of NOX2+ and IL-1β+ microglia were determined using quadrants as shown in the representative dot plots (Figure 4.5A). Briefly, Q1 + Q2 is IL-1β+, Q2 + Q3 is NOX2+, Q2 is the double positive for NOX2+ IL-1β+ and Q4 is the double negative. The populations were gated using the fluorescent minus one (FMO) control, which contained the same staining as the samples apart from the staining of interest to indicate the positive gate. ROS production was measured as the mean fluorescent intensity (MFI) as this was the clearest way to visualise the data for this specific dye.

CCI induced increased #NOX2+ microglia at 3- and 7 DPI as shown by the representative dot plots (Figure 4.5A) and corresponding quantifications (Figure 4.5B; p<0.0001 vs sham). The number of NOX2+ microglia peaked at 3 DPI as demonstrated by significantly lower numbers at 1- and 7 DPI (Figure 4.5B; p<0.0001 vs 1 d; p<0.01 vs 7 DPI). Although non-significant, there was a trend towards increased IL-1β+ microglia at 3 DPI (Figure 4.5C). CCI upregulated the double positive (Q2) NOX2+ IL-1β+ at 3- and 7 DPI (Figure 4.5D; p<0.05; p<0.001 vs sham). As well as increased NOX2 and IL-1β expression, CCI induced microglial ROS production across all timepoints as illustrated by the representative MFI histograms and corresponding quantification (Figure 4.5E; p<0.05; p<0.001; p<0.0001 vs sham).
Microglial ROS production was highest at 3 DPI, followed by decreased ROS observed at 7 DPI (Figure 4.5E; p<0.05 vs 3 DPI). These data highlight 3 DPI as the peak of microglial activation through NOX2/ROS and IL-1β production.

Figure 4.5: Microglial NOX2/ROS and IL-1β production peaked at 3 DPI in CCI mice. Adult male C57BL6/J mice were subjected to sham or CCI surgery and euthanised at 1-, 3- or 7 DPI. Mononuclear cells isolated from the ipsilateral cortex were stained with surface markers, DHR123, an indicator of ROS production followed by intracellular staining of NOX2 and IL-1β and analysed by flow cytometry. CCI increased #NOX2⁺ and #NOX2⁺ IL-1β⁺ microglia at 3- and 7 DPI as shown in the representative quadrants (A) and corresponding quantifications (B, D; *p<0.05; ***p<0.001; ****p<0.0001 vs sham). CCI induced ROS production, as shown by increased DHR123 MFI, in microglia across all time-points (E; *p<0.05; ***p<0.001; ****p<0.0001 vs sham) which peaked at 3 DPI (E; *p<0.05 vs 7 DPI). Data are mean ± SEM (n=4-5 per group). *p<0.05; ***p<0.001; ****p<0.0001 vs sham; *p<0.05; ****p<0.0001 vs 1 DPI; *p<0.05; **p<0.01; vs 3 DPI; by One-Way ANOVA with Tukey’s multiple comparisons test.

4.2.6 Neutrophil NOX2/ROS and IL-1β production peaked at 1 DPI in CCI mice.

As the previous data on absolute number assessment depicted neutrophils as the early infiltrating cells into the brain following CCI, the role of these neutrophils was next evaluated.
TBI has been shown to increase oxidative burst in circulating neutrophils in humans after 24h [109]. Given respiratory burst is an important function of neutrophils in the innate immune response and preventing infection [53], evaluating temporal NOX2/ROS levels was therefore a primary output in this study. Analysis of neutrophil function was carried out in the same manner as microglia in the previous figure.

CCI induced increased #NOX2⁺ neutrophils at 1- and 3 DPI as shown by the representative quadrants (Figure 4.6A; Q2 + Q3) and corresponding quantification (Figure 4.6B; p<0.05; p<0.001 vs sham). There was a significant reduction in #NOX2⁺ neutrophils at 7 DPI (Figure 4.6B; p<0.05 vs 3 DPI) which was due to an overall decreased number of neutrophils at this timepoint. CCI increased #IL-1β⁺ (Q1 + Q2) and #NOX2⁺ IL-1β⁺ neutrophils (Q2) at 1 DPI which were significantly lower at 7 DPI (Figure 4.6C, D; p<0.01 vs sham; p<0.05 vs 1 DPI). CCI induced neutrophil ROS production at 1 DPI as illustrated by the representative MFI histograms and corresponding quantification (Figure 4.6E; p<0.05 vs sham). Similar to #NOX2⁺ IL-1β⁺, there was significantly lower ROS production in neutrophils at 7 DPI (Figure 4.6E; p<0.05 vs 1 DPI). These findings highlight 1 DPI as the peak of neutrophil activation through NOX2/ROS and IL-1β production.
Figure 4.6: Neutrophil NOX2/ROS and IL-1β production peaked at 1 DPI in CCI mice. Adult male C57BL6/J mice were subjected to sham or CCI surgery and euthanised at 1-, 3- or 7 DPI. Mononuclear cells isolated from the ipsilateral cortex were stained with surface markers, DHR123, an indicator of ROS production followed by intracellular staining of NOX2 and IL-1β and analysed by flow cytometry. CCI increased #NOX2+, #IL-1β+ and #NOX2+IL-1β+ neutrophil at 1 DPI as shown in the representative quadrants (A) and corresponding quantifications (B-D; **p<0.01; ***p<0.001 vs sham). #NOX2+ neutrophils were also increased at 3 DPI (B; *p<0.05 vs sham) with numbers reduced to sham levels at 7 DPI (B; *p<0.05 vs 3 DPI; C, D; *p<0.05 vs 1 DPI). Neutrophils induced ROS production at 1 DPI, as shown by increased DHR123 MFI (E; *p<0.05 vs sham) which was reduced at 7 DPI (E; *p<0.05 vs 1 DPI). Data are mean ± SEM (n=4-5 per group). 

4.2.7 Monocyte IL-1β production and NOX2/ROS activation peaked at 1- and 3 DPI respectfully in CCI mice.

Given the pro-inflammatory profile of neutrophils predominantly at 1 DPI and the fact the number of monocytes peaked at 3 DPI, the functional role of monocytes following CCI was next examined. Analysis for monocytic function was carried out in the same manner as microglia and neutrophils as shown in the previous figures.
CCI induced increased \#NOX2\(^+\) monocytes at 1- and 3 DPI as shown by the representative quadrants (Figure 4.7A; Q2 + Q3) and corresponding quantification (Figure 4.7B; \(p<0.001; p<0.0001\) vs sham). There was a significant reduction in \#NOX2\(^+\) monocytes at 7 DPI (Figure 4.7B; \(p<0.001\) vs 3 d). CCI increased \#IL-1\(\beta\)\(^+\) (Q1 + Q2) and \#NOX2\(^+\) IL-1\(\beta\)\(^+\) monocytes (Q2) at 1 DPI (Figure 4.7C, D; \(p<0.0001\) vs sham) which was significantly lower at 3- and 7 DPI (Figure 4.7C, D; \(p<0.001; p<0.0001\) vs 1 DPI). Notably, the number of IL-1\(\beta\)\(^+\) monocytes was higher than the number of IL-1\(\beta\)\(^+\) neutrophils (Figure 4.6). CCI induced increased monocytic ROS production at 1 DPI which peaked at 3 DPI as demonstrated by the representative MFI histograms and corresponding quantification (Figure 4.7E; \(p<0.05; p<0.0001\) vs sham; \(p<0.01\) vs 1 DPI). Similar to \#NOX2\(^+\) IL-1\(\beta\)\(^+\), there was significantly lower ROS production in monocytes at 7 DPI (Figure 4.7E; \(p<0.001\) vs 3 DPI). These results suggest activated monocytes induce NOX2/ROS production starting at 1 DPI which is sustained until 3 DPI.

**Figure: 4.7:** Monocyte IL-1\(\beta\) production and NOX2/ROS activation peaked at 1- and 3 DPI respectfully in CCI mice.

Adult male C57BL6/J mice were subjected to sham or CCI surgery and euthanised at 1-, 3- or 7 DPI. Mononuclear cells isolated from the ipsilateral cortex were stained with surface markers,
DHR123, an indicator of ROS production followed by intracellular staining of NOX2 and IL-1β and analysed by flow cytometry. CCI increased #NOX2+ monocytes at 1- and 3 DPI as shown in the representative dot plots and quantification (B; ***p<0.001; ****p<0.0001 vs sham) with reduced levels at 7 DPI (B; ***p<0.001 vs 3 d). CCI increased #IL-1β+ and #NOX2+ IL-1β+ monocytes at 1 DPI (C, D; ****p<0.0001 vs sham) which was lower at 3- and 7 DPI (C, D; "p<0.01; ***p<0.001 vs 1 DPI). CCI induced monocytic ROS production at 1 DPI (E; *p<0.05 vs sham) which peaked at 3 DPI (E; **p<0.01 vs 1 DPI) and there was reduced MFI at 7 DPI (E; ***p<0.001 vs 3 DPI). Data are mean ± SEM (n=4-5 per group). *p<0.05; ***p<0.001; ****p<0.0001 vs sham; **p<0.01; ***p<0.001 vs 1 DPI; ****p<0.0001 vs 3 DPI; by One-Way ANOVA with Tukey’s multiple comparisons test.

4.2.8 CD4+ and CD8+ T cells peaked at 3- and 7 DPI respectfully in CCI mice.

Having shown the neuroinflammatory contribution of the resident and infiltrating innate immune cells after CCI, it was next examined if the adaptive immune system becomes activated at 1, 3 and 7 DPI. Evidence from the literature has demonstrated that T cell activation persists during chronic TBI pathology [137], which may be mediated through microglial chemotactic recruitment [107]. To determine T cell responses to IL-1β+ production, as shown by the myeloid cell population, the absolute number and the IL-1R+ expression of CD3+, CD4+ and CD8+ T cells was evaluated. The absolute number of T cells at 1-, 3- and 7 DPI was first determined to identify the kinetic immune response to CCI.

In measuring absolute numbers, CD3+ T cells were gated from CD45hi+ cells as demonstrated by the full gating strategy illustrated in detail in Chapter 2: Materials and Methods, section 2.2.6. CD4+ and CD8+ T cells are then gated from total CD3+ T cells. Assessment of CD3+ T cells revealed a significant increase at 3- and 7 DPI as shown by the representative dot plots (Figure 4.8A) and corresponding quantifications (Figure 4.8B; p<0.01; p<0.001 vs sham; p<0.01 vs 1 DPI). Of these CD3+ T cells, there was upregulation of CD4+ T cells following CCI which peaked at 3 d (Figure 4.8C; p<0.0001 vs sham; p<0.05 vs 1 DPI; p<0.05 vs 7 DPI) and CD8+ T cells at 7 d (Figure 4.8D; p<0.01 vs sham, 1 d).
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Figure 4.8: CD4⁺ and CD8⁺ T cells peaked at 3- and 7 DPI respectfully in CCI mice.
Adult male C57BL6/J mice were subjected to sham or CCI surgery and euthanised at 1-, 3- or 7 DPI. Mononuclear cells isolated from the ipsilateral cortex were stained with surface markers including CD3, CD4 and CD8 and analysed by flow cytometry. CCI induced increased #CD3⁺ T cells at 3- and 7 DPI as shown in the representative dot plots and corresponding quantification (B; **p<0.01; ***p<0.001 vs sham; **p<0.01 vs 1 DPI). #CD4⁺ T cells peaked at 3 DPI (C; ****p<0.0001 vs sham; *p<0.05 vs 3 DPI). #CD8⁺ T cells increased at 7 DPI (D; **p<0.01 vs sham, 1 DPI). Data are mean ± SEM (n=4-5 per group). **p<0.01; ***p<0.001 vs sham; ****p<0.0001 vs sham; *p<0.05; **p<0.01; vs 1 DPI; *p<0.05 vs 3 DPI; by One-Way ANOVA with Tukey’s multiple comparisons test.

4.2.9 CCI induced IL1R⁺ on CD4⁺ and CD8⁺ T cells at 3- and 7 DPI.
The expression of IL-1R on these T cell populations was next examined to understand if the receptor expression was elevated following injury and if this was a T cell response from the TBI-elicited secretion of IL-1β in the brain. This study hypothesised that T cells would become activated as shown by increased IL-1R⁺ expression, following pro-inflammatory release from innate cells, with the aim of promoting an immune response.

In parallel to the absolute number of T cells, it was observed that the majority of the T cells are IL-1R⁺ in CCI mice. The results revealed increased #IL-1R⁺ CD3⁺ T cells at 3- and 7 DPI as shown by the representative dot plots (Figure 4.9A) and corresponding
quantification (Figure 4.9B; p<0.05; p<0.001 vs sham; p<0.01 vs 1 DPI). Of these IL-1R+ CD3+ T cells, there was upregulation of #IL-1R+ CD4+ T cells at 3 DPI (Figure 4.9C; p<0.0001 vs sham; p<0.05 vs 1 DPI; p<0.01 vs 7 DPI) and #IL-1R+ CD8+ T cells at 7 DPI (Figure 4.9D; p<0.05 vs sham, 1 DPI).

The findings from this time-course study elucidated the pro-inflammatory profile of resident and infiltrating myeloid cells as well as IL-1R+ T cells, thus suggesting a possible microglial/macrophage – T cell crosstalk following CCI.

**Figure 4.9:** CCI induced IL1R+ on CD4+ and CD8+ T cells at 3- and 7 DPI.

Adult male C57BL6/J mice were subjected to sham or CCI surgery and euthanised at 1-, 3- or 7 DPI. Mononuclear cells isolated from the ipsilateral cortex were stained with surface markers including CD3, CD4, CD8 and IL-1R and analysed by flow cytometry. CCI induced increased #IL-1R+ CD3+ T cells at 3- and 7 DPI as shown in the representative dot plots and corresponding quantification (B; *p<0.05; ***p<0.001 vs sham; **p<0.01 vs 1 d). Of these IL-1R+ CD3+ T cells, the #IL1R+ CD4+ T cells was elevated at 3 DPI (C; ****p<0.0001 vs sham; *p<0.05 vs 1 DPI; **p<0.01 vs 7 DPI) and the #IL-1R+ CD8+ T cells increased at 7 DPI (D; *p<0.05 vs sham, 1 DPI). Data are mean
± SEM (n=4-5 per group). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 vs sham; *p<0.05; ′′p<0.01; vs 1 DPI; ′′′p<0.01 vs 3 DPI; by One-Way ANOVA with Tukey’s multiple comparisons test.

4.2.10 Determining IL-1R\(^+\) expression on myeloid cells and IL-1\(\beta^+\) expressing T cells.

The results so far have indicated that resident and even more so infiltrating neutrophils and monocytes are the key producers of pro-inflammatory mediators such as IL-1\(\beta\) production following CCI. The data from the T cells suggested there was an upregulated IL-1R\(^+\) expression following CCI and that this may indicate T cell activation following IL-1\(\beta^+\) production by microglia, neutrophils, and monocytes. It was important to assess the converse; IL-1R\(^+\) expression on the myeloid population and IL-1\(\beta^+\) T cells to better understand the relationship between the innate and adaptive immune cells in response to injury. The table below shows the absolute number of cells ± SEM and the statistical analysis performed comparing CCI mice to sham over the temporal course of the study. Overall, it was observed that there were low numbers of IL-1\(\beta^+\) T cells, although a significant difference in IL-1\(\beta^+\) CD3\(^+\) and CD8\(^+\) T cells at 7 DPI (Table 4.1A, C; p<0.05; p<0.01 vs sham) and no difference IL-1\(\beta^+\) CD4\(^+\) cells. There was no difference in IL-1R\(^+\) microglia between groups. Indeed, there was an upregulation of IL-1R\(^+\) neutrophils and monocytes at 1- and 3 DPI (Table 4.1E, F; p<0.05; p<0.0001 vs sham) but this may be due to the overall increased number of cells observed at these time-points. Combined, these results demonstrated that although relatively low number of cells there was significant CCI-elicited upregulation of IL-1\(\beta\) in CD8\(^+\) T cells and IL-1R\(^+\) neutrophils and monocytes. However, IL-1\(\beta\) production by these different cell population is time-dependent and during the acute phase of injury it was the myeloid cell population acting as the dominant source of IL-1\(\beta^+\) in this present study.
### Table 4.1 Determining IL-1R\(^+\) expression on myeloid cells and IL-1\(\beta\)+ expressing T cells.

The absolute cell number (± SEM) of IL-1\(\beta\)+ CD3, CD4 and CD8\(^+\) T cells and IL-1R\(^+\) microglia, neutrophils and monocytes were calculated. CCI increased #IL-1\(\beta\)+ CD3 and CD8\(^+\) T cells at 7 DPI (A, C; *p<0.05 vs sham) and there was no difference in the # IL-1\(\beta\)+ CD4\(^+\) T cells at any timepoint. IL-1R\(^+\) microglia were unchanged between groups, whereas there was increased IL-1R\(^+\) neutrophils and monocytes at 1- and 3 DPI (E, F; *p<0.05; ****p<0.0001 vs sham). Data are mean ± SEM (n=4-5 per group). *p<0.05; ***p<0.001; ****p<0.0001 vs sham; by One-Way ANOVA with Dunnett’s multiple comparisons test vs sham.

<table>
<thead>
<tr>
<th>Cell populations</th>
<th>Sham</th>
<th>1d</th>
<th>3d</th>
<th>7d</th>
<th>Statistical analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>A IL-1(\beta)+ CD3 (^+) T cells</td>
<td>32.38 ± 7.82</td>
<td>14.20 ± 4.95</td>
<td>23.44 ± 5.79</td>
<td>81.02 ± 15.64</td>
<td>*p&lt;0.01 sham vs 7d One-Way ANOVA; Dunnett post hoc vs sham</td>
</tr>
<tr>
<td>B IL-1(\beta)+ CD4(^+) T cells</td>
<td>4.53 ± 1.11</td>
<td>3.37 ± 1.65</td>
<td>3.73 ± 1.35</td>
<td>7.7 ± 2.6</td>
<td>ns (p=0.13); One-Way ANOVA; Dunnett post hoc vs sham</td>
</tr>
<tr>
<td>C IL-1(\beta)+ CD8(^+) T cells</td>
<td>20.59 ± 7.04</td>
<td>8.79 ± 2.9</td>
<td>5.71 ± 4.03</td>
<td>51.96 ± 9.21</td>
<td>*p&lt;0.05 sham vs 7d One-Way ANOVA; Dunnett post hoc vs sham</td>
</tr>
<tr>
<td>D IL-1R Microglia</td>
<td>297.8 ± 143.1</td>
<td>178.4 ± 14.41</td>
<td>199.7 ± 17.47</td>
<td>117.5 ± 11.74</td>
<td>ns (p=0.37); One-Way ANOVA; Dunnett post hoc vs sham</td>
</tr>
<tr>
<td>E IL-1R Neutrophils</td>
<td>4.39 ± 1.77</td>
<td>40.93 ± 9.32</td>
<td>27.92 ± 3.16</td>
<td>0.82 ± 0.42</td>
<td>*p&lt;0.05 sham vs 3d; ****p&lt;0.0001 sham vs 1d One-Way ANOVA; Dunnett post hoc vs sham</td>
</tr>
<tr>
<td>F IL-1R Monocytes</td>
<td>6.53 ± 1.82</td>
<td>123.2 ± 13.14</td>
<td>119.4 ± 27.5</td>
<td>8.39 ± 4</td>
<td>****p&lt;0.0001 sham vs 1- and 3d One-Way ANOVA; Dunnett post hoc vs sham</td>
</tr>
</tbody>
</table>

### 4.2.11 Reduced ROS production in neutrophils in the blood 1 DPI in CCI mice.

There is emerging evidence that TBI can cause multiple organ dysfunction in humans [109], and that there are chronic changes to the immune system following experimental brain injury in mice [30]. Ritzel et al., demonstrated elevated ROS levels in neutrophils 60 days post CCI indicating sustained inflammation. It is for these reasons that this present study next investigated the effect of CCI on the systemic immune response of the circulating leukocytes. Neutrophils and monocytes were stained with NOX2 and ROS dye DHR123, an indicator of oxidative stress. Notably, DHR123 is currently used clinically as the diagnostic test for Chronic Granulomatous Disease (CGD) which is an inherited NOX2-deficient disease resulting in increased susceptibility to infection [143].

Blood was collected in anti-coagulated EDTA tubes through cardiac puncture, harvested and stained for CD11b, CD45, Ly6G and Ly6C, and analysed for flow cytometry. The MFI histograms below are gated on live single CD11b\(^+\)CD45\(^{hi}\) cells. CCI induced a trend towards increased NOX2 MFI in Ly6G\(^+\) neutrophils and Ly6C\(^+\) monocytes (Figure 4.10A,
C) at 1 DPI but levels failed to reach statistical analysis. There was a significant reduction in NOX2 MFI neutrophils at 7 DPI (Figure 4.10A; \(p<0.05\) vs 1 DPI). In terms of oxidative burst, there was a significant reduction in ROS, measured by DHR123 MFI, produced by neutrophils at 1 DPI (Figure 4.10B; \(p<0.05\) vs sham) and no difference in monocytic ROS at any timepoints (Figure 4.10D). This result contradicts the increased ROS levels observed in neutrophils at 60 DPI [30]. The reduced ROS seen acutely may be due to the neutrophils trafficking from the blood into the brain to the site of injury to promote an immune response. These results highlight the importance of time-course studies in determining the function of cells throughout the course of TBI pathology.

**Figure 4.10: Reduced ROS production in neutrophils in the blood 1 DPI in CCI mice.**

Adult male C57BL6/J mice were subjected to sham or CCI surgery and euthanised at 1-, 3- or 7 DPI. Blood was collected through cardiac puncture, harvested, and stained for DHR123 followed by surface markers; CD11b, CD45, Ly6G and Ly6C, followed by intracellular NOX2 staining and analysed by flow cytometry. CCI induced a trend towards increased NOX2 MFI in neutrophils (A) and monocytes (C) at 1 DPI, but levels failed to reach statistical significance. CCI reduced DHR123 MFI in neutrophils at 1 DPI (B; \(\ast p<0.05\) vs sham) and had no effect on DHR123 monocytes. Data are mean ± SEM (n=4-5 per group). \(\ast p<0.05\) vs sham; \(\ast\ast p<0.05\); \(\ast\ast\ast p<0.05\); vs 1 DPI; by One-Way ANOVA with Tukey’s multiple comparisons test.
4.2.12 CCI induced increased caspase-1 activity and IL-1β production in CCI mice at 3 DPI.

The results from this chapter have so far demonstrated that CCI induced NOX2/ROS activation which peaked in microglia and infiltrating monocytes at 3 DPI. Consistent with the in vitro results outlined in chapter 3, previous studies have shown NOX2 acts upstream of the inflammasome, whereby NOX2 deficient mice had reduced NLRP3 inflammasome activation and improved neurological outcomes [94]. Others highlighted caspase-1 activation as a driving factor for TBI-induced pyroptotic cell death [128]. Thus, this led to the rationale to investigate the effect of CCI on caspase-1 and IL-1β activity, and ultimately the link with NOX2 activation, in resident microglia and infiltrating myeloid cells; neutrophils and monocytes at 3 DPI.

To assess cell-specific NLRP3 inflammasome activation, cells were stained with surface markers as previously mentioned, FLICA, an indicator of caspase-1 activity, followed by intracellular staining of IL-1β and analysed by flow cytometry. The results revealed that although there was no difference in the #FLICA+ (Q1 + Q2; B) or #IL-1β+FLICA+ (Q2; D) microglia between sham and CCI, there was a significant increase in the #IL-1β+ (Q2 + Q3) microglia as shown by the representative quadrants (Figure 4.11A) and corresponding quantification (Figure 4.11C; p<0.05 vs sham). The total infiltrating myeloid cells (CD11b+ CD45hi) had significantly upregulated #FLICA+, #IL-1β+ and #IL-1β+FLICA+ in CCI mice as seen in the dot plots and quantifications (Figure 4.11F-H; p<0.01; p<0.001; p<0.0001 vs sham), suggesting an increased caspase-1 activity of the infiltrating compared to resident microglia. Indeed, there was a higher absolute number of microglia, but the frequency of IL-1β+ cells is greater in the infiltrating compared to resident as demonstrated in the fluorescent shift of cells to the right in the quadrants (Q2 + Q3). This would coincide with previous studies indicating infiltrating cells are more pro-inflammatory once trafficked into the brain compared to microglia [16] [116].

The mean fluorescence intensity (MFI) of IL-1β and FLICA was assessed in microglia and infiltrating myeloid cells and a linear regression analysis was carried out. The results revealed a positive correlation between IL-1β and FLICA MFI in the infiltrating
population (Figure 4.11; p<0.001 vs sham), but not in microglia. Although a significant increase in IL-1β production, there was no positive correlation in caspase-1 activity in the microglia of CCI mice.

Figure 4.11: CCI induced increased caspase-1 activity and IL-1β production in CCI mice at 3 DPI.

Adult male C57BL6/J mice were subjected to sham or CCI surgery and euthanised at 3 DPI. Mononuclear cells isolated from the ipsilateral cortex were stained with FLICA_FAM to measure Caspase-1 activity, surface markers including CD11b, CD45 to measure microglia and infiltrating myeloid cells, followed by intracellular IL-1β staining and analysed by flow cytometry. CCI induced increased FLICA+ microglia (D; Q2) but levels failed to reach statistical significance. CCI induced increased FLICA+ (Q1 + Q2), #IL-1β+ (Q2 + Q3) and IL-1β FLICA+ (Q2) CD11b+ CD45hi as shown in the representative quadrants (E) and corresponding quantification (F-H) with **p<0.01; ***p<0.001; ****p<0.0001 vs sham). There was a positive correlation between FLICA MFI and IL-1β MFI in CD11b+CD45hi (J; ***p<0.001) but not microglia (I; R² = 0.3174). Data are mean ± SEM (n=6 per group). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 vs sham; Unpaired Student t-test (B-G, F-H) and Pearson r correlation (I, J).
4.2.13 Neutrophils and monocytes upregulate caspase-1 activity and IL-1β production in CCI mice at 3 DPI.

To further investigate the contribution of infiltrating cells to the pro-inflammatory phenotype, the expression of IL-1β and FLICA in neutrophils and monocytes was determined. CCI induced significant upregulation of #FLICA (Q1 + Q2), #IL-1β (Q2 + Q3) and IL-1β FLICA (Q2) in neutrophils (A-D) and monocytes (E-H) as shown in the representative quadrants (Figure 4.12A, E) and corresponding quantification (Figure 4.12B-D, F-H; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 vs sham). As expected from the time-course study, there was significantly more monocytes than neutrophils in the brains of CCI mice at 3 DPI. Moreover, there was more than double the #FLICA and 20x more #IL-1β monocytes compared to neutrophils at 3 DPI. Within monocytes, IL-1β is predominantly expressed at a higher level compared to caspase-1 activity. These findings indicate CCI-elicited increased NOX2/ROS and NLRP3 inflammasome activation in resident microglia and infiltrating neutrophils and monocytes at 3 DPI in CCI mice.

**Figure 4.12: Neutrophils and monocytes upregulate caspase-1 activity and IL-1β production in CCI mice at 3 DPI.**

Adult male C57BL6/J mice were subjected to sham or CCI surgery and euthanised at 3 DPI. Mononuclear cells isolated from the ipsilateral cortex were stained with FLICA_FAM to measure Caspase-1 activity, surface markers including Ly6G (neutrophils) and Ly6C (monocytes) followed by intracellular IL-1β staining and analysed by flow cytometry. CCI induced increased #FLICA (Q1 + Q2), #IL-1β (Q2 + Q3) and IL-1β FLICA (Q2) in neutrophils (A-D) and monocytes (E-H) as...
shown in the representative quadrants (A, E) and corresponding quantification (B-D, F-H; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 vs sham). Data are mean ± SEM (n=6 per group). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 vs sham; Unpaired Student t-test.

4.2.14 Neuro-behavioural outline and surgery day parameters in male and female mice in a 28 d TBI study.

The results so far have focused on NOX2/NLRP3 inflammasome activation at the cellular (flow cytometry data) and tissue (RT-PCR data) level at acute time-points post CCI. To determine the effect of CCI on neuro-behavioural responses at a more chronic timepoint, a pilot study was conducted to optimise an array of behavioural tasks over the course of 28 days.

The role of sex differences in neuro-behavioural responses in rodents is widely debated. Some studies report the effects of CCI on cognitive and motor function are equal between male and female mice [144]. Others have demonstrated improved motor function in female rats, irrespective of oestrous cycle stage, assessed by the beam walk [145]. Given the conflicting reports, CCI-elicited sex differences in behavioural responses were considered at this point in the project. This pilot study consisted of 8 males and 8 females; 5 CCI and 3 sham per sex. The first objective was to optimise the neuro-behavioural tasks for the lab and secondly, to identify any sex differences among these tests.

Adult male and female C57BL6/J mice were randomly assigned injury groups prior to the day of CCI surgeries. The predefined assignment ensured there was no weight, age, or cage effect before starting the study. All mice entering the study were handled (5 min/mouse) 14d before injury and had 10 or less foot-faults on the beam walk at -1d prior to CCI. Throughout the 28d study, mice underwent a battery of behavioural tests including beam walk at 1, 3, 7, 14, 21 and 28 DPI. The two-trial Y-maze was assessed at 7 DPI, the novel object recognition (NOR) test between 14-17 DPI, and finally the Morris Water Maze (MWM) was tested at 21-25 DPI.

To ensure rigor and reproducibility of these experiments, a multitude of parameters were recorded including the duration of the surgery, the time it took the mice to right
themselves and the weight over the 28 days. It was revealed that there was no
difference in surgery duration (Figure 4.13B), meaning the mice spent equal time under
anaesthetic. Albeit not significant, CCI mice took longer to right themselves after the
surgery (Figure 4.13C). Male mice weighed more than female mice at each time-point
throughout this study (Figure 4.13D; p<0.05; p<0.01; p<0.001; p<0.0001) with black stars
comparing sham male vs sham female and teal stars comparing CCI males vs CCI
females. Irrespective of sham or CCI surgery, all mice had reduced weight at 1 DPI
compared to baseline (Day 0) as shown by absolute weight (g) (Figure 4.13D; p<0.05)
and the weight as a percentage of day 0 (Figure 4.13E; p<0.05; p<0.01). These data
suggest mice were exposed to isoflurane for the same amount of time and mice had a
consistent rate of weight gain over-time which suggests no issues on surgery day
therefore indicating that these factors should not impact behavioural results.
Figure 4.13: Neuro-behavioural outline and surgery day parameters in male and female mice in a 28 d TBI study.

Adult male and female C57BL6/J mice were subjected to sham or CCI surgery, underwent an array of neuro-behavioural tests including Beam walk, Two-trials Y-maze, Novel Object Recognition (NOR) and Morris Water Maze (MWM) and euthanised at 28 DPI. The surgery duration and righting reflex time was recorded on the day of the surgery. The weight was recorded at each day of the study. There was no difference in the duration of the surgery across all groups (B). CCI mice took longer to right themselves after surgery, but levels failed to reach statistical significance (C). Male mice weighted more than female mice at each time-point throughout this study (D; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001). All mice had reduced weight at 1 DPI, measured as a percentage of post-injury day 0 (PID0; E; *p<0.05 vs PID0; **p<0.01 sham female vs CCI female). Data are mean ± SEM (n=3-5 per group). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 male vs female; *p<0.05 1 DPI vs 0 DPI; **p<0.01 sham female vs CCI female; Two-Way ANOVA with Tukey's multiple comparisons test (B, C); Two-Way ANOVA repeated measures with Tukey's post hoc test (D, E).
4.2.15 Females had reduced foot-faults on the Beam walk compared to male CCI mice. The fine motor function and co-ordination of mice was assessed by the Beam walk longitudinally to identify any improvements over the course of the study. Mice were trained on the beam walk for 3 days prior to the surgery day and all mice entering the study had less than ten foot-faults on -1 d as shown in the combined graph (Figure 4.14A). The number of foot-faults, out of a total of 50 steps, from the right hind limb of the mouse was recorded.

As expected CCI induced a significant increase in the number of foot-faults on the beam walk in male and female mice, with CCI mice scoring a maximum of 50 foot-faults on 1 DPI (Figure 4.14A; p<0.01 vs sham). At 7 DPI, sham males did not perform as expected and showed mean foot-faults greater than 10 which skewed results as shown in the combined graph (Figure 4.14A) and males only graph (Figure 4.14B). CCI males had an increased number of foot-faults again at 14 DPI, whereas CCI females had a lower number of foot-faults, as seen in the combined graph (Figure 4.14A; p<0.05 male vs female CCI). CCI females showed higher number of foot-faults compared to sham females over the course of the study as shown in the combined graph (Figure 4.14A; p<0.01; p<0.001 vs sham female) and the female only graph (Figure 4.14C; p<0.01; p<0.001; p<0.0001 vs sham female).

The findings from this pilot study suggest female mice performed better on the Beam walk compared to males, even at baseline sham levels. However, due to low numerosity, this data should be repeated to improve rigor.
Figure 4.14: Females had reduced foot-faults on the Beam walk compared to male CCI mice.

Adult male and female C57BL6/J mice were subjected to sham or CCI surgery. Fine motor coordination was assessed by Beam Walk at 1, 3, 7, 14, 21 and 28 DPI. CCI induced increased number of foot-faults in male and female mice at 1 and 2 DPI (A; **p<0.01; ***p<0.001 vs sham) where the pink stars compare sham vs CCI female, and the teal stars compare sham vs CCI males. CCI males had increased number of foot-faults at 14 DPI (A; +p<0.05 vs CCI females). Sham males did not perform as expected and had increased number of foot-faults by 7 DPI which skewed results, as shown in the male only graph (B). CCI females continued to have increased number of foot-faults throughout the study as shown in the combined graph (A; **p<0.01; ***p<0.001 vs sham female) and the female only graph (C; **p<0.01; ***p<0.001; ****p<0.0001 vs sham female). Data are mean ± SEM (n=3-5 per group). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 sham vs CCI; *p<0.05 CCI male vs female; Two-Way ANOVA repeated measures with Tukey’s multiple comparisons test (D, E).

4.2.16 Males had improved spatial memory compared to females at baseline and CCI reduced spatial memory in males.

Cognition of mice was then evaluated by the two-trail Y-maze which specifically assessed spatial learning memory. Briefly, mice were introduced into the Y maze arena with one arm blocked for 5 minutes and then placed back into their holding cage for an
inter-trial interval of 1 h. After this period, mice were placed back into the maze with the arm unblocked and the amount of time the mouse spends in the novel arm was recorded.

At baseline levels, sham males spent more time in the novel arm compared to the familiar arm indicating working spatial memory, however this did not reach statistical significance (Figure 4.15A). In contrast, sham females did not show any trend towards spending more time in the novel compared to familiar arm (Figure 4.15A). CCI males showed a trend towards reduced time spent in the novel arm, but levels failed to reach statistical significance (Figure 4.15A). There was no effect of CCI on the spatial working memory of females. However, the fold difference of the novel over the familiar arm showed a significant reduction in sham females and CCI males compared to sham males (Figure 4.15B; p<0.001; p<0.0001). As control measures of the two-trial Y-maze, there was no significant difference in the distance travelled (mean ± SEM) during the test between sham males (12.35 ± 2.543 m), sham females (16.66 ± 2.204 m), CCI males (16.32 ± 1.472 m) and CCI females (19.91 ± 1.533 m).

These results suggest there was a sex effect between shams in spatial learning memory along with an injury effect between males.

**Figure 4.15**: Males had improved spatial memory compared to females at baseline and CCI reduced spatial memory in males.

Adult male and female C57BL6/J mice were subjected to sham or CCI surgery. Spatial learning memory was assessed by the two-trials Y-maze at 7 DPI. Sham males showed a trend towards spending more time in the novel compared to the familiar arm, but levels failed to reach statistical significance (A). The fold difference of the novel over familiar arm revealed a
significantly reduction in sham females and CCI males (B; ***p<0.001; ****p<0.0001 vs sham males). Data are mean ± SEM (n=3-5 per group). ***p<0.001; ****p<0.0001 vs sham male; Two-way ANOVA with Uncorrected Fisher’s LSD test (B), paired t-test (A).

4.2.17 Males showed a trend towards better recognition memory compared to females which was reduced following CCI.

It was next examined, what effect CCI had on recognition memory, assessed by the novel object recognition (NOR) test on days 14-17 post-injury. NOR consisted of two habituation days, a third familiarisation day with two identical objects and a fourth test day where one object set is replaced with a novel object. The greater the discrimination index (DI; more time spent with novel object) the better the performance in the task. Mice with a DI greater or equal to 20% have intact cognitive function.

On the familiarisation day, where the objects were identical, it was observed that sham females showed a preference for the left side of the arena (Figure 4.16A; p<0.05 vs right). This was accounted for by randomising the side that the novel object will be placed on the following testing day between all groups. On the test day, there was a trend towards reduced DI in sham female and CCI males compared to sham males, but levels did not reach statistical significance due to low numerosity (Figure 4.16B).

Although no significant differences in the distance travelled (mean ± SEM) during the open field stage/habituation day between sham males (20.82 ± 7.703 m), CCI males (23.92 ± 1.219 m), sham females (24.10 ± 4.271 m) and CCI females (35.59 ± 3.0 m), CCI females did show a trend towards increased distance compared to CCI males which is indicative of increased activity.
Figure 4.16: Males showed a trend towards better recognition memory compared to females which was reduced following CCI.

Adult male and female C57BL6/J mice were subjected to sham or CCI surgery. Recognition memory was assessed by a 4-day Novel Object recognition (NOR) test at 14-17 DPI. Sham female mice showed a preference for the left side of the arena on familiarisation day 3 (A; *p<0.05 vs right side) which was accounted for prior to testing day. On the testing day, there was a trend towards reduced DI in sham female and CCI males compared to sham males, but levels failed to reach statistical significance. Data are mean ± SEM (n=3-5 per group). *p<0.05 vs left; paired t-test (A); Two-way ANOVA with Uncorrected Fisher’s LSD test (B).

4.2.18 Males showed a non-significant trend towards improved hippocampal-dependent spatial memory compared to females which appeared reduced following CCI.

The final neuro-behavioural test of this pilot study measured hippocampal dependent memory using the Morris Water Maze (MWM) at 21-25 DPI. The objective was to record the latency to find a hidden platform submerged 0.5 cm below the water’s surface during the acquisition days (1-4) and to measure the time spent in the platform quadrant when the platform was removed in the probe test (day 5). In the optimisation of the MWM protocol, this first test kept the platform in the same quadrant (Northwest) for all mice and the mice entered different quadrants for each trial. This was important to note as future MWM tests utilised a slightly different protocol where the platform was placed in different quadrants across experimental groups.
The results revealed that sham males, but not females, showed a learning curve over the acquisition days and therefore had a reduced latency to platform, but levels failed to reach statistical significance due to variability of the data as demonstrated in the combined male and female graph (Figure 4.17A) and in the isolated male graph. Similarly, the data from the probe test is scattered, most likely due to the low n numbers, and does not show a sex or injury effect on hippocampal-dependent memory in this test.

In conclusion, the neuro-behavioural results from this pilot study indicate that males perform better than females in spatial learning memory, recognition memory and hippocampal dependent memory, which is decreased following moderate level TBI. However, due to low n numbers it is understood that this study was underpowered and should not be over-interpreted. The goal of this pilot study was to establish the behavioural tasks within the CCI model, identify trends in mice behaviours and seek any CCI-elicited behavioural sex differences.

**Learning**

**Probe test**

![Graphs showing learning and probe test results](image-url)

Figure 4.17: Males showed a non-significant trend towards improved hippocampal-dependent spatial memory compared to females which appeared reduced following CCI.

Adult male and female C57BL6/J mice were subjected to sham or CCI surgery. Hippocampal dependent memory was assessed by Morris Water Maze (MWM) at 21-25 DPI. Sham males but
not females showed a trend towards a reduced latency to platform during the acquisition days, but levels failed to reach statistical significance as shown in the combined graph (A) or males only graph (B). There was no significant difference between groups in the time spent and number of entries in the platform quadrant or the speed during the probe test (day 5). Data are mean ± SEM (n=3-5 per group). Analysed by Two-Way ANOVA with repeated measures and Tukey’s multiple comparison’s test (A, B); Two-Way ANOVA with Tukey’s multiple comparison’s test (C-E).

4.2.19 Sham outlier identified which contributed to reduced recognition and hippocampal dependent memory.
Given the aim of this pilot study was to optimise behavioural tasks in male and female mice, it was next investigated where the variability was coming from. There was an obvious sham male outlier in the beam walk, NOR and MWM and it was noted that the mouse became slower to move over-time which was skewing the results. Once sacrificed, this outlier presented with an enlarged liver (hepatomegaly), double the size its sham male counterpart, and splenomegaly as demonstrated in the scaled pictures below (Figure 4.18A). Since the liver and spleen act as sites for extramedullary haematopoiesis, these enlarged organs observed may be due to infection and inflammation [146, 147]. Notably, no post-mortem tests were carried out on this mouse.

Following this finding, the data was re-analysed with the outlier removed and revealed sham males were distinctly superior to CCI males in both the NOR and MWM (Figure 4.18B, C). Indeed, the number of mice in the group was reduced to n=2 and therefore no statistical analysis was performed. However, it gave an indication that the behaviour tests were working, and future experiments should incorporate greater number of mice to accommodate uncontrollable infections in mice.
Figure 4.18: Sham outlier identified which contributed to reduced recognition and hippocampal dependent memory.

On day 28 mice were sacrificed and the sham male outlier from the behaviour tests presented with hepatomegaly and splenomegaly considerably larger than it’s sham counterpart. No further post-mortem test were carried out but the qualitative results were suggestive of increased infection or inflammation. The outlier was removed from the NOR and MWM which then showed improved cognition although low n numbers (n=2) did not allow for statistical analysis.
4.3 Discussion

This chapter set out to optimise the CCI murine model of TBI. Animal models are essential for studying neuroimmune interactions at the cellular and tissue level which mimic human TBI, that cannot be investigated in the clinic and ultimately, for the discovery of novel therapeutics. CCI induces neuronal cell death and brain atrophy [39] as well as cognitive dysfunction [148], thus mimicking various aspects of human TBI [27]. With this model, the speed, depth and dwell time is controlled for making it more accurate and reproducible than other TBI models, such as the weight-drop model, for biochemical analysis. In all experiments, CCI did not induce mortality and mice did not have considerable weight loss which could result in the requirement of a humane endpoint.

In TBI, the primary injury occurs as a result of a direct mechanical head impact which causes immediate neuronal and axonal damage, microglia activation and pro-inflammatory release. The secondary injury occurs minutes to months after the initial insult and induces mitochondrial dysfunction, glutamate excitotoxicity, cell-death mechanisms, and oedema [14]. These factors drive increased neuroinflammation which gives rise to increased neurological impairment [149] and sustained systemic immune activation. The focus of this project was to investigate the role of the innate and adaptive immune system in response to CCI. TBI has been shown to cause blood brain barrier dysfunction and increased permeability [150]. Following BBB breakdown, there is influx of neutrophils and monocytes into the brain, promoting microglial activation and the release of pro-inflammatory mediators ultimately leading to a pro-inflammatory cascade [15, 16, 39].

This present study indicated a temporal progression of the immune system in that different cell populations at different timepoints played a crucial role in promoting neuroinflammation following CCI. Microglial activation peaked at 3 DPI but persisted by 7 DPI with increased NOX2/ROS and IL-1β production. In contrast, the infiltrating CD11b^+ CD45^{hi} myeloid cells were short-lived and therefore little to no infiltrating cells were observed at 7 DPI (Figure 4.4). This is consistent with the literature which showed even though the number of infiltrating cells are reduced by 7 DPI, these cells have
upregulated NOX2 and DHR123 MFI which may be the contributing factor towards increased neuroinflammation following TBI [116]. Furthermore, these authors showed greater pro-inflammatory responses in the infiltrating cells compared to the resident microglia. A similar result was identified in this present study whereby CCI induced greater caspase-1 activity and IL-1β production in total infiltrating myeloid cells compared to microglia. Of these infiltrating cells, it was the pro-inflammatory monocytes (Ly6C+) with greater NLRP3 inflammasome activation compared to the neutrophils at 3 DPI (Figure 4.12). In agreement, preliminary data from the lab showed increased NOX2/ROS in the infiltrating cells compared with microglia after TBI using image stream which incorporates morphological and functional analysis. The data from this present study and published literature therefore provides rationale for targeting NOX2/ROS and NLRP3 inflammasome activation acutely following TBI.

The adaptive arm of the immune system was also activated following CCI as shown by upregulated absolute numbers of CD3+ T cells and IL-1R expression on CD4+ and CD8+ T cells. The IL-1R antibody used in this study was Type I Interleukin-1 Receptor (IL-1RI), which is expressed by T cells and it is capable of binding to IL-1α and IL-1β. Although it does not react with Type II (IL-1RII) which is present on macrophages and B-cells, converse analysis was carried out and provided no strong evidence for IL-1R expressed on the myeloid population or IL-1β-producing T cells. Therefore, the working hypothesis states IL-1β-producing resident and infiltrating myeloid cells trigger T cell activation demonstrated by increased expression of IL-1R, leading to an immune response acutely at 3 DPI.

Coinciding with previous literature linking the brain and peripheral immune system following TBI, this present study found reduced ROS producing neutrophils in the blood at 1 DPI. This is inversely related to the increased oxidative burst seen in the neutrophils in the brain perhaps indicating trafficking of neutrophils into the CNS, towards the site of the injury, as early as 1 DPI. Previous work showed neutrophilia at 60 DPI, whereby the neutrophils and monocytes exhibited respiratory burst and phagocytic dysfunctions [30] thus proving the importance of timing when performing cellular analysis post TBI.
The final aim of this results chapter was to investigate sex differences in behavioural tasks following CCI. In this pilot study it was decided to use identical injury parameters even though females were approximately 5 g lighter than their age-matched males. CCI females showed improved fine motor function on the beam walk at 14 DPI compared to males. Other researchers have also shown females perform better than males on the beam walk irrespective of their oestrous cycle [145] which was an interesting finding because others report oestrogen to have neuroprotective effects. This improvement in motor function is consistent with the literature stating females perform better on the rotarod compared to males [116]. Rotarod performance was coupled with a reduced number of highly reactive and pro-inflammatory infiltrating myeloid cells into the brain at 1 DPI compared to males. However, this was an acute transient sex difference because the authors showed equal number of cells at 7 DPI with no sex difference in the pro-inflammatory profile in microglia or infiltrating myeloid cells, highlight again the importance of timing post injury. Similarly, in a separate paper, males have a more robust initial pro-inflammatory profile associated with increased lesion volume but after 1 week post CCI, female inflammatory responses become similar to that of the males. This present study of the two-trial Y-maze coincides with the idea that females are not protected 1-week post CCI. The Y-maze gave the most convincing data from this pilot study and revealed both an injury effect (Sham vs CCI males) and sex effect (male vs female sham) at 7 DPI. Others have shown no significant sex difference in TBI-elicited motor and cognitive deficits apart from sham males performing better in the Y-maze compared to female mice [144]. Therefore, in understanding the complicated reports on how sex differences effect the neuroinflammatory and behavioural response, a temporal approach should be considered. Moreover, these underpowered preliminary results could be followed up to cement these findings, but it is promising that they mirror the literature quite well. This is important for model development and to understand male vs female differences in neuroimmunology and neurobehavioral.

This pilot study experienced variability within the behavioural tasks despite the surgery duration and rate of weight gain being consistent among groups. The main variability came from the sham males group and following euthanasia the reason for the NOR and MWM outlier was evidently due to the hepatosplenomegaly which resulted in the
slowing down of the mouse. Hepatosplenomegaly describes the simultaneous enlargement of both the liver and spleen and has been shown to be upregulated in rodents due to viral or bacterial infections or haematologically when haematopoiesis takes place in outside of the bone marrow, called extramedullary haematopoiesis [147] [146]. Previous work showed, infecting mice with murine gamma herpes virus (respiratory infection) resulted in a 2-3-fold increase in spleen cell numbers which was associated with increased CD4+ T cells [151]. If sham mice become infected throughout the study, they do not act as relative controls. Notably, this was not the same sham mouse who performed progressively worse in the beam walk skewing the data. This therefore shows the necessity for a greater number of mice in future experiments, especially when carrying out behavioural studies.

In conclusion of this results chapter, as 3 DPI illustrated peak NOX2 gene expression, microglial and monocytic NOX2/ROS production and increased number of CD3+ and IL-1R+CD4+ T cells, this timepoint was chosen for the future acute CCI intervention experiments. Indeed, the role of sex-differences in the TBI field is an on-going debate and further research is needed to consolidate effective individualised therapeutics. Having said that, males were used for the following intervention experiments outlined in the next chapter due to the increased number of mice required per experiment and the timing practicality of the CCI model.
Chapter 5: Pharmacological inhibition of NOX2 activation using the small molecule inhibitor, GSK2795039, in an experimental TBI model in mice
5.1 Introduction

The results from chapter 3 and 4 demonstrated that the NOX2/ROS and NLRP3 inflammasome pathway is upregulated in vitro using microglial models and in an in vivo mouse model of TBI. Results from chapter 3 showed promising effects of GSK2795039 on NLRP3 inflammasome-stimulated microglia in reducing pro-inflammatory mediators, such as NOX2, ROS, cleaved caspase-1 and IL-1β. Once the CCI model was optimised and shown to produce a robust neuroinflammatory response in resident and infiltrating peripheral cells acutely post CCI, the next experiments aimed to investigate GSK2795039 treatment in vivo.

Preclinical studies from the Loane lab have shown microglial-mediated mechanisms involved in chronic neurodegeneration following CCI. Increased lesion volume and hippocampal neurodegeneration was associated with upregulated NOX2 expression in iba1+CD68+ activated microglia up to 1 year post injury [39]. A separate study demonstrated accumulation of NOX2+ inflammatory macrophages in the injured cortex 7d post CCI. NOX2−/− mice had a significantly reduced lesion volume and improvements in fine motor coordination as demonstrated by beam walk. Therefore, NOX2 inhibition provided neuroprotection and enhanced functional recovery [16]. These combined results indicate neuroinflammatory changes in the resident and infiltrating myeloid cells of the brain following CCI and highlight the importance of attenuating NOX2 in preventing chronic neuroinflammation.

Pharmacological inhibition of NOX2 inhibits oxidative stress and neuronal damage which has been implicated in many neurodegenerative diseases and plays a role in secondary brain damage. CCI mice injected with Apocynin, a broad NOX2 inhibitor, in combination with mGluR5 receptor agonist, CHPG, showed reduced lesion volume and improved motor and cognitive function as assessed by beam walk and Morris Water Maze [78]. Notably, the combined treatment did not enhance protective functional outcomes more than CHPG treatment alone indicating that both treatments target the same pathway of NOX2 activation. In parallel, CCI mice treated with gp91ds-tat, a selective NOX2 peptide inhibitor, revealed reduced NOX2+ colocalization in P2Y12+ microglia and F4/80+ macrophages [16].
The current study aimed to target secondary induced neuroinflammation using GSK2795039 as a potential therapeutic intervention mitigating NOX2-mediated neuroinflammation post-CCI. GSK2795039 is a small molecule, brain penetrable, specific NOX2 inhibitor. Hirano et al., illustrated how GSK2795039 significantly reduced NOX2-dependent ROS production to levels similar to that of the positive control gp91<sup>−/−</sup> in a paw inflammation model, which was not observed with broader antioxidants Apocynin or DPI [74]. Furthermore, GSK2795039 inhibited NOX2 in a NADPH competitive manner. In a weight-drop mouse model of TBI, pre-treatment of GSK2795039 showed reduced NOX2 activity at 24h and NOX2 expression up to 48h post injury in total brain, associated with improved performance in the Morris Water Maze [80]. These data suggest GSK2795039 has promising effects on NOX2-mediated oxidative stress and neuroinflammation in systemic and acute models of neuroinflammation. A more novel approach was taken in this present study, and it was decided to administer GSK2795039 (i.p) post injury, as opposed to pre-injury. This ensured the study was designed clinically relevant by mimicking an individual arriving at the emergency department requiring treatment following a head injury.

The objective of this chapter was to pharmacologically inhibit NOX2, using GSK2795039 following CCI in C57BL6/J male mice. The hypothesis being tested was that targeting secondary neuroinflammation, which gives rise to sustained microglial activation and release of pro-inflammatory cytokines, would reduce NOX2-mediated neuroinflammation and improve neurological outcomes. The pharmacokinetics of GSK2795039 in the brain was also examined.

5.2 Results

5.2.1 Vehicle optimisation to ensure safety and welfare of mice.

Prior to GSK2795039 interventions it was necessary to find the most suitable vehicle composition that reconstitutes the drug and has the least adverse effects following repeated injections in mice. In the first experiment, mice were injected with vehicle A (20% DMSO, 20% Tween 80, 60% Polyethylene glycol 200) as this was used in the paper outlining the discovery of GSK2795039 [74]. The weights of mice were taken each day
for three consecutive days. In this first experiment, mice lost 10-15% of their body weight and one mouse did not survive past 1 day post injection (Figure 5.1B). This was not a safe option because the loss of weight was too much for naïve mice, given that CCI induced weight loss. DMSO 20% was predicted to be too high, especially for repeated doses, and may be causing this loss of weight. A second optimisation experiment was carried out and mice were injected with either vehicle B (10% DMSO, 40% PEG 300, 5% Tween 80, 45% saline) or vehicle C (10% DMSO, 90% Corn oil). In the vehicle B injected group, one mouse had more than 20% weight loss and required humane endpoint (Figure 5.1C). Whereas with vehicle C, there was no loss of body weight over the 3 days of repeated dosing (Figure 5.1D). Therefore, as GSK2795039 could be reconstituted in this formula, and no toxicity was experienced, all the drug intervention studies utilised Vehicle C.

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>Formula</th>
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<tbody>
<tr>
<td>A</td>
<td>20% DMSO + 20% Tween 80 + 60% Polyethylene glycol 200</td>
</tr>
<tr>
<td>B</td>
<td>10% DMSO + 40% PEG 300 + 5% Tween 80 + 45% Saline</td>
</tr>
<tr>
<td>C</td>
<td>10% DMSO + 90% Corn Oil</td>
</tr>
</tbody>
</table>

**Figure 5.1:** Vehicle consisting of 10% DMSO + 90% Corn Oil resulted in less than 5% weight loss during repeated injections administered to mice over 3 days.

Mice were injected intraperitoneally with Vehicle A, B or C as outlined in the table (A) once every day for 3 consecutive days. Weights were recorded each day and graphed as a % of baseline day 0. Vehicle A and B resulted in 15 and 25% weight loss respectfully and the sacrifice of one mouse before the end of the study (B, C). Vehicle C did not alter weight and was chosen as the vehicle for further intervention studies (D). Data are mean ±SEM (n=4-5 per group).
5.2.2 Pharmacological inhibition of NOX2 by GSK2795039 did not reduce gross motor function deficits in mice subjected to CCI.

Once the CCI model of TBI was optimised (Chapter 4), the next experiments investigated the inhibitory potential of GSK2795039 on NOX2-mediated neuroinflammation in mice. An acute timepoint of 3 DPI was chosen based on the time-course experiment described in the model optimisation results chapter 4, whereby there was upregulation of pro-inflammatory resident microglia and infiltrating monocytes at this timepoint. To make this study as clinically relevant as possible, GSK2795039 was administered post-injury, as opposed to a pre-injury treatment observed in previous literature [74, 80]. Here, GSK2795039 was administered intraperitoneally (i.p) starting at 4 h post CCI, a second dose was given at 24h and a final administration at 48h post injury. Gross motor function was assessed at 1-, 2- and 3 DPI. Mice were euthanised at 3 DPI and a tissue punch of the ipsilateral hippocampus was flash frozen for analysis by qPCR. The remaining ipsilateral cortex was collected in media and processed for flow cytometry analysis, as outlined in the experimental design below (Figure 5.2A).

Given that mice are subjected to isoflurane prior to sham or CCI surgeries, it was vital that all surgery times were consistent to ensure that mice spent equal amounts of time under anaesthetic and no results were due to the isoflurane. In this experiment there was no difference in surgery duration (Figure 5.2B). CCI induced an increase in righting reflex time when compared to righting reflex times in sham mice (Figure 5.2C; p<0.01 vs sham veh). At the time of righting reflex, there was no treatment administered to injured mice, but mice are broken down according to treatment group for comparison. This result depicts no difference in the righting reflexes between CCI groups (veh and GSK2795039). The weight of the mice was recorded throughout the study. Sham mice had consistent weight throughout the study and CCI mice had reduced weight at 1-3 DPI. GSK2795039 treated CCI mice showed increased weight gain following 1 DPI, however levels failed to reach statistical significance (Figure 5.2D). Importantly no mouse lost more than 20% of its body weight, which would have initiated a humane endpoint.

Gross motor function was assessed using an accelerating rotarod whereby mice are placed on a rotarod at 4 RPM which increases up to 60 RPM over a ramp of 180 seconds
as previously described [116]. The percentage of time that the mouse spends holding on, or before two consecutive rotations on the rod, from its baseline day is determined. Following CCI, mice showed reduced time spent on the rotarod, but levels failed to reach statistical significance compared to sham veh (Figure 5.2E). CCI mice showed an improved performance at 3 DPI (Figure 5.2E; p<0.05 vs CCI veh 1 DPI (teal star)). GSK2795039 treated mice also showed a significantly reduced time on the rotarod at 1-, 2- and 3 DPI (Figure 5.2E; p<0.001; p<0.0001 vs sham GSK2795039 (pink stars)). These findings indicate that GSK2795039, at this dosing regime, does not provide protective effects on gross motor function acutely after TBI.

Figure 5.2: GSK2795039 administration had no effect on gross motor function in mice subjected to CCI in an acute 3 DPI study.

GSK2795039 was administered 4h, 12h and 24h post CCI. The surgery duration and righting reflex time was recorded on the day of the surgery. The weight was recorded each day of the
study and gross motor function was measured by an accelerating rotarod at 1, 2- and 3 DPI as shown in the schematic (A). There was no difference in the duration of the surgery across all groups (B). Mice subjected to CCI showed increased righting reflex time (C; **p<0.01 vs sham veh). Mice showed a decrease in weight 1 DPI, but levels failed to reach statistical significance (D). CCI mice had reduced time spent on the rotarod at 1-, 2- and 3 DPI, but levels failed to reach statistical significance. CCI mice showed an improved performance at 3 DPI (E; *p<0.05 vs CCI veh 1 DPI). GSK2795039 treated CCI mice showed a significant reduction in the time spent on the rotarod (E; **p<0.01; ****p<0.0001 vs sham GSK2795039). Data are mean ±SEM (n=3-5 per group). *p<0.05; **p<0.01; vs sham veh, **p<0.01; ****p<0.0001 vs sham GSK2795039) by Two-Way ANOVA with uncorrected Fisher’s LSD (B, C); Two-way Mixed effect analysis with repeated measures and uncorrected Fisher’s LSD (D, E).

5.2.3 GSK2795039 did not alter absolute numbers of microglia and infiltrating myeloid cells in CCI mice at 3 DPI.

As well as investigating the role of GSK2795039 on injury-induced gross motor deficits, the effect on cell specific neuroinflammation was also assessed. The first question was whether GSK2795039 had any effect on the absolute numbers of resident microglia and infiltrating cells following injury. To evaluate this, mononuclear cells isolated from the ipsilateral hemisphere of sham and TBI mice were stained with CD11b, CD45, Ly6G and Ly6C and analysed by flow cytometry. The flow plots below are gated on live single cells and show the absolute number of cells as shown in the prior results chapter 4.

CCI mice had an increased number of CD11b⁺CD45⁻ microglia cells compared to sham veh, but levels failed to reach statistical significance as shown by the representative dot plots (Figure 5.3A) and corresponding quantification (Figure 5.3C). Consistent with the previous chapter, following CCI there was an upregulation of CD11b⁺CD45⁻ infiltrating cells into the brain as shown in the dot plot and quantification (Figure 5.3A, D; p<0.05 vs sham veh). Of these total CD11b⁺ infiltrating cells in CCI mice, there was increased Ly6G⁺ neutrophils and Ly6C⁺ monocytes (Figure 5.3E, F; p<0.05; p<0.001 vs sham veh). GSK2795039 treated CCI mice did not significantly alter the absolute numbers of microglia and infiltrating myeloids; neutrophils and monocytes (Figure 5.3C-F).
Figure 5.3: GSK2795039 did not alter the absolute number of resident and infiltrating myeloids in CCI mice at 3 DPI.

GSK2795039 was administered 4h, 12h and 24h post CCI. Mononuclear cells isolated from the ipsilateral cortex were stained with CD11b, CD45, Ly6G and Ly6C for flow cytometry analysis. CCI increased the number of CD11b⁺CD45<sup>int</sup> microglia but failed to reach statistical significance (C) and increased CD11b⁺CD45<sup>hi</sup> infiltrating cells (D; *p<0.05 vs sham veh) including Ly6G<sup>+</sup> neutrophils and Ly6C<sup>+</sup> monocytes (E, F; *p<0.05; **p<0.01 vs sham veh). GSK2795039 did not alter the number of resident and infiltrating cells in CCI mice. Data are mean ±SEM (n=3-5 per group). *p<0.05; **p<0.01 versus sham veh; Two-Way ANOVA with uncorrected Fisher’s LSD.
5.2.4 GSK2795039 showed modest reductions in NOX2⁺ ROS⁺ microglia in CCI mice at 3 DPI.

Even though GSK2795039 did not alter the number of cells it was next examined if this drug intervention could attenuate pro-inflammatory profiles of these cells. Given that multi-dimensional flow cytometry allows for the assessment of cell specific functional output, the number of NOX2⁺ and DHR123⁺ cells and IL-1β mean fluorescence intensity (MFI) in microglia was determined.

Firstly, cells were stained with DHR123, a ROS indicator dye, prior to surface staining. Following this, cells are permeabilised and stained intracellularly for NOX2 and IL-1β production. Microglial quadrants allow for analysis of both NOX2⁺ and DHR123⁺ cells. Q1 and Q3 are single positives, Q2 is double positive and Q4 is the double negative. MFI allows for the comparisons of IL-1β across the mode or “peak” of each population. Fluorescent minus one (FMO) controls contained the same antibody staining except one fluorophore and were used for gating purposes.

As expected, CCI induced increased NOX2⁺ (Q2 + Q3) CD11b⁺CD45int microglia, but levels failed to reach statistical significance (Figure 5.4B). DHR123⁺ (Q1 + Q2) and NOX2⁺DHR123⁺ (Q2) microglia were upregulated following CCI as demonstrated by the representative dot plot quadrants and corresponding quantifications (Figure 5.4C; D; p<0.05 vs sham veh). CCI increased IL-1β MFI as shown in the fluorescent shift to the right on the histogram, and corresponding quantification (Figure 5.4E; p<0.01 vs sham veh). GSK2795039 showed a modest reduction in CCI induced NOX2⁺ and ROS⁺ microglia, but levels failed to reach statistical significance (Figure 5.4B-D). GSK2795039 had no effect on microglial IL-1β MFI (Figure 5.4E).
Figure 5.4: GSK2795039 showed a modest reduction in NOX2\(^+\) and ROS\(^+\) microglia in CCI mice at 3 DPI.

GSK2795039 was administered 4h, 12h and 24h post CCI. Mononuclear cells isolated from the ipsilateral cortex were first stained with DHR123, followed by intracellular staining of NOX2 and IL-1\(\beta\) for flow cytometry analysis using quadrants for NOX2 vs DHR123 and mean fluorescent intensity (MFI) of IL-1\(\beta\). Fluorescent minus one (FMO) controls were used for gating. CCI increased NOX2\(^+\) (Q2+Q3), but levels failed to reach significance (B), DHR123\(^+\) (Q1+Q2) and NOX2\(^+\)DHR123\(^+\) (Q2) in CD11b\(^+\)CD45\(^{\text{Int}}\) microglia (C, D; \(^*p<0.05\) vs sham veh). CCI increased microglial IL-1\(\beta\) MFI (E; \(^{**}p<0.01\) vs sham veh). GSK2795039 showed modest reductions in DHR123\(^+\) and NOX2\(^+\) microglia (C, D) but levels failed to reach statistical significance. GSK2795039 had no effect on microglial IL-1\(\beta\) MFI (E). Data are mean ±SEM (n=3-5 per group). \(^*p<0.05; \(^{**}p<0.01\) vs sham veh; Two-Way ANOVA with uncorrected Fisher’s LSD.

5.2.5 GSK2795039 showed modest reductions in NOX2\(^+\) IL-1\(\beta\)^+ neutrophils but had no effect on monocytes in CCI mice at 3 DPI.

GSK2795039 treatment showed minor attenuation of injury induced pro-inflammatory microglia at 3DPI. To test if GSK2795039 affected the pro-inflammatory profile of infiltrating myeloid cells, absolute numbers of NOX2 and IL-1\(\beta\), and MFI of DHR123 and IL-1\(\beta\) were assessed in neutrophils and monocytes respectfully. Infiltrating myeloids,
expressing CD11b<sup>+</sup>CD45<sup>hi</sup>, were stained in the same way as the previous microglia data, with included surface staining of Ly6G for neutrophils and Ly6C for monocytes.

CCI upregulated absolute numbers of IL-1β<sup>+</sup> (Q1+Q2), NOX2 (Q2+Q3) and NOX2<sup>+</sup> IL-1β<sup>+</sup> (Q2) neutrophils as demonstrated in representative quadrants and corresponding quantifications (Figure 5.5B-D; p<0.05; p<0.01 vs sham veh). Furthermore, CCI increased DHR123 MFI in monocytes as demonstrated by the fluorescent shift to the right in the representative histograms and corresponding quantifications (Figure 5.5E; p<0.0001 vs sham veh). There was a slight increase in IL-1β MFI in monocytes, but levels failed to reach statistical significance (Figure 5.5F). GSK2795039 treated CCI mice showed reduced NOX2<sup>+</sup> and IL-1β<sup>+</sup> neutrophils but levels failed to reach significance (Figure 5.5B-D). GSK2795039 provided no strong data to show protective effects in injury elicited inflammation in monocytes.

These findings, along with the microglial data, infer modest inhibitory abilities of GSK2795039 at the cellular level. Further drug intervention studies with a larger sample size and optimised treatment scheme were warranted to see improved inhibition of NOX2-mediated neuroinflammation in resident and infiltrating myeloid cells.
Figure 5.5 GSK2795039 showed modest reductions in NOX2+ IL-1β+ neutrophils but had no effect on pro-inflammatory monocytes in CCI mice at 3 DPI.

GSK2795039 was administered 4h, 12h and 24h post CCI. Mononuclear cells isolated from the ipsilateral cortex were first stained with DHR123, surfaced staining with Ly6G for neutrophils and Ly6C for monocytes, followed by intracellular staining of NOX2 and IL-1β for flow cytometry analysis using quadrants for NOX2 vs IL-1β and mean fluorescent intensity (MFI) of DHR123 and IL-1β. CCI increased #IL-1β+ (Q1+Q2), #NOX2+ (Q2+Q3) and #NOX2+IL-1β+ (Q2) Ly6G+ neutrophils (B-D; *p<0.05; **p<0.01 vs sham veh). CCI increased DHR123 MFI in monocytes (E; ****p<0.0001 vs sham veh) and IL-1β but levels failed to reach significance (F). GSK2795039 showed reduced NOX2+ and IL-1β+ neutrophils in CCI mice but levels failed to reach significance (B-D). GSK2795039 had no effect on DHR123 and IL-1β MFI in monocytes (E, F). Data are mean ±SEM (n=3-5 per group). *p<0.05; **p<0.01; ****p<0.0001 vs sham veh; Two-Way ANOVA with uncorrected Fisher’s LSD.
5.2.6 GSK2795039 reduced pro-inflammatory gene expression in CCI mice, but these effects did not reach statistical significance.

The previous results illustrated the effects of GSK2795039 at the cellular level, specifically on the mononuclear cells from the ipsilateral cortex. A secondary aim was to investigate the effect of GSK2795039 at the tissue level. Hippocampal tissue punches were taken from the ipsilateral cortex of sham or CCI mice treated with vehicle or GSK2795039 and RT-qPCR was carried out. The following Taqman mRNA probes were measured; *Cyba* and *Cybb* which are NOX2 membrane bound subunits, *Tnf* and *Il6* which are general pro-inflammatory markers and *Nlrp3* and *Il1b* which indicate NLRP3 inflammasome activation at the gene level.

CCI mice showed an upregulation of *Cyba* and *Nlrp3* gene expression (Figure 5.6A, D; p<0.05; p<0.01 vs sham veh), and increased *Cybb*, *Tnf*, *Il1b* and *Il6* (Figure 5.6B, C, E, F) but levels failed to reach statistical significance. GSK2795039 showed a modest reduction of CCI-induced increase of pro-inflammatory genes (Figure 5.6A-F) in hippocampal tissue punches, but these effects did not reach statistical significance.

These findings suggest a modest reduction in NOX2-mediated inflammation with GSK2795039 treatment. Indeed, the level of GSK2795039 inhibition at the cellular and tissue level is not robust which makes it difficult to conclude the results across all parameters of this study. Further information on how the drug interacts with the body and enters the brain was required to understand the ability of GSK2795039 inhibition following CCI. The next experiment aimed to optimise the drug bioavailability and penetration.
Figure 5.6 GSK2795039 showed reduced pro-inflammatory gene expression in CCI mice, but these effects did not reach statistical significance.

Hippocampal tissue punches were taken from the ipsilateral cortex of mice subjected to CCI treated with vehicle or GSK2795039. RT-qPCR amplification was carried out which measured Cyba, Cybb, Tnf, Nlrp3, Il1b and Il6. CCI induced Cyba and Nlrp3 upregulation (A, D; *p<0.05; **p<0.01) and Cybb (B), Tnf (C), Il1b (E) and Il6 (F) but levels failed to reach statistical significance, compared to sham veh. GSK2795039 showed reduced expression of these pro-inflammatory genes (A-F) but levels failed to reach statistical significance. Data are mean ±SEM (n=3-5 per group). *p<0.05; **p<0.0001 vs sham veh; Two-Way ANOVA with uncorrected Fisher’s LSD.

5.2.7 Pharmacokinetic analysis demonstrates that GSK2795039 enters the cortex and hippocampus of injured mice.

The previous experiment indicated a modest reduction in NOX2-mediated neuroinflammation in mice treated with GSK2795039 following TBI. However, more information on GSK2795039 pharmacology was necessary to optimise the drug administration and dosing time-points to obtain more robust findings with GSK2795039 treatment. Therefore, the concentration of GSK2795039 entering the brain was assessed.
Pharmacokinetics (PK) is the study of how a drug administered reacts in the body over time. PK analysis demonstrates drug concentration-time courses in the different organs of the body, following administration of a drug [152]. Understanding the biodistribution and the half-life of the drug is important in developing the drug dosing regimen and avoiding toxicity. As GSK2795039 is a small molecule drug with a relatively short half-life, as seen where NOX2 activity returned to baseline levels 24h post dose [82], it was vital to prove that GSK2795039 enters the brain following TBI, in order to understand it’s functions thereafter.

To assess this, C57BL6/J male mice (8-10 weeks) were subjected to TBI using the CCI model. GSK2795039 (100mg; kg) or Vehicle (10% DMSO and 90% Corn oil) was administered intra-peritoneally (i.p) 2h following CCI, and mice were humanely euthanized 30 min later, as outlined in the schematic (Figure 5.7A). Blood was taken by cardiac puncture and collected in anti-coagulated EDTA tubes. The ipsilateral cortex and hippocampus were dissected from the total brain and flash frozen. One lobe of the liver was also collected in case drug levels in the plasma were not detectable. These tissue were isolated, collected and flash frozen in Trinity College Dublin before being shipped to collaborators for further analysis. An Ultimate 3000 LC system interfaced to an Orbitrap Exploris 480 mass spectrometer was used for liquid chromatography and mass spectrometry (LC–MS/MS) analysis. This analysis was carried out by Dr. Joseph Ojo and his team at The Roskamp Institute, Sarasota, FL, USA.

This PK analysis demonstrated that GSK2795039 penetrated the brain, as shown by levels in the cortex (8.19% of plasma) and hippocampus (7.01% of plasma) (Figure 5.7B). Spearman correlations suggest a relationship between GSK2795039 levels in the plasma positively correlated with levels in the cortex (Figure 5.7C; p=0.4194) and in the hippocampus (Figure 5.7D; p=0.6583). According to the literature, 5-10% of plasma levels indicate that the drug gets into the brain [153]. The results also demonstrate that high levels of the GSK2795039 enter the liver which is where one would expect drug metabolism to take place.
Figure 5.7: Pharmacokinetic analysis demonstrates that GSK2795039 enters the cortex and hippocampus of injured mice. C57BL6/J adult male mice were subjected to a controlled cortical impact (CCI), GSK2795039 (100mg; kg) was administered intra-peritoneally (i.p) 2h following CCI and mice were sacrificed 30 min later, as outlined in the schematic (A). The concentration of GSK2795039 (ng/ml) in the plasma, liver, cortex and hippocampus was quantified using liquid chromatography mass spectrometry (LC–MS) analysis. The concentration of GSK2795039 (ng/ml) was shown as a percentage of the plasma concentration in the liver (37.09%), cortex (8.19%) and hippocampus (7.01%) (B). GSK2795039 levels in the plasma positively correlated with levels in the cortex (C) and hippocampus (D). Data are mean ± (n=6). p= 0.4194 (C) and p=0.6583 (D); by Spearman Correlations and linear regression analysis. LC-MS analysis was carried out by Joseph Ojo at The Roskamp Institute, Sarasota, FL, USA.

5.2.8 GSK2795039 showed minor improvements in CCI related motor dysfunction and spatial memory deficits.

As the previous drug intervention indicated limited effects of GSK2795039 treatment at the cellular and tissue level (Figures 5.2-5.6), a different drug administering regime was warranted. Given the results of the pharmacokinetic analysis, it was next investigated if GSK2795039 intervention starting at 2h post-injury could attenuate NOX2/NLRP3 inflammasome activation in resident microglia and infiltrating cells acutely 3 DPI. This
2h window following injury mimics what would occur in the clinical setting, allowing an individual to get to the hospital and begin treatment. It was hypothesised that GSK2795039 intervention at 2h ensures brain penetration from the peripheral system. Moreover, GSK2795039 inhibition would improve motor and cognitive behavioural deficits and reduce NOX2-mediated pro-inflammation at the cellular level.

Adult male C57Bl6/J mice were administered GSK2795039 (100mg/kg) intraperitoneally at 2h, 12h, 1d, and 2d post-injury, as shown in the experimental outline (Figure 5.8A). Mice were assessed for gross motor function on the accelerating rotarod at 1-, 2- and 3 DPI and for spatial memory using the two-trials Y-Maze at 2 DPI. Mice underwent training for the rotarod for three consecutive days prior to CCI and the percentage of time spent on the rotarod of the baseline day (-1d) was analysed. CCI induced a significant decrease in the amount of time spent holding onto the rotarod at 1-, 2- and 3 DPI (Figure 5.8B; p<0.05 vs sham veh). GSK2795039 treated CCI mice showed reduced time spent on the rotarod at 1 DPI (Figure 5.8B; p<0.05 vs sham GSK2795039) but these mice showed an improvement at 2- and 3 DPI, with levels not statistically different to sham. Furthermore, the time spent (in seconds) on the rotarod at 3 DPI was isolated and graphed to show CCI induced reduction (Figure 5.8C; p<0.05 vs sham veh) and GSK2795039 treated CCI mice demonstrated increased time spent on the rotarod, but levels failed to reach statistical significance. In terms of spatial memory assessed by the two-trials Y-Maze test, sham mice (veh and GSK2795039 groups were pooled) spent more time in the novel arm compared to the familiar arm (Figure 5.8D; p<0.05 vs sham familiar). CCI mice did not differentiate the novel arm from the familiar, showing no difference between the time spent in either arm (Figure 5.8D). As illustrated in the graph, only half of the CCI veh group spent more than 50% in the novel arm. GSK2795039 treated mice showed an increase towards spending more time in the novel arm but levels did not reach statistical significance (Figure 5.8D). However, 5 out of the 6 GSK2795039 treated mice spent more than 50% of the time in the novel arm, indicating the time spent in the arm was not by chance and suggests intact cognitive function. The ratio of the novel over the familiar arm was analysed to compare the fold differences across sham, CCI veh and CCI GSK2795039 (Figure 5.8E). CCI induced a decrease in the novel/familiar arm (Figure 5.8E; p<0.01 vs sham) and GSK2795039 treated mice showed...
a minor increase in this ratio but levels failed to reach statistical significance. As a control measure of the 2-trials Y-Maze test, there was no statistical difference in the distance travelled (mean ± SEM) between groups for sham (12.31 ± 0.7895 m), CCI veh (13.69 ± 1.411 m) and CCI GSK2795039 (11.80 ± 0.9463 m).

These findings suggest that GSK2795039 intervention indicated positive trends in improving CCI elicited motor and spatial deficits, albeit not statistically significant.

Figure 5.8: GSK2795039 showed improved motor and spatial learning in CCI mice at 3 DPI but these effects did not reach statistical significance.

GSK2795039 was administered systemically (i.p) starting at 2h, 12h, 24h, and 48h post CCI in adult male C57BL6/J mice. Gross motor function was measured by an accelerating rotarod at 1-, 2- and 3 DPI. Spatial learning and memory were assessed by two-trials Y-Maze at 2 DPI. CCI mice
showed reduced time spent on the rotarod at 1-, 2- and 3 DPI (B; *p<0.05 vs sham veh). GSK2795039 treated CCI mice showed increased time on the rotarod at 3DPI, but levels failed to reach significance (C). Sham mice spent more time in the novel arm of the Y-Maze (D; *p<0.05) compared to the familiar arm, calculated by the % of time of novel/ time of novel and familiar. CCI mice did not show any difference in the time spent in the familiar vs novel arm. GSK2795039 treated mice showed more time spent in the novel arm but levels failed to reach statistical significance (D). The fold difference of the novel arm (over the familiar arm) was significantly lower in CCI mice (E; **p<0.01 vs sham). GSK2795039 showed a trend towards sham levels but this did not reach statistical significance. Dotted lines are mean ± SEM of the time spent in the familiar arm. Data are mean ± SEM (n=4-6 per group). *p<0.05 vs sham veh/sham GSK2795039; **p<0.01 vs sham; Two-Way ANOVA with repeated measures and uncorrected Fisher’s LSD (B, C); paired t-test (D); One-Way ANOVA with Dunnett’s post hoc test (E).

5.2.9 GSK2795039 reduced the number of infiltrating myeloid cells in CCI mice at 3 DPI.

Like the first intervention study, the role of GSK2795039 on NOX2-mediated neuroinflammation in resident and infiltrating myeloid cells was investigated. Mononuclear cells were stained with a viability dye and surface markers (CD11b, CD45, Ly6C, Ly6G, CD3, CD4, CD8, IL-1R), followed by intracellular staining of NOX2 and IL-1β for analysis by flow cytometry. The absolute number of cells were calculated.

As shown previously, CCI induced a significant increase in the absolute number of CD11b+CD45int microglia, CD11b+CD45hi infiltrating cells including Ly6G+ neutrophils and Ly6C+ monocytes as demonstrated in the representative dot plots (Figure 5.9A, B) and the corresponding quantification (Figure 5.9C-F; p<0.0001 vs sham veh). GSK2795039 treated CCI mice had modest effects against microglial number (Figure 5.9C) but significantly reduced the number of CD11b+CD45hi infiltrating cells (Figure 5.9D; p<0.01 vs CCI veh). Of these infiltrating cells, GSK2795039 reduced Ly6G+ neutrophils but levels failed to reach statistical significance (Figure 5.9E). However, GSK2795039 significantly attenuated number of Ly6C+ monocytes (Figure 5.9F; p<0.05 vs CCI veh).

These findings suggest GSK2795039 plays a role in reducing the number of cells that traffic into the brain following CCI. The next question was whether the CCI induced NOX2+ and IL-1β+ resident and infiltrating cells could be attenuated following GSK2795039 treatment and ultimately lead to reduced neuroinflammation.
Figure 5.9: GSK2795039 reduced absolute number of CD11b<sup>+</CD45<sup>hi</sup> cells in CCI mice at 3 DPI. GSK2795039 was administered at 2h, 12h, 24h and 48h post CCI. Mononuclear cells isolated from the ipsilateral cortex were stained for CD11b, CD45, Ly6G and Ly6C, and analysed by flow cytometry. CCI increased the absolute number of CD11b<sup>+</CD45<sup>Int</sup> microglia, CD11b<sup>+</CD45<sup>hi</sup> infiltrating cells, including Ly6G<sup>+</sup> neutrophils and Ly6C<sup>+</sup> monocytes (C-F; ****p<0.0001 vs sham veh). GSK2795039 reduced CD11b<sup>+</CD45<sup>hi</sup> infiltrating cells and of these, Ly6C<sup>+</sup> monocytes (D, F; *p<0.05; **p<0.01 vs CCI veh). Data are mean ± SEM (n=4-6 per group). ****p<0.0001 vs sham veh, *p<0.05; **p<0.01 vs CCI veh; Two-Way ANOVA with uncorrected Fisher’s LSD.
5.2.10 GSK2795039 reduced NOX2$^+$ and IL-1β$^+$ infiltrating myeloid cells in CCI mice at 3 DPI.

To examine if GSK2795039 treatment elicited protective effects in the brain after TBI, the total number of NOX2$^+$ and IL-1β$^+$ microglia and infiltrating cells was determined following treatment. To assess the pro-inflammatory phenotype, cells were stained with surface markers followed by intracellular staining of NOX2 and IL-1β and analysed by flow cytometry. This allowed for cell-specific functional assessment.

As previously shown, CCI induced increased numbers of NOX2$^+$ and IL-1β$^+$ microglia (CD11b$^+$CD45$^{\text{Int}}$) as shown in the representative dot plots (Figure 5.10A, B) and corresponding quantifications (Figure 5.10C, D; p<0.0001 vs sham veh). GSK2795039 treated CCI mice had reduced numbers of NOX2$^+$ and IL-1β$^+$ microglia but levels failed to reach statistical significance (Figure 5.10C, D). CCI upregulated NOX2$^+$ and IL-1β$^+$ infiltrating myeloid cells (CD11b$^+$CD45$^{hi}$) as shown in the representative dot plots (Figure 5.10A, B) and corresponding quantifications (Figure 5.10E, F; p<0.0001 vs sham veh). GSK2795039 significantly reduced the number of NOX2$^+$ and IL-1β$^+$ infiltrating myeloids (Figure 5.10E, F; p<0.001 versus CCI Veh).

Together, these results demonstrated that GSK2795039 administration starting at 2h post CCI, attenuated NOX2/NLRP3 activation in cells trafficking to the brain following moderate-level CCI. This important finding was not observed in the initial intervention study where GSK2795039 was administered at 4h, 24h and 48h post CCI.
Figure: 5.10 GSK2795039 reduced NOX2\(^+\) and IL-1\(\beta\)^+ in infiltrating myeloid cells in mice following CCI.
GSK2795039 was administered at 2h, 12h, 24h and 48h post CCI. Mononuclear cells isolated from the ipsilateral cortex were stained with surface markers followed by intracellular staining.
of NOX2 and IL-1β and analysed by flow cytometry. CCI increased the absolute number of NOX2+ and IL-1β+ CD11b+CD45int microglia and CD11b+CD45hi infiltrating cells (C-F; ****p<0.0001 vs sham veh). GSK2795039 showed reduced #NOX2+ #IL-1β+ microglia but levels failed to reach significance (C, D). GSK2795039 significantly reduced #NOX2+ IL-1β+ infiltrating cells (E, F; +++p<0.001 vs CCI veh). Data are mean ± SEM (n=4-6 per group). ****p<0.0001 vs sham veh, +++p<0.001 vs CCI veh; Two-Way ANOVA with uncorrected Fisher’s LSD.

5.2.11 GSK2795039 reduced absolute numbers of NOX2+ and IL-1β+ neutrophils in CCI mice at 3 DPI.
As GSK2795039 reduced NOX2+ and IL-1β+ total infiltrating myeloid cells after TBI, the role of this NOX2 inhibitor on the specific populations of infiltrating cells was further examined. As demonstrated by the time-course experiment in the previous results chapter, neutrophils were the first responders to injury in the brain and release a respiratory burst (ROS) to induce an inflammatory response. In this present study, the absolute number of IL-1β+ (B), NOX2+ (C) and NOX2+IL-1β+ (D) Ly6G+ neutrophils were increased following CCI as shown by representative quadrants of NOX2 vs IL-1β+ (A) whereby Q1+Q2 is IL-1β+, Q2+Q3 is NOX2+ and Q2 is the double positive for NOX2+ IL-1β+, along with the corresponding quantifications (Figure 5.11B-D; p<0.0001 vs sham veh). GSK2795039 treated CCI mice had significantly reduced numbers of IL-1β+, NOX2+, and NOX2+IL-1β+ neutrophils as demonstrated in the representative dot plots and quantifications (Figure 5.11A-D; p<0.05; p<0.01; p<0.0001 vs CCI veh).

These findings indicated an inhibitory potential of GSK2795039 on NOX2/NLRP3 inflammatory neutrophils which is crucial as sustained ROS release induces oxidative stress and upregulates a pro-inflammatory cascade. Therefore, in targeting neutrophil activation, GSK2795039 alleviated CCI elicited neuroinflammation. Of note, GSK2795039 did not completely inhibit NOX2+ neutrophils (Figure 5.11). This was important because low levels of ROS are necessary to prevent infection [53] but sustained immune activation may increase neuropathological outcomes.
Figure 5.11: GSK2795039 reduced absolute numbers of NOX2+ and IL-1β+ neutrophils in CCI mice at 3 DPI.

GSK2795039 was administered at 2h, 12h, 24h and 48h post CCI. Mononuclear cells isolated from the ipsilateral cortex were stained with surface markers, including Ly6G to stain for neutrophils, followed by intracellular staining of NOX2 and IL-1β, and analysed by flow cytometry. CCI increased the absolute number of IL-1β+ (Q1+Q2), NOX2+ (Q2+Q3) and NOX2+IL-1β+ (Q2) Ly6G+ neutrophils as shown in the representative quadrants of NOX2 vs IL-1β (A) and corresponding quantifications (B-D; ****p<0.0001 vs sham veh). GSK2795039 reduced the number of IL-1β+, NOX2+ and NOX2+IL-1β+ neutrophils (A-B; *p<0.05; **p<0.01; ****p<0.0001 vs sham veh). Data are mean ±SEM (n=4-6 per group). ****p<0.0001 vs sham veh, *p<0.05; **p<0.01; ****p<0.0001 vs CCI veh; Two-Way ANOVA with uncorrected Fisher’s LSD.
5.2.12 GSK2795039 reduced absolute numbers of NOX2\(^+\) monocytes in CCI mice at 3 DPI.

Given that GSK2795039 reduced neutrophil activation, the next step was to investigate the role of NOX2 inhibition in infiltrating monocytes, which were demonstrated in the time-course study to peak at 3 DPI. Furthermore, preliminary data from the lab suggested that monocytes trafficking into the brain are more pro-inflammatory compared to the resident microglia following TBI.

In this present study, the absolute number of IL-1\(\beta\)\(^+\) (B), NOX2\(^+\) (C) and NOX2\(^+\)IL-1\(\beta\)\(^+\) (D) Ly6C\(^+\) monocytes were increased following CCI, as shown by representative quadrants of NOX2 vs IL-1\(\beta\)\(^+\) (Figure 5.12A) whereby Q1+Q2 is IL-1\(\beta\)\(^+\), Q2+Q3 is NOX2\(^+\) and Q2 is the double positive for NOX2\(^+\) IL-1\(\beta\)\(^+\), along with the corresponding quantifications (Figure 5.12B-D; p<0.0001 vs sham veh). GSK2795039 treated CCI mice had significantly reduced numbers of NOX2\(^+\) monocytes (Figure 5.12C; p<0.05 vs sham veh) and showed reduced #IL-1\(\beta\)\(^+\) and #NOX2\(^+\)IL-1\(\beta\)\(^+\) monocytes, but levels failed to reach statistical significance (Figure 5.12C, D).

Although the initial hypothesis for this project stated GSK2795039 attenuates NOX2 activation in microglia, this study clearly indicated that GSK2795039 had inhibitory effects against neutrophil and monocytes NOX2/NLRP3 inflammasome activation. These findings indicate that GSK2795039 plays a role in attenuating pro-inflammation in the innate immune cells trafficking to the brain.
5.12 GSK2795039 reduced absolute numbers of NOX2+ monocytes in CCI mice at 3 DPI.

GSK2795039 was administered at 2h, 12h, 24h and 48h post CCI. Mononuclear cells isolated from the ipsilateral cortex were stained with surface markers, including Ly6C to stain for monocytes, followed by intracellular staining of NOX2 and IL-1β, and analysed by flow cytometry. CCI increased the absolute number of IL-1β+ (Q1+Q2), NOX2+ (Q2+Q3) and NOX2+IL-1β+ (Q2) Ly6C+ monocytes as shown in the representative quadrants of NOX2 vs IL-1β (A) and corresponding quantifications (B-D; ****p<0.0001 vs sham veh). GSK2795039 significantly reduced the number of NOX2+ monocytes (C; *p<0.05 vs sham veh) and the number of IL-1β+ and NOX2+IL-1β+ monocytes, but levels failed to reach statistical significance (C, D). Data are mean ±SEM (n=4-6 per group). ****p<0.0001 vs sham veh, *p<0.05; vs CCI veh; Two-Way ANOVA with uncorrected Fisher’s LSD.
5.2.13 GSK2795039 reduced absolute numbers of CD3+ T cells in CCI mice at 3 DPI.

To determine whether the effect of GSK2795039 was specific to resident and infiltrating myeloid cells, the adaptive arm of the immune system was also examined. Cytotoxic T cells have been shown to aggregate in the injury lesion, promote chronic neuroinflammation, and worsen neuropathological outcomes following TBI [137], thus proving a therapeutic target post TBI. The following experiment investigated the role of GSK2795039 in CD3+ T cell activation.

To assess this, mononuclear cells were stained with CD3, CD4 and CD8 antibodies as T cell markers and the flow plots below are gated on live single CD45+CD11b-CD45hiCD3+ T cells. CCI induced upregulation of the number of CD3, CD4, CD8 and CD4-CD8- T cells as shown by the representative dot plots (Figure 5.13A) and corresponding quantifications (Figure 5.13B-E; p<0.0001 vs sham veh). GSK2795039 treated mice significantly reduced the numbers of CD3+, CD4+, CD8+ and CD4-CD8- T cells (Figure 5.13B-E; p<0.01; p<0.0001 vs CCI veh). The CD4-CD8- T cell population was speculated to be gamma delta (γΔ) T cells; however, this marker was not in the flow cytometry panel and so future studies are required to confirm this. Nevertheless, it was worth reporting that GSK2795039 effectively reduced this population.

Indeed, the reduction of the number of T cells by GSK2795039 was unexpected at this acute 3 DPI timepoint. In conjunction, previous work demonstrated genetic depletion of CD4+ and CD8+ T cells resulted in reduced infarct size 1d post-ischemic stroke and improved functional output [154]. This highlights the importance of targeting T cell activation and how GSK2795039 may be a therapeutic intervention after TBI.
Figure 5.13: GSK2795039 reduced absolute numbers of CD3⁺, CD4⁺, CD8⁺ and CD4⁻CD8⁻ T cells in CCI mice at 3 DPI.

GSK2795039 was administered at 2h, 12h, 24h and 48h post CCI. Mononuclear cells isolated from the ipsilateral cortex were stained for CD3, CD4 and CD8, and analysed by flow cytometry. CCI increased absolute numbers of CD3⁺, CD4⁺, CD8⁺ and CD4⁻CD8⁻ T cells as shown by representative dot plots (A) and corresponding quantifications (B-E; ****p<0.0001 vs sham veh). GSK2795039 treated CCI mice significantly reduced absolute numbers of CD3⁺, CD4⁺, CD8⁺ and CD4⁻CD8⁻ T cells (B-E; **p<0.01; ****p<0.0001 vs CCI veh). Data are mean ± SEM (n=4-6 per group). ****p<0.0001 vs sham veh, **p<0.01; ****p<0.0001 vs CCI veh; Two-Way ANOVA with uncorrected Fisher’s LSD.
5.2.14 GSK2795039 reduced IL-1R expression on T cells in CCI mice at 3 DPI.

Along with evaluating the effect GSK2795039 has on the absolute numbers of infiltrating T cells following injury, it was next investigated if GSK2795039 could also impact IL-1 receptor (IL-1R) expression on the T cells. T cells expressing IL-1R respond to IL-1β released from cells of myeloid origin such as microglia or monocytes. This ligand binding complex triggers and facilitates T cell responses [155]. IL-1 signalling may act as an inducer of adaptive immune responses.

To evaluate this, IL-1R+ expression was first gated from total CD3+ T cells, as shown in the upper panel (Figure 5.14A). Of the IL-1R+CD3+ T cells, the number of CD4+, CD8+ and CD4CD8- were determined, as shown in the lower panel (Figure 5.14A). CCI upregulated IL-1R expression on CD3+, CD4+, CD8+ and CD4CD8+ T cells, demonstrated in the representative dot plots (Figure 5.14A) and corresponding quantifications (Figure 5.14B-E; p<0.0001 vs sham veh). GSK2795039 treated CCI mice significantly decreased the number of IL-1R+CD3+, IL-1R+CD4+, IL-1R+CD8+ and IL-1R+CD4+CD8+ T cells (Figure 5.14B-E; p<0.05; p<0.01; p<0.0001 vs CCI veh). The significant reduction in IL-1R+ T cells indicates that microglial-T cell crosstalk was altered by GSK2795039 treatment following injury.
Figure 5.14: GSK2795039 reduced absolute numbers of IL-1R+ T cells in CCI mice at 3 DPI.

GSK2795039 was administered at 2h, 12h, 24h and 48h post CCI. Mononuclear cells isolated from the ipsilateral cortex were stained for CD3, CD4, CD8 and IL-1R, and analysed by flow cytometry. CCI increased absolute numbers of IL-1R expression on CD3+, CD4+, CD8+ and CD4-CD8- T cells as shown by representative dot plots (A) and corresponding quantifications (B-E; ****p<0.0001 vs sham veh). GSK2795039 treated CCI mice significantly reduced absolute numbers of IL-1R+CD3+, IL-1R+CD4+, IL-1R+CD8+ and IL-1R-CD4-CD8- T cells (B-E; *p<0.05; **p<0.01; ****p<0.0001 vs CCI veh). Data are mean ±SEM (n=4-6 per group). ****p<0.0001 versus Sham, *p<0.05; **p<0.01; +++p<0.0001 vs CCI Veh; Two-Way ANOVA with uncorrected Fisher’s LSD.
5.2.15 GSK2795039 reduced CD11b⁺CD45⁺myeloid cells in the blood of CCI mice at 3 DPI.

The main goal of these experiments was investigating the effect of GSK2795039 on NOX2-mediated neuroinflammation following TBI. Previous studies have demonstrated systemic immune dysregulation following injury, including chronically elevated oxidative stress levels in myeloid cells of the blood [30], thus indicating the link between the brain and immune system is disrupted following injury. Given GSK2795039 was administered systemically, it was important to investigate its role in the peripheral system and therefore, the absolute numbers of CD11b⁺CD45⁺myeloid cells were determined in the blood of CCI mice.

To evaluate this, blood was collected in anti-coagulated EDTA tubes through cardiac puncture, harvested and stained for CD11b, CD45, Ly6G and Ly6C, and analysed for flow cytometry. The flow plots below are gated on live single CD11b⁺CD45⁺cells. Firstly, there was a greater percentage of neutrophils to monocytes across all groups (Figure 5.15A). Secondly, it was observed that GSK2795039 promoted an innate immune response at the level of the sham. Sham GSK2795039 mice had an increased number of CD11b⁺CD45⁺Ly6C⁺ monocytes as shown in the representative dot plots (Figure 5.15A) and corresponding quantification (Figure 5.15E; p<0.05 vs sham veh). Sham GSK2795039 mice showed increased number of neutrophils compared to sham veh, but levels failed to reach statistical significance (Figure 5.15D). GSK2795039 treated CCI mice had significantly reduced number of total CD11b⁺CD45⁺, neutrophils and monocytes (Figure 5.15C-E; p<0.05; p<0.01 vs sham GSK2795039) and reduced number of neutrophils compared to vehicle treated CCI mice (Figure 5.15D; p<0.05 vs CCI veh).

These findings suggest GSK2795039 attenuated number of myeloid cells in the blood of CCI mice which may be due to the cells trafficking to the brain following blood–brain barrier breakdown after injury.
GSK2795039 was administered at 2h, 12h, 24h and 48h post CCI. Blood was collected through cardiac puncture, harvested, and stained for CD11b, CD45, Ly6G and Ly6C, and analysed by flow cytometry. There was a greater neutrophils/monocytes ratio across all groups (B). Sham GSK2795039 mice had increased number of CD11b^+CD45^{hi} monocytes (E; *p<0.05 vs sham veh). CCI GSK2795039 mice had reduced number of total CD11b^+CD45^{hi}, neutrophils and monocytes (C-E; *p<0.05; **p<0.01 vs sham GSK). CCI GSK2795039 mice had significantly lower #neutrophils (D; *p<0.05 vs CCI veh). Data are mean ±SEM (n=4-6 per group). *p<0.05; **p<0.01 versus sham GSK, +p<0.05 vs CCI veh; Two-Way ANOVA with uncorrected Fisher’s LSD.

5.2.16 GSK2795039 reduced CD11b^+CD45^{hi} lymphocytes in the blood of CCI mice at 3 DPI.

To further investigate the effects of GSK2795039 treatment on systemic immune function, and to determine if the adaptive immune system was altered, the number of lymphocytes in the blood was assessed.

The blood was stained for CD3, CD4 and CD8, and analysed by flow cytometry. The flow plots below are gated on live single CD11b^+CD45^{hi} CD3^+ cells. Like the previous result, GSK2795039 promoted an adaptive immune response at the level of the sham. Sham GSK2795039 mice showed an increased number of CD8^+ T cells as shown by the representative dot plot (Figure 5.16A) and corresponding quantifications (Figure 5.16D; p<0.05 vs sham veh). Sham GSK2795039 mice had increased number of CD3^+ and CD4^+ T cells compared to sham veh, but levels failed to reach statistical significance (Figure
5.16B, C). GSK2795039 treated CCI mice had significantly reduced number of CD3+, CD4+ and CD8+ T cells compared to sham GSK2795039 (Figure 5.16B-D; p<0.05; p<0.01 vs sham GSK2795039). There was no significant difference in number of T cells between GSK2795039 or vehicle treated CCI mice.

These findings did not indicate a robust GSK2795039 effect with injury, but GSK2795039 did have an effect in the absolute number of cells in the blood at baseline levels. These results simply state the cell number in the blood, but investigating the function of these cells following treatment will enable better understanding of the role of GSK2795039 in the immune system.

Figure 5.16: GSK2795039 increased number of T cells at baseline which were reduced with injury in the blood.

GSK2795039 was administered at 2h, 12h, 24h and 48h post CCI. Blood was collected through cardiac puncture, harvested, and stained for CD3, CD4 and CD8, and analysed by flow cytometry. Sham GSK2795039 mice had increased number of CD8+ T cells as shown in the representative dot plots (A) and corresponding quantification (D; *p<0.05 vs sham veh). CCI GSK2795039 mice had significantly reduced number of CD3+, CD4+ and CD8+ T cells (B-D; *p<0.05; **p<0.01; ***p<0.001 vs sham GSK2795039). Data are mean ±SEM (n=4-6 per group). *p<0.05; **p<0.01; ***p<0.001 versus sham GSK2795039; Two-Way ANOVA with uncorrected Fisher’s LSD.
5.2.17 Neuro-behavioural outline and GSK2795039 administration regime for chronic TBI study over a 28-day timepoint.

Given that GSK2795039 showed promising effects on motor function, spatial learning and memory, and NOX2-mediated neuroinflammation of infiltrating cells acutely at 3 DPI, it was next investigated what effect GSK2795039 would have chronically up to 28 DPI in CCI mice. The hypothesis stated that GSK2795039 treatment would attenuate CCI elicited cortical lesion volume and would improve neurological outcomes in the form of cognitive and motor functions post-injury.

Adult male C57BL6/J mice were randomly assigned to injury and treatment groups prior to the day of CCI surgeries. The predefined assignment ensured there was no weight, age, or cage effect before starting the study. All mice entering the study were handled (5 min/ mouse) 14d before injury and had ten or less foot-faults on the beam walk at 1d prior to CCI. Throughout the 28d study, mice underwent a battery of behavioural tests. Both the beam walk, which measures fine motor coordination [114] and the Simple Neuro-assessment of Asymmetric Impairment (SNAP), which is a composite of eight tests used to measure neurological deficits after injury [117], were measured longitudinally as shown in the experimental outline below (Figure 5.17A). The 2-trial Y-maze measured spatial learning and memory at 10 DPI. Both the Novel Object Recognition (NOR) test, which measured recognition memory [114] over a 4d test, and Morris Water Maze (MWM), which measured hippocampal dependent memory [114] over a 5d test, were measured at a more chronic time-point post-injury.

In the previous intervention study, four GSK2795039 doses were administered over the 3-day time-point and this was tolerated well in the mice. Other studies pharmacokinetic analysis also proved complete NOX2 inhibition up to 24h post injection [74]. Therefore, in this present study it was decided to keep four GSK2795039 doses in the first 48h, in line with the acute TBI study, with additional GSK2793059 administration at 3- and 7 DPI, as depicted in the administration regime (Figure 5.17B). It was hypothesised that with higher amounts of GSK2795039 in the system, the behavioural deficits and neuroinflammation could be rescued in CCI mice.
A Neuro-behavioural outline for chronic TBI study

![Diagram showing the neuro-behavioural outline and GSK2795039 administration regime for chronic TBI study over a 28-day timepoint.]

**Figure 5.17:** Neuro-behavioural outline and GSK2795039 administration regime for chronic TBI study over a 28-day timepoint.

Adult male C57BL6/J mice were handled (5 min/mouse) 14d prior to CCI. Mice underwent behavioural training on the beam walk 3 days prior to injury. Following CCI, mice underwent a battery of neuro-behavioural tests such as SNAP, Beam Walk, Y-Maze, novel object recognition (NOR) and Morris Water Maze (MWM) over 28 days. GSK2795039 (100mg; kg) or Vehicle (DMSO 10% + Corn oil 90%) was administered intraperitoneally starting at 2h post CCI, 12h, 24h, 48h, 3d and 7d post-injury.

B GSK2795039 (100mg/kg; i.p) administration regime

![Diagram showing the GSK2795039 administration regime.]

5.2.18 Surgery duration and weight were consistent across groups and righting reflex was increased with injury.

It was important to record all parameters on the day of the surgery in case results appeared unexpected due to factors not linked to the experimental question. Therefore, the duration of the surgery, the time it took the mice to right itself after injury, and the weight throughout the study was recorded. In this present study, all sham mice received vehicle treatment as there was no difference in the brains of sham vehicle vs GSK2795039 mice in the previous experiments, and increasing the sample size N was a primary objective for behavioural tests.

Surgery duration was consistent across all groups (Figure 5.18A) which meant that all mice were under isoflurane for the same amount of time. This consistency ensured any effects due to the length of time the mouse spent under the anaesthetic was accounted for. The time it took the mice to right themselves after injury was recorded and showed
an injury effect. CCI mice had an increased righting reflex time when compared to sham operated mice (Figure 5.18B; \( p<0.05; p<0.001 \) vs sham). At the time of righting reflex, no treatment was administered (because it was 2h post injury) so CCI GSK is the group that will receive the GSK2795039. There were no differences in the righting reflex between CCI groups (veh and GSK2795039). Mice were weighed every day (following behavioural tests) and expressed as a percentage of their baseline weight on CCI day. All mice showed a significant loss of weight 1 DPI compared to the day of the surgeries (Figure 5.18C; \( p<0.05; p<0.001; p<0.0001 \)). Within 14d, all mice restored their weight loss and showed increased weight over the course of the study. There was no significant difference in weight between groups.

**Figure 5.18:** The surgery duration, righting reflex, and weight over the 28d timepoint were recorded.

The duration of the surgery and the time it took the mice to right themselves were measured on the day of the CCI surgeries. The weight was recorded each day and expressed as a percentage of the baseline weight on the CCI day. There was no difference between groups in the duration of the surgery (A). CCI mice took longer to right themselves (B; ***\( p<0.001; ^* p<0.05 \)) compared to sham mice. Weight was significantly reduced in all mice at 1DPI (C; **\( p<0.01; \)
***p<0.001; ****p<0.0001) compared to baseline. Data are mean ±SEM (n=8-12 per group).
*p<0.05; **p<0.01 vs sham; One-Way ANOVA with post hoc Tukey’s multiple comparisons test (A, B); **p<0.01; ***p<0.001; ****p<0.0001 vs baseline levels; Two-Way ANOVA mixed effect analysis with uncorrected Fisher’s LSD (C).

5.2.19 GSK2795039 showed minor improvements in the beam walk and had no effect on sensorimotor function in the SNAP test in CCI mice.

Longitudinal studies are important to identify changes in mice behaviour over the course of the study. CCI-induced fine motor coordination and sensorimotor deficits were assessed longitudinally by the beam walk and SNAP test respectfully.

Fine motor coordination and balance was assessed on the beam walk and neurological function of mice was examined using SNAP, described in detail in Chapter 2: Materials and Methods. Briefly, the greater the score suggests asymmetric deficits [117].

Assessment of fine motor coordination revealed CCI-induced increase in the number of foot-faults from 1-28 DPI with gradual improvements over the course of the study (Figure 5.19A; p<0.01; p<0.001; p<0.0001 vs sham, green stars). GSK2795039 treated CCI mice also had increased number of foot-faults from 1-28 DPI (Figure 5.19A; p<0.01; p<0.0001 vs sham, pink stars). GSK2795039 treated CCI mice showed minor improvements in reduced number of foot-faults compared to CCI veh, but levels did not reach statistical significance. Neurological assessment by SNAP revealed CCI-induced long-lasting asymmetric deficits from 3-28 DPI (Figure 5.19B; p<0.01; p<0.001; p<0.0001 vs sham, green stars). GSK2795039 treated CCI mice had no effect on preserving sensorimotor function and were significantly worse compared to sham (Figure 5.19B; p<0.01; p<0.001 vs sham, pink stars). CCI mice, irrespective of treatment, showed improved performance over time but levels were significantly different to sham mice.

These findings indicate GSK2795039 had modest effects on fine motor coordination and did not restore CCI-elicited sensorimotor deficits up to 28 DPI.
Figure 5.19: GSK2795039 showed minor improvements in fine motor deficits but had no effect on sensorimotor function the SNAP test in CCI mice. GSK2795039 was administered at 2h, 12h, 24h, 48h, 3d and 7d post CCI. Fine motor coordination and sensorimotor dysfunction were measured longitudinally by beam walk and SNAP. CCI, irrespective of treatment, induced increased number of foot-faults from 1-28 DPI (A; **p<0.01; ***p<0.001; ****p<0.0001 vs sham). GSK2795039 treated CCI mice showed a minor reduction in the number of foot-faults compared to vehicle treated, but levels failed to reach statistical significance (A). CCI, irrespective of treatment, induced an increased SNAP score from 1-28 DPI (B; **p<0.01; ***p<0.001; ****p<0.0001 vs sham). GSK2795039 treated CCI mice did not improve sensorimotor function compared to vehicle treated, but all CCI mice did show improvements over the 28d. Data are mean ±SEM (n=8-12 per group). **p<0.01; ***p<0.001; ****p<0.0001 vs sham; Two-Way ANOVA with repeated measures and uncorrected Fisher’s LSD.

5.2.20 GSK2795039 did not protect CCI elicited spatial learning deficits in the Y-maze at 10 DPI.

The next behavioural test was the two-trial Y-maze which assessed spatial learning and memory at 10 DPI. The main outcome was to determine the amount of time spent in the novel arm as a percentage of the total time (Figure 5.20A) or the fold difference of the novel arm over the familiar arm (Figure 5.20B).

Sham mice did not perform as expected and showed no preference for exploring the novel arm compared to the familiar arm (Figure 5.20A). CCI mice, treated with GSK2795039 or vehicle, did not differentiate the novel arm and showed no difference between the time spent in either arm (Figure 5.20A). The fold difference of novel/familiar arm was consistent across all groups (Figure 5.20B). As control measures of the Y-maze test, there was no difference in the distance travelled (mean ± SEM)
between groups for sham (13.24 ± 0.8174m), CCI veh (15.78 ± 0.9430m) and CCI GSK2795039 (16.33 ± 1.062m).

**Figure 5.20: GSK2795039 did not protect CCI elicited spatial learning deficits in the Y-maze at 10 DPI.**

GSK2795039 was administered at 2h, 12h, 24h, 48h, 3d and 7d post CCI. Spatial learning memory was assessed by the two-trials Y-maze at 10 DPI. Sham or CCI mice did not show any difference in the time spent in the familiar vs novel arm as shown by the percentage of time (A) and fold difference of the novel arm (over the familiar arm) (B). GSK2795039 treated CCI mice did not alter the time spent in the novel arm. Data are mean ±SEM (n=8-12 per group). Paired t-test (A); One-Way ANOVA with Dunnett’s multiple comparisons test vs CCI veh (B).

**5.2.21 GSK2795039 showed modest improvements in novel object recognition in CCI mice.**

It was next examined what effect GSK2795039 had on cognitive function 10 days following the final GSK2795039 administration. Recognition memory was assessed by the novel object recognition (NOR) test which consisted of two habituation days, a third familiarisation day with two identical objects and a fourth test day where one object set is replaced with a novel object. The greater the discrimination index (DI; more time spent with novel object) the better the performance in the task. Mice with a DI greater or equal to 20% have intact cognitive function.

On the familiarisation day, where the objects are identical, it was observed that CCI mice preferred the right side of the arena (Figure 5.21A; p<0.05 vs left). Although unexpected,
this was accounted for prior to the following testing day by randomising the side the novel object was placed across all groups. The recognition test revealed a reduced DI in CCI mice compared to sham, but levels failed to reach statistical significance (Figure 5.21B). GSK2795039 treated CCI mice showed improved discrimination for the novel object, but these effects did not reach statistical significance.

The habituation day also served as the open field test and revealed no difference in the distance from the border (thigmotaxis; mean ± SEM) across groups; sham (4.963 ± 0.2203 cm), CCI veh (4.517 ± 0.1878 cm) and CCI GSK2795039 (4.542 ± 0.1828 cm). There was an expectation to observe sham mice exploring and therefore spending more time away from the sides of the arena. This leads to speculation that the sham mice were not performing as anticipated.

Figure 5.21: GSK2795039 showed modest improvements in novel object recognition in CCI mice.

GSK2795039 was administered at 2h, 12h, 24h, 48h, 3d and 7d post CCI. Recognition memory was assessed by a 4-day Novel Object recognition (NOR) test at 17-20 DPI. CCI mice showed a preference for the right side of the arena on familiarisation day 3 (A; *p<0.05 vs left side) which was accounted for prior to testing day. CCI mice had reduced DI compared to sham, but levels failed to reach statistical significance (A). GSK2795039 treated CCI mice showed improved DI, but levels did not reach significance (A). Data are mean ±SEM (n=8-12 per group). *p<0.05 vs left side; Paired t test (A); Kruskal-Wallis test with Dunn’s multiple comparisons test vs CCI veh (B).
5.2.22 GSK2795039 had no effect on CCI-induced hippocampal dependent memory deficits in the Morris Water Maze.

The final neuro-behavioural test of this study measured hippocampal dependent memory using the Morris Water Maze (MWM). The objective was to record the latency to find a hidden platform submerged 0.5cm below the water’s surface during the acquisition days (1-4) and to measure the time spent in the platform quadrant when the platform was removed in the probe test (day 5). In a mouse model of TBI, genetic depletion of NOX2−/− improved motor recovery post-injury [16]. It was next evaluated if pharmacological inhibition by GSK2795039 could have similar cognitive improvements.

In this present study, sham mice showed a learning curve over the four acquisition days, illustrated in a reduced latency to find the hidden platform (Figure 5.22A). CCI mice required more time to find the platform and showed an increased latency on acquisition days 1- 3 (Figure 5.22A; p<0.05; p<0.01; p<0.0001 vs sham) with reduced latency similar to sham mice on day 4. GSK2795039 treated CCI mice had an increased latency to platform over the 4 acquisition days (Figure 5.22A; p<0.05; p<0.01 vs sham) and showed no differences compared with vehicle treated CCI mice. In the probe test (day 5), there was no significant differences in the time spent in the platform quadrant or the number of entries between groups (Figure 5.22C, D). As a control measure, the swim speed of all mice was consistent indicating no motor impairments in finding the platform (Figure 5.22E). Finally, the visual acuity test was performed after the probe test and consisted of a flag on the platform to assess visibility. Of note, three CCI mice did not find the platform before the 20 second threshold, and therefore were excluded from all MWM analysis (Figure 5.22A, C-E).

These findings suggest GSK2795039 did not have any protective effects in cognitive abilities chronically and may be due to insufficient number of doses resulting in levels of NOX2 being restored.
Figure 5.22: GSK2795039 had no effect on CCI-induced hippocampal dependent memory deficits in the Morris Water maze.

GSK2795039 was administered at 2h, 12h, 24h, 48h, 3d and 7d post CCI. Hippocampal dependent memory was assessed by Morris Water Maze (MWM) at 22-26 DPI. Sham mice showed a learning curve over the four acquisition days illustrating a reduced latency to the platform (A). CCI mice showed an increased latency to the hidden platform on acquisition days 1-3 (A; *p<0.05; **p<0.01; ****p<0.0001 vs sham). GSK2795039 treated CCI mice had increased latency to the platform over the 4 acquisition days (A; *p<0.05; **p<0.01 vs sham). There was no significant difference between groups in time spent (C) or number of entries in the platform quadrant (D) in the probe test. The average speed was consistent between groups (E). The final visual acuity test indicated three CCI veh mice that took more than 20 seconds to find the platform (B) and so were excluded from the entire MWM analysis. Data are mean ±SEM (n=8-12 per group). *p<0.05; **p<0.01; ****p<0.0001 vs sham; Two-Way ANOVA with repeated measures and uncorrected Fisher’s LSD (A); One-Way ANOVA with Dunnett’s multiple comparisons test vs CCI veh (C-E).
5.2.23 GSK2795039 showed a modest reduction in total lesion volume in CCI mice at 28 DPI.

Along with determining the role of GSK2795039 on CCI induced neuro-behavioural deficits, the next study aimed to investigate the effect of GSK2795039 on neuropathology at 28 DPI. Previous work demonstrated that NOX2−/− mice exhibited reduced TBI-induced lesion volume and cortical neurodegeneration compared to WT mice [16]. This gave rationale for pharmacological NOX2 inhibition by GSK2795039 to reduce histological markers of neurodegeneration.

To evaluate this, brains were perfused with saline and 4% PFA, cryopreserved in sucrose and snap frozen before sectioning at 40µm on the cryostat. Slices were collected onto slides from +0.6mm from Bregma through to -3.6mm and were 0.2mm apart. Sections were stained with Cresyl violet, acquired on a brightfield microscope and lesion volume was quantified using FIJI software. The total volume (mm³) of the lesion on the ipsilateral side was calculated as the tissue loss compared to the contralateral side.

As predicted, CCI induced a large cortical lesion as shown by the representative brain images across the anterior-posterior axis (Figure 5.23A). GSK2795039 treated CCI mice showed a modest reduction in lesion volume, but levels failed to reach statistical significance (Figure 5.23B). The next question was whether GSK2795039 could preserve hippocampal volume following injury. CCI induced a significant reduction in hippocampal volume of the ipsilateral (ispi) hemisphere compared to sham and the contralateral (contra) side within group (Figure 5.23C; p<0.05; p<0.01 vs sham ipsi; p<0.01; p<0.001 vs contra within group). Although no statistically significant difference between veh and GSK2795039 treated CCI groups was observed, GSK2795039 treated CCI mice were statistically less different from sham (p=0.05) than vehicle treated CCI mice (p=0.007). Thus, the results of this GSK2795039 intervention 28d study showed modest improvements in attenuating CCI induced behavioural deficits and neuropathology over a chronic timepoint.
Figure 5.23: GSK2795039 showed a modest reduction in total lesion volume in CCI mice at 28 DPI.

GSK2795039 was administered at 2h, 12h, 24h, 48h, 3d and 7d post CCI. Whole brains were perfused, isolated, cryopreserved, and snap-frozen before sectioning on the cryostat. Slices were stained with Cresyl violet, acquired on a brightfield microscope capturing brain regions across the anterior-posterior axis. The total volume (mm$^3$) of the lesion on the ipsilateral side was calculated as the tissue loss compared to the contralateral side using FIJI. The hippocampal volume of both the ipsi and contra was also calculated. CCI induced a total cortical lesion and GSK2795039 treatment showed reduced lesion volume, but levels failed to reach statistical significance (B; $p=0.1755$ vs CCI veh). CCI reduced ipsi hippocampal volume (C; $^*p<0.05$; $^{**}p<0.01$ vs sham ipsi; $^{***}p<0.0001$ vs contra within group). GSK2795039 treated CCI mice were statistically less different from sham than vehicle treated. Data are mean ±SEM ($n=8$-$12$ per group). $^*p<0.05$; $^{**}p<0.01$ vs sham ipsi; $^{***}p<0.001$ ipsi vs contra within group; unpaired One-tailed t-test (B); Two-Way ANOVA with with Šidák’s multiple comparisons test. The hippocampal volume analysis (C) was carried out by undergraduate student Katherine Falcon and Dr. Gloria Vegliante.
5.3 Discussion

To date, and to the best of my knowledge, this present study is the first to report promising results of a small molecule NOX2 inhibitor, GSK2795039, on NOX2-mediated NLRP3 inflammasome activation in resident and infiltrating peripheral cells in experimental TBI. The initial hypothesis for this project was that GSK2795039 would inhibit NOX2 activation in microglia following brain injury in mice. As NOX2 is upstream of the NLRP3 inflammasome, the inhibition of GSK2795039 would consequently attenuate pro-inflammatory release downstream of the NLRP3 inflammasome and reduce neuroinflammation. However, an important finding of this chapter was that GSK2795039 had an inhibitory effect on the NOX2+ and IL-1β+CD11b+CD45hi infiltrating cells, which was more robust than in the microglial population. Furthermore, of the CD11b-CD45hiCD3+ infiltrating cells, GSK2795039 showed attenuation of absolute numbers and IL-1R+ expressing T cells. Whilst this result was unexpected, it showed the effect GSK2795039 had on the adaptive immune system at just 3 DPI, the acute phase of the disease and indicated that microglial-T cell crosstalk may be altered by post-injury GSK2795039 treatment. These findings suggest a more complicated system following brain injury, than solely the activation of resident microglia cells, highlighting the complexity of the neuroimmune milieu in injured tissue.

An important question in this chapter was whether systemic administration of GSK2795039 penetrates the brain. As a rule of thumb brain-to-plasma concentration ratio below 1% indicates little to no brain permeability potential, and 10% represents brain penetration [153]. This present study showed 7.01% and 8.19% in the hippocampus and cortex, respectfully. Once it was shown that it entered the brain, the next question was to determine the potency of GSK2795039 in inhibiting NOX2 activation. The IC50 is the half maximal inhibitory concentration, and it is a measure of the potency of a substance to inhibit a biological system [156]. This output gives an informative prediction of a drug’s efficacy levels. It is known that the IC50 of NOX2 inhibition is 0.74 µM [157]. In this present study, the pharmacokinetic analysis of NOX2 inhibition in the brain following systemic administration showed levels of GSK2795039 in the hippocampus (1.06 ± 0.43 µM) and cortex (1.24 ± 0.44 µM). As the concentration
of GSK2795039 detected in the brain is higher than the known IC50 NOX2 inhibition then it was likely that GSK2795039 had an effect in the brain.

In this treatment scheme, administering GSK2795039 post injury was a novel approach compared to the existing literature and ensured the experiment was designed as clinically relevant as possible. Prior GSK2795039 studies focused on NOX2 inhibition in a paw inflammation model [74], or inhibiting NOX2/NLRP3 signalling pathway in a pyroptotic model of liver fibrosis [158]. In the TBI field, a study investigating the efficacy of GSK2795039 in a weight drop model, administered the drug pre-injury and showed improved neurological outcomes and long-term potentiation through electrophysiology of coronal hippocampal slices [81]. Whereas, in this present study, the effect of GSK2795039 on NOX2/NLRP3 inflammatory signalling was determined at the cellular level because each cell type plays an important role throughout the temporal progression of TBI disease. In the first experiment, comprising of only three doses which began 4h post injury, GSK2795039 showed minor reduction in pro-inflammatory expressing genes at the tissue level of the hippocampus by RT-PCR. Following increased knowledge of the drug bioavailability and half-life through pharmacokinetic analysis, experiments thereafter consisted of earlier (2h post injury) and extra GSK2795039 administrations which demonstrated more robust inhibition of NOX2 and downstream pro-inflammatory mediators.

Multi-dimensional flow cytometry demonstrated the effect of GSK2795039 across many cell populations, such as resident microglia and infiltrating cells, including neutrophils, monocytes, and T cells. More precisely, flow cytometry allowed for cell specific functional analysis and revealed that GSK2795039 significantly attenuated NOX2\(^{+}\)IL-1\(\beta\)\(^{+}\) expressing neutrophils and monocytes. These findings add to the existing literature which showed that inhibiting the influx of CCR2\(^{+}\) macrophages into the brain following injury, using a CCR2\(^{+}\) antagonist RS102895, resulted in reduced NOX2 and TNF\(\alpha\) mRNA [16]. Therefore, a reduced number and pro-inflammatory phenotype of infiltrating myeloid cells may alleviate injury-induced neuroinflammation. Not only does NOX2 activation in myeloid cells drive neuropathogenesis after TBI, sustained activation of CD8\(^{+}\)T cells have also been shown to accumulate in the lesion at 8- and 32 weeks post CCI and cause long-term neurological impairments in adult male C57BL6/J mice [137].
The authors depleted CD8+ T cells first by genetic depletion of CD8+T cell β2m−/− mice, and in an independent experiment using a CD8+ T cell neutralising antibody, which resulted in reduced behavioural deficits, improved neurological scores, and increased anti-inflammatory responses compared to relative controls. Subtypes of CD4+ T helper (Th) cells have contradictory functions as Th1 and Th17 responses drive neuroinflammation whereas Th2 cells promote repair. Daglas et al., also showed upregulated IL17+ IFN-γ producing CD4+T cells at 1 week following CCI. Therefore, the results of this chapter coincide with current literature to conclude that infiltration and prolonged activation of neutrophils, monocytes, CD4+ and CD8+ T cells play a pivotal role in the progression of neuropathology and may act as a target for TBI treatment.

In this present study, GSK2795039 showed reduced numbers of IL-1β+ myeloid cells and decreased IL-1R+T cells. Therefore, GSK2795039 may be a promising therapeutic drug for mitigating the damaging effects of NOX2-mediated neuroinflammation in microglia and infiltrating cells in the context of moderate-severe experimental TBI in mice. Although this present study focused on the inhibitory potential of GSK2795039 in NOX2-induced pro-inflammatory mediators, future research perspectives should evaluate the anti-inflammatory properties of GSK2795039. Emerging evidence in the literature revealed CCI mice treated with gp91ds-tat, a specific peptide inhibitor, had upregulated anti-inflammatory markers Arginase 1 and YM-1 in P2Y12+ microglia and CD11b+ macrophages respectfully [16]. Independent studies demonstrated that bone marrow-derived macrophages (BMDMs) from NOX2−/− mice stimulated with LPS/IL-4, had significantly reduced pro-inflammatory markers but also upregulated IL-10, Arginase-1 and YM1 expression compared to WT BMDMs [63]. Moreover, this study showed upregulated anti-inflammatory markers in the cortex of NOX2−/−TBI mice compared to WT, which was IL-10 mediated. Therefore, opposed to a complete ablation of the immune system as is the case with steroid treatment, more targeted therapy such as NOX2 inhibition, may be needed to regulate microglia/infiltrating cells to a more homeostatic, protective phenotype to ensure an optimal neurorestorative treatment approach.

The brain is not an immune privileged organ and systemic immune dysfunction has been linked to severity and poor outcomes following TBI [30]. Furthermore, given that
GSK2795039 was systemically administered, determining effects of the drug in the blood was of interest in this study. The literature states that blood-based biomarkers are necessary to indicate the severity and phenotype of a TBI [159]. A side study to this project was to investigate if DHR123 expression in the blood could act as a blood-based biomarker. Although we did not see significant treatment differences, understanding the phenotype of cells in the blood following injury would promote diagnostic accuracy. This present work showed increased number of CD45$^{hi}$ myeloids (CD11b$^+$) and T cells (CD11b$^-$CD3$^+$) in the blood of sham mice treated with GSK2795039 compared to vehicle. This drug only effect in the blood was an important observation because this was not the case in the brain. There was a reduced number of cells in the blood of CCI mice treated with GSK2795039. The literature evidently shows CCI induces BBB breakdown as demonstrated by injecting Evan’s blue 15 min prior to CCI and observing this staining throughout the lesion area 1h post injury [150]. Furthermore, in a mild closed-head injury model of TBI, systemic injections of Lucifer yellow and FITC-albumin indicated vascular leakage at 1.5h and 6h post injury [160]. Together these findings indicate that BBB breakdown also correlates to worsened outcomes post injury. In this present study, the reduced number of cells in the blood may be due to CCI induced decreased brain permeability and therefore an influx of cells from the blood into the brain. In contrast, sham GSK2795039 mice do not have a breached BBB and so a higher number of cells remain in the bloodstream. What this data does not tell us is the role of these CD45$^{hi}$ cells. It would be imperative to investigate the phenotypic function of these cells following GSK2795039 administration. Whether the altered number of cells between sham and CCI mice are anti- or pro-inflammatory will enable understanding the role of GSK2795039 on TBI-elicited systemic inflammatory response.

Despite the fact we see promising results with GSK2795039 at both 3- and perhaps less at 28 DPI, there are limitations to GSK2795039 treatment. Hirano and colleagues showed that 24h following systemic administration of GSK2795039, levels of NOX2 inhibition were reversed as indicated by undetectable levels of the compound in the blood along with increased ROS levels similar to that in vehicle-treated mice [74]. This suggests more frequent dosing of GSK2795039 is necessary to ensure consistent reduction on NOX2 activation following TBI. The behaviour results from this 28 DPI
experiment mirror this finding given that we do not see robust improvements with GSK2795039 in the Y-Maze, NOR and MWM which all take place three or more days after the final and sixth dose of GSK2795039, administered on 7 DPI. Similarly, in terms of total lesion volume analysis, perhaps more dosing of GSK2795039 would lead to a greater reduction in lesion size and possible neuroprotection. Indeed, the high rate of drug clearance may be the reason previous studies required repeated daily dosing of GSK2795039 to see cognitive improvements over 14 days post TBI [81]. Additionally, in a mouse model of spared-nerve injury, GSK2795039 was administered twice daily for 2d which began 1h prior to surgery and demonstrated reduced microglial activation at 2 but not 11 DPI suggesting NOX2 inhibition was reversed at this later timepoint [82].

Indeed, the timing of GSK2795039 administration and the day post injury for behaviour and cellular assessment was important. There is a balance between a safe small molecule inhibitor that is tolerated well in mice with no adverse effects following multiple injections, and the lack of potency whereby NOX2 inhibition is reversed as early as 24h post administration. [74]. The drug dosing is therefore crucial and alternative routes of administration should be explored to guarantee optimal drug efficacy.
Chapter 6: General Discussion
The main findings of this project demonstrated first *in vitro*, that NOX2 is upstream of NLRP3 in microglia and upregulated inflammasome activation and IL-1β production. These effects were modulated with GSK2795039, a specific small molecule NOX2 inhibitor (Chapter 3). Translating these findings into an *in vivo* model of moderate-severe TBI using CCI, resulted in a more complex activation of NOX2/NLRP3 inflammatory axis in the injured brain as demonstrated by infiltration of neutrophils, monocytes and T cells and resident glial activation (Chapter 4). NOX2 inhibition had a more pronounced effect on the infiltrating innate and adaptive immune cells that may contribute to improved neurological outcomes in TBI mice (Chapter 5). In particular, GSK2795039 treatment reduced the absolute numbers of CD3+ T cells and IL-1R expression on T cells, indicating these effects may be related to microglial-T cell interactions. This is summarised in the schematic below (Figure 6.1).

Figure 6.1: Proposed mechanism of how GSK2795039 attenuates NOX2-NLRP3 inflammasome activation following experimental TBI.

Following TBI, there is upregulation of microglial activation leading to the release of pro-inflammatory cytokines and chemokines. TBI-induced BBB disruption allows for the infiltration
of peripheral immune cells into the brain including neutrophils, monocytes, and T cells. Activated microglia, neutrophils and monocytes induced NOX2/ROS signalling upstream of the NLRP3 inflammasome leading to caspase-1, pyroptosis and proinflammatory cytokine (IL-1β, IL-18) release. The production of IL-1β from the myeloid population triggered an adaptive immune response by increased IL-1R expression on the surface of T cells. This image was created using BioRender.

This present study first aimed at mimicking TBI in vitro to study the mechanisms of NOX2-mediated microglial NLRP3 inflammasome and downstream neuroinflammation. Prior to setting up the in vivo lab, the in vitro experiments outlined in Chapter 3 enabled early mechanistic detection such as the reduction of NOX2 activity and NLRP3-mediated neuroinflammatory cytokines by GSK2795039. By taking this approach, my PhD project adhered with the renowned 3Rs concept; Replacement, Reduction and Refinement [161]. In vitro methods were used to replace and reduce the use of multiple animals to identify important pathways to later pursue in vivo. Experiments in the cell line were consistent and reproducible due to the high yield of cells obtained. To further ensure rigorous results, experiments were then performed in primary microglia. Once mechanistic results were observed, the project was more focused and only then were experiments translated into the animal model to better understand the more complex molecular and cellular neuroimmune microenvironment that is promoted in response to moderate-severe TBI. In my experimental approach, the use of in vitro systems and in my initial setup experiments in the TBI model consequently refined experiments as less mice were initially used which therefore minimised unnecessary suffering. This point adheres to the 4th newly added R, Responsibility, by Charles River [161]. Indeed, animal studies provide huge advantages in studying cell specific functional outputs in the more complicated neuro-environment closely related to the complexity of the human brain. It is impossible to reproduce the complex brain injury in 2/3D cell systems or slice preparation and therefore it is important to utilise animal models such as CCI which mimics many important features of secondary injury in human TBI including widespread cortical and hippocampal degeneration [27].

Despite ongoing preclinical and clinical research into therapeutic advances, TBI, affecting 50-60 million people worldwide remains a public health concern with a huge
burden on the health and economic systems [2]. Global networks of researchers are working together to improve research studies, clinical management, raise awareness for TBI research and ultimately highlight the urgent need for therapeutic interventions. In recent years there has been great advances in biomarkers and in particular protein blood-based biomarkers for injury detection and severity determination. One of the main benefits of blood-based biomarker S100B is the prevention of excess CT scanning in patients who don’t necessarily require it, hence avoiding unnecessary exposure to radiation [162]. Biomarkers can ultimately lead to outcome prediction with the aim of developing a pitch-side strategy to do a blood test immediately following a sports-related concussion and therefore could be used for the return to safe play. However, development of inflammatory biomarkers such as NOX2 and inflammasomes in the serum could better link acute injury with chronic TBI. Previous studies on the potential of systemic immune biomarkers in TBI demonstrated, using cytokine load score, that circulating cytokines correlated with worsened cognition. Cytokines IL-1β, IL-6, IL-8, IL-10, and TNFα were elevated over 3 months post-injury which was predictive of low Glasgow Outcome Scale, up to 12 months, and therefore identified individuals at risk for unfavourable outcomes following severe TBI [163]. In this way, perhaps systemic inflammation can predict abnormalities picked up by PET imaging [20]. Although not a direct treatment for TBI patients, biomarkers indicate common elevated proteins in the circulation of TBI patients which present potential therapeutic targets for this disease. Researchers found upregulated APOE gene expression in TBI patients which could contribute to the possible link between TBI and AD, seeing as APOE4 is the greatest genetic risk factor for AD [164].

Another plausible link between TBI-mediated oxidative stress and AD is the critical role of NOX2, further emphasising the importance of targeting this inflammatory signalling pathway in this present study. Zhang and colleagues showed pharmacological NOX2 inhibition, with apocynin, reduced TBI-elicited AD proteins β-amyloid in the cortex and hippocampus of mice [62]. Similarly, others have shown NOX2 activation plays a pivotal role in oxidative neuronal damage in neurodegenerative diseases such as AD [165]. The expression of NOX2 subunit p47phox mRNA was upregulated in the frontal cortex of post-mortem AD brains [65]. Moreover, mouse models of AD lacking the NOX2 membrane
bound subunit, gp91phox, did not show oxidative stress and exhibited improved behavioural performance in the Y-maze spatial memory test [66]. Additionally, microglial NOX2 activation is a key factor in the pathogenesis of experimental autoimmune encephalomyelitis (EAE), a mouse model of Multiple Sclerosis [166]. Moreover, NOX2−/− mice showed significantly reduced number of autoreactive T cells infiltrating into the brain, reduced microglial activation, and EAE-elicited oxidative stress. Therefore, NOX2 is an important molecular driver of neuroinflammation [64] as this complex enzyme system is implicated in many neuroinflammatory and neurodegenerating diseases.

NOX2 has been recently demonstrated to regulate microglial response to Aβ in ageing [167]. In ageing, which is known to be the greatest risk factor for AD [168], Geng and colleagues reported increased Aβ deposition in the midbrains of aged mice (20-22 m) compared to their young controls (3-4 m). However, NOX2−/− aged mice had reduced Aβ plaque accumulation compared to aged WT mice. Furthermore, post-mortem midbrain tissue revealed increased p47phox phosphorylation and ROS production in old age (61-85 years) compared to young (25-38 years) none of which had diagnosed NDD [167]. Thus, confirming an age-related NOX2-dependent shift in redox state towards an oxidising environment. This has also been observed preclinically whereby aged WT mice had augmented NOX2 expression and increased regulatory subunits p22phox, p47phox and p67phox, along with increased ROS production in the midbrain compared to young WT mice [165]. The absence of NOX2 revealed decreased ROS production compared to aged WT mice. Moreover, NOX2−/− preserved locomotor activity in aged mice, measured by total activities relative to young WT mice [165]. In a separate study, aged TBI mice (24-month-old) showed significantly higher NOX2 expression in microglia/macrophages in the cortex compared to young mice (3-month-old) and this was associated with attenuated antioxidants, superoxide dismutase (SOD1) and glutathione peroxidase 1 (GPX1), and increased lesion size [169]. This is also the case in aged SCI whereby microglial NOX2-dependent ROS is significantly increased in 14- but not 4-month-old mice [68]. Furthermore, NOX2 inhibition reduced ROS as well as lesion volume in middle-aged but not young SCI mice. Combined, these findings indicate NOX2 contributes to age-related oxidative damage and neuroinflammation.
Not solely does NOX2 activation contribute to increased neuroinflammation and TBI pathogenesis. This present study illustrated NOX2-mediated regulation of the NLRP3 inflammasome in vitro (chapter 3) and in vivo (chapter 5). NLRP3 inflammasome-stimulated microglia induced pro-inflammatory cytokines (IL-1β, IL-18) and had significant cell death and pyroptotic release. Previous work in the Loane lab showed NLRP3 inflammasome activation contributes to TBI disease progression. Delayed chronic microglia depletion, using PLX5622, alleviated NLRP3 inflammasome-associated neuroinflammation as illustrated by reduced caspase-1 activity and IL-1β release [45]. This was correlated with improved motor and cognitive function. Independently, a model of penetrating ballistic-like brain injury (PBBI) in rats showed increased NLRP3 inflammasome proteins, cytokines and pyroptosis up to 72h post injury [170]. The researchers found upregulated ASC in the perilesional and overtime the intact dorsal regions of the cortex and demonstrated increased cell-specific ASC expression in the activated microglia, which persisted until 12 weeks following PBBI, similar to what Henry and colleagues found. In chapter 3, the reverse effect of NLRP3 on NOX2 in microglia was also investigated (Figure 3.11). The NLRP3 inhibitor, MCC950, had no effect on LPS/nigericin induced NOX2 protein expression, but did reduce NOX2 activity which may imply a protective cyclic effect whereby inhibited NLRP3 inflammasome-induced pro-inflammatory release also attenuated NOX2 activity. Indeed, NLRP3 inhibitors (MCC950 and JC124) have shown promising effects in reducing TBI-elicited inflammatory responses and neurological disorders [100]. Precisely in the TBI field, MCC950 administration post-injury resulted in preserved BBB permeability, reduced lesion volume, and improved neurological deficits including increased time spent on the rotarod and reduced latency to the platform in MWM in CCI mice [93]. Moreover, administration of JC124 post-TBI alleviated cortical lesion damage 2 DPI and thus neuroinflammation [171]. Separately, treatment with a pan caspase inhibitor, z-VAD-fmk, 15 min post-injury, improved motor and spatial learning in a fluid-percussion model of TBI in rats [172]. Together, these findings emphasise the NLRP3 inflammasome and its downstream activation as important targets in preventing the chronic trajectory of neurodegeneration.
Combined, the described evidence from several neuroinflammatory and NDD thus far emphasise both NOX2 and NLRP3 inflammasome as key players in the pathogenesis of disease. This present study primarily focused on the inhibition of NOX2-mediated oxidative stress upstream of the NLRP3 inflammasome by GSK2795039 both in vitro and in vivo. Identifying a link between NOX2-mediated oxidative stress and NLRP3 inflammasome activation may indicate how GSK2795039 is acting. A molecule called Thioredoxin-Interacting Protein (TXNIP) may be a mechanistic link between oxidative stress and inflammation. TXNIP is a molecule that senses oxidative stress and is critical for driving NLRP3 inflammasome activation [173]. TXNIP binds to and inhibits an important antioxidant protein, thioredoxin (TRX), which therefore reduces its resistance to oxidative stress. In the presence of ROS, which is evidently upregulated following TBI, there is induced dissociated of TXNIP and TRX binding and the TXNIP is free to bind to NLRP3. This protein-to-protein interaction enhances caspase activity, IL-1β release and therefore neuroinflammation [174]. In AD there is an age-related reduction in TRX and upregulation of TXNIP along with heightened inflammation. Moreover, TXNIP has been identified 7 months before tau protein in the cortex of mouse models of AD, and its interaction with Aβ is associated with cognitive deficits [175]. TBI has also been shown to induce TXNIP expression [94]. NOX2^-/- mice had reduced TXNIP-NLRP3 interaction and expression in the proximity ligand assay compared to WT TBI mice [94]. Furthermore, activation of TXNIP stimulates downstream signaling of MAPK and NFκB, which further enhance oxidative stress [174]. As microglia express TXNIP/NLRP3 interactions [174], these findings suggest TXNIP may link NOX2-dependent oxidative stress and NLRP3 inflammasome activation-induced inflammation, which is a plausible link to the in vitro results shown in Chapter 3.

As shown here and in the literature discussed, NOX2 has detrimental effects in neuroinflammation and promoting TBI pathogenesis. In this present study, it was demonstrated that GSK2795039 reduced NOX2^+ IL-1β^+ neutrophils compared to vehicle treated CCI mice. Notably, NOX2 expression levels did not go below sham levels meaning GSK2795039 did not completely ablate NOX2 nor entirely dimmish ROS production. This is particularly serious in CGD patients with inherited immunodeficiency of NOX2 due to reduced oxidative burst whom as a result experience recurrent infection. Loss-of-
function mutation in the NOX2 subunit of macrophages [72] and B cells [176] increased NFκB signaling which drives NLRP3 inflammasome, contrary to our findings. NOX2 deficient B cells had an accumulation of endosomal compartments, due to limited degradation, which lead to increased TLR signalling, resulting in an overall increased risk to systemic lupus erythematosus and other autoimmune diseases [176]. This highlights the crucial balance of NOX2-mediated ROS. In one regard, complete abolishment will lead to increased susceptibility to infection and/or enhancement of autoantibodies and autoimmunity. On the other hand, chronic NOX2 activation upregulated neuroinflammatory responses and contributes to worsened outcomes in the TBI field but also in several NDD. Therefore, there is an urgent need to balance NOX2 activation in disease, as depicted in the schematic below. To achieve this, the identification of feasible drugs is vital and pharmacology approaches need to be tightly regulated.

![Balancing NOX2 activation is crucial in disease](image)

Figure 6.2: Balancing NOX2 is crucial in neurological disease.

Complete ablation of NOX2 results in insufficient oxidative burst, which is vital for host defence, leading to an increased susceptibility to infections. NOX2 deficiency has also been associated with increased autoimmunity. In contrast, sustained chronic NOX2 activation promotes oxidative stress, neuronal damage, and hippocampal degeneration. Overproduction of NOX2 plays a pivotal role in the secondary injury after TBI and contributes to cognitive dysfunction and neurodegenerative diseases.
In this present study, GSK2795039 proved to be a non-toxic, brain penetrable, small molecule NOX2-specific inhibitor. Chapter 3 highlights the ability of GSK2795039 to attenuate NOX2 protein expression and activity along with reducing cleaved caspase-1 and IL-1β, major outputs of the NLRP3 inflammasome. However, the effect of in vivo GSK2795039 treatment post-TBI was not optimal, particularly in the chronic phase. With a short half-life of 0.5h in mice, the pharmacokinetic analysis of GSK2795039 (Chapter 5; Figure 5.7) showed high clearance which may be limiting as a neurotherapeutic drug to treat chronic TBI. This high clearance, in which the concentration of GSK2795039 was almost 5-fold greater in the liver compared to the brain, could be due to GSK2795039 being metabolised by liver microsomal fractions and preventing the dehydrogenation of methylindoline moiety to reduce rodent metabolism [79]. Therefore, modest neuroprotective results observed in the acute (3 DPI), but not chronic (28 DPI) study suggest the effect of GSK2795039 had diminished due to the high clearance rate and short half-life in mice. Improving the GSK2795039 treatment scheme through increased dosing may have been more successful, albeit not feasible in this present study due to high costs for a largely powered animal study and limited time to repeat the studies during my PhD work. The short half-life of this drug also explains why previous GSK2795039 intervention studies administered the drug daily thus highlighting its weak efficacy [81, 82]. Moreover, due to poor potency, many groups administered GSK2795039 as a pre-treatment in acute, < 3 DPI biochemical studies, as the drug was still in the system [80]. Recently, a small molecule optimised from GSK2795039, called NCATS-SM7270, showed improved inhibitory specificity to NOX2 and neuroprotection after mild TBI in mice using a meningeal crush injury model [177]. However, researchers administered this novel inhibitor transcranially directly to the site of injury immediately following mild TBI, and sacrificed mice 6h post injury for analysis. Therefore, this study does not provide information on the half-life of this drug remaining in the system. More chronic phase TBI studies are needed to understand its efficacy in mild TBI and other more severe injury conditions. It also emphasises the importance of the route of administration. In this current study, the i.p injection of GSK2795039 required entry into the CNS from the periphery, which did induce a complex neuroimmune response of the peripherally derived innate and adaptive immune cells. Accordingly, the pharmacokinetics, half-life, efficacy, safety, and route of administration are the utmost
important factors in the development of long-term therapy for many NOX2-mediated neuroinflammatory and neurodegenerative diseases including TBI.

Nevertheless, GSK2795039 did show promising results in the adaptive immune system acutely after TBI as outlined in chapter 5. The reduction in absolute numbers as well as IL-1R expression in CD4+ and CD8+ T cells was a surprising finding, particularly at only 3 DPI. Previous studies have also shown that depleting CD4+ and CD8+ T cells reduced infarct size and neurological score 1d post stroke [154]. More specifically, Granzyme B-producing CD8+ cytotoxic T cells have been shown to drive long-term neurological impairment after TBI [137]. CD8+ T cell-deficient mice (β2m−/−) induced an anti-inflammatory, IL-13, response. In this present study, there was an indirect link to GSK2795039 altering microglial-T cell crosstalk because of reduced IL-1β-producing myeloid cells and a simultaneous reduction in IL-1R-expressing T cells. Indeed, there is evidence surrounding the phenomenon of crosstalk between the CNS and immune systems whereby innate immune cells of the brain recruit T cells. Recently, it has been demonstrated that microglia drive transient insult-induced brain injury by chemotactic recruitment of CD8+ T lymphocytes in radiation-induced brain injury (RIBI) patients [107]. Using chemotactic mechanisms, pro-inflammatory microglial clusters expressing CCL2 and CCL8 mediated CCR2 and CCR5 expressing CD8+ T cells recruitment. CD8α neutralizing antibody ablated CD8+ T cells and showed reduced brain lesion volume in RIBI mice, indicating CD8+ T cells play a pathological role in brain injury. Furthermore, an in vitro trans-well system allowing infiltration of T cells towards BV-2 microglia was blocked with either CCR or CCL neutralising antibody, thus highlighting the cell-cell interaction. Not only does attenuating CD8+ T cell activation alleviate neuroinflammation, but augmenting Treg cells has demonstrated therapeutic potential. IL-2 has been shown to drive Treg cells which regulates and protects the inflammatory brain following TBI. Gene-delivery of IL-2 to the brain resulted in a reduced lesion size and reduced latency to platform in the MWM in CCI mice [108]. These findings imply IL-2 mediated regulation of the adaptive immune system following TBI.

One of the ways GSK2795039 may be having an effect on T cell population in vivo is that NOX2 inhibition interferes with antigen presentation and hence alters T cell recruitment, which has downstream effects on T cell/microglial interactions. It has been shown in
EAE, that NOX2 expressing dendritic cells regulate and support myelin oligodendrocyte protein (MOG) antigen processing and presentation to T cells [178]. Gene ablation of Cybb, the gene encoding NOX2, specifically in dendritic cells (using cybb$^{fl/fl}$-Itgax-Cre mice) was sufficient in restricting T cell recruitment into the CNS and ameliorated disease development as demonstrated by reduced immune invasion, demyelination, and axonal damage. Moreover, post-mortem MS patients exhibit increased NOX components such as Cyba, Cybb in resident, predominantly microglia, and infiltrating cells of the CNS, particularly at the site of tissue damage and at perivascular areas in close proximity to T cells [179]. Combined, these studies shed a light on the pathogenic role of the adaptive immune system in the CNS.

It had previously been suggested that adaptive immunity was not crucial for the sustained neuroinflammation experienced post-TBI. Studies in Rag 1$^{-/-}$ mice devoid of mature B and T cells showed no protection in neurological score or in BBB impairments following TBI [180]. However, more recent studies in humans and animals using more advanced neuroimmunological models and tools challenged this view. Emerging evidence of the role of adaptive immunity and neuroimmune activation post TBI quickly followed. Needham and colleagues found, using protein micro array technology, increased autoantibody, and in particular IgM responses, in the serum of moderate-severe TBI patients. Subacute IgM autoantibody responses persisted for several years and was associated with worsened outcomes [181]. This begs the question, could antigen driven autoantibodies, such as IgM and IgG act as prognostic biomarkers in chronic TBI? Sustained autoantibody production contributes to the adaptive immune response which evidently plays a part in ongoing CNS injury and neurodegeneration. Targeting specific genes responsible for initiating the adaptive immune response, such as MHC II expressed on innate cells and responsible for significant T cell influx into the CNS, would help tolerate uncontrolled immunological activation [182]. To conclude, despite its importance, neuroimmunology remains under-investigated in the TBI field. Addressing the many research gaps of the innate and adaptive immune responses is essential for future TBI therapies. The findings from this thesis imply the NOX2-NLRP3 inflammatory axis along with microglial-T cell crosstalk as effective targets in the neuroinflammatory response following TBI.
Chapter 7: Bibliography


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Chapter 8: Appendix
8.1 Statistical breakdown of parametric and non-parametric *in vitro* data.

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## 8.2 The ARRIVE guidelines 2.0

### The ARRIVE Essential 10

These items are the basic minimum to include in a manuscript. Without this information, readers and reviewers cannot assess the reliability of the findings.

<table>
<thead>
<tr>
<th>Item</th>
<th>Description</th>
</tr>
</thead>
</table>
| Study design| For each experiment, provide brief details of study design including:  
|             | a. The groups being compared, including control groups. If no control group has been used, the rationale should be stated.  
|             | b. The experimental unit (e.g. a single animal, litter, or cage of animals).  
| Sample size | 2. Specify the exact number of experimental units allocated to each group, and the total number in each experiment. Also indicate the total number of animals used.  
|             | b. Explain how the sample size was decided. Provide details of any a priori sample size calculation, if done.  
| Inclusion and exclusion criteria | 3. a. Describe any criteria used for including and excluding animals (or experimental units) during the experiment, and data points during the analysis. Specify if these criteria were established a priori. If no criteria were set, state this explicitly.  
|             | b. For each experimental group, report any animals, experimental units or data points not included in the analysis and explain why. If there were no exclusions, state so.  
|             | c. For each analysis, report the exact value of n in each experimental group.  
| Randomisation | 4. a. State whether randomisation was used to allocate experimental units to control and treatment groups. If done, provide the method used to generate the randomisation sequence.  
|             | b. Describe the strategy used to minimise potential confounders such as the order of treatments and measurements, or animal/cage location. If confounders were not controlled, state this explicitly.  
| Blinding | 5. Describe who was aware of the group allocation at the different stages of the experiment (during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis).  
| Outcome measures | 6. a. Clearly define all outcome measures assessed (e.g. cell death, molecular markers, or behavioural changes).  
|             | b. For hypothesis-testing studies, specify the primary outcome measure, i.e. the outcome measure that was used to determine the sample size.  
| Statistical methods | 7. a. Provide details of the statistical methods used for each analysis, including software used.  
|             | b. Describe any methods used to assess whether the data met the assumptions of the statistical approach, and what was done if the assumptions were not met.  
| Experimental animals | 8. a. Provide species-appropriate details of the animals used, including species, strain and substrain, sex, age or developmental stage, and, if relevant, weight.  
|             | b. Provide further relevant information on the provenance of animals, health/immune status, genetic modification status, genotype, and any previous procedures.  
| Experimental procedures | 9. For each experimental group, including controls, describe the procedures in enough detail to allow others to replicate them, including:  
|             | a. What was done, how it was done and what was used.  
|             | b. When and how often.  
|             | c. Where (including detail of any acclimatisation periods).  
|             | d. Why (provide rationale for procedures).  
| Results | 10. For each experiment conducted, including independent replications, report:  
|             | a. Summary/descriptive statistics for each experimental group, with a measure of variability where applicable (e.g. mean and SD, or median and range).  
|             | b. If applicable, the effect size with a confidence interval.  


<table>
<thead>
<tr>
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<tr>
<td>These items complement the Essential 10 and add important context to the study. Reporting the items in both sets represents best practice.</td>
</tr>
<tr>
<td><strong>Abstract</strong></td>
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<tr>
<td><strong>Background</strong></td>
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<td><strong>Interpretation/scientific implications</strong></td>
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<td><strong>Protocol registration</strong></td>
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<td><strong>Data access</strong></td>
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<td><strong>Declaration of interests</strong></td>
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*The ARRIVE guidelines adapted from Du Sert et al., Plos Biol, 2020 [113].*