Genetic, molecular, and neurobiological determinants of post-traumatic stress disorder-related behaviour in mice

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Statement of originality

This thesis is submitted to the University of Sydney in fulfilment of the requirements for the degree of Doctor of Philosophy.

I certify that, to the best of my knowledge, the content of this thesis is original and assert that any assistance received in preparing this thesis has been acknowledged. I have not submitted this material, either in full or part, for a degree at this or any other institution.

Kristie Leigh Smith

Author attribution statement

I, Kristie Leigh Smith, completed the majority of work in this thesis, and any assistance that I received is outlined in detail below. Investigations were carried out at the Brain and Mind Centre at the University of Sydney. All experimental work was completed under the direction of my supervisor, Associate Professor Jonathon Arnold who, as corresponding author of the three manuscripts that compose the body of this thesis, has granted permission for the use of materials herein.

For the publication in **Chapter 2**, under the supervision of A/Prof Arnold I designed and conducted all behavioural and immunohistological experiments and analysed all experimental data. I acknowledge Stephanie Todd, David Clarke, and Mustafa Kassem who provided training and continued guidance for certain behavioural and histological experiments. I further acknowledge Dr Michael Kuligowski who provided critical microscopy support, and Dr Miguel Bedoya-Perez in providing statistical analysis assistance. I solely composed the manuscript for publication with editing assistance from A/Prof Arnold.

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Table of contents

Acknowledgements ..... **vi**

Publications, presentations, and awards ..... **vii**

Additional publications ..... **viii**

List of figures and tables ..... **ix**

List of abbreviations ..... **xii**

Abstract ..... **xv**

Chapter 1 - Introduction **2**

1.1. Stress and stress related disorders **2**

1.1.1. What is stress? 2

1.1.2. Post-traumatic stress disorder 4

1.1.3. The modern picture of PTSD 6

1.1.4. Current treatment of PTSD 8

1.2. Advancing the understanding of neurobiological and genetic bases of PTSD and stress disorders **12**

1.2.1. PSTD neurobiology: fear circuits and loss of grey matter 12

1.2.2. Animal models of PTSD 15

1.2.3. Face, construct and predictive validity of an animal model of PTSD 17

1.2.4. Positive and negative neuroadaptive consequences of stress 20

1.3. PTSD and neuroinflammation: Is there a link between loss of synaptic connectivity and microglia? **23**

1.3.1. A brief history of microglia 24

1.3.2. Structure and function of microglia 26

1.3.3. The complement system and microglia 28

1.3.4. Stress and microglia..... 30

1.4. Could genetic factors predispose or confer resilience to PTSD? 33

1.4.1. Development of novel serenic agents for PTSD and beyond 36

1.5. Aims..... 38

Chapter 2 - Microglial cell hyper-ramification and neuronal dendritic spine loss in the hippocampus and medial prefrontal cortex in a mouse model of PTSD**. 41**

Chapter 3 - Genetic deletion of P-glycoprotein alters stress responsivity and increases depression-like behavior, social withdrawal and microglial activation in the hippocampus of female mice **.....53**

Chapter 4 - $P2X_7$ rececptor knockout mice display less aggressive biting behaviour correlating with increased brain activation in the piriform cortex**.....65**

Chapter 5 – General discussion 73

5.1. Conclusion 92

6. References 94

7. Supplementary material..... 127

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Publications, presentations, and awards

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*First co-authors.

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International engagements

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Awards

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Additional publications

During my PhD candidature I contributed to the following publications which are complimentary to, however do not form the body of this thesis:

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Clarke, D. J., Chohan, T. W., Kassem, M. S., **Smith, K. L.,** Chesworth, R., Karl, T., . . . Arnold, J. C. (2018). Neuregulin 1 Deficiency Modulates Adolescent Stress-Induced Dendritic Spine Loss in a Brain Region-Specific Manner and Increases Complement 4 Expression in the Hippocampus. *Schizophr Bull*. doi:10.1093/schbul/sby029

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List of figures and tables

Chapter 1

Table 1: Diagnostic and Statistical Manual 5 criteria for Post-traumatic stress disorder.

Table 2: Perceived efficacy from evidence-based research of pharmacotherapy interventions in the treatment of PTSD

Figure 1: Simplified diagram of the fear circuitry in the human brain and corresponding rodent brain regions

Table 3: Criteria for the establishment of an animal model of PTSD….

Table 4: A sample of neurobiological studies employing the Wotjak mouse model of PTSD

Figure 2: Progression of the histological characterisation of microglial cells**.**

Figure 3: Microglia control a variety of physiological processes during neurodevelopment and throughout the life span

Table 5: Psychosocial stressors and their effect on Iba-1-demarcated microglial cells

Figure 4. Stages of microglial remodelling following pathological (classical) and non-pathological stimuli

Figure 5. P-gp transporters located on the luminal and abluminal membranes of endothelial cells of the BBB, pericytes and astrocytic feet limit the brain uptake of glucocorticoids such as cortisol and corticosterone

Chapter 2

Figure 1: Overview of the experimental protocol and behavioural batter over 32 days.

Figure 2: Representations from Paxinos & Franklin mouse atlas of regions of interest analysed.

Figure 3: Shock exposure increases contextual conditioned freezing, decreases locomotor activity, and induces exaggerated startle responses representative of PTSD symptomology.

Figure 4: Shock exposure elevated microglial cell density in the CA1 and CA3 regions of the dorsal hippocampus and induced robust microglial cell process elongation, complexity, and volumetric expansion at 32 days.

Figure 5: Shock exposure elevated microglial cell density in the IL cortex and induced process elongation, complexity and volumetric expansion across the medial prefrontal cortex (both IL and PrL regions).
Figure 6: Shock exposure does not promote marked changes in microglial cell density and morphology in the BLA at 32 days post-shock exposure
Figure 7: Shock reduced the density of dendritic spines on apical dendrites of pyramidal neurons of the dorsal hippocampus and medial prefrontal cortex, but not in the basolateral amygdala.
Figure 8: Shock upregulates C1q expression in the striatum pyramidale of the CA1 region of the dorsal hippocampus.

Chapter 3
Figure 1: Germline P-gp and Bcrp KO increased PPI and reduced startle reactivity in female mice which was not influenced by injection stress. P-gp but not Bcrp KO in female mice induced frantic anxiety-related behavior in response to injection stress in the light-dark test.
Figure 2: P-gp KO in female mice tended to impair fear conditioning in the short-term but not long-term. P-gp KO increased darting behavior and foot-shock decreased social interaction.
Figure 3: Female P-gp KO mice displayed decreased startle responses to loud acoustic stimuli and increased depressive-like behavior in the forced swim test.
Table 1: Mean microglial cell count 30 days after foot-shock stress in the frontal cortex, hippocampus, and amygdala of female WT and P-go KO mice.
Figure 4: Microglial cell processes of female P-go KO mice had significantly less surface area and volume, were shorter in length, and were less ramified than microglia of WT mice in the CA3 region of the hippocampus.
Figure 5: Female P-gp KO mice had increased GR expression compared to WT in the pyramidal layer of the CA3 region of the hippocampus.

Chapter 4
Figure 1: Germline deletion of *P2rx7* did not influence social behaviour or locomotor activity but reduced repetitive behaviour in early adulthood.

Table 1: Germline deletion of *P2rx7* did not influence social behaviour or locomotor activity but reduced repetitive behaviour in early adulthood.
Figure 2: Germline *P2rx7* deletion in mice decreases aggressive biting behaviour in the resident-intruder test.
Figure 3: Resident *P2rx7* knockout mice display enhanced brain activation but identical microglial cell density in the piriform cortex compared to WT mice following an aggressive encounter in the resident-intruder test.

Chapter 5
Figure 1: The corticolimbic system is susceptible to neuronal dendritic spine loss and elevated hyper-ramified microglial cell densities 32 days following footshock exposure.
Table 1: Summary of the methods, behavioural outcomes and neurobiological alterations observed in **Chapter 2**, **Chapter 3**, and **Chapter 4**.
Figure 2: In contrast to **Chapter 2**, microglial cells in the limbic system of FVB strain WT and P-gp$^{-/-}$ mice are differentially affected by footshock exposure at 30 days in **Chapter 3**.

Appendix:
Supplementary material Chapter 3
Figure S1: P-gp KO or footshock stress did not influence anxiety-related behaviour in the light-dark test. P-gp KO mice displayed locomotor hyperactivity which was more clearly expressed on Day 22.
Table S1: Microglial cell body morphology features in WT and P-gp KO *(mdr1a/b*$^{-/-}$) mice in the CA3 region of the hippocampus.
Figure S2: Female P-gp KO mice had increased GR expression compared to WT mice in the pyramidal layer of the CA3 region of the hippocampus.

Supplementary material Chapter 4
Table S1: c-Fos^{+} cell densities between WT and *P2rx7* knockout experimental groups following the resident-intruder test.
Table S2: Density of Iba-1^{+} microglial cells between *P2rx7* knockout and WT animals.

List of abbreviations

ABC	ATP-binding cassette
ACTH	Adrenocorticotropic hormone
AAV	Adeno-associated virus
AMG	Amygdala
ANOVA	Analysis of variance
APA	American Psychological Association
ASR	Acoustic startle response
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BCRP	Breast cancer resistance protein
BDNF	Brain derived neurotrophic factor
BLA	Basolateral amygdala
BSA	Bovine serum albumin
C1q	Complement component 1q
C3	Complement component 3
C4	Complement component 4
CA1	Cornu Ammonis 1
CA3	Cornu Ammonis 3
CBT	Cognitive behavioural therapy
CNS	Central nervous system
CRH	Corticotropin-releasing hormone
CSF1	Colony stimulating factor 1
dlPAG	Dorsolateral periaqueductal grey
dmPAG	Dorsomedial periaqueductal grey
DSM	Diagnostic and statistical manual of mental disorders
FC	Fear conditioning
FDA	Food and Drug Administration
FPS	Fear potentiated startle
FKBP5	FK506 binding protein
FVB	Friend virus B-type mice
GR	Glucocorticoid receptor
HC	Homecage
HPC	Hippocampus
HPA	Hypothalamic-pituitary axis
Iba-1	Ionized calcium-binding adapter molecule 1
IL	Infralimbic cortex
IL-1β	Interleukin 1β
IL-6	Interleukin 6
IL-12	Interleukin 12
I.P	Intraperitoneal
I.V	Intravenous
KO	Knockout
LDT	Light dark test

LSM	Laser scanning microscope
LMM	Linear mixed model
lPAG	Lateral periaqueductal grey
LSV	Ventrolateral septum
MDR	Multidrug resistance
MDRP	Multidrug resistance related protein
mRNA	Messenger ribonucleic acid
MFI	Mean fluorescence intensity
NO	Nitric oxide
OCD	Obsessive-compulsive disorder
P-gp	P-glycoprotein
PPI	Prepulse inhibition
PrL	Prelimbic cortex
PND	Post-natal day
PTSD	Post-traumatic stress disorder
PV	Paraventricular thalamic nucleus
PVA	Anterior paraventricular thalamic nucleus
PFC	Prefrontal cortex
P_2X_7	Purinergic receptor X7
S.E.M	Standard error of the mean
SSRI	Selective serotonin reuptake inhibitor
SNK	Student-Newman-Keuls
SNP	Single nucleotide polymorphism
sTNF-RII	Soluble receptor II for tumor necrosis factor
TNF-α	Tumor necrosis factor alpha
WT	Wild-type

‘Histology is an odd-tasting dish, repulsive as a medicament to students who must be examined in it, and little liked by physicians who consider their schooling finished. Taken in large quantities under compulsion it is not absorbed, but if tasted in little sips it finally becomes a delight to the palate and even a cause of addiction.’

- Pío del Río-Hortega

Abstract

Post-traumatic stress disorder (PTSD) is a debilitating mental health condition that occurs as a consequence of the experience of a traumatic, potentially life-threatening event or series of events. PTSD is commonly observed in combat veterans and emergency service workers, however it also afflicts victims of natural disasters, terrorism, sexual assault, and interpersonal violence. PTSD is incorporated into the Traumatic and Stressor Related Disorders category of the Diagnostic and Statistical Manual of Mental Disorders V (DSM-V). PTSD is characterised by four clusters of hallmark symptoms incorporating intrusive thoughts of the traumatic event/s, hyperarousal, nightmares, anxiety, depression, and attentional and cognitive disturbances.

A key feature of PTSD is the persistent nature of symptoms which may progressively become worse over time. Exposure therapy is considered the gold standard of clinical treatment for PTSD however 40% of patients do not respond to therapy. Pharmacological therapies are also utilised, however the only Federal Drug Administration (FDA) approved drugs are sertraline and paroxetine, both selective serotonin reuptake inhibitors (SSRIs) which were not designed to treat PTSD and demonstrate only modest efficacy. There is a crisis in PTSD drug development that necessitates the discovery of novel pharmacological targets for treatment of the disorder.

To achieve this better animal models of the disorder are required which mirror genetic variability in the disorder and its underlying neurobiological determinants. Chapter 1 reviews our current understanding of PTSD and animal models of PTSD that

have been developed. It provides a framework for the current thesis, which aims to extend research on a mouse model of PTSD developed by Wotjak and colleagues. Understanding of the neurobiology of the model is further developed with Chapter 2 providing a deeper examination of alterations in microglia, the brain's immune cells, and pyramidal neuron dendritic spines, the small protrusions that harbour excitatory synapses. In Chapter 3 the thesis explores a possible mechanism for genetic variation in PTSD by exploring whether knockout (KO) of P-glycoprotein (P-gp) augments neurobehavioural features in the mouse model of PTSD. Finally, aggression is an undermanaged feature of PTSD and other mental disorders, so in Chapter 4 we explored whether the purinergic receptor $P2X_7$, which is expressed on microglia, may offer a new drug target for the development of novel serenic compounds.

Few animal models fail to comprehensively model the numerous core associative and non-associative behaviours relevant to PTSD, such as long-lasting fear reactions, hyperarousal, and subtle attentional and cognitive dysfunction. A more adequate PTSD model would also have improved construct validity by reproducing core pathophysiological features such as central nervous system grey matter loss and neuroinflammation. In Chapter 2 we aimed to investigate in a mouse model of PTSD whether contextual fear conditioning exposure induces loss of neuronal dendritic spines in various corticolimbic brain regions, as their regression may help explain grey matter reductions in PTSD patients. Further, we aimed to observe whether these changes were accompanied by alterations in microglial cell number and morphology, and increased expression of complement factors implicated in the mediation of microglial cell-mediated engulfment of dendritic spines.

We report in Chapter 2 that shock exposure in male C57Bl/6 mice promoted a lasting contextual fear response, decreased locomotor activity, exaggerated hyperarousal responses, and a short-term facilitation of sensorimotor gating function. The shock-triggered loss of dendritic spines on pyramidal neurons was accompanied by increased microglial cell number and complexity in the medial prefrontal cortex and hippocampus. Shock also increased expression of C1q in the CA1 region of the hippocampus. These results further elaborate on the face and construct validity of a mouse model of PTSD and provides a good foundation to explore potential molecular interactions between microglia and dendritic spines.

Not all individuals exposed to a traumatic event will develop PTSD, therefore the search for susceptibility genes conferring vulnerability for PTSD is an area of intense research focus. ATP-binding cassette transporters such as P-gp are membrane bound efflux proteins located at several important biological barriers in the body. These transporters regulate brain uptake of xenobiotics and endogenous mediators including glucocorticoid hormones which are critical to the stress response. P-gp localised at the blood brain barrier regulates the brain uptake of corticosteroid stress hormones which are dysregulated in PTSD pathophysiology. P-gp is also localised on microglia and may modulate microglial cell responses to stress. In Chapter 3 we hypothesised that germline deletion of P-gp may moderate the behavioural and microglial response of mice in a model of PTSD.

In Chapter 3 we show that female P-gp KO mice on an FVB background strain displayed an unusual, frantic anxiety response to intraperitoneal injection stress in the light-dark test. P-gp KO mice also tended to display reduced conditioned fear responses compared to wild-type mice. Footshock stress reduced social interaction and decreased

microglia cell density in the amygdala which was not varied by P-gp genotype. Independently of stressor exposure, P-gp deficiency increased depression-like behaviour, idiosyncratic darting, age-related social withdrawal and hyperactivity, facilitated sensorimotor gating and altered startle reactivity. In addition, P-gp deletion increased microglial cell density in the CA3 region of the dorsal hippocampus, with microglial cells exhibiting a hypo-ramified morphology. In conclusion, Chapter 3 shows that germline P-gp deletion affected several behaviours of relevance to psychiatric conditions including PTSD, schizophrenia and depression. We also show for the first time that P-gp KO and footshock stress independently affects microglial cell activity in the hippocampus and amygdala respectively.

$P2X_7$ receptors are implicated in the pathophysiology of psychiatric conditions such as depression and bipolar disorder, however their role in PTSD pathophysiology has yet to be investigated. $P2X_7$ receptors are predominantly localised on microglial cells and facilitate the synthesis and release of pro-inflammatory cytokines such as IL-1β. Converging evidence from clinical and animal studies highlights heightened IL-1β signalling in aggressive behaviour. In Chapter 4 we therefore hypothesised that genetic knockout of $P2X_7$ receptors may reduce aggressive behaviour in mice. P2rx7 KO mice displayed reduced obsessive-compulsive disorder (OCD)-related marble burying behaviour which was most pronounced in late adolescence/early adulthood. P2rx7 KO mice also exhibited reduced aggressive attack behaviours in adulthood in the resident-intruder test. Reduced aggression in *P2xr7* KO mice did not coincide with changes to microglial cell densities, however c-Fos expression was elevated in the piriform cortex of P2rx7 KO mice compared to wildtype mice. Our results suggest

that the $P2X_7$ receptor might serve as a novel target for serenic and anti-OCD therapeutics.

Finally, **Chapter 5** summarises the main findings of this thesis in regard to neurobiological and genetic determinants of PTSD and examines future directions with implications novel drug targets.

Chapter 1

Introduction

Chapter 1 - Introduction

1.1. Stress and stress related disorders

1.1.1. What is stress?

Stress, or the stress response is an evolutionarily conserved mechanism activated when an individual, organism, or biological system faces homeostatic disruption or survival challenge. Stress is not inherently negative and, at times, can be positive as responding to a stressful challenge allows an organism to adapt to their environment and develop the capacity to handle a stressor on subsequent re-exposures [1]. As such, stress can be beneficial. Despite our understanding of this and the increasing reference to stress in modern society, the construct of stress is hard to define due to high subjectivity [2]. It is recognised, however, that the brain is the key organ for the stress response. The brain delineates between what is a potentially threatening stimulus and what is not, and subsequently triggers the appropriate physiological and behavioural responses within an organism.

The "fight or flight response", mediated by the autonomic nervous system, describes the physiological characteristics of the stress response including increased heart rate and blood pressure, dilated pupils and decreased digestive activity. This is an evolutionarily conserved "alarm" mechanism imperative for survival. While originally evolving to assist the survival of an organism when faced with a stressor such as a predator, the same response is activated when faced with modern challenges such as relational, health, or financial concerns, unemployment, bereavement, unpredictability, and other psychosocial demands [1].

Stress theory, proposed by Hans Selye in 1936, states stress is a syndrome, the response to a nonspecific nocuous agent; “the non-specific response of the body to any demand for change” [3]. Selye also stated that “stress is a perception”. Holyroyd & Lazarus (1982) opined that “psychological stress requires a judgement that environmental and/or internal demands exceed the individual’s resources for managing them” [4]. More recently stress was defined as, “conditions where an environmental demand exceeds the natural regulatory capacity of an organism” [5].

Fundamentally, stressors can be organised into categories of which two primary types consist of physical: where infliction of injury threatens or overwhelms homeostatic balance, i.e. haemorrhage or infection [6], and psychological: the threat or potential for threat to the subject i.e. social conflict, predator cues, or adverse environmental stimuli [6]. Stress can be an acute (i.e. a work deadline), or chronic challenge (i.e. long-lasting poverty). Stressors can be experienced in a single exposure or be repetitive/cyclical, and can be mild, moderate, or severe. Additionally, stress can also be anticipated or unexpected and uncontrolled. These factors demonstrate the complex and heterogeneous nature of stressors and highlight that differential exposure may elicit alternate effects on an animal or organism.

In response to stress, the body activates a number of biological systems including the hypothalamic-pituitary-adrenal (HPA) axis and the autonomic nervous system, as well as metabolic and immune functions to promote allostasis, the attempt to maintain homeostasis [7]. Allostasis allows for adaptation to stress, and the allostatic load is the cumulative impact of multiple stressors on the body. However, when the magnitude or duration of a stressor is prolonged or too great an organism can experience allostatic overload, which can result in serious pathophysiology [8].

1.1.2. Post-traumatic stress disorder

Post-traumatic stress disorder (PTSD) is a debilitating mental health condition occurring in a subset of individuals following exposure to a traumatic, potentially life-threatening event or series of events [9]. While social awareness of PTSD has predominantly developed within the early 20th century, literary reference to the behavioural characteristics of the disorder extend back many thousands of years [10]. In 1980, PTSD was officially introduced into the third edition of the American Psychological Association's (APA) Diagnostic and Statistical Manual (DSM) and is today conceptually quite well engrained in western society. The industrialised military of World War I and II are outstanding examples of the devastating effects of trauma and total war on human mentality. War-mediated trauma carried the monikers war neurosis and shell shock, and would be established as combat fatigue and gross stress reactions in the 1952 DSM-I. The subsequent Vietnam War was the tipping point of both medical and cultural awareness of PTSD, then "Post-Vietnam syndrome". Post-Vietnam syndrome afflicted an estimated 700,000 soldiers (a quarter of total persons deployed), and was the catalyst for the eventual classification of PTSD within the DSM-III [10].

Table 1. Diagnostic and Statistical Manual 5 criteria for Post-traumatic stress disorder.

Diagnostic criteria (DSM-V) for Post-Traumatic Stress Disorder (PTSD)		
Criterion A	**Trauma** (one required): The person was exposed to: death, threatened death, actual or threatened serious injury, or actual or threatened sexual violence in the following way(s):	
	1. Direct exposure 2. Witnessing the trauma 3. Learning that a relative or close friend was exposed to a trauma	4. Indirect exposure to aversive details of the trauma, usually in the course of professional duties (e.g., first responders, medics)
Criterion B	**Re-experiencing** (one required): The traumatic event is persistently re-experienced, in the following way(s):	
	1. Unwanted upsetting memories 2. Nightmares 3. Flashbacks	4. Emotional distress after exposure to traumatic reminders 5. Physical reactivity after exposure to traumatic reminders
Criterion C	**Avoidance** (one required): Avoidance of trauma-related stimuli after the trauma, in the following way(s):	
	1. Trauma-related thoughts or feelings	2. Trauma-related reminders
Criterion D	**Negative alterations in cognition & mood** (two required): Negative thoughts or feelings that began or worsened after the trauma, in the following way(s):	
	1. Inability to recall key features of the trauma 2. Overly negative thoughts and assumptions about oneself or the world 3. Exaggerated blame of self or others for causing the trauma	4. Negative affect 5. Decreased interest in activities 6. Feeling isolated 7. Difficulty experiencing positive affect
Criterion E	**Alterations in arousal and reactivity** (two required): Trauma-related arousal and reactivity that began or worsened after the trauma, in the following way(s):	
	1. Irritability or aggression 2. Risky or destructive behaviour 3. Hypervigilance	4. Heightened startle reaction 5. Difficulty concentrating 6. Difficulty sleeping
Criterion F	**Persistence of symptoms** (required):	
	○ **Symptoms last for more than 1 month**	
Criterion G	**Functional impact** (required): ○ **Symptoms create distress or functional impairment (e.g., social, occupational)**	
Criterion H	**Exclusion** (required): ○ **Symptoms are not due to medication, substance use, or other illness.**	

1.1.3. The modern picture of PTSD

The DSM-III (1980) initially classified PTSD within the anxiety disorders category alongside generalised anxiety, social phobias, and panic disorders. However, the evolution of PTSD classification through evidence-based research has led to constant refinement of the diagnostic criteria delineating PTSD from acute stress and adjustment disorders, incorporating a broader spectrum of symptomology. Currently the DSM-V (2013) [9] categorises PTSD within Trauma and Stressor Related Disorders with diagnosis requiring presentation of symptoms from four main clusters incorporating both physiological and psychological disturbances (refer to DSM-V criteria in Table 1). In addition to exposure to an initiator trauma, clusters of hallmark symptoms include intrusive thoughts of the traumatic event/s, exaggerated startle reactivity/hyperarousal, nightmares, anxiety, depression, aggression, and attentional and cognitive disturbances. To be classified as PTSD the symptoms manifest must persist for longer than a month, create functional impairment, and not be due to substance abuse or other mental health conditions (see Table 1).

PTSD was originally associated with returning military personnel and emergency service workers, however today the condition is understood to also afflict victims of terrorism, natural disasters, sexual assault, child abuse, and interpersonal violence [11-14]. PTSD has an estimated lifetime prevalence across the western world of 1.9 to 6.8%, and accounts for 40% of the economic burden of anxiety disorders [15]. Interestingly accumulating evidence highlights that factors including gender, race, personality, education, IQ, genetics and prior traumatic experience influence risk or resilience to developing PTSD [16-18]. One aspect of the present thesis is to explore genetic variation

in the development of PTSD-related behaviours using an animal model of PTSD (**Chapter 3**).

Beyond primary burdens on an individual, PTSD disturbs socio-economic functioning leading to negative secondary effects including disruption of occupational status, breakdown of social relationships, and development of drug dependency behaviours. Substance use and abuse is prevalent amongst PTSD sufferers, with chronic substance abuse often leading to the development of a co-morbid Substance Use Disorder diagnosis [19]. Escapism from a patient's reality and numbing of mental anguish are the primarily reported reason for risky drug and intoxicant use behaviours [20], which highlights the ineffective nature of currently utilised pharmaceutical methods for treating PTSD. Predominantly reported drugs/intoxicants used and abused by PTSD sufferers to self-medicate include alcohol and opioids, as well as sedatives, amphetamine, and cannabis [21]. Excessive use of these drugs has devastating effects on both health of an individual as well as secondary social effects impacting occupation and interpersonal relationships [21]. Heightened symptom severity is associated with increased self-medication and alcohol abuse [20, 22], with self-reports of lower quality of life [23] and increased incidence of suicide attempts and heightened suicidal ideation [24]. Substance use and abuse are particularly problematic as these self-medicating, secondary behaviours can feed back into and aggravate core PTSD symptomology such as negative alterations in cognition and mood (Criterion D) and altered arousal and reactivity (Criterion E).

Aggression (Criterion E), and anger issues rank as one of the highest concerns related to clinical treatment of PTSD [25, 26]. Evidence suggests trauma exposure and PTSD negatively alters processing of social information that can result in cognitive

deficits such as the hostile attributional bias – the tendency to interpret or ascribe behaviours of others as hostile when in reality are ambiguous or even benign [27], rendering an individual at a heightened risk of aggression [25]. Additionally, problematic substance abuse potentially serves as a disinhibiting factor between PTSD and aggression. That is, in the presence of an emotionally salient situation (i.e. a conflict reminiscent of a trauma), the presence of intoxicants can interact with PTSD resulting in heightened arousal and aggression [25]. As there is only modest efficacy for clinical therapy for the minimisation of anger and aggression symptoms of PTSD, there remains a need for targeted treatments of aggression and violence in PTSD patients. **Chapter 4** of the present thesis will explore a novel drug target for the development of serenic agents that might be utilised for treating PTSD patients.

1.1.4. Current treatment of PTSD

The treatment options for PTSD primarily incorporate psychological and/or pharmacological interventions. The forefront clinical therapy approaches include exposure-based therapies (which are considered gold standard treatment options), as well as cognitive behavioural therapy (CBT), and eye movement desensitisation and reprocessing [28]. Primary aims of exposure and CBT based therapies is the modification of negative or distorted belief systems surrounding a traumatic event and a patient's sense of self which, ultimately, influences behaviour [29]. Therapy-mediated reductions in negative thoughts associated with one's self, an experienced trauma, and the surrounding world are associated with symptom reduction [30]. This is approached

through identifying and restructuring negative thought patterns relating to the initiating traumatic event/s [31].

Exposure therapy is considered the gold standard of clinical therapeutic techniques for the treatment of PTSD. Exposure therapy incorporates repetitive, cyclical revisiting of traumatic memories, guided by a clinician, and is often followed up with *in vivo* exposure to traumatic stimuli that can incorporate the environment and traumatic cues avoided by the patient. In some cases, this can lead to a diminished fear response to a traumatic memory and associated stimuli through extinction processes [30]. However, 40% of patients do not respond to psychotherapy [32, 33], therefore other treatments options such as pharmacological therapies are often utilised.

Despite the prescription of pharmacotherapy regimes for the treatment of PTSD there are few drugs available that are considered somewhat beneficial to treat the disorder (see Table 2). At present, the only Federal Drug Administration approved drugs for the treatment of PTSD are the selective serotonin reuptake inhibitors (SSRIs) sertraline (Zoloft) and paroxetine (Paxil) [34, 35]. Perceived benefits of SSRIs include promoting changes in regulation of mood, reducing anxiety and sleep disturbances, however, sertraline and paroxetine are not specifically designed for PTSD treatment. These drugs arguably only mask symptoms in the cases where any significant results are observed rather than eliciting an actual remission of the disorder, and often fail to exhibit effects greater than placebo [36, 37]. Such mediocre outcomes have often led to the reliance on polypharmacological regimes with off-label prescription medications.

In contrast to the SSRIs fluoxetine and sertraline, serotonin-noradrenaline reuptake inhibitors (SNRIs) such as venlafaxine, are not approved for the treatment of PTSD, however have been prescribed as an off label treatment [37]. Administered at low

doses, the SNRIs mode of action is serotonin reuptake inhibition, akin to fluoxetine and sertraline. Administered at a higher dose, however, SNRIs also limits the reuptake of noradrenaline from the synaptic cleft. While the SNRI drug class demonstrates some efficacy for the treatment of PTSD, there is some evidence to suggest drugs such as venlafaxine may even promote heightened arousal levels [37-40]. Noradrenergic and specific serotonergic antidepressants (NaSSAs) such as mirtazapine also modulate serotonin and noradrenaline within the synaptic cleft in a more targeted manner, specifically antagonising 2-adrenergic and serotonergic receptor subtypes such as 5-HT_{2A} and 5-HT_{2C} [41]. NaSSAs drugs may also be somewhat beneficial to PTSD treatment when used as a monotherapy [42], or as an adjunct treatment with SSRIs on patient remission rates [43]. However, further research with greater sample sizes are required to determine the full efficacy of individual PTSD symptoms.

Beyond SSRIs, mood stabilisers such as quetiapine and risperidone are at times prescribed as off label treatments for PTSD, however are traditionally designed for treatment of schizophrenia and bipolar disorder. Aside from diminishing any co-occurring psychotic symptoms present in a PTSD patient, neither drug shows greater efficacy than that reported for SSRIs [37] (Table 2). Benzodiazepines are a highly controversial drug class prescribed to PTSD patients, however there is some evidence of benzodiazepines worsening PTSD symptom severity [44]. A 2015 meta-analysis highlighted seventeen studies that determined this class of drug was, overall, ineffective in the treatment of PTSD and even aggravated mood and sleep disturbances [44]. Moreover, benzodiazepine administration was found to elevate hyperarousal, aggression, and substance use, impair social functioning, and negatively affected psychotherapy outcomes [45-62] Overall, benzodiazepines have an adverse impact in

the prevention and treatment of PTSD [44]. The United States Department of Veterans Affairs and Department of Defence highlighted in the 2017 clinical practice guideline for the management of PTSD that benzodiazepines are harmful and should not be administered [63].

Table 2. Perceived efficacy from evidence-based research of pharmacotherapy interventions in the treatment of PTSD. Amended from Alexander (2012) [37].

FDA approved	Some benefit	Unknown benefit	No benefit
▪ SSRIs ▪	▪ MAO inhibitors (phenelzine) ▪ Mirtazapine ▪ Nefazodone ▪ Prazosin (for sleep/nightmares) ▪ TCAs ▪ SNRIs	▪ Atypical antipsychotics (monotherapy) ▪ Atypical antipsychotics (adjunctive) ▪ Bupropion ▪ Buspirone ▪ Clonidine ▪ Conventional antipsychotics ▪ Gabapentin ▪ Lamotrigine ▪ Non-benzodiazepine hypnotics ▪ Prazosin (for global PTSD) ▪ Propranolol ▪ Trazodone (adjunctive)	▪ Benzodiazepines [harmful] ▪ Guanfacine ▪ Risperidone ▪ Tiagabine ▪ Topiramate ▪ Valproate

The unsatisfactory efficacy of pharmacotherapeutic options for the treatment of PTSD alongside incidence of high-risk self-medicating drug behaviours highlights the urgency for targeted novel therapeutics. Polypharmacotherapeutic approaches for the treatment of PTSD may prove advantageous and efficacious, particularly given the heterogeneity of the disorder, by directly targeting the individual pathophysiological aspects of PTSD directly. For this approach to be successful, revealing genetic factors

conferring vulnerability or resilience to PTSD development, and subsequent neurobiological alterations that underlie specific symptom clusters is required. It may be that focusing on and treating specific neurobiological aspects (i.e. inflammation), and debilitating symptoms of the disorder (i.e. heightened aggression), with polypharmacy drug regimens will provide the best benefit outcome for PTSD sufferers. Accordingly, the research that comprises this thesis investigates genes that may confer vulnerability to both stress and aggression (a highly detrimental symptom of PTSD), and also aims to advance understanding of the associated neurobiological alterations of the disorder.

1.2. Advancing the understanding of neurobiological and genetic bases of PTSD and stress disorders

1.2.1. PSTD neurobiology: fear circuits and loss of grey matter

Understanding neurobiological changes that occur in response to traumatic stimuli may help assist in the development of novel treatments for PTSD. Animal and human studies demonstrate great complexity in the neurobiological bases of the acquisition, expression, consolidation and extinction of fear memories. Fear memory is predominantly controlled by a series of brain pathways with reciprocal innervations between the amygdala, hippocampus and medial prefrontal cortex regions, which will be reviewed briefly below. A diagrammatic representation of the human and rodent brain regions implicated in PTSD-related behaviour is shown in Figure 1.

The amygdala is integral for both the acquisition and expression of fear memories [64-66]. Both sensory and contextual information relating to fearful stimuli converge on the input region, the basolateral amygdala, via both cortical and subcortical routes [66]. Fear learning and emotional memory formation via N-methyl-D-aspartate receptor-dependent long-term plasticity occurs at basolateral amygdala synapses from thalamic and cortical afferents [67, 68]. Structural imaging studies of PTSD patients show that both increased [69, 70] and decreased grey matter volume [71, 72] have been reported in the amygdala, however meta-analyses have shown overall that significant volumetric changes are not found in PTSD patients compared to healthy or trauma-exposed controls [73-76]. Interestingly however, functional imaging studies show that the amygdala appears functionally hyper responsive to both trauma and non-trauma related stimuli in the PTSD population [77], highlighting that this region is critically implicated in the expression of exaggerated fear response.

Establishment of contextual representations of newly formed fearful associations occurs through bidirectional innervations between the amygdala and hippocampus, with the strength of the memory modulated by the basolateral amygdala [78]. The hippocampus forms a neural representation of a contextual environment, of which the encoded memory ultimately influences behaviour [79, 80]. One of the most consistently recorded structural alterations in the PTSD brain is reduced grey matter volume loss in the hippocampus [81-83]. This hallmark feature of PTSD is not necessarily an indicator of trauma damage per se but may actually serve as a predisposing risk factor for the development of the disorder [74, 84, 85]. Monozygotic twins discordant for trauma exposure have demonstrated reduced hippocampal volume is a risk factor for PTSD development [74]. Reduced hippocampal volume in PTSD cohorts coincides with

decreased positron emission tomography measured blood flow [73, 82], and hypo activation of the region in response to both trauma and non-trauma related stimuli [77].

The medial prefrontal cortex mediates cognitive processes such as decision making and emotional regulation, is implicated in the gating and facilitation of fear expression, as well as extinction of fear memories [66]. Pharmacological blockade and microstimulation studies targeting individual medial prefrontal regions have made important distinctions between the contribution of the infralimbic and prelimbic cortices to fear expression and extinction [66]. Consolidation and recall of fear memories is facilitated by the prelimbic cortex, through activating excitatory projections to the amygdala [86]. Conversely, the adjacent infralimbic cortex is not involved in the expression of fear, but rather the consolidation of learned extinction, with activation of this region inhibiting the amygdala [86]. Akin to the hippocampus, the medial prefrontal cortex is another region identified to be subject to grey matter reductions in the PTSD brain [82], and demonstrates hypo-activation in response to both trauma and non-trauma related stimuli [77]. Interestingly, neuronal dendritic retraction in this region causes resistance to fear extinction in mice [87], suggesting that atrophy in this region may contribute to PTSD-related deficits in fear memory control and extinction.

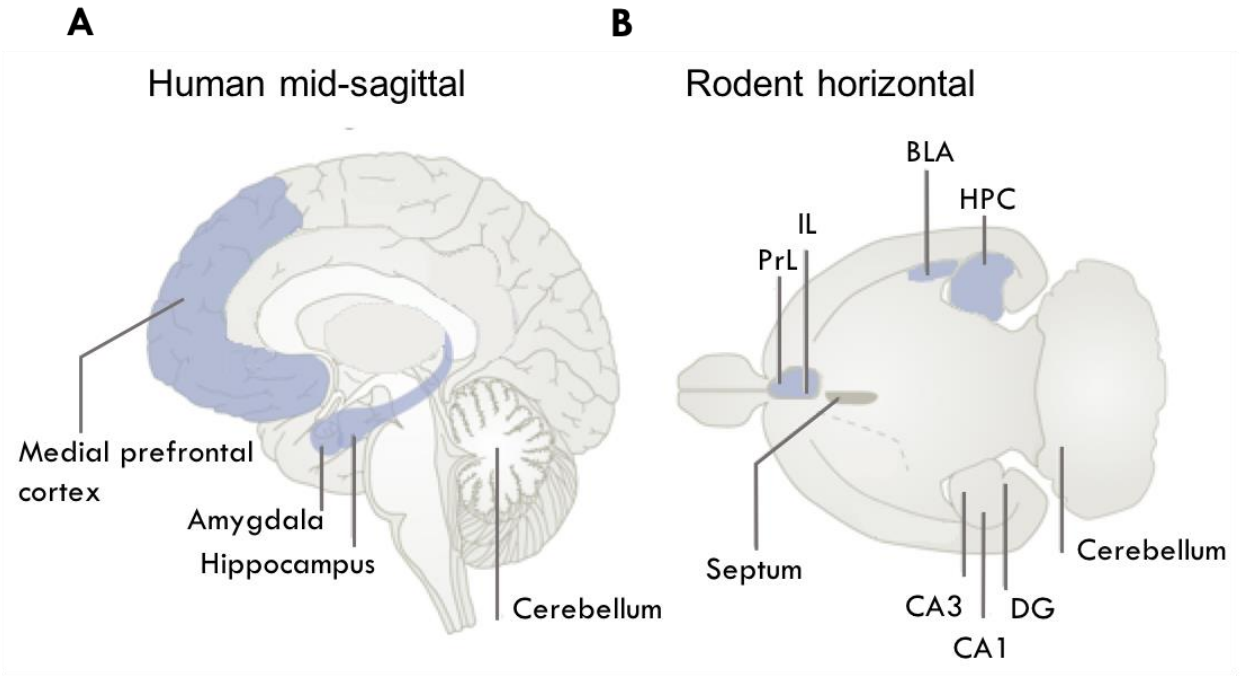

Figure 1. Simplified diagram of the fear circuitry in the human brain and corresponding rodent brain regions. A) Mid-sagittal schematic depicting the amygdala, hippocampus, and medial prefrontal cortex (cerebellum for reference). **B)** Horizontal view of the rodent brain highlighting the basolateral amygdala (BLA) and hippocampus (HPC), as well as the PrL (prelimbic), and IL (infralimbic) regions of the medial prefrontal cortex. Also specified are the hippocampal sub regions cornu ammonis 1 (CA1), cornu ammonis 3 (CA3), and dentate gyrus (DG). Adapted from Fenster et al., (2018) [88].

1.2.2. Animal models of PTSD

The ethical limitations of human research hinder deeper exploration into the role stress exposure plays in the genesis of psychiatric conditions. Epidemiological research repeatedly demonstrates that experiencing stress in both childhood and adulthood can predispose san individual to the development of psychiatric disorders, and that specific genetic variants may increase one's risk of developing psychiatric disorders [89, 90]. However, such research is limited to providing correlational evidence. Deeper insights into the role of stress in psychiatric conditions like PTSD may be achieved using animal models. These studies provide more adequate scientific control and the ability to more

deeply probe the genetic, molecular, and cellular determinants of stress-related behaviour.

Developing animal models of PTSD that have high translational value is challenging as it is a heterogeneous disorder with more ≥ 20 symptoms. Improved animal models are therefore required which more comprehensively model PTSD's core symptomology [91, 92]. These animal models must also evoke a fearful response to a traumatic event that is both long lasting [93]. Several acute and chronic physical and psychological stress models have been implemented in an attempt to elucidate the neurobiological disturbances underlying PTSD and to develop novel pharmacological treatments. These models include including immobilisation, footshock, underwater submersion, social defeat, and predator stress manipulations [94, 95].

Animal models of stress can be acute or chronic in nature dependent on the type of traumatic situations they are trying to model. For example, social defeat and unpredictable variable stress both produce behavioural and biological phenotypes relevant to PTSD, however require animals to undergo multiple exposures to the stressor/s (days to weeks). The chronicity of these designs may therefore be better suited to modelling disorders such as complex PTSD which occurs following exposure to repeated traumatic events such as torture, slavery, or repeated childhood assault [96]. When attempting to model acute traumatic situations (e.g. assault/battery, a natural disaster), the stimulus should be brief but severe enough to elicit long-lasting inappropriate fear responses. Models of single prolonged stress, while designed to mimic the single trauma exposure relevant to PTSD, are susceptible to habituation as the intensity can only be modified through prolonging the duration or repeating exposure [97]. For detailed reviews of these models see [92, 93].

Table 3. Criteria for the establishment of an animal model of PTSD. Siegmund & Wotjak (2006) **[97]**.

General criteria ▪ The model should allow to assess behavioral correlates of both associative and nonassociative trauma-related memories. ▪ Behavioral tasks should allow to perform longitudinal studies of PTSD-like symptoms in order to assess incubation, extinction, habituation, and desensitization of fear responses.
Traumatic incident ▪ The phenotype should be induced by an aversive stimulus (stressor). ▪ Brief stressors should be sufficient for the induction of PTSD-like symptoms. ▪ The intensity of the stressor should define the severity of the symptoms.
PTSD-like symptoms ▪ Symptoms should persist for several weeks or even increase with the passage of time (delayed onset, fear incubation). ▪ Symptoms should include exaggerated fear responses to trauma-related cues, hypervigilance, and hyperarousal. ▪ Symptoms should include signs of hyporesponding (emotional blunting, social withdrawal). ▪ There should be considerable inter-individual variability of PTSD-like symptoms to study factors of vulnerability and resilience.
Predictive validity ▪ Chronic SSRI treatment should ameliorate at least part of the manifested PTSD symptoms.

1.2.3. Face, construct and predictive validity of an animal model of PTSD

PTSD has a complex symptomology which encompasses more than simply exaggerated fear memory and anxiety. To more faithfully represent the human condition improved animal models are needed which incorporate multiple behavioural phenotypes. Table 3 presents the validity criteria outlined by Siegmund & Wotjak that a prospective animal model of PTSD should meet to more adequately represent the disorder [97, 98]. The

defined face validity criterion calls for an intense yet short lived stressor that provokes both associative and non-associative aspects of the condition. That is, there should be measurable fear expression to both fear-associated cues as well as generalised fear responses, hyperarousal, emotional blunting, and social withdrawal [97, 98]. The authors also define that predictive validity is required where SSRIs might reduce PTSD-related phenotypes in the animal model. PTSD-related symptomology must be long-lasting and potentially undergo an incubation process where the severity of the symptoms worsens over time.

The model developed by Siegmund & Wotjak [97, 98] provides a useful model for translational PTSD research as summarised in Table 4. These studies strongly demonstrate the Wotjak model addresses face, predictive, and construct validity measures. In all of these studies, a brief inescapable "traumatic" footshock produced long lasting fear memories and fear incubation, while also generating measurable hyperarousal, anxiety, and emotional blunting behaviours (face validity) [99-102]. Moreover, sleep disturbances are a commonly reported by PTSD patients and this has been replicated using the Wotjak model of PTSD [102], where footshock stress evoked long lasting changes to sleep cycle architecture with early and sustained REM cycles associated with fear and hyperarousal [102]. The behavioural effects generated by footshock trauma, such as freezing and generalised fear, were ameliorated by administration of the SSRI fluoxetine supporting the predictive (therapeutic) validity of the model [100, 101].

While the biological basis of PTSD is yet to be fully delineated, neurobiological effects reminiscent of the human condition are evoked in this model and thus provide an element of construct validity. This is evident where long term grey matter volume

abnormalities reported in the hippocampus of PTSD sufferers are observable within the footshocked mice [99]. Molecular changes within the hippocampus indicating loss of synaptic integrity and dendritic terminals were found that may contribute to PTSD-related phenotypes. Long-term reductions in the synaptic markers synapsin Ia-b/IIa, synaptophysin and homer 1b/c occurred in the hippocampus 60 days after footshock trauma [101]. There was also reductions in the neuronal plasticity marker GAP43 and hippocampal volume at ~74 days post-shock exposure [99].

Also relevant to the current thesis, the Wotjak model has established long-lasting upregulation of neuroinflammatory pathways in the CNS following footshock trauma at 64 days post-shock exposure [100]. Inflammatory gene expression with concurrent elevated microglial cell densities were evident within the anterior cingulate cortex, adjacent to the medial prefrontal cortex, coinciding with decreased gene expression for hippocampal neurotransmitter secretion and membrane integrity [100]. This is important as it demonstrates that inflammatory responses become active alongside the early PTSD symptom development trajectory. Collectively prior studies have shown that there is likely loss of synaptic terminals and altered microglial cell activity in the Wotjak model, however an explicit demonstration of the dendritic spine regression and microglial cell morphological changes remains unresolved. Accordingly, **Chapter 2** of the present thesis addresses this gap in the literature, as well as examining possible molecular links between dendritic spine loss and microglial cell alterations. The following sections of this introductory chapter will overview the prior research on the effects of stress on synapses and microglial cells which are instructive to the research conducted in the present thesis.

Table 4. A sample of neurobiological studies employing the Wotjak mouse model of PTSD. Arrows represent direction of traumatic shock induced change. D = day; ↻ Effects blocked or reversed by intervention.

Face validity		
Diagnostic criteria (DSM-V) for PTSD	**PTSD-like behaviour in mice**	
Criterion B) Persistent and long-lasting traumatic memory	↑ Long-lasting exaggerated fear response [99-103]	
Criterion B) Nightmares	↑ REM sleep [102]	
Criterion C) Avoidance	↑ Generalised avoidance behaviour [99, 101, 102, 104]	
Criterion E) Heightened startle reaction/hypervigilance	↑ Fear potentiated startle [99, 101, 103]	
Predictive validity		
	Intervention	**Outcome**
Enduring fear memory; hippocampal volume loss	Environmental enrichment [99]	↻
Long lasting fear memory	Fluoxetine [100, 101]	↻
Hippocampal synaptic protein loss (grey matter deficits)	Fluoxetine [101]	↻
Inflammatory gene expression	Fluoxetine [100]	↻
Construct validity		
Neurobiological	**PTSD-like alterations in mice**	**End point**
Hippocampal volume loss and other related grey matter alterations	↓ Hippocampal volume ↓ Hippocampal GAP43 ↓ Central amygdala volume [99]	D 74-88
	↓ HPC synapsin Ia-b/IIa [101]	D 2> 60-74
	↓ Gene cluster neurotransmitter secretion and membrane integrity CA1 [100]	D 64
Inflammation	↑ Gene cluster inflammatory genes Anterior cingulate cortex ↑ Iba-1^{+} cells ACC [100]	D 64

1.2.4. Positive and negative neuroadaptive consequences of stress

Brains are computational organs that process environmental information via electrochemical transmission through a network of neurons [105]. Neurons are made up

of cell bodies, axons and dendritic processes that form connections with other neurons at the level of the synapse, where chemical transmission occurs. In response to stress exposure the brain demonstrates dynamic structural and functional plasticity, particularly dendritic remodelling. Information processing is influenced by the dendritic arborisation and size of the dendritic field, as well as the density and integrity of dendritic spines [105]. Dendritic spines are small protrusions localised to dendritic processes and, while only at a general length of 0.5 – 2 μm, they are critical to excitatory synaptic input throughout the brain [106]. Under normal conditions, the density of dendritic spines is increased by long-term potentiation and reduced by long-term depression, the two mechanisms of synaptic plasticity that subserves memory [107].

Dendritic processes and dendritic spines are incredibly plastic, modifiable neuronal extensions which exhibit morphologic alterations under various circumstances, including as a normal physiological function. An example of this is upon entering and leaving hibernation, where several mammals undergo a degree of dendritic regression that returns to baseline within hours following waking and is a normal, adaptive physiological process [108]. Although, under states of stress, higher cognitive brain regions that mediate functions such as memory, decision making, and anxiety can be adversely affected, due to brain regions such as the hippocampus and medial prefrontal cortex undergoing neuroadaptive changes in response to stress which includes decreases in dendritic length, branching, and dendritic spine density [109-114]. Dendritic process hypotrophy as well as dendritic spine loss can even become so significant they can lead to observable grey matter volume loss in magnetic resonance imaging studies [115, 116]. Importantly, stress mediated neuronal dendritic remodelling is not always retractory. This is apparent in the amygdala where stress can increase both

dendritic length and dendritic spine densities [117, 118], and further emphasised by distinct dendritic spine remodelling patterns on branches proximal or distal to the cell soma within different amygdalar nuclei [117].

Stress modifications to neuronal architecture are associated with maladaptive behavioural responses in rodents causing anxiety and social avoidance, memory impairment, and cognitive inflexibility [119]. One factor that is known to contribute to dendritic hypotrophy is stress mediated glucocorticoid signalling, as excess glucocorticoid activity results in glucocorticoid toxicity [120]. This effect is particularly observable within the hippocampus, a region enriched with a high concentration of receptors for glucocorticoids [121]. In the absence of stress, excessive administration of glucocorticoids results in significant grey matter damage within the hippocampus leading to loss of dendritic branching and length [122] as well as neuronal cell loss [123], and grey matter loss [124]. It was considered that lower grey matter volume in the hippocampus of PTSD sufferers may be explained by the glucocorticoid toxicity hypothesis, as PTSD patients have dysregulated HPA axis function, including lower plasma cortisol concentrations and elevated sensitivity and number of glucocorticoid receptors [125-127]. Future research is needed to explain why PTSD patients show grey matter loss in various brain regions. Here we hypothesise that the retraction of dendritic spines may at least partially explain the grey matter losses (**Chapter 2**). In **Chapter 3** we also explore whether genetic variation in transporters that regulate brain corticosterone exposure alter responses to stress and trauma using a mouse model of PTSD.

1.3. PTSD and neuroinflammation: Is there a link between loss of synaptic connectivity and microglia?

It is becoming increasingly evident that at least part of the underlying pathophysiology of several psychiatric and neurodegenerative disorders is attributable to dysregulation of the immune system. Psychiatric disorders of which stress exposure is shown to predispose individuals, such as major depression, bipolar disorder, and schizophrenia, have been repeatedly linked to both peripheral inflammation and microglial cell activation. Patients with depression present with elevated serum levels of a range of pro-inflammatory cytokines including interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-12 (IL-12), as well as interferon-γ and tumour necrosis factor alpha (TNF-α) [128]. Similar to this, PTSD is also characterised by cytokinemia, which is an upregulation of pro-inflammatory cytokines including IL-1β, IL-6, and TNF-α [129]. Length of illness and PTSD symptom severity are found to be positively correlated with peripheral IL-1β and IL-6, respectively [129-132]. There is also evidence that plasma concentrations of the inflammatory marker c reactive protein are elevated in PTSD cohorts, and that elevated levels maybe predictive of those more vulnerable to developing PTSD [133, 134]. In addition, PTSD is associated with elevated peripheral expression of nuclear factor-κB, a transcription factor that regulates pro-inflammatory cytokines [135]. As major depressive and bipolar disorder are also characterised by inflammatory states, a current theory in psychiatry is that neuroinflammation may be pathogenic to these conditions [136].

The recognition that inflammation may contribute to the pathophysiology of PTSD has led to studies investigating links between pro-inflammatory cytokines and

hallmark symptoms of the disorder such as hippocampal grey matter volume loss. This was, in part, driven by the fact elevated peripheral IL-6 is linked to hippocampal atrophy and lower hippocampal grey matter volumes in critically ill [137], depressed [138], and healthy populations [139]. Indeed, an association exists between increased soluble receptor II for TNF (sTNF-RII) upregulation and reduced bilateral hippocampal volume in Gulf War veterans with PTSD [140]. Neuroinflammation appears to negatively influence function in brain regions involved in emotional regulation and cognitive processing [33, 34]. To this end, the current thesis is dedicated to investigating changes in microglia in stress-related brain regions, as these cells are the major cells mediating immune function and inflammation in the central nervous system (CNS).

1.3.1. A brief history of microglia

The origins of glia (from the Greek γλία, meaning glue) can be traced back to the work of Rudolph Virchow (1821 – 102) who in 1846 described a connective substance throughout the CNS which he termed "nevernkitt" or nerve-glue (later neuroglia) [141-144]. The histological visualisation of glial cells proved a difficult task and it was more than 75 years later that neuroglia were determined to be composed of what we today refer to as astrocytes, oligodendrocytes, and microglial cells [145]. Gluge (1812 – 1898) in 1841 first characterised phagocytic cells in the CNS naming them inflammatory corpuscles [146]. However, the initial discovery of microglia is attributed to Franz Nissl (1860 – 1919) in 1898 who described them when studying "rod cells" in the cortex of dementia patients (Figure 2A) [147]. Nissl theorised rod cells were reactive neuroglia with phagocytotic and migratory properties. In 1919 Pío del Río Hortega (1882 – 1945)

would eventually characterise microglial cells, as we know them today, delineating them from astrocytes and oligodendrocytes [148]. Río Hortega advanced research into the morphology and function of microglial cells, characterising microglial cell distribution throughout the brain, specifying their motility, proliferation, and phagocytic properties under pathological conditions (Figure 2B).

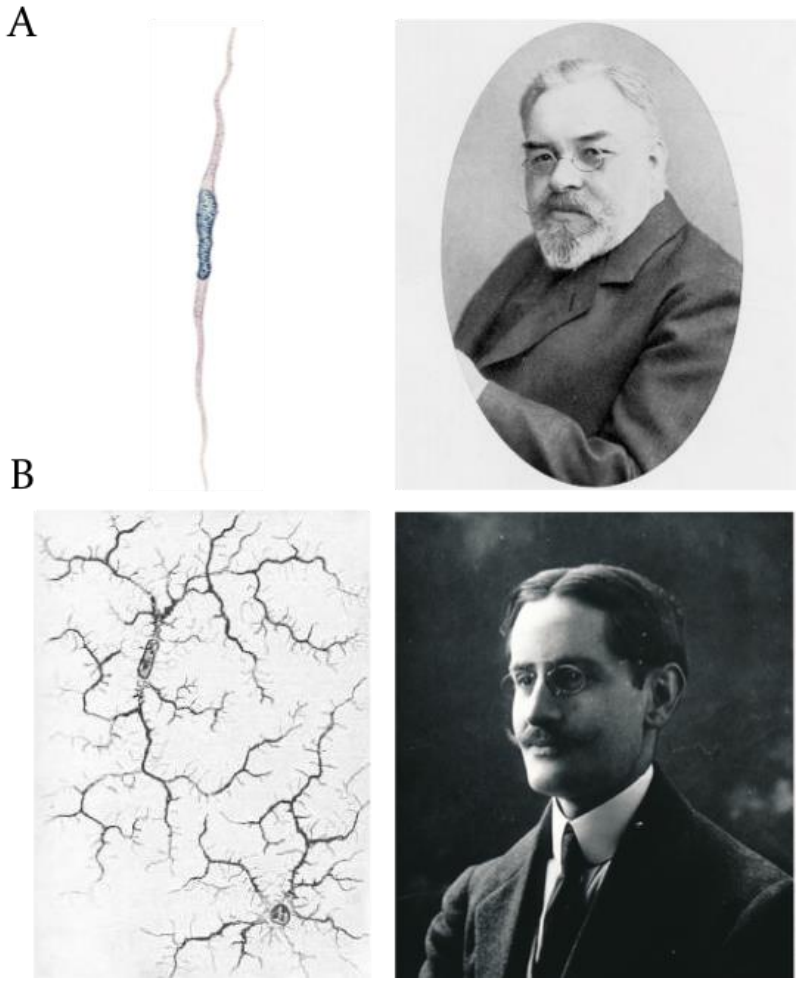

Figure 2. Progression of the histological characterisation of microglial cells. **A)** The initial visualisation of "Stäbchenzellen", or rod cells, illustrated in 1898 by Professor Franz Nissl, were described as reactive neuroglial cells with migratory properties. **B)** Silver carbonate staining by Pío del Río Hortega in 1919 who completely visualised the intricate details of microglial cells and, after much research, would conclude rod cells and microglia to be the same cell type. Adapted from Rio-Hortega (1919) [149] and Taylor et al.,2014 [150].

1.3.2. Structure and function of microglia

Microglial cells are the innate resident macrophage immune cells within the brain parenchyma. Distributed throughout the brain and spine, microglial cells account for approximately 10-15% of the total cell population in the mature CNS [151]. Microglial cells infiltrate the developing CNS during embryogenesis and take up residency forming a self-sustaining population [152]. As immunocompetent phagocytic cells, microglia are classically recognised for orchestration of immune responses to invading pathogens, responsivity to traumatic brain injury and ischaemic insult [153], and act as the resident housekeepers cleaning up cellular debris.

Resting state microglial cells within the healthy, mature brain were once considered quiescent, only becoming responsive or "active" to CNS injury or immunological threat. The development of advanced *in vivo* imaging modalities has since corrected these inaccurate assumptions with two-photon microscopy revealing microglial cells are in a constant state of surveillance of their surrounding environment [154]. The extension and retraction of de novo formed filopodium-like protrusions from highly motile ramified processes occurs in a cyclical manner over a matter of minutes [154, 155], at an average velocity of ~2.5 μm/min (range 0.2 - 6.5 μm/min dependent on branch order) [154, 156]. In contrast to the highly motile processes, resting state microglial cell somata shift minimally (1 - 2 μm per hour), if at all [154]. At resting state microglial cells possess three to five primary processes extending from the cell soma which, in turn, possess subsidiary branches [157, 158]. Microglial cells are spatially distributed into individual territories that are repulsively maintained at a distance of

~50 μm from neighbouring cells [154, 159]. Despite repulsion from neighbouring cells, there is overlap of territorial environmental sampling [160].

Despite accounting for only 1.3% of cortical grey matter [161], within the healthy CNS microglial cells are intrinsically involved in the formation and maintenance of neuronal networks and homeostatic maintenance of the CNS environment. During homeostasis microglial cells make contact and interact with local astrocytes and neuronal cells, as well as blood vessels [154]. Microglia are highly sensitive to pharmacological modulation and changes in the extracellular matrix with highly motile processes serving to sample surrounding extracellular space in search for environmental cues [154]. Microglial cells are the first line of defence within the CNS, detecting local environment alterations including increased extracellular calcium (Ca^{2+}) concentrations [162] or release of adenosine triphosphate (ATP) from surrounding cells [163]. Microglia mobilise in response to these signals by directing their processes to the source site and can elicit subsequent inflammatory signalling. High extracellular concentrations of ATP activate the purinergic $P2X_7$ receptor on microglial cells leading to the synthesis and release of IL-1β [164]. However, IL-1β release can be heightened by gain-of-function polymorphisms at *P2RX7* which also increase the risk for mental health conditions including depression, bipolar, and anxiety disorder [165, 166]. As the contribution of $P2X_7$ receptors to PTSD-related symptoms has not yet been investigated **Chapter 4** of this thesis aims to fill this gap in the literature.

Microglial cells perform an array of critical activities during neurodevelopment and early wiring of the CNS. Microglia regulate the survival and proliferation of neural progenitor cells and guide axonal tracts [167], phagocytose apoptotic cells and also contribute to the development and refinement of the synaptic network (Figure 3) [167].

Under homeostatic conditions, microglial cell processes make frequent contact with neuronal synaptic elements assessing their functional state [155]. In addition to early embryonic development and the post-natal period, microglia within the mature CNS are active in remodelling synaptic elements via both secreted and contact-dependent signalling. This holds implications for learning, memory, and behaviour in both the healthy brain and for neuropsychiatric and neurodegenerative disorders. Targeted Cre/lox depletion approaches have highlighted the role of microglial cells in learning-dependent synapse formation in the healthy mouse brain [168]. Conditional gene inactivation of microglial-derived brain derived neurotrophic factor (BDNF) exerts similar effects suggesting that learning related postsynaptic dendritic spine development is mediated by microglial BDNF in the healthy brain [168]. Genetic ablation of microglia in the retina shows a maintenance role for the cells in synaptic structure and visual function [169]. Just as microglial cells modulate synaptic plasticity by contributing to experience-dependent synapse formation within the healthy brain, so do they interact with synaptic elements under major pathological conditions.

1.3.3. The complement system and microglia

During early wiring of the neural network neurons acquire supernumerary synaptic connections that are neither required nor retained in the mature CNS. Microglial cells play an active role in synaptic remodelling during the postnatal period by engulfing synapses through a C3-dependent complement signalling pathway [156, 170]. Complement, a component of the innate immune system, is comprised of a series of over 30 soluble and cell-associated factors which serve as a host's first defence in the

recognition and elimination of infiltrating pathogens [171, 172]. Of the three known complement activation pathways (classical, lectin, alternative), the classical cascade is implicated as a molecular mechanism underlying removal of supernumerary synapses during development and neurological disease states later in life [173].

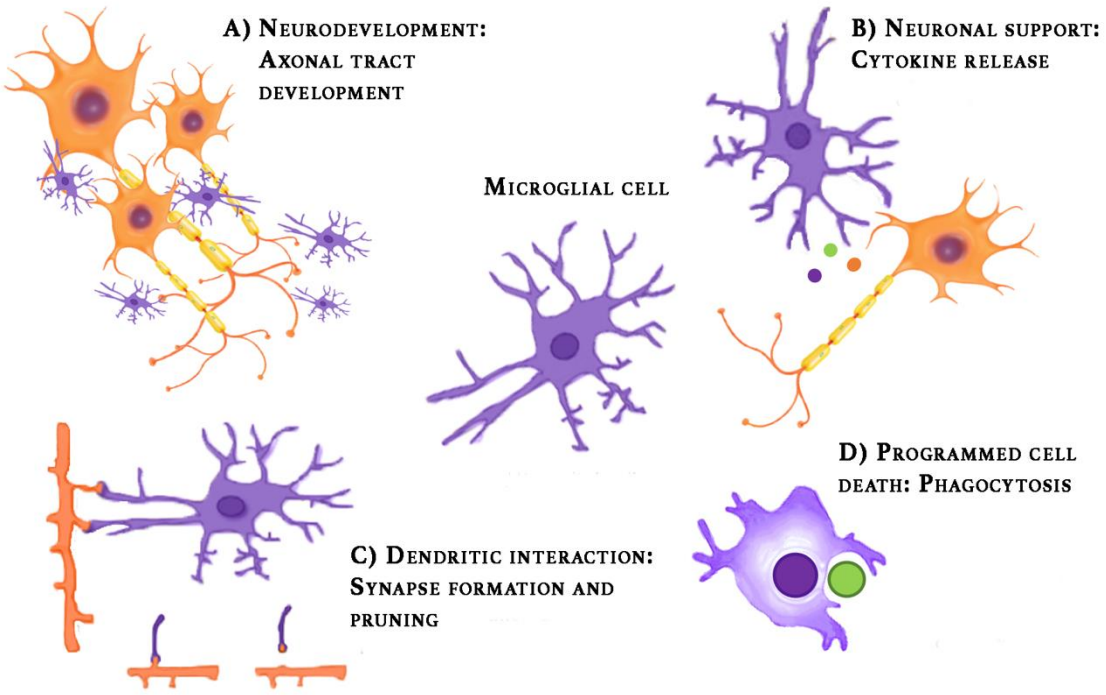

Figure 3. Microglia control a variety of physiological processes during neurodevelopment and throughout the life span. A) Microglia guide axons during neuronal development playing a pivotal role in CNS wiring. **B)** Neurotransmitter signalling and cytokine release between neurons and microglial cells assists both normal functioning and response to homeostasis disruption. **C)** Microglia interact with dendritic processes transiently surveying the environment, assisting the development of synapses and pruning of supernumerary or tagged dendritic spines. **D)** The phagocytosis of apoptotic cells within the CNS is performed by amoeboid microglial cells. Adapted from Sominsky et al., 2018 [174].

The initiating component of the classical pathway, C1q, is a 460kDa hexameric protein possessing a globular domain that functions as a recognition pattern molecule [175]. Candidate synaptic spines for elimination are tagged by C1q and the complement protein C3, ligand for the phagocytic surface receptor CR3 localised solely on microglial cells [170]. The CNS locally synthesises complement components independent from the peripheral circulation [171], with complement sourced from glial cells as well as *de novo*

synthesis of complement proteins by neurons [176]. The removal of synapses through microglial stripping has been demonstrated to occur via a C1q-C3/C4 complement-microglial mediated pathway in models of Alzheimer's Disease and schizophrenia [177, 178]. Hyperactivation of the classical pathway is found in PTSD patients, evidenced by elevated plasma levels of complement C2 and C4 factors [179], therefore in **Chapter 2** complement signalling was selected for analysis as a potential mechanism for microglial-mediated dendritic spine loss.

1.3.4. Stress and microglia

That stress can modify neuronal dendritic arborisation and the density of dendritic spines is well established, however it is now appreciated that microglial cells are also disrupted by and responsive to stress [180]. Robust microglial cell cytoskeletal rearrangement occurs in response to stress both in the presence and absence of inflammatory marker release or apoptosis [181-184]. The diversity of microglial cell changes in response to stress differs as a function of the brain region analysed, the type of stressor, as well as the stressor's intensity and duration (see Table 5).

It is well established that a variety of different stressors, such as prenatal and chronic stress, can trigger increases in the reactivity of microglial cells across the medial prefrontal cortex and hippocampus, both regions implicated in the pathophysiology of PTSD [181, 183-185]. The effects of stress on microglial cells can range from shifts in regional densities to morphology changes targeting cell body size, process length, volume and ramification. These effects occur both with [186, 187], and in the absence of inflammatory mediators [182]. Chronic restraint and repeated social defeat stress

robustly elevate microglial cell density within the hippocampus [188, 189], while footshock can evoke a transient increase in microglial cell counts albeit to a lesser degree [190].

Table 5. Psychosocial stressors and their effect on Iba-1-demarcated microglial cells. Modified from Calcia et al. 2016 [191].

ROI	Species	Stressor	Effect on Iba-1	Reference
Amygdala	Mouse	Repeated social defeat stress	↑↑↑	Wohleb et al. (2011, 2012)
	Rat	Chronic restraint stress	NE	Tynan et al. (2010)
	Mouse	Chronic restraint stress	NE	Clarke et al., (2019)
Hippocampus	Mouse	Repeated social defeat stress	↑↑↑	Wohleb et al. (2011, 2012)
	Mouse	Varying unpredictable stress	↑↑↑	Bian et al. (2012)
	Rat	Prenatal stress	↑↑↑	Ślusarczyk et al. (2015)
	Mouse	Chronic restraint stress	↑↑↑	Clarke et al., (2019)
	Rat	Chronic restraint stress	↑↑	Tynan et al. (2010)
	Mouse	Prenatal stress	↑↑	Diz-Chaves (2012, 2013)
	Mouse/rat	Varying unpredictable stress	↑	Kreisel et al. (2014), Giovanoli et al. (2013)
	Mouse	Occlusal disharmony stress	↑	Kojo et al. (2010)
	Mouse	Footshock	↑	Brevet et al. (2010)
	Mouse/rat	Chronic varying unpredictable stress	↓	Kreisel et al. (2014)
Prefrontal Cortex	Mouse	Repeated social defeat stress	↑↑↑	Wohleb et al. (2011, 2012)
	Mouse	Varying unpredictable stress	↑↑↑	Bian et al. (2012)
	Rat	Prenatal stress	↑↑	Ślursarczyk et al. (2015)
	Mouse	Chronic restraint stress	↑↑	Clarke et al., (2019)
	Rat	Social isolation stress	↑	Schiavone et al. (2009)
	Rat	Chronic restraint stress	↑	Tynan et al. (2010), Hinwood et al. (2012, 2013)
	Mouse	Varying unpredictable stress	↑	Kopp et al. (2013), Couch et al. (2013)
	Mouse	Varying unpredictable stress	NE	Kopp et al. (2013)
	Mouse	Varying unpredictable stress	NE	Giovanoli et al. (2013)
	Mouse/rat	Varying unpredictable stress	NE	Kreisel et al. (2014)

Measured change in Iba-1 activity in stressed animals (compared to control): NE indicates no significant effect, ↓ >5% decrease, ↑ 5 – 30% increase, ↑↑ 30 – 70% increase, and ↑↑↑ > 70% increase. Results predominantly based on quantifying histology preparation of microglial cell densities and/or changes in percentage area occupied by Iba-1 immunostaining. Ślursarczyk et al. (2015) and Schiavone et al. (2009) measured Iba-1 via western blot and Kreisel et al. (2014), by real-time PCR.

The prefrontal cortex is also affected by stress with elevated microglial cell density being reported in response to chronic restraint [181, 185] and repeated social defeat in this brain region [188, 189]. However, this has not been consistently observed in all studies suggesting the intensity and duration of a stressor in combination with factors such as biological sex can influence these effects [192, 193].

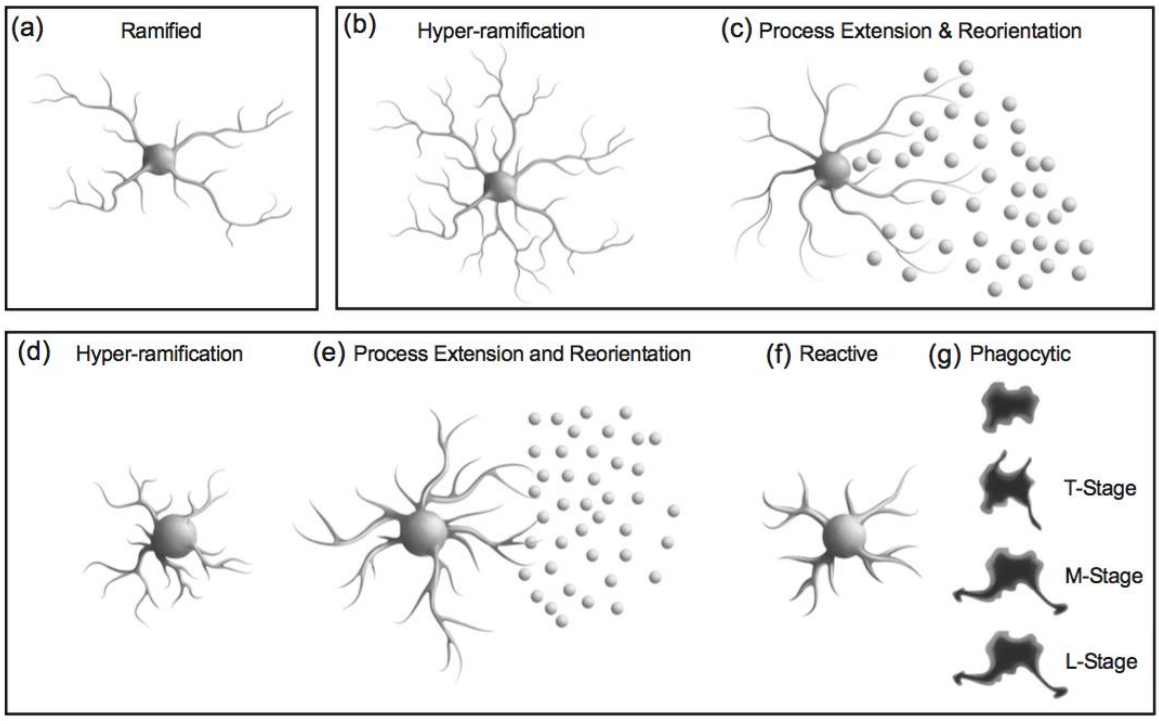

Figure 4. Stages of microglial remodelling following pathological (classical) and non-pathological stimuli. A) Ramified resting state cell. Morphological alterations to experience-dependent stimuli include hyper-ramification and process elongation **B)** as well as reorientation of processes towards synaptic elements **C)**. Morphology changes to microglial cells appears highly stereotyped **D)**. In response to injury microglial cells extend and reorientate their processes to the site of injury **E)**. Microglial cells can then shift into a reactive phase characterised by retraction and thickening of processes **F)**. Finally, microglial cells reactive cells can transition to phagocytic amoeboid cells which can be differentiated in several stages **G)**. Amended from Walker et al., (2013) [180].

One of the most striking attributes of microglial cells is their dynamic plasticity and cytoskeletal restructuring. Due to the lack of permanent physical or electrical connectivity with the surrounding neural elements, microglia freely transform their morphology from a ramified resting state on functional demand [180]. Microglial cell

activation in response to injury or inflammation coincides with a series of morphology transformations. For example, in detection of injury, microglia can migrate to the source of injury and then transform their morphology into an amoeboid form in which they phagocytose cellular debris or dying neuronal cells [180]. Experience-dependent changes, like exposure to stressors, can alter microglial cell morphology in which the morphology become ramified or even hyper-ramified (see Figure 4).

Chronic restraint stress causes microglial cells in the prefrontal cortex to undergo structural modifications where the microglial cells adopt a hyper-ramified state possessing an increased number of processes that become significantly elongated [182]. Similar changes have also been described following chronic unpredictable stress in the hippocampus [184]. However, akin to changes in microglial cell densities, structural modifications are not always observed [192]. While there are some discrepancies between studies it is clear that repeated stressors disrupt microglial cells in regions responsible for memory processing, cognition and emotional regulation. In both **Chapter 2** and **Chapter 3** of the current thesis, we contribute to this field of research by examining microglia in terms of both the density of cells, as well as morphological alterations in response to an acute, traumatic experience.

1.4. Could genetic factors predispose or confer resilience to PTSD?

Determining the modifying factors that underlie an individual's susceptibility or resilience to developing PTSD is also a key area of research with a major focus on genetic variants. Genetic influences are estimated to account for 30% - 72% of vulnerability to developing PTSD [194-196], and many gene variants have been identified.

Advancements in high-throughput genotyping platforms has allowed for the isolation of single nucleotide polymorphisms (SNPs) of risk loci in genes that confer vulnerability to developing PTSD.

One compelling susceptibility gene for PTSD is the FK506 binding protein 5 (*FKBP5*) [197-200]. *FKBP5* regulates glucocorticoid receptor sensitivity and function of the HPA axis and several *FKBP5* SNP's were associated with increased expression of glucocorticoid receptors. *FKBP5* expression predicts PTSD symptoms related to childhood abuse severity [197] with allele-specific DNA demethylation mediating gene-childhood trauma interactions [201]. Further, the functional variants of *FKBP5* are also associated with biologically distinct subtypes of PTSD [202]. Epigenetic modification of the glucocorticoid receptor gene *NR3C1* were observed in Rwandan genocide survivors that had PTSD [203]. It follows then that any gene that regulates components of the HPA axis might predispose to PTSD.

Activation of the HPA axis by stress leads to the secretion of corticotropin-releasing hormone (CRH) from the hypothalamus which triggers adrenocorticotrophic hormone (ACTH) release from the pituitary gland and subsequent adrenal gland secretion of glucocorticoid hormones (cortisol or corticosterone; human and rodent, respectively) and mineralocorticoids [204]. Secreted cortisol primarily exerts effects within the brain via glucocorticoid receptors or mineralocorticoid receptors to inhibit the HPA axis through an inhibitory negative feedback circuit [205, 206]. In contrast to the normal physiological stress response, PTSD is characterised by dysregulation of the HPA axis which arguably contributes to the development of the disorder. Both urinary and plasma concentrations indicate abnormal regulation of cortisol in PTSD cohorts including combat veterans and Holocaust survivors [207]. While PTSD subjects have

elevated levels of CRH [208-210], cortisol levels are significantly lower than healthy controls or trauma exposed groups [211, 212]. Reduced cortisol levels are due to enhanced negative feedback inhibition as a result of elevated glucocorticoid receptor sensitivity [213].

One factor that might regulate brain levels of cortisol and therefore alter the susceptibility to PTSD is the activity of ATP-binding cassette (ABC) transporter, P-glycoprotein (P-gp). P-gp is an efflux pump that utilises the energy of ATP hydrolysis to transport substrates across membranes [214]. P-gp is localised along biological barriers to protect organs through removal of potentially hazardous substrate compounds [215]. At the blood brain barrier P-gp facilitates efflux of various xenobiotics from the brain, a mode of action that contributes to drug resistance by hindering delivery of therapeutic drugs [216]. The xenobiotic substrates for P-gp range from antidepressants, antipsychotics, anticonvulsants, antibiotics, and chemotherapies among others [217-220].

P-gp is also an efflux transporter for endogenous products such as testosterone, and progesterone analogues, as well as the stress hormones cortisol and corticosterone [219]. Pharmacological inhibition of P-gp decreases anxiety-related behaviours by increasing brain corticosterone accumulation and enhancing negative feedback, providing evidence that this transporter may modulate the HPA axis [221]. As activation of glucocorticoid receptors and HPA axis stimulation enhance consolidation of emotionally arousing memories [204], P-gp dysfunction and dysregulation of central cortisol may enhance vulnerability to PTSD. In **Chapter 3** this will be explored by examining whether P-gp KO mice display altered susceptibility to footshock trauma and microglial cell responses in a mouse model of PTSD.

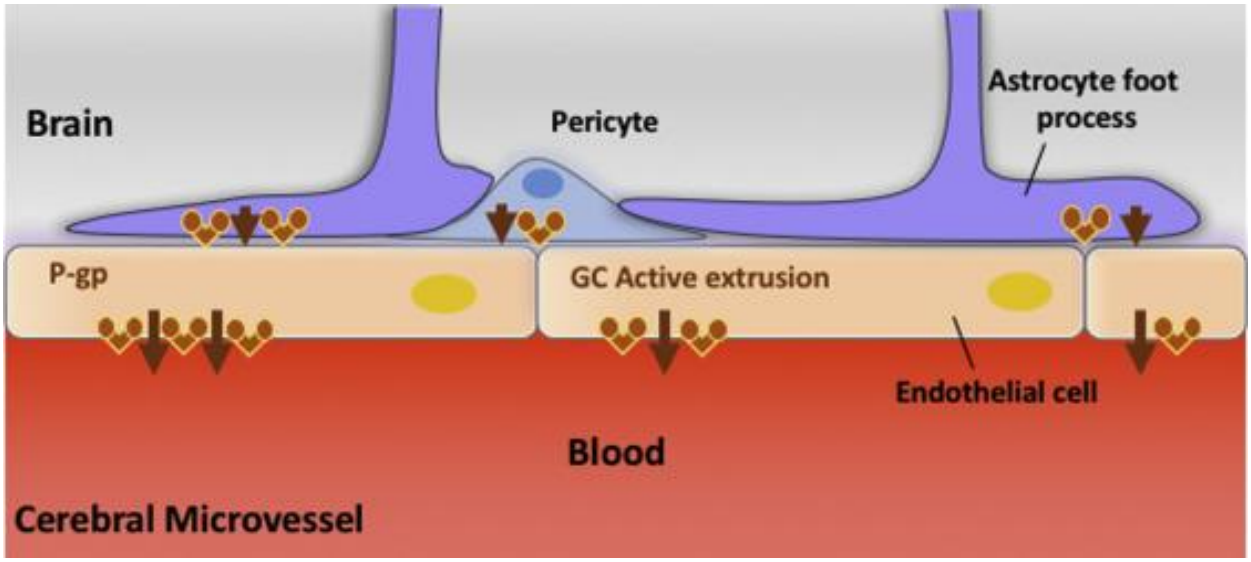

Figure 5. P-gp transporters located on the luminal and abluminal membranes of endothelial cells of the BBB, pericytes and astrocytic feet limit the brain uptake of glucocorticoids such as cortisol and corticosterone. Amended from Bloise & Matthews (2019) [222].

1.4.1. Development of novel serenic agents for PTSD and beyond

Heightened aggression in PTSD is an indicator of altered arousal and reactivity (Criterion E) [9], and is one of the highest concerns for PTSD patients receiving clinical treatment [25, 26]. There are no approved serenic drugs for the treatment of aggression in PTSD rendering patients vulnerable to the negative outcomes of these destructive behaviours. Treatment of neuropsychiatric-related aggression (including PTSD) has predominantly relied on off-label neuroleptic agents, however low perceived efficacy and high incidence of extrapyramidal side-effects make them an unattractive treatment option [223, 224]. Improving treatment options for aggression in PTSD and other neuropsychiatric conditions is therefore required.

There is converging evidence from human and animal studies that proinflammatory cytokines are mediators of aggressive behaviour [225-227]. For

example, direct injection of IL-1β into the hypothalamus of cats elicits aggressive behaviour in cats [228], while state anger in rugby players is associated with increased plasma levels of IL-1β [229]. In the CNS, the synthesis and release of IL-1β is facilitated by ATP-mediated activation of $P2X_7$ receptors, which are predominantly localised on microglial cells [230, 231]. While ATP can be neuronally released as a co-transmitter along with classical neurotransmitters such as noradrenaline [232], psychosocial immobilisation stress studies demonstrated that stress-induced elevations in neuronal glutamate signalling triggers release of ATP from astrocytes, subsequently activating $P2X_7$ receptors [233]. Elevated corticolimbic release of glutamate has been observed in animal models of PTSD contributing to long lasting aberrant effects on behaviour and brain function [234]. Clinical studies have also identified links between glutamatergic system modulation and hyperglutamatergic transmission and negative trauma-mediated behavioural alterations, particularly dissociative symptoms [234]. It may, therefore translate that cells in the PTSD brain may undergo elevated ATP secretion in response to enhanced glutamatergic-mediated ATP release via astrocytes.

The *P2RX7* gene (chromosomal region 12q24.31) is located on a susceptibility locus associated with psychiatric conditions including both affective and anxiety disorders [235]. *P2RX7* gain-of-function polymorphisms are linked with both elevated IL-1β levels and increased risk of depression and bipolar disorder [165, 236-239]. Importantly, acute restraint and chronic unpredictable stress both activate $P2X_7$ receptors via elevated ATP in the hippocampus and prefrontal cortex, facilitating the subsequent synthesis and release of IL-1β [233]. Considering this evidence, it is reasonable to consider that $P2X_7$ receptors may play a role in heightened PTSD aggression and that development of novel serenic therapeutics for treating aggression

and violence in PTSD patients may be achieved by directly targeting $P2X_7$ receptors. To this end, **Chapter 4** of this thesis will investigate the effect germline genetic deletion of the P2X7 receptor in mice on aggressive behaviours.

1.5. Aims

In summary, the present thesis specifically aims to:

Investigate the impact of acute traumatic footshock stress on microglial cell density and morphology and concurrent dendritic spine loss in an animal model of PTSD (Chapter 2). Preclinical modelling of core emotional disturbances representative of human PTSD symptomology offers insight into neurobiological correlates of the disorder. There is currently a crisis in the access to efficacious drug therapies for PTSD, and such models provide a platform for the development of targeted pharmacological treatment. A recent model of PTSD is the Wotjak model which demonstrates excellent face, predictive validity and construct validity. Systemic immune dysregulation and CNS grey matter volume loss are established clinical PTSD features however further research is required to understand the causal mechanisms. One explanation may be microglial cell activation and dendritic spine density reductions. In **Chapter 2**, using the Wotjak model of PTSD, we aim to determine if traumatic shock exposure promotes dendritic spine regression that is associated with microglial cell activation in various stress-related corticolimbic regions. Here, we hypothesise that acute trauma exposure will have a negative effect on dendritic spine densities which may be accompanied with upregulated microglial cell activity.

Investigate the role of P-gp in stress-mediated behavioural deficits and microglial cell activity in an animal model of PTSD (Chapter 3). Research into potential biological risk factors for development of PTSD includes searching for underlying susceptibility genes of which may explain why not all persons exposed to a traumatic event develop PTSD. Understanding a genetic risk for development of a disease or disorder, such as PTSD, may provide a platform for early intervention and targeted treatments. At the BBB the ABC transporter P-gp regulates brain uptake of corticosteroid stress hormones. P-gp is also localised on microglial cells in the CNS and, in understanding these cells are highly responsive to stress, P-gp may exert a modulatory role in the stress response. In **Chapter 3** aims to examine whether P-gp modulates the behavioural and microglial response to stress in an animal model of PTSD. We hypothesise that germline deletion in mice will alter PTSD-related behaviours and microglial activation. In addition, we hypothesise germline P-gp KO itself may influence behaviour and microglia in mice independently of stress exposure.

Investigate the role of genetic deletion of the $P2X_7$ receptor on aggressive behaviour and subsequent effects on microglial cells (Chapter 4). Elevated levels of the proinflammatory cytokine IL-1β are consistently reported in the pathology of patients with mood disorders, such as depression and bipolar disorder, as well as PTSD. Increased IL-1β is positively associated with heightened levels of aggression, an undermanaged symptom of PTSD. $P2X_7$ receptors located on microglial cells are a major source of IL-1β in the CNS. In **Chapter 4** we aim to determine whether genetic knockout of the $P2X_7$ receptor, and subsequent muting of microglial-mediated IL-1β signalling, attenuates aggression in mice. We further aim to evaluating any impact of $P2X_7$ receptor

knockout on microglial cells. We hypothesise that $P2X_7$ receptor knockout will reduce aggressive behaviours in mice in the stressful resident-intruder environment and which may be reflected in altered, observable microglial activity.

Chapter 2. Microglial cell hyper-ramification and neuronal dendritic spine loss in the hippocampus and medial prefrontal cortex in a mouse model of PTSD

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Full-length Article

Microglial cell hyper-ramification and neuronal dendritic spine loss in the hippocampus and medial prefrontal cortex in a mouse model of PTSD

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ABSTRACT

Few animal models exist that successfully reproduce several core associative and non-associative behaviours relevant to post-traumatic stress disorder (PTSD), such as long-lasting fear reactions, hyperarousal, and subtle attentional and cognitive dysfunction. As such, these models may lack the face validity required to adequately model pathophysiological features of PTSD such as CNS grey matter loss and neuroinflammation. Here we aimed to investigate in a mouse model of PTSD whether contextual fear conditioning associated with a relatively high intensity footshock exposure induces loss of neuronal dendritic spines in various corticolimbic brain regions, as their regression may help explain grey matter reductions in PTSD patients. Further, we aimed to observe whether these changes were accompanied by alterations in microglial cell number and morphology, and increased expression of complement factors implicated in the mediation of microglial cell-mediated engulfment of dendritic spines. Adult male C57Bl6J mice were exposed to a single electric footshock and subsequently underwent phenotyping of various PTSD-relevant behaviours in the short (day 2–4) and longer-term (day 29–31). 32 days post-exposure the brains of these animals were subjected to Golgi staining of dendritic spines, microglial cell Iba-1 immunohistochemistry and immunofluorescent staining of the complement factors C1q and C4. Shock exposure promoted a lasting contextual fear response, decreased locomotor activity, exaggerated acoustic startle responses indicative of hyperarousal, and a short-term facilitation of sensorimotor gating function. The shock triggered loss of dendritic spines on pyramidal neurons was accompanied by increased microglial cell number and complexity in the medial prefrontal cortex and dorsal hippocampus, but not in the amygdala. Shock also increased expression of C1q in the pyramidal layer of the CA1 region of the hippocampus but not in other brain regions. The present study further elaborates on the face and construct validity of a mouse model of PTSD and provides a good foundation to explore potential molecular interactions between microglia and dendritic spines.

1. Introduction

Post-traumatic stress disorder (PTSD) is a debilitating mental health condition that occurs as a consequence of the experience of a traumatic event, and has a lifetime prevalence of 1.9–6.8% (Australian Bureau of Statistics, 2007). Commonly observed in combat veterans and emergency service workers, PTSD also afflicts victims of terrorism, natural disasters, sexual assault, and interpersonal violence (Pietrzak, 2014; Skogstad, 2013; Jaycox et al., 2002; Middleton and Craig, 2012). PTSD is incorporated into a Trauma and Stressor Related Disorders category of the Diagnostic and Statistical Manual of Mental Disorders V (American Psychiatric Association, 2013), and is characterised by four clusters of hallmark symptoms incorporatingintrusive thoughts of the traumatic event/s, hyperarousal, nightmares, anxiety, depression, and attentional and cognitive disturbances (American Psychiatric Association, 2013). A feature of PTSD is the persistent nature of these symptoms which may become progressively worse over time (Andrews, 2007; Bryant, 2013; Smid, 2009). Exposure therapy is considered the

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gold standard psychological treatment for PTSD, however 40% of patients fail to respond to this therapeutic approach (Stines et al., 2009; Joseph and Gray, 2008). Pharmacological therapies are also utilised but there are few drugs available and these have modest efficacy. Indeed, the only FDA approved drugs are the selective serotonin reuptake inhibitors (SSRIs) sertraline and paroxetine, which were not developed to specifically treat PTSD pathophysiology (Krystal, 2017). The crisis in PTSD drug development necessitates the discovery of novel pharmacological approaches (Krystal, 2017).

PTSD drug development would be improved by utilising better animal models that more faithfully reproduce the diversity of PTSD symptoms and their underlying neurobiology. One such model is that developed by Wotjak and colleagues which involves exposure of mice to a single "traumatic" electric footshock (Siegmund and Wotjak, 2006; Siegmund and Wotjak, 2007). This animal model demonstrates excellent face validity with trauma exposure triggering a constellation of behavioural phenotypes relevant to PTSD, such as long-lasting fear memory, hyperarousal, sleep disturbance, increased anxiety and depression-related behaviour, as well as social withdrawal (Siegmund and Wotjak, 2007; Golub, 2009, 2011; Polta, 2013; Sauerhofer et al., 2012; Pamplona, 2011; Kao, 2015). The Wotjak et al. model also demonstrates predictive validity as chronic fluoxetine reduced long-lasting contextual fear memories in this model, which was associated with reduced inflammatory gene expression and increased microglial cell density in the anterior cingulate cortex (Kao, 2016). The latter observations also provide a degree of construct validity for this model, as there is an emerging role for immune dysfunction in PTSD. PTSD patients are well-established to have increased plasma concentrations of proinflammatory cytokines including interleukin 1β (IL-1β), interleukin 6 (IL-6), and tumour necrosis factor alpha (TNF-α) (Passos, 2015; Maes, 1999; Gill, 2010), with the length of illness and symptom severity correlating with IL-1β and IL-6 plasma concentrations (Spivak, 1997; Gola, 2013; Uddin, 2010).

There is an emerging role for the complement cascade, a biochemical process of the innate immune system, in the pathophysiological response to psychological stress and trauma. Comprised of proteins in the blood and CNS, complement mediates immune system activation to eliminate invading pathogens (Hajishengallis, 2017). Activation of the classical pathway, initiated by the recognition molecule C1q, signals cleavage of downstream complement fragments (e.g. C4 and C3) increasing responsivity and chemotaxis of immune cells (Janeway et al., 2001). Dysregulation of complement can drive inflammatory responses which has been implicated in the pathogenesis of various mental disorders and neurodegenerative diseases (Hong et al., 2016; Clarke, 2018). Psychological stressors increase plasma concentrations of complement fragments C3a, C3c, C4, and C5a, and may contribute to stress-induced inflammation (Maes, 1997; Burns, 2008). Hyperactivation of the classical pathway has been found in PTSD patients evidenced by elevated plasma levels of complement C2 and C4 factors (Hovhannisyan, 2010). Complement component C4 is also increased in the brain of schizophrenia patients and is hypothesised to play a role in microglial cell-mediated engulfment of dendritic spines, the small protrusions found on neuronal dendrites that harbour excitatory synapses (Sekar, 2016; Nimgaonkar, 2017; Sellgren, 2016; Hakobyan et al., 2005; Mayilyan et al., 2008). It is noteworthy that stress is implicated both in the aetiology of schizophrenia and the worsening of the clinical course of the disorder (Schmitt, 2014).

Immune dysregulation and microglial cell-mediated loss of excitatory synapses may also play a role in stress and trauma-related disorders such as PTSD. Hippocampal volume reductions and loss of grey matter in PTSD patients are well described which might be explained by stress-induced synaptic regression (Kassem, 2013; O'Doherty, 2015). Here we investigated in an animal of PTSD whether contextual fear conditioning associated with a relatively high intensity footshock exposure promoted long-lasting dendritic spine regression in various stress-related brain regions. We also sought to determine whether this was associated with altered microglial cell density and morphology, and altered expression of the complement recognition molecule C1q and complement factor C4.

2. Methods

2.1. Mice

All procedures described herein have been approved by the University of Sydney's Animal Ethics Committee and were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (AEC# K21/3–2013/3/5923). Adult (~10 weeks old) male C57BL6J mice were housed in groups of 4–6, in a temperature-controlled facility on an inverse light/dark cycle. All procedures were undertaken during the animals active/awake dark phase (10:00–17:00). Animals were kept under specific pathogen free conditions, underwent routine health monitoring schedules, and were subject to a health sentinel programme. Animals were provided with food and water *ad libitum*, and environmental enrichment housing features such as cardboard tubing, climbing ring, and a mouse house. Mice were received from Animal Resources Centre, Perth and left to habituate for one week prior to experimental procedures. Animals were handled for 3 min for three consecutive days prior to commencing the experiment.

2.2. Experimental conditions and design

Mice were randomly allocated to one of two groups: control or shock (n = 18 per condition). A behavioural battery was conducted over a 32 day period (see Fig. 1). On the first day of experimentation animals were subjected to the fear conditioning protocol (see 2.2.2 below for detailed protocol). On day 2 (24 h following fear conditioning), both cohorts of mice were re-exposed to the conditioned context. Anxiety measures in the light-dark test were also assessed on day 2 approximately 2.5 h following re-exposure to the conditioned context. Mice were examined on day 3 for acoustic startle responses (ASR), and on day 4 in the prepulse inhibition of startle (PPI) paradigm (Fig. 1). Both conditions underwent an incubation period from days 5 to 27 where they remained undisturbed in their homecages. The behavioural battery was re-administered in the same format as described above on days 28–31. Twenty four hours after the final behavioural testing mice were sacrificed via cardiac perfusion or cervical dislocation (Fig. 1).

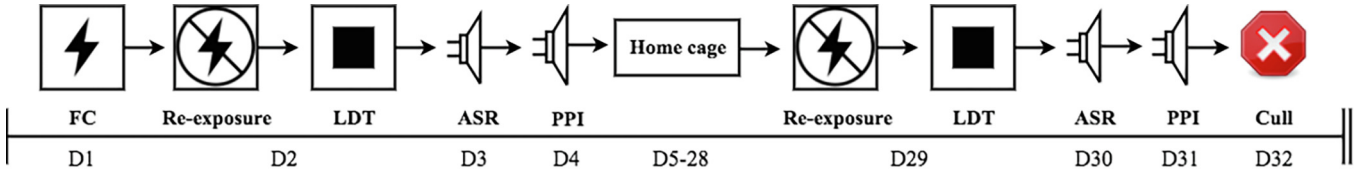

Fig. 1. Overview of the experimental protocol and behavioural battery over 32 days. Days 1–32 (D1-32); Contextual fear conditioning (FC); Re-exposure to shock-paired context (Re-exposure); Light-dark testing (LDT); Acoustic startle response (ASR); Prepulse inhibition of startle test (PPI); Home cage incubation period; Animals were culled on day 32 by cardiac perfusion or cervical dislocation (Cull).

2.2.1. Fear conditioning

Mice underwent contextual fear conditioning in a transparent rectangular plexiglass shock chamber with metal grid flooring as previously described (Golub, 2011; Brzozowska, 2017) (Clever Sys. Inc. USA). Briefly, animals were introduced into the conditioning chamber for 198 s, after which a single, inescapable scrambled electric foot shock (2 s, 1.5 mA) was administered via the metal grid flooring. Animals remained in the chamber for a further 60 s before being returned to their home cages. Mice in the control cohort followed the same design, with the exclusion of the shock. Chambers were cleaned with 70% ethanol between trials. For re-exposure, animals were introduced into the shock chamber for 3 min (in the absence of a shock) and freezing behaviours were measured. Chambers were cleaned with 70% ethanol between trials.

2.2.2. Light-dark test

Anxiety-related behaviours were analysed in the light-dark test. Testing was performed as previously described (Brzozowska, 2017; Chohan, 2014; Clarke, 2019). Briefly, animals were placed in the centre hide box insert (8.5 × 10 cm^2) at the beginning of testing before they freely explored the arena (45 × 45 × 26 cm^3) illuminated under a red light (40 lux) for 10 min. Animals were recorded, and videos subsequently quantified by the video analysis system TopScan (Clever Sys. Inc. USA), calculating total hide time and total distance travelled. Transitions into the hide box were scored manually by an experimenter blind to conditions. Arenas were cleaned with 70% ethanol between trials.

2.2.3. Acoustic startle responses

Acoustic startle testing was conducted in acoustic startle chambers (SR-Lab: San Diego Instruments, San Diego, USA) based on previously published methods (Golub, 2011). Animals were positioned in a small restraint tube inside the testing chambers and, after a 5 min acclimation period with a 70 dB constant background noise, they were exposed to the startle stimuli. A total of 70 trials were administered consisting of 10 control trials and 20 startle stimuli of varied intensity (75, 105, and 115 dB). Stimuli were presented in pseudorandom order in each testing session, with an average inter-trial interval of 15 s. Startle stimuli were presented as white noise bursts of 20 ms in duration. A piezoelectric transducer quantified startle responses and were recorded using SR-LAB software. All chambers were cleaned with 70% ethanol between sessions.

2.2.4. Prepulse inhibition of startle

PPI testing was performed to determine the effects of shock on sensorimotor gating as previously described by Brzozowska et al., (Brzozowska, 2017). Following a 5 min acclimation period, the mice underwent a session of 42 trials consisting of the following: no stimuli, acoustic startle only (120 dB of 40 ms duration), and the experimental PPI trials. The PPI stimuli were presented 100 ms before the 120 dB startle stimulus. The prepulse stimuli, 74, 78, and 82 dB, were presented in pseudorandomised order and were 20 ms in duration, with an average inter-trial interval of 15 s. %PPI was calculated as [(startle response 120 dB–PPI response) × 100/startle response 120 dB]. All chambers were cleaned with 70% ethanol between sessions.

2.3. Neurobiology and microscopy

2.3.1. Iba-1 immunohistochemistry and microglial cell analysis

For all immunostaining protocols described herein, mice were deeply anesthetised with isoflurane and transcardially perfused with phosphate-buffered saline, followed by 4% paraformaldehyde. Brains were removed and cryoprotected in graded sucrose solutions, initially submerged in a 15% concentration solution for 24 h, followed by a 30% solution over 72 h or until the brains sank. Brains were sliced coronally on a cryostat in 40 µm sections. For microglial analysis brain slices were incubated in ionized calcium-binding adapter molecule 1 (Iba-1) primary antibody (Iba-1 1:2000; WakoChem) overnight at 4 °C, followed by a biotinylated secondary antibody incubation (BA-1000 1:500; Vector Laboratories, USA) for 1 h at room temperature (Brzozowska, 2017). A peroxidase reaction was visualised with 3,3′-diaminobenzidine and tissue incubated in glucose oxidase (Sigma-Aldrich, Australia) for 10 min. Tissue was mounted on gelatinised glass slides prior to dehydration in graded alcohol solutions, clearing in xylene, and coverslipped with DPX (Sigma-Aldrich, Australia).

Iba-1^+ cell counts were performed manually using a light microscope by an experimenter blind to conditions with the use of a square graticule (0.5 mm × 0.5 mm), equating to 0.25 mm^2 when observed under a 20× objective. Brain regions analysed were identified using the Paxinos & Franklin mouse brain atlas (Paxinos, 2004). The specific locations of regions are as follows: dorsal hippocampus – situated in the segment of the cornu ammonis 1 (CA1) and cornu ammonis 3 (CA3) sub regions (bregma approximately −1.70 to −2.18); medial prefrontal cortex - selected from layers II/III in the infralimbic (IL) and prelimbic (PrL) regions (bregma approximately between 1.98 and 1.54); basolateral amygdala (BLA) – (bregma approximately between −0.82 and 1.82; see Fig. 2). Z stack images were captured by an experimenter blind to conditions with a 63x oil immersion objective (NA 1.40) via brightfield on a Zeiss confocal microscope 710 (LSM710) and were processed using the Zen 2010 software package (Carl Zeiss Microimaging, Jena, Germany). Confocal z stack images were imported into Neurolucida (MicroBrightField, Colchester, VT), where cells were automatically traced. Tracings were imported into Neurolucida Explorer to calculate length, volume, and number of intersections of microglial cell processes, with Sholl analysis data obtained through the application of concentric circles extending from cell soma every 5 µm.

2.3.2. Golgi Cox staining and dendritic spine analysis

For Golgi-Cox staining mice were deeply anesthetised with isoflurane and sacrificed via cervical dislocation. Whole brains were processed using an FD Rapid GolgiStain Kit™ (Neurotechnologies Inc. USA), as per the methods provided by the manufacturer. Brains were sliced at 100 µm using a cryostat, and sections collected on gelatinised glass slides before undergoing dehydration in absolute ethanol and being cleared in xylene, and coverslipping with Permount medium (Bioworld, USA) (Clarke, 2018; Chohan, 2014). Z stack images of Golgi-stained neurons were captured using a 40x water immersion objective via brightfield on a Zeiss Confocal Microscope 710 (LSM710) and were processed using the Zen 2010 software (Carl Zeiss Microimaging, Jena, Germany). Quantitative differences in the density of dendritic spines on individual dendritic branch orders between conditions was determined as previously described (Kassem, 2013). Briefly, dendritic spine densities were analysed by manual counts using Neurolucida and software (MicroBrightField, Colchester, VT) by an observer blind to conditions, from pyramidal neurons selected from the same regions as Iba-1^+ cell analyses. For each mouse, dendritic spines on apical dendritic protrusions were counted as a density per µm on orders 2 through 5. Six segments of dendrites were sampled per branch order. Spines were determined as being a minimum of 0.25 µm in length, not detached from the dendrite by more than 0.25 µm, and a maximum of 3 µm in width (Keifer, 2015; Harris and Kater, 1994; Stephan, 2013).

2.3.3. Complement immunofluorescence staining and analysis

For complement cascade immunostaining, anti-C1q (Abcam ab182451; 1:500; C1qKO validated) (Stevens, 2007), and anti-C4 (Abcam ab11863; 1:150) primary antibodies were used, with goat anti-rabbit Alexa Fluor 488 (ThermoFisher; 1:2000), and donkey anti-rat Alexa Fluor 647 (ThermoFisher; 1:1500) secondary antibodies, respectively (Clarke, 2018). Free floating paraformaldehyde fixed tissue sections were washed in phosphate buffer and blocked overnight in 3% bovine serum albumin (BSA; Sigma-Aldrich, Australia) with 0.1% sodium azide. Tissue was incubated in primary antibody for 48 h, washed

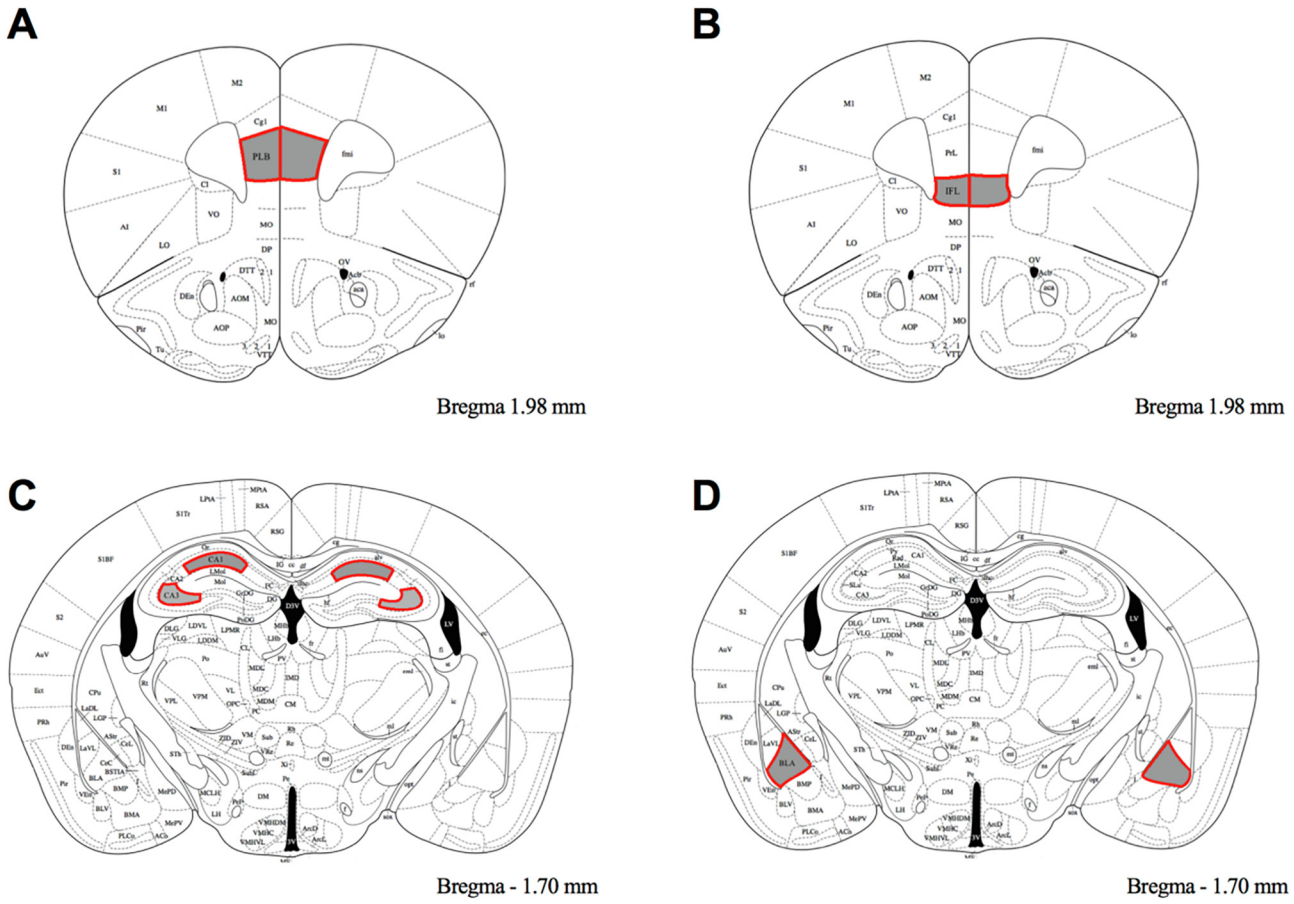

Fig. 2. Representations from Paxinos & Franklin mouse atlas of regions of interest analysed. (A) Prelimbic, (B) Infralimbic, (C) CA1 and CA3 regions of the dorsal hippocampus, and (D) Basolateral amygdala.

in phosphate buffer, and placed in secondary antibody for 24 h. Tissue samples were then washed in phosphate buffer, mounted on gelatinised slides and coverslipped with ProLong Gold Antifade mounting medium (Invitrogen). Immunofluorescent images were captured using a 20× water immersion objective (NA 0.95), on a Zeiss confocal microscope (LSM710). 488 and 633 nm lasers were utilised for fluorophore excitation of Alexa 488 and Alexa 647, and emissions were collected between 493 and 630 nm, and 638 nm and 755 nm, respectively. C1q and C4 image samples were macro batch processed using FiJi (Free Open Source Software, NIH, USA; Schindelin et al., 2012). The macro automated the process of splitting the RGB channel into individual channels and then measured the mean fluorescence intensity (MFI) in each respective channel. Following this, a region of interest was selected encompassing pyramidal cell bodies within the CA1 stratum pyramidale. Images were again macro batch processed.

2.4. Data analysis

Statistical analyses were performed using SPSS v25 (IBM) software. Behavioural testing was analysed with two-way repeated measures ANOVAs or linear mixed models (LMM) when data violated normality assumptions or subjects had missing data points. A between-subjects factor of condition (control verses shock), and within-subjects factors of time, intensity, radii etc were used. For acoustic startle testing, when a significant trauma by day of testing interaction was observed, LMM was performed to analyse individual days. Microglial cell counts and complement component fluorescence intensity were compared using independent samples t tests between the shock and control conditions, while Sholl analysis of microglial cell morphology and dendritic spine counts were analysed using LMM to accommodate normality violations and empty data points due to cell size differences between conditions. Results were deemed significant at $p < 0.05$. Multiple comparisons were adjusted using Bonferroni corrections.

3. Results

3.1. Foot shock promotes long-lasting conditioned fear, reduced locomotor activity, and increased startle responses in mice.

We first examined the effects of contextual fear conditioning as induced by a single electric footshock on behavioural responses in the short (2–4 days) and longer term (29–31 days post-shock exposure). A two-way repeated measures ANOVA demonstrated that, overall, fear conditioned mice displayed significantly higher freezing responses compared to controls, evidenced by a main effect of shock ($F_{1,34} = 46.51$, $p < 0.001$; Fig. 3A). Further, the conditioned fear response became more pronounced over days as supported by a condition by day of testing interaction on % freezing to the shock-paired context ($F_{1,34} = 6.33$, $p = 0.017$; Fig. 3A). We then assessed the impact of shock on anxiety-related behaviours in the light-dark test. A two-way repeated measures ANOVA indicated that shock exposure did not influence the total time spent hiding in the hide box ($p = 0.920$; Fig. 3B), however shocked animals displayed reduced distance travelled in the light-dark test arena as indicated by a significant main effect of shock ($F_{1,34} = 20.99$, $p < 0.0001$; Fig. 3C). The mice appeared to habituate to the light–dark test chambers over days as evidenced by an effect of day of testing ($F_{1.34} = 49.46$, $p < 0.0001$; Fig. 3C).

We then examined the effect of shock on startle responses to loud acoustic stimuli. LMM showed a significant main effect of acoustic stimulus intensity ($F_{2,65} = 106.42$, $p < 0.0001$; Fig. 3D), and more

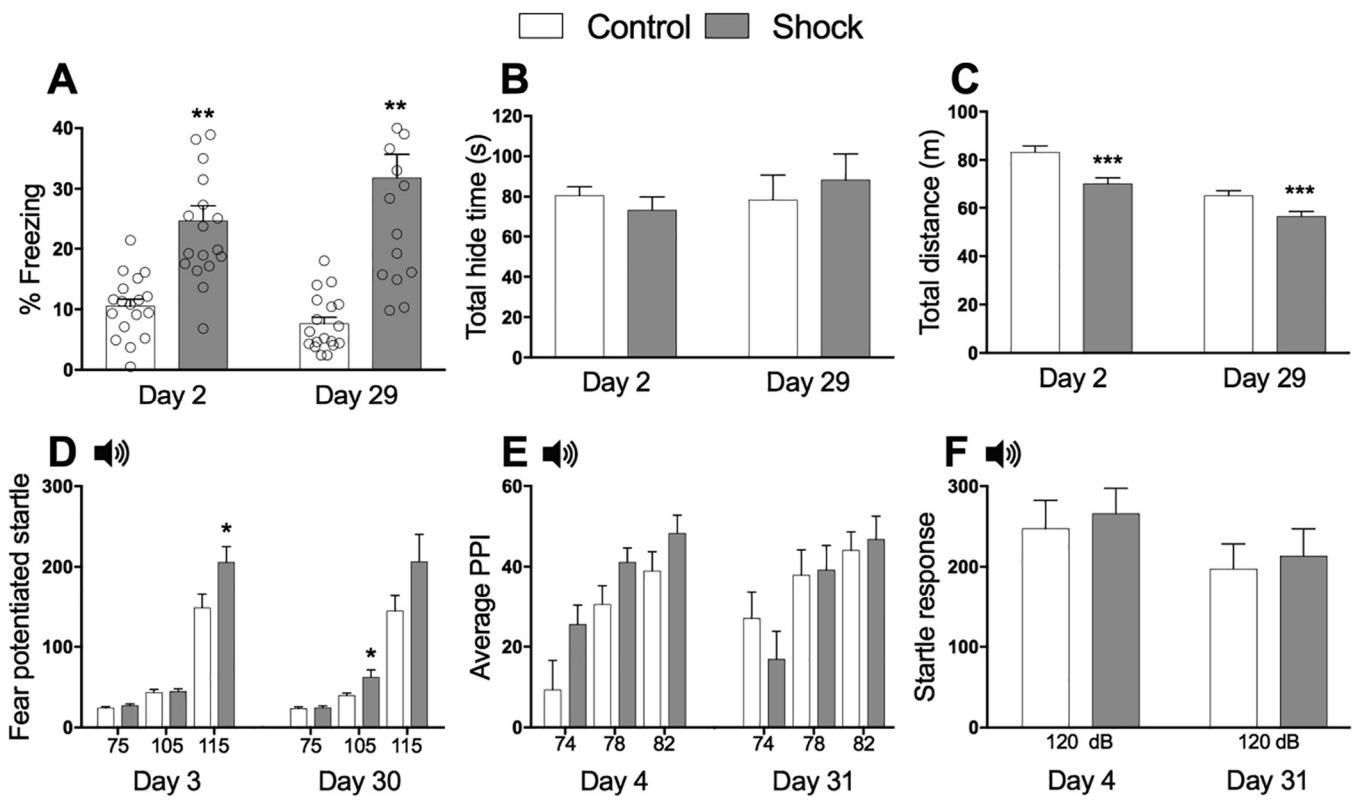

Fig. 3. Shock exposure increases contextual conditioned freezing, decreases locomotor activity, and induces exaggerated startle responses representative of PTSD symptomology. (A) Contextual freezing behaviours on days 2 and 29. (B) Total hide time and (C) total distance travelled in the light-dark test on days 2 and 29. (D) ASR to 75 dB, 105 dB, and 115 dB stimuli on days 3 and 30. (E) Average %PPI response to 74 dB, 78 dB, and 82 dB prepulse intensities and (F) startle response to a 120 dB acoustic stimulus, both on days 4 and 31. Data presented as mean + SEM *p < 0.05, ** < 0.01, *** < 0.001 (shock versus control). n = 18 per condition.

importantly a main effect of shock ($F_{1,67}$ = 8.99, p = 0.004; Fig. 3D), as shock generally increased the startle response to loud acoustic stimuli. A shock by acoustic stimulus intensity interaction was also observed ($F_{2,65}$ = 4.19, p = 0.019; Fig. 3D). Bonferroni comparisons confirmed that, averaged over intensity, shocked mice demonstrated significantly exaggerated startle responses to higher intensity acoustic stimuli than non-shocked control mice (i.e. at 115 dB on day 3 and 105 dB on day 30; Fig. 3D).

Examination of sensorimotor gating function as operationalised by PPI showed that average PPI increased with prepulse-intensity (LMM: main effect prepulse intensity: $F_{2,129}$ = 18.52, p < 0.0001; Fig. 3E). A shock by day of testing interaction was also observed ($F_{1,168}$ = 4.72, p = 0.031; Fig. 3E). As we observed a shock by day interaction we performed further analyses of the individual days tested. A main effect of shock was observed on day 4 consistent with shock facilitating PPI (LMM: $F_{1,83}$ = 8.56, p = 0.004). There was no effect of shock on day 31 (p = 0.685) (Fig. 3E). During PPI testing we also compared the startle responses of mice to the 120 dB acoustic stimulus, however no significant effects of shock were observed on either day of testing (LMM: p = 0.126; Fig. 3F).

Collectively, our results here show that shock-exposed mice demonstrated long-lasting PTSD-related behaviours, such as contextual fear conditioning, decreased locomotor activity, and exaggerated ASR to acoustic stimuli. We also report here for the first time that traumatic shock exposure promoted a short-term increase in PPI. These behavioural results provide a strong platform from which to explore associated neurobiological changes such as alterations in microglial cells and pyramidal neuron dendritic spine densities in various brain regions.

3.2. Shock increased microglial cell density and altered microglial cell morphology in the dorsal hippocampus and medial prefrontal cortex, but not in the basolateral amygdala

Following the behavioural battery mice were sacrificed to assess the effects of shock on neurobiology. Microglial cells demarcated by Iba-1 were examined in several stress-relevant brain regions. We quantified the density of microglial cells in each region, as well as conducting 3D reconstructions of the cells and performing Sholl analyses to assess changes in the morphology of the microglial cells. Sholl analysis involved superimposing concentric circle radii in 5 µm increments over microglial cell tracings and were used to compare the length, volume, and complexity of cellular processes between conditions.

Traumatic shock exposure significantly elevated the density of microglial cells in the CA1 and CA3 regions of the dorsal hippocampus (independent samples t-tests: t (10) = 5.08, p < 0.0001; t (10) = 2.35, p = 0.040; Fig. 4A and E). Further, shock increased microglial cell process length, volume and complexity as indicated by intersections in the CA1 and CA3 regions (Fig. 4B–D, F–H). This was supported by LMM analysis which showed significant main effects of shock, radius, and significant shock by radius interactions for process length, volume and intersections in both the CA1 and CA3 regions (see Fig. 4 for specific F and P values). Bonferroni comparisons confirmed that shock increased microglial process length, complexity and volume in the dorsal hippocampus across radii extending from the cell soma (Fig. 4B–D, F–H). Taken together, contextual fear conditioning associated with a traumatic shock exposure promoted robust changes in the density and morphology of microglial cells in the dorsal hippocampus.

In the medial prefrontal cortex, traumatic shock also altered the density and morphology of microglial cells, although the effects were more subtle in this region compared to the dorsal hippocampus. Shock significantly increased the density of microglial cells in the IL region (t

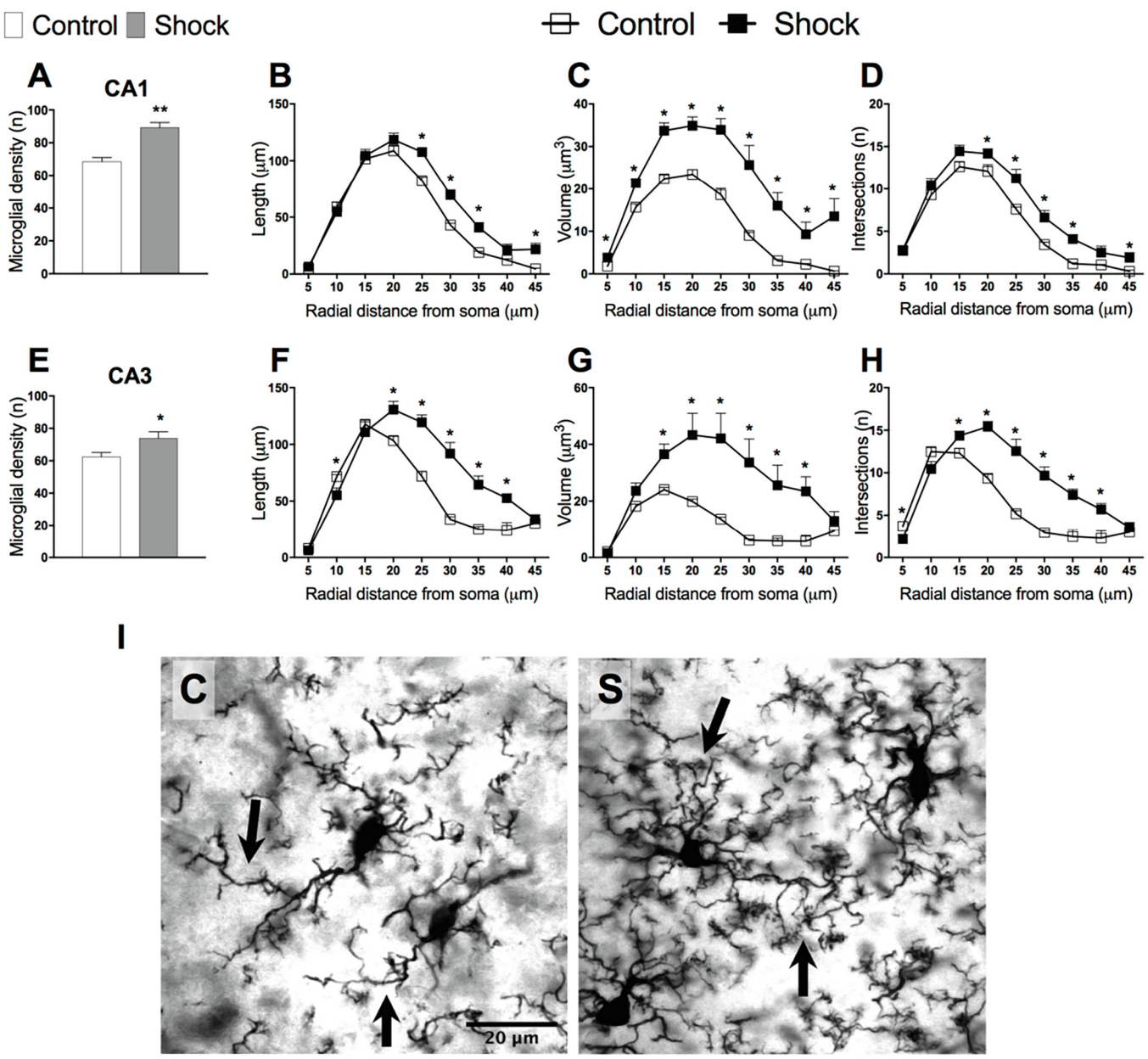

Fig. 4. Shock exposure elevated microglial cell density in the CA1 and CA3 regions of the dorsal hippocampus and induced robust microglial cell process elongation, complexity and volumetric expansion at 32 days. (A) CA1 microglial cell count and (B) radial process length (C) volume and (D) intersections. (E) CA3 microglial cell count and (F) radial process length (G) volume and (H) intersections. (I) Representative images of microglial cells in the CA1 region of the dorsal hippocampus from control and shock mice. Data presented as mean + SEM *$p < 0.05$. ** < 0.01 n = 6 per condition. For process length LMM analysis showed there were main effects of shock (CA1: $F_{1,67} = 41.79$, $p < 0.0001$; CA3: $F_{1,76} = 62.71$, $p < 0.0001$), and radius (CA1: $F_{8,19} = 365.04$, $p < 0.0001$; CA3: $F_{8,20} = 214.19$, $p < 0.0001$), and a shock by radius interaction (CA1: $F_{8,19} = 9.59$, $p < 0.0001$; CA3: $F_{8,20} = 14.24$, $p < 0.0001$). For volume there were main effects of shock (CA1: $F_{1,55} = 121.67$, $p < 0.0001$; CA3: $F_{1,57} = 58.23$, $p < 0.0001$), and radius (CA1: $F_{8,19} = 166.47$, $p < 0.0001$; CA3: $F_{8,24} = 68.72$, $p < 0.0001$), and a shock by radius interaction (CA1: $F_{8,19} = 8.70$, $p < 0.0001$; CA3: $F_{8,24} = 8.15$, $p < 0.0001$). Finally for complexity (intersections) there were main effects of shock (CA1: $F_{1,68} = 48.71$, $p < 0.0001$; CA3: $F_{1,70} = 83.03$, $p < 0.0001$), and radius (CA1: $F_{8,23} = 147.90$, $p < 0.0001$; CA3: $F_{8,20} = 109.60$, $p < 0.0001$), and a shock by radius interaction (CA1: $F_{8,22} = 3.74$, $p = 0.006$; CA3: $F_{8,20} = 15.85$, $p < 0.0001$).

(10) = 2.54, p = 0.029 Fig. 5A) but not in the PrL (p = 0.062, Fig. 5E). Moreover, shock increased microglial cell process length, volume and complexity (intersections) in both the IL and PrL (Fig. 5B–D, F–H). This was supported by LMM analysis which showed significant main effects of shock, radius, and significant shock by radius interactions for these parameters in both the IL and PrL (see Fig. 5 for specific F and P values). Bonferroni corrected pairwise comparisons confirmed that contextual fear conditioning associated with exposure to a traumatic shock increased microglial process length, complexity and volume across radii extending from the cell soma in the medial prefrontal cortex (see Fig. 5B–D, F–H).

Traumatic shock exposure did not influence the density of microglial cells in the BLA (Fig. 6A). LMM analysis showed that shock significantly influenced the length of the processes as supported by main effects of shock, radius and a shock by radius interaction (see Fig. 6 for specific F and P values). Bonferroni comparisons showed there were subtle increases in process length in proximal (10 μm) and distal processes (40 μm) from the microglial cell soma, although at intermediate distances from the soma (15 and 25 μm), where the microglial cells processes are most complex and dense, shock exposure decreased

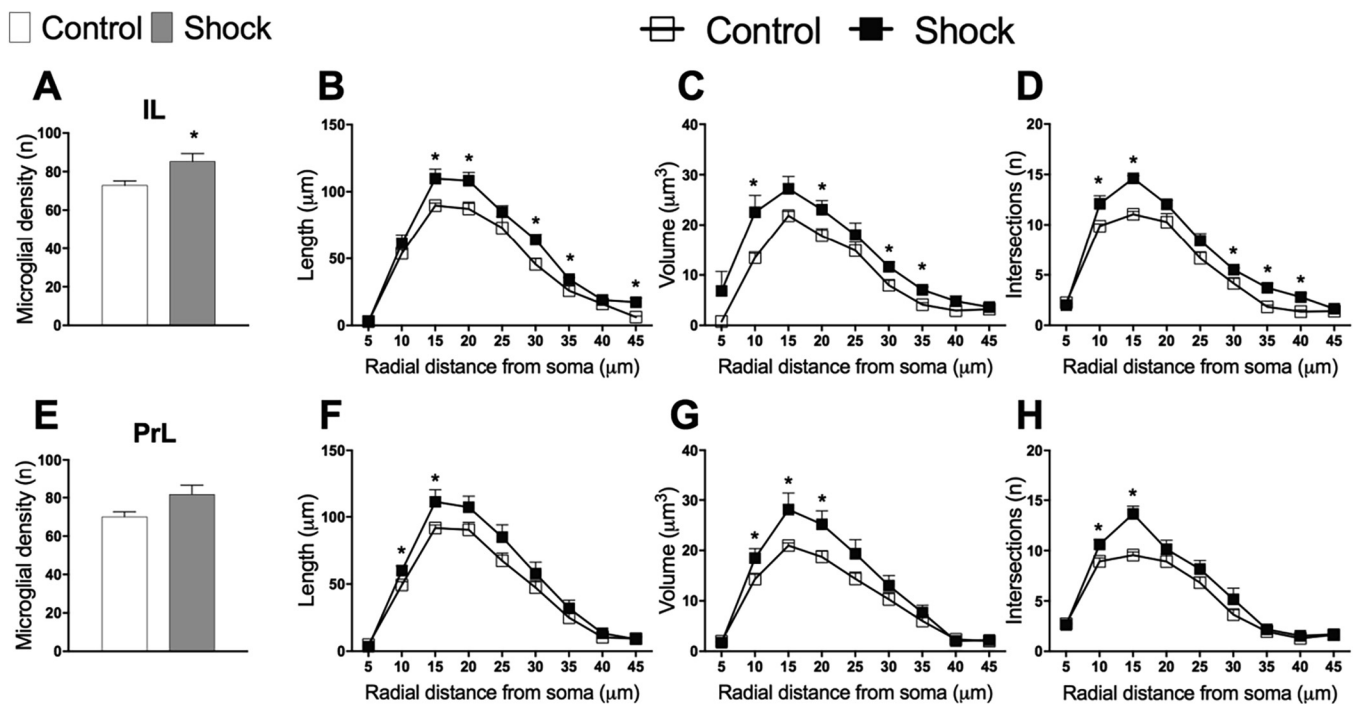

Fig. 5. Shock exposure elevated microglial cell density in the IL cortex and induced process elongation, complexity and volumetric expansion across the medial prefrontal cortex (both IL and PrL regions) at 32 days post-shock exposure. (A) IL microglial cell count and (B) radial process length (C) volume and (D) intersections. (E) PrL microglial cell count and (F) radial process length (G) volume and (H) intersections. Data presented as mean + SEM *p < 0.05. n = 6 per condition. For process length LMM analysis showed there were main effects of shock (IL: $F_{1,53} = 31.84$, p < 0.0001; PrL: $F_{1,54} = 14.17$, p < 0.0001), and radius (IL: $F_{8,24} = 287.79$, p < 0.0001; PrL: $F_{8,20} = 193.16$, p < 0.0001), and a shock by radius interaction (IL: $F_{8,24} = 5.297$, p = 0.001; PrL: $F_{8,20} = 2.80$, p = 0.03). For volume there were main effects of shock (IL: $F_{1,51} = 28.74$, p < 0.0001; PrL: $F_{1,54} = 19.29$, p < 0.0001), and radius ($F_{8,26} = 67.33$, p < 0.0001; $F_{8,13} = 85.64$, p < 0.0001). For complexity (intersections) there were main effects of shock (IL: $F_{1,55} = 54.86$, p < 0.0001; PrL: $F_{1,55} = 18.63$, p < 0.0001) and radius (IL: $F_{8,21} = 367.12$, p < 0.0001; PrL: $F_{8,19} = 18.93$, p < 0.0001), and a shock by radius interaction (IL: $F_{8,21} = 8.72$, p < 0.0001; PrL: $F_{8,19} = 3.63$, p = 0.01).

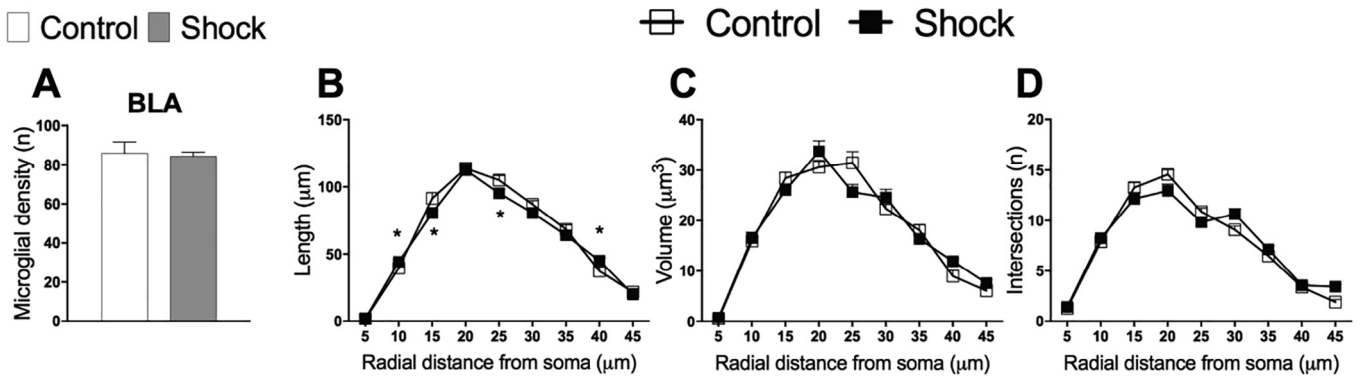

Fig. 6. Shock exposure does not promote marked changes in microglial cell density and morphology in the BLA at 32 days post-shock exposure. (A) BLA microglial cell count and (B) radial process length (C) volume and (D) intersections. Data presented as mean + SEM *p < 0.05. n = 6 per condition. For process length, LMM analysis showed main effects of shock ($F_{1,69} = 5.480$, p < 0.022), and radius ($F_{8,20} = 1342.09$, p < 0.0001), and a shock by radius interaction ($F_{8,20} = 3.58$, p = 0.009). A main effect of radius was also found for volume ($F_{8,20} = 650.18$, p < 0.0001) and intersections ($F_{8,23} = 463.84$, p < 0.0001) in the BLA, as well as a shock by radius interaction for both volume ($F_{8,20} = 2.67$, p = 0.036) and intersections ($F_{8,23} = 4.75$, p = 0.002).

process length (Fig. 6B). LMM analysis showed that shock did not influence volume or complexity of microglial cells.

3.3. Shock reduced the density of dendritic spines on the apical dendrites of pyramidal neurons in the dorsal hippocampus and medial prefrontal cortex

We then assessed whether traumatic shock exposure modified the density of dendritic spines on apical dendrites of pyramidal neurons in the dorsal hippocampus, medial prefrontal cortex and basolateral amygdala. As evidenced by main effects of shock in the LMM analysis, shock decreased dendritic spine densities in all sub-regions of the dorsal hippocampus and medial prefrontal cortex, but not in the BLA (see Fig. 7 for specific F and P values). Bonferroni comparisons indicated that dendritic spine loss occurred on higher fourth and fifth order dendrites within both the dorsal hippocampus and medial prefrontal cortex (Fig. 7A–D).

3.4. Shock elevated complement component C1q levels in the CA1 region of the dorsal hippocampus

Accumulating research points to a role of the immune system in the pruning of dendritic spines. One hypothesis is that the complement cascade plays a role in dendritic spine removal via tagging and signaling mechanisms that instruct microglia to engulf dendritic spines

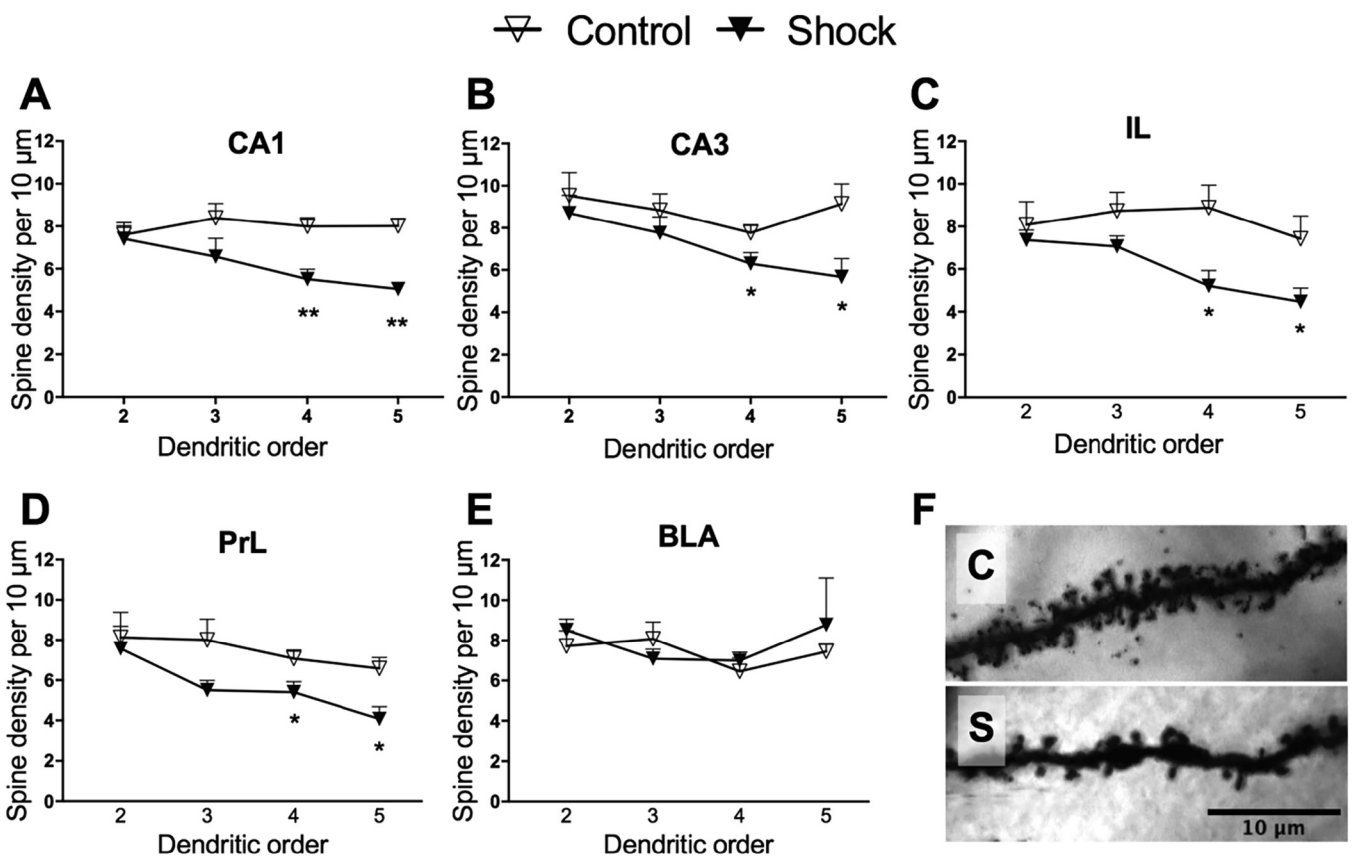

Fig. 7. Shock reduced the density of dendritic spines on apical dendrites of pyramidal neurons of the dorsal hippocampus and medial prefrontal cortex, but not in the basolateral amygdala. Line graphs displaying spine density reductions in trauma-exposed mice across dendritic orders 2 to 5 in the (A) CA1, (B) CA3, (C) IL, (D) PrL, and (E) BLA. (F) Representative images of fourth order dendrites in the CA1 region of the dorsal hippocampus. Data are presented as mean + SEM *p < 0.05 ** < 0.01, n = 6 per group. LMM analysis showed traumatic shock decreased dendritic spine densities (main effect of shock – CA1: $F_{1,\ 29}$ = 22.45, p < 0.0001; CA3: $F_{1,35}$ = 15.73, p < 0.0001; IL: $F_{1,39}$ = 14.41, p = 0.001; PrL: $F_{1,26}$ = 9.97, p = 0.004). There was a main effect of dendritic branch order in the CA3 and PrL regions (CA3: $F_{3,20}$ = 6.04, p = 0.004 and PrL: $F_{3,25}$ = 3.11, p = 0.044).

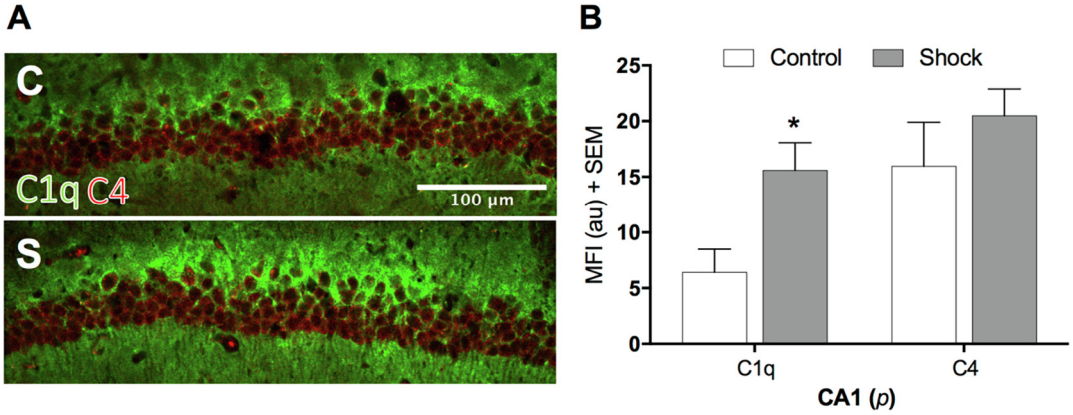

Fig. 8. Shock upregulates C1q expression in the stratum pyramidale of the CA1 region of the dorsal hippocampus. (A) Mean fluorescence intensity expression levels of C1q (green) and C4 (red) in the stratum pyramidale of the CA1 region between control (C) and shocked (S) mice. (B) Mean fluorescence intensity of C1q and C4 expression between conditions. *p < 0.05 for significant differences between control and shocked mice. Data are presented as mean + SEM, n = 6 per group (or interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

(Dykman et al., 1997). To determine a possible molecular link between microglial cell changes and concurrent dendritic spine loss, we analysed expression of the complement cascade components C1q and C4. Shock did not influence C1q or C4 expression in a given field of view in any of the brain regions analysed (CA1 (p = 0.21; 0.75), CA3 (p = 0.49; 0.84), IL (p = 0.71; 0.80), PrL (p = 0.79; 0.67) or BLA regions (p = 0.43, 0.50 respectively)). However, when narrowing in specifically on the cell body layer of the CA1 region of the dorsal hippocampus (stratum pyramidale; Fig. 8A) we observed that shock significantly increased C1q expression (t (10) = 2.8, p = 0.018, Fig. 8B) but not C4 expression (p > 0.35; Fig. 8B).

4. Discussion

Exposure to a single electric footshock led to enduring contextual fear memory, hyperarousal and reduced locomotor activity in mice. In

addition, shock transiently facilitated sensorimotor gating function. These PTSD-related behavioural phenotypes were associated with pronounced increases in microglial cell density and hyper-ramification, as well as dendritic spine regression on pyramidal neurons in the medial prefrontal cortex and the dorsal hippocampus. In addition, shock increased expression of the complement recognition molecule C1q in the pyramidal cell layer of the CA1 region of the dorsal hippocampus.

Our findings further reinforce research showing that exposure to a single electric footshock in C57Bl6J mice produces a wide range of behaviours with excellent face validity in modelling PTSD symptoms (Siegmund and Wotjak, 2006, 2007; Golub, 2011; Kao, 2016). PTSD patients show enduring fear responses with some evidence of sensitization of these responses over time (Bremner, 1995; Lissek and van Meurs, 2015; Poulos, 2016). Here we show that a single pairing of an electric footshock with a particular context led to a long-lasting conditioned fear response up to 29 days post-shock exposure, and further that this response appeared to undergo a degree of sensitization during the incubation period (Siegmund and Wotjak, 2007; Balogh and Wehner, 2003; Balogh, 2002; Shalev, 2000). Hyperarousal is commonly observed in PTSD patients (Kohl, 2013; Herrmann, 2012) and was reproduced here, as shocked mice displayed greater startle responses to loud acoustic stimuli than control mice in the short (3 days post-shock exposure) and longer term (30 days post-shock exposure). This accords with prior research demonstrating this phenomenon in mice (Golub, 2011; Grillon, 1998). It should be noted that ethanol was used to clean both the contextual fear chambers and the acoustic startle chambers in our study. Hence, the residual ethanol odour in the startle chamber may have inadvertently served as a conditioned olfactory stimulus that triggered the hyperarousal response, rather than the stress of the shock exposure *per se* (Pamplona, 2011).

There are conflicting findings on the nature of sensorimotor gating in PTSD patients, with studies reporting either improved, disrupted or normal function (Herrmann, 2012; Grillon, 1996, 1998; Ornitz and Pynoos, 1989; Butler, 1990; Holstein, 2010; Lipschitz, 2005; Bakshi, 2012). A novel behavioural finding reported in the present study is that traumatic shock exposure promoted a transient enhancement in sensorimotor gating function, with the shocked mice displaying facilitated PPI on day 4 but not later on day 31 of testing. This suggests that shock in the short-term may have increased the salience of the prepulse stimulus thus enhancing PPI. Few studies have assessed whether stress exposure or fear conditioning impacts upon sensorimotor gating function. Our own laboratory has assessed the effects of injection stress or restraint stress on PPI in mice but could not resolve any effects of these different types of stressful stimuli (Brzozowska, 2017; Chohan, 2014). It might be that traumatic stressors of greater magnitude are required to influence PPI. Consistent with this is the observation that predator exposure in rats was able to modulate PPI function (Hinwood, 2013). Our findings here suggest that, at least in mice, footshock exposure transiently facilitates sensorimotor gating function. This could not be explained by a shock-induced increase in startle responsivity, as the mice did not display an increased startle response to the 120 dB startling stimulus used in the PPI paradigm.

We then sought to examine whether the enduring PTSD-related behavioural deficits observed were associated with alterations in microglial cells and dendritic spine densities in various fear and stress-related brain regions. Other stressors such as chronic restraint stress are well-established to promote microglial cell activation and loss of dendritic spines in the medial prefrontal cortex and hippocampus (Clarke, 2018; Tynan, 2010; Radley, 2006; Milior, 2016). Here we aimed to assess whether a single footshock stressor could have similar effects. Overall, shock had pronounced effects upon microglial cells in the dorsal hippocampus and medial prefrontal cortex. Shock exposure increased microglial cell density, as well as increasing the length and volume of microglial cell processes and their overall complexity consistent with a hyper-ramified phenotype. However, traumatic shock exposure had very little impact on microglial cells in the amygdala, at least in the basolateral nuclei. Correlating well with the profile of results on microglial cells, shock reduced dendritic spine density on pyramidal neurons in the medial prefrontal cortex and dorsal hippocampus, however it did not influence dendritic spine density in the BLA. These results are consistent with research showing that stress exposure promotes concomitant microglial cell activation and dendritic spine regression in the medial prefrontal cortex and hippocampus but not in the amygdala (Clarke, 2018; Bian, 2012).

Our results elaborate on research showing traumatic shock exposure in mice increased the density of microglial cells in the anterior cingulate cortex 64 days post-shock exposure (Kao, 2016). Here we provide evidence of shock-induced microglial cell changes in the adjacent structures of the medial prefrontal cortex, that is, the PrL and IL cortices, as well as in the CA1 and CA3 regions of the dorsal hippocampus (Clarke, 2018; Radley, 2006; Walkera et al., 2013). The exact functional role of microglial cells on the PTSD-related behavioural changes observed in this mouse model of PTSD need to be better elucidated. One theory is that stress-induced activation of microglial cells causes the release of inflammatory cytokines that disturbs neuronal function, a theory consistent with the emerging role of inflammation in PTSD (Passos, 2015; Maes, 1999; Gill, 2010). Indeed, increased inflammatory gene expression in the anterior cingulate cortex has been observed in this mouse model of PTSD, and chronic fluoxetine treatment normalised these inflammatory changes and abolished long-term conditioned fear (Kao, 2016). However conflicting with these findings, here we report using the same model that shock exposure promotes microglial cell hyper-ramification, a phenotype that is not usually associated with neuroinflammation, but rather non-pathological, experience-dependent changes (Gupta, 2018). Hypo-ramified reactive microglial cells secrete pro-inflammatory mediators and are commonly observed in brain injury models; although there are rare reports of hyper-ramified microglial cells being observed (Bremner, et al., 2007). Future studies are needed to reconcile the hyper-ramified microglial cell morphology observed here with the increased neuroinflammatory status that prior research has demonstrated in this mouse model of PTSD.

Using an identical mouse model of PTSD, Golub (2011) reported that shock exposure reduced the volume of the hippocampus in mice, mirroring changes that have been reported in PTSD patients (O'Doherty, 2015; Preston and Eichenbaum, 2013). In addition, shock exposure in this model was associated with a long-term reduction in various hippocampal synaptic proteins such as synapsin Ia-b/IIa, synaptophysin and homer 1b/c (Grillon, 1998), as well as transcriptomic-quantified synaptic vesicle dysfunction (Kao, 2016). Consistent with these findings, here we demonstrate for the first time that shock reduces the density of dendritic spines in the CA1 and CA3 regions of the dorsal hippocampus. In addition, we report that shock reduced dendritic spine densities in the PrL and IL regions of the medial prefrontal cortex. Both the dendritic spine losses in the medial prefrontal cortex and the dorsal hippocampus may contribute to the behavioural disturbances observed in response to stress exposure (Frankfurt and Luine, 2015; Vidal-Gonzalez, 2006; Paolicelli, 2011).

The shock-induced microglial cell changes might directly contribute to the dendritic spine regression we observed in the medial prefrontal cortex and the dorsal hippocampus. Microglial cells phagocytose synaptic elements and contribute to synaptic remodelling during early stages of neurodevelopment and in response to environmental challenges such as stress (Schafer, 2012; Tremblay et al., 2010; Wohleb, 2018). Indeed, recent studies using the chronic unpredictable stress model (CUS) demonstrated that stress exposure triggers microglial cell engulfment of synapses which subserve disturbances in anxiety and depression-related behaviour (Bian, 2012; Vertes, 2006). The present study extends on these results by showing that another type of stressor, that is footshock exposure, also promotes microglial cell hyper-ramification and reductions in dendritic spines in the medial prefrontal cortex and dorsal hippocampus, both regions of the brain that are well-established to regulate emotional, cognitive and behavioural responses to

stress (Alexander, 2008). A limitation of the current study is that we can not draw any causal inferences between the shock-induced changes in microglial cells and pyramidal neuron dendritic spines. One possible means to address this would be to utilise agents that inactivate microglial cells such as minocycline. Indeed, minocycline inhibits chronic restraint stress-induced hyper-ramification of microglial cells (Tynan, 2010); therefore a future study could attempt to observe whether minocycline treatment reverses both shock-induced microglia cell hyper-ramification and dendritic spine atrophy in this mouse model of PTSD.

Recent studies have begun probing the molecular basis for microglia-synapse interactions by disrupting neuronal and microglial signalling molecules. For instance, viral knockdown of neuronal colony stimulating factor 1 (CSF1) reduced stress-induced microglial cell-mediated phagocytosis of neuronal elements (Vertes, 2006). Further, disruption of fractalkine signalling through knockout of the chemokine CX3CR1 in mice reduced stress-induced microglia-neuron interactions (Bian, 2012). The complement pathway might also be involved in microglial cell-synapse interactions, as this pathway plays a role in microglial cell engulfment of dendritic spines during both early life development and in response to disease (Hong, et al., 2016; Dykman et al., 1997; Qing, 2018). Our results here provide evidence that the complement factor C1q is involved in the stress response, as shock increased the expression of C1q in the pyramidal cell layer of the CA1 region of the dorsal hippocampus. This confirms a recent report of chronic stress increasing the expression of C1q mRNA in the hippocampus of mice (Michailidou, 2015). C1q has been suggested to be a signalling molecule responsible for the loss of hippocampal synapses due to the strong correlation between increased C1q expression and decreased synaptic density in multiple sclerosis patients (Nava, 2017). Moreover, C1q was found in microglia that had close proximity to the cell body of neurons, which is interesting given that in the present study the increased C1q expression was only resolved in the pyramidal cell body layer of the CA1 region of the hippocampus. Future studies are needed to better elucidate the role of C1q signalling in the stress response, and to demonstrate unequivocally that C1q is involved in microglia mediated engulfment of neuronal dendritic spines in response to shock.

Another limitation of the present study is that it can not clearly dissociate the effects of stress versus simple fear learning on the neurobiological changes observed. However, the pattern of increased microglial cell density, as well as losses of dendritic spines in the hippocampus and medial prefrontal cortex, are highly reminiscent of changes observed following chronic stressors rather than changes induced by contextual fear conditioning (Clarke, 2018; Kassem, 2013). Similar to the present profile of results, we have shown that chronic restraint stress robustly reduces pyramidal neuron dendritic spine density associated with increased microglial cell counts and size in the medial prefrontal cortex and the dorsal hippocampus but not in the basolateral amygdala (Clarke, 2018; Kassem, 2013). Contextual fear learning at lower shock intensities (e.g. 0.3–0.8 mA) transiently *increases* dendritic spine densities in the hippocampus, prefrontal cortex and amygdala (Abate, 2018; Trabalza, 2012; Restivo, 2009), and has no impact in the longer-term, even when the mice were re-exposed to shock-paired context 36 days post-conditioning (albeit longer term exposure was associated with an increased dendritic spine density in the anterior cingulate cortex) [93]. Therefore, while a contextual learning component can not be ruled out, our findings are most likely explained by the stress associated with exposure to a higher intensity shock stimulus.

In conclusion, the present study further elaborates on our understanding of a mouse model of PTSD by showing that traumatic shock exposure promotes long-term behavioural disturbances relevant to PTSD, as well as defining concomitant microglial cell changes and dendritic spine regression in the medial prefrontal cortex and dorsal hippocampus. It also provides further evidence that stress increases dorsal hippocampal C1q expression, suggesting a role for the complement cascade in traumatic stress responses.

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Chapter 3. Genetic deletion of P-glycoprotein alters stress responsivity and increases depression-like behavior, social withdrawal and microglial activation in the hippocampus of female mice

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Genetic deletion of P-glycoprotein alters stress responsivity and increases depression-like behavior, social withdrawal and microglial activation in the hippocampus of female mice

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ABSTRACT

P-glycoprotein (P-gp) is an ABC transporter expressed at the blood brain barrier and regulates the brain uptake of various xenobiotics and endogenous mediators including glucocorticoid hormones which are critically important to the stress response. Moreover, P-gp is expressed on microglia, the brain's immune cells, which are activated by stressors and have an emerging role in psychiatric disorders. We therefore hypothesised that germline P-gp deletion in mice might alter the behavioral and microglial response to stressors. Female P-gp knockout mice displayed an unusual, frantic anxiety response to intraperitoneal injection stress in the light-dark test. They also tended to display reduced conditioned fear responses compared to wild-type (WT) mice in a paradigm where a single electric foot-shock stressor was paired to a context. Foot-shock stress reduced social interaction and decreased microglia cell density in the amygdala which was not varied by P-gp genotype. Independently of stressor exposure, female P-gp deficient mice displayed increased depression-like behavior, idiosyncratic darting behavior, age-related social withdrawal and hyperactivity, facilitated sensorimotor gating and altered startle reactivity. In addition, P-gp deletion increased microglia cell density in the CA3 region of the hippocampus, and the microglial cells exhibited a reactive, hypo-ramified morphology. Further, female P-gp KO mice displayed increased glucocorticoid receptor (GR) expression in the hippocampus. In conclusion, this research shows that germline P-gp deletion affected various behaviors of relevance to psychiatric conditions, and that altered microglial cell activity and enhanced GR expression in the hippocampus may play a role in mediating these behaviors.

1. Introduction

P-glycoprotein (P-gp) belongs to the ATP-binding cassette (ABC) superfamily of transporter proteins and is expressed at chemical barriers in the body. Encoded by *MDR1* in humans, the two rodent gene isoforms *mdr1a* and *mdr1b*, together encompass the total expression pattern of *MDR1* (Devault and Gros, 1990). Expressed in brain endothelial cells that comprise the blood brain barrier (BBB), P-gp actively extrudes various xenobiotics and endogenous mediators from brain tissue back into the peripheral blood compartment (Chen et al., 2016; Szakács et al., 2008). Many CNS drugs including antidepressants, anticonvulsants and antipsychotic drugs are P-gp substrates, which limits their brain accumulation and may contribute to treatment-resistance in patients (Doran et al., 2005; Suzuki et al., 2013). In addition, there is a growing list of endogenous P-gp substrates, such as the hormones cortisol, progesterone and testosterone, all of which facilitate normal neurodevelopment and various physiological processes in mature animals including moderation of the stress response (Deak et al., 2015; Mason et al., 2012; Uhr et al., 2002; Van Kalken et al., 1993).

Accumulating evidence reinforces the notion that P-gp dysfunction might alter normal neurobehavioral function and tolerability to stress (de Klerk et al., 2010; Muller et al., 2003; Schoenfelder et al., 2012; Thoeringer et al., 2007). Pharmacological inhibition of P-gp decreased anxiety-related behaviors that are associated with increased brain accumulation of corticosterone, the rodent

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form of cortisol (Thoeringer et al., 2007). Germline genetic deletion of P-gp involving double knockout (KO) of both *mdr1a* and *mdr1b* in mice altered stress-coping behavior in conventional animal models of depression (Schoenfelder et al., 2012). Although, P-gp KO mice did not differ from wild-type (WT) mice in anhedonic models of depression implying the effects of P-gp deletion may be specific to stress (Klein et al., 2015). P-gp KO mice displayed resilience to stress, as these mice had reduced stress-induced adrenocorticotropic hormone (ACTH) and corticosterone concentrations compared to wild-type (WT) mice (Muller et al., 2003). In addition, P-gp KO mice exhibited reduced expression of corticotropin-releasing hormone in the paraventricular nucleus of the hypothalamus and increased whole brain glucocorticoid receptor (GR) expression (Schoenfelder et al., 2012). These results pointing to dysregulation of the glucocorticoid-GR system in P-gp KO mice were obtained from studies that solely used male mice, therefore an additional aim of the current study is to confirm such dysregulation in female mice.

Apart from its localisation at the BBB, P-gp is also expressed on microglia, the brain's innate immune cells (Ballerini et al., 2002; Dallas et al., 2004, 2003; Gibson et al., 2012; Lee et al., 2001). Little is known about the consequences of P-gp dysfunction in microglial cells, and whether it affects their cellular function in the brain. Interestingly, immune challenges may impair P-gp-mediated efflux in microglial cells, favouring the intracellular accumulation of P-gp substrates (Lee and Bendayan, 2004). More research is needed to delineate the role of P-gp on microglial cell function. One possibility is that P-gp facilitates chemical communication between microglia and neurons. Another is that P-gp may moderate stress-induced activation of microglia (Calcia et al., 2016; Kao et al., 2016; Reader et al., 2015; Tynan et al., 2010). Aberrant glucocorticoid-GR system signalling in P-gp KO mice provides an alternative means to alter microglial function in these mice, given GRs modulate microglial cell function (Barrientos et al., 2015; Nakatani et al., 2012).

The first aim of the present study is to provide a more comprehensive behavioral characterisation of P-gp KO mice in the presence and absence of stress. Thus far the neurobehavioral profile of P-gp KO mice has been restricted to male mice, so here we aim to extend this work by examining female mice to address whether the effects of P-gp deletion might be sexually dimorphic. Moreover, we will examine brain GR expression to assess whether the finding of upregulated GR expression in male mice extends to female P-gp KO mice (Schoenfelder et al., 2012). We will assess whether P-gp deletion moderates the response to a single intraperitoneal (i.p) injection stressor. The impact of deletion of another closely related ABC transporter, breast cancer resistance protein (Bcrp), will also be assessed to determine whether stress effects are P-gp specific. In addition, we will assess the impact of P-gp deletion in an animal model of post-traumatic stress disorder (PTSD). Mice in this model are exposed to a single-electric foot-shock and develop an enduring conditioned fear response to the stress-related environment, as well as social withdrawal and hyperarousal (Siegmund and Wotjak, 2007). The final aim of the current study is to examine whether P-gp deletion influences microglial cell density and morphology, alone or in response to stress.

2. Methods

2.1. Mice

46 P-gp KO (*Mdr1a/b-/-)* mice, 24 Bcrp KO *(Bcrp1-/-)* mice and 42 wild-type (WT, FVB/NJ background strain) mice were used in this study (Brzozowska et al., 2016; Spiro et al., 2012). The ABC transporter KO and WT mice were bred in-house at the University of Sydney under identical rearing conditions. Founder mice were purchased from Taconic farms (New York, USA) and were originally developed at the Netherlands Cancer Institute (Schinkel et al., 1997). Mice were all female and age-matched to be between 3 and 4 months of age. They were housed 3–4 mice per cage in a reversed 12:12 light-dark cycle in a temperature-controlled environment. All behavioral testing was conducted at approximately the same time each day between 10 am and 4 pm. Animals had unrestricted access to food and water and were housed in an enriched environment (igloo, nesting material, sunflower seeds and toilet paper roll). Mice were handled daily for seven days prior to the commencement of experimentation and habituated to the testing room for at least one hour before testing. All research and animal care procedures were approved by the University of Sydney Animal Ethics Committee and complied with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.*

2.2. Experimental design

2.2.1. Experiment 1: The effects of acute injection stress on sensorimotor gating and anxiety-related behavior in P-gp and Bcrp KO mice

We first examined whether germline deletion of P-gp and Bcrp modulated the effects of acute intraperitoneal (i.p.) injection on sensorimotor gating in the prepulse inhibition of startle (PPI) paradigm and anxiety-related behavior in the light-dark test. Details of each of the behavioral measures are described below. WT, P-gp and Bcrp KO mice received no injection or an i.p injection of saline 5 min before behavioral testing in the PPI chambers and were tested for 30 min followed by 10 min in the light-dark test.

2.2.2. Experiment 2: The effects of foot-shock stress on fear memory, acoustic startle, and anxiety, social and depression-related behavior in P-gp KO mice

We assessed the short-term and long-term behavioral effects of exposure to a single electric foot-shock, an animal model of PTSD, in WT and P-gp KO mice (Siegmund and Wotjak, 2007). Mice did or did not receive an electric foot-shock in a fear conditioning chamber on day 0. 24 h later on day 1 the animals' expression of contextual fear conditioning was tested by placing the mice in the previously shock-paired context in the absence of shock and measuring freezing. On the same day approximately 10 min after the fear conditioning test, mice underwent testing in the light-dark test. On day 2 mice were examined for social interaction, on day 3 for acoustic startle responses and on day 4 for depression-related behavior in the forced swim test. Mice remained in their home cages for the following 18 days and then underwent another battery of behavioral tests with the expression of fear conditioning and light-dark test being conducted on day 22, social interaction on day 23, acoustic startle testing on day 24, and the forced swim test on day 25. Mice were sacrificed on day 30 for microglial cell analysis.

2.3. Behavioral tests

2.3.1. Prepulse inhibition of startle (PPI)

PPI is a phenomenon whereby exposure to a weak auditory stimulus (prepulse) reduces the extent of the startle reaction to a subsequent startling auditory stimulus. Mice were first habituated to the PPI chambers for 3 days prior to testing. We used our standard paradigm where on the test day mice were placed in the chambers and acclimatised for 5 min to a 70 dB background noise, followed by a test session of 105 trials in a pseudorandomised order (Boucher et al., 2007; Chohan et al., 2014). The trials presented included no stimulus, acoustic startle stimuli alone (70,

80, 100, 120 dB) and PPI trials consisting of a 20 ms prepulse stimulus (prepulse intensities (PI): 74, 82 and 86 dB) presented before a 40 ms 120 dB startle stimulus. Various PI-startle interstimulus intervals were used (32, 64, 128, 256 or 512 ms) which varied randomly from 10 to 20 ms. Startle responses were transduced by a piezoelectric accelerometer and sampled at 1 mHz to yield a peak after the presentation of an acoustic stimulus (Geyer and Swerdlow, 2001). Startle response was measured as the mean amplitude of these peaks across all trials and the %PPI was calculated as [(startle response 120 dB – PPI response) × 100/startle response 120 dB].

2.3.2. Light-dark test

The light-dark test exploits the natural conflict between the propensity of mice to explore a novel environment and to avoid an exposed, illuminated open area (Bourin and Hascoët, 2003). Anxiety related behaviors were evaluated for 10 min by placing the mouse in an automated open field activity chamber ($45 \times 45 \times 26\ cm^3$) illuminated under red light (40 lux), and equipped with a dark box insert ($8.5 \times 10\ cm^2$) placed in the centre of the chamber (Chohan et al., 2014). Mice were placed in the hide box at the beginning of the test and allowed to freely explore the chamber for the duration of the experiment. Anxiety was measured as the time spent in the hide box of the chamber. Due to tracking complications in Experiment 1, number of entries into the hide box and the time spent in the hide box data was scored manually by a researcher blind to experimental conditions. In Experiment 2, the number of entries into the hide box, the time spent in the hide box and locomotor activity in the light-dark test were recorded by the computer software Trackmate 2.1 (Motion Mensura, Australia) (Chohan et al., 2014).

2.3.3. Fear conditioning

Fear conditioning was conducted in fear conditioning chambers (Clever Sys. Inc. USA). Animals were placed into the chamber where they remained for 198s before being administered a single, inescapable scrambled electric foot-shock (duration 2 s, 1.5 mA) via the metal grid floor (Golub et al., 2009; Siegmund and Wotjak, 2007). To counter the reported retinal degeneration in FVB/NJ mice (Voikar et al., 2001), additional sensory stimuli were introduced to the operant chamber in order to create a memorable context that wasn't dependent solely on sight. The cages were heavily scented with a cleaning product (Sparkle for Windows; Pascoe's, Australia) and a tone (80 dB, 5 kHz) was played at 170 s for 30 s. Animals remained in the chamber for a total of 260 s before being returned to their home cages. Freezing behavior was measured using the Freezescan video tracking system (Clever Sys. Inc. USA), which registered and quantified the time the mice spent immobile. This immobility, referred to as freezing, is the natural response of mice in fear-eliciting situations and is used as an index of fear conditioning (Blanchard and Blanchard, 1988). Freezing is defined as the absence of any movement except for respiratory-related movements and was reported as the percentage of time spent immobile during the 3 min exposure time.

Darting behavior was also measured in the fear conditioning chambers upon re-exposure to the shock-paired context using the DrugEffectsScan module of the TopScan video analysis system (Clever Sys. Inc. USA). Darting was narrowly defined as satisfying all the following conditions: locomotion lasting >1 s; >10 cm of distance travelled; and reaching a velocity of >24 cm/s (Gruene et al., 2015).

2.3.4. Social interaction

Social withdrawal is a common symptom of PTSD and can be modelled in rodents using the social interaction test as previously described in our lab (Boucher et al., 2007). Social interaction of the experimental mice was tested with female A/J mice for 10 min in red-light illuminated Plexiglas open field chambers (43 cm × 43 cm × 25 cm; illumination 20 lx). A/J mice were used as they are passive and non-aggressive (Boucher et al., 2007). The mice were placed facing towards the wall in opposite corners of the open field test chamber at the commencement of the test session. Active socio-positive behaviors were scored such as general sniffing, anogenital sniffing, allogrooming, following and climbing over or under the A/J mouse. For each mouse, the frequency of interactions initiated by the experimental mice, along with the latency to the first interaction and total duration of time spent interacting was measured manually by a researcher blind to experimental conditions.

2.3.5. Acoustic startle response

The acoustic startle response (ASR) test was used to assess the mice's fear-potentiated startle responses. Hyperarousal is a key feature of PTSD and involves hyperreactivity to acoustic stimuli which can be easily reproduced and measured in animals. Mice were tested using a San Diego Instruments SR-Lab system (USA) according to the procedure described by Golub et al. (2009). Mice were placed in a small restraint tube in the testing chambers that transduce the startle response. After a 5 min acclimation period, 10 control trials and 20 startle stimuli of each intensity were presented in pseudorandom order in each test session. The interstimulus interval was 15 s on average and the 4 different startle stimuli consisted of white noise bursts of 20 ms duration and intensities of 75, 105 and 115 dB, which were presented in a constant background noise of 70 dB. On control trials only background noise was presented. A piezoelectric transducer was used to quantify the vibrations and the startle response was recorded with SR-LAB software. Before startle measurements, response sensitivities for each chamber were calibrated in order to assure identical output levels.

2.3.6. Forced swim test

PTSD patients may display comorbid depression (Elhai et al., 2011; Kessler et al., 1995). Depressive-like behavior was tested using a modified version of Porsolt's forced swim test (Porsolt et al., 1977). Each mouse was placed in a clear Plexiglas cylinder (height, 41 cm; diameter, 17 cm) filled with 15 cm of water at 24–25 °C for 6 min as described previously (Boucher et al., 2011). The cylinder was placed in a soundproof cabinet under red light and the animal's behavior recorded with a video camera. Immediately following the swim, each mouse was placed into a dry plastic holding cage with numerous tissues and paper towels and allowed to dry itself. Immobility was defined as passive floating with the exception of minimal movements necessary for the mouse to keep its head above water. Immobility time was recorded as seconds per minute spent immobile by the computer software Trackmate Quad 2.1 (Motion Mensura, Australia).

2.4. Iba-1 immunohistochemistry and microglial cell analysis

Mice were anaesthetized using isoflurane before being transcardially perfused with 0.09% phosphate-buffered saline followed by 4% paraformaldehyde. Brains were removed and cryoprotected in graded sucrose solutions, first at 15% concentration over 24 h and then 30% over 72 h or until they sunk. Brains were sliced coronally at 40 μm using a cryostat. Sliced brain tissue was stained with an Iba-1 primary antibody (Novachem, Australia) diluted at 1:500 overnight in 4 °C. After incubation at 1:500 with secondary antibody (Vector Laboratories, USA), a peroxidase reaction was visualized with diaminobenzidine and incubated with glucose oxidase (Sigma, Australia) for 10 min. Brain slices were mounted on gela-

tinized glass slides before they were dehydrated with ethanol, cleaned with xylene and coverslipped.

Iba-1 immunoreactive cells were manually counted under a light microscope using a 20 × objective, by an observer blind to experimental conditions. Subnuclei of the amygdala were counted within the region of interest. The hippocampus and prefrontal cortex were counted within a 0.25 mm^2 field of view with the use of a graticule. Regions of interest were identified using the mouse brain atlas of Paxinos (Paxinos and Franklin, 2004). The prelimbic, infralimbic and cingulate cortices were analysed at Plate 16 (Bregma 1.78 mm). The lateral, basolateral, central, posterodorsal medial, posteroventral medial amygdaloid nuclei were analysed at Plate 44 (Bregma -1.58 mm). The dentate gyrus, and CA1 and CA3 of Ammon's horn were analysed at Plate 45 (Bregma -1.70 mm).

Further morphological analysis of Iba1-positive microglial cells from the CA3 region of the hippocampus was conducted by reconstructing captured Z stack images obtained on a Stereo Investigator Microscope (MBF Biosciences) using brightfield imaging with a 40× objective with a 2 μm step size. Seven animals from FVB WT and P-gp KO conditions were randomly selected with six CA3 hippocampal microglial cells per animal analysed. This yielded a total of 84 microglial cells that were analysed. The area of interest was located at Bregma -1.7 mm. Neurolucida software (MBF Biosciences) was used for interactive tracing and analysis of reconstructed microglial cells. Analysis was conducted on the cell body (cell body perimeter, volume, and surface area) followed by Sholl analysis of process surface area, volume, length, and number of intersections originating from the cell soma at (5 μm intervals).

2.5. GR immunofluorescence

To determine whether P-gp deletion altered GR expression we performed immunofluorescence analysis. To prevent non-specific binding tissue was incubated in blocking solution containing 3% BSA and 0.1% sodium azide in PB overnight. The tissue was then incubated with the primary polyclonal mouse antibody anti-glucocorticoid receptor (Abcam ab3578) for 48 h before the secondary goat anti-rabbit IgG Alexa Fluor488 (Invitrogen) for 24 h. Tissue slices were then co-stained with DAPI. Sections were mounted onto slides and coverslipped using ProLong Gold Antifade (Invitrogen).

Immunofluorescent images were captured on a Zeiss Confocal Microscope 710 (LSM710) using a 20× water immersion objective (NA 0.95). 405 nm and 488 nm lasers were utilised for fluorophore excitation of DAPI and Alexa 488, and emissions were collected between 410 and 507 nm, and 493 and 629 nm respectively. The images were then deconvolved using Huygens Professional software (Scientific Volume Imaging (SVI), The Netherlands). Images were manually cropped to the region of interest (CA3 stratum pyramidale) using a 70 μm × 160 μm rectangle, to ensure that subsequent analysis was uniform per area measurement. Images were analysed using macro batch processing using Fiji software (Free Open Source Software, NIH, USA) (Schindelin et al., 2012). The macro automated the process of splitting the RGB channel into individual channels and then measured either the mean fluorescence intensity or the fluorescent area above background staining within the green channel.

2.6. Data analysis

All statistical tests were undertaken in PASW 21.0 (SPSS Inc. USA). All behavioral and microglial data were analysed using two, three or four-way ANOVA where appropriate with between subject factors of genotype (WT versus ABC transporter KO) and stressor (control versus stress) and in some cases with within-subjects factors (time, acoustic intensity etc). Results that did not meet the equality of variance and normality assumptions of ANOVA were log transformed. In the cases where log transformation failed to fix assumption violations, data was transformed using the aligned rank transformation (Wobbrock et al., 2011). Post-hoc analyses were performed in instances where significant interaction effects were observed using Bonferroni comparisons or t-tests. The results of all analyses were deemed significant at $P < 0.05$.

3. Results

3.1. Experiment 1: The effects of acute injection stress on sensorimotor gating and anxiety-related behavior in P-gp and Bcrp KO mice

We first examined the impact of stress of injection on sensorimotor gating and anxiety-related behavior in WT, P-gp and Bcrp KO mice. A summary of PPI data is shown in Fig. 1A. Two-way ANOVA including all WT, P-gp and Bcrp genotypes revealed main effects of genotype and injection stress with both conditions increasing average PPI [$F(2,65) = 4.9$, $P = 0.01$ and $F(1,65) = 5.6$, $P = 0.02$, respectively] (Fig. 1A). There was no significant interaction between the factors of genotype and injection stress. Separate two-way ANOVA of WT and P-gp KO or WT and Bcrp KO data showed main effects of P-gp or Bcrp genotype with both genotypes increasing average PPI [$F(1,43) = 4.1$, $P = 0.04$ and $F(1,45) = 10.3$, $P = 0.003$, respectively]. However, no effects of stress, or genotype by stress interactions were observed.

During the PPI test, we also measured the startle response of the mice to a 120 dB acoustic stimulus (Fig. 1B). Two-way ANOVA including all genotypes showed a main effect of genotype [$F(2,65) = 18.7$, $P < 0.0001$], as there was a significant reduction in startle amplitude in P-gp and Bcrp KO mice. Startle response was not affected by injection stress and there was no interaction between genotype and injection stress. Separate two-way ANOVA of WT versus P-gp KO mice or WT versus Bcrp KO mice confirmed the significant decrease in startle response in P-gp and Bcrp KO mice [main effects of P-gp or Bcrp genotype: $F(1,43) = 14.5$, $P = 0.0004$ and $F(1,45) = 27.1$, $P < 0.0001$ respectively].

The effect of injection stress on anxiety related behaviors of WT, P-gp KO and Bcrp KO mice as measured in the light-dark test is shown in Fig. 1C–E. Two-way ANOVA indicated a main effect of ABC transporter genotype in the number of entries into the hide box [$F(2,64) = 14.2$, $P < 0.0001$] (Fig. 1C) and a differential effect of stress on WT, P-gp KO and Bcrp KO mice as supported by a genotype by stress interaction [$F(2,64) = 15$, $P < 0.0001$]. Analysis of only WT and P-gp KO mice data using two-way ANOVA showed a main effect of P-gp genotype on entries into the hide box [$F(1,42) = 18.3$, $P = 0.0001$], as well as a P-gp genotype by stress interaction [$F(1,42) = 18.3$, $P = 0.0001$]. Bonferroni post-hoc analysis showed stress increased the frequency of hide box entries in P-gp KO mice compared to P-gp KO controls and stressed WT mice ($P = 0.012$ and $P < 0.0001$, respectively), whereas stress had the opposite effect on WT and Bcrp KO mice, decreasing hide box entries. Two-way ANOVA of WT and Bcrp KO mice data showed stress significantly reduced the number of entries into the hide box [main effect of stress: $F(1,44) = 28$, $P < 0.0001$], however there was no effect of Bcrp genotype or a genotype by stress interaction. Bonferroni post-hoc analysis showed stressed Bcrp KO mice entered the hide box significantly less than Bcrp KO controls ($P = 0.02$). P-gp or Bcrp deletion did not influence the number of entries in non-stressed, control mice ($Ps > 0.05$).

We also examined the amount of time the mice spent in the hide box and two-way ANOVA of all genotypes revealed injection stress overall increased the hide time of WT, P-gp KO and Bcrp KO mice [main effect of stress: $F(1,64) = 15.9$, $P = 0.0002$]

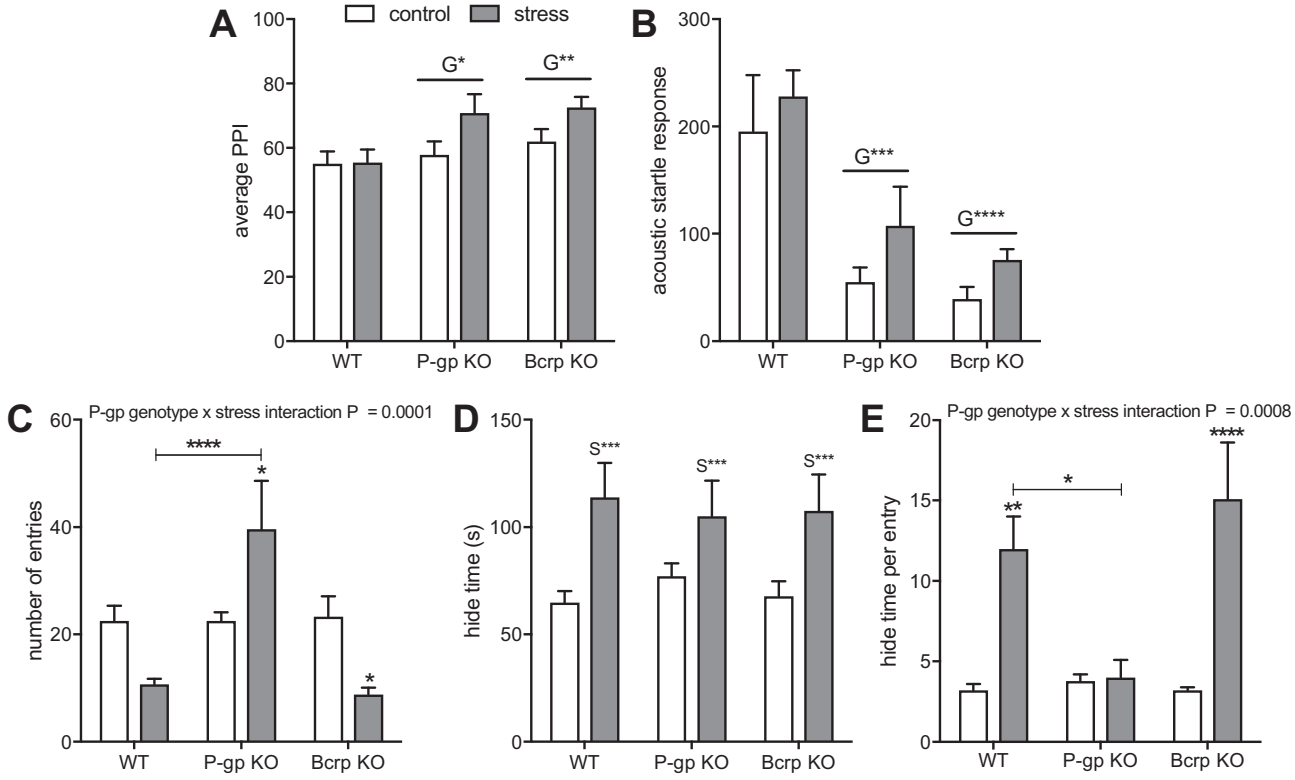

Fig. 1. Germline P-gp and Bcrp KO increased PPI and reduced startle reactivity in female mice which was not influenced by injection stress. P-gp but not Bcrp KO in female mice induced frantic anxiety-related behavior in response to injection stress in the light-dark test. A) Average %PPI and B) Startle response to a 120 dB acoustic stimulus. C) Entries into the hide box. D) Time spent in the hide box. E) Average time spent in hide box per visit (hide time/number of entries). Data are presented as mean + S.E.M. n per group: WT control = 12; WT stress = 12; P-gp KO control = 15, P-gp KO stress = 7, Bcrp KO control = 12, Bcrp KO stress = 12. G = main effect of genotype, S = main effect of stress, * P < 0.05, ** P < 0.01, *** P < 0.001. Bonferroni post-hoc analyses, control versus stress group within a specific genotype or as otherwise indicated * P < 0.05, **** P < 0.0001.

(Fig. 1D). Genotype did not influence the time hiding and there was no interaction of genotype and stress. Separate ANOVAs of P-gp KO or Bcrp KO data confirmed stress increased the time spent in the hide box [main effect of stress in P-gp or Bcrp KO mice: $F(1,42) = 11.7$, $P = 0.001$ and $F(1,44) = 12.7$, $P = 0.0009$ respectively]. We further examined time spent in the hide box per entry (Fig. 1E). Two-way ANOVA showed an overall main effect of stress [$F(1,64) = 22.8$, $P < 0.0001$], ABC transporter genotype [$F(2,64) = 4.6$, $P = 0.01$], and an ABC transporter genotype by stress interaction [$F(2,64) = 5.8$, $P = 0.005$]. When analysing WT and P-gp KO mice separately on this measure, two-way ANOVA showed a main effect of stress [$F(1,42) = 14.3$, $P = 0.0005$], P-gp genotype [$F(1,42) = 9.7$, $P = 0.0034$] and a genotype by stress interaction [$F(1,42) = 13$, $P = 0.0008$]. There was also a main effect of stress on the hide time/number of entries in WT and Bcrp KO mice [$F(1,44) = 26$, $P < 0.0001$] but no effect of Bcrp genotype or interaction effects. Bonferroni post-hoc analysis showed stress did not influence time in the hide box per entry in P-gp KO mice, unlike WT and Bcrp KO mice where stress increased the amount of time hiding per entry in both genotypes ($P = 0.0084$ and $P < 0.0001$, respectively). Stressed P-gp KO mice were no different to the P-gp KO controls and spent a significantly shorter amount of time in the hide box per visit than stressed WT mice (Bonferroni post-hoc: $P = 0.04$).

3.2. Experiment 2: the effects of foot-shock stress on behavior and microglial cell number in P-gp KO mice

We then examined WT and P-gp KO mice in an animal model of PTSD where mice are exposed to a single electric foot-shock to produce a myriad of long-lasting phenotypes such as increased fear conditioned freezing responses and hyperarousal (Golub et al., 2009; Siegmund and Wotjak, 2007). The conditioned freezing response to foot-shock paired to a compound auditory, olfactory and contextual stimulus was measured on day 1 and day 22 (Fig. 2A) prior to a battery of other behavioral tests that were conducted over days. For ease and efficiency of expression the results section refers to "stress" as a factor, however it must be pointed out that any behavioral or neurobiological effects reported hereafter might not only be explained by purely foot-shock stress itself, but by the conditioned fear provoked by re-exposure to the shock-paired context.

Overall stressed mice demonstrated a significantly higher freezing response than control mice supported by a main effect of foot-shock stress in the three-way repeated measures ANOVA [$F(1,37) = 5.98$, $P = 0.019$]. There was also a significant main effect of genotype, with P-gp KO mice having significantly reduced freezing responses in comparison to WT mice [$F(1,37) = 9.54$, $P = 0.0038$]. In addition, there was a main effect of day [$F(1,37) = 8.32$, $P = 0.0065$], with higher freezing on day 22 than day 1. There was no significant interaction between the factors of genotype, stress or day. Two-way ANOVA of the day 1 data alone confirmed main effects of stress [$F(1,37) = 11.9$, $P = 0.0014$] and P-gp genotype [$F(1,37) = 9.6$, $P = 0.0037$]. Interestingly, there was a trend for a significant P-gp genotype by stress interaction [$F(1,37) = 4.1$, $P = 0.05$], as it appeared that P-gp KO mice displayed reduced fear conditioning compared to WT mice. Analysis of day 22 data with two-way ANOVA revealed a main effect of genotype, as P-gp KO mice mice displayed less freezing than non-stressed mice [$F(1,37) = 4.9$, $P = 0.033$]. There was no stress or genotype by stress interaction.

Darting behavior has been recently characterised as an alternative stress coping mechanism to freezing in response to a condi-

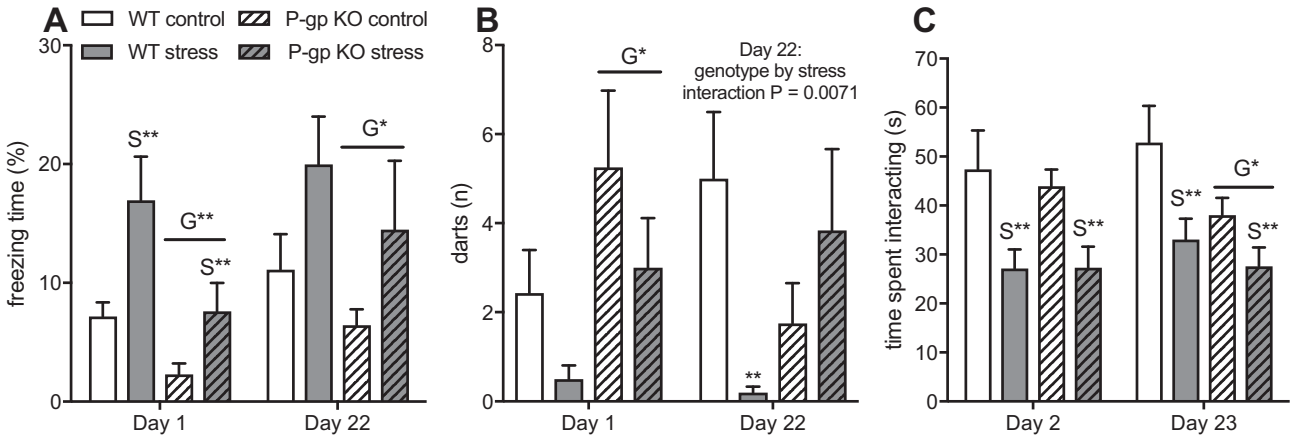

Fig. 2. P-gp KO in female mice tended to impair fear conditioning in the short-term but not long-term. P-gp KO increased darting behavior and foot-shock decreased social interaction. A) Fear conditioning data in WT and P-gp KO mice when introduced to a foot-shock paired context in the absence of shock, 1 and 22 days after initial foot-shock exposure. B) Darting behavior measured in the fear conditioning test. C) Mean time spent interacting with the novel mouse 2 and 23 days after foot-shock exposure. Data are presented as mean + S.E.M n per group: WT control = 7; WT stress = 10; P-gp KO control = 12, P-gp KO stress = 12. G = main effect of genotype; S = main effect of stress, * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$. Bonferroni post-hoc analyses, control versus stress group within a specific genotype, ** $P < 0.01$.

tioned fear stimulus (Gruene et al., 2015). The darting behavior was interpreted as a more active coping response than the passive freezing behavior, and interestingly was more prevalent in female than male rats. As we showed that injection stress increased darting in and out of the hide box in the light-dark test it is possible that the reduced freezing response we observed in female P-gp KO mice might have been influenced by an increased incidence of darting behavior in the test. It could be then that female P-gp KO mice display a qualitatively different "active" fear response than the passive freezing fear response exhibited by WT mice. The data did not support this hypothesis. A three-way repeated measures ANOVA found a significant main effect of foot-shock on the number of darts [$F(1,36) = 4.97$, $P = 0.032$] (Fig. 2B) but no main effects of P-gp genotype or day in the fear conditioning test. Interestingly, there was a significant interaction of P-gp genotype, stress and day [$F(1,36) = 4.28$, $P = 0.046$]. Two-way ANOVA of day 1 data alone showed a significant main effect of P-gp genotype [$F(1,37) = 4.29$, $P = 0.045$] indicating more darting behavior in P-gp KO mice compared to WT mice, which might help explain the overall reduced freezing behavior observed in P-gp KO mice on day 1. But there was no significant effect of stress or a genotype by stress interaction on the number of darts on day 1, which is inconsistent with the notion that P-gp KO mice display less foot-shock-induced freezing due to a concomitant increase in darting. Two-way ANOVA of day 22 data showed a significant genotype by stress interaction [$F(1,36) = 8.16$, $P = 0.0071$], indicating that while stressed WT mice darted less than non-stressed WT mice (Bonferroni post-hoc test, $P = 0.0071$), stressed P-gp KO mice did not show any such decrease in darting behavior.

The effect of stress on anxiety-related behaviors of WT and P-gp KO mice as measured in the light-dark test and the social interaction paradigm was examined. P-gp KO did not influence the time spent hiding in the hide box in the light-dark test (see Supplementary data, Fig. S1). We also analysed locomotor activity in the light-dark test by measuring the distance the mice travelled in this test and the P-gp KO mice displayed a subtle, locomotor hyperactivity that was more pronounced on day 22 (Supplementary data Fig. S1). Social interactions initiated by the test mouse were quantified when interacting with a passive AJ mouse (Fig. 2C). Three-way repeated measures ANOVA indicated foot-shock reduced the total time spent socially interacting [main effect of stress: [$F(1,32) = 10.5$; $P = 0.003$]. There were no significant main effects of P-gp genotype or any interaction effects between genotype, foot-shock stress and day on this measure. Two-way ANOVAs on days 2 and 23 confirmed that stress reduced the total time spent interacting [main effect of stress: $F(1,32) = 13.2$, $P = 0.001$ and $F(1,32) = 9.9$, $P = 0.004$, respectively]. On day 23 there was a main effect of P-gp genotype on the total time spent socially interacting [$F(1,32) = 4.4$, $P = 0.04$), with P-gp KO mice socially interacting significantly less than WT mice.

The effect of stress on the startle response to various acoustic stimuli intensities in WT and P-gp KO mice was measured on days 3 and day 24 (Fig. 3A and B). Four-way repeated measures ANOVA with between subjects factors of genotype and stress and within-subjects factors of acoustic intensity and day showed there was an effect of acoustic intensity [$F(2,74) = 379.76$, $P < 0.0001$], confirming that the startle response increased in a stimulus intensity dependent manner. There was also a significant interaction between P-gp genotype and acoustic stimulus intensity [$F(2,74) = 23.1$, $P < 0.0001$]. We then separately analysed the individual stimulus intensities using two-way ANOVA. Interestingly, P-gp KO mice displayed a greater startle response than WT mice at the lower stimulus intensity of 75 dB [main effect of genotype day 3: $F(1,37) = 24$, $P < 0.0001$ and day 24: $F(1,37) = 12.6$, $P = 0.001$]. Whereas at the higher stimulus intensities of 105 and 115 dB opposite results were observed; P-gp KO mice displayed reduced startle responses compared to WT mice on day 3 [main effects of genotype: $F(1,37) = 8$, $P = 0.008$ and $F(1,37) = 18.8$, $P = 0.0001$, respectively] and on day 24 [main effect of genotype: $F(1,37) = 4.3$, $P = 0.04$ and $F(1,37) = 18.7$, $P = 0.0001$].

Depression-like behavior was tested in the forced swim test by measuring the time spent floating in each minute of the test on days 4 and 25 (Fig. 3C and D). Four-way repeated measures ANOVA with between factors of genotype and stress, and within-subjects factors of both minutes and day found an overall interaction between genotype and minutes [$F(5,185) = 4.5$, $P = 0.001$], suggesting P-gp KO mice displayed a greater increase in floating time over minutes than WT mice. There was also an effect of minute [$F(5,185) = 12.4$, $P = 0.0008$] demonstrating an overall increase in floating behavior over time in the test. This was further confirmed with separate three-way repeated measures ANOVA, with both days showing a minute [Day 4: $F(5,185) = 13.2$, $P < 0.0001$ and Day 25: $F(5,185)$ 12.4, $P < 0.0001$] and genotype by minute interaction [Day 4: ($F(5,185) = 5.0$, $P = 0.0003$ and day 25: $F(5,185) = 4.5$, $P = 0.0007$]. The increased depression like behavior of P-gp KO mice was also more pronounced on day 4 than day 25 as seen by

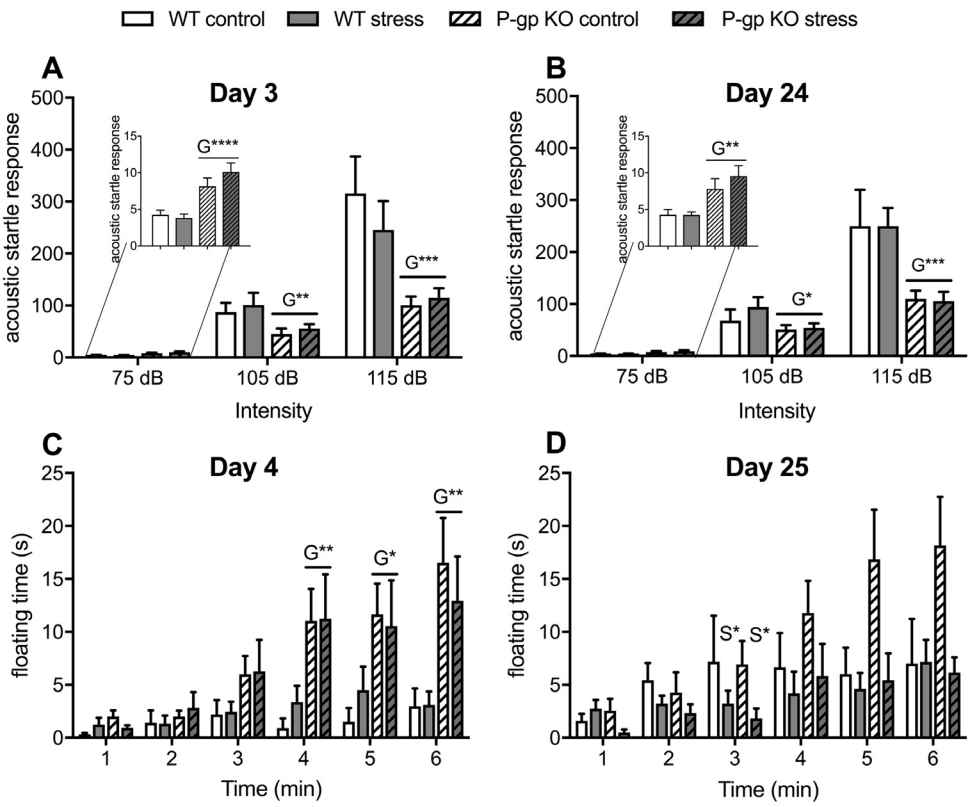

Fig. 3. Female P-gp KO mice displayed decreased startle responses to loud acoustic stimuli and increased depressive-like behavior in the forced swim test. A) Mean acoustic startle response (at 75, 105 and 115 dB) for WT and P-gp KO mice 3 days after foot-shock exposure. B) Mean acoustic startle response (at 75, 105 and 115 dB) for WT and P-gp KO mice 24 days after foot-shock exposure. C) Mean floating time per minute for WT and P-gp KO mice 4 days after foot-shock exposure. D) Mean floating time per minute for WT and P-gp KO mice 25 days after foot-shock exposure. Data are presented as mean + S.E.M. n per group: WT control = 7; WT stress = 10; P-gp KO control = 12, P-gp KO stress = 12. G = main effect of genotype, S = main effect of stress, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

a main effect of genotype on day 4 [$F(1,37) = 13.7$, $P = 0.0007$] but not day 25. This effect was further confirmed by two-way ANOVA of individual minutes on day 4, which showed a main effect of genotype at minutes four, five and six [$F(1,37) = 7.8$, $P = 0.008$, $F(1,37) = 5.8$, $P = 0.02$ and $F(1,37) = 10$, $P = 0.003$ respectively]. In addition, individual analysis of minutes on day 25 revealed a main effect of stress at minute three [$F(1,37) = 4.3$, $P = 0.04$]. The data tended to show that the long-term effects of shock may have protected against the increased depression-like behavior observed in P-gp KO mice, however there was no significant P-gp genotype by stress interaction effects to statistically support this viewpoint.

We then examined the effects of stress on microglial cell density in the prefrontal cortex, hippocampus and amygdala in WT or P-gp KO mice (Table 1). Two-way ANOVA showed that stress significantly reduced the number of microglial cells compared to controls in the lateral amygdala [main effect of stress: $F(1,28) = 8.6$; $P = 0.007$], the basolateral amygdala [main effect of stress: $F(1,28) = 10.4$; $P = 0.003$] and the posteroventral medial amygdala [main effect of stress: $F(1,28) = 6.7$; $P = 0.015$] (Table 1). No significant effects were observed in the posterodorsal medial amygdala or the central amygdala. P-gp KO, stress or their combination did not alter the number of microglial cells within the anterior cingulate cortex, prelimbic cortex or infralimbic cortex (Table 1). Two-way ANOVA showed that microglial cell density in the CA3 region of the hippocampus was significantly higher in P-gp KO mice than WT mice [main effect of genotype: $F(1,28) = 4.9$; $P = 0.035$].

As the P-gp KO mice displayed significantly more microglial cells than WT mice in the CA3 region of the hippocampus irrespective of stress, we decided to further examine the impact of P-gp deletion on microglial cell morphology by conducting detailed 3D microglial cell reconstructions of control P-gp KO and WT mice in this region. Two-way ANOVA showed no significant differences between P-gp KO and WT mice in microglial cell soma size, as there were no significant differences between P-gp KO and WT mice in microglial cell body perimeter, cell body volume and cell body surface area (see Supplementary data, Table S1). However, Sholl analysis (Fig. 4) demonstrated the microglial cell processes of P-gp KO mice had significantly less surface area and volume, were shorter in length and made fewer intersections than microglia of WT mice [main effect of genotype in two-way repeated measures ANOVA: $F(1,60) = 8.7$, $P = 0.012$; $F(1,60) = 7.4$, $P = 0.02$; $F(1,60) = 7.9$, $P = 0.016$; $F(1,60) = 8.8$, $P = 0.012$ respectively]. The measures of surface area, volume, length and intersections also showed significant genotype by distance interactions [$F(5,60) = 3.1$, $P = 0.015$; $F(5,60) = 2.8$, $P = 0.026$; $(5,60) = 3.7$, $P = 0.006$; $F(5,60) = 3.4$, $P = 0.009$ respectively] implying that the effect of P-gp deletion on the microglial cell process morphology varied as a function of the radial distance from the soma. This was confirmed by unpaired t-tests which showed the differences between P-gp KO and WT mice became significant at radial distances at equal to and greater than 15 μm.

Prior studies reported dysregulation of glucocorticoid signalling in P-gp KO mice including increased GR expression (Muller et al., 2003; Schoenfelder et al., 2012). These studies we conducted exclusively in male mice and so here we assessed whether these findings extend to female mice. To achieve this we analysed GR immunofluorescence in the pyramidal layer of the CA3 region of the hippocampus of control WT and P-gp KO mice (Fig. 5). We found that, similarly to males, female P-gp KO mice had significantly increased GR expression compared to WT using both inten-

Table 1
Mean microglial cell count 30 days after foot-shock stress in the frontal cortex, hippocampus and amygdala of female WT and P-gp KO mice. Data are presented as mean ± S.E.M. n per group: WT control = 7; WT stress = 10; P-gp KO control = 7, P-gp KO stress = 8. G = a main effect of genotype, S = a main effect of foot-shock stress.

	WT control	WT stress	P-gp KO control	P-gp KO stress	Stats
Frontal regions					
1. Anterior cingulate cortex	84.3 ± 1.7	85.6 ± 3.1	87.3 ± 3.0	94.4 ± 4.8	
2. Prelimbic cortex	78.4 ± 2.2	85.6 ± 4.6	84.4 ± 2.9	82.6 ± 4.1	
3. Infralimbic cortex	76.3 ± 3.1	84.8 ± 3.9	83.6 ± 2.4	89.9 ± 5.0	
Hippocampus					
4. Dentate gyrus	81.3 ± 1.9	78.7 ± 2.9	88.3 ± 4.8	85.5 ± 3.7	
5. CA1	85.1 ± 2.8	84.9 ± 4.0	94.6 ± 5.8	92.5 ± 4.4	
6. CA3	78.7 ± 2.7	76.4 ± 2.4	87.7 ± 5.2	82.6 ± 3.6	G
Amygdala					
7. Basolateral	92.0 ± 5.7	82.7 ± 4.0	102.9 ± 7.0	81.6 ± 2.9	S
8. Lateral	102.1 ± 9.4	82.6 ± 4.8	99.1 ± 8.1	82.6 ± 2.7	S
9. Medial, posteroventral	76.4 ± 6.4	67.6 ± 4.6	81.9 ± 6.2	65.6 ± 3.0	S
10. Medial, posterodorsal	77.7 ± 5.8	68.4 ± 3.1	79.0 ± 5.1	72.6 ± 2.8	
11. Central	87.3 ± 5.5	76.1 ± 3.5	78.7 ± 3.5	75.5 ± 3.8	

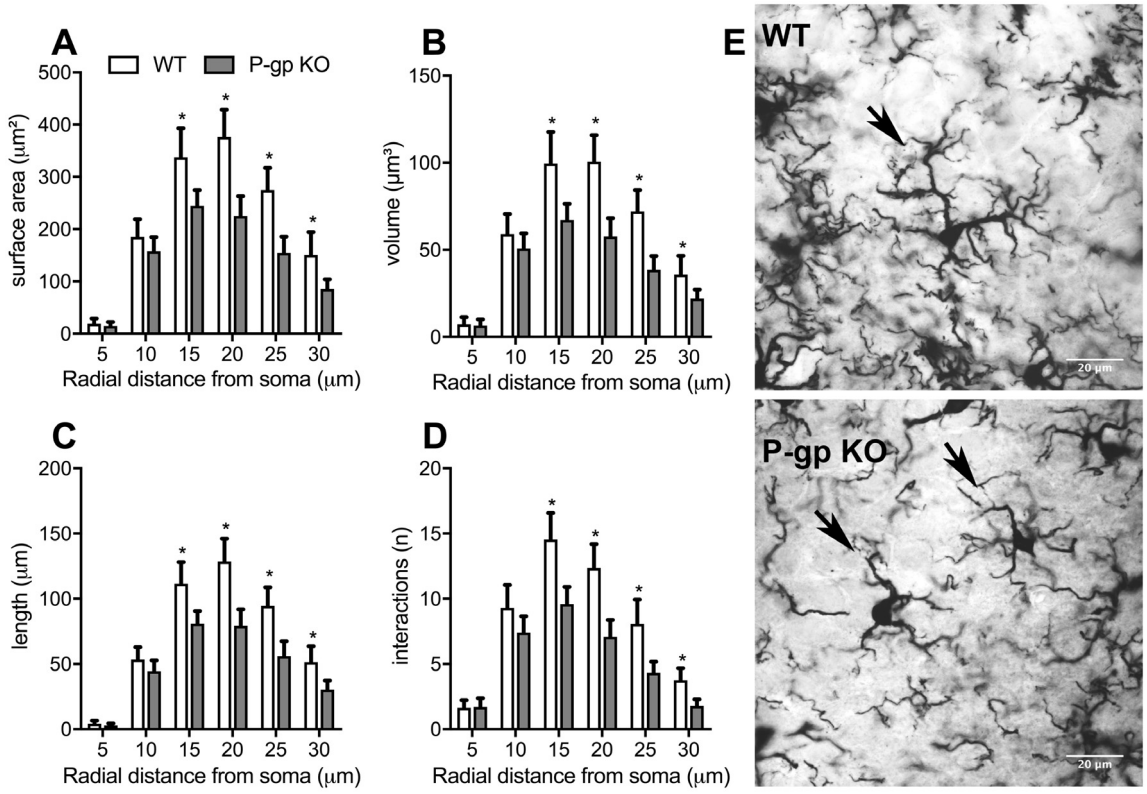

Fig. 4. Microglial cell processes of female P-gp KO mice had significantly less surface area and volume, were shorter in length and were less ramified than microglia of WT mice in the CA3 region of the hippocampus. Process A) surface area B) volume C) length D) interactions. E) Representative images of microglial cells from WT and P-gp KO mice. Unpaired t-tests indicated by * $p < 0.05$ for significant differences between WT and P-gp KO mice. Data are presented as mean + S.E.M, n = 7 per group.

sity ($t = -2.5$, df = 12, P = 0.029) and area measures (see Supplementary data, Fig. S2).

4. Discussion

Germline genetic deletion of the ABC transporter P-gp in female mice altered a range of behaviors in the absence of stress and in response to explicit stressors. P-gp KO in female mice increased depression-like behavior, produced age-related social withdrawal and locomotor hyperactivity, promoted distinctive darting behavior, facilitated sensorimotor gating and reduced startle responses. Moreover, the behavioral phenotypes of P-gp KO mice were associated with increased microglial cell density in the CA3 region of the hippocampus, and the microglial cells exhibited a hypo-ramified morphology compared to WT mice. Consistent with prior observations in male mice, female P-gp KO mice displayed higher GR expression than WT mice in the CA3 region (Schoenfelder et al., 2012), suggesting that dysregulation of the glucocorticoid-GR system in P-gp KO mice is not sexually dimorphic.

Female P-gp KO mice also showed an acute, atypical response to stress, with injection stress triggering frantic behavior in P-gp KO mice. That is, while P-gp KO mice displayed an equivalent stress-induced increase in the total time spent hiding in the light-dark test to WT mice, the P-gp KO mice spent less time hiding per visit and more frequently darted in and out of the hide box. P-gp KO

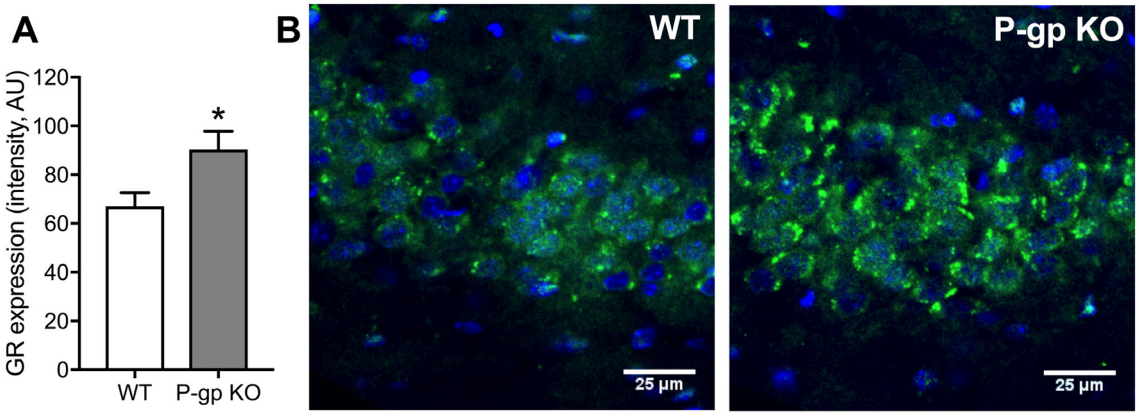

Fig. 5. Female P-gp KO mice had increased GR expression compared to WT in the pyramidal layer of the CA3 region of the hippocampus. A) Summary data of GR expression as measured by mean fluorescence intensity in WT control and P-gp KO control mice. Green denotes GR immunostaining, blue is for DAPI. Unpaired *t*-test indicated * $p < 0.05$. Data are presented as mean + S.E.M. n = 7 per group.

mice also tended to display less fear conditioning when re-exposed to the shock-paired context 1 day, but not 22 days, post-shock exposure, at least based on the conventional, passive fear measure of freezing. Interesting, P-gp KO also displayed idiosyncratic darting behavior in the fear conditioning test, an alternative active fear response that is more prevalent in female animals (Gruene et al., 2015). Further, P-gp KO mice were resilient to fear-induced reductions in darting behavior observed on day 22 which was clearly observed in WT mice.

Both P-gp and Bcrp are both ABC transporter efflux pumps and have overlapping substrate specificity and tissue distributions including localisation on the luminal side of brain endothelial cells (Hong, 2016; Szakács et al., 2008). Despite this, injection stress induced frantic, darting behavior in P-gp KO but not Bcrp KO mice, highlighting that the aberrant effects of ABC transporter deletion on the stress response are P-gp specific. Future studies are required to explain this disparity. One explanation for this is that the stress hormone corticosterone is a P-gp but not Bcrp substrate, and so dysregulation of the glucocorticoid stress system that occurs with germline ABC transporter deletion may only be relevant to P-gp and not Bcrp (Muller et al., 2003; Pavek et al., 2005; Uhr et al., 2002; Wolf and Horwitz, 1992). Corticosterone was not transported by Bcrp in cells overexpressing Bcrp (Pavek et al., 2005), so to confirm our hypothesis future *in vivo* studies are needed to verify a lack of altered corticosterone brain uptake and GR expression in Bcrp KO mice.

Germline genetic deletion of P-gp dysregulates glucocorticoid signalling in the brain which may subserve some of the distinctive behavioral phenotypes exhibited by these animals in the present study. We confirmed that P-gp KO mice display increased GR expression in whole brain tissue (Schoenfelder et al., 2012), here specifically in the pyramidal layer of the CA3 region of the hippocampus. Previous studies have also reported altered glucocorticoid signalling in P-gp KO mice, as these mice had decreased expression of the ACTH precursors corticotropin releasing hormone (CRH) and proopiomelanocortin (POMC) in the anterior pituitary gland (Muller et al., 2003). Moreover, P-gp KO mice displayed lower basal and stress-induced corticosterone concentrations than WT mice (Muller et al., 2003; Schoenfelder et al., 2012). It is possible that these changes in adult P-gp KO mice reflect a neuroadaptive response to enhanced brain accumulation of endogenous corticosteroids early in life, as P-gp KO mice retain higher brain uptake of exogenously administered corticosteroids (Uhr et al., 2002). This could be examined in future developmental studies, mapping plasma corticosterone concentrations and GR expression over the lifespan of P-gp KO mice.

Some of our behavioral results, at least at face value, conflict with prior research on P-gp KO mice. We observed a subtle locomotor hyperactivity and increased depression-like behavior in female P-gp KO mice, whereas others reported that male P-gp KO mice had decreased locomotor activity and decreased depression-like behavior in the forced swim test (Schoenfelder et al., 2012). Sexual dimorphism provides one explanation for these discrepancies, although here we showed increased brain GR expression also occurs in female P-gp KO mice. Schoenfelder et al. (2012) also reported that over days the locomotor activity of P-gp KO mice increased, while the WT mice activity decreased. This suggests that P-gp KO mice may exhibit impaired habituation to a novel context or alternatively that locomotor hyperactivity might develop with increasing age. Schoenfelder et al. (2012) tested mice at 8 weeks of age, however our mice were 12–16 weeks of age. In addition, the locomotor hyperactivity we observed in P-gp KO mice appeared more pronounced on day 22 of testing than on day 1. Further reinforcing the hypothesis of age-dependent expression of behavioral phenotypes in P-gp KO mice was our novel observation that P-gp knockout mice displayed social withdrawal on day 24 but not on day 2 of testing. Our finding in the forced swim test also contradicts Schoenfelder et al. (2012) who showed male P-gp KO mice displayed less depression-like behavior in this test. Although contradicting their own forced swim test results, Schoenfelder et al. (2012) in the same manuscript reported that P-gp KO mice displayed increased depression-like behavior in the tail suspension test (Schoenfelder et al., 2012). Consistent with P-gp deletion increasing depression-like behavior is the demonstration that loss-of-function single nucleotide polymorphisms in *MDR1* increase the risk of developing depression in human females (Enokido et al., 2014; Qian et al., 2006).

We report here for the first time that P-gp KO mice display distorted startle responses to loud acoustic stimuli. At the lowest stimulus intensity of 75 dB, P-gp KO mice displayed a higher startle response than WT mice, highlighting that these animals are unlikely to have impaired hearing. Surprisingly, P-gp KO mice displayed a lower startle response than WT mice at the higher 105 and 115 dB acoustic stimuli. P-gp is expressed at the blood-inner ear barrier which is functionally similar to the blood-brain barrier (Saito et al., 1997). Disruption of this barrier in P-gp KO mice resulted in hearing deficits to chemotherapeutic drugs (Zhang et al., 2000), indicating that P-gp plays a protective role in the inner ear. Therefore, P-gp deletion may distort normal hearing function through the alteration of endogenous molecules vital to normal hearing development or via enhanced inner ear exposure to oto-

toxins. These hypotheses could be examined in future more detailed studies.

Another original finding reported here is that P-gp KO mice display greater microglial cell density and hyporamification in the hippocampus. Microglial cells adopt a different morphology dependent on functional demand and various physiological challenges cause microglia to adopt a hypo-ramified morphology where the cells proliferate and retract their processes (Biber et al., 2007; Kettenmann et al., 2011; Stankovic et al., 2016; Tynan et al., 2010; Walker et al., 2013, 2014). Future studies are needed to explain the abnormal microglial cell number and morphology in P-gp KO mice. It may be a stress triggered phenotype as the control "non-stressed" mice that were analysed here were also recently exposed to the forced swim test, a stressful test that increases microglial cell density in the hippocampus (Iwata et al., 2016). Interestingly, the hippocampus may be particularly sensitive to the effects of P-gp deletion, as the *mdr1b* isoform of P-gp is only expressed in the hippocampus, likely offering this structure another level of protection from toxic chemicals (Kwan et al., 2003). We hypothesise then that germline knockout of the *mdr1b* isoform in P-gp KO mice renders the hippocampus uniquely vulnerable to abnormal microglial cell activity. Dysregulation of the glucocorticoid system in P-gp KO mice may play a role given corticosteroids influence microglial cell proliferation via activation of corticosteroid receptors on microglia, thus future studies might assess whether P-gp deletion alters microglial cell GR expression (Ganter et al., 1992; Tanaka et al., 1997). P-gp might directly modify microglial cell structure and function as P-gp is expressed on the cell and nuclear membranes of microglial cells, however the exact physiological role of P-gp on microglia is unclear and requires further examination (Ballerini et al., 2002; Dallas et al., 2004, 2003; Gibson et al., 2012; Lee and Bendayan, 2004; Lee et al., 2001; Ronaldson et al., 2004).

Our results re-affirmed that a single electric foot-shock promotes lasting fear conditioning up to 22 days after shock exposure in mice (Golub et al., 2009; Siegmund and Wotjak, 2007). Further, we showed for the first time that the lasting conditioned fear response was associated with a decreased density of microglial cells in various areas of the amygdala 48 h after re-exposure to the shock-paired context on day 22. This is of interest as the amygdala is implicated in the neurobiology of conditioned fear (Maren and Quirk, 2004; Zelikowsky et al., 2014; Zhang et al., 2015) and microglia influence synaptic function (Ekdahl, 2012; Ji et al., 2013; Tremblay et al., 2011). Therefore, future studies could explore the role of microglia in the regulation of fear memory circuits in the amygdala. We also report for the first time that enduring fear conditioning was associated with reduced social behavior. This decrease in sociability may also be mediated by altered amygdalar microglial cell densities, as the amygdala is a neural hub for social interaction (Amaral, 2006; Bickart et al., 2014).

To conclude, we show that germline P-gp deletion altered behavior and stress responsivity in female mice, further underscoring that this ABC transporter may play an important role in conferring risk to physical and psychiatric conditions. Further, the abnormal behavioral phenotype of P-gp KO mice was associated with increased microglial cell number and hypo-ramification in the CA3 region of the hippocampus, and increased GR expression in this region.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2017.05.008.

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Chapter 4. $P2X_7$ receptor knockout mice display less aggressive biting behaviour correlating with increased brain activation in the piriform cortex

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Research article

$P2X_7$ receptor knockout mice display less aggressive biting behaviour correlating with increased brain activation in the piriform cortex

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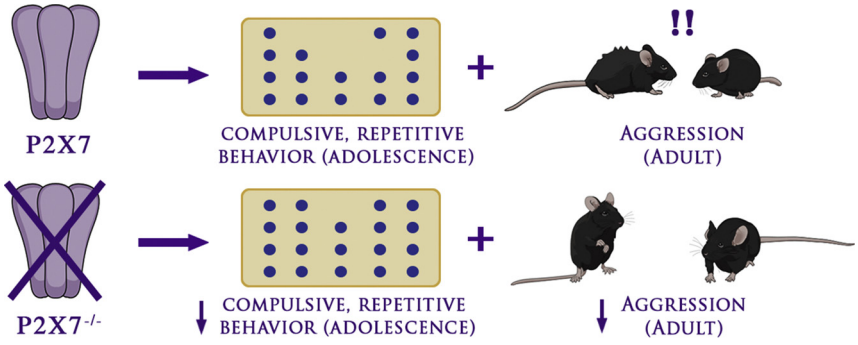
GRAPHICAL ABSTRACT



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ABSTRACT

$P2X_7$ receptors are implicated in the pathophysiology of psychiatric conditions such as depression and bipolar disorder. $P2X_7$ receptors regulate the release of pro-inflammatory cytokines from microglia, and gain-of-function $P2X_7$ mutations may contribute to the neuroinflammation found in affective disorders. However, the role of this receptor in mediating other mental health conditions and aberrant behaviours requires further examination. The current study we investigated the effects of germline genetic deletion of *P2xr7* on social and marble burying behaviours in mice throughout the critical adolescent developmental period. Marble burying behaviour is thought to provide a mouse model of obsessive-compulsive disorder (OCD). We also characterised the effects of *P2rx7* deletion on aggressive attack behaviour in adult mice and subsequently quantifieded microglial cell densities and c-Fos expression, a marker of neuronal activation. *P2rx7* knockout mice displayed reduced OCD-related marble burying behaviour which was most pronounced in late adolescence/early adulthood. *P2rx7* knockout mice also exhibited reduced aggressive attack behaviours in adulthood in the resident-intruder test. Reduced aggression in *P2xr7* knockout mice did not coincide with changes to microglial cell densities, however c-Fos expression was elevated in the piriform cortex of *P2rx7* knockout mice compared to wildtype mice. This study suggests that the $P2X_7$ receptor might serve as a novel target for serenic or anti−OCD therapeutics.

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1. Introduction

The purinergic $P2X_7$ receptor is an ATP-gated, nonselective cation channel localised predominantly on microglial cells in the central nervous system (CNS) [1–3]. These receptors are also detected on macroglial cells, and there is continued debate surrounding whether they are expressed on neurons (see [4]). The $P2X_7$ receptor possesses unique properties compared to $P2X_{1\text{-}6}$ receptors, as they require high extracellular ATP concentrations ($EC_{50} \geq 500$ μM) for activation [5,6]. Prolonged $P2X_7$ receptor activation results in the formation of a transmembrane pore allowing the permeation of molecules up to 1 kDa [7]. The channel is activated on demand in response to tissue damage, trauma, or pathology that results in elevated extracellular ATP [8].

$P2X_7$ receptor activation plays an important function in the cytokine response by facilitating synthesis and release of proinflammatory signalling molecules such interleukin-1β (IL-1β) through recruitment of the NLRP3 inflammasome complex [2,9]. This has led to $P2X_7$ being examined for its involvement in IL-1β-associated inflammatory conditions such as rheumatoid arthritis [10]. $P2X_7$ receptors have also been implicated in various pathologies including neurodegeneration and neuropathic pain [11,12]. For example, *P2rx7* knockout mice display reduced responses to chronic inflammation and neuropathic pain [11]. In addition, genetic variation in $P2X_7$ receptors has been associated with an increased risk of developing various psychiatric conditions. The *P2RX7* gene is located on a susceptibility locus (chromosomal region 12q24.31) for affective disorders [13], and gain-of-function polymorphisms have been associated with an increased risk of developing depression and bipolar disorder, and elevated IL-1β secretion [14–18]. Preclinically, IL-1β administration produces depressive and anxiogenic behaviours in mice [19,20] while, conversely, germline *P2rx7* knockout results in an anti-depressant phenotype in both the forced swim and tail suspension tests [21–23].

Despite evidence for the role of $P2X_7$ receptors in affective disorders, the involvement of $P2X_7$ receptors in other mental health conditions and aberrant behaviours has not been extensively investigated. Interestingly, obsessive-compulsive disorder (OCD) may have a neuroinflammatory basis as OCD patients have increased plasma concentrations of cytokines such as IL-1β, IL-6 and tumour necrosis factor-α (TNF-α) [24]. It is possible then that $P2X_7$ receptors play a role in driving the enhanced inflammatory status of these patients. In addition, increased aggression has been linked to affective disorders and OCD [25,26], and proinflammatory cytokines are thought to be mediators of aggressive behaviour [27,28]. We therefore hypothesised that $P2X_7$ receptors are involved in aggressive behaviour. The current study aimed to assess whether germline genetic knockout of the $P2X_7$ receptor affects compulsive and aggressive behaviours in mice. As the developmental trajectory for mental illness often begins during adolescence, we first assessed the impact of $P2X_7$ deletion on social and compulsive behaviours during early to late adolescence. We then examined the aggressive phenotype of $P2X_7$ knockout mice during early adulthood and investigated neurobiological changes in microglial cell density and regional c-Fos expression.

2. Methods

2.1. Mice

Male *P2rx7* knockout (*P2rx7*$^{-/-}$) and wildtype (WT) C57Bl/6 mice were bred at the Brain and Mind Centre as previously described [22]. *P2rx7*$^{-/-}$ mice are homozygous null mutants for the *P2rx7* gene that are healthy and fertile with no overt phenotype. Mice were housed 2–5 mice per cage in a temperature-controlled facility on a reversed light-dark cycle. All procedures were undertaken during the animals active/awake phase (10:00 – 17:00). Mice were provided food and water *ad libitum* and environment enrichment such as an igloo, running wheel, nesting material, sunflower seeds, and toilet paper roll. For the resident-intruder test, both resident and intruder mice were individually housed for 3 weeks prior to the test. Mice were handled for three days prior to the commencement of experimentation and habituated to the procedure room for at least one hour before testing. All research and animal care procedures were approved by the University of Sydney Animal Ethics Committee and agree with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*.

2.2. Behavioural testing

2.2.1. Social interaction and marble burying behaviour during adolescence and early adulthood

WT (n = 14) and *P2rx7*$^{-/-}$ (n = 8) mice was assessed for social and compulsive behaviours in the social interaction and marble burying tests at three developmental time points (ie. at post-natal day (PND) 28, 42, and 56). Mice are adolescent at PND 28 and have reached early adulthood by PND 56 [29,30]. The social interaction test was conducted in an open field apparatus that consisted of a red transparent box (43 cm x 43 cm x 25 cm), illuminated under a white light [31,32]. Individual WT or *P2rx7*$^{-/-}$ mice were paired with an unfamiliar docile A/J strain mouse. At commencement of the test an experimental and A/J strain mouse were placed in opposite corners of the open field and testing was conducted over a 10 min period. Behavioural measures including total interaction time and frequency of social behaviours (anogenital sniffing, nosing, following, and allogrooming) were scored by an experimenter blind to experimental condition. Locomotor activity in the social interaction test was quantified by tracking software (TrackMate, Motion Mensura, Australia).

The marble burying test models repetitive, compulsive behaviours observed in OCD and autism spectrum disorders [33]. Twenty-four hours following social interaction testing the WT and *P2rx7*$^{-/-}$ mice were tested in the marble burying test which was conducted in clean perspex homecage filled with 5 cm of dried corncob litter under white lighting. Mice were individually habituated in the test cage for 10 min before being returned to their homecage with their cage mates. A grid of 20 glass marbles were then placed in the corncob litter of the test cage before mice were returned to this cage for 20 min. Burying behaviours were quantified by an experimenter blind to experimental conditions with a marble considered buried if two thirds of its surface area was beneath the litter. Bedding was replaced and marbles were cleaned with 70 % ethanol solution between trials.

2.2.2. Territorial aggression of adult mice in the resident-intruder test

The resident-intruder test is an animal model of aggression and is based on a resident mouse establishing their homecage territory and aggressively defending it against an intruder. Weight and age-matched adult male WT (n = 48; 19 resident) and *P2rx7*$^{-/-}$ mice (n = 44; 27 resident) of 9 weeks age were used for this experiment. Our experimental design for this model used 3 pairings of animals: 1) WT resident vs. WT intruder, 2) *P2rx7*$^{-/-}$ resident vs. *P2rx7*$^{-/-}$ intruder, 3) *P2rx7*$^{-/-}$ resident vs WT intruder. All mice were single-housed for three weeks prior to experimentation. All items were removed from the resident's cage 10 min prior to testing. Intruder animals were introduced to the resident's homecage under white lighting, and behaviours were scored by two observers blind to genotype. The social behaviours of anogenital sniffing, nosing, and following were scored, as well as the aggressive behaviours of tail rattling, pinning of the intruder mouse, aggressive grooming, sexual domination, sideways threat, aggressive posture, pursuit and aggressive biting. The experiment was terminated if 15 bites occurred or when 10 min had elapsed.

Ninety minutes following the resident-intruder test, mice were deeply anesthetised with isoflurane and transcardially perfused with phosphate-buffered saline, followed by 4% paraformaldehyde [34]. Brains were removed and cryoprotected in graded sucrose solutions. The brains were initially submerged in a 15 % concentration solution for 24 h, followed by a 30 % solution over 72 h or until the brains sank.

Brains were sliced in 40 µm coronal sections with a cryostat.

2.3. c-Fos and Iba-1 immunohistochemistry

Brain tissue underwent c-Fos and Iba-1 immunohistochemical staining as previously described [34,35]. Briefly, brain slices were incubated in c-Fos primary antibody (Santa Cruz Biotechnology, USA) at a 1:10,000 dilution for 72 h or Iba-1 antibody at a 1:2000 dilution (WakoChem) overnight at 4 °C. Tissues were then incubated in a biotinylated secondary antibody (BA-1000, 1:500, Vector Laboratories, USA) for 1 h at room temperature. A peroxidase reaction was visualised with 3,3′-diaminobenzidine and tissue was incubated in glucose oxidase for 10 min (Sigma-Aldrich, Australia). Tissue was mounted on gelatinised glass slides prior to dehydration in graded alcohol solutions, clearing in xylene, and coverslipping with DPX mountant (Sigma-Aldrich, Australia).

2.4. Cell quantification

c-Fos$^+$ and Iba-1$^+$ cell quantification was performed manually as previously described [34–36]. Cell counts were performed by an experimenter blind to conditions with the use of a square graticule (0.5 mm × 0.5 mm), equating to 0.25 mm^2 when observed under a 20× objective on a light microscope. Brain regions analysed were identified using the Paxinos & Franklin mouse brain atlas [37]. The specific locations analysed are as follows: the prelimbic cortex (Bregma +1.98), the lateral septum, nucleus accumbens shell, and piriform cortex (Bregma +0.62 mm); the anterior paraventricular thalamic nucleus (Bregma -0.46 mm); paraventricular thalamic nucleus (Bregma −0.94 mm); the ventral and dorsal medial amygdala, basolateral amygdala, central amygdala (Bregma −1.58 mm); the lateral, dorsomedial and ventromedial hypothalamus (Bregma −1.58 mm); the CA1 and CA3 regions of the dorsal hippocampus and the dentate gyrus (Bregma −1.70 mm); the dorsomedial, dorsolateral, and lateral periaqueductal grey (Bregma −4.72 mm).

2.5. Data analysis

All tests were performed using SPSS (version 24; SPSS Inc. USA). Developmental behavioural data was analysed with two-way repeated measures ANOVA with genotype as the between subjects variable and age the repeated variable. Independent samples *t*-tests compared WT and *P2rx7*$^{-/-}$ mice data on individual days. For the resident-intruder test, behavioural parameters of resident WT and *P2rx7*$^{-/-}$ were compared using Independent samples *t*-tests and Mann Whitney U for continuous measures. Due to cell counts < 5, a Fishers Exact-test was used to compare the proportions of resident mice that bit intruders. c-Fos$^+$ and Iba-1$^+$ cell counts of WT v *P2rx7*$^{-/-}$ mice were compared using independent samples t-tests.

3. Results

3.1. Germline deletion of P2rx7 does not modify social behaviours or locomotion however does reduce marble burying behaviour

We first examined the effects of homozygous deletion of *P2rx7* on social and compulsive behaviours in mice from early adolescence through to early adulthood (at PND28, 42 and 56). *P2rx7* deletion did not influence social behaviour during adolescence through to adulthood, with no differences between WT and *P2rx7*$^{-/-}$ mice on the total time or frequency of social interactions observed on PND28, 42 or 56 (Fig. 1A & B). The age of testing influenced the total duration and frequency of social encounters (main effect of age: $F_{1,22} = 6.068$, $p < 0.01$; $F_{1,29} = 25.508$, $p < 0.001$ respectively), with mice interacting with passive A/J mice for significantly longer period of time and more frequently at PND28 compared to PND42 and 56. We also assessed locomotor activity in the social interaction test. Overall locomotor activity did not differ between WT and *P2rx7*$^{-/-}$ mice (two-way repeated measures ANOVA: no effect of genotype or age p's > 0.05; Fig. 1C).

Obsessive, repetitive behaviour was analysed in the marble burying test by quantifying the total number of marbles buried per session. Homozygous knockout of *P2rx7* in mice reduced the number of marbles buried (main effect of genotype: $F_{1,20} = 4.736$, $p < 0.05$, Fig. 1D). Despite *P2rx7*$^{-/-}$ mice burying less marbles overall, both genotypes buried more marbles with increasing age (main effect of age: $F_{2,40} = 7.143$, $p < 0.05$) and there was no genotype by age interaction ($p > 0.05$). Comparison of individual timepoints found *P2rx7*$^{-/-}$ mice buried significantly fewer marbles than WT mice at PND56 ($t(20) = 2.339$, $p < 0.05$; Fig. 1D).

3.2. Germline deletion of P2rx7 decreases the propensity of mice to perpetrate severe aggressive attacks compared to WT

We then sought to examine the effects of *P2rx7* deletion on aggressive behaviour in mice in adulthood in the resident-intruder test. Resident *P2rx7*$^{-/-}$ mice were confronted by either a WT or *P2rx7*$^{-/-}$ intruder mouse, however as there was no differences between these groups on the various social and aggressive measures recorded in the resident-intruder test (Independent samples *t*-test and Mann-Whitney U tests: p's > 0.05), the groups were collapsed and data subsequently compared to that of WT residents who opposed a WT intruder mouse. WT and *P2rx7*$^{-/-}$ mice did not differ in social or aggressive measures (Independent samples *t*-test and Mann-Whitney U test respectively p's > 0.05; Table 1 and Fig. 2A), the latency to first aggressive encounter nor the total frequency of aggressive interactions (Independent samples *t*-test and Mann-Whitney U test respectively p's > 0.05; Fig. 2B and 2C). However, resident *P2rx7*$^{-/-}$ mice perpetrated significantly fewer biting attacks than resident WT animals (Mann-Whitney U test, $U = 857.50$, $p < 0.05$; Fig. 2D). Moreover, the percentage of resident *P2rx7*$^{-/-}$ mice that bit an intruder mouse was also significantly lower than the percentage of resident WT mice that did this (Fisher's Exact Test $p < 0.05$; Fig. 2E).

WT and *P2rx7*$^{-/-}$ animals also did not differ in the latency to, or total time spent defensive burying in the resident-intruder test (Independent samples *t*-test and Mann-Whitney U test p's > 0.05; see Table 1). Social behaviours such as nosing and anogenital sniffing were also not significantly different between genotypes in the resident-intruder test (Independent samples t-tests p's > 0.05; Table 1).

3.3. Resident P2rx7 knockout mice display increased c-Fos expression in the piriform cortex compared to resident WT mice following agonistic encounters in the resident-intruder test

We then characterised the impact of *P2rx7* deletion on brain activation of resident animals following agonistic encounters by quantifying the density of c-Fos$^+$ cells in several aggression-related brain structures. As with the behavioural analyses, there was no effect of varying the genotype of the intruder on c-Fos counts in the *P2rx7*$^{-/-}$ resident group, therefore the c-Fos data were collated (Independent samples t-tests; p's > 0.05). The resident *P2rx7*$^{-/-}$ mice had significantly higher c-Fos positive neuron counts in the piriform cortex than resident WT animals ($t(26) = 2.136$, $p = < 0.05$; Fig. 3A). No other significant differences in c-Fos counts were observed between resident *P2rx7*$^{-/-}$ mice and resident WT mice in the 20 other brain regions counts (see supplementary Table S1). As $P2X_7$ receptors are predominantly located on microglial cells within CNS we then compared Iba-1$^+$ cell densities between resident *P2rx7*$^{-/-}$ and WT mice in a sub-set of mice. *P2rx7* deletion had no effect upon Iba-1 microglial cell densities in the piriform cortex (independent samples *t*-test, $p > 0.05$, see Fig. 3B) or any other regions examined (Independent samples t-tests, p's > 0.05; see supplementary Table S2).

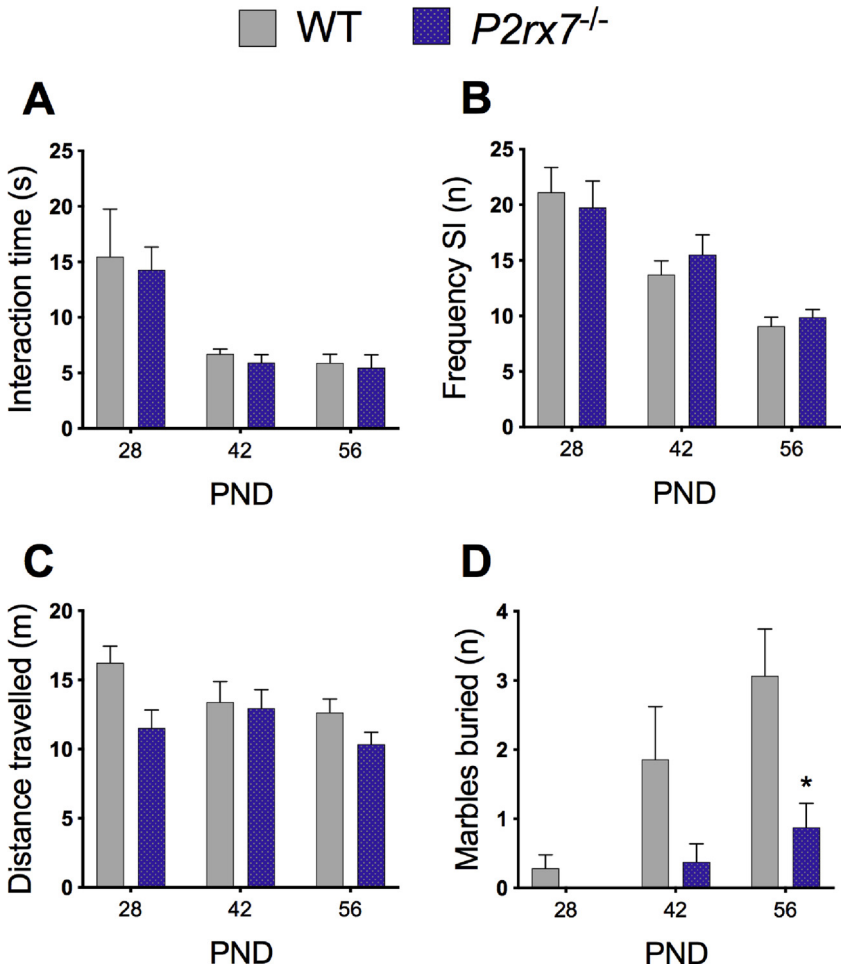

Fig. 1. Germline deletion of *P2rx7* did not influence social behaviour or locomotor activity but reduced repetitive behaviour in early adulthood (A) Total time spent interacting, and **(B)** the frequency of social interactions (SI); **(C)** Total locomotor activity during social interaction testing; **(D)** Total number of marbles buried during marble burying testing. Data are represented as mean + SEM (WT n = 14, *P2rx7*$^{-/-}$ n = 8). *p < 0.05, WT versus *P2rx7*$^{-/-}$.

Table 1
The defensive and social behaviours of resident WT and *P2rx7* knockout mice in the resident-intruder test (WT n = 19, *P2rx7*$^{-/-}$ n = 27). Data presented as mean ± SEM.

Behaviour	Genotype	
	WT	P2 × 7$^{-/-}$
Defensive burying		
1. Latency to burying (s)	418.37 ± 40.92	367.26 ± 31.36
2. Total burying time (s)	18.11 ± 8.67	13.91 ± 4.01
Social interaction		
3. Total interactions (n)	25.11 ± 2.42	26.15 ± 2.27
4. Nosing (n)	6.37 ± 0.66	8.33 ± 0.96
5. Anogenital sniffing (n)	9.37 ± 1.01	8.93 ± 1.12
6. Following (n)	6.95 ± 0.81	6.37 ± 0.66

4. Discussion

Genetic knockout and antagonism of P2X$_7$ receptors in mice has anti-depressant effects in mouse models of depression such as the forced swim test, tail suspension and sucrose preference tests [21,22,38,39]. Here we extend on current knowledge of the behavioural phenotype of *P2rx7* knockout mice by providing unprecedented evidence that *P2rx7* mediates OCD-related and aggressive behaviour. *P2rx7* knockout mice displayed less marble burying behaviours than WT mice. Further, *P2rx7* knockout mice displayed less aggressive biting behaviour than WT mice in the resident-intruder test. The reduced aggressive behaviour of *P2rx7* knockout mice was associated with increased activation of the piriform cortex as measured by c-Fos expression. Interestingly, the piriform cortex contains dense expression of P2X$_7$ receptors, and is implicated in the neurobiology of aggression [40].

Research is required to elucidate the neurodevelopmental role of P2X$_7$ receptors [41]. Male *P2rx7* knockout mice display increased sociability compared to WT mice in adulthood (2–3 months of age) [42], however no studies have addressed social behaviour during adolescence, a period in which various neuropsychiatric conditions are manifest. Here we characterised the behavioural phenotype of *P2rx7* knockout mice during adolescence (PND 28 – PND 56) and could not find any evidence of altered social behaviour compared to WT mice. Our findings in adolescent mice are inconsistent with studies in adult mice and rats which showed that P2X$_7$ receptor antagonists or germline genetic deletion of *P2rx7* receptors increased social behaviour [42,43]. It appears the prosocial effects of *P2rx7* knockout are only evident in adulthood, and that P2X$_7$ receptors do not subserve adolescent social behaviours.

The current study provides unprecedented evidence that the P2X$_7$ receptor mediates repetitive and compulsive behaviours as measured in the marble burying test. WT mice displayed a clear increase in marble burying behaviour from PND 28 to 56, which was significantly less pronounced in *P2rx7* knockout mice. The reduced marble burying behaviour of the *P2rx7* knockout mice was most evident in late adolescence/early adulthood at PND 56. There is debate about the specific cellular expression of *P2rx7* receptors in the brain [44,45], although it is clear that these receptors are found on microglial cells and influence

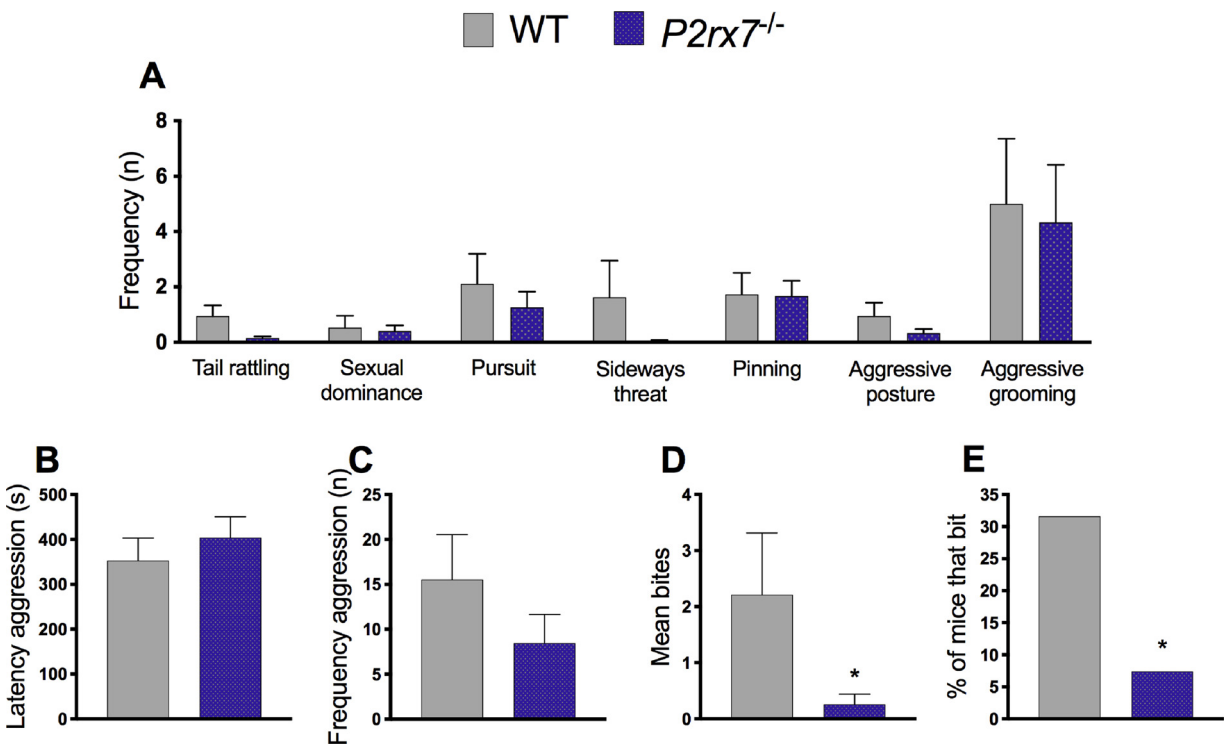

Fig. 2. Germline *P2rx7* deletion in mice decreases aggressive biting behaviour in the resident-intruder test. (A) Frequency of individual aggressive behaviours scored in the resident-intruder test. **(B)** Mean latency to and **(C)** frequency of aggressive behaviours in the resident-intruder test. **(D)** Mean number of bites perpetrated by resident WT and *P2rx7*$^{-/-}$ mice and **(E)** the % of mice that bit when paired against intruder mice in the resident-intruder test (WT n = 19, *P2rx7*$^{-/-}$ n = 27). * $p < 0.05$. Data presented as mean + SEM.

the release of inflammatory cytokines [7,46]. Future studies could examine whether cell-specific genetic deletion of *P2rx7* on microglial cells reproduces the effects of germline *P2rx7* knockout on marble burying. The marble burying test is considered a mouse model of OCD demonstrating face validity through compulsive behaviours, construct validity as behaviours are modulated by dysregulated steroid, monoamine transmitter, and nitric oxide activity, and finally predictive validity with compulsive marble burying being attenuated by SSRIs but not anxiolytic drugs [47,48]. Interestingly, there is emerging data that neuroinflammation and increased microglial cell activation occurs in the brain of OCD patients [24,49,50]. Future studies could examine whether single nucleotide polymorphisms in *P2rx7* are associated with

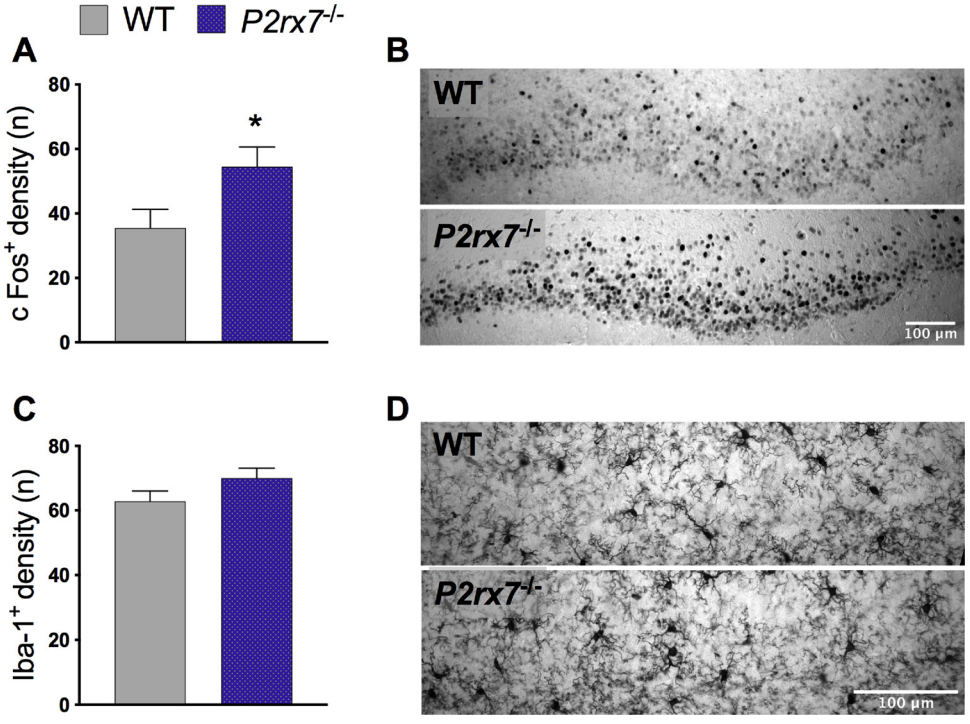

Fig. 3. Resident *P2rx7* knockout mice display enhanced brain activation but identical microglial cell density in the piriform cortex compared to resident WT mice following an aggressive encounter in the resident-intruder test A) Density of c-Fos positive cells in the piriform cortex in WT and *P2rx7*$^{-/-}$ mice (WT n = 9, *P2rx7*$^{-/-}$ n = 20), and **(B)** representative images of c-Fos in the piriform cortex. **(C)** Density of Iba-1 positive cells in the piriform cortex in WT and *P2rx7*$^{-/-}$ mice (WT n = 7, *P2rx7*$^{-/-}$ n = 8), and **(D)** representative images of Iba-1^{+} cells in the piriform cortex of resident WT and *P2rx7*$^{-/-}$ mice. Data presented as mean + SEM. * $p < 0.05$.

altered risk of developing OCD.

Here we report for the first time that genetic knockout of *P2rx7* in mice decreases aggressive biting behaviours in the resident-intruder test. We found that the reduced aggression of resident *P2rx7* knockout mice was associated with greater c-Fos expression in the piriform cortex compared to WT mice. The piriform cortex is a relatively understudied brain region in aggression research, however a study in cats showed that electrical stimulation of this region reduced biting attack behaviour driven by hypothalamic stimulation [40]. Notably, the piriform cortex contains a relatively high density of $P2X_7$ receptors as demarcated by in situ hybridisation [51]. Future studies are required to clarify the precise role of $P2X_7$ receptors in the piriform cortex in aggressive behaviour. There is converging evidence from human and animal studies that proinflammatory cytokines are mediators of aggressive behaviour [28,52]. For example, state anger in rugby players is associated with increased plasma concentrations of IL-1β, and direct brain injection of IL-1β increased aggressive behaviour in cats [27,53]. Thus, our working hypothesis is that activation of $P2X_7$ receptors expressed on microglial cells triggers the release of inflammatory cytokines such as IL-1β which inhibits the piriform cortex and facilitates aggressive biting behaviour.

In conclusion, this study elaborates on the behavioural profile of *P2rx7* knockout mice by showing that these animals display reduced repetitive, compulsive burying behaviours and aggression. These results suggest that $P2X_7$ antagonists might serve as novel serenic or anti − OCD agents.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.neulet.2019.134575.

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Chapter 5

General Discussion

Chapter 5 – General discussion

The present thesis reports a series of studies that aimed to enhance our understanding of neurobiological and genetic determinants of behaviour relevant to PTSD. The experimental work in **Chapters 2** and **3** were designed primarily to further our understanding of a mouse model of PTSD developed by Wotjak and colleagues, and are summarised in Table 1. In **Chapter 2** the neurobiological understanding of this model was advanced through showing that the observed long-term PTSD-relevant behavioural disturbances observed were accompanied by losses of dendritic spines densities on pyramidal neurons, as well as microglial cell hyper-ramification, in the medial prefrontal cortex and the hippocampus. In **Chapter 3** genetic vulnerability to stress and trauma was explored by examining the impacts of genetic deletion of the ABC transporter P-gp in the mouse model of PTSD. Most notably, P-gp KO mice displayed increased depressive-like behaviour, social withdrawal, sensorimotor gating, locomotor activity, and reduced startle responses and initial, but not long-lasting reductions in fear conditioning. In **Chapter 4**, we extended on the known role of $P2X_7$ receptors in mediating depression-like behaviour to other psychiatric diseases-relevant behaviours by showing that $P2X_7$ receptor KO mice displayed decreased OCD-related behaviour and aggression.

Neurobiological disturbances underlying the genesis and maintenance of PTSD are not well understood, however both peripheral inflammation [129-132] and volumetric grey matter loss [81-83] are often cited as potential clinical biomarkers. To this end, in **Chapter 2** we investigated whether long-term molecular, synaptic, and microglial cell alterations occurred in response to footshock trauma in an animal model of PTSD. Prior research has shown that mice in this model have reduced hippocampal

volumes [99], and increased expression of various inflammatory markers [100]. We therefore hypothesised that the loss of hippocampal volume may be related to loss of dendritic spine densities, and that increased brain inflammation might also be explained by increased microglial cell activation.

We first replicated PTSD-related behavioural disturbances that have previously reported in this model [98-101]. In **Chapter 2** we found exposure to a single, relatively high intensity electric footshock led to the formation and long-term retention of fear memory. We found that there appeared to be a sensitised fear response following incubation in the homecage for 23 days. This is reminiscent of clinical PTSD presentation where a patient's symptoms worsen over time [9]. Footshock trauma also evoked increased startle reactivity to a loud acoustic stimulus which models hyperarousal symptoms observed in PTSD patients. It is noteworthy that in **Chapter 2** we provided unprecedented data showing that that mice undergo a transient facilitation of sensorimotor gating function following exposure to the traumatic footshock stimulus. Further investigation is required to provide a deeper understanding of this effect, particularly as the few studies that show abnormal sensorimotor gating in clinical PTSD populations report loss of inhibitory modulation of startle response in combat veterans [240] and traumatised children [241]. Taken together, the associative fear memory retention and development of non-associative startle responses reported in **Chapter 2** satisfied the symptomatic face validity of the PTSD model.

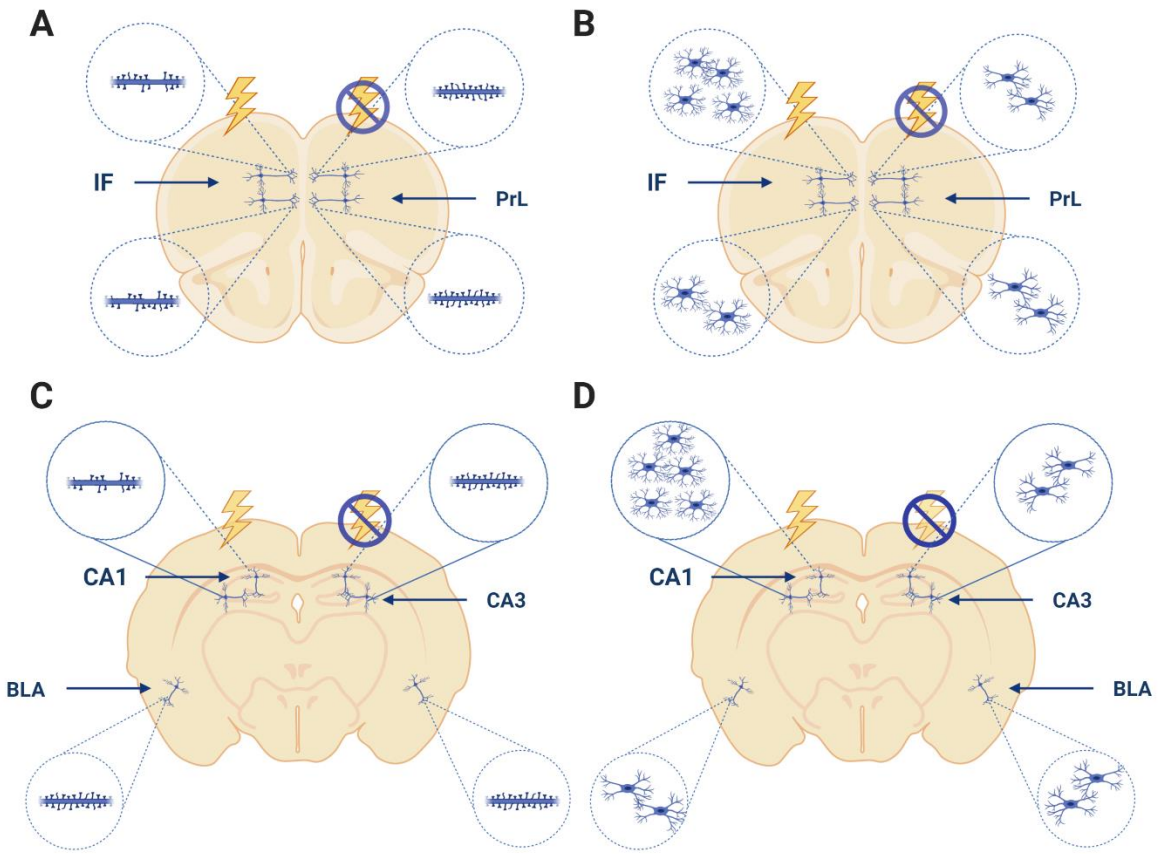

Figure 1. The corticolimbic system is susceptible to neuronal dendritic spine loss and elevated hyper-ramified microglial cell densities 32 days following footshock exposure. In the prefrontal cortex, pyramidal neuron dendritic spine loss **A)** is observed alongside an increased density of hyper-ramified microglial cells **B)**. A similar effect is observed in the CA1 and CA3 regions of the hippocampus but the magnitude of dendritic spine loss **C)** and increased hyper-ramified microglial cell activity **D)** is greater than that observed in the prefrontal cortex. Dendritic spines and microglia were not affected within the basolateral amygdala.

The corticolimbic structures examined in **Chapter 2** subserve and modulate fear memory formation, retention and expression [242], and are also the site of structural and functional deficits that might underlie PTSD symptoms [83]. In this PTSD model, three interesting and novel neurobiological alterations were identified across these structures. First, the medial prefrontal cortex and hippocampus were susceptible to a robust, long-lasting microglial cell response following footshock trauma. While microglial cell densities were elevated at 32 days post shock exposure, perhaps the most striking finding of **Chapter 2** was the magnitude of concomitant microglial cytoskeletal

restructuring. Despite the comparative brevity of the footshock in this PTSD model, the microglial morphology in experimental mice was reminiscent of the hyper-ramified phenotype that has been reported following 21 days of chronic restraint or 28 days of chronic unpredictable stress [182, 184]. Collectively these studies show that different stressors both produce structurally similar hyper-ramified microglial cell morphology.

The results of **Chapter 2** compliment prior reports of increased microglial cell densities and concomitant inflammatory gene expression in the anterior cingulate cortex in this PTSD model [100]. Reactive or "activated" microglial cells, particularly associated with an M1 or pro-inflammatory phenotype, have been characterised to undergo morphology changes including retraction and simplification of processes [243-245], in contrast to microglial cell measurements in **Chapter 2** which showed microglial cells adopt a hyper-ramified morphology with elongated processes. Accumulating evidence indicates microglial cell morphology and function is more complex than appreciated by categorical phenotyping, such as into M1/M2 polarisation [246]. We conclude that hyper-ramified microglial cells characterised in **Chapter 2** cannot be ruled out from mediating pro-inflammatory signalling based on morphology, which is supported by recent evidence of hyper-ramified microglial cells mediating pro-inflammatory signalling in the hippocampus in response to chronic unpredictable stress [184]. Moreover, Kao et al., [100] showed using RNA-seq analysis that neuroinflammation was evident in tissue from the anterior cingulate cortex of mice in the model of PTSD used in this thesis [100].

Future studies might examine the molecular determinants of trauma-induced alterations in microglial cell densities and morphology. Chronic unpredictable stress has shown persistent and long-lasting hyper-ramification of microglia in the CA3 region

of the hippocampus with concurrent elevation of RAGE and HMGB1 mRNA [184]. This is important as HMBG1 is an effector of inflammation that sensitises microglia [247]. Moreover, it is elevated in the prefrontal cortex and hippocampus of mice 31 days after predator exposure in an animal model of PTSD [248], and is also increased in the plasma of trauma patients who go on to develop PTSD [249]. Future research could then determine whether RAGE-HMGB1 signalling is a mechanism for the long-lasting microglial cell response observed in **Chapter 2**. Microglial cell surface RAGE expression in this PTSD model could be quantified using flow cytometry. Moreover quantitative real-time PCR measurements for mRNA of RAGE and HMGB1 could be measured, as well as plasma HMBG1 levels. Clinical research has reported elevations in plasma HMBG1 concentrations in trauma patients who developed PTSD [249]. Therefore preclinical models of PTSD could be used to evaluate whether temporal changes occur HMBG1 plasma levels, and how they correlate with long-lasting microglial cell activity (see **Chapter 2**). This could potentially assist in the development of a clinical biomarker to predict PTSD development.

Another novel observation made in **Chapter 2** was that long-lasting microglial cell activity coincided with regionally-matched neuronal dendritic spine loss in the medial prefrontal cortex and hippocampus. The plasticity of dendritic spines renders them highly responsive to experience-dependent stimuli, as we have repeatedly demonstrated with chronic stress models [114, 115, 183]. Here, we show for the first time that dendritic spines atrophy occurs in response to footshock trauma in this mouse PTSD model, or at least in response to re-exposure to the previously shock-paired context 32 days after conditioning. We hypothesise that the behavioural disturbances observed in the PTSD model may owe, at least in part, to the dendritic spine atrophy

observed in **Chapter 2**. A failure of the hippocampus to process contextual cues and signal safety to the medial prefrontal cortex is hypothesised to underlie exaggerated hyperarousal and fear states experienced by patients with PTSD [83, 250], and dendritic spine modifications could contribute to these functional deficits. Future studies could seek to explore the specific relationship between dendritic spine loss and the behavioural disturbances observed.

In vivo two-photon imaging may help achieve this. This technique allows for repeated monitoring of dendritic processes in real-time and could be utilised to delineate the temporal aspects of dendritic spine dynamics in this model of PTSD. Thinned-skull preparation [251] or imaging cranial windows from implanted glass coverslips [252] in Thy-1 transgenic mice provides a method to repeatedly track dendritic changes over time, targeting the same dendritic branches and spines originally sampled. While this method is readily achievable in the prefrontal cortex, challenges arise for long term *in vivo* imaging of dendritic spines in the hippocampus. Despite being a powerful tool, two-photon microscopy is at present limited to an imaging depth of ~700 μm. While protocols for creating cranial windows for hippocampal imaging have been established [253], this requires removal of overlaying cortical tissue. However, this induces acute microgliosis and, with microglial activity a primary aim of this PTSD model in **Chapter 2** may introduce confounds. Cortical removal from a single hemisphere is not found to influence hippocampal-dependent behaviour (such as contextual fear conditioning [253]) which might be exploited to advance the understanding the relationship between trauma-induced dendritic spine regression and behaviour.

In **Chapter 2** we showed for the first time that expression of the complement factor C1q was increased in the CA1 region of the hippocampus in the mouse model of PTSD used in this thesis. The removal of synapses in the CNS during development occurs via a classical complement cascade mechanism [170], and the deposition of C1q and then C3 at synapses signals for microglial cell engulfment of synaptic elements [156, 170]. The classical complement cascade has been implicated in the pathophysiology of multiple sclerosis, schizophrenia, and epilepsy [177, 254, 255]. However, little research has been conducted on the role of the complement system in PSTD, with only one study showing that PTSD patients have increased expression of the classical complement pathway in serum samples [179]. Our findings here support further investigation of the complement C1q system this mouse model of PTSD. With no changes in C4 immunoreactivity, the presence of C1q is insufficient to support our hypothesis of complement-mediated microglial stripping of dendritic spines, as C1 cleavage of C4 is critical for activation of downstream components that initiate microglial engulfment of dendritic spines [170, 177, 178, 256].

An alternative hypothesis that may explain the elevated hippocampal C1q observed in **Chapter 2** is that this may serve an anti-inflammatory function. C1q secretion, in the absence of other complement factors, is shown to decrease inflammatory gene expression and chemotaxis of microglial cells [257, 258]. In the context of **Chapter 2,** an increase of C1q in the hippocampus may function to counter microglial cell activation and increased neuroinflammatory signalling that occurs in response to trauma [257]. This hypothesis does not explain the absence of C1q elevation in other brain structures analysed, however the hippocampus is known to be one of the most susceptible brain regions to the detrimental impacts of stress. Multiple time point

tracking of C1q would provide an improved temporal window into when C1q levels become elevated. Further, screening for additional downstream fragments (i.e. complement factor C3 and C5) may also shed light into the role of the classical complement cascade in this animal model of PTSD. Cell specific analyses of complement factors in neuronal and microglial cells might also help illuminate the role of this system in response to stress.

Interventions blocking long-lasting microglial cell activity, such as that following footshock in **Chapter 2**, could be utilised to determine whether dendritic spine loss in this model of PTSD occurs independently of elevated microglial cell density and morphology alterations. The tetracycline antibiotic minocycline has been shown to block chronic stress-induced microglial cell hyper-ramification [182]. Similarly, the colony stimulating factor 1 (CSF1) antagonist PLX5622, which depletes microglia from the brain, was found to reduce the effects of chronic stress [259]. Therefore, the administration of the minocycline or PLX5622 could be administered to observe whether it reverses behavioural and neurobiological changes observed in response to trauma in the present thesis. More broadly, future studies should investigate the negative influence of a heightened immune response and dendritic spine loss on bidirectional neural connectivity between hippocampus and medial prefrontal cortex [260]. The integrity of this network is required for accurate context-specificity relating to fear memories [261]. Dysfunction in this pathway may create difficulty assessing a threat and generating an appropriate response [260], which may underlie lasting inappropriate fear responses in PTSD.

Chapter 3 extended on the research of **Chapter 2** and aimed to determine whether genetic deletion of the ABC transporter P-gp modified an animal's behaviour

and conferred vulnerability to the effects of traumatic footshock stress in the mouse model of PTSD. P-gp was hypothesised to modify the stress response, as it has been previously shown to regulate brain levels of the stress hormones corticosterone and cortisol via its expression at the blood brain barrier [262]. As we demonstrated trauma influenced microglial cell activity in **Chapter 2** we also continued our microglial cell analysis in **Chapter 3**.

On a behavioural level P-gp appeared to influence fear conditioning and stress responses. P-gp KO mice demonstrated lower conditioned fear responses compared to the WT mice. However, the reduced fear conditioning exhibited by P-gp KO mice on day 1 was not observed at day 22. Somewhat inconsistent with these results, P-gp KO mice appeared more susceptible to injection stress which selectively triggered frantic anxiety behaviour in this strain and not in WT or Bcrp KO mice. Further research is required to explain these effects with one potential factor being that corticosterone is a substrate of P-gp but not Bcrp transporters, therefore the stress effects of ABC transporters on glucocorticoid signalling and the HPA axis may only be observable in P-gp KO animals.

In **Chapter 3**, irrespective of stress or trauma exposure, we observed that genetic deletion of P-gp in mice increased depression-like behaviour, social withdrawal, sensorimotor gating, locomotor activity, and reduced startle responses in mice. We hypothesised that an accumulation of corticosterone in the brain of P-gp KO mice during the early developmental phase may have contributed to a depressive phenotype by disrupting neurodevelopmental trajectories and also impacting function of the HPA axis [221]. Indeed, adult P-gp KO mice demonstrate a perturbed HPA axis in adulthood with lower levels of circulating corticosterone and concomitant increased GR expression

[263, 264]. Additionally, loss-of-function SNPs in *MDR1* are associated with development and symptom severity of depression and schizophrenia [265-267]. To further elucidate the role of P-gp KO in the development of depression and social withdrawal behaviour, future studies should emphasise tracking the HPA axis development and dysregulation in early development (i.e. PND1), through to adulthood.

Table 1. Summary of the methods, behavioural outcomes and neurobiological alterations observed in Chapter 2, Chapter 3, and Chapter 4. ↑ Increase ↓ Decrease ↔ No change; KO = knockout, WT = Wild type; ASR = Acoustic startle response, PPI = Prepulse inhibition, P-gp = P-glycoprotein, *P2rx7*$^{-/-}$ = Purinergic receptor 7 KO.

Chapter	**2**	**3**	**4**
Strain	C57BL/6j	FVB	C57BL/6j
Sex	Male	Female	Male
n + age	**WT (~12w)** control = 18 shock = 18	**WT (~12-16w)** WT control = 7 WT shock = 10 **KO** P-gp$^{-/-}$ control = 12 P-gp$^{-/-}$ shock = 12	**Developmental(PND 28-56)** WT = 14 *P2rx7* $^{-/-}$ = 8 **Adult (~9w):** WT = 19 *P2rx7*$^{-/-}$ = 27
Experimental Intervention	➢ 2 sec, 1.5mA foot shock ➢ 32 day protocol	➢ Germline genetic deletion of P-gp. ➢ 2 sec, 1.5mA foot shock + auditory cue ➢ 30 day protocol	➢ Germline genetic deletion of *P2rx7*
Behavioural results	↑ Freezing ↓ Locomotor activity ↑ Transient facilitation of PPI ↑ Long term increased ASR	↓ Freezing in shock Pgp-KO (vs WT shock) ↑ P-gp KO depression-like behaviour (independent of shock) + ↑ Darting + ↑ Social withdrawal and hyperactivity + ↑ PPI + ↑ ASR	***P2rx7*** $^{-/-}$ **(vs WT)** **Age: PND 28 - 56** ↔ Social interaction ↓ Marble burying (OCD-like) ***P2rx7*** $^{-/-}$ **(vs WT)** **Age: 9 weeks (adult)** ↓ Aggressive territorial biting
Neurobiology **Dendritic spines**	**Shock (vs control) at 32 days** ↓ dendritic spines: CA1, CA3, IF, PrL ↔ dendritic spines: BLA		
Microglial cells	**Shock (vs control) at 32 days** ↑ Iba-1^{+} cell density: CA1, CA3, IF ↔ Iba-1^{+}cell density: PrL, BLA ↑ Iba-1^{+} cell process length, volume, complexity: CA1, CA3, IF, PrL	**P-gp KO (vs WT) at 30 days** ↑ Iba-1^{+} cells in CA3; hypo-ramified **P-gp KO shock + WT shock (vs P-gp KO + control WT) at 30 days** ↓ Iba-1^{+} cell density in BLA, LA, MePV	***P2rx7*** $^{-/-}$ **(vs WT)** ↔ Iba-1^{+} cell density
c-Fos			***P2rx7*** $^{-/-}$ **(vs WT)** ↑ c-Fos piriform cortex
Glucocorticoid receptor		**P-gp KO (vs WT)** ↓ Glucocorticoid receptor density in the CA3	

While P-gp deletion did not influence trauma-induced changes in microglial cell activation, it did on its own increase microglial cell densities in the CA3 region of the hippocampus. Interestingly, on further inspecting their morphology, microglial cells demonstrated a hypo-ramified morphology analogous with reactive microglial cell activity, that is reduced branch complexity and length. We speculated that lower concentrations of circulating corticosterone may result in microglial proliferation and activity as glucocorticoid receptors localised on microglia are shown to inhibit microglial cell activity [268].

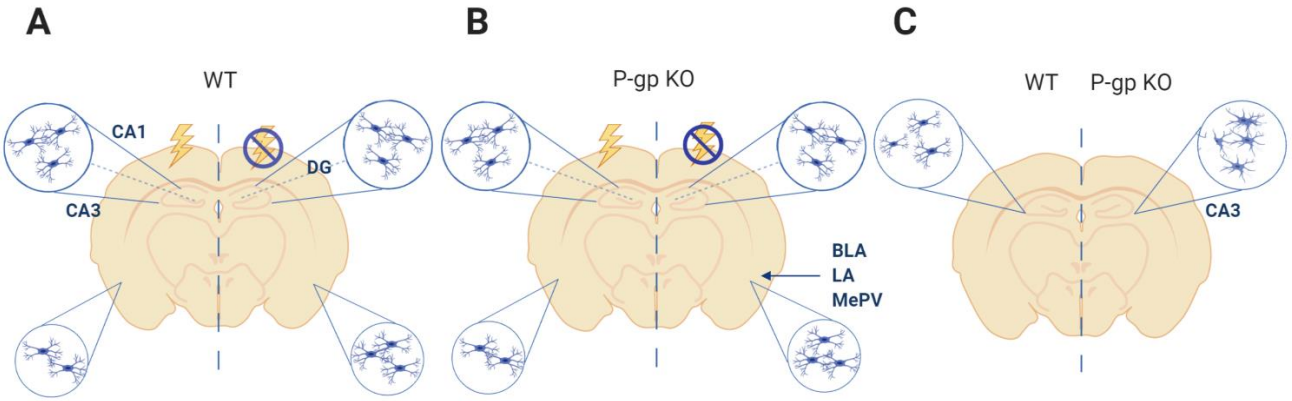

Figure 2. In contrast to Chapter 2, microglial cells in the limbic system of FVB strain WT and P-gp$^{-/-}$ mice are differentially affected by footshock exposure at 30 days in Chapter 3. In the basolateral, lateral, and medial posteroventral nuclei of the amygdala reduced density of microglial cells in WT mice **A)** is also observed in P-gp KO mice **B)**, however the hippocampal formation was not affected. A genotype effect was observed in the CA3 where, independent of footshock, P-gp KO mice demonstrated an increased density of microglial cells which displayed a hypo-ramified morphology.

As to why the dorsal CA3 appeared vulnerable to these effects requires further research, however one explanation may revolve around the fact the *mdr1b* isoform of P-gp is only expressed within the hippocampus [269]. We hypothesise here that germline knockout of the *mdr1b* isoform in P-gp KO mice may results in the hippocampus being particularly vulnerable to abnormal microglial cell activity. As P-gp is expressed on both the nuclear

and cell membranes of microglial cells it is plausible that P-gp can modulate the activity of microglia and therefore influence the abnormalities observed in **Chapter 3** such as depressive behaviour and social withdrawal.

The conditional knockdown of P-gp on microglia would specifically show the effects the transporter has on microglial cell activity and morphology both at baseline and under experimental stress conditions. Importantly, this would address the intrinsic issue of germline deletion of P-gp and counter-adaptive mechanisms that might occur during development. An alternative approach is developing a Cre/lox model where mice with loxP flanking *Mdr1a* are administered viral vectors for Cre recombination into targeted brain structures via direct cannulation. Going beyond brain region-specific deletion, cell specific Cre-lox deletion strategies might be implemented to selectively delete *Mdr1a* on brain endothelial cells or on microglia cells, with time-specific recombination using a tamoxifen-inducible system.

Noteworthy inconsistencies in results were observed in **Chapter 2** and **Chapter 3** likely due to the different background strains and the sex of the mice used across the chapters. In **Chapter 2** male C57BL/6 mice were used, whereas in **Chapter 3** female P-gp KO mice on an FVB background were utilised. Unlike male C57BL/6 mice, female FVB mice showed fear conditioning in the short-term but failed to express fear memory or exaggerated hyperarousal responses in the longer-term. This accords with Farley et al., [270] who showed that FVB mice are less susceptible to fear conditioning as they demonstrated significantly lower freezing responses than C57BL/6 mice. Interestingly, another strain or sex-based discrepancy was observed in the effects of footshock exposure on microglial cell density: while female FVB mice in **Chapter 3** displayed a footshock-induced decrease in microglial cell density in the BLA, no such effect was

observed in male C56BL/6 mice in **Chapter 2.** We hypothesise that discrepancies between **Chapters 2** and **3** may afford to strain differences, sexual dimorphism, or both. Indeed, stress promoted alterations in microglial cell activity in corticolimbic system has been shown to occur in a sex and strain-dependent manner [193].

Additionally, FVB mice in **Chapter 3** showed no long-term footshock mediated changes to microglial cells in either the dorsal hippocampal regions or the anterior cingulate cortex, disagreeing with both the outcomes of **Chapter 2**, as well as the previously published Kao et al., who also utilised male C57BL/6 mice in this PTSD model [100]. Further research is required to explain these conflicting findings, with one potential explanation being the time between final behavioural testing and microglial cell analysis. Mice in **Chapter 2** were culled within 24 h of the end of the behavioural battery, while mice in **Chapter 3** remained in their homecage for 5 days until the experiment was terminated and this may have therefore influenced the results. Temporal analysis of elevated microglial cell activity following a stressor shows a return to baseline in 30-40 days. Chronic unpredictable stress in rats leading to elevated microglial cell densities in the CA3 and PrL which returned to baseline after 40 days of recovery [271], while elevated Iba-1+ cell densities within the HPC following repeated footshock stress were ameliorated following a month of recovery [190]. Based on this it appears unlikely then that the different sampling times post-stressor could explain the discrepancies in microglial density or morphology, however analyses performed on brains sampled at the same time post-stressor are necessary to rule out this explanation. An alternative is that differences in mouse strain or sex used across these chapters might explain these discrepant findings. The experiment in **Chapter 3** could be repeated with

FVB WT and P-gp KO animals being restricted to the same timeline as mice in **Chapter 2** to determine if differential outcomes are due to a time delay.

An experimental flaw present in **Chapter 3** and **Chapter 4** of this thesis that must be addressed in future studies is that knockout and WT animals were derived from independent breeding stocks. While the mice were of the same background strain and bred under identical conditions there is a possibility that the study might be confounded by epigenetic changes in the mice. Future studies should breed heterozygous breeding pairs to avoid this potential issue.

While SSRIs exert moderate anti-inflammatory effects via an unknown mechanism, a significant number of PTSD sufferers administered this drug still present with detrimentally elevated pro-inflammatory cytokine concentrations which are linked to disturbed mood and cognitive deficits [129]. This highlights that SSRI treatment alone is not sufficient to address inflammation and may help explain why the SSRI drug class is, overall, not more efficacious than placebo or psychotherapy in the treatment of PTSD. Despite evidence of neuroinflammation in PTSD, anti-inflammatory agents are not currently used to treat the disorder. The efficacy of off-label anti-inflammatory drugs for the treatment of PTSD has recently been reviewed in detail [272]. This review analysed non-steroidal anti-inflammatory drugs (NSAIDs), cytokine-targeted monoclonal antibodies, glucocorticoids, angiotensin-converting enzyme (ACE), and angiotensin receptor blockers (ARB) [272].

The efficacy of NSAIDs and cytokine-targeted monoclonal antibodies have not directly been tested in PTSD populations, however both show promise in reducing pro-inflammatory cytokine levels in depressed cohorts [273, 274]. Ibuprofen administration in a rat model of PTSD (single prolonged stress), has shown promise by reducing

elevated levels of the pro-inflammatory cytokines TNF-α and IL-1β, and also minimising anxiety behaviours [275]. In PTSD patients, exogenous glucocorticoid administration attenuated PTSD symptoms [276, 277], and has also been considered somewhat efficacious in reducing risk of developing PTSD following trauma [278]. However, this field requires further research as there remains contentious debate around the role of glucocorticoids in the neuropathophysiology of PTSD.

There is evidence that stress and anxiety may be modulated by the renin-angiotensin pathway, an essential pathway for cardiovascular system regulation [22]. In line with this, ACE inhibitors and ARBs, predominantly used to treat hypertension and with potential off target anti-inflammatory effects, have been investigated [279]. ACE inhibitors and ARBs, which block the synthesis of angiotensin II and its target receptors, also reduce PTSD symptom severity in humans [279]. Importantly, ARBs also show promise in ameliorating inflammation-associated fear extinction impairments in rats [280].

Modulation of endocannabinoid system in PTSD is also relevant to the current thesis. The importance of endocannabinoid signalling in the treatment of PTSD has received attention with researchers investigating the effects of both endogenous and synthetic cannabinoids in animal models of stress and trauma [281]. The cannabinoid-based pharmaceutical nabilone (a synthetic analogue of Δ9-tetrahydrocannabinol (THC)), is efficacious in reducing nightmares and improving sleep in PTSD patients [282], and may hold anti-inflammatory potential by targeting both CB1 and CB2 receptors. However, CB1 receptor activation introduces unwanted psychotropic side effects, thus selectively targeting the CB2 receptor which are expressed on microglial cells could be explored in future investigations.

When activated by extracellular ATP, $P2X_7$ receptors localised on microglial cells facilitate the release of the pro-inflammatory cytokine IL-1β [230, 231]. While gain-of-function $P2X_7$ polymorphisms and overexpression of IL-1β are characterised in affective disorders their role in other mental health and neurodevelopmental conditions remains largely unexamined. We proposed that germline genetic deletion of the $P2X_7$ receptor would influence aggression in animals, as aggression has repeatedly been demonstrated to be associated with elevated IL-1β levels, and this may have implicated for the treatment of heightened aggression in PTSD patients. The experimental work presented in **Chapter 4** therefore investigated behavioural and neurobiological effects of homozygous genetic deletion of $P2X_7$, an ATP-gated purinergic receptor primarily localised on microglia. **Chapter 4** firstly characterised the neurodevelopmental effects of constitutive *P2rx7* deletion in mice on social and compulsive/repetitive behaviour from early to late adolescence, as well as territorial aggression in the resident-intruder test during adulthood.

One major finding of **Chapter 4** was that genetic knockout of *P2rx7* reduced marble burying by mice, an indicator of OCD-like behaviour, in late adolescence. Our observations support prior claims of a role for purinergic receptors in repetitive behaviours [283]. **Chapter 4** highlights for the first time that the purinergic $P2X_7$ receptor may serve as a novel target for the development of therapeutics for OCD-related behaviours. Current pharmacological treatment for OCD incorporates SSRIs, anti-epileptics and, in some cases, antipsychotics, however one third of patients do not respond to treatment and many more experience adverse side effects (see [284]). The present thesis also provided unprecedented evidence validating $P2X_7$ receptors as a novel target for the development of serenic agents to manage aggression. **Chapter 4**

reported that homozygous deletion of the $P2X_7$ receptor decreases the propensity of territorial-driven aggressive biting behaviours by resident animals in the resident-intruder test. It is interesting to speculate that the combined reduction in compulsive and aggressive behaviour in *P2rx7* KO mice might mean that $P2X_7$ receptor antagonists could be useful agents to treat impulsive aggression. It has recently been argued that novel drugs are sorely needed to treat maladaptive forms of aggression as no drugs are registered for this specific application [224]. Nevertheless, human and murine $P2X_7$ receptors share ~80% sequence homology and have distinct functional properties which may limit the clinical translation of these research findings [285].

One mechanistic hypothesis for the observed anti-OCD and anti-aggressive effects in **Chapter 4** is the functional loss if IL-1β, however it is important to note we did not measure the levels of this pro-inflammatory cytokine. Future studies should implement measurements of cytokine levels, with a focus on IL-1β to ensure germline deletion of *P2rx7* reliably mutes the synthesis and release of IL-1β in the central nervous system. Our current ubiquitous approach of genetic deletion of *P2rx7* makes it difficult to discern whether the observed anti-compulsive and anti-aggressive phenotypes are not due to any neurodevelopmental compensatory mechanisms. It is also unknown whether the present effects of homozygous deletion of *P2rx7* on aggression and OCD-like behaviour are specifically due to the expression of this receptor on microglial cells or neurons. However, these questions could be answered using a cell-type specific short interfering RNA (siRNA; [286]), packaged in an adeno-associated virus (AAV). This AVV could be used with a promotor for the cluster of differentiation 68 (CD68), protein expressed by microglial cells however absent in neurons. Alternatively, a promotor for the synapsin 1 gene, which is specific for neurons but absent in microglia could be used

to investigate the inverse. This AAV-CD68/hSyn1-$P2X_7$/siRNA virus would then enable silencing of $P2X_7$ receptor signalling specifically in microglia or neurons, and from a time-point defined by the surgical procedure.

As $P2X_7$ receptors localised on microglial cells mediate synthesis and release of IL-1β [231] in **Chapter 4** we investigated activity of microglial cells immediately following antagonistic encounters in the resident-intruder test. No differences in the density of microglial cells were observed across numerous aggression-related brain regions. However, brains were collected at 90 minutes post behavioural testing and this could potentially be either too early or late to observe any changes. In contrast to this, *P2rx7* KO mice that demonstrated reduced aggressive attack behaviours had elevated c-Fos staining in the piriform cortex following resident-intruder testing. This is interesting as the piriform cortex is highly dense with $P2X_7$ receptors and activation of this region has previously been shown to elicit predatory aggression in cats [287]. We hypothesise that $P2X_7$ receptors in the piriform may modulate aggressive behaviour, potentially through IL-1β signalling, however further research is required to consolidate the results of this study with the existing literature.

5.1. Conclusion

The research presented in this thesis broadens our understanding of molecular, neurobiological and genetic determinants of behaviour relevant to PTSD, OCD and aggression. This research showed robust dendritic spine atrophy and microglial cell hyper-ramification was observed in the hippocampus and medial prefrontal cortex of a PTSD mouse model. Moreover, the study showed that these mice had increased hippocampal expression of the immune-related complement factor C1q. The thesis also reports that germline deletion of P-gp increased depression-like behaviour and social withdrawal which was associated with elevated microglial cell activity in the hippocampus, suggesting genetic variation in *MDR1* could predispose individuals to neurodevelopmental and psychiatric disorders. Lastly, the thesis showed that the purinergic $P2X_7$ receptor may serve as a novel target for serenic or anti-OCD therapeutics as germline genetic deletion of *P2rx7* reduced both compulsive and aggressive biting behaviour.

Chapter 6

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6. References

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Appendix

7. Supplementary material

Chapter 3

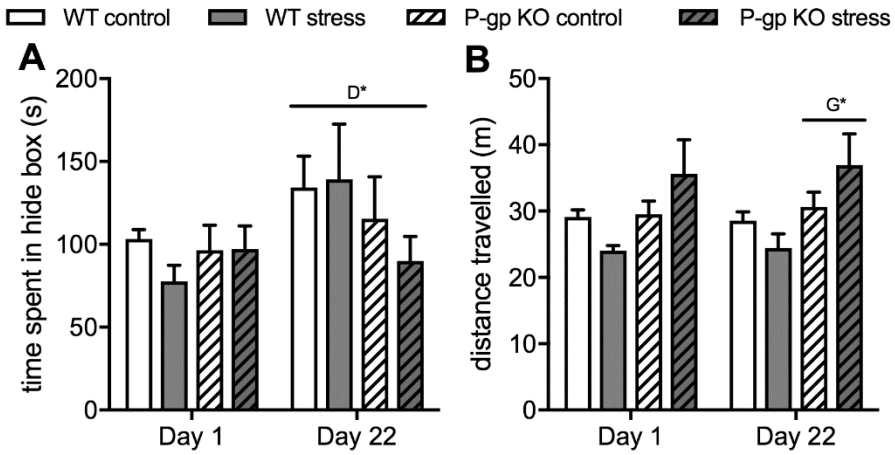

Figure S1. P-gp KO or footshock stress did not influence anxiety-related behaviour in the light-dark test. P-gp KO mice displayed locomotor hyperactivity which was more clearly expressed on Day 22. A) Mean anxiety response as measured by time spent hiding in a novel environment 1 and 22 days after foot-shock exposure. B) Mean locomotor activity as measured by distance travelled in a novel environment 1 and 22 days after foot-shock exposure. Data are presented as mean + S.E.M. n per group: WT control = 7; WT stress = 10; P-gp KO control = 12, P-gp KO stress = 12. WT = wild-type, KO = knockout. G = main effect of genotype; D = main effect of day * $P < 0.05$.

A three-way repeated measures ANOVA found a significant main effect of day [$F(1,37) = 5$, $P = 0.031$], with mice showing higher hide times on day 22 than day 1 in the light-dark test There were no significant effects of genotype or foot-shock stress, nor were there any interaction effects. As there were no significant effects of genotype or stress on time spent in the hide box, we did not drill down further by conducting separate two-way ANOVAs on day 1 and day 22. Three-way repeated measures ANOVA showed P-gp KO mice travelled further than WT mice over days 1 and 22 as supported by a significant main effect of genotype [$F(1,37) = 4.2$, $P = 0.047$]. There was no main effect of foot-shock stress and no interaction effects. Separate two-way ANOVAs confirmed a main effect of P-gp genotype on day 22 [$F(1,37) = 4.7$, $P = 0.04$] but not on day 1.

Table S1. Microglial cell body morphology features in WT and P-gp KO (*mdr1a/b*$^{-/-}$) mice in the CA3 region of the hippocampus. WT = wild-type, KO = knockout. Data presented as mean ± S.E.M, n = 7 per group.

	WT	P-gp KO
Cell body perimeter (μm)	26.5 ± 1.6	23.8 ± 1.1
Cell body volume (μm^3)	1454.3 ± 271.8	1142.5 ± 127.3
Cell body surface area (μm^2)	858.5 ± 125.2	748.6 ± 67.5

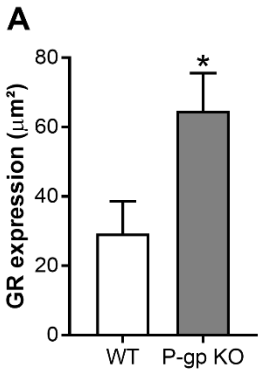

Figure S2. Female P-gp KO mice had increased GR expression compared to WT mice in the pyramidal layer of the CA3 region of the hippocampus. A) Summary data of GR expression as measured by area (μm^2) of GR staining. Unpaired t-test indicated * $p < 0.05$. Data are presented as mean + S.E.M, n = 7 per group.

Area above threshold staining analysis revealed a significant increase in GR staining in P-gp KO mice compared to WT mice (t = -2.5, df = 12 , p = .030).

Chapter 4

Table S1. c-Fos$^+$ cell densities between WT and *P2rx7* knockout experimental groups following the resident-intruder test. Data presented as Mean ± SEM (*P2rx7*$^{-/-}$ n = 20, WT n = 9). * p < 0.05

Region	Bregma	Genotype		*t*	p
		P2rX7$^{-/-}$	WT		
Frontal regions					
1. Prelimbic cortex	+1.98	140.8 ± 7.83	158.78 ± 14.66	-1.181	0.248
2. Lateral ventral septum	+0.62	125.75 ± 7.39	144.33 ± 16.54	-1.195	0.242
Striatum and olfaction					
3. Piriform cortex	+0.62	54.5263 ± 5.36	35.44 ± 6.26	2.136	0.042*
4. Nucleus accumbens, shell	+0.62	32.35 ± 3.87	31.56 ± 6.24	0.112	0.912
Thalamus					
5. PVA	-0.46	211.55 ± 10.75	223.22 ± 15.83	-0.607	0.549
6. PV	-0.94	122.4 ± 9.52	126.78 ± 17.87	-0.236	0.815
Hippocampus					
7. CA1	-1.70	8.5 ± 1.05	36.78 ± 3.19	-0.94	0.356
8. CA3	-1.70	19.65 ± 1.67	27.89 ± 4.61	-0.414	0.682
9. Dentate gyrus	-1.70	17.35 ± 1.46	24.44 ± 14.11	-1.253	0.221
Hypothalamus					
10. Lateral	-1.58	66 ± 3.15	58.78 ± 5.35	0.929	0.361
11. Dorsomedial	-1.58	38.3 ± 2.85	42.56 ± 7.71	0.318	0.753
12. Ventromedial	-1.58	27.55 ± 2.93	28.44 ± 5.85	-0.063	0.95
Amygdala					
13. Medial, posteroventral	-1.58	63.7 ± 6.24	20.78 ± 1.55	0.491	0.628
14. Medial, posterodorsal	-1.58	52.95 ± 4.36	10.44 ± 2.02	1.375	0.18
15. Central	-1.58	29.2 ± 2.83	61.22 ± 3.02	0.132	0.896
16. Basolateral	-1.58	37.65 ± 4.37	20.56 ± 1.96	0.314	0.756
Periaqueductal Grey					
17. dmPAG	-4.60	53.45 ± 4.17	45.67 ± 5.97	1.051	0.303
18. dlPAG	-4.60	39.65 ± 3.26	31.56 ± 4.48	1.414	0.169
19. lPAG	-4.60	38.7 ± 4.14	32.11 ± 3.05	1.005	0.324

Table S2. Density of Iba-1$^+$ microglial cells between *P2rx7* knockout and WT animals. Data presented as Mean ± SEM (*P2rx7*$^{-/-}$ n = 8, WT n = 7).

Region	Bregma	Genotype		*t*	p
		P2rX7$^{-/-}$	WT		
Frontal regions					
1. Prelimbic cortex	+1.98	106.63±2.21	109.42 ± 6.67	-.873	.398
2. Lateral ventral septum	+0.62	51.88 ± 1.95	50.29 ± 4.96	.298	.773
Striatum and olfaction					
3. Piriform cortex	+0.62	70.00 ± 2.54	62.86 ± 4.26	1.484	.162
4. Nucleus accumbens, shell	+0.62	71.63 ± 2.78	72.14 ± 5.82	-.084	.935
Thalamus					
5. PVA	-0.46	55.38 ± 2.33	51.86 ± 3.58	.844	.414
6. PV	-0.94	62.50 ± 1.96	61.14 ± 3.96	.320	.754
Hippocampus					
7. CA1	-1.70	78.75 ± 3.33	84.29 ± 7.37	-.716	.487
8. CA3	-1.70	70.88 ± 4.24	69.71 ± 4.51	.187	.854
9. Dentate gyrus	-1.70	73.00 ± 4.19	70.57 ± 4.51	.386	.706
Hypothalamus					
10. Lateral	-1.58	49.75 ± 2.48	45.86 ± 4.21	.822	.426
11. Dorsomedial	-1.58	61.13 ± 5.02	54.00 ± 3.02	1.172	.262
12. Ventromedial	-1.58	67.75 ± 4.07	53.57 ± 6.46	1.907	.079
Amygdala					
13. Medial, posteroventral	-1.58	71.50 ± 1.83	70.86 ± 7.49	.084	.930
14. Medial, posterodorsal	-1.58	72.00 ± 2.11	68.29 ± 5.17	.619	.526
15. Central	-1.58	73.75 ± 2.54	75.00 ± 5.25	-.223	.827
16. Basolateral	-1.58	88.13 ± 6.22	93.86 ± 5.52	-.680	.508
Periaqueductal Grey					
17. dmPAG	-4.60	59.62 ± 2.31	56.71 ± 4.29	.619	.547
18. dlPAG	-4.60	40.25 ± 2.27	39.43 ± 5.22	.151	.882
19. lPAG	-4.60	55.13 ± 2.49	56.71 ± 4.29	.997	.337