Behavioural and neuroimmune responses to early life stress on contextual fear and extinction memory.

A thesis presented for the degree of

Doctor of Philosophy

By Peter E Richardson

30/04/2024

1 Acknowledgements

The work in this thesis is an achievement that was only possible due to the support of many individuals and groups.

I extend my greatest thanks to Dr Kerrie Thomas. Without her support throughout the thesis this would not have been possible. She has helped me by providing support and motivation as well as practically guiding me through what can only be described as unprecedented challenges. Thank you also to Prof Lawrence Wilkinson and Prof Jeremy Hall who together have given me good advice and helped me get this thesis over the finish line.

I must make a specific thanks to Dr Esther Lin who supported me with long days completing my behavioural data. Thanks also to Dr Nichola Thurvelshomen who trained me in the techniques that would bring this thesis to life.

Further thanks must be given to Dr Jack Reddaway who has assisted greatly with the microglia morphology pipeline and as friend when the coding and the microglia themselves were not acting as they should. Thanks to Dr Dan Gallichan and Dr Marco Palombo who have provided fascinating insights into the microglia morphology element of this thesis.

I thank Dr Cezar Tigaret for giving me support during this time writing my thesis and instilling the mantra to never give up. For sure there are times when I needed to hear that.

I must extend my thanks to the Hall Thomas lab. Despite changing over the years, the people working in this department are amazing individuals to work with. Not only exceptionally creative, smart, and driven but also kind and supportive. Thank you to

my family who never gave up believing in me and where I have gotten to. Thanks to my friends in the Wellcome trust, especially Matt and Steve, the curries were a great time. I thank my family for the support they have given me, since I was young, I had a passion for science, and they were always there for me supporting me. This thesis would not have been possible without their support. Finally, thank you to Caitlin Lloyd who was there for me when all experiments were going wrong, and the writing was a lot to think about. I am sorry for the absences, the long days and nights and the weekends working in the lab.

I note a lot of people mention their pets in these acknowledgements, so I too mention Splotdge, the cat, here. While he didn't contribute to the science much, he is a good cat and I think he would like his name in this.

The work was generously funded by the Wellcome Trust, and I am grateful that I was given an opportunity to complete such a challenging, fulfilling and rewarding PhD programme. This thesis is a culmination of support from so many people and shows how scientific achievement cannot, and should not, be done alone.

2 Abstract

Early life stress (ELS) profoundly affects brain development and increases the risk of psychiatric disorders such as anxiety and PTSD, which are associated with abnormal fear learning, memory and extinction Despite this, the mechanisms—especially those involving neuro-immune and stress system interactions in fear memory formation and extinction—are not well understood. This thesis investigates how ELS affects contextual fear conditioning (CFC), contextual fear memory (CFM) and extinction in adulthood with an emphasis on exploring sex differences. Chapter 3 demonstrates that rats, having undergone a period of ELS exhibit heightened fear memories compared to their non-stressed counterparts. Notably, male animals exposed to ELS show evidence of a resistance to extinction of CFM. During extinction recall, ELS-exposed males exhibit potentially enhanced recall, indicated by lower freezing levels, suggesting a resilience to stress, while females, show very low levels of conditioned freezing under the same CFC training. Chapter 4 explores variations in fear expression through ultrasonic vocalisations and shows that measuring 22kHz reveals an effect of ELS on CFC in females and males as an alternative measure of fear memory. ELS enhances CFM in males, but levels were too low in females to conduct meaningful analysis past CFM recall. Furthermore, this work delves into neuro-immunological mechanisms in Chapters 5 and 6, investigating whether the neuroimmune system contributes to the changes in CFM and extinction observed after ELS. While cytokine levels in the CA1 of the hippocampus after CFM recall and extinction differed, there was no additional effect of ELS (Chapter 5). There were changes in the morphology of microglia in the IL region of the PFC after CFC that were both ELS and sex dependent (Chapter 6). This research advances our understanding of how ELS modifies CFC and extinction learning and underscores the potential involvement of neuro-immunological mechanisms. The identified differences suggest potential therapeutic targets for intervening in adulthood following a stratification system focusing on exposure to ELS and with the goal of ameliorating the fear memory and extinction deficits characterising certain neuropsychiatric disorders.

3 Abbreviations

DAPI	4’,6-diamidino-2-phenylindode
ANOVA	Analysis of variance
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
CZI	Carl Zeiss image
CSV	Comma separated values
CS	Conditioned stimulus
CFC	Contextual fear conditioning
CFM	Contextual fear memory
CNN	Convolutional neural network
DAMP	Damage associated molecular pattern
ELS	Early life stress
FIJI	Fiji is just imageJ
GNB	Gaussian Naïve Bayes
GBM	Gradient boosted machine
GRO-KC	Growth Regulated Oncogene - Keratinocyte Chemoattractant
HPA	Hypothalamic-pituitary adrenal
IL 1 - 12	Interleukin 1, Interleukin 2 …
IBA1	ionised calcium-binding adapter molecule 1
LPS	Lipopolysaccharide
LTP	Long term potentiation
MCSF	Macrophage colony-stimulating factor
MIP1- A	Macrophage Inflammatory Protein-1 Alpha
MG	microgram
ML	microlitre
MM	micrometre
MS	Multiple sclerosis
NMDA	N-Methyl-D-aspartate
NR	No recall
NS	Non-stressed
NDS	Normal donkey serum
OCT	Optimal cutting temperature
PAMP	Pathogen associated molecular pattern
PAG	Peri aquiductal grey
PMSF	phenylmethylsulphonyl fluoride
PBS	Phosphate-buffered saline
PND	Post natal day
PTSD	Post traumatic stress disorder
RF R	Random forest
ROI	Region of interest
RANTES	Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted

SVM/SVC	Support vector machine/classifier
TIFF/TIF	Tagged image (file) format
TNF	Tumour necrosis factor
US	Unconditioned stimulus
VEGF	Vascular endothelial growth factor

4 Contents

5 Figures

6 Tables

7 Equations

1 Chapter 1: Literature review

Post-traumatic stress disorder (PTSD) is a highly debilitating disorder which can occur at any point throughout an individual's lifetime. Onset occurs following exposure to a traumatic event. The symptoms of this disorder can include the presence of intrusive experiences such as: flashbacks, intrusive memories, nightmares, disassociation and intense distress when exposed to cues [1]. As well as these symptoms there are also mood and cognitive disturbances. The effects of such symptoms can last from a few months to a lifetime [2]. Despite this symptom profile not everybody who experiences trauma goes on to develop PTSD. A major unanswered question in PTSD research is which risk factors predispose an individual to develop PTSD. Economically, PTSD carries a significant financial burden, with direct costs through individuals being hospitalised suggested to be around 10,000 - 18,000 USD in the USA per patient per year [3] and similar amounts in Europe [4]. Indirect costs such as individuals needing sick leave, or being unable to gain and keep employment compound the economic burden caused by this disorder[5].

Despite this high financial burden, and the severe physiological and psychological costs, advancements in various treatments for PTSD have stagnated. Many in the field of psychiatry are suggesting that there is a crisis in pharmacotherapy for PTSD [6]. Indeed, pharmacological treatment for PTSD consists of selective serotonin reuptake inhibitors (SSRI's), which were not developed to target specific facets of PTSD and new treatments have stalled with the last new treatment having been approved in 2001[6]. Furthermore, while psychological therapies have been shown to be more effective than pharmacological therapies, as shown in a meta review [7] there are still issues with individual factors such as early life experience which mediate effectiveness

either by conferring a sensitivity to further trauma [8-10] or a resilience [11], the latter a phenomenon which is examined in detail throughout this thesis.

Psychological treatment programmes also suffer from high participant dropout rates and non-compliance, as shown in a meta review detailing a pooled dropout rate of 16% for those participating in psychological PTSD treatment [12]. This can be further compounded by comorbidities that are often diagnosed in patients with PTSD such as substance misuse [13], major depression disorder (MDD) and other anxiety disorders [7, 14]. The disorder is highly disabling, yet the complex presentation of disparate symptoms, as well as the contribution of external stressors throughout life as risk factors, may suggest why PTSD is so difficult to treat. Despite these challenges, understanding the biological mechanisms by which risk factors increase the chances of developing PTSD has been mounting. These risk factors are described below.

1.1 Risk factors for PTSD.

Many individuals throughout their life will be exposed to a potentially traumatic event (PTE). The DSM-5 describes a PTE to mean being exposed to actual or threatened death, severe injury, or sexual violence [15]. Despite many individuals being exposed to such an event most individuals who do experience a PTE will not develop any enduring pathology [1, 16].

There are different factors that mitigate or potentiate PTSD risk. A summary of relevant factors are shown in Figure 1. Briefly, these are split into: pre trauma, including factors such as genetic risk factors (described further in Subsection 1.1.4), sex [17-19], demographic factors[20], education[21, 22], socio-economic status(SES)[23],and during or post trauma factors such as magnitude of the PTE[21], levels of social support[24], directed post trauma therapeutic support[25], and exposure to any further

trauma. Each of these factors are not isolated and likely interact with each other. For example, demography and SES influence the level of education that an individual will be exposed to which in turn can increase PTSD risk. Immediate reactions, magnitude of trauma and post trauma situations are difficult to control as they either relate to the event that has already passed or requires prediction of how the individual will respond. However, knowing the individuals background could allow stratification of treatment according to the individuals previous history and may increase effectiveness. Despite the importance of these factors, predicting who will be resilient to PTSD following exposure to a PTE and who will not, is still significantly challenging.

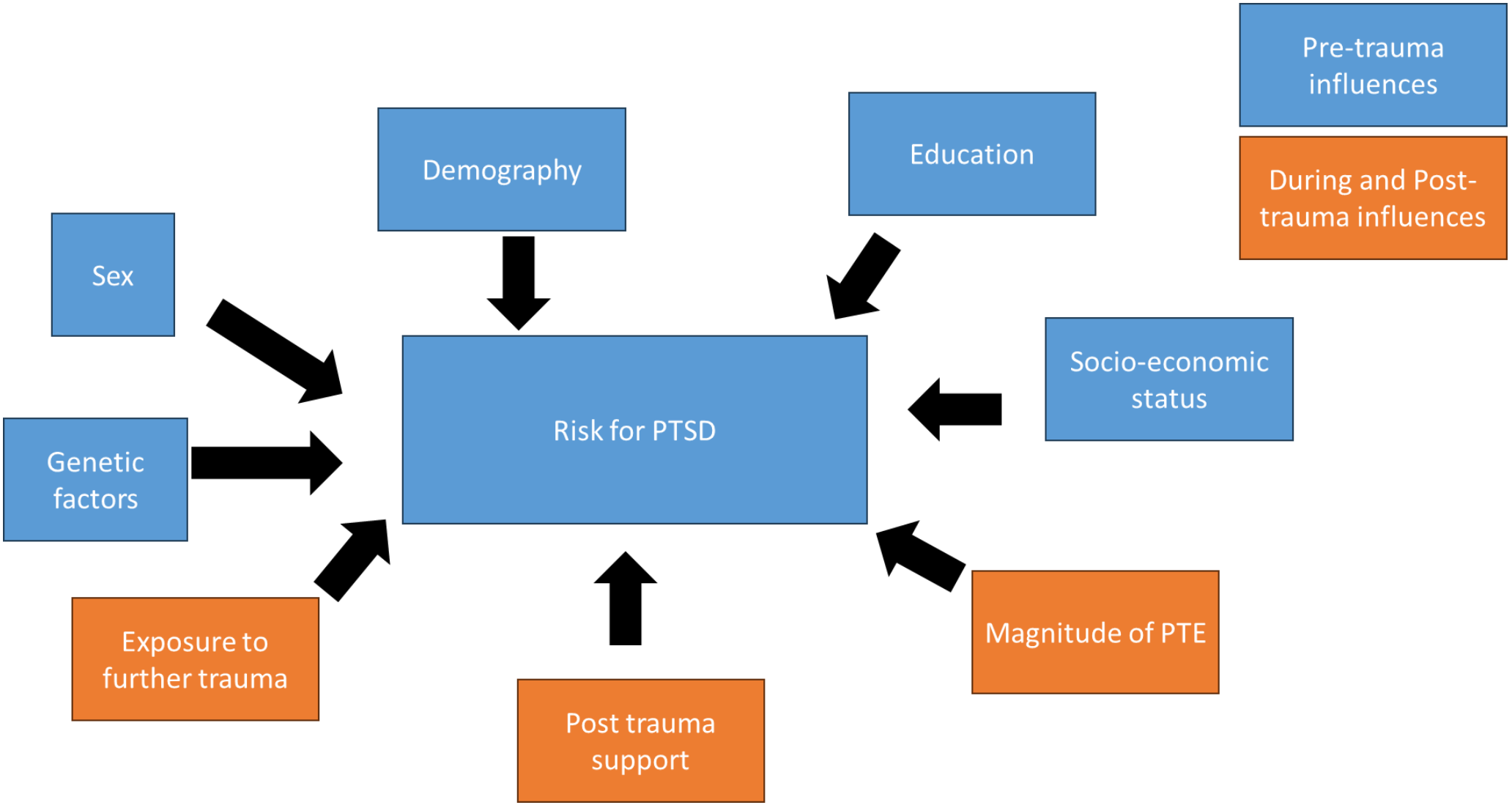


Figure 1: A non-exhaustive selection of environmental factors which can influence the risk for PTSD. References for these links are given in text.

Nonetheless, despite these challenges research is beginning to show key risk factors for PTSD. It is highly unlikely that one single factor makes an individual more likely to develop PTSD. However, together several factors may confer a heightened risk. Sex, age, and exposure to an early life stressor are three such factors that have received much attention for example, sex is a primary factor in a meta review by Nievergelt et al [26]. A review by [27] suggests that stress at different timepoints can have confer different levels of risk for PTSD suggesting the importance of examining early life adversity, and the importance of the age at which the adversity occurred. Individually however, these factors differ in their propensity to predict PTSD across different subgroups of those with PTSD.

1.1.1 Biological sex as a risk factor.

There is an increasing amount of research suggesting that females are more likely than males to develop PTSD[28-30]. The influence that biological sex has on the likelihood to develop PTSD following a traumatic experience is likely to be complex and multifaceted. For example, sex may predispose an individual to experience an early life stressful event. However, in the meta review by Tolin and Foa [28] they suggest that males are more likely than females to be exposed to a trauma that could lead onto development of PTSD. Furthermore, Breslau [17] shows that exposure to PTE's do not differ, throughout an individual's lifetime according to sex. However, the kind of trauma an individual is exposed to is also influenced by sex. For example, female children are more likely than male children to be exposed to sexual abuse[28]. Despite the other factors it appears that the increased risk for females, compared to males, to develop PTSD is robust to type of trauma. Females are more likely to be diagnosed with the disorder than men, which is upheld when controlling for

environmental factors [29, 31] (for example males more likely to experience war as a traumatic event, or females being more likely to experience childhood sexual trauma).

Building on previous studies indicating differences in PTSD risk between males and females[17, 19, 26, 32, 33], research [34] suggests that such differences are specifically observable in the thickness of the rostral anterior cingulate cortex (rACC). In females, a greater thickness of the rACC is associated with higher PTSD symptom severity six months after a traumatic event. This relationship was not observed in males, suggesting to an extent that structural brain characteristics, particularly the thickness of the rACC, may play a role in the sex-dependent risk and manifestation of PTSD symptoms. The ACC is thought to have two key functions relevant to PTSD. First, it exerts top-down control over the amygdala [35] - an area of the brain deeply involved in fear memory processing (as discussed further in subsection 1.5.1). Second, the ACC plays a role in processing the salience of cues and contexts [36]. Changes in circulating hormone levels, both during critical developmental milestones and following heightened stress, differ according to sex which is discussed in greater detail in section 1.9. These hormonal differences may affect the maturity of these cortical areas, thereby altering the networks that mediate fear memory processing as well as cue and context salience and thus has an influence on the risk for development of PTSD.

1.1.2 Stress as a risk factor.

Alterations in the homeostatic functioning of the stress system, which is described in greater detail below (subsection 1.7), plays a significant role in the risk of developing PTSD. Research has consistently shown that individuals with PTSD exhibit an increased sensitivity to the activity of glucocorticoids (GCs) [37, 38].

Studies have shown that PTSD patients often exhibit greater glucocorticoid receptor (GR) sensitivity[39]. This means that their GRs are more responsive to cortisol, requiring lower levels of the hormone to become activated and initiate a cellular response. This heightened sensitivity suggests that the stress system in individuals with PTSD responds more intensely to stress hormones, which could explain the increased stress response and hypervigilance observed in these individuals. Furthermore, McFarlane et al. [40] found that lower cortisol excretion in the morning and higher levels in the afternoon were associated with more symptoms six months post-trauma, suggesting a complex interaction between cortisol levels and PTSD symptoms.

Advances in epigenetics have provided insights into the molecular mechanisms underlying the effects of GR sensitivity in PTSD. For instance, research has found that GR promoter methylation is reduced in individuals with PTSD compared to controls following the administration of dexamethasone, a synthetic glucocorticoid. This finding, as highlighted by Somvanshi et al [39], indicates that epigenetic modifications can influence the stress response system's sensitivity to GCs, further implicating the stress system's role in PTSD.

In addition to alterations in the stress-hormone system described below, enhanced inflammation is another critical aspect linked to increased stress and PTSD risk. This well-known finding suggests that stress not only affects hormonal pathways but also has significant immunological consequences [27, 41], which can exacerbate the symptoms of PTSD. The relationship between stress, inflammation, and PTSD is complex and reinforces the idea that the stress response system's dysregulation is central to the risk of developing PTSD.

1.1.3 ELS as a risk factor for adult-onset PTSD.

There are periods during development when individuals are particularly sensitive to stress, these time periods have a protracted period of development and where there is increased plasticity [42, 43]. During this time the brain is undergoing neuronal, immune system and network maturation (described in more detail in subsection 1.9) and these areas become either sensitive to or attenuated to a neuroendocrine responses to stress, leading to an altered state [44-47].

Studies have shown that exposure to ELS can be retrospectively associated with a higher risk of developing psychiatric conditions such as depression and PTSD [48, 49], with effects that are widespread and significant. Additionally, the effects of ELS are profound across various systems, including alterations to the immune system (described more in subsection 1.14), increased circulating stress hormones during development, and changes in neural network development—such as accelerated maturation and plasticity in the hippocampus [50]—which can impact adult behaviour by sensitising individuals to stress later in life.

1.1.4 Genetic contribution to PTSD risk

Twin studies have shown evidence for the influence of genetics in PTSD risk. For example, work by Wolf et al[51], showed that PTSD has a 49% heritability, other studies such as that by Sartor et al [52] have found similar results even when comparing low and high risk trauma. Interestingly, in the genome wide association study (GWAS) described in further detail below [26], heritability was stronger for females than for males. However, this finding was dependent upon the demographics of the population examined where the Psychiatric Genomics Consortium (PGC) - showed similar results to that presented above, but when looking at only the UK

biobank data there was no differences in heritability between males and females. Taken together however these results suggest PTSD risk may be heritable.

Currently the link between copy number variants (CNV) to PTSD is modest [53], however, the psychiatric genetics consortium-PTSD analysed datasets from multiple ethnicities as well as including UK biobank and have conducted GWAS analysis and development of a polygenic risk score that could predict the re-experiencing of symptoms which is a hallmark of PTSD [26]. They showed that variants in the loci ZDHHC14, which is associated with β-adrenergic receptors, could play a role in determining PTSD risk onset. Interestingly, β-adrenergic receptors have also been shown to play a role in the ability to deal with stressful events in both animal models of stress induced depression [54] and a human social stressor test [55].

Furthermore, data [56] shows that single nucleotide polymorphisms (SNPs) in the ADRB2 gene, a gene also involved in regulation of β-adrenergic receptors has a complex interaction with childhood adversity in that individuals with a specific allele (rs2400707 *A*) may experience more childhood adversities but not develop PTSD while other groups show a heightened vulnerability to developing the disorder suggesting the SNP can modulate resilience and sensitivity respectively. Other single genetic polymorphisms have also been shown to affect directly or interact with the environment to increase risk of PTSD development. For example, it has been shown that levels of FK506 binding protein 5 (FKBP5) expression links childhood abuse and occurrence of PTSD as an adult [57]. FKBP5 is a chaperone protein that binds to glucocorticoid receptors and prevents translocation to the nucleus, thus stopping GR mediated downstream effects on transcription. These downstream effects might also include regulation of immune system functioning which is discussed as an element of PTSD risk later in subsection 1.1.5.

The meta-analysis by Nievergelt et al [26] which shows genetic risk related to sex and ancestry is also supported by a pre-print of a GWAS study suggesting that a genetic profile is altered in those with PTSD [58]. Genes related to ion channel modulation, development and synaptic transmission were all modulated, but importantly, immune regulatory genes (such as the tumour necrosis factor (TNF) genes) were also found to be altered.

A large-scale analysis of DNA methylation patterns in individuals from a Detroit PTSD population (Detroit Neighbourhood health study, DNHS) found that clusters of uniquely non-methylated genes in individuals with PTSD were related to immune system function such as Toll-like receptor (TLR, 1 and 3) and IL-8 [59]. Epigenetics clusters may be involved in immune signalling and the influence of the immune system on PTSD risk is described below. The methylation patterns seen in individuals with PTSD also reflect the symptom profile. For example in the work by Uddin et al [59] it was found that one of the highest affected cluster of uniquely methylated genes was related to sensory perception of sound which is directly related to the hypersensitivity aspect of the disorder.

1.1.5 Immune system dysregulation as a risk factor

Inflammation is intimately linked to fear and anxiety disorders and research is beginning to show the importance of a dysfunctional immune system in psychiatric disease. This so called neuroimmune hypothesis is proposed to be involved in many psychiatric diseases such as depression[60], anxiety [61] and PTSD[61-63].

Recent studies are shedding light on the close connection between the central and peripheral immune systems, which were previously regarded as distinct, separate entities. They reveal how the peripheral immune system can impact the function of the

central nervous system (CNS), and in turn the CNS can signal, for example through Vagus nerve signalling [64] to the peripheral systems.

As an example, sickness behaviours being a direct signal from the CNS to the peripheral system [65] and the ability of many cytokines being able to cross the blood brain barrier (BBB) from the periphery to the CNS[66]. The immune system and neuro-inflammation is intimately linked to the risk of developing PTSD [67]. Exposure to trauma can increase the levels of key cytokines involved in pro-inflammatory signalling such as IL-1 β, IL-6 and TNF- α circulating peripherally[61]. Indeed, many of these cytokines were found to be increased in a recent meta review which examines inflammatory markers for PTSD[62, 68]. C-reactive protein (CRP) has also been shown to be upregulated in individuals with PTSD[69-71]. A summary of studies examining the up or downregulation of cytokines in individuals with PTSD is shown in Table 1. There are some inconstancies in the direction of cytokine levels which may be related to the sample method, the demographics, or the influence of confounds. Nonetheless, cytokines such as IL-6, TNF–α and IL-1β are consistently found to be upregulated in individuals with PTSD.

Table 1: A summary of studies analysing cytokine levels in individuals with PTSD and controls.

Authors	Ref	Type of ref	Number of patients/ number of studies (meta review).	Demographics	Time of sampling	Cytokines analysed and direction (in those with PTSD vs controls), NS = non-significant	Outcome Summary
Passos et al	[62]	Meta - Review	Various	Various ages and demographics	Various	IL-1 β, increased; IL – 6, increased; IFN -γ, increased;	Pro-inflammatory cytokines generally increased
von Känel, et al	[72]	Age and gender matched PTSD/Control group	28	Mean age 33 +/- 10, PTSD, 33 +/- 11 controls.	Morning or afternoon.	IL-1β, higher; IL-6, NS; TNF-α, higher; IL-4, lower; IL-10, NS;	Mixed results with significant increases in IL-1β and TNF-α
Tursich et al	[73]	Meta analysis	Various (36 samples, 14,991 individuals	Various ages and demographics	Various	CRP, IL-1β, IL-6	Wide population scope; summary of cytokines provided
Guo et al	[74]	Age and gender matched	100	Mean age 42 (sd 12)	Within one week of PTSD diagnosis	IL-2, higher; IL-4, higher; IL-6, higher;	Multiple cytokines significantly elevated

		PTSD/Control group				IL-8, higher; IL-10, higher; TNF – α higher;	
Plantinga et al	[75]	Matched PTSD and control twins.	238 male twin pairs	Age: PTSD 55, control 57	Same date for both PTSD and control. Vietnam era twins were used.	High sensitivity CRP, higher; Intercellular Adhesion Molecule 1, higher; IL-6, higher, when adjusted for other factors;	Significant elevation of inflammation markers

Individuals with PTSD have also been shown to have a hyperactive immune response to further immune challenges. For example, individuals with PTSD react with a pronounced IL-6 following exposure to endotoxin [76] or an increase in IL-6 and TNF-α in blood co-incubated with a T-cell activator (phytohemagglutinin) when compared with controls [77].

The above study suggests that ex-vivo, the behaviour of T cells is altered in individuals with PTSD. Further to this, both the numbers of T-cells and lymphocytes have been observed to be above the normal range in individuals with PTSD compared with controls [78]. Furthermore, the number of CD8 + T-cells are decreased which coincides with an increased level of CD3+ cells [79], and a decreased number of regulatory T-cells [80] , indicating a complex role of the adaptive immune system, but suggest strongly a pro-inflammatory profile, in individuals with PTSD.

The aforementioned research indicates that there are numerous risk factors for PTSD, involving complex interactions among genetic, environmental, cultural, and demographic elements. These factors can collectively increase the likelihood that acute trauma exposure will manifest as PTSD. However, whether the development of PTSD is irrelevant of the specific traumatic event or if there are distinct mechanisms in response to particular types of trauma remains an area of ongoing investigation. Studies often focus on targeted groups—such as veterans, survivors of natural disasters, or rape victims—which can introduce bias and limit the generalizability of the findings. For example Yehuda and LeDoux [38] discusses the complex interactions between genetic, environmental, and biological factors in the development of PTSD, emphasizing that different traumatic events may trigger specific mechanisms. While work by Olff et al [19] show that women are much more likely than men to develop PTSD This suggests that certain traumatic events may have unique psychological or

biological impacts that influence the onset and progression of PTSD. As described above in section 1.1, studies on PTSD groups often focus on a targeted group of individuals (veterans, survivors of a natural disaster, rape victims), and thus some bias is introduced. To explore these nuances in greater detail, modelling the disorder using animal models—or examining specific facets of the disorder—serves as a useful tool. Such models allow for controlled investigation into whether different traumatic events elicit distinct mechanisms leading to PTSD, thereby enhancing our understanding of the disorder's complexity.

1.2 Dysfunctional fear memory as a facet of PTSD

The ability to predict and deal effectively with threatening events and circumstances confers an evolutionary advantage[81] and this is still pertinent in dealing with threats in the modern world. However, just as it is important to deal with an actual threat in an appropriate way, it is highly important that this response is not triggered when there is no real threat. Indeed, the inability to dampen fearful, unwanted or anxiety inducing memories is a highly characteristic trait/hallmark of various neuropsychiatric disorders including PTSD [1, 82], adult onset anxiety disorders [83] and substance misuse [84]. In the case of PTSD, a person may experience vivid negative memories when exposed to a context (for example a room or event), where they previously experienced some significant trauma, eliciting fear and panic[85]. Despite a lack of actual threat, the individual feels fear when they return to the context, or a similar context to that which was paired to the traumatic event. For those with PTSD there is a struggle to extinguish the fearful association between the context and a harmful stimulus; essentially a struggle to learn that the context is in fact now safe.

Extinction memory, also known as extinction learning, involves the reduction of a conditioned fear response through repeated exposure to the same cue or context without the noxious stimulus. Over time or with multiple exposures, the fear response gradually diminishes. The importance of studying extinction memory is important as this provides a good target for intervention. Intervening during the acquisition of a fearful memory presents significant challenges, whereas later recollection and subsequent extinction of the memory can be more easily managed. As examples, in war environments, or during a natural disaster (both common causes of PTSD) the focus may be on physical safety and thoughts of conducting exercises to prevent acquisition of fearful memories would be impractical. Consider a soldier who develops PTSD following exposure to an explosion caused by roadside bomb. On the road at the time there was rubbish. Inside one pile of rubbish was the explosive device. This is a real-world example following the invasion of Iraq by US military personnel [86]. Intervening during deployment becomes very impractical, not least because the time of acquisition is unknown. However, interventions to improve the ability to learn that rubbish on the side of the road is not directly linked to explosions when back home becomes a more practical approach. If therapy (for example exposure therapy, which is widely used as a therapy for PTSD [87-89]) could focus on augmenting the extinction of a link between the safe context and the traumatic event the therapy could be conducted when there is no risk of actual physical harm, ultimately reducing the person's symptoms and improving their ability to function in contexts which once were triggers.

1.3 Fear learning, contextual fear conditioning and extinction training to model facets of PTSD.

At the outset, it is vital to consider that developing a model for PTSD is challenging. There is, currently, no way to effectively model the full spectrum of symptoms that occur in PTSD within a single animal model. Instead, certain aspects of the disease can be modelled individually, there are many methods of eliciting stress for modelling PTE exposure. Examples include predator exposure stress, such as exposing rodents to a cat [90] or the scent of a cat [91] or ferret [92], as reviewed by Verbitsky et al [93] for modelling facets of PTSD. While the flashbacks, nightmares and disassociation are undoubtedly important and highly distressing for individuals with PTSD, this element is especially challenging to model in animals, as it is simply not possible for rodents to verbalise these experiences and so we have to rely on indices of changes in internal states such as fear. Further issues include co-morbidity present within PTSD which include depression, substance abuse [13, 94] and anxiety [95]. However, sensitivity to cues hyper-arousal, and dysfunctional extinction memory learning are facets of anxiety disorders which can be modelled successfully. PTSD can be seen as a disorder of fear dysregulation where either a heightened sensitivity to fearful stimulus or cues, or an inability to extinguish fearful memories as described above in subsection 1.2. While not a model of PTSD exactly, the use of fear conditioning in rodents has been invaluable in elucidating this facet of PTSD and has been useful in determining the structural [96-98], molecular [99, 100] and environmental[101] factors which contribute to the risk of developing PTSD (or related anxiety disorders). One highly important facet of neuronal communication is Long-Term Potentiation (LTP) which is defined as a long-lasting enhancement in signal transmission between two neurons that results from specific patterns (often high frequency) of stimulation [102]. It is widely

considered one of the primary mechanisms underlying learning and memory. Induction of LTP following contextual fear conditioning (CFC) has been demonstrated in previous work [103] and is discussed further in the context of neuroanatomical areas involved in fear memory below in subsection 1.5.

As a form of behavioural learning, fear conditioning can be broadly divided into two main categories: cued fear conditioning and contextual fear conditioning (which are both described in detail below). Both cued and contextual fear conditioning have translational validity for the investigation of PTSD and there are many comprehensive reviews on the two methods [84, 86, 104, 105].

Cued fear conditioning is where a discrete but neutral stimulus (common examples are lights and tones) termed the neutral stimulus is paired with a noxious stimulus termed the unconditioned stimulus or US (a common example is a foot shock or puff of air to the eye) which elicits an unlearnt natural response to the stimulus, this is termed the unconditioned response (UR). The animal forms an association between the US and the neutral stimulus which causes the neutral stimulus to become a conditioned stimulus (CS) which in turn elicits a conditioned response (CR; examples include freezing, jumping and vocalisation). Once this association is achieved, a gamut of different tests can be conducted. Testing the CR following the learned association can assess how well the animal learns the fear association, and testing the CR after time can assess how persistent the memory is. This methodology is powerful, simple, and very widely used as a translational means of studying disorders of fear memory.

Contextual fear conditioning (CFC) follows a similar methodology. The US is paired with a NS which then becomes a CS, but here the NS is a context comprising of many facets of the environment in which the animal is placed. This can include both the

external environment (and further includes physical attributes such as the walls, floor, smells and lights, as well as non-physical attributes such as the time of day) and internal state. The removal of one of these features would not render the context changed, instead the animal forms a gestalt concept of the context. The representation of the context itself does need adequate time to form before being presented with a US in order to condition a context to a US [106].

Fear memory extinction is a complex process that is significantly influenced by context, exhibits a high degree of plasticity and is less stable than the fear memory association. This is evidenced by phenomena such as spontaneous recovery, reinstatement, and renewal [107-109]. Spontaneous recovery refers to the reappearance of a fear response after a period of time, reinstatement occurs when the fear response is triggered by exposure to an un-signalled noxious stimulus, and renewal happens when a fear response re-emerges in a context different from the one where extinction was learned. These are shown schematically in Figure 2.

There is a debate at present as to whether extinction learning is a weakening of the original fear memory association or the formation of a new memory that inhibits or competes with the original fear association [110]. This new learning involves the formation of neural associations, primarily localised in the mPFC, which either increase excitatory inputs to inhibitory interneurons, reducing basolateral amygdala (BLA) activation, or directly increase inhibition of the BLA [111, 112]. Despite this, extinction learning is less stable than the original fear memory and is susceptible to interference, evidenced by generalisation and the previously mentioned phenomena of spontaneous recovery and renewal.

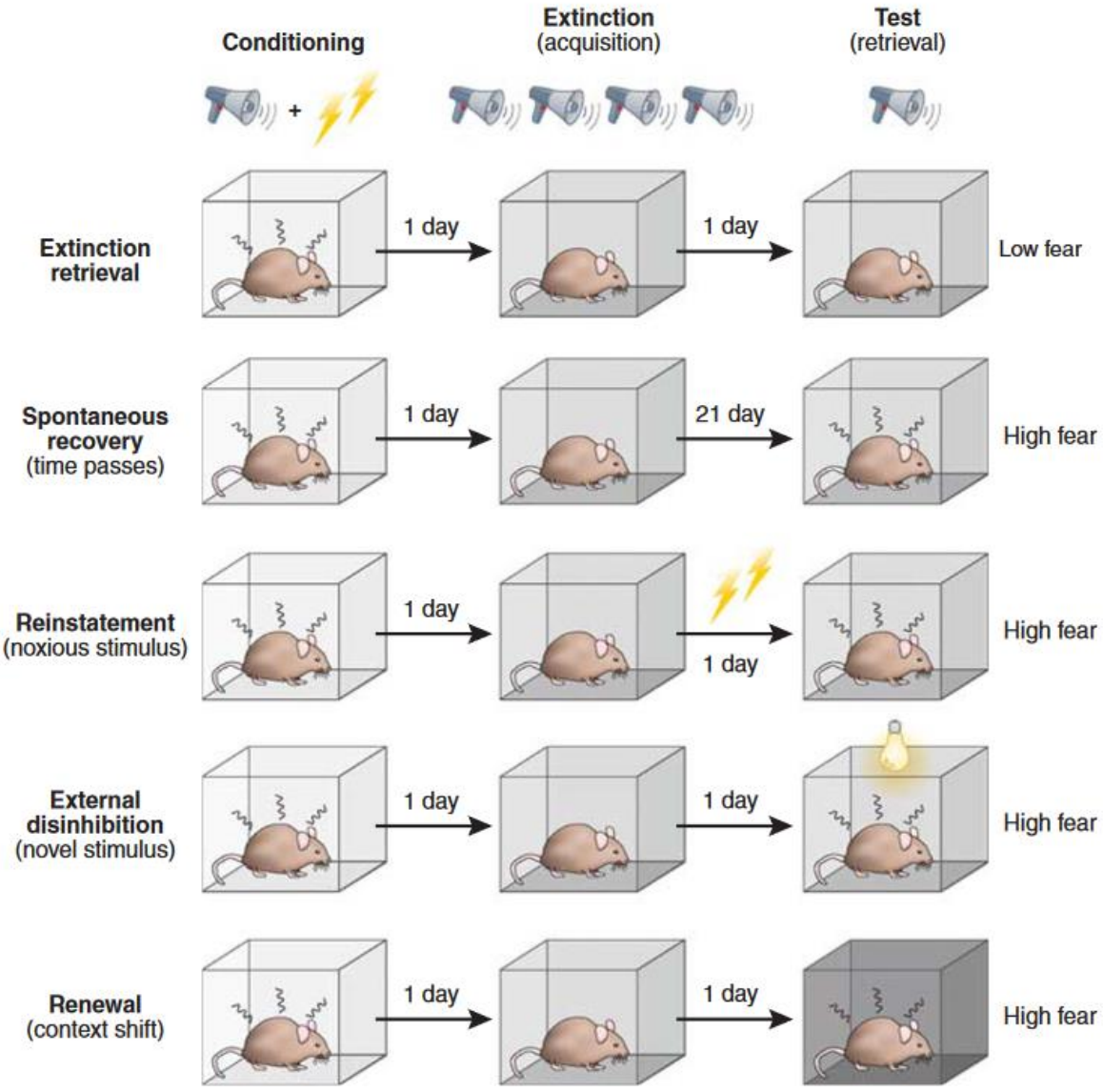


Figure 2: Various forms of relapse following extinction memory learning. Extinction memory retrieval results in low fear expression upon testing however all of the others show examples of where extinction learning is superseded by a different phenomenon which causes a high fear expression. Figure adapted from Maren and Holmes [31].

It has been established that extinction is not due to erasure of the fear memory, but instead a learning of an extinction memory [31, 84, 113]. Indeed, there is some debate as to what constitutes extinction memory. Firstly, research suggests that extinction of a memory does not merely mean forgetting the fear associated memory[114]. Instead, there is a learning of another memory which interacts/interferes with the first memory. Indeed, Bouton et al [115] suggests that extinction is a type of retroactive interference. This is supported by research showing that other forms of retroactive interference such as counterconditioning are more sensitive to renewal and spontaneous recovery than extinction[116]. Throughout all these kinds of learning, context appears to be a highly important determinant as to if the fear response returns. The hippocampus is highly implicated in contextual representation (as described in subsection 1.5.2) and so serves a major role in the mediation of hippocampal dependant contextual fear learning and extinction [117, 118] but see work by McNish [119] who shows that lesions of the hippocampus after training does not affect fear potentiated startle in the training context (while preserving contextual freezing).

1.4 Plasticity and learning

To describe fear learning and the involvement of different brain regions it is useful to first briefly discuss plasticity. Neural plasticity refers to the ability of the CNS to reorganise itself and form new neural connections throughout life. This adaptability is crucial for learning and memory, as it allows the brain to adjust and optimise the connections and neural networks between brain regions in response to new experiences, environmental changes, and information. Synaptic plasticity, a key mechanism within this process, involves the strengthening or weakening of synapses, based on their activity. When a new memory is formed, specific synapses are repeatedly activated, leading to long-term potentiation (LTP), which strengthens the

connections and enhances communication between neurons. Conversely, less active connections may undergo long-term depression (LTD), weakening the synapses. This dynamic process underpins the ability of the CNS to adapt, remember, and acquire new memories, making neural plasticity a fundamental aspect of learning and memory.

1.5 Neuroanatomy of fear memory

To discuss deficits in extinction learning as a potential factor in development of PTSD it is important to gain an understanding of the neuroanatomical pathways that underlie fear memory and the process of extinction learning in a physiologically healthy brain. By examining the underlying neural circuits involved in the acquisition, recall and extinction of fear memories, particularly circuits within the amygdala and hippocampus, we can begin to understand how these processes are critical for fear conditioning and extinction learning.

1.5.1 Amygdala

The amygdala is an almond shaped structure laying within the temporal lobe. It can be sub-divided into different sub-regions with the BLA and the central amygdala CeA having a well-defined roll in fear memory.[120] While this area was not directly examined in the current thesis, a discussion of CFC cannot be had without considering how the other brain areas such as the mPFC and the hippocampus described below interact with the amygdala. The role of the amygdala role in anxiety and fear has been shown by numerous lesion experiments including the seminal work by Kluver and Bucy using Rhesus Macaques [121] where lesions lead to a reduction in fear and anger responses, albeit with the caveat that these lesions affected surrounding areas also. More recent papers however also show that damage to the amygdala can cause a reduction in fear responses [122, 123], (for example those with Urbach–Wiethe

disease (UWD), [124]). Imaging studies in humans both physiologically healthy[125] and with PTSD[36] have also contributed to an understanding of the importance of the amygdala to fear memory. The BLA is intimately linked with plasticity and LTP (discussed in detail in Chapter 1 subsection 1.4) underlying the formation of long-term fear memories and extinction memory [126]. Work by Gale et al [126], where rats were given BLA lesions do not learn to extinguish contextual conditioned or cued conditioned fear. These results are in line work which examines the effects of blocking protein synthesis in the BLA through direct injection of inhibitors [127]. Auditory fear memory consolidation was blocked in a dose dependant manner but only when given immediately following the conditioning, not at a later (6 hours) timepoint. This supports the hypothesis that, in this context, the BLA underlies early- plasticity but not late plasticity, as well as activation of the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway, or the c-Adenosine monophosphate (c-AMP) dependant protein kinase A (PKA) pathway at specific timepoints following fear conditioning is suggested to underlie some of this mechanism [128]. Further work, for example that by McKernan and Shinnick-Gallagher [129], finds that there is an increase in evoked EPSC's measured in LA neurons in those which have experienced fear conditioning, which supports the notion that plasticity is occurring in the amygdala following fear conditioning. These all point strongly towards the importance of the amygdala in fear conditioning and extinction. Further to this, inactivation of the BLA with muscimol has shown that the BLA is necessary for both fear expression and extinction recall following auditory fear conditioning [112]. Numerous reviews have explored the intricate neuronal network of the amygdala, specifically how the BLA and CeA subdivisions regulate and influence different aspects of fear conditioning [130-133].

1.5.2 Hippocampus

The hippocampus, with its seahorse shaped structure in humans from which it derives its name, is located in the medial temporal lobe. The hippocampus can be divided into cornum ammonis (CA) regions with three (CA1, CA2 and CA3) leading to the granule cell layer of the dentate gyrus (DG) [134]. Within these regions there are cell and fibre layers stratified into well-defined bands. Importantly, there is also a distinction between the dorsal and ventral hippocampus with the dorsal hippocampus being essential for context processing [135] and the ventral hippocampus having a role in stress and emotional processing[136, 137]. The neuronal makeup of the hippocampus is 90% excitatory neurons and 10% inhibitory GABAergic neurons[138]. The canonical connections of the hippocampus form what is often referred to as the trisynaptic loop which links outputs from the entorhinal cortex to the DG The DG projects to the CA3 via what is known as the perforent path and then from the CA3 projections go to the CA1 via Schaffer collaterals. The CA1 projects to the EC to complete the loop. In addition to this loop there are other pathways, such as direct projections from the entorhinal cortex to the CA1, CA2 and CA3 as well as CA3 self-projection. Connections from the CA1 to the anterior cingulate cortex (ACC) are also pertinent to the transfer of fear memory from the hippocampus to the mPFC and are described further below. The trisynaptic loop and other connections are shown diagrammatically in Figure 3.

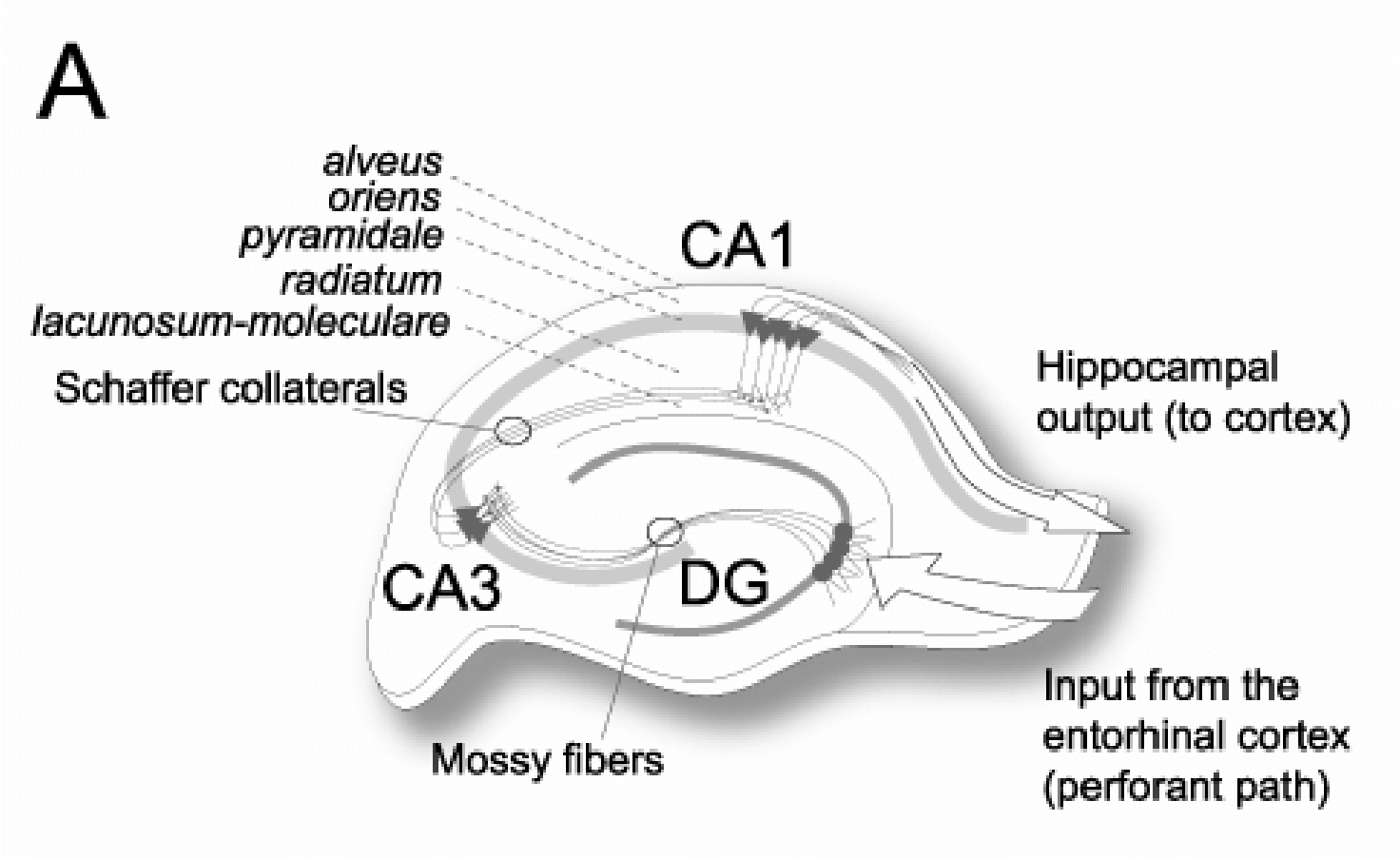


Figure 3: Hippocampal network showing directions of primary neuronal signalling paths. The trisynaptic loop connects the DG CA1 and CA3.

The hippocampal sub regions CA1 and CA3 have been implicated in the consolidation of contextual fear conditioning memory [135, 139]and its subsequent recall/extinction.

The importance of the hippocampus in encoding context is shown by Hirsch [140] who posits that many associative memories are encoded by a network that is not hippocampal dependant. Hirsch's theory, in the context of memory systems, suggests that some associative memories do not rely on the hippocampus. However, when a memory is dependent on the context in which it was formed, the hippocampus becomes crucial. The role of the hippocampus in this scenario is to create a kind of "context map" that ties together various elements of an experience. So, when the recall of a memory relies on context, the hippocampus helps reconstruct the circumstances surrounding that memory, making it easier to retrieve. This is shown schematically in Figure 4. Plasticity in the hippocampus appears to be a key mechanism for contextual learning. This is demonstrated by experiments where knocking out NMDARs in the CA1 region of the hippocampus leads to deficits in trace fear acquisition [141]. Similar deficits are observed when downstream mechanisms involved in synaptic plasticity and long-term LTP, such as CREB [142] and BDNF[143], are disrupted.

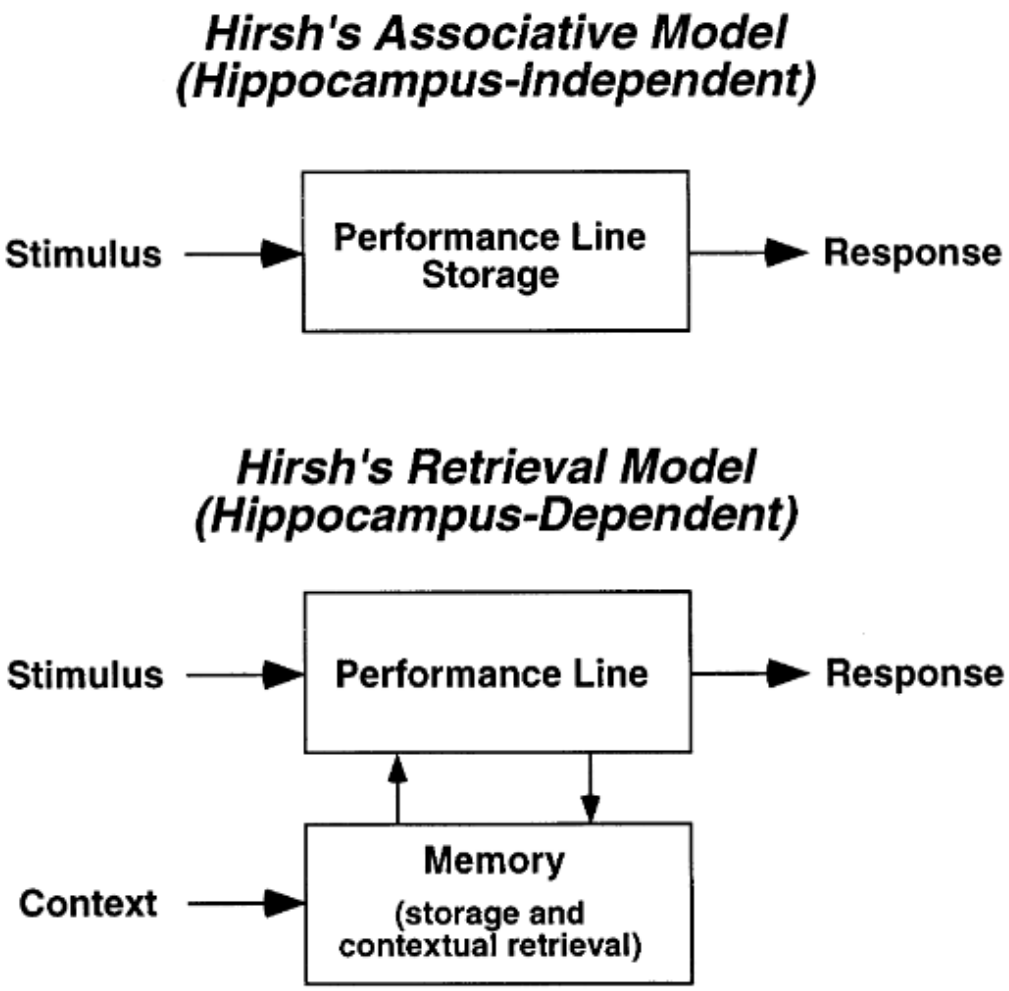


Figure 4: A theory posited by Hirsch suggests that most association memories are hippocampus independent. When the memory is context dependant, the role of the hippocampus becomes important, and the contextual association is encoded there. Figure adapted from Maren and Holt[144].

Studies where the hippocampus is lesioned pre-training show that associations between contextual stimuli and a foot shock are impacted, with less freezing observed, but not following the pairing of cued stimuli with foot shock [119, 145, 146]. Much research suggests that the hippocampus “gates” complex fear memories and contextual associations [119, 140, 147].

The hippocampus is also intimately linked to the encoding of extinction memory[148]. For example the role of the dorsal hippocampus in extinction learning has been supported by a study[148] that utilized muscimol-induced inactivation during a cued fear conditioning task. It was shown that when given after fear conditioning, but before extinction training there was a lack of extinction learning. Furthermore, hippocampal inactivation before testing disrupts renewal of fear after a single extinction training session[117]. The subtle differences in the roles of the CA1 and CA3 is further elucidated by work which probed the effects of the sodium channel blocker lidocaine on a cued conditioning task[149]. Injection into the CA3 region inhibited rapid encoding of contextual memory while injection into the CA1 region inhibited the consolidation of contextual memory.

The use of optogenetic studies in recent years has further elucidated convincingly, the role of the hippocampus in fear memory with Liu et al [150] showing that activation of a pattern of specific cells (a memory engram) in a mouse hippocampus, can elicit a contextually specific light induced fear memory in a context never before seen.

1.5.3 Prefrontal cortex

The dorsolateral pre-frontal cortex in humans is purported to be a uniquely primate structure [151]. This area, being not an anatomical location and instead a functional location, primarily consists of the regions which receive projections from the thalamic mediodorsal nucleus[152]. There is much debate around the existence of an exact homologue between the human dorsolateral cortex and a functional area in rodents[153]. Despite this debate, much research suggests that there are some areas in the rodent mPFC that are homologous, at least in part, to the human PFC [154]. These are described as the area of the mPFC being, broadly divided into 3 sections: Anterior cingulate cortex (ACC), Pre-limbic cortex (PL), and Infra-limbic cortex (IL). As with the hippocampus most of the neurons in the mPFC are excitatory with a small population of inhibitory GABAergic neurons [155].

The mPFC exerts top-down control over the behavioural expression of fear with evidence indicating that the PL and the IL controlling response to fear memory recall and response to extinction recall respectively. This was shown by inactivation of either the PL or the IL with muscimol whereby inactivation of the PL 30 minutes prior to extinction training (and recall, as indexed by the first minute of extinction training) reduced freezing at the recall of fear memory (a day following fear conditioning) [156]. In contrast injection of muscimol into the IL (at the same timepoint) had no effect on the recall of fear memory, but instead reduced both extinction learning (as indexed by higher freezing during the later extinction timepoints) as well as long term recall of fear memory following extinction training. Firing rates in IL neurons are increased following extinction training [156] suggesting that this increased rate of firing may be necessary for increased synaptic plasticity in downstream regions. Further electrophysiological

data has shown that giving high frequency stimulation, of the IL cortex, before extinction training facilitated the recall of extinction later [157].

This work supports that shown by Laurent and Westbrook [158] where muscimol injected into the PL inhibited freezing in the recall of contextual fear but had no effect on the extinction while injection into the IL had no effect on CFM recall but negatively impacted extinction learning. These areas exert their effects primarily through signalling to different subregions of the amygdala and are shown diagrammatically in Figure 5a. While the PL projects to the BA, through the CeA and further during CFM recall, the IL signals through the ITC (which is inhibitory as described in subsection 1.5.1). Upon extinction learning, synaptic potentiation or LTP occurs at the areas shown in Figure5b, and thus signals from the ITC (which themselves are inhibitory) are upregulated.

Despite much evidence showing that manipulations in the mPFC, hippocampus and amygdala impact extinction learning, the synaptic mechanisms which underly extinction learning are still not yet fully understood. Indeed, it is not yet known whether the widely accepted notion of extinction memory being a new, and competing memory is always the case, or alternatively if the extinction memory is encoded as a weakening of the original association.

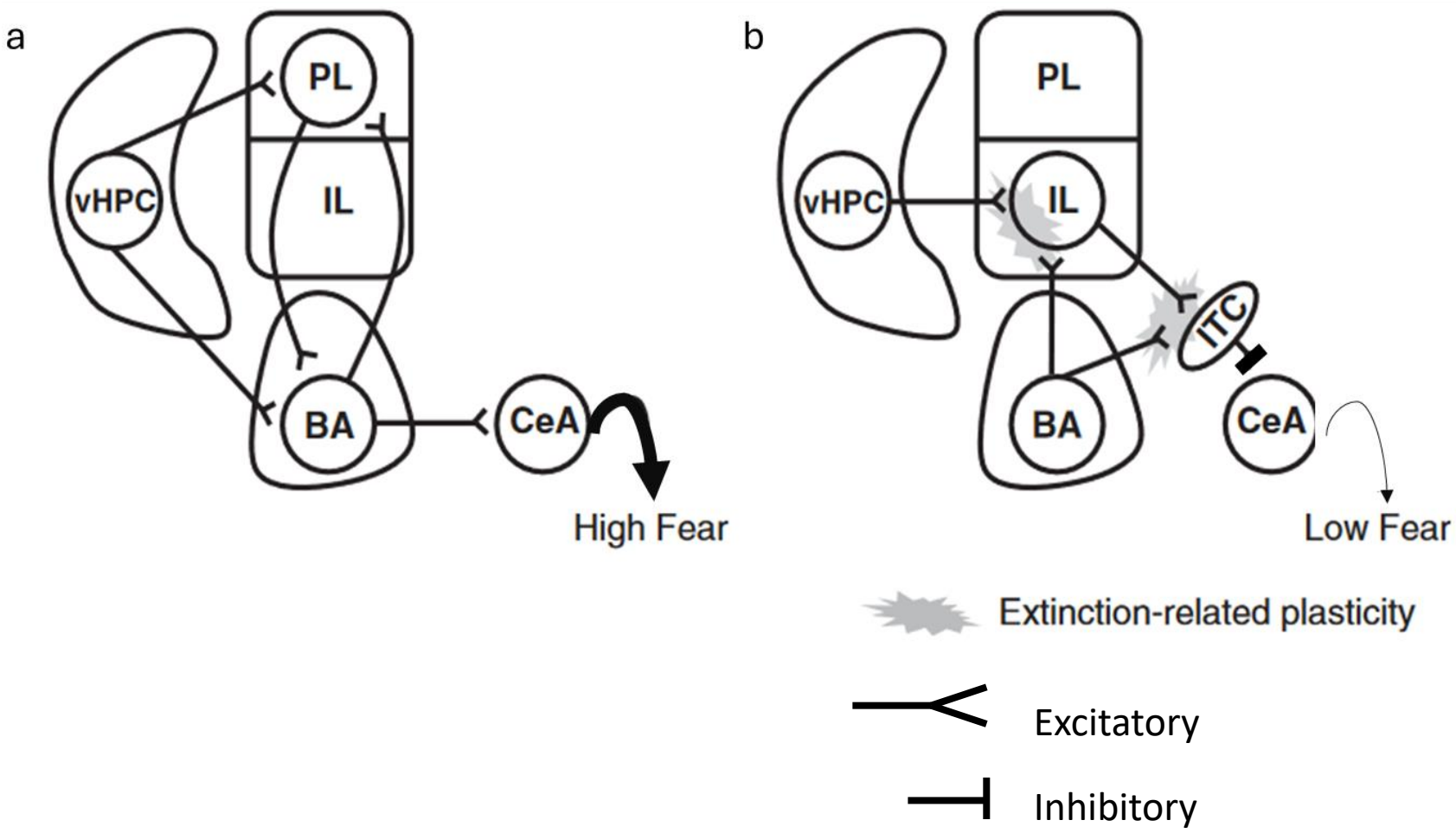


Figure 5: Connections from the prelimbic cortex (PL) converge on the basal amygdala (BA) subregion of the amygdala which activates the central nucleus of the Amygdala (CeA), a. In contrast following extinction projections from the ventral hippocampus (vHPC), the infralimbic support (IL) projects to the intercalated cells of the amygdala (ITC) which inhibits activation of the CeA,b.

1.6 Sex differences in fear conditioning and extinction.

In humans there are sex differences in various measures of fear conditioning, much literature has shown differences in skin conductance response (SCR), with mixed results. For example, work by Lonsdorf et al [159] shows that males show a stronger CS/ US association, as indexed by SCR than females suggesting stronger fear acquisition. In contrast, research by Lebron-Millad et al[160] finds no sex differences in SCR but identifies distinct fMRI activity in certain brain regions. These varying outcomes may stem from methodological differences, such as the intensity or modality of the US, and differences in timing. Such discrepancies highlight the need for further research.

It is hypothesised that changes in brain structure occur during development because of sex hormones leading to long term sensitivities or resistance to fear memory acquisition [161]. With regards to neuronal activity, fear conditioning experiments highlight changes in activity in the amygdala, anterior cingulate cortex (ACC) insula cortex and hippocampus and hypothalamus with females showing higher amygdala and ACC activity[160].

For extinction there is also evidence of differential activation of certain brain areas following extinction for example Lebron-Millad et al[160] found that women show a greater activation in the insular cortex while men conversely show activation in the ACC. Once again circulating sex hormones have been shown to be linked to these sex differences, Zeidan et al [162] has shown that higher oestradiol levels increase extinction retention as well as lowering SCR and coincides with upregulated activity in the vmPFC and amygdala.

There are also many different types of behavioural response to fear in rodent models, these include freezing, calling, darting, escaping, fighting reduced locomotion and environmental scanning [163]. Multiple lines of evidence suggest that there are sex dependant differences in both the magnitude of response to fear conditioning as well as differences in the actual fear response type. These are discussed in more detail in Chapter 3 and Chapter 4 but briefly, key work includes that carried out by Brydges et al [96, 164] showing that in general female rats freeze less than male rats following contextual fear conditioning. Gruene et al [165] has effectively shown that male rats are more likely to exhibit fear as an increase in 22kHz vocalisations. Kosten et al [166] has shown that ELS in the form of maternal separation can have a sex specific effect on fear conditioning in rats, this was further dependant on the measure of fear conditioning where 22kHz vocalisation was affected but not freezing. 22kHz vocalisation is discussed in detail in Chapter 4.

1.7 The stress system

Stress is generally defined as the physiological and psychological response to a challenge or threat be they implied or actual. Much literature discusses stress in terms of balance with homeostasis being a normal state and allostasis being an altered either negative state (cacostasis) or positive state (eustasis) [167]. Being out of balance is dependent on activation or inactivation (as many mechanisms in the stress system have negative feedback loops) of any single, or a combination of the stress systems. The stress system can be primarily subdivided into central and peripheral components and is shown schematically in Figure 6. The central systems affect the peripheral systems (but there is also some feedback in the opposite direction, for example inhibition of hypothalamic signalling via stress hormone secretion as part of the hypothalamic-pituitary adrenal axis, (HPA) axis) which in turn affect peripheral tissues

and further influence brain activity and development [168]. The central systems can be affected by a variety of moderators such as sex, age, demographics comorbidities and genetics. Furthermore, stressors can have different effects depending upon the intensity of the stress, be those differences in duration, magnitude, or type of stress.

Central regulation of the stress system primarily revolves around interactions between the hypothalamus and various other brain regions, such as subregions of the pons and brain stem including the corticotrophin releasing hormone (CRH) releasing neurons of the para-gigantocellular nucleus (PGC). The paraventricular nucleus (PVN) in the hypothalamus primarily controls the HPA axis by releasing arginine vasopressin (AVP). Additionally, the autonomic nervous system (ANS) also contributes to regulating the HPA axis, particularly through vagal pathways and is, at least in part, mediated by signalling from the medulla oblongata. This vagal regulation is crucial not only for the direct control between the central nervous system and the immune system but also for facilitating communication from the periphery back to the brain [169]. Vagal signalling from the periphery also enhances arousal and vigilance [170, 171], behaviour that is modulated by the HPA axis.

The peripheral regulation is made up of the ANS which can be further subdivided into the peripheral nervous system (PNS), sympathetic nervous system (SNS) and sympathetic adrenal medullary system (SAM). These regulate peripheral tissues through the secretion of acetylcholine, noradrenaline, and adrenaline respectively. The other primary element of this response is the activation of the HPA axis, made up of the anterior pituitary gland and the adrenal cortex which has a key role in producing stress hormones such as cortisol in humans or corticosterone in rodent models[172]. Stress hormones initiate what is termed a "second wave" of responses, characterized by widespread effects determined by the distribution of glucocorticoid receptors (GRs)

and mineralocorticoid receptors (MRs). These receptors are located in the brain (among other areas), with GRs found across many brain regions and MRs more specifically concentrated in the limbic system [173]. Response to GCs include regulation of gene expression and feedback to the hypothalamus to inhibit activation of the HPA axis. There are many reviews which cover the hormonal response and potential resulting disorders [167], the evolutionary purpose in humans and cross species comparison [172, 174].

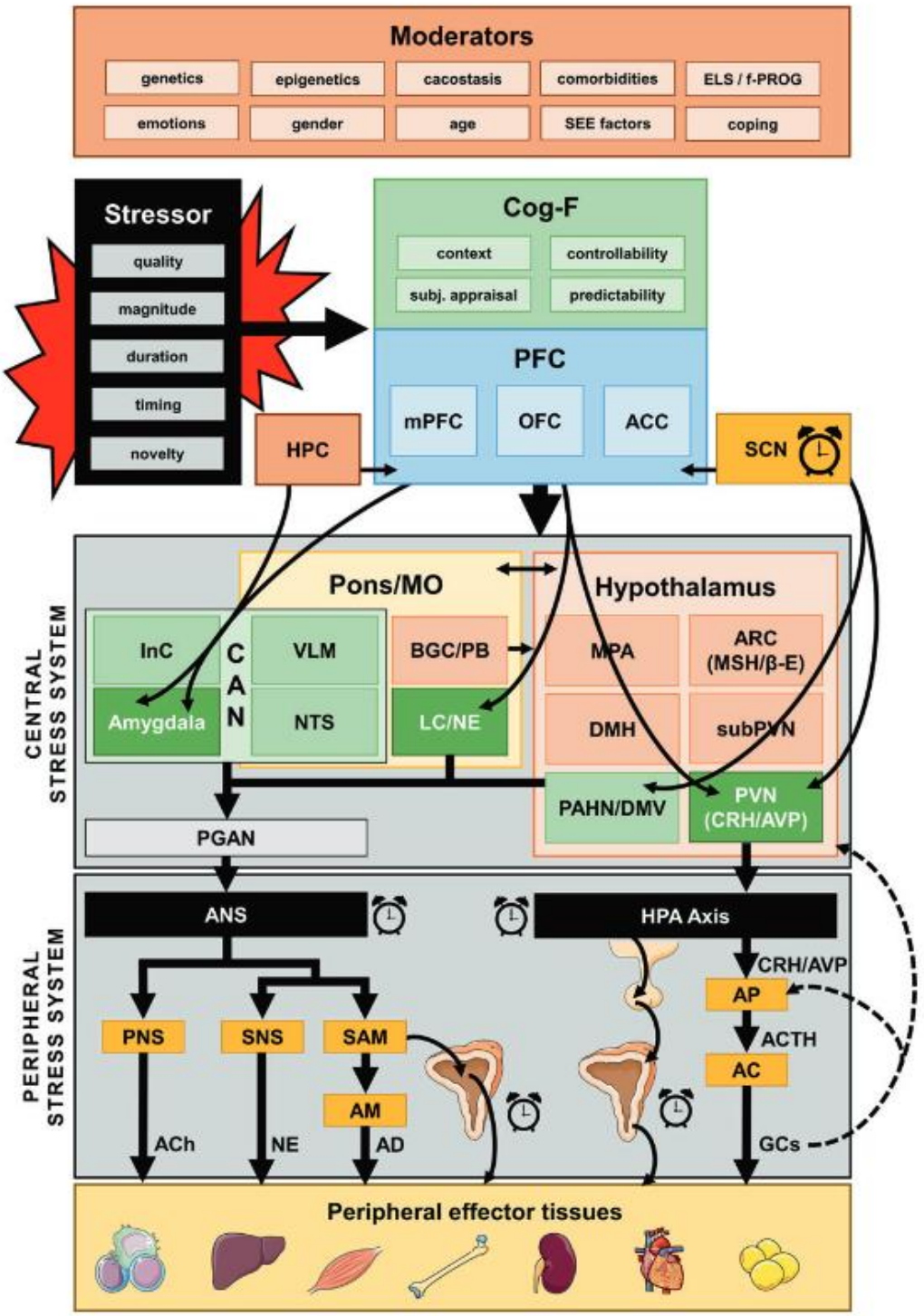


Figure 6: Schematic overview of the central and peripheral stress systems and downstream effector systems. A gamut of environmental and variations in stress are presented at the top of the figure which can modulate the stress response. C adrenal cortex, ACC anterior cingulate cortex, ACh acetylcholine, ACTH adrenocorticotropic hormone, AD adrenalin, AM adrenal medulla, ANS autonomic nervous system, AP anterior pituitary, ARC arcuate nucleus, AVP arginine vasopressin, CAN central autonomic network, Cog-F cognitive filter, CRH corticotropin-releasing hormone/corticoliberin, DMH dorsomedial hypothalamus, DMV dorsal motor nucleus of the vagus nerve, ELS early life stress, f-PROG fetal programming, GCs

glucocorticoids, HPA axis hypothalamic-pituitary-adrenal axis, HPC hippocampus, InC insular cortex, LC locus caeruleus, MPA medial preoptic area, mPFC medial prefrontal cortex, MSH melanocyte-stimulating hormone/melanocortin, β-E β-endorphin, NE norepinephrine, NTS nucleus of the solitary tract, OFC orbitofrontal cortex, PAHN preautonomic hypothalamic nucleus, PB parabrachial nuclei, PFC prefrontal cortex, PGC paragigantocellular nuclei, PNS parasympathetic nervous system, PGAN preganglionic autonomic neurons, PVN paraventricular nucleus, SCN suprachiasmatic nucleus, SEE factors social, economic and environmental factors, SNS sympathetic nervous system, subPVN subparaventricular area, VLM ventrolateral medulla. Solid line: top-downregulation/stimulation, dashed line: bottom-up feedback regulation/inhibition; clocks: crucial circadian regulation. Adapted from [168]

1.8 Effects of Stress on learning, memory and plasticity

The CNS is able to respond to the effects of stress and is sensitive to levels of glucocorticoids [175] which has varying effects downstream. Effects on memory is one such functional consequence. The hippocampus, has been shown to be highly implicated in CFC (especially the encoding of contextual memories, see subsection 1.5.2) and express both GR and MR. Importantly, in human work, it has been shown that levels of circulating cortisol can influence and learning in an “inverted U” curve. Stress can improve memory [176] for example when the intensity of a stimulus is very high (approaching life threatening situations) stress can sharpen memory [177]. But stress can also have a negative impact on memory, and this is often seen with chronic stressors which is described more in Chapter 3. However, as with many aspects of memory learning and retention, the timing of the stressor is crucial in determining whether the stress impairs the memory [178]. Furthermore, stress in early life could improve memory when the conditions are stressful at a later time-point. This is something described as the match mismatch hypothesis [179] and is discussed in detail below in subsection 1.10.2. Finally, stress does not act as a lone modulator of memory and behaviour. The effects of stress have been shown to be sex dependant

[180-183], age dependant [181, 183] and have modulatory effects on the immune system [184, 185], which can either potentiate or attenuate the effects of stress on memory and behaviour. All of these are areas of study in the current thesis.

Stressors occurring at different stages of life can modulate the ability to acquire, recall or extinguish memories in complex ways (and interestingly, not always in a negative way, see Figure 20) [186]. Research is beginning to elucidate sensitive periods in an individual's life when they are more sensitive to the effects of stressors or trauma with those impacting brain development, for example through modulation of hippocampal development [43]. Indeed, the effects of trauma during early life (one such sensitive period) in humans appear to impact the risk of developing PTSD [37]. This is detailed further in subsection 1.9. As mentioned above, stress or trauma during a sensitive period interact with other factors that can confer/mitigate risk for PTSD.

These sensitive periods have been explored in animal models in detail also. For example, Bowman et al [181] show that when aged (21 months) both female and male rats do not show stress dependant impairments in object recognition tasks while younger animals exposed to stress, do show the impairments. This suggests that there are periods where stress has an effect while other age time points there is less effect. Indeed, these periods of sensitivity are characterised as periods where there are CNS developmental changes occurring at a rate that is not seen in adulthood. Exposure to stress during these timepoints interferes with memory processes such as LTP and synaptic plasticity [187]. Other work such as that by Brydges et al [96, 164] has shown that exposure to ELS during the pre-adolescent stage can influence the recall of fear memory. This work highlights the importance how ELS occurring during a sensitive period can influence memory processes. It has been well documented that in humans exposed to stressors during childhood (for example malnutrition, trauma and maternal

infection during pregnancy) there is an increased susceptibility to develop serious neuropsychiatric diseases later in life [10, 188, 189]. It is an intriguing possibility that this increased risk is due to modulation of fear memory processing, either through increased recall of such a fear memory or dysfunctional extinction memory learning (described more below).

1.8.1 Effects of chronic stress on fear conditioning

Stress can manifest as different types, for example there is a distinction between acute and chronic stress, where stress occurs for a short period of time, or is ongoing for a lengthy period, respectively. This distinction influences both how the HPA axis and downstream systems are activated and the effects that this has on systems such as the immune system. There are also effects on learning and memory, but these are more complex. Acute stress has been shown to improve selective attention[190, 191], working memory and associative learning[192]. However, the intensity of such a stressor is important as very intense stressors can lead to maladaptive hypervigilance and attention to innocuous stimuli as is seen in PTSD models[90, 193].

For chronic stress, which can be modelled by repeatedly exposing animals to the same stressor, for example work by Baran et al [194] and work by Sandi et al [195] exposed animals to restraint for several hours a day for many days, often longer than a week. Or this can be chronic unpredictable stress where a stressor from a list is chosen randomly (stressors include, forced swim, exposure to loud noise, water/food deprivation, social isolation, short cage rotations) such as that conducted by Hill et al[196]. A review of various stressors, both chronic and acute and their effects of different forms of attention, memory and extinction learning is shown in Graybeal and Holmes [197]. Chronic stress can lead to detrimental effects on brain structure and function, particularly in the hippocampus, prefrontal cortex, and amygdala which were

described above (subsection 1.5) to be highly important for the encoding of fear memory and extinction. This prolonged stress response can also lead to an alteration of the immune system, which in turn can lead to further detrimental changes in brain structure and function.

1.8.2 Effect of stress on areas of the brain related to fear memory.

1.8.2.1 ELS and hippocampus

Seminal work by McEwen et al [175] has shown that stress early in life alters the volume, function and structure of the hippocampus, in a rat model, later in life. Low levels of maternal care, a common form of early life stress can lead to reduced glucocorticoid receptors in the hippocampus. Certain levels of stress, especially that during childhood, can alter levels of plasticity. Interestingly, it has been shown that intervention during adult stress can reverse the negative effects of stress on HPA axis function later in life while intervention during stress in childhood has less effects [198]. Other work such as that by Youseff et al [199] has shown that exposure to ELS can delay hippocampal development. This could be modulated via effects on the immune system and is discussed in greater detail in subsection 1.15.

The effects of ELS can have different impacts on hippocampal development with some studies, which use animal models, suggesting that stress can impact adult hippocampal volume [200], impairments in plasticity and effects on dendritic spines[201] in the hippocampus, reductions in neurogenesis [202-204] and others suggesting ELS can increase neurogenesis[199]. All of which can lead to phenotypic deficits later in life (although Youseff et al [199] notes that there is a recovery from the phenotype that somewhat dampens negative effect in adulthood).

1.8.2.2 ELS and mPFC

Research by Choyck et al has shown that following maternal separation there was a decrease in spine density on pyramidal neurons [205]. This was concomitant with increased AMPA subunits, αCaMKII and PSD -95 a post synaptic scaffolding protein with an important role in synaptic plasticity. Other work such as that by Chen et al[45], and by Ohta et al [206] have shown that ELS can induce temporally specific alterations in neuronal excitability. For example, Chen et al show that stress increases excitability of neurons in the mPFC during adolescence, while that stress decreases excitability in that same area during adulthood. Otherwise, work by Ohta shows that the expression of mRNA, as well as expression of proteins, related to inhibitory synapses in the mPFC was reduced following maternal stress [206].

1.9 Early life stress and the link to disease in adulthood.

Many areas of the brain are undergoing extensive remodelling and maturation during childhood up to puberty [44, 207] (here defined as the pre-pubertal stage, see Figure 7 for a schematic of rodent and human periods of age). There are periods when cell maturation (of neurons and other cells such as microglia and astrocytes), synaptic pruning and myelination occurs. These periods are described as highly sensitive to stressors [42] which can profoundly impact development, for example altering the cell type proportions, or delaying maturation which can alter network development (as shown by the coloured lines in Figure 7) and influence risk for disease later in adult life, by for example increasing sensitivity to stress later in life. ELS in humans has been associated with reduced hippocampal volume in adulthood [96, 164] as well as having a negative effect on hippocampus dependant spatial memory tasks (elevated plus maze) in animal studies [208] and human. ELS exposure also interferes with microglia

maturation (via alterations in transcription factors) leading to changes in phagocytic activity[209, 210]. The effects on stress on microglia are described further in Chapter 6. Taken together this suggests that ELS has an influencing role in development and is considered a major risk factor for developing both psychological [211] and physical [212] disease.

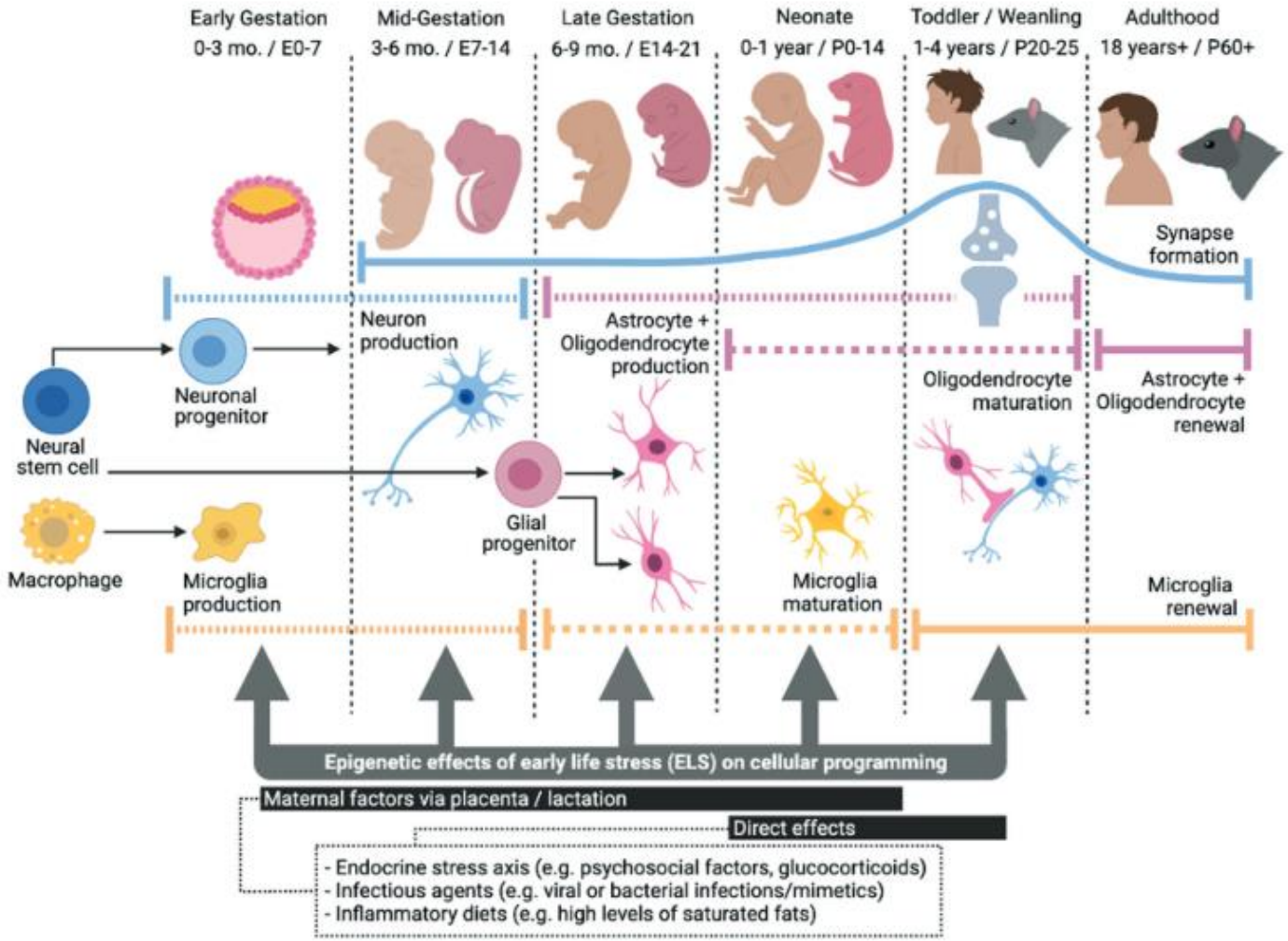


Figure 7: Early life stress can influence the differentiation, and maturation of different cell types (neurons, blue, glia, pink and immune cells (discussed further in subsection 1.12), yellow). E = embryonic day, P = post-natal day. Figure adapted from Rahman and McGowan [213].

The risk for adult onset of disease is further recognised when considering a cumulative effect model (also sometimes defined as an allostatic model, described in 1.10.1) where a stressor early in life can have an effect on the response to stress later in life [214]. Priming in the context of the HPA axis refers to the process by which prior exposure to stress can enhance the responsiveness of the HPA axis to subsequent stressors [215] (priming can also refer to the sensitisation of microglia or elements of the immune system to stimuli later in life, this is described in subsection 1.14). This modification usually results in an enhanced or exaggerated response to a new stressor, indicating that the system has been 'primed' by previous experiences. ELS can prime the HPA axis which is one of the key biological systems regulating response to stress as described above in subsection 1.7 [216, 217], importantly the work presented by Brydges et al [216] suggests that the effect of ELS on the HPA axis is sex specific with females showing an upregulation of HPA axis associated components while in males this was unaffected.

1.10 Theories of fear and behavioural response: allosteric load hypothesis and the match-mismatch hypothesis.

The Allostatic Load Model (ALM) was originally developed to explain how chronic exposure to stressors affects the body's physiological systems, leading to a cumulative burden that impacts overall health [218]. It emphasises the attempt to maintain stability through change, a process known as allostasis [168]. On the other hand, the Match-Mismatch Hypotheses (MMH) focus on the consequences of discrepancies between the environment an individual has become adapted to and the one they currently inhabit. While neither the ALM nor the MMH were specifically devised to explore the

ramifications of ELS, they offer frameworks for examining its effects in the pathophysiology of stress and PTSD.

The allostatic load hypothesis and the match-mismatch hypotheses were not specifically developed to address early life stress (ELS), they provide valuable frameworks for understanding its potential effects on fear conditioning processes, such as contextual fear conditioning (CFC), in adulthood. These theories and hypotheses relate to the longitudinal effects of adverse or beneficial environments at successive times in the individual's life.

1.10.1 Allostatic load hypothesis

The allostatic load hypothesis (ALH) posits that the combined effect of genetic predisposition and repeated or chronic stressors (collectively referred to as the "load") accumulates over time, potentially surpassing a critical threshold that triggers a physiological response. The regulation of the stress response system is crucial for maintaining allostatic balance, a concept elaborated upon in subsection 1.7. Dysregulation of this balance can lead to disease onset, exemplified by PTSD which can occur after a single, severe stress event[88], or from prolonged high-stress levels[218]. Individuals' responses to stress vary significantly, influenced by different risk factors and stressors as well as the other environmental factors which were presented in subsection 1.1 and in Figure 1, with each stressor contributing to an increased risk of disease.

Genetic risk factors have been described above in subsection 1.1.4 are a potential mediator of the stress system that can modulate the allostatic load. They can be in the form of genetic variants which impact the HPA axis [219, 220] or can impact CNS regulators of stress such as the amygdala or hypothalamus. They can cause sensitivity

to the stress hormones such as glucocorticoids and thus lead to an increased sensitivity to further stressors and physiological responses (such as a lower BMI) [221]. Other mediators such as epigenetics, age and other comorbidities are likely to add to the allostatic load [135].

The ALH also emphasises that factors influencing an individual's health or stress responses extend beyond genetic or biological predispositions [218]. It posits that environmental factors, particularly early life stressors such as childhood neglect or abuse, play a crucial role in shaping an individual's sensitivity to future stress[9, 222]. These early experiences can have lasting effects on a person's development, increasing their vulnerability to stress-related disorders or diseases later in life [223]. Environmental mediators do not always increase the load towards the hypothetical threshold point. Mediators such as having a strong social support network [224], a specific coping mechanism [225], and engaging in exercise[226] can reduce the load. The ALH is well defined and provides a potential way to ultimately understand how each of the factors influence the development of disorders such as PTSD.

1.10.2 Match mismatch hypothesis.

In contrast to the ALH, an alternative hypothesis explaining the effects of stress on adult functioning is the match mismatch hypothesis (MMH) described by Schmidt [179]. This hypothesis emphasizes the importance of congruence between early life conditions and those encountered later. It theorizes that when an adverse environment is matched with benefit in later life (and a beneficial environment is matched later with an adverse environment) there is a mismatch between the two and can lead to an increased risk for formation of disease [179]. A simple diagram of this is shown in Figure 8. It is important to consider here that the two environments are polar: either “beneficial” or “adverse” and there is no mention about an in between state (e.g. a

“normal” environment where neither exposure to stressors, nor exposure to positive stimulus is considered). The effects of early life adversity and early life beneficial environments and the corresponding adult life has been tested [227] and supports the proposed match-mismatch hypothesis. However, examination of a normal state has not been examined. If exposure to an early life stressor was paired with a “normal” environment later in life this would still be a mismatch and so it would be expected that this would confer a negative state. However, this is not adequality explained in the work presented. A dichotomy of mismatch is always presented with no “middle ground”. Finally, although this hypothesis posits a mechanism that can explain how some individuals show resilience in the face of a significant stressor later in life, the theory lacks much empirical evidence.

The “dosage” of stressful events is highlighted in a study by Seery et al [228] which examines the effects of cumulative lifetime adversity and measures such as catastrophising and post-exposure negative effect, following the cold pressor test (a cardiovascular test to measure pain threshold and intensity). They show that moderate adversity was linked to reduced negative reactions than either strong or high adversity. This was replicated in the second part of their study using a psychophysiological simulated test. Again, individuals who had experienced some adversity showed resilience compared to no adversity or high adversity. They suggest resilience is more effectively built up when exposed to some adversity, as opposed to repeated intense adversity or to no adversity and that a U-shaped curve is apparent for resilience.

Work conducted by Ricon et al. [229] explores how early life stress, modelled in animals, can lead to resilience development, aligning with the MMH. Specifically, the research indicates that animals subjected to maternal deprivation (MD) during postnatal days (PND) 7-14, followed by exposure to unpredictable chronic stress

(UCS) involving elevated platform, forced swim, and food deprivation, show improved performance in tests for anxiety (such as the open field test) and learning (including active avoidance test and Morris water maze (MWM) test) compared to animals that experienced MD alone. This finding underscores the concept of resilience as proposed in the MMH. Additionally, the study suggests that stress encountered during the juvenile stage, as opposed to adulthood, has contrasting effects on resilience, particularly concerning anxiety and learning in male subjects. It implies that rats undergoing chronic stress from a young age develop distinct coping mechanisms compared to those raised under less stressful conditions, highlighting the nuanced impact of timing and nature of stress on resilience outcomes.

Work by Avital and Richter-Levin [230] does suggest that exposure to ELS and subsequent adult stress has a linear effect on anxiety with exposure to both ELS and subsequent adult stress having a more detrimental effect than either ELS or adult stress alone. Importantly, however, when examining memory in the MWM task, stress appeared to have a facilitating effect with animals exposed to both ELS and adult stress, performing better than either control, ELS alone or adult stressed alone. This further highlights the importance of “dosage” of stress in determining resilience vs vulnerability outcome as well as highlighting the importance of which measure is examined.

The MMH suggests an important notion that exposure to stress may not have a linear effect on behavioural measures. Indeed, the work presented above suggests that a U-shaped curve better explains the combined effects of ELS and adult stressors. The results seen in humans is mirrored in that observed through animal models and the MMH is an important consideration for forming hypotheses regarding the effects of ELS on fear memory and extinction the latter of which has not yet been examined.

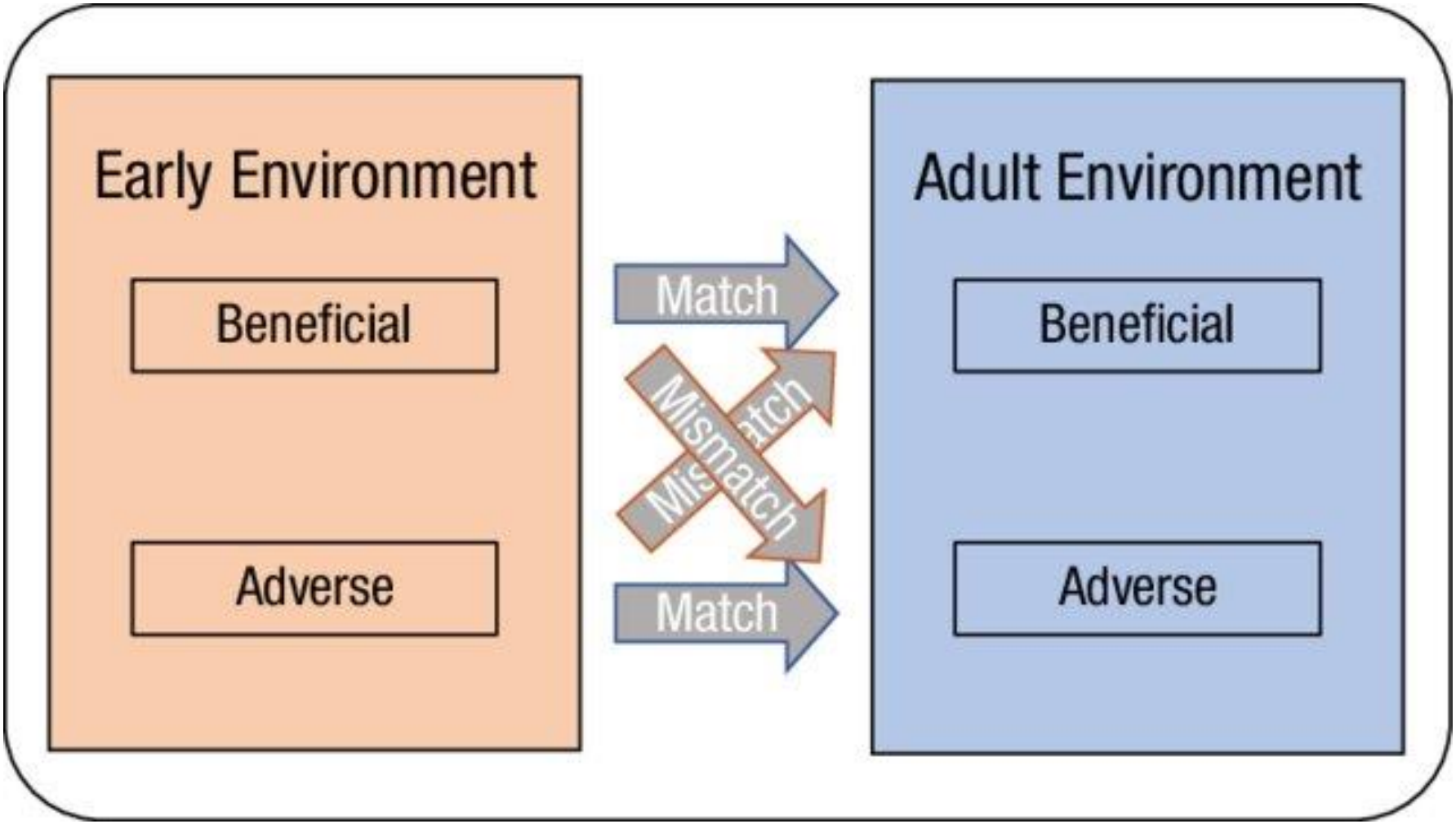


Figure 8: A schematic of the mismatch hypothesis developed by Schmidt [179] and shown diagrammatically from Hoogland and Ploeger [231]. It is hypothesized that when there is a match between the early environment and adulthood in terms of both being adversity or beneficial (blue arrows), a decreased risk for disease is expected. In contrast if is a mismatch between the early and late environment an increased risk for disease is expected (red arrows).

The hypotheses described above can elucidate the effect of ELS on fear memory. The ALH which suggests that a stressor early in life “primes” the system for a stronger response later and the MMH which suggests that if there is a stressor early in life when a stressor is presented later in life these scenarios are a “match” and there is some resilience that is present. This may help explain why some individuals demonstrate resilience after experiencing trauma, while others develop PTSD.

1.11 Neuroinflammation as a continuum

Neuroinflammation is a complex response within the CNS to either harmful stimuli, such as infections, trauma, or autoimmune disorders or as a response to various neuropsychiatric events. Depending on its intensity and duration, neuroinflammation can be both protective and damaging suggesting that the role of neuroinflammation is so-called double-edged sword[232]. The concept of a "continuum of inflammation" reflects this dynamic nature, ranging from mild or localized inflammation to chronic, widespread, and potentially harmful responses.

Sterile and non-sterile inflammation are two distinct types of neuroinflammatory responses which are discussed more in section 5.4.4. Briefly, sterile inflammation occurs in the absence of pathogens and is typically triggered by damage-associated molecular patterns (DAMPs) released from injured or dying neurons. This type of inflammation is commonly observed in neurodegenerative diseases and following brain trauma[233]. Non-sterile inflammation, on the other hand, is triggered by pathogen-associated molecular patterns (PAMPs) from infectious agents such as bacteria or viruses and is observed in conditions like bacterial meningitis or encephalitis.

This complex interplay of cytokines and cellular responses highlights the multifaceted nature of neuroinflammation, which can vary significantly depending on the underlying cause and extent of immune activation.

1.12 Immune cell types and the CNS

The ability of the immune system to conduct a vast array of functions is made possible by the gamut of cells that make up the system each of which has a specific role. There is a broad divide between the two main classes of immune cells: the innate immune cells respond generally to signals of infection or damage (pathogen associated molecular patterns [PAMPs] and damage associated molecular patterns [DAMPs] respectively), cells within this class are macrophages, monocytes and neutrophils. The second class is the adaptive immune cells, these are more specialised to a specific threat and are made up predominantly of lymphocytes which themselves are subdivided into B-cell and T-cells. The involvement of the innate immune system in CNS functioning is complex, with much debate, and has become a major topic for examination [234-237] however, this is beyond the scope of the current thesis. The CNS exhibits a phenomenon known as immune privilege, whereby, the general functioning of the immune system is markedly different in the brain compared to the peripheral organs. Instead immune functioning in the brain (with the caveat that with major infection, or neurodegenerative disease the BBB can become leaky and immune cells from both the adaptive and innate immune system can infiltrate the CNS [234, 238], is regulated by astrocytes and microglia. Astrocytes have a multifaceted role, producing and regulating cytokine production, maintaining BBB integrity and can become directly involved in responding to damage, forming glial scars [239]. Microglia are also multifaceted, with roles spanning the response to CNS infection to regulating

synaptic pruning during development. This is discussed further below in subsection 1.13.

1.13 Microglia overview and discussion on nomenclature.

Before discussing the nuanced effects of stress on the immune system, it is worth introducing microglia, as these cells make up the primary element of the immune system in the CNS. In particular it is useful to discuss microglia development and review some of the debate regarding microglia classification depending on activation state.

Microglia are the resident macrophages of the CNS in the brain, spinal cord, and retina [240]. Microglia form from precursors in the yolk sac which travel to the brain at E9.5 in animal models, where they mature into microglia cells[241]. The population undergoes self-renewal and has very little interaction with peripheral cells except in case of injury or infection when the BBB becomes permeable [234].

Knowledge of the function of microglia, as well as the nomenclature used to describe them as reviewed by has changed drastically over time. At one time it was suggested that microglia solely deal with the response to infection and diseases of the CNS [242]. Now it has been shown that microglia have a major role in maintaining homeostasis of the brain [243], regulation of synaptic pruning [244], plasticity [245] and long term potentiation (LTP, [246]). Classically research has suggested that microglia adopt either an M1 or an M2 phenotype. M1 is characterised as the “pro-inflammatory state” (these terms can be somewhat misleading due to many cytokines having a pleiotropic role (pro- and anti- inflammatory) depending upon location and temporal stage of inflammation, with secretion of pro-inflammatory cytokines such as IL-6, TNF- α and

IL-1β [247-250] as well as engaging in phagocytosis and generation of reactive oxygen species. M2 in contrast is characterised as “anti-inflammatory” where there is a release of anti-inflammatory cytokines such as IL-10 and IL-13. However, this model has now been suggested to be reductive and simplistic [251, 252]. More recently, there has been a move to five distinct classes which can be classified according to their distinct morphology, Homeostatic, reactive, amoeboid, hyper-ramified and rod (shown in Figure 9, [252]). These are observed as follows: homeostatic microglia contain long processes with small somas; hyper-ramified microglia have longer processes and more complex branching patterns; reactive cells have reduced process length and larger somas; ameboid cells have very few processes and a much larger soma; finally rod microglia show a polarised morphology with two long processes giving the rod like appearance[252]. Recently, a review by Paoliccini [251] suggests that classifying microglia in terms such as activated/non-activated M1/M2 or into one of the five classes above is reductionist and that microglia function is more complex than this. Much of the literature to this date involves classification into these five classes, or into an M1/M2 dichotomy. Here, classes will be used to describe microglia, but with explanation of what the features of the class entails.

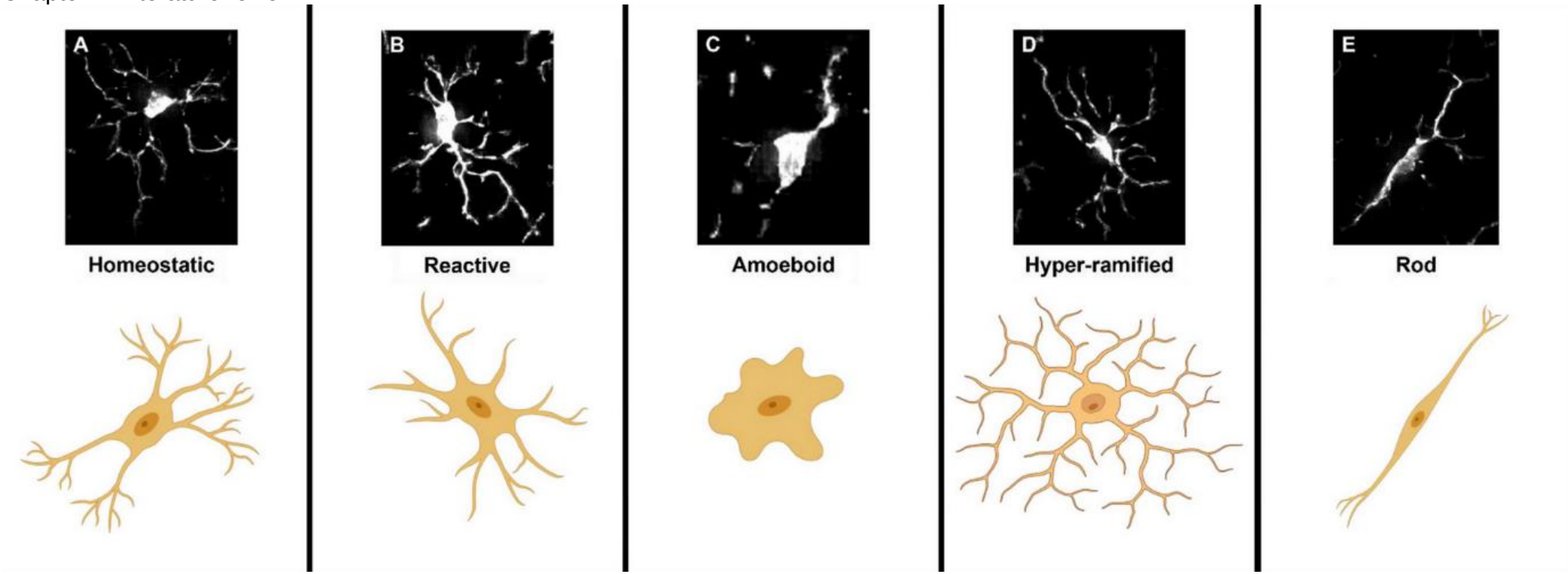


Figure 9: Five distinct classes of microglia can be distinguished due to characteristic morphological profiles. A) Homeostatic microglia, which are often referred to as ramified in the literature, B) Reactive microglia show retracted and shorter processes as well as larger somas C) Ameboid microglia lack processes and have a greatly enlarged soma, D) Hyper-ramified are less well defined in the literature, however they are defined as an intermediate “stage” between homeostatic and reactive. E) Rod microglia are highly polarised with long somas in two opposing directions giving the characteristic rod shape. Image taken from [252].

1.14 Interaction of stress with the neuro-immune system

The interaction between stress and the immune system is well described. For example the SAM and HPA axis (as described in subsection 1.7) controls the release of stress hormones which have an influence on the immune system [185, 253] with specific effects such as a reduction in white blood cell (WBC) count in those with elevated cortisol [254] and modulation of cytokine networks [237]. The mechanisms underlying these interactions however is less well known [255]. The brain also interacts with the immune system in response to immune activation by activating “sickness behaviour” which results in fatigue, depression, reduced appetite, and increased sensitivity to pain [256]. A putative consequence for this mechanism could be to conserve energy and to ensure that resources are dedicated towards combatting infection/disease and not towards digestion and movement. The effects of stress can also supress the immune system on a global scale, Selye [257] suggests that chronic stress supresses the immune system globally.

It has been suggested that the type of stress is important. Acute stress elicits a different immune response to chronic stress [258, 259] and the age at which the individual experiences the stressor has also been shown to influence the response of the immune system[260] potentially due to the interplay between stress and the immune system having more of an effect on development during critical periods described in work by Brydges et al [164]. The effect of stress on development has already been described in detail in subsection 1.9, however, it is important to note that the immune system is regulated by stress and thus is a key system that can be involved in the effects of stress on early life development.

1.15 Developmental stress effects and the neuroimmune system.

The effects of ELS on the neuroimmune system can be observed from the time at which the stressor occurred. Both the CNS and immune system are developing throughout early life and any infection, stressor or traumatic event can have long lasting effects [261]. Increases in proinflammatory cytokines can result from maternal separation. This elevation can have several effects, such as affecting synaptic pruning during development, as noted by Hennessy et al [262]. Additionally, elevated cytokines can modulate plasticity and the homeostasis of brain function. Together, these impacts can significantly influence adult neural functioning and behaviour [263]. The downstream effects of excess inflammation, for example increased cytokine circulation or resulting oxidative stress can also impact upon these developmental patterns. Furthermore, microglia which are described in more detail in subsection 1.13 have been shown to exhibit a more reactive, immunogenic and pro-inflammatory profile during early development [264], suggesting that they are more sensitive to immunogenic, and importantly, stress related, stimuli[265]. This phenomenon is defined as priming (not to be confused with the priming observed in the stress system described in subsection 1.9, although the two mechanisms share some similarities). A schematic of microglial priming is shown in Figure 10. Priming occurring in the hippocampus or the mPFC can lead to a cascade of effects. Some important elements of this to highlight are the increased ROS which is secreted, which can alter LTP, plasticity and network development [266], the increased sensitivity described above as priming and resulting morphology changes and increased levels of peripheral circulating cytokines. Considering the multitude of work which examined the

interactions between ELS and its consequences on the developing neuro-immune system the ramifications of such stress are profound and enduring.

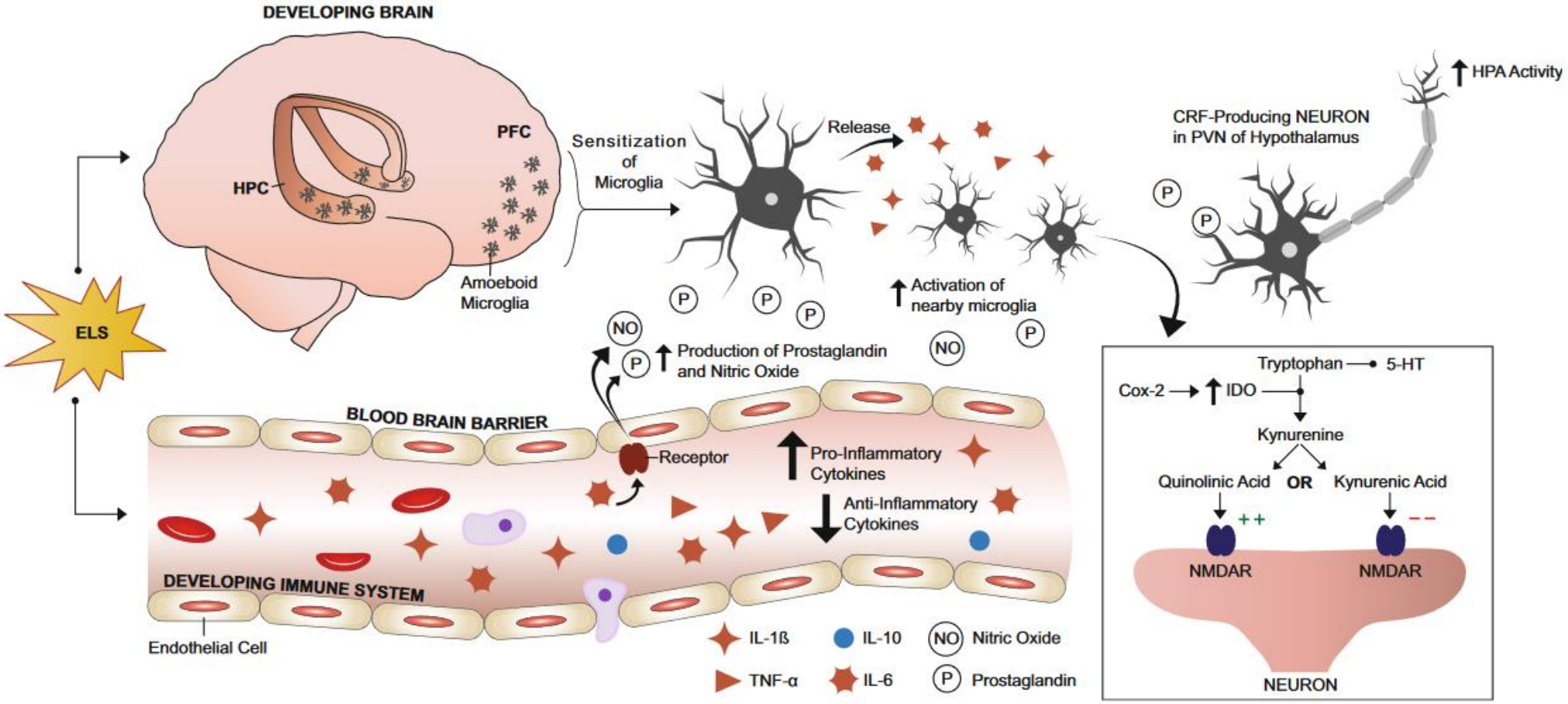


Figure 10:The effects of ELS on the developing neuroimmune system are widespread. Microglia sensitisation and increased ROS, proinflammatory cytokines and prostaglandins can have impacts of CNS development. Figure adapted from Brenhouse et al[41].

1.16 Interaction of biological sex with the neuroimmune system

The development of the central nervous system (CNS) follows sex-specific trajectories with notable differences in the microglial, and cytokine response to stimuli between sexes [264, 267-270]. There are also differences between the sexes in terms of microglial response to a stimulus. For example, following LPS injection Berkiks et al found that there was an increase in the number of microglia in the hippocampus in females and this effect was less pronounced for males [271]. Work by Loram et al [272] shows that in ex vivo microglia exposure to LPS elicits a complex sex dependant response. Males produce more pro-inflammatory cytokine IL-1β mRNA, and CD14 (related to pro-inflammation during detection of bacterial PAMPS) mRNA than females, however when exposed to oestradiol in the presence of LPS the sex effect is essentially reversed (higher IL-1 β and CD14 in females than males). Unfortunately, morphological characteristics, which are a major determinant of microglia function [252], were not analysed here. Other work by Schwarz et al [264] has shown that there are differences in cytokine profiles between the sexes at baseline. These include higher levels of IL-10, IL10 receptor (IL-10R) and IL-1β in females compared to males. Other cytokines such as CCL20 and CCL4 are upregulated in males specifically over females, but only at P0. CD11b, however, which has been shown to be highly implicated in cell migration and activating the pro-inflammatory phagocytosis mechanism, as well as a microglia activation marker, shows higher levels in females compared to males at the adult age of P60.

Microglia differ between sexes in terms of number as well as activation state during development from PND 0 to PND 60 in rodent models [264, 273].In adults, during

aging, there are also differences in microglia in the hippocampus with changes in genes related to cytokine expression and phagocytosis, as well as genes that characterise activation state towards activated, being more upregulated in females than in males [274, 275].

There are sex differences in how microglia respond to different forms of inflammatory stimuli, for example male microglia have been shown to respond with a stronger inflammatory state, releasing more IL-1β than females in response to LPS exposure[272] and a stronger response from male microglia to amyloid β plaques than females [276].Interestingly, there is also evidence that there is an interaction between sex and stress on microglia. One study showed that sex has an effect on both acute and chronic stress, with males showing increased priming following acute stress and females showing the opposite effect, with chronic stress priming (as described in subsection 1.9) was decreased in females with no effects in males [277].

1.17 Effects of stress on microglia

Microglia within the hippocampus and amygdala are activated upon increases in corticosterone levels induced by both acute and chronic stress [278]. This stress can lead to changes in cytokine expression patterns [279] (and are described in more detail in Chapter 5) and changes in morphology [280, 281] (and described further in Chapter 6). A stressor early in life can confer a priming or memory effect [282] which could mean that response to a pro-inflammatory response, or a behaviour that causes activation of the microglia, at a later time point is potentiated. Research by Nie et al [281] has shown that chronic social defeat (CSD) stress can elicit an activated morphology. Other work by Weber et al [279] has shown that expression of cytokines such as IL-1β and IL-6 can be upregulated for up to 24 days following this primed state

induced through repeated social defeat stress. Interestingly, in the work by Nie et al [281] microglia that are exposed to CSD stress show some differences in receptor expression, such as no change in IL-1R1 expression, in comparison to microglia that are activated following a more immunologically inflammatory stimulus (such as TBI or exposure to amyloid beta) where expression of such a receptor is greatly upregulated [280, 283, 284]. This highlights a difference in “pro-inflammatory” between injury/infection and how changes in microglia following psychological stress or during behavioural paradigms may elicit more subtle changes.

1.18 Cytokine interaction with CFC and extinction learning.

The complexity of mechanisms underlying CFC and extinction learning, with the complex anatomical and functional network involved (as described in subsection 1.5) as well as the intricate involvement of the stress system (as described in subsection 1.7) suggests that the regulation of no single cytokine regulation will explain the complex symptom profile. Nonetheless, studies have elucidated potential important cytokines in the regulation of CFC and extinction memory [247, 283, 285, 286]. There is convergence in the literature on several cytokines, namely, TNF- α, IL-1 β and IL-6 that appear to be key for fear behaviour.

It has been shown that CFC memory extinction is associated with an increase in hippocampal pro-inflammatory cytokines such as IL-1 and IL-6 [286] both of these cytokines lead to further inflammation and activation of microglia, the primary immune cells of the brain. It has also been shown that inhibiting IL-1R signalling in the hippocampus can precipitate extinction [285]. Also, inhibiting IL-6 signalling in the amygdala can rescue extinction deficits in discreet cue conditioning [287]. Evidence

suggests that the immune system implicated in regulating fear conditioning and the extinction of fear memories [286] therefore it can be envisioned that targeting the immune system may be a novel therapeutic strategy for those with pathology arising from those with extinction resistant fear memories.

1.19 An overview of machine learning concepts.

The importance of analysing microglia morphology as a measure of function is described above and to do this, complex statistical models can be leveraged. To analyse datasets with many cells contained in them it becomes useful to use machine learning. Some concepts are essential to introduce to highlight the importance of using these models to answer interesting questions about microglia biology.

1.19.1 The bias variance trade-off.

Throughout this thesis each of the models used will be trying to equalise loss in either bias or variance, the so-called bias-variance trade-off. The bias is a measurement of loss whereby the model does not learn to predict the classes effectively enough and therefore makes erroneous predictions even with the training dataset (underfitting). In contrast variance is the measure of the model learning very specific elements of noise in the data to the training dataset and not being able to generalise to an unseen test dataset (overfitting).

Each of the models described below have been implemented successfully in image classification as well as a whole host of other classification problems, this was the reason they were selected as candidates for microglia morphology classification.

1.19.2 Feature space.

This space is essentially multidimensional, with each dimension representing a specific feature that describes the objects under consideration.

For instance, as an example to classify fruits, the features taste, smell, and colour can be used. Each of these features represents a dimension in the feature space. Thus, by considering these three features, there would be a three-dimensional feature space. A single fruit would be represented as a point, where its position along the taste dimension reflects its taste (e.g., sweet to sour), its position along the smell dimension reflects its smell (e.g., fragrant to odourless), and its position along the colour dimension reflects its colour (e.g., red to green).

Machine learning algorithms that work in this feature space aim to find patterns or separations among different classes of objects (e.g., different types of fruits, or microglia according to different morphological parameters) based on their feature representations.

1.19.3 Random forest

The random decision forest method employs an ensemble of decision trees, each comprising branches that split the data and leaves that represent decision outcomes. Decision trees are highly flexible regarding the inputs and are good at categorising data with mixed datatypes. However, decision trees alone are prone to overfitting and can misclassify when used on unseen data [288]. Random forests overcome this by building many trees from a bootstrapped dataset (Figure 11 shows a hypothetical example). Bootstrapped data is where a random selection of the data is put aside (the same datapoints can be chosen more than once, choosing samples with replacement). Trees are built on this bootstrapped dataset, but these trees have different numbers

of leaves and branches, where a subset of the features is selected to build the tree, at random. The selection of the subset of features generates diversity of, and reduces the correlation between, the individual trees. New samples are then run through the random selection of trees (the random forest) and are checked for accuracy.

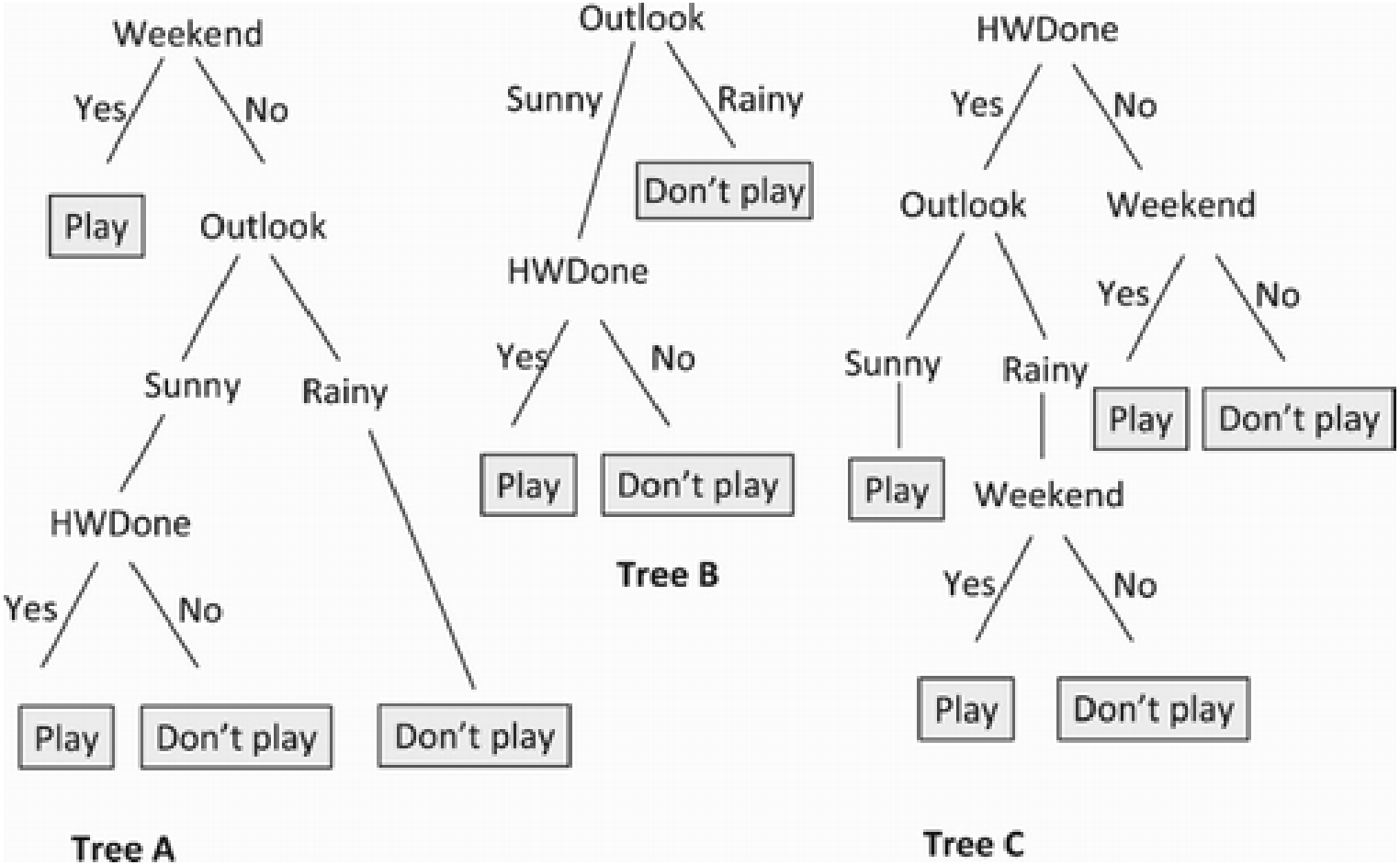


Figure 11: Hypothetical example of a random forest adapted from Fawagreh et al [289]. Tree A B and C show different depths and may contain branches that are duplicated (for example weekend in tree C).

By using an average of the multiple trees an improvement in generalisability and accuracy can be achieved. This approach of bootstrapping and assessing against the aggregated forest of trees is termed bagging. When building these forests, we can control certain hyper-parameters such as the number of trees for the aggregated forest and the maximum depth that any single tree can attain. Adjusting these parameters in the hyperparameter tuning phase of model building is essential to develop a highly performing model (reducing both over and underfitting). Random forests are utilised to classify biomedical images and generate predictions from genetic analysis.

1.19.4 Support vector machine

Support vector machines (SVM) consist of both the support vector classifier and support vector regressor. The SVM classifier is more commonly used and employs an algorithm to separate two classes according to a best fit hyperplane. The data points closest to the hyperplane, which influence its position and orientation, are known as the support vectors, from which the model derives its name.

Within the classification model there are two types of classifiers, hard margin, and soft margin. A hard margin classifier does not allow for misclassifications or can mean that outliers highly influence the position of the plane making the model much less generalisable (Figure 12), while a soft margin classifier would allow misclassifications, thus allowing some bias with a determent to the variance. The best preservation of the bias/variance trade-off described above is selected. The choice between using a soft margin classifier and a hard margin classifier depends upon the dataset and how important the sensitivity to outliers is for the problem.

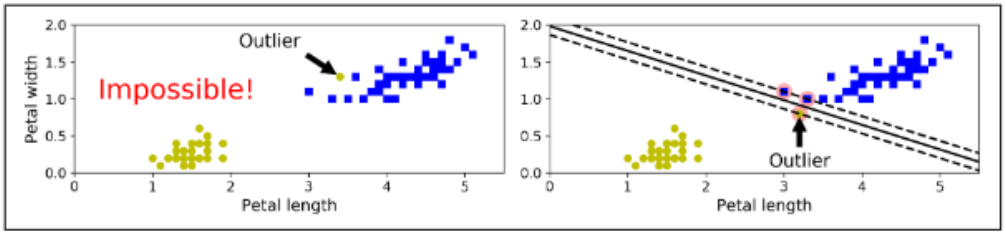


Figure 12: Hard margin classifiers are very sensitive to outliers, either being impossible to fit (as is seen in the first image) or highly non-generalisable (as is seen in the second image). Image adapted from Aurelion [290]

When the number of parameters is higher than 2 or 3 it becomes difficult to visualise. Furthermore, when the classes cannot be linearly separated, which is the majority of complex data, SVM can utilize what is known as the kernel trick which allows you to get the same result as if many polynomial features were added without adding them to the model[290]. This allows SVM classifiers to deal with non-linearly separable data by mapping the data into a higher-dimensional space. There are different kernels that can be used such as linear, polynomial, radial basis function and each of these allows for the linear separation of the data in their own ways.

SVM classifiers are commonly used in image classification and are applied to bioinformatics data, however they do suffer from a drop in accuracy when scaling to very large datasets[290].

1.19.5 K nearest neighbours

K nearest neighbours (KNN) is a relatively simple classification algorithm where the function is only approximated locally, and all computation is deferred until classification. K represents the number of nearest values in a training dataset that will be used to classify a new unseen observation. KNN classifies a new data point based on the majority class among its nearest neighbours in the training dataset.

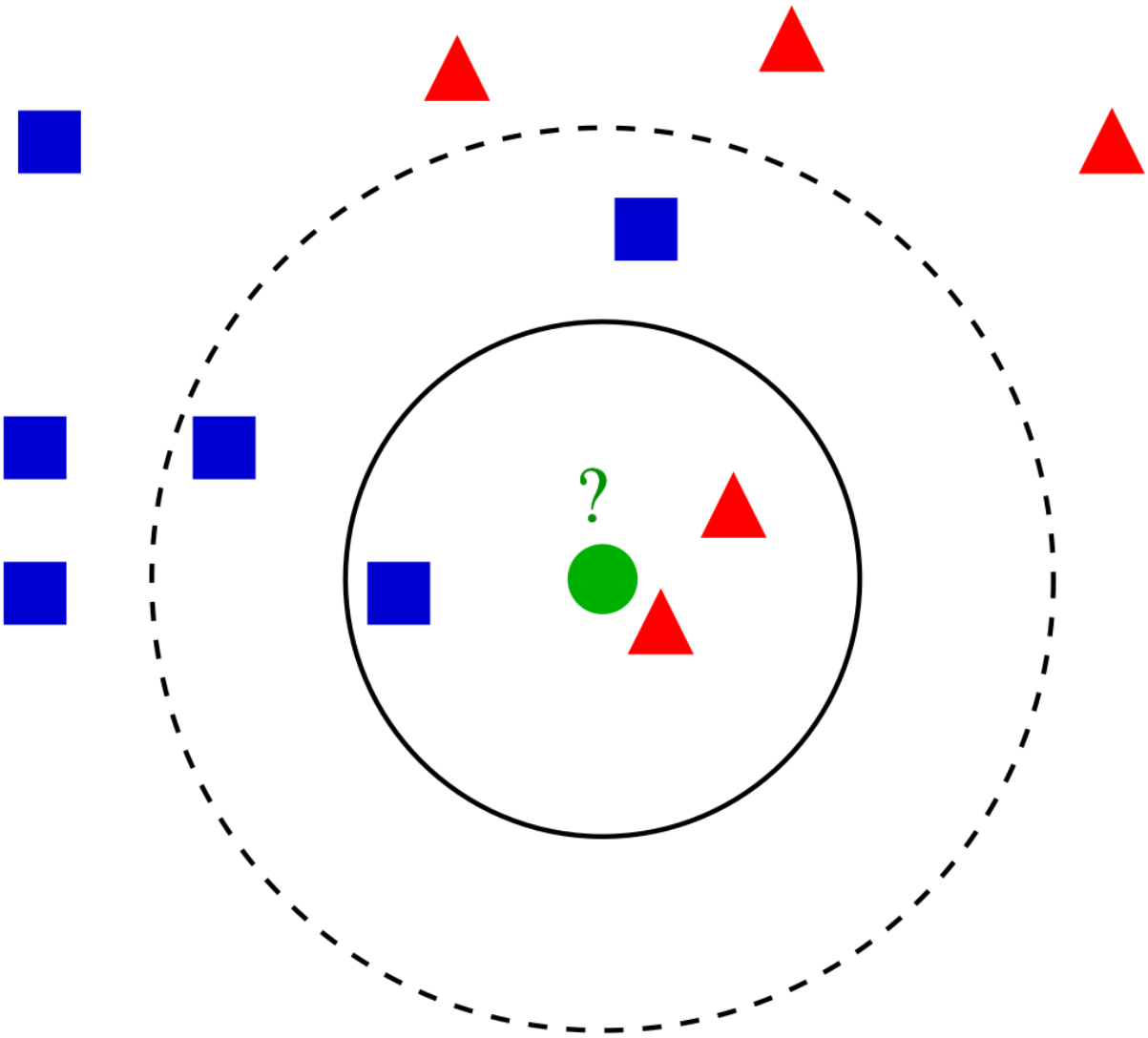


Figure 13: Schematic showing a two class (blue squares and red triangles) KNN algorithm. Here the green point is to be classified. The algorithm iterates through the closest points in feature space. If k is set at 3 (solid line), the majority class of red triangles (2 red triangles vs 1 blue square) would mean the point gets classified as a red triangle class, if instead k is set to 5 the majority class would be blue squares and the point would be classified as a blue square.

This model is generally strong but suffers from misclassification when the dataset is unbalanced as the class with the greater number of observations tends to skew the classification towards that. To overcome this bias, a weighted KNN classifier (where weights can be assigned inversely proportional to the distance) can be used where the under sampled class is assigned a higher weight to balance out the class imbalance. The model also is highly sensitive to issues of scale as the model calculates the distance between points. To accurately classify samples the features must be normalised or scaled, features on larger scales can disproportionately influence the classification.

As with all models choosing hyperparameters that balance over and underfitting is important and the K value is essential. Choosing a k-value that is too small can make the model more sensitive to overfitting while the converse (too large a k-value) can lead to underfitting.

1.19.6 Gaussian Naïve Bayes

Gaussian Naïve Bayes is a classifier that extends the Naïve Bayes algorithm to handle continuous features by assuming that these features follow a Gaussian distribution. Probabilities are estimated using maximum likelihood in that class based on the Gaussian distribution of each feature.

Has been debate as to if GNB classifiers are more effective than Logistic regression. GNB tends to perform better in cases where the assumption of feature independence holds true and when the dataset is small or when the dimensionality of the input space is high. Logistic regression, on the other hand, can perform better when these assumptions do not hold. Features presented in the microglia model may be correlated (for example branch length and number of branches) and GNB is also dependant on

the models following a Gaussian distribution. This will need to be carefully considered if GNB is the more dominant model.

1.19.7 Gradient boosted model

These models gather multiple weak models (typically decision trees), which alone do better than chance, that when combined into an ensemble model can give a stronger prediction than any single weaker model. The ensemble model generated for a GBM is generated additively and sequentially as follows. The first weak model is typically a decision stump (a decision tree with a single split). The loss of this model is calculated, and the area where high loss is detected is focused on for subsequent model development. The next model is added to minimise the error of the overall model. This is the “boosting” part of the GBM. Predictions are made and a second model is generated. The process is conducted iteratively until either a set stopping criteria (for example a number of trees) is met or that the addition of trees does not lead to an improvement in the loss function. This is done to prevent overfitting of the model.

1.19.8 Logistic Regression

Logistic regression is a simple classification algorithm which is highly suited to binary classification. Logistic regression models can be built using both discrete and continuous variables which is a key strength for this model. To understand how logistic regression can be used for binary classification it is important to consider the S shaped sigmoid function which defines the logistic regression as follows:

$$\mathrm{P}(\mathrm{Y}=1)=\frac{1}{1+\ e^{-x}}$$

Equation i

where x is a linear combination of the independent variables, and e is Eulers number (approximately equal to 2.71828). The equation is used to map real values with a wide range of values to values between 0 and 1. The curve is fit to the data using a maximum likelihood metric which aims to maximise the probability of observing the given set of data (both Y= 1and Y=0 cases) under the model parameters.

1.19.9 Hyperparameter tuning.

Hyperparameters are the elements of a statistical model that can be modified to best fit a specific dataset. Examples of such hyperparameters include the number of trees in a random forest, the C value, or kernel type, in an SVM, the k value in KNN or the learning rate in many different models. They are different than the parameters of a model in the sense that they are set before training the model and are not learned from a specific dataset. Adjusting these hyperparameters can lead to the model having a stronger fit to the data. There are various methods to search for the optimal hyperparameters which fit the data. These include grid search which is a comprehensive search of every variation (or a predefined range) of a single or set of hyperparameters, randomised search, which works like grid search but selects a random selection of these hyperparameter values and gradient based optimisation (which is used more in deep learning models). Each method has advantages and disadvantages such as time taken to run, and computational complexity vs completeness. A consideration of over and underfitting should be considered when using these methods and modulation of hyperparameters often influences complexity of the model which has a direct effect on the model's propensity to under or overfit. Balancing the two is a highly essential consideration when developing any model. Cross validation, which is where the dataset is split into multiple parts to train on, and

then the model is tested on the left-out part, is often employed when running methods to find the optimal hyperparameters in a model to reduce overfitting.

1.19.10 Synthetic minority oversampling technique

Synthetic minority oversampling (SMOTE) is a method to correct for imbalanced datasets where one class contains more samples than the other class (or classes). SMOTE works by selecting samples, from the under-represented class that are close together in the feature space (defined as the combination of the dataset parameters as a space). Samples are then interpolated from these selected samples. An example of SMOTE sampling, with a schematic, is shown in Figure 14. This method improves the performance of the classifier by equalising the ratio of samples per class. This reduces any assigned weights that would result from imbalanced classes. There is a risk that generated data might lead to some overfitting. However, this method has an advantage over just duplicating samples, another common method to correct for imbalance as it provides variance and potentially limits overfitting.

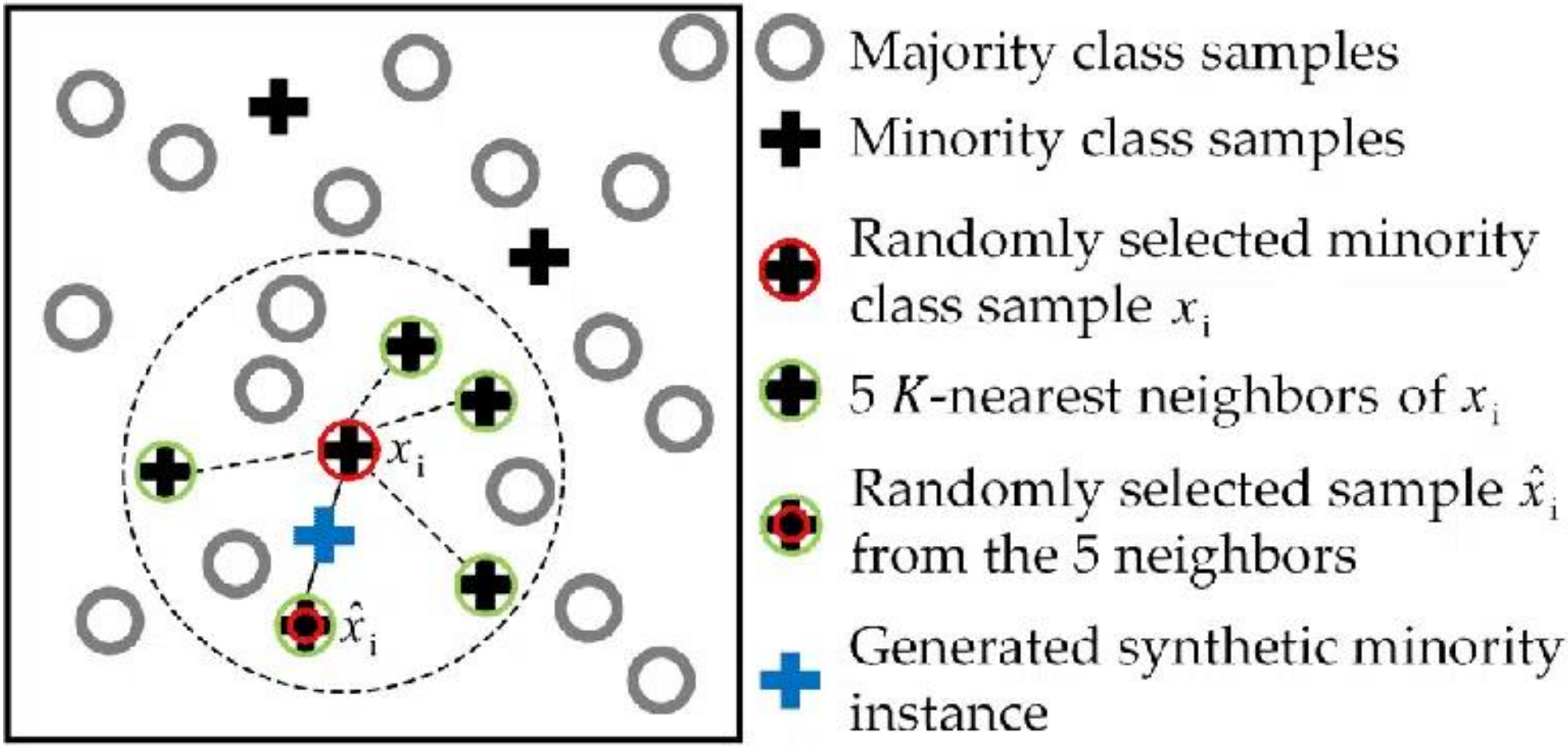


Figure 14: SMOTE sampling example. Two are represented as grey circles (majority class) and black crosses (minority class). To generate a synthetic sample a minority class sample is randomly selected (cross with red circle). A number of nearest neighbours (k) is selected (here k=5). One of the nearest neighbours is selected (cross with green and blue circle). The blue cross represents a synthetic minority sample generated between this selected sample and the first randomly selected sample.

1.20 Experimental plan and aims.

ELS has been shown to be a sex dependant risk factor implicated in the development of various psychiatric disorders including schizophrenia, depression, and PTSD. The underlying mechanisms driving this risk is suggested to be related to CNS immune activation and the subsequent interaction with ELS and an effect on fear memory and extinction.

In this thesis the effects of ELS on CFC on extinction memory was analysed. This was examined by utilizing two different measures of fear. The thesis also focused on the role of the immune system as a potential biological mechanism interacting with behavioural processes. My aims were as follows:

1. Determine the effects of ELS on CFC and extinction with a focus on sex differences in order to confirm and expand upon the findings of Brydges et al [96].

2. Explore the measurement of 22 kHz vocalisations as a different index of fear to freezing behaviour. Elucidate stress or sex differences that may be hidden when focusing on a sole behavioural metric.

3. Investigate the role of the cytokine immunological profile on the sex dependant effects of ELS on CFM and extinction.

4. Explore microglia morphology using an updated version of the pipeline developed by Reddaway using an LPS protocol to confirm and expand upon the work conducted by Reddaway [291]. Furthermore, the model will be used to examine morphologic profiles of microglia in animals exposed to an ELS with CFC model.

2 Chapter 2: General methods

2.1 Animals

All animal experimental methods were carried out in accordance with relevant guidelines and regulations of the European regulations on animal experimentation (Directive 2010/63/EU) and the UK Home Office Animals (Scientific Procedures) Act 1986. All experimental protocols were approved by the local ethical review body (AWERB) of Cardiff University.

Lister Hooded rats either bred in-house from animals obtained from Charles River (CR) to be used in experiments (Chapters 3,4,5) or from animals obtained from Charles River that were used for breeding (Chapter 6). Animals were housed in conventional cages (32 × 50 × 21 cm) lined with sawdust and were given bedding, free access to tap water and standard chow. 1-2 cardboard tubes and a wooden chew stick were provided as enrichment. Lights were kept on a 12h reverse light–dark cycle with lights switching off at 10:00 and on at 20:00.

After habituating from transport for at least 7 days the rats were paired with 1 male and 1 female per cage for breeding. Animals were monitored for signs of pregnancy and litter birth. At PND 21 animals were weaned and checked for sex. An overview of numbers for each experiment is given in Figure 21. All animals from each litter were used. Males and females were separated into individual cages with all males in one litter going in one cage and all females going into another. Female mothers were then paired with another female which had given birth (with cages up to 4 females) and were either culled shortly after or used for microglia experiments as described in more

detail in chapter 6. After PND 27 all animals were split further into pairs or groups of three.

For animals born in-house after PND 27 animals were given either a single left, right or both ear notch. For cages with paired animals, animals were given a left or right ear notch. For cages with three animals, animals were given left, right and both ear notches. This was to ID animals later for correct placement into the contextual fear conditioning (CFC) chambers and due to the long time periods between conditioning (>30 days) was considered a less stressful method of ID than tail marking[292]. These animals, which underwent ELS, were used for experiments in chapters three, four and five. For chapter 6, for the administration of compounds experiments, breeders giving rise to animals for chapters 3, 4 and 5 were used. Females were paired as described above, in twos or threes, however males which were used for breeding were then housed individually due to high chance of post breeding fighting.

2.2 Behavioural protocols

2.2.1 Complete behavioural timeline

The overall schematic, for experiments spanning chapters 3,4 and 5 and 6 which includes ELS contextual fear conditioning, a modified fear conditioning paradigm for cytokine and microglia morphology analysis is shown in Figure 15. The Coronavirus disease 2019 (COVID-19) pandemic caused some animals to be terminated early which impacted upon animals used for Chapter 3 and Chapter 4. Where this occurred, it is described further in the relevant chapters.

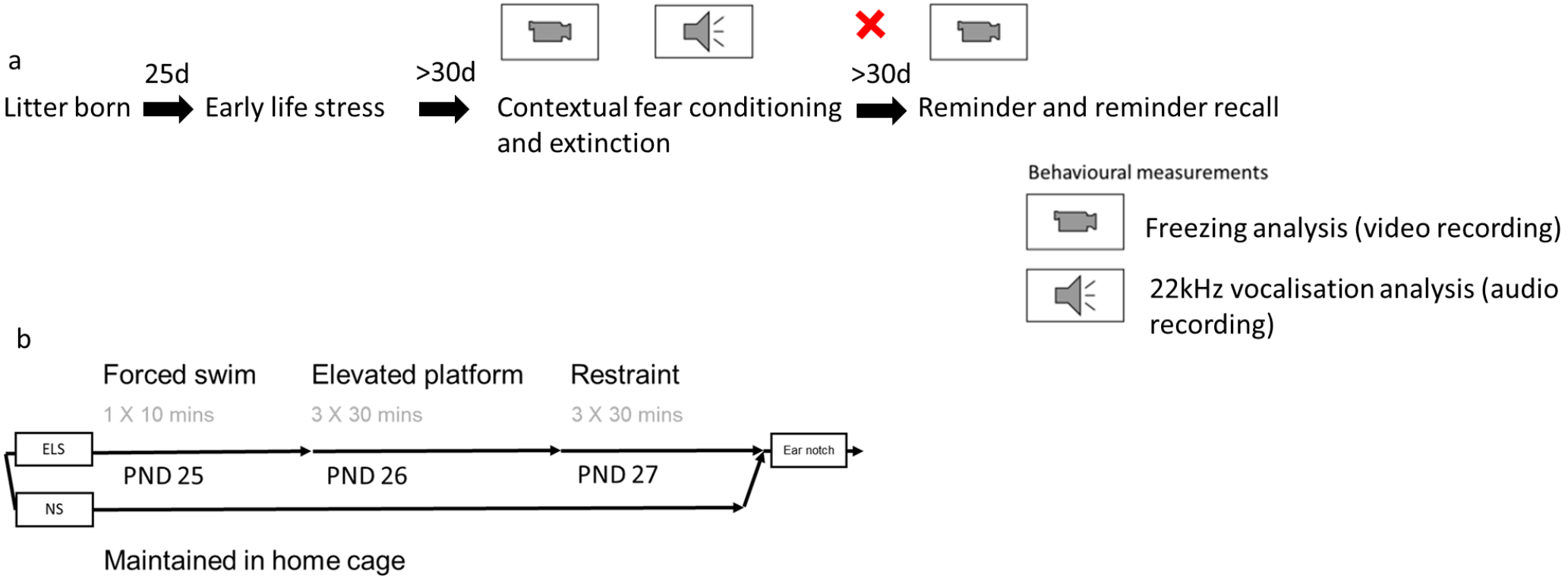


Figure 15: a: Overall schematic showing the time course from the birth of the animals up to the termination of the behavioural experiments. Restrictions due to the COVID-19 pandemic required that some animals be terminated prematurely (depicted by the red cross). Post COVID-19 pandemic, animals underwent the complete protocol shown. b: Schematic of early life stress protocol.

2.2.2 Early life stress

Rats underwent ELS via an established protocol [293] originally developed by Tsoory et al (Figure 15b) [294]. Animals were destined to be used for CFC where foot shock is an integral part of the experiment. Foot shock which was one arm of the ELS protocol in previous work by Brydges et al [293] was therefore replaced with the elevated platform stressor as has been suggested previously [164]. Animals were transported in their home cages, on a trolley (note, a different trolley was used for that used for CFC later) animals underwent three variable stress procedures occurring over three days, always, at PND25, PND26 and PND27 and took place in a separate room from the holding room. Firstly, at PND25 animals were placed into plastic, opaque, 12L buckets (25 cm high, 34 cm diameter) containing water maintained at 27 ± 2 °C. The water level was sufficient to prevent them from touching the bottom of the bucket with their tails or feet. Animals were placed into this water bucket for 10 minutes. They were then towel dried and monitored for any adverse effects such as rapid drop in body temperature (through feel), long periods of shivering, lack of movement and not forming a huddle with cage mates (such adverse effects were absent during monitoring in all conditions) and returned to their home cages. The next day, at PND26 animals were placed in clear Perspex boxes (250mm height, width, and depth) elevated approximately 100 cm from the floor, for 30 minutes followed by a one-hour rest in the home cage in between, this was repeated twice, leading to 3 X 30 minutes with 1 hour rest between each. The next day, (PND27) animals were placed into flat bottom rod restraint tubes (15 cm length, 5 cm diameter, Harvard Apparatus) for 30 minutes and then returned to their home cage for 30 minutes. This was repeated 3 times leading to 3 X 30 minutes with 20 minutes rest between each. Animals then went back into the home cage. Additionally, it should be noted that during this time animals

are exposed to light within the room for the duration of the stress thus disrupting the reverse light cycle. Animals which underwent ELS were then maintained in the holding room until further experiments.

Weighing animals following exposure to ELS is not a stipulation covered under the licence at the time of experimentation. A change in weight following ELS has already been observed previously [295]. Furthermore, frequent weighing could potentially introduce additional stress to the animals, as repeated handling is often considered a significant stressor in itself [296].

Animals underwent ELS as part of experiments for all experimental Chapters (3, 4, 5 and 6).

2.2.3 Contextual fear conditioning equipment and software

Contextual fear conditioning (CFC) took place in two standard conditioning chambers (dimensions: 32 cm × 25.5 cm × 27 cm) with clear polycarbonate sides and door (Med Associates Inc., Vermont, USA). The chambers differed in location with a 1-meter distance between the chambers and orientation, with one chamber being at 90° to the other and a different pattern presented via a piece of paper with black stars, which was present on the rear wall of one of the chambers. The chambers were enclosed within sound dampening cubicles (56 cm x 56 x 36 cm; Med Associates Inc., Vermont USA). Individual animals underwent conditioning and where required recall, extinction training, reminder-shock exposure, and reminder-recall in the same box. A video camera (SP Electronics Limited Co., Guangdong, China) and ultrasonic vocalisation microphone (CM16/CMPA, Avisoft bioacoustics) was mounted above the box for acquisition of videos and sound files respectively. The floor (interior dimensions: 30.5 cm x 24.1 cm x 21.0 cm) consisted of 19 equally spaced metal bars placed 1.6 cm above the floor, that were connected to stimulators that deliver a pulse-scrambled electric shock (ENV-414S, Med Associates Inc., Vermont USA). During the experiments a light was on, and a fan was activated to mildly attenuate any external noise including from one animal being shocked in the other box. Some cross over was noticed, vocalisations at human detectable frequencies, especially during shock, within the box could be heard outside the box. The chambers were cleaned with 50% ethanol between each trial.

Visual recording

A video camera (SP Electronics Limited Co., Guangdong, China) was mounted above the box for acquisition of videos files which were saved as AVI files. Visual recording was performed with Numeriscope software (ViewPoint, Ain, France) and the

automated contextual fear conditioning programme was generated with MedPC software (Med Associates, Fairfax, Vermont).

Audio recording

An ultrasonic vocalisation microphone (CM16/CMPA, Avisoft bioacoustics) was attached facing into the box as shown in Figure 16 and Audio recordings were taken using Avisoft-Bioacoustics microphones (CM16/CMPA) and were wired into an UltraSoundGate 416H (Avisoft bioacoustics) using XLR cables.

Vocalisations were recorded with hardware which is described above. The software used was Avisoft-RECORDER USGH (ultra-sound gate hardware), v4.3.03. Details on the programme and specific parameter settings are described further in Chapter 4. An issue arose between the acquisition of the USV recordings with the microphones, the mixing hardware, and the software interface. This meant that the automatic USV counter, which is built into Avisoft- RECORDER USGH, was detecting wavelets in the USV range even when no animals were present and at high numbers. This likely was due to the proximity to CRT monitors and computer hardware which was unavoidable. The frequency range and duration of noise artifacts was known and so filters, and post processing was employed (and is discussed in further detail in Chapter 4), to mitigate this. Spectrograms were monitored via the online spectrogram visualisation online to confirm that the pipeline was working and not picking up any noise (beside that described above), but detailed analysis was conducted offline on the saved Waveform Audio File (WAV) files that were generated by the software. Analysis of these files is described in 2.4.2

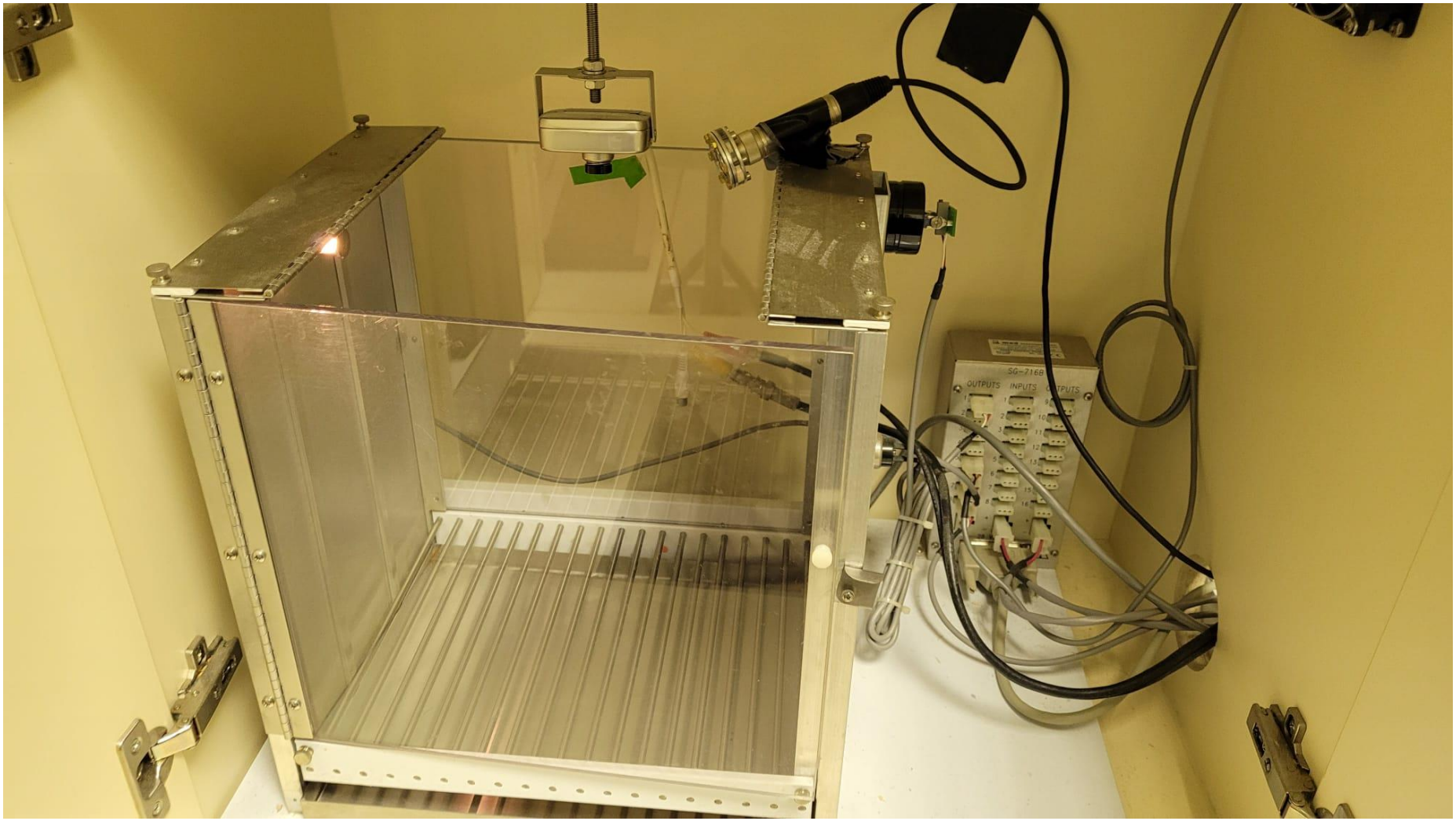


Figure 16: Positioning of the camera and USV mic inside the CFC. The USV mic was taped to the corner of the box. Despite there being areas in the box where the microphone was not facing directly, the microphones were very sensitive and calls could still be picked up from these areas (data not shown).

Transport: Each rat was transported alone from their holding room to a behavioural room, using a trolley, in individual white opaque plastic boxes. For the whole experiment each animal was transported in the same large transport box.

2.3 Contextual fear conditioning protocol

2.3.1 CFM acquisition

For the CFC trial each animal received a single scrambled foot shock (US: either 0.5mA, 2s or 0.7mA, defined as low intensity or high intensity shock, respectively) after being placed in the conditioning chamber (context) for 2 min (with fan and light on). Following this the animal remained in the conditioning chamber, while being monitored for another minute. The trial would terminate by shutting off the lights and fan. Video and audio recording continues throughout the two minutes pre-shock, inclusive of the foot shock and the one minute after. Animals were immediately removed from the conditioning chambers, placed back into their respective transport boxes, and taken back to the holding room.

2.3.2 Extinction training and extinction control

After 48 hours, animals were transported, as described above, to the conditioning chambers, and then placed back into the same conditioning chamber where they underwent CFC. Animals then were exposed to the context of the conditioning chamber for either 2 or 10 minutes, which was determined randomly from the cages, where both visual and audio recordings were taken and now defined as extinction control (2 minutes) or extinction training (10 minutes). During this time animals experienced no foot shocks. Note, the 2-minute condition does not extinguish CFM here. Instead, the animals exposed to the two minute protocol are defined as the extinction control group and are likely to experience CFC recall as two minutes has

been shown to be insufficient amount of time to develop a robust extinction memory[297]. Throughout the thesis I will also describe the analysis of the freezing behaviour during the extinction control protocol (2 minutes) as contextual fear memory recall following consolidation. This protocol serves both an extinction control and way to analyse CFM recall.

2.3.3 Extinction recall

Animals were then removed and transported back to the holding room as above. 48 hours later animals were transported as above to the same conditioning chamber and the next programme (recall) was run. Here, 2 minutes of recording took places for all animals (lights and fan on). All animals underwent the same programme for recall. Animals were then transported back to the home cages as above.

2.3.4 Reminder and reminder recall

For animals which went on to conduct further behavioural testing animals were kept in their home cages for 32 days where they then underwent a reminder protocol. The reminder protocol is as follows: all animals (for all groups) were placed into the conditioning chamber for two minutes recording (fan and lights on) which then terminated in a 2 second scrambled 0.25mA (sub-threshold) foot shock. This foot shock is weak enough to not cause any *de novo* conditioning [297]. In contrast the reminder will probe any reinstatement that has been elicited which is suggested to be a reversal of extinction[298]. They were then immediately removed from the chamber and placed back in their home cages for 48 hours. The animals were placed into the chamber (fan and light on) for 2 minutes (reminder recall). Animals were taken back to the holding room and soon after euthanized.

2.4 Behavioural data analysis:

2.4.1 Freezing behaviour

Freezing during contextual fear conditioning, recall, extinction, and reminder phase as shown in Figure 15 was manually analysed from video recordings by an observer blind to group. Freezing was defined as immobility (besides respiration) for 1 second, with all 4 limbs on the bars on the floor of the box. Freezing was not counted if it was obvious to the observer the animal was sniffing a part of the cage (e.g. nose movement while still stationary). 1 second was sampled every 10 seconds for freezing behaviour with the previous and post second being considered. A second observer examined a subset of the videos for accuracy with less than 10% discrepancy. A percentage of freezing was then calculated as:

$$Freezing\ percentage = \left(\frac{number\ of\ samples\ containing\ freezing}{Total\ number\ of\ samples}\right) \times 100$$

Equation ii

For contextual fear memory acquisition there were two epochs assessed, pre shock and post shock. Pre shock was for two minutes before the shock was given and post shock was the following one minute of recording. Results gathered from the first two minutes of the extinction protocol were pooled together with the results from the 2-minute group defined as CFM recall following consolidation as there was no experimental difference between the groups at this stage (extinction control group + the first 2 minutes of the 10-minute extinction trained group).

2.4.2 Ultrasonic vocalisation analysis

Audio files were saved as varying lengths without a clear start of the recording (namely the initiation of the protocol). Files were cropped according to the length of the protocol,

measured back from the shutdown of the fan which signalled the end of the programme. In future work it would be advised to have an audio cue signalling the start of the protocol, however, this could also act as a cue which would confound behavioural testing. The method described here was sufficient for these experiments. A python script was generated to crop sound files into only that which should be recorded during the running of the programme, for example not recording sounds which could be present while handling animals to put them into the conditioning chambers and not while taking the animals out of the chambers. The library pydub (James Robert, 2011) was used to allow manipulation of WAV files and crop files accordingly. For files that required a split for different time dependant analyses, for example the pre and post shock timepoints at contextual fear memory acquisition and the five two-minute bins used for extinction training the files were also divided into these bins using this script. Files were checked to see that each 2-minute bin was an accurate split of the 10-minute clip using Audacity (v3.0.0) and were then run in batch.

Offline analysis of the vocalisations is discussed in detail in Chapter 4. Briefly a script in R allowed Raven, a software developed by the K. Lisa Yang Center for Conservation Bioacoustics (https://ravensoundsoftware.com/about/), to be run in a high throughput manner and USV calls in the 22kHz range to be counted. The number of calls per session as well as the percentage of callers compared to non-callers was analysed.

2.5 LPS administration

Animals were injected with lipopolysaccharide (LPS) from Escheria. coli 0111:B4 (InvivoGen, California, USA). LPS was dissolved in 0.1 M PBS (pH 7.4) to 5 mg/ml and administered (250 μg/kg) intraperitoneally. This solution was filtered, to remove particulates, aggregations and potential contaminants, before injection. Control

animals received a single intraperitoneal injection of 0.1 M PBS (5 ml/kg). Following injection rats were returned to their home cages and were monitored for clinical signs of perforation of visceral organs or abnormal reactions to injection. All LPS injected animals did show LPS induced sickness behaviour as was measured on the score sheet, adapted from Wolfson and Lloyd [299] (lethargy, reduced locomotion and lack of grooming) but none showed excessive sickness (anything beyond a 2 in any parameter) or signs of perforation of visceral organs or extreme adverse reactions to the injection.

2.6 Cytokine analysis

2.6.1 Tissue collection, processing, and storage

Brains were extracted rapidly after euthanasia with rising concentration of CO_2 (8-minute programme, four minutes at 4L per minute and four minutes at 8L per minute) in a Semi-Automatic Home Cage Culling Unit (Clinipath Equipment Limited, East Yorkshire, UK). Brains were placed on foil inside an insulated box containing dry ice. Once frozen brains were wrapped in parafilm, and this wrapped in foil, labelled, and then stored at -75°C until further use.

2.6.2 Tissue punch collection

Brains collected as above were placed into a pre-chilled Rodent Coronal Brain Matrix (Rat 175-300 g, RBMS-300C, 1mm steel, World Precision Instruments, Hertfordshire, UK) for sectioning. Feather razor blades (Feather FA-10, 0.1mm) were wetted with water and inserted every second jig position (2mm inter-blade distance) dividing the brain in the coronal plane from the most rostral appearance of the forceps minor (Bregma + 5.16) to the formation of the genu of the corpus callosum (Bregma + 2.52 mm) which captures the PFC. Slices were taken from the formation of the third

ventricle (Bregma -0.36 mm) to the rostral beginning of the mesencephalon (bregma -6.60 mm) which encapsulates the hippocampus. A precision brain punch with diameter of 1.5 (±5%) mm (Ted Pella Inc., California, USA) was used to take tissue punches using the Palkovits punch technique [300] from the PL and IL cortex of the mPFC with the forceps minor acting as a guide for correct positioning of punch holes see Figure 17A. Punches from the CA1, CA3 and dentate gyrus of the dorsal hippocampus were also taken and are shown in Figure 17B. All punches were kept at -75 until needed for further use.

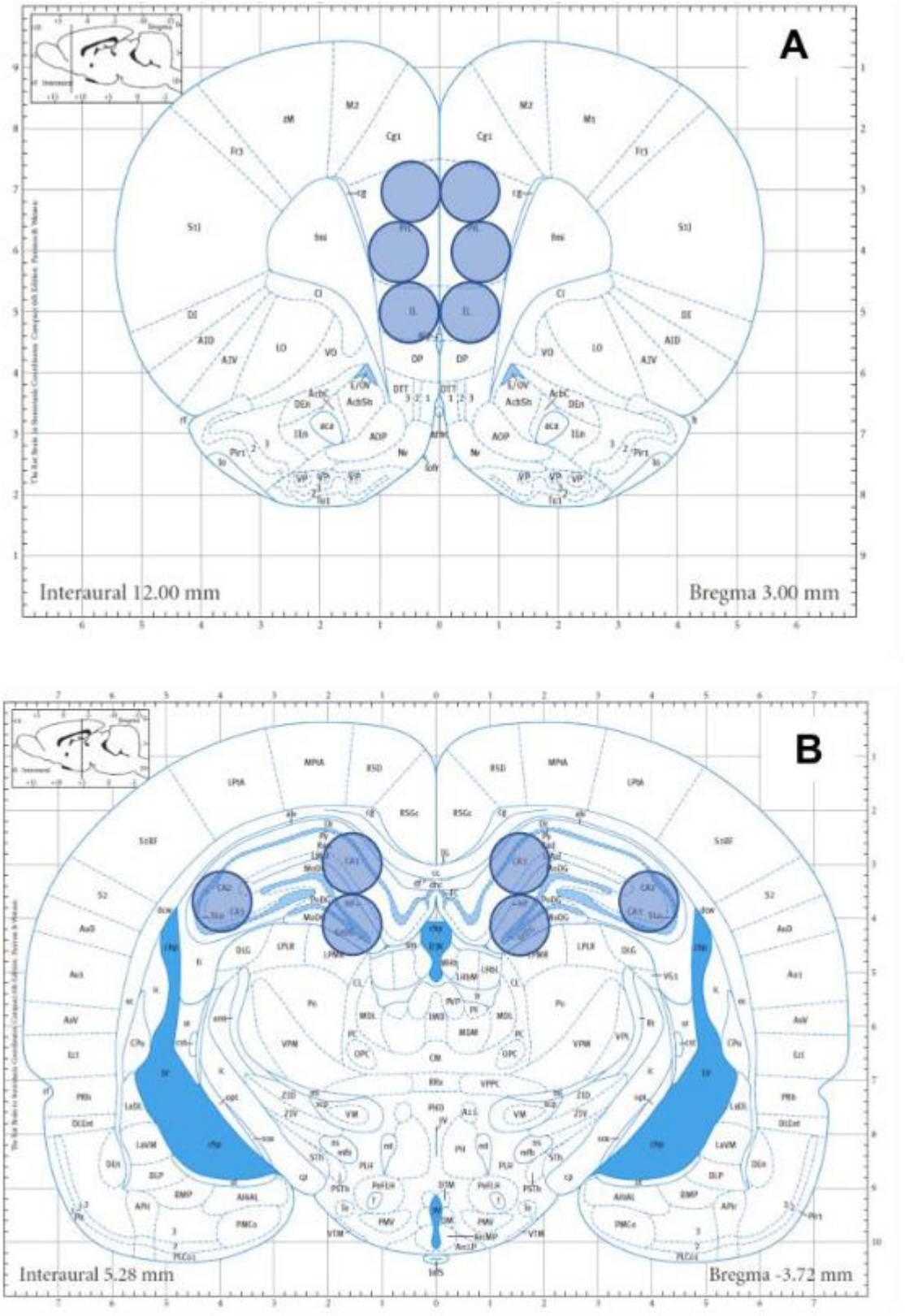


Figure 17: Locations of punches taken from the 2 mm coronal slices to isolate the PL and IL cortex (A) and the CA1, CA3 and Dentate gyrus subregions of the hippocampus (B). Figure adapted from Reddaway[291]

2.6.3 Protein concentration assay

Tissue punches were homogenised using ice cold cell lysis buffer (Tris-HCl, 10mM; NaCl, 10mM; IGEPAL CA-630, 0.2%; Sodium Butyrate, 10mM; phenylmethylsulfonyl fluoride (PMSF), 50ug/ml; Leupeptin, 1ug/ml). Punches and 400µl of the lysis buffer were added to a Dounce homogenizer and the plunger inserted 30 times. Samples were left on ice for 20 minutes and then centrifuged at 4 °C for 20 minutes at 12,000 rpm (16099 RCF, Centrifuge 5424 R). Protein concentration in each sample was measured using a Pierce BCA assay (23225, ThermoScientific). Concentration was read using a microplate reader (Clariostar, BMG Labtech) with absorption measured at 595 nm. Samples which were above the threshold range of 900 µg/ml were diluted with the lysis buffer described above to bring them close to 700ug/ml as possible which was then in the detection range recommended by Bio-Rad (200-900 µg/ml of protein) all samples contained protein concentrations within this range. Samples were kept frozen at -75°C until further analysis.

2.6.4 Cytokine analysis

Samples were analysed using a comprehensive secretomic analysis performed using a Bio-Plex Pro Rat Cytokine 23-plex Assay (BIO-RAD systems). The plate works using 6.5 µm magnetic beads coupled to antibodies targeting the 23 antigen targets. Detection antibodies are included for the assay and the detection method is via streptavidin - Phycoerythrin. A more list of cytokines is shown in Chapter 5. Three brains, with samples from IL cortex and the CA1 region from the hippocampus, from each group were randomly selected and run due to time and money constraints The assay was run on a BioPlex 200 system by Indoor Biotechnologies Ltd, Cardiff. Results from the analysis were collected as picogram of cytokine per sample (pg/sample).

Samples were normalised according to the total protein concentrations the full concentration list is described in Chapter 5; this yields a mg/ml concentration level.

2.7 Immunohistochemistry

2.7.1 Perfusion

Animals were sacrificed either 24 hours following LPS/PBS injection or 24- or 72-hours following CFC for microglia analysis, with overdose of pentobarbital solution (200 mg/kg, Euthatal, Boehringer Ingelheim Animal Health, Surrey, UK). Upon sacrifice animals were transcardially perfused with 0.1 M PB followed by ice cold 4% paraformaldehyde (PFA) dissolved in the PB solution (flowrate: 20 ml/min) for approximately 8 minutes. Animals were decapitated, and the brains removed and placed into ice cold 4% PFA and kept for 24 hours at 4°C. Brains were transferred to 30% sucrose in 0.1M PBS. The brains were monitored for buoyancy, once the brains sank to the bottom of the tube, they were completely embedded in OCT media (Scigen, California, USA), and stored at -75 °C until required.

2.7.2 Tissue preparation

Brains were sectioned using a NX50 cryostat (Leica Microsystems, Buckinghamshire, UK). 40 µm slices were sectioned coronally, going from rostral to caudal. Slices were collected, rostral to caudal, from the appearance of the forceps minor (Bregma + 4.20 mm) to the genu of the corpus callosum (Bregma + 2.52 mm) for the PFC, and slices were collected, rostral to caudal, from the appearance of the dorsal hippocampus (Bregma -1.72 mm) to the appearance of the ventral hippocampus (Bregma -4.68 mm). A 1/12 series was taken, and sections were placed in 0.1 M PBS in 12 well plates. Plates were stored at 4°C until needed.

2.7.3 Immunohistochemistry

Slices were washed 3 times with 0.1 M PBS for five minutes on a plate rocker. Blocking was conducted with 500µl of blocking solution made up of 10% (1/10) normal donkey serum (NDS) (Thermo-Fisher, Massachusetts, USA), 1% volume per volume (v/v) Triton X-100 (Bio-Rad Laboratories, California, USA) in 0.1 M PBS. Rabbit, anti-rat Iba1 (product no: 019-1974, FUJIFILM, Wako, Tokyo, Japan) was used at a concentration of 1/1000 diluted with blocking solution (0.1 M PBS containing 1/500 NDS and 0.1% v/v Triton X-100). This primary antibody was incubated at 4 °C for 24 hours on a see-saw rocker. Slices were then washed 3 times with 0.1 M PBS containing 1/500 NDS and 0.01% v/v Triton X-100 with agitation for 5 minutes. A secondary antibody Alexa Fluor 488, (Thermo-Fisher, Massachusetts, USA) was incubated for two hours at room temperature at a concentration of 1/1000 diluted with 0.1 M PBS containing 1/500 NDS and 0.01% v/v Triton X-100. Slices were washed three times as above. During the second wash 4’,6-diamidino-2-phenylindode (DAPI) (0.1 ng/ml, 62248, Thermo-Fisher, Massachusetts, USA) was added to the PBS wash (1µl per 500µl PBS) and removed with the third wash. Sections were mounted on Superwhite glass coverslips (Landon LaboQuip, London, UK) and cover slips applied with MOWIOL 4-88 (Merck & Co., New Jersey, USA) or Flurosave (Merck, 345789-20ML). Slides were stored at 4°C in opaque slide boxes.

2.8 NueMorph microglia analysis pipeline

To analyse the morphology of microglia in a high throughput manner, development and improvement of many features of a pipeline described previously by Reddaway [291] are described below. Figure 18 shows a schematic of the pipeline with changes to the workflow since Reddaway’s work detailed in Figure 19. This new pipeline

(containing preprocessing, skeletonization and analysis steps) was named NueMorph and is described as such below.

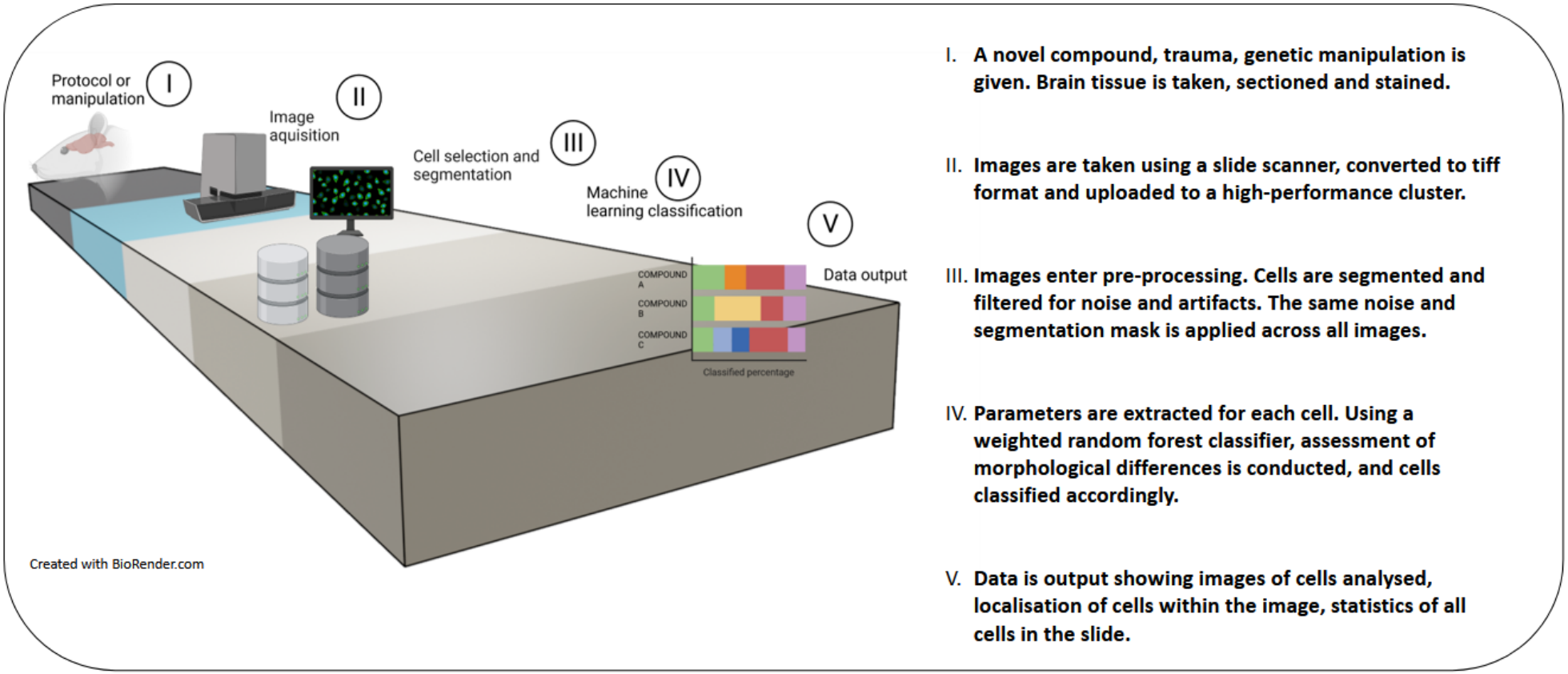
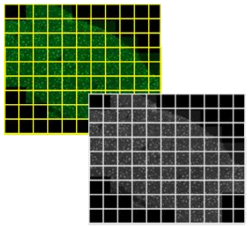


Figure 18: Schematic of microglia analysis pipeline from brain isolation to data analysis. Each section is described in further detail in Chapter 6 subsection 6.2.

Imaging

Z stack imaging
Zeiss software

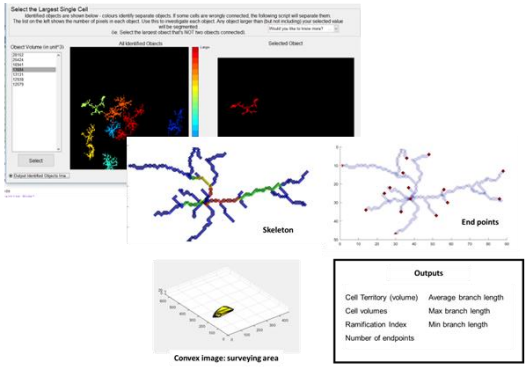
No major modifications



Pre-Processing

Image dividing
8-bit conversion
Python

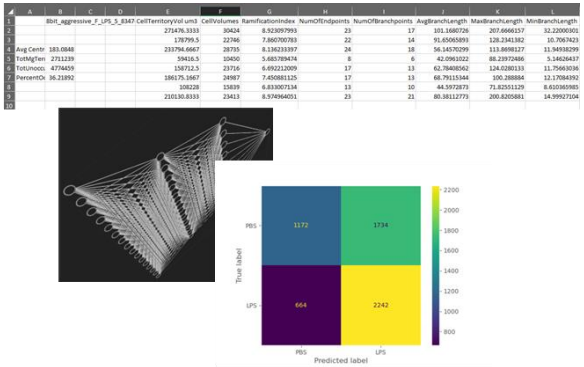
Converted from slow Image J process written by myself and Reddaway to python.



Segmentation and skeletonization

Skeletonization script 3DMorph
MATLAB
Dataset combination
R script provided by Clifton (reference in legend)

No major modifications



Machine learning pipeline

Preprocessing and model training
Python with sci-kit learn
Inferential statistics and visualisation
R

Major modifications from pipeline developed by Reddaway.

Figure 19: Summary of analysis pipeline with languages and software in blue. N.Clifton reference (https://github.com/NiCl2/Morph3D). Modifications to the pipeline, which are shown in the bottom row of the figure, are described in further detail in Chapter 6.

2.8.1 Image acquisition

A ZEISS Axio Scan.Z1 Slidescanner (Carl Zeiss AG, Baden- Württemberg, Germany) and ZEISS ZEN imaging software (v3.1 blue edition) was used to acquire 20X images containing a region or subregion of interest which was manually drawn in (the barrel cortex, IL and PL cortex). These ROI's are shown in figures presented in Chapter 6 subsection 6.2.5.1 (Figure 49). Between 6 to 13 ROI's were taken per animal. Images every 1 µm along the z-plane were collected. 4 dimensional (width, length, depth, channel), 32bit images were acquired. The scanner acquires focus through DAPI imaging but only the channel of interest (wavelength 488, for IBA1) was selected for further analysis and exported as an Open Microscopy Environment - Tagged Image File Format (OME-TIFF) file. Images were saved for further processing.

2.8.2 Pre-Processing

A python script was constructed which uses the TIFFFILE package (tifffile 2022.5.4, Christoph Gohlke, Laboratory for Fluorescence Dynamics, University of California, Irvine) to open and modulate Tiff images quickly. The script runs similar to that described by Reddaway[291] and is explained in more detail in Chapter 6 subsection 6.2.5.1. Briefly, a size of square is selected (here we chose 500pixel) and the programme calculates how many divisions can be made along the X and Y plane using those dimensions. The Z plane dimension is conserved. Images are converted into 8bit and are saved for further analysis with the pipeline.

2.8.3 Microglia morphological assessment

3DMorph image analysis software was obtained from GitHub (https://github.com/ElisaYork/3DMorph) running in MATLAB R2019b (MathWorks, California, USA) and was used as the basis for all morphometric analyses. An unedited

version of the 3D-Morph release was used to generate a parameters file for use in automated analyses. The parameters file contains an optimal threshold value to input into a global Otsu thresholding algorithm as well as, limits on the maximum and minimum size of microglia, complexity of outputs (full branch structure or not, image output or not) and instructions to not analyse cells that touch the image border. Large scale and high throughput generation of microglial morphometrics required the 3DMorph script to be run on Cardiff University's high-performance computing cluster HAWK. The 12,736 cores of HAWK allow the morphological analysis of ~50,000 microglia to be completed in four hours compared to over a month on a standard four-core desktop PC. With this development image segmentation, morphological analysis and data output was adapted to run in a UNIX environment. This required the adaptation of the segmentation script, removal of all graphical user interfaces, inclusion of parallel processing at all stages of the pipeline, the introduction of a try loop accounts for errors occurring in images which contain no microglia and combining the (comma-separated value) CSV data files output from each image with a final CSV containing all of the parameters needed for further analysis (https://github.com/NiCl2/Morph3D).

2.8.4 Morphological output parameters

The output from NueMorph contains eight morphological parameters from each microglia: cell volume, cell territory, number of branch points, number of endpoints, average process length, minimum process length, maximum process length and ramification index. Cell volume is the total volume of the cell including the soma and all processes. Cell territory is the volume in space the cell is in contact with and thus able to survey. Number of branch points is the number point where a new process emerges from either the soma or another process of the cell. Number of endpoints is

the count of terminal points on a cell's processes. Average process length is the mean length of primary, secondary and tertiary processes. Minimum process length is the shortest length of uninterrupted process emanating from the cell. Maximum process length is longest length of uninterrupted process emanating from the cell. Ramification index is the ratio between cell territory and cell volume and is regularly used within the literature as a metric of microglial activation, with a higher ramification index reflecting a lower degree of activation.

Cells that contained 0's on the continuous variables (cell territory volume, cell volume, average branch length, max branch length, min branch length) were eliminated as this is highly indicative of an artefact or noise: cells cannot have an area of 0 μm^2 and we did not expect amoeboid cells whereby branch length equalled zero.

Classes were given the name PBS-like and non-PBS-like to refer to the change that can occur in morphology following stress which can lead to hyper ramification [301] which follows the opposite pattern of the canonical ramified -> activated -> ameboid pattern of activation that results in shortening processes and larger cell body, instead resulting in increased process length and complexity[252].

2.8.5 Machine learning classification

A detailed overview of the machine learning models, optimisation techniques and SMOTE correction for imbalanced sampling, is given in Chapter 1 subsection 1.19. SMOTE correction was conducted to train the model and each of the models described in more detail in subsection 1.19.10 were tested. Models were compared for the highest accuracy value. The best model (optimised random forest) was used for further analysis of morphology following ELS and CFC exposure. This process is described in more detail in Chapter 6.

2.9 Statistics and software

Chapter 3 – Freezing behaviour.

Exploratory data analysis, data visualisation and inferential statistical analysis was conducted using R running in R-studio 1.4.1. A general linear mixed model was applied to assess differences in the groups when multiple time points were assessed in that protocol. These consisted of contextual fear conditioning with the time points: pre shock and post-shock, and the ten-minute extinction protocol with five two-minute recordings. To analyse differences between timepoints in the individual sex and stress groups independently (Male ELS, Male NS, Female ELS, Female NS) repeated measures ANOVA was applied. For protocols without a repeated time course (recall, extinction recall, reminder test and reminder recall) an ANOVA model was fit. Significant results were further investigated with Tukey honest significant difference (HSD). For the extinction recall groups the important comparisons were between the 2- and 10-minute groups and unpaired t-tests were conducted to assess the differences. A significance value for rejection of the null hypothesis was set as $p < 0.05$.

Chapter 4 – USV analysis

R running in R-studio 1.4.1 was used to automatically calculate USV counts. Exploratory data analysis, data visualisation and inferential statistical analysis was conducted using R running in R-studio 1.4.1. Two types of hurdle models were used due to the large number of zero responders. The details are described further in Chapter 4 subsection 4.2.3.

Chapter 5 – Molecular analysis

Chapter 2: General methods

Exploratory data analysis, data visualisation and inferential statistical analysis was conducted using R running in R-studio 1.4.1. Freezing analysis is conducted as above for Chapter 3 where protocols are identical. ANOVA models were applied for analysis of differences between cytokine concentrations post hoc testing was conducted with Tukey HSD multiple comparison correction.

Chapter 6 - Microglia analysis

Python v 3 was primarily used for analysis of microglia morphology in Chapter 6. Analysis of freezing during CFC is identical to that described above for Chapter 3. A detailed description of the models used for microglia analysis is found in Chapter 1 subsection 1.19

3 Chapter 3: Behavioural analysis of ELS on CFC and extinction memory.

3.1 Introduction

Early life stress (ELS) has been shown to increase the risk of developing psychiatric disorders [302] often characterized in a form of diathetic stress model [10, 188]. These include adult onset depression [303], schizophrenia [304, 305], anxiety [306] and post-traumatic stress disorder (PTSD) [307]. PTSD is characterised by four primary symptoms (intrusive memories/ re-experiencing, avoidance and emotional numbing, negative changes in thinking and mood, and changes in physical and emotional reactions including hyper-arousal) as well as a dysregulation of associative fear learning and fear memory extinction[308]. Dysregulated fear memory is a characteristic of many psychiatric disorders such as anxiety [309] , schizophrenia [310] and PTSD [56, 311] and is highly pertinent for research as it can be modelled in animals [312]. Work has shown that individuals with PTSD show impaired extinction and that it is likely that impaired extinction learning plays a role in the development and course of PTSD[82, 313]. This is discussed in more detail in Chapter 1 subsection 1.2.

Importantly, stress, including that experienced early in life, has an effect on the ability to acquire, recall and extinguish fear memories in both humans [314] and non-human animal species [315]. The lasting effects of stress are widespread and include impacting upon learning and memory, neuronal plasticity and on the development of areas involved in these areas, see Figure 20. The specific nature of ELS, being either

chronic or acute, appears to have a major effect on the direction of impact that the stress has on both contextual fear memory conditioning and extinction. For example Conrad et al [98] have shown chronic stress enhances the acquisition of CFM, In contrast, other work has shown that maternal separation has no effect on acquisition [205]. Nonetheless, one meta review [316] suggests that exposure to handling, limited nest bedding and maternal stress (all of which are widely used protocols for the experimental study of ELS) all have a similar reduction effect on conditioned contextual freezing.

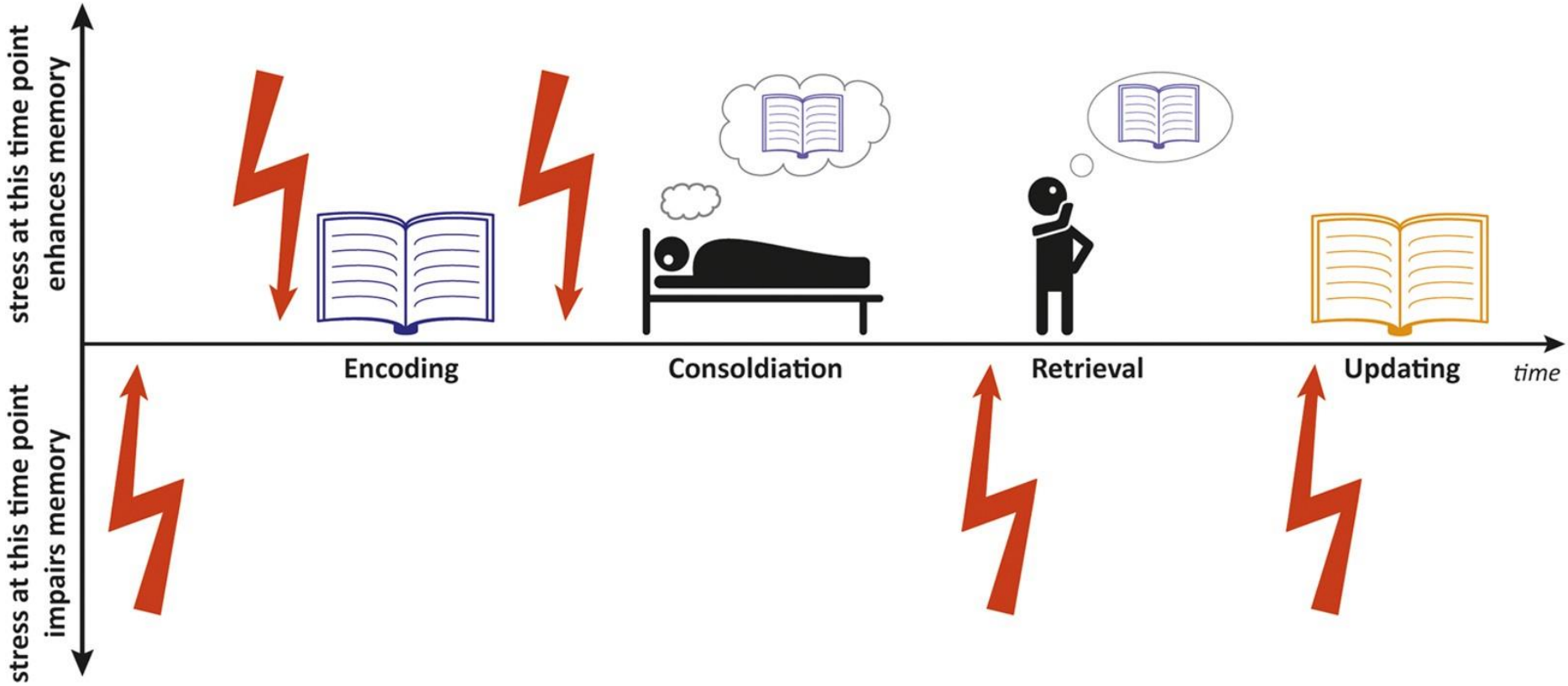


Figure 20: The effects of stress at different timepoints on learning and memory can have impairing or enhancing effects taken from work by Vogel and Schwabe [186]. Less work examines the effects of exposure to early life stressors and how these impact learning later in life.

The number of stressors early in life can influence the risk for developing disorders later in life [218]. Priming of the HPA axis can be induced by ELS [306], this is discussed further in Chapter 1 subsection 1.7. This could suggest why single stress models such as the limited nest bedding and maternal separation show different results to that which uses a variable stressor, defined as different stress protocols applied over different timepoints (usually on different days), such as work by Brydges et al [96]. A single stressor may not be enough to elicit significant demonstrable adverse issues later in life. Nonetheless, the priming effect of ELS on the HPA axis and the effect that this can have in later life has implications for stratification of PTSD treatment, namely via exposure to and, indeed, differences in levels of, early life stress. Two prominent hypotheses that can explain some of the behavioural effects of ELS and adversity later in adult life are the allostatic hypothesis where stress compounds, and the effects of a second stressor are amplified following the first stressor and the match mismatch hypothesis, where stress confers either a resilience to a matching adult situation or sensitivity to the adult situation when the scenarios don’t match.

Both the match-mismatch hypothesis and to some extent the allostatic hypothesis are dependent on being able to determine how intense a stressor is (these theories are discussed in more detail in Chapter 1 subsection 1.10). For example, the match-mismatch hypothesis suggests that if an individual is exposed to a very stressful environment during upbringing, they will be able to better adapt to a very stressful environment in adulthood. In the same vein if the individual is within a mildly stressful environment, they are suited only to mildly stressful environments later in life[179]. The hypothesis is reductive here in that stressful environments can take many forms (one only needs to look at the various forms of stressor given to be considered ELS[316]) as well as many levels of severity. There is also no consensus as to what constitutes

a mild or severe (or moderate) stressor, or indeed if the scale is fluid, without categories. In the animal model literature for example, work by Bali et al [317] suggests that 0.5mA is a moderate stressor for rodents while other work suggests 0.5mA is a mild or weak stressor[318, 319]. This is present across much of the work using CFC and is further compounded by differences in number of shocks, length of shock, amount of time between shocks and between shock sessions which will all influence severity.

The neuroanatomy underlying CFC and any subsequent recall, extinction and relapse is complex with many circuitry systems working in tandem at each stage. Relevant neuroanatomy is discussed in some detail in Chapter 1 subsection 1.5. However, some points are salient for explaining the choice of both ELS paradigm and CFC paradigm in the experiments in this thesis.

Firstly, it is well documented through lesion studies [97, 320] that CFC is highly dorsal, and not ventral [321], hippocampal dependant. However, note McNish et al [119] suggested through lessoning of the dorsal hippocampus that it is the freezing response to CFC that is affected and not the recall of the CFM itself as fear potentiated startle was preserved. For extinction memory the involvement of the hippocampus is more complex still. The hippocampus both drives pre-frontal cortex (PFC) inhibition of the amygdala during exposure to the stimulus when in the presence of an extinction context [322], as well as driving amygdala activation which occurs during renewal [148]. Brydges [323] has shown that ELS influences the expression of genes known to be implicated in risk for neuropsychiatric disease (DISC1, GSK3ß and NRG1) within the hippocampus. Other work has shown that ELS changes the proliferation rate of cells in dentate gyrus sub region of the hippocampus [316].Overall, previous work strongly suggests the hippocampus is a highly important brain region for CFC and

subsequent extinction as well as being an area which is highly susceptible to changes following ELS.

Table 2: Sex differences in behavioural and neurobiological responses to ELS and fear conditioning

Factor	Males	Females	Modality	Duration
Freezing behaviour in fear conditioning	Males show more freezing behaviour during contextual and cued fear conditioning recall [324] [325] [113] [165].	Females show less freezing, sometimes exhibit darting behaviour in response to fear stimuli [324] [325] [326] [113] [165]. But note [327, 328] show conflicting results.	Contextual & cued fear conditioning	Short-term & long-term
Pre-exposure effect	Short pre-exposures lead to more freezing behaviour in males [329]].	Longer pre-exposures lead to more freezing behaviour in females[329].	Fear conditioning	Varies
Locomotion	Males show less locomotion, [96] [325]	Females show more locomotion, conflicting with freezing behaviour [96] [325]	Locomotion and freezing	N/A
Fear extinction	Males generally show higher fear retention following fear conditioning[326].	Females show reduced retention of fear extinction when measured by fear-potentiated startle but not by freezing behaviour[326].	Fear extinction	Long-term
Response to ELS in CFM	ELS decreases freezing response and impairs contextual fear memory (CFM) in males [96].	Females are less affected by ELS in terms of freezing response during CFM recall [96].	Contextual fear memory (CFM)	Long-term
Response to ELS in spatial navigation	ELS does not improve spatial navigation in males [164].	ELS can improve spatial navigation abilities in females[164].	Spatial tasks	Long-term
Contextual fear conditioning	Males are impaired by ELS in CFC tasks, showing decreased freezing following ELS [164].	Females do not show impairment in CFC tasks under ELS, sometimes showing improvements[164].	Contextual fear conditioning (CFC)	Long-term
Pattern separation & memory deficits	Males show deficits in hippocampus-dependent tasks such as pattern separation and trace fear conditioning after ELS exposure [96] [330].	Females show fewer deficits in pattern separation and trace fear conditioning, even under ELS exposure [96] [330].	Hippocampus-dependent memory tasks	Long-term
Anxiety & social behaviour	ELS increases anxiety, impairs social behaviour, and alters choice behaviour in males [216] [208].	Females may experience less anxiety and fewer social behaviour impairments following ELS, but exact outcomes depend on specific paradigms used [216] [208].	Social behaviour, anxiety	N/A
Fear acquisition & retention	Males generally acquire fear memory more strongly, with increased freezing behaviour in fear acquisition and recall compared to females [324].	Females acquire fear memory differently, sometimes showing less freezing or using alternate behaviours like darting in response to fear stimuli [326].	Fear memory acquisition & retention	Short-term & long-term

Work assessing the effects of ELS (using the limited nesting and bedding paradigm) on synaptic plasticity previously found that LTP and pre-pulse inhibition was reduced in the CA1 in those animals exposed to ELS compared to control animals [187]. Moreover, this was concurrent with behavioural deficits in the form of impaired cued fear conditioning. The exact neurochemical process which causes the ELS to have an impact on plasticity and brain development is less well known and more research is needed to elucidate the way stress can modulate memory formation in the way it clearly does. Certain neuroanatomical areas of the brain such as the mPFC and the hippocampus continue to develop post-natally and only mature later in life [331]. Importantly, these areas play key roles in various aspects of contextual fear memory recall, retrieval and extinction[332]. Due to the longer period of maturation this could leave these areas open, potentially, to the influence of ELS.

3.1.1 Experimental aims

The effects of varying shock intensities on CFM and extinction learning were investigated for two main reasons: First, higher shock intensities are known to be associated with resistance to extinction, a characteristic seen in anxiety disorders like PTSD, making it crucial to compare higher and lower shock intensities. Additionally, while the impact of shock intensity on extinction recall, a therapeutically important time point, is underexplored, this study addresses that gap. Second, females typically exhibit low levels of freezing with a standard 0.5mA foot shock, which may result in a floor effect as noted by Brydges et al[164]. Research has shown that an increase in shock does not interact with either ELS [330] or sex [333] to increase sensitivity and acquisition of contextual fear memory. Furthermore, dos Santos Corrêa et al [334] has shown that increased foot shock increases freezing post training. However, it should be noted that this study was only done in males. However, this is not a universal

finding, for example work by Laine et al [335] have shown that females can show levels of freezing following recall of CFM. In the initial experiments of this chapter, low freezing levels were observed in female rats with a 0.5mA shock, prompting further experiments with a higher 0.7mA shock intensity. The hypothesis is that this increase will elevate freezing levels and potentially address the floor effect in females.

This Chapter is focused around three primary aims:

Aim1: Replicate the results of Brydges et al [164] by investigating how early life stress (ELS) affects fear memory (CFM) recall, with a focus on sex differences. It was hypothesised that ELS increases sensitivity to fear memory acquisition and that females will show less freezing than males.

Aim 2: Explore the impact of ELS on extinction memory training and recall, hypothesizing that ELS negatively affects these processes.

Aim 3: Examine the effects of varying shock intensities on CFM and extinction learning. This includes assessing whether higher shock intensities, which are linked to resistance to extinction (a key feature in anxiety disorders like PTSD), influence freezing levels differently in males and females, particularly addressing potential floor effects in female freezing behaviour.

3.2 Methods

3.2.1 Animals

Animals strain, licencing and housing characteristics are described at length in Chapter 2 subsection 2.1. Here a total of 294 animals were used (150 males and 144 females). Animals were kept on reverse day/night cycle with lights switching off at 8:00 and switching on at 20:00. When litters reached weaning age at PND21 animals were housed in same sex groups of two or three.

3.2.2 Behavioural protocol

An overview of the experimental schematic is shown in Figure 21. At PND25 animals that were assigned to ELS were exposed to the ELS protocol. Briefly, the early life stress protocol consists of variable short-term stressors: 10 min forced swim, 3 X 30 min restraint and 3 X 30 min elevated platform occurred on PND 25, PND 26 and PND 27, respectively and took place in a separate room from the holding room. Animals were given ear notches which in itself is a stressor[336], but this provides a longer lasting ID than tail marking and a single stressor as opposed to repeatedly stressing the animals to label their tails. Animals were then maintained in the holding room until fear conditioning at PND 60 and PND 90 (Figure 21).

After at least 30 days, all animals underwent CFC which was conducted during the light period of the reverse light cycle. CFC was performed as described in detail in Chapter 2 subsection 2.3. A schematic of the protocol is shown in Figure 21. Animals were transported in the boxes and manner described in Chapter 2. Rats experienced one of two different shock intensities 0.5mA or 0.7mA during the CFC phase and are then referred to as low intensity shock and high intensity shock groups respectively. 48 hours later animals were returned to the context to elicit the recall of the CFM.

Animals that were assigned to extinction training were placed into the context for 10 minutes (extinction trained) while animals assigned to extinction control were placed into the context for 2 minutes (extinction control). 48 hours following this, all rats were exposed to the context for 2 minutes to test for extinction recall. In some experiments animals were exposed to the conditioning chamber for two minutes after a period of 32-64 days, this two minutes co terminated with a two second 0.25mA scrambled foot shock as a reminder stimulus. 48 hours later all animals were exposed to the conditioning chamber for two minutes which served as a recall test for the reminder. Freezing behaviour was recorded as the index of conditioned fear response and was defined as the cessation of movement excluding respiration within a one second period [105]. Freezing was assessed, in all trials above, and sampled every ten seconds with the scorer blinded to group consistent with previous studies [285, 291, 297]. It was planned that all animals would follow the entire CFC, extinction and reminder recall protocol, however, due to the COVID-19 pandemic some animals had to be culled earlier, just after extinction recall.

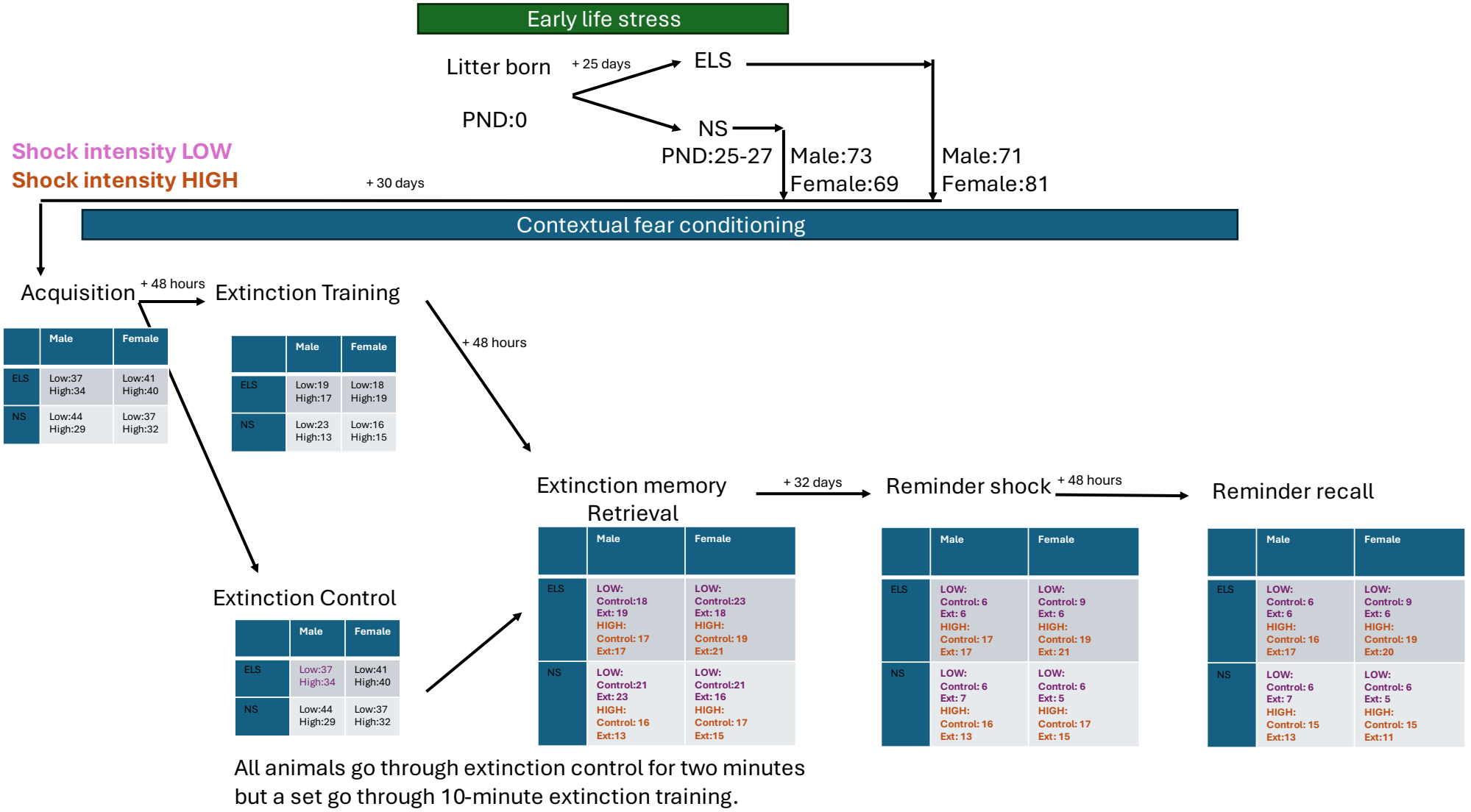


Figure 21: Schematic for experimental timeline. Black arrows show number of days between experimental protocols where animals were kept in their home cages. Freezing behaviour was measured during contextual fear conditioning (CFC) and extinction and reminder training and reminder recall. All animals experienced CFC but some animals had to be culled before experiencing reminder and reminder recall due to the COVID-19 pandemic.

3.2.3 Data analysis and acquisition:

Freezing during contextual fear conditioning, CFM recall, extinction training, extinction recall, reminder test and reminder recall, was manually analysed from video recordings by an observer blind to group. Definition of freezing and method of recording is described in detail in Chapter 2. For freezing percentage during CFC, the two minutes before the shock and the one minute after shock, results are binned into a two-minute pre-shock (US) and one-minute post-shock bin. For extinction training the ten minutes of recording is subdivided into 5 two-minute bins where each is a successive two-minute bin. For extinction recall the first two minutes of the extinction trial is pooled with the 2-minute extinction control as there are no differences between the groups at this point. All other periods of recording consisted of a single 2-minute recording. Between subjects ANOVA was used to assess significant differences between the groups at CFM recall, reminder test, and reminder recall. For statistical analysis of protocols with repeated measures a mixed linear model was fitted using the lme4 library. Repeated measures ANOVA was conducted to assess differences in the groups individually (Male ELS, Male NS, Female ELS, Female NS) across the successive time points in CFC and in extinction training. Significant interactions were further analysed with Tukey HSD. For the extinction training recall groups, comparisons were made between the two- and ten-minute groups, these were independent groups and therefore unpaired Students t-tests were conducted to assess the differences. Where repeated measures ANOVA was uses sphericity was tested with Mauchly's test and where sphericity was not assumed Greenhouse Geisser (GG) corrections were applied and where sphericity was non-significant the GG corrected values are presented. All statistical analysis was conducted with using R in R-studio 1.4.1(Tiger Daylily).

3.3 Results

Throughout the results presented below, 0.5mA will be described as a low intensity shock. The 0.7mA level will be described as a high intensity shock. These different levels of foot shock were not run concurrently; therefore, analysis of shock intensity as an independent variable would not be appropriate and was not conducted here. Animals were randomly assigned to each condition to minimise selection bias and any litter effects. Reported t values for the mixed linear models (calculated through Satterthwaite's method) are the tests for if each parameter influences freezing behaviour, degrees of freedom are complex due to the inclusion of subject (individual animal) as a random effect. For results without repeated measures the purple circle shows the mean, individual samples are shown with black dots. For results with repeated measures the results are shown here without individual points for clarity.

3.3.1 Contextual fear conditioning

Low intensity shock: There was an effect of two second 0.5mA foot shock on freezing behaviour with animals freezing more during post shock than pre shock (Estimate = 54.453, t (155) = 13.121, $p < 0.001$). Analysis across both pre and post shock, as a repeated measures model showed that there were no interactions between Stress and Sex present (Estimate = 4.218, t (309) = 0.702, $p > 0.05$, mixed linear model). There was an effect of Sex (Estimate = 8.855, t (309) = 2.068, $p < 0.05$) on freezing with males freezing more than females. There was an effect of Stress (Estimate = 0.514, t (309) = 2.456, $p < 0.05$, mixed linear model, Figure 22a) with animals exposed to ELS freezing more than non-stressed animals.

High intensity shock: There was a significant effect of two second 0.7mA foot shock on freezing behaviour with animals freezing more during post shock than pre shock

(Estimate = 72.1400, t (129.7) = 20.089, $p < 0.001$, mixed linear model). There was no interaction between Stress and Sex (Estimate = 0.7723, t (258) = 0.136, $p > 0.05$, mixed linear model) and no differences between Sex (Estimate = 5.0842, t (258) = 1.331$p > 0.05$, mixed linear model) or Stress groups (Estimate = 6.8643, t (258) = 1.768, $p > 0.05$, mixed linear model, Figure 22b).

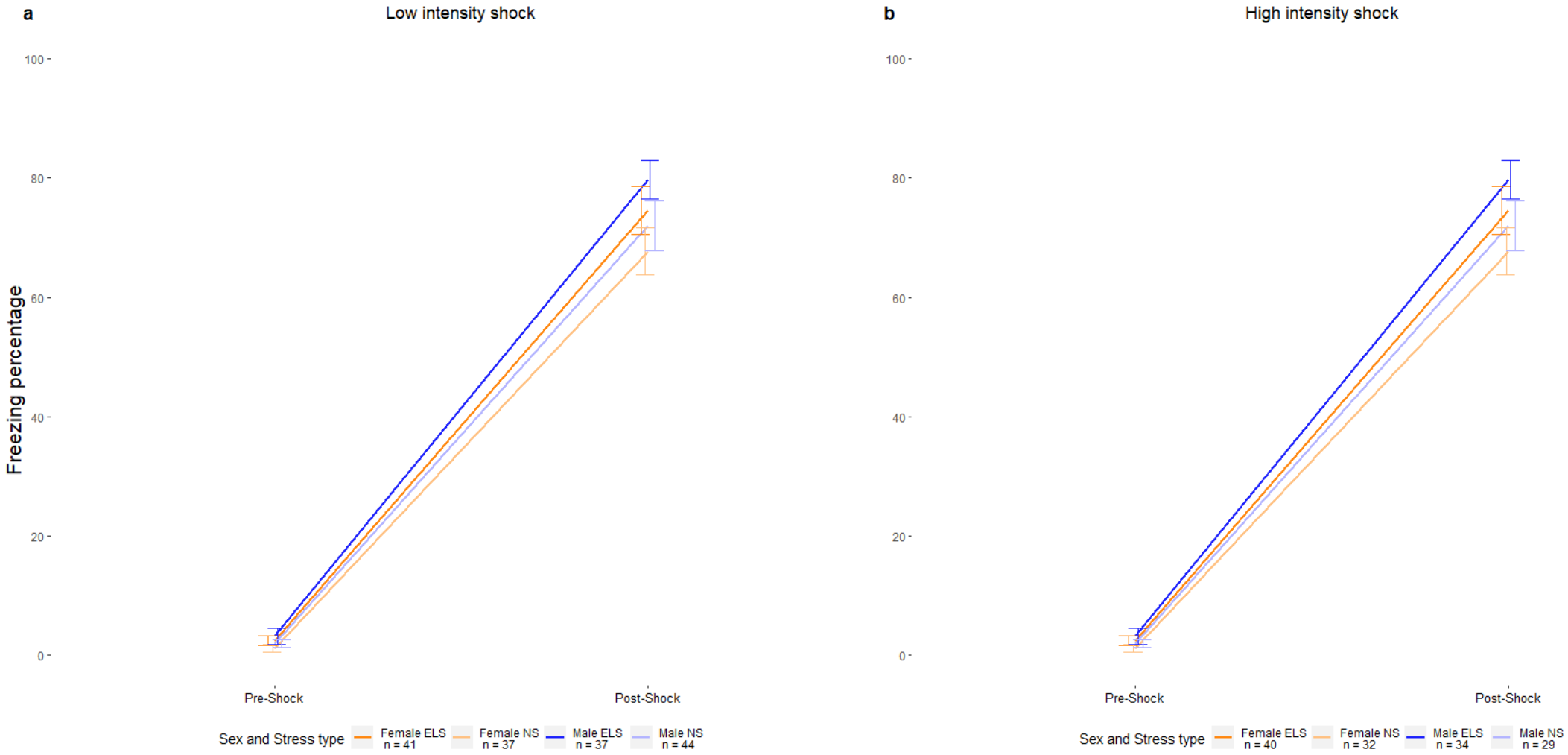


Figure 22 Males and females both in the ELS group and NS group showed strong freezing behaviour following foot shock. All rats were exposed to either: (a) low intensity shock (0.5mA) or (b) high intensity shock(0.7mA). All groups showed a higher percentage of freezing behaviour after foot shock in both the low intensity shock group (a) and the high intensity shock group (b). Values are shown as means, error bars represent mean ± SEM. ELS = Early life stress. NS = non-stressed.

3.3.2 Recall test following CFC.

Low intensity shock: There was no interaction effect of Sex and Stress ($F(1,155) = 0.015$, $p > 0.05$, ANOVA). There was a main effect of Sex, with male animals freezing more following CFM consolidation than females ($F(1,155) = 10.665$, $p < 0.01$, ANOVA). There was a main effect of Stress with animals that had experienced ELS showing greater levels of freezing behaviour than animals which had not experienced ELS, ($F(1,155) = 15.869$, $p < 0.01$, ANOVA, Figure 23a).

High intensity shock: There was no interaction between the effect of Sex and Stress on freezing ($F(1,129) = 0.455$, $p > 0.05$, ANOVA). There was a main effect of sex with male animals show greater levels of freezing behaviour than female animals following CFM consolidation ($F(1,129) = 4.691$, $p < 0.05$ ANOVA).There was a trend towards a main effect of stress with animals which experienced ELS showing a trend to higher levels of freezing behaviour than animals which had not experienced ELS ($F(1,129) = 3.812$, $p=0.0530$, ANOVA, Figure 23b).

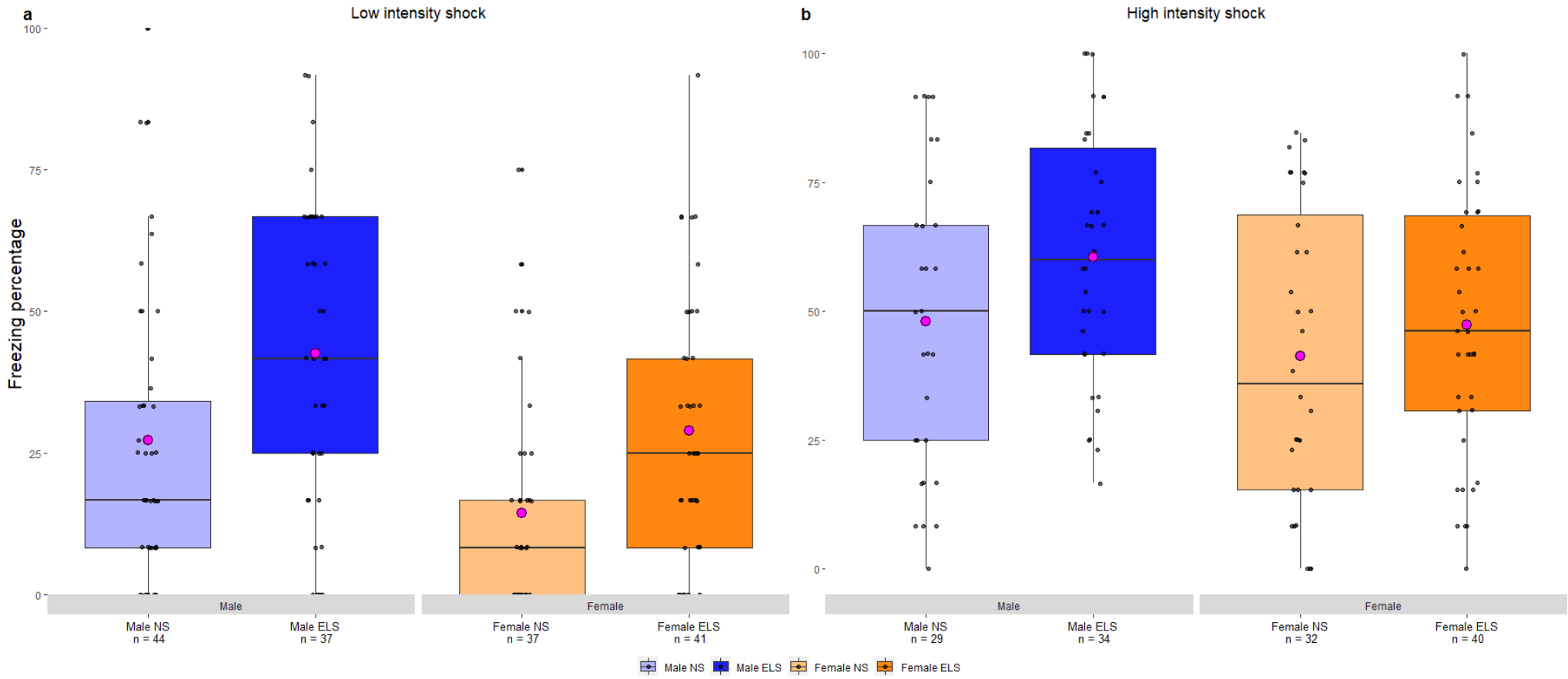


Figure 23: During 2-minute recall animals exposed to stress freeze more than non-stressed animals and males freeze more than females. Animals exposed to low shock (a) show a main effect of sex and stress on freezing behaviour. Animals exposed to a high shock (b) also show a main effect for sex while stress trends towards a main effect. Males are shown in shades of blue and females are orange (Horizontal lines show median with IQR as the hinges, the mean is shown as a solid magenta circle, top whisker shows up to 1.5 X IQR up to the maximum level bottom whisker shows down to 1.5 X IQR). Black dots depict individual data-points. Group numbers shown as n.

3.3.3 Extinction training

Low intensity shock: There was no interaction between Sex and Stress (Estimate = 2.60, t (222) = p > 0.05, mixed linear model). There was a main effect of extinction training with animals freezing less between the last two min of extinction training and the first two min of extinction training with the last two minutes being lower than the first two (Estimate = -21.6330, t (288) = -4.72, p < 0.01, mixed linear model). Individual analysis of extinction (RM-ANOVA's) within each group is shown in Table 3.There was a significant effect of extinction training for both males exposed to ELS and males not exposed to ELS. For females that were exposed to ELS there was an effect of extinction while for non-stressed animals there was no effect. There was no main effect of Sex (Estimate = 9.38, t (222) = 1.616, p > 0.05, mixed linear model). There was an overall main effect of Stress with animals which had experienced ELS showing higher freezing behaviour than animals which had not experienced ELS (Estimate = 17.07, t (222) = 2.816, p <0.05, mixed linear model, Figure 24a).

High intensity shock: There was no interaction between Sex and Stress (Estimate = -12.22, t (146) = 0.936, p >0.05, mixed linear model). There was an effect of extinction training with animals freezing less at time point five against time point one (Estimate = -21.47, t (240) = -3.66, p < 0.01, mixed linear model). Individual analysis of extinction (RM-ANOVA's) within each group is shown in Table 3. There was a significant effect of extinction training for both males exposed to ELS while for males not exposed to ELS there was no effect. For females, there was no effect of extinction training on either animals exposed to ELS or non-stressed. There was a main effect of Sex with males overall freezing more than females (Estimate = 17.86, t (146) = 2.07, p< 0.05, mixed linear model). There was no main effect of Stress (Estimate = 5.51, t (146) = 0.617, p > 0.05, mixed linear model, Figure 24b).

Table 3: Repeated measures analysis of freezing behaviour during the 10 min extinction training session. Green shows significant effect of extinction white shows non-significance.

Low intensity shock	High intensity shock
Group	
NS Male	F (4, 110) = 6.1043, p < 0.01	F (4,60) = 1.495, p >0.05
ELS Male	F (4,90) = 6.6336, p < 0.01	F (4,80) = 9.537, p < 0.01
NS Female	F (4,75) = 1.3521, p > 0.05	F (7,70) = 1.4417, p > 0.05
ELS Female	F (4,85) = 5.5755, p < 0.01	F (4,90) = 2.1402, p = 0.082

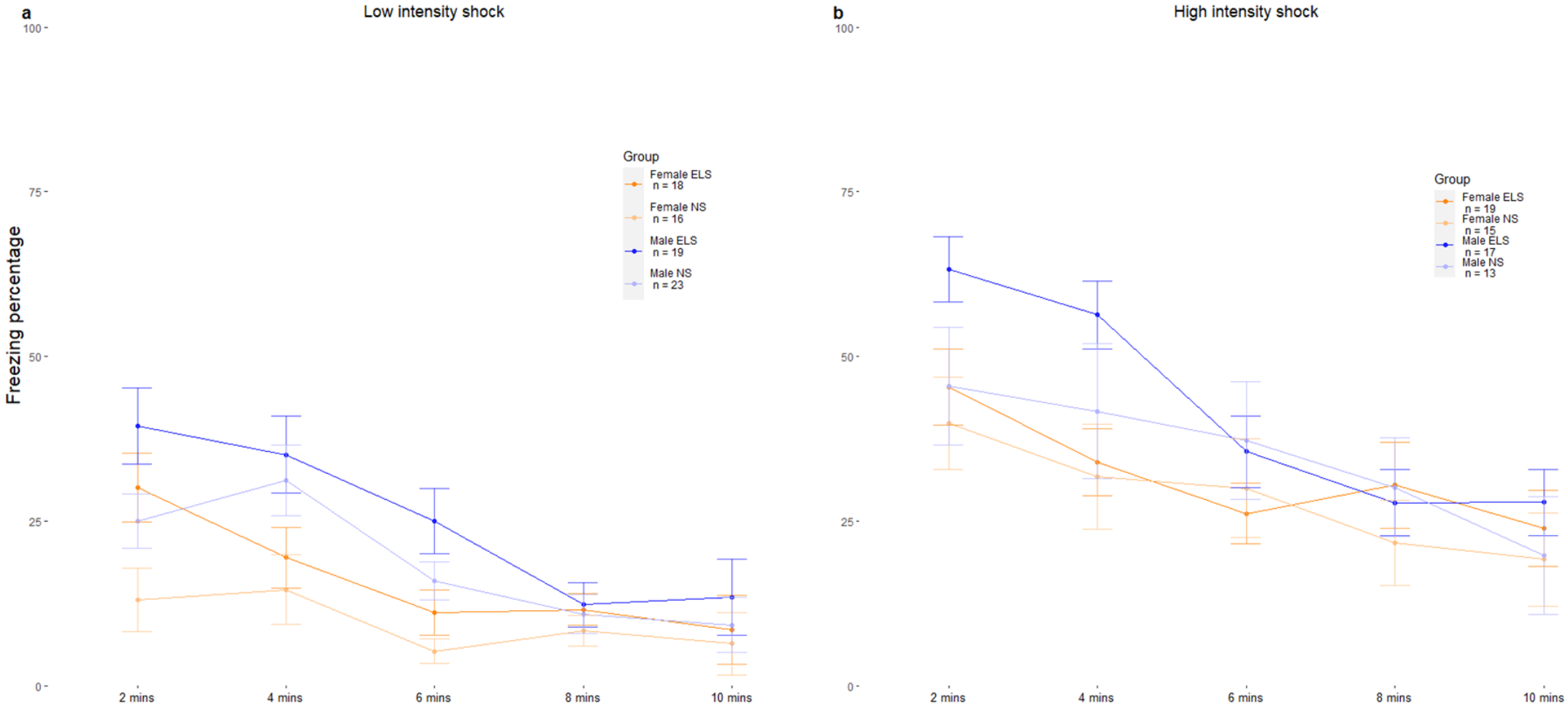


Figure 24 Animals exposed to either high or low shock freeze less during the last two minutes and the first two minutes of extinction training indicative of within session extinction. Animals exposed to the low shock show a main sex and main stress effect (a). Animals exposed to the high shock intensity (b) showed a main effect of sex. Males are shown in shades of blue and females are orange (values shown as means, error bars represent mean ± SEM). ELS = Early life stress. NS = Non-stressed

3.3.4 Extinction recall

Animals which were exposed to 2-minute recall following CFM consolidation were compared against animals which underwent 10-minute extinction. To compare extinction within each sex and stress group (male ELS, male NS, female ELS, female NS) independent t-tests were conducted. As each group was separate and independent there was no need to correct for multiple comparisons. All tests for extinction effects (between extinction control and extinction training) are unpaired t-tests unless specified otherwise.

Low intensity shock: There were no interactions present (all $p > 0.05$, Figure 25a, Table 4 shows all interaction effects). There was a main effect extinction training ($F(1, 149) = 9.357$, $p < 0.05$, ANOVA,) with those which experienced the extinction control showing more freezing than those which experienced extinction training. To further probe the effects of condition, independent t-tests were conducted (Table 2). In males those which experienced ELS show a lower level of freezing in the extinction trained, compared to the extinction control group, while those which have not been exposed to stress don't show differences between the extinction trained and extinction control group. In females neither stressed or non-stressed animals showed a difference in freezing between the extinction control and extinction groups. There was a main effect of Sex ($F(1,149) = 12.314$, $p < 0.05$, ANOVA) with males showing a higher amount of freezing than females. For Stress there was a trend towards a main effect with stressed animals showing more freezing than non-stressed animals ($F(1, 149) = 3.59$, $p = 0.06$, ANOVA, Figure 25a).

High intensity shock: There were no interactions present (all $p < 0.05$, Figure 25b, Table 4 shows all interaction effects). For extinction condition There was a main effect

with those which experienced the extinction control show more freezing than those which experienced extinction training ($F(1, 127) = 5.349$, $p < 0.05$). To further probe the effects of condition, independent t-tests were conducted as described above, where the two minute and ten-minute group are analysed across each group independently, results are shown in Table 5. Males that experienced early life stress showed extinction of fear memory. Males that did not experience stress did not show extinction, but results suggest a trend towards extinction. For females which experienced no stress there was a trend towards extinction while those that experienced ELS showed no extinction. There was a main effect of Sex, with males showing a higher amount of freezing than females ($F(1,127) = 6.505$, $p < 0.05$, ANOVA). There were no effects of Stress ($F(1,127) = 3.210$, $p > 0.05$, Figure 25b).

Table 4: Complete interaction terms for extinction recall. None of the interactions were significant.

Interaction	Low shock	High shock
Sex * Stress	$F(1,151) = 0.01$, $p > 0.05$	$F(1,125) = 0.44$, $p > 0.05$
Sex * Condition	$F(1,151) = 0.50$, $p > 0.05$	$F(1,125) = 0.11$, $p > 0.05$
Stress * Condition	$F(1,151) = 0.03$, $p > 0.05$	$F(1,125) = 0.09$, $p > 0.05$
Sex * Stress * Condition	$F(1,151) = 0.18$, $p > 0.05$	$F(1,125) = 0.24$, $p > 0.05$

Table 5: Inferential analysis via unpaired students t-test of the difference in conditioned freezing between extinction trained and extinction control in each group at extinction recall. Green shows significant effect of extinction, white shows non-significance and orange shows a trend towards significance.

Group	Low shock	High shock
Male NS	t (30.78) = 1.7578, p = 0.09	t (19.565) = 0.753, p = 0.460
Male ELS	t (35) = 2.4164, p < 0.05	t (30.7) = 2.284, p < 0.05
Female NS	t (20.447) = 1.705, p = 0.1033	t (29.77) = 1.8294, p = 0.077
Female ELS	t (31.92) = -0.02, p = 0.9877	t (36.5) = -0.216, p = 0.83

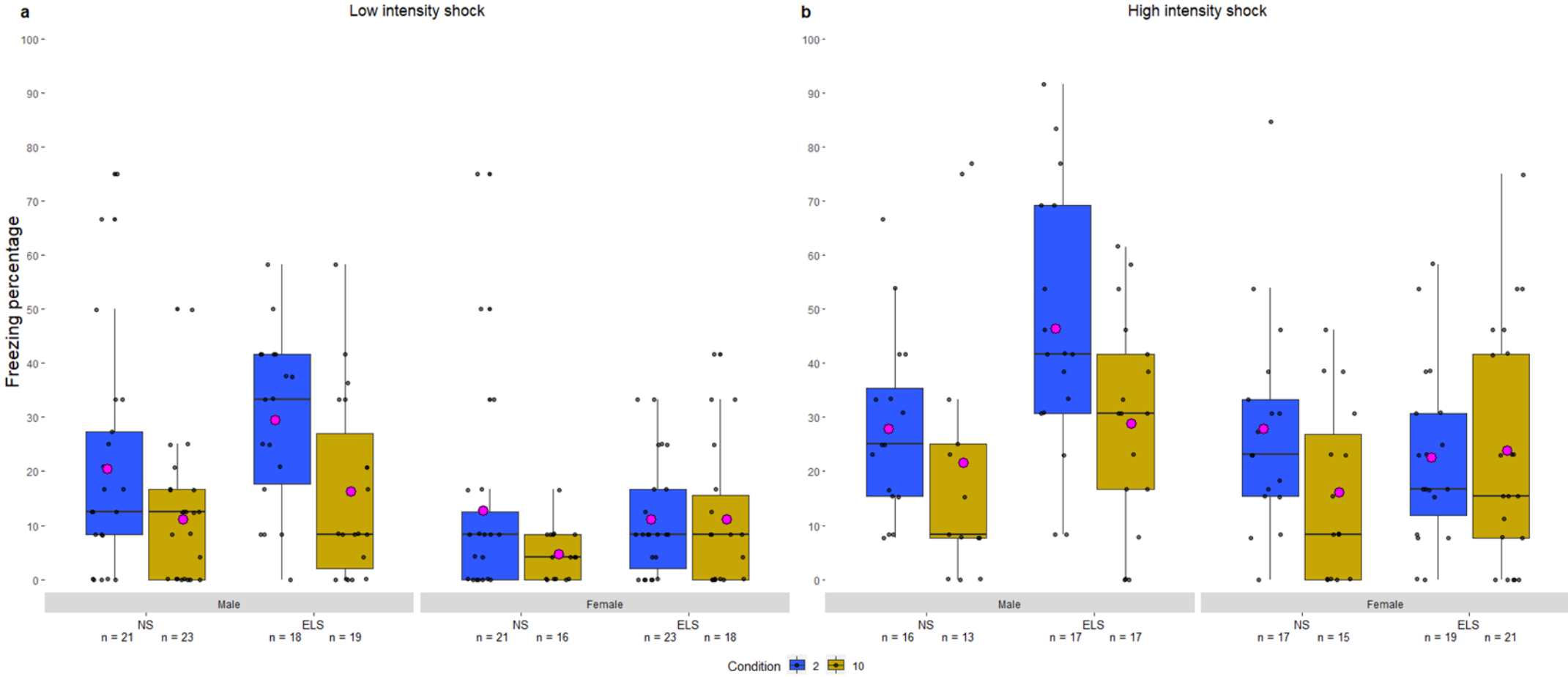


Figure 25: Sex and exposure to stress influence freezing percentage during recall following extinction training or extinction control: Animals experiencing the low intensity shock (a) show a sex and stress effect. With high shock intensity (b) there is a sex dependant effect of stress on extinction recall. Animals which have undergone 2-minute non- extinction are shown in blue with animals which went through 10-minute extinction in yellow (Horizontal lines show median with IQR as the hinges, the mean is shown as a solid magenta circle, top whisker shows up to 1.5 * IQR up to the maximum level bottom whisker shows down to 1.5* IQR). Black dots depict individual data-points. Group numbers shown as n.

3.3.5 Reminder training

Freezing was recorded for two minutes 32-48 days following the extinction recall trial recall to assess the levels of spontaneous recovery. At the end of the training, the animals are given a 0.25mA shock as a reminder stimulus and removed immediately from the context.

Low intensity shock: There is a sex by extinction condition (extinction control or exposure to extinction training) interaction present ($F(1,43) = 4.56$, $p < 0.05$, ANOVA), but no other interactions present (Table 6). Analysis of the extinction condition within sex and stress show no differences between extinction trained and extinction control (all $p > 0.05$, unpaired t-tests, Table 7) with all four groups (Female ELS, Female NS, Male ELS, Male NS). There was a Sex main effect ($F(1,43) = 7.528$, $p < 0.05$, ANOVA, Figure 26a) with males freezing more than females. There were no effects of Stress ($F(1,43) = 0.003$, $p > 0.05$, ANOVA).

High intensity shock: For animals exposed to the high intensity foot shock there was no interactions present (Table 6). There was no main effect of Sex ($F(1,125) = 3.482$, $p > 0.05$). There were no main effects of Stress ($F(1,125) = 2.833$, $p > 0.05$, ANOVA). None of the independent t-tests showed significant differences between extinction trained and extinction control (p's > 0.05, ANOVA Figure 26b and Table 7).

Table 6: Complete interaction terms for reminder training. Significant results shown in green.

Interaction	Low shock	High shock
Sex * Stress	$F(1,43) = 1.432$, $p > 0.05$	$F(1,125) = 0.140$, $p > 0.05$
Sex * Condition	$F(1,43) = 4.564$, $p < 0.05$	$F(1,125) = 0.324$, $p > 0.05$
Stress * Condition	$F(1,43) = 0.858$, $p > 0.05$	$F(1,125) = 0.00$, $p > 0.05$
Sex * Stress * Condition	$F(1,43) = 0.531$, $p > 0.05$	$F(1,125) = 2.978$, $p > 0.05$

Table 7: Inferential analysis via unpaired student's t-test of the difference between extinction control and extinction trained in each group at reminder training. All results are non-significant.

Group	Low shock	High shock
Male NS	t (10.926) = 0.930, p > 0.05	t (17.089) = -0.219, p >0.05
Male ELS	t (9.067) = 0.711, p >0.05	t (30.058) = 1.462, p >0.05
Female NS	t (7.056) = -0.233, p > 0.05	t (21.448) = 1.075, p >0.05
Female ELS	t (5.720) = -2.329, p > 0.05	t (37.994) = -0.789, p >0.05

.

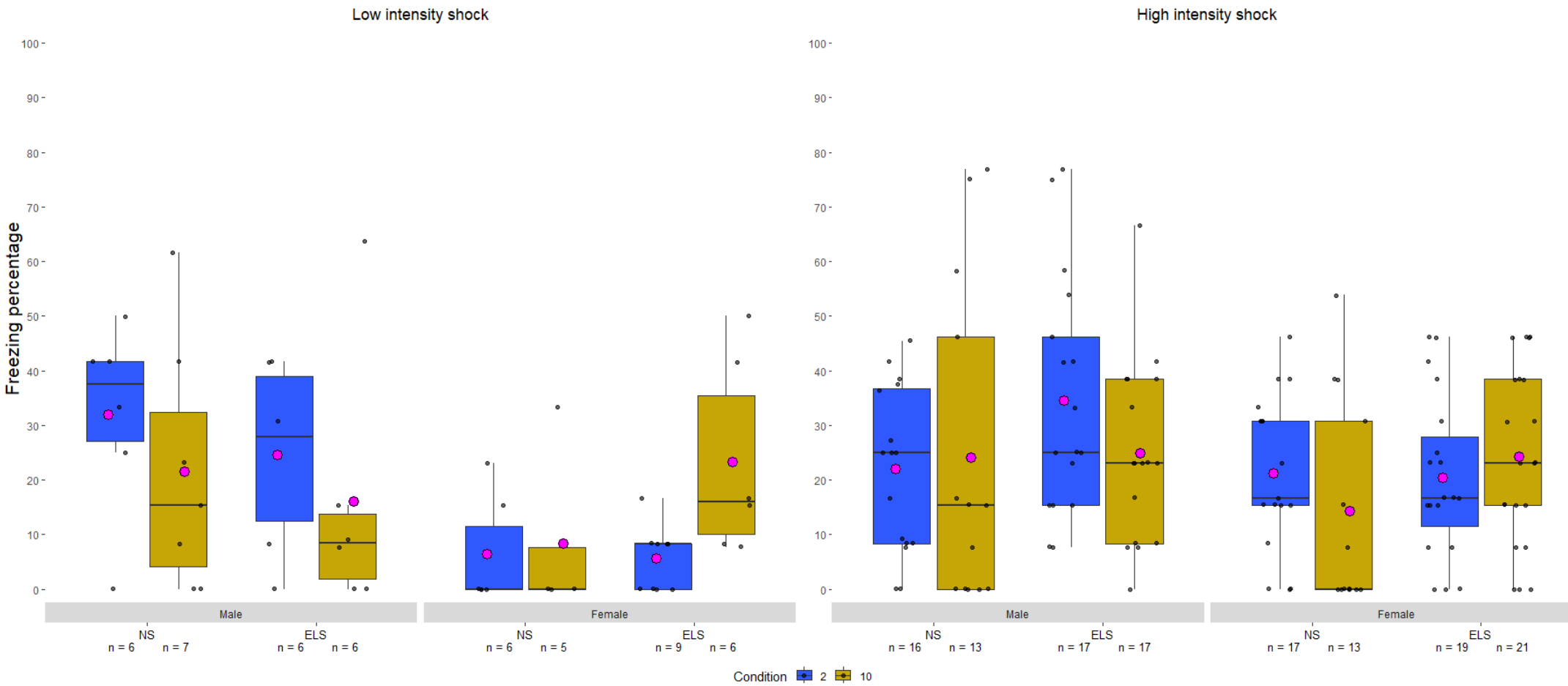


Figure 26:The effects of an extended period in the home cage were used to assess spontaneous recovery during reminder training and extinction memory consolidation. Animals which have undergone extinction control are shown in blue, animals which went through extinction training are shown in yellow. ELS = Early life stress, NS = No stress (Horizontal lines show median with IQR as the hinges, the mean is shown as a solid magenta circle, top whisker shows up to 1.5 * IQR up to the maximum level bottom whisker shows down to 1.5* IQR). Black dots depict individual data-points. Group numbers shown as n.

3.3.6 Reminder recall

Freezing behaviour was recorded for two minutes 48 hours following the reminder shock.

Low intensity shock: There is a Sex by Stress interaction effect on freezing behaviour present ($F(1,43) = 5.442$, $p < 0.05$, ANOVA, Figure 27a), there were no other interaction effects present (Table 8). There was a main effect of Sex ($F(1,43) = 14.971$, $p < 0.05$, ANOVA) with males freezing more than females. However, focused post hoc analysis shows that for both sexes and both stressed and non-stressed there was no difference between the extinction control and extinction trained groups (all p's > 0.05, unpaired t-test, Figure 27a and Table 9). There was no main effect of Stress ($F(1,43) = 0.002$, $p > 0.05$, ANOVA).

High intensity shock: There are no interaction effects present (Table 8). There is a main effect of Stress ($F(1,118) = 4.96$, $p < 0.05$, ANOVA, Figure 27b). There is a main effect of Sex ($F(1,118) = 4.24$, $p < 0.05$, ANOVA) males freeze more than females and ELS exposed animals freeze more than non-stressed animals. Focused post hoc testing shows that there are no effects of stress or sex on the difference between extinction control and extinction trained groups at reminder recall (p's > 0.05, unpaired t-test, Figure 27b and Table 9).

Table 8: Complete interaction terms for reminder recall. None of the interactions were significant.

Interaction	Low shock	High shock
Sex * Stress	F (1,43) = 5.442, p < 0.05	F (1,118) = 2.662, p >0.05
Sex * Condition	F (1,43) = 0.288, p >0.05	F (1,118) = 0.000, p >0.05
Stress * Condition	F (1,43) = 0.426, p >0.05	F (1,118) = 0.165, p >0.05
Sex * Stress * Condition	F (1,43) = 0.174, p >0.05	F (1,118) = 2.970, p >0.05

Table 9: Inferential analysis via unpaired student's t-test of the difference between extinction control and extinction trained in each group at reminder recall. All results are non-significant.

Group	Low shock	High shock
Male NS	t (9.688) = 0.860, p > 0.05	t (24.307) = -0.500, p >0.05
Male ELS	t (5.765) = 0.00, p >0.05	t (27.126) = 1.508, p >0.05
Female NS	t (7.959) = 0.343, p > 0.05	t (23.995) = 1.886, p >0.05
Female ELS	t (12.05) = -0.103, p > 0.05	t (28.708) = 0.127, p >0.05

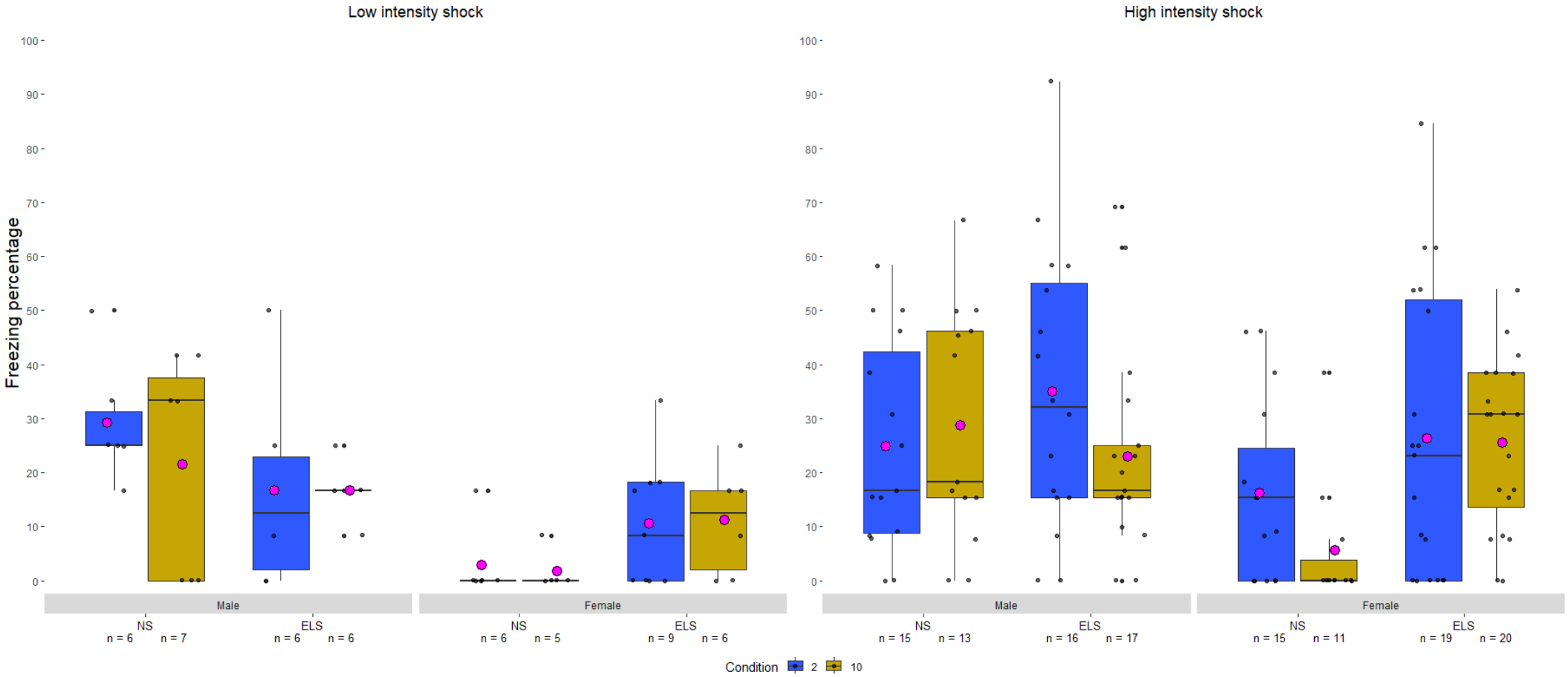


Figure 27: Two days following the reminder shock (0.25mA, two seconds) reminder recall was analysed to assess fear memory reinstatement. Animals which have undergone 2-minute extinction are shown in blue with animals which went through 10-minute extinction in yellow. M = male, F = female, ELS = Early life stress, NS = No stress (Horizontal lines show median with IQR as the hinges, the mean is shown as a solid magenta circle, top whisker shows up to 1.5 * IQR up to the maximum level bottom whisker shows down to 1.5* IQR) Group numbers shown as n. Statistical analysis shown in text. Black dots depict individual data-points. Group numbers shown as n.

3.3.7 Longitudinal analysis

A longitudinal analysis was conducted on CFC recall, extinction recall, reminder session, and reminder recall (Figure 28). Comprehensive inferential statistics can be found in Table 10.

Low shock intensity: For animals that were exposed to the low shock intensity and had not undergone extinction training no interaction effects were present (Table 10, Figure 28a). There was a significant effect of time point ($F(2.29, 52.72) = 6.716$, $p < 0.05$, RM ANOVA). There was a significant effect of Sex ($F(1,23) = 14.204$, $p < 0.05$, RM ANOVA) with males freezing more than females.

For animals exposed to low shock intensity who had undergone extinction training, a significant effect of time point was observed ($F(3, 60) = 7.727$, $p < 0.05$, RM ANOVA). While other factors did not show a main effect, there was an interaction between Stress and Time-Point ($F(3, 60) = 2.855$, $p < 0.05$, RM ANOVA). No other interaction effects were present (Table 10, Figure 28b).

High shock intensity: For animals exposed to high shock intensity and which had not been exposed to extinction, there was a main effect of Sex with males freezing more than females ($F(1.00, 61.0) = 5.324$, $p < 0.05$) and a main effect of Stress ($F(1.00, 61.0) = 4.236$, $p < 0.05$) with animals exposed to ELS freezing more than non-stressed. There was a main effect of time point present. There were no interaction effects present (Table 10, Figure 28c).

For animals that were exposed to a high shock intensity as well as extinction training there were no interaction effects present (Table 10, Figure 28d). There was a main effect of time point. There was a main effect of Sex with males freezing more than

females, main effect of Stress with animals exposed to ELS freezing more than non-stressed animals.

Table 10: Overall inferential analysis of longitudinal data. Significant differences are shown in green, non-significant results are shown in white.

	Low shock intensity		High shock intensity	
Factor	Extinction control	Extinction trained	Extinction control	Extinction trained
Sex	F (1,23) = 14.204, p < 0.05	F (1, 20) = 2.418, p > 0.05	F (1.00, 61.0) = 5.324, p < 0.05	F (1, 55) = 5.776, p < 0.05
Stress	F (1,23) = 0.154, p > 0.05	F (1, 20) = 2.276, p > 0.05	F (1.00, 61.0) = 4.236, p < 0.05	F (1, 55) = 4.716, p < 0.05
Time point	F (2.29, 52.72) = 6.716, p < 0.05	F (3, 60) = 7.727 p <0.05	F (2.59, 158.2) = 24.408, p <0.05	F (3, 165) = 30.108, p < 0.05
Sex X Stress	F (1, 23) = 0.522, p > 0.05	F (1, 20) = 1.059, p > 0.05	F (1.00, 61.0) = 1.583, p > 0.05	F (1, 55) = 1.067, p > 0.05
Sex X Time point	F (2.29, 52.72) = 1.311, p >0.05	F (3, 60) = 0.453, p > 0.05	F (2.59, 158.2) = 0.110, p > 0.05	F (3, 165) = 1.163, p > 0.05
Stress X Time point	F (2.29 52.72) = 0.199, p >0.05	F (3, 60) = 2.855 p < 0.05	F (2.59, 158.2) = 0.146, p > 0.05	F (3, 165) = 1.677, p > 0.05
Sex X Stress X Time point	F (2.29, 52.72) = 1.278, p >0.05	F (3, 60) = 0.248, p > 0.05	F (2.59, 158.2) = 1.911, p > 0.05	F (3, 165) = 1.351, p > 0.05

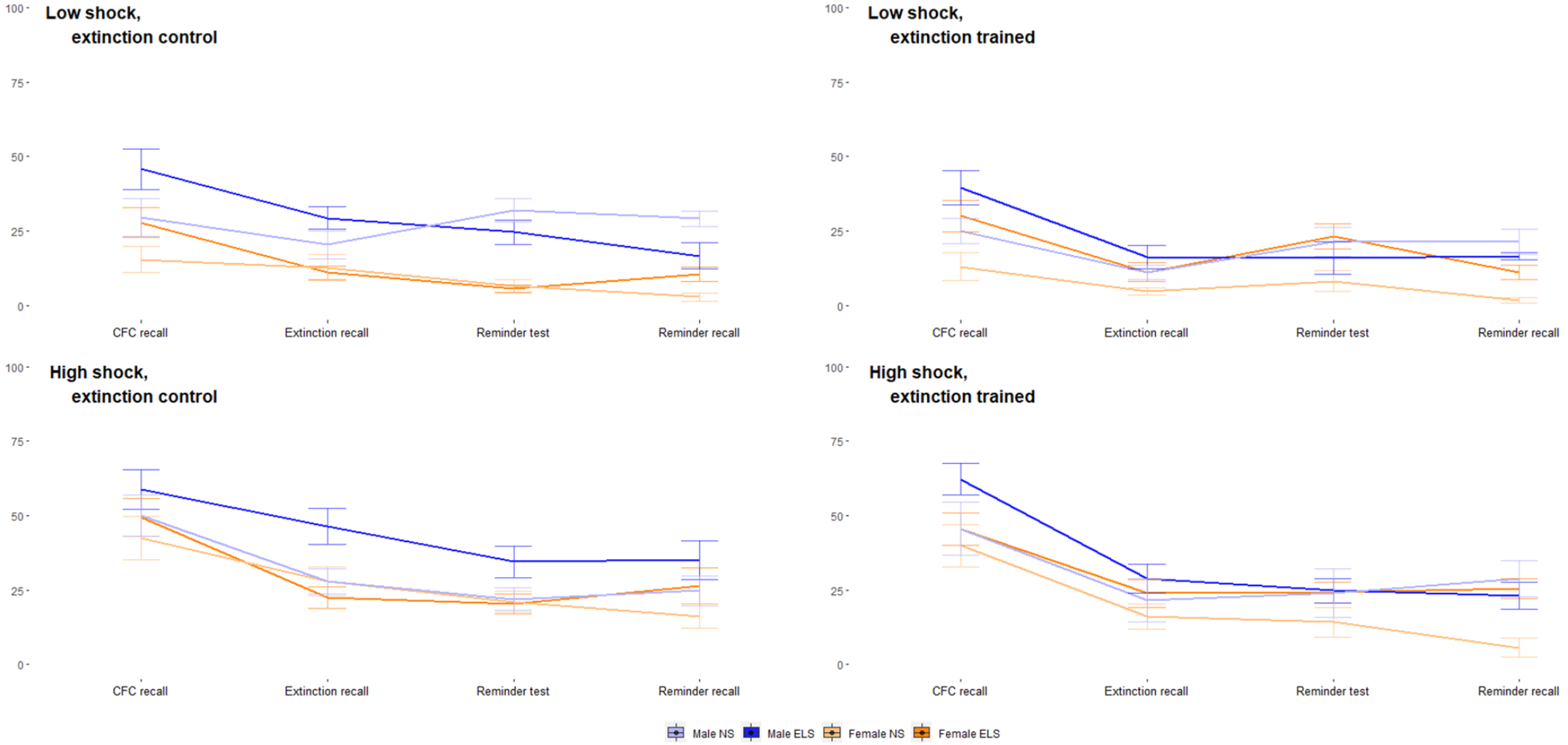


Figure 28: Longitudinal analysis of CFC recall, extinction recall, reminder test and reminder recall show that freezing decreases over time and highlights the sex differences present. Males are shown in shades of blue and females are orange (values shown as means, error bars represent mean ± SEM). ELS = Early life stress

3.4 Discussion

The current work suggests that ELS and sex have effects on CFC and its subsequent extinction. Each section of CFC is broken down within Table 11 with effects of sex, extinction and stress shown. The results are discussed further below.

Table 11: Effects of sex, stress and extinction condition on freezing during CFC, extinction, long-term memory and reminder recall. ELS = early life stress, NS = non-stressed. ExtControl = extinction control, ExtTrained = extinction trained.

Condition	Low shock	High shock
CFM acquisition	Pre < post ELS > NS Male > Female	Pre < post
Recall	ELS > NS Male > Female	Trend ELS > NS Male > Female
Extinction training	2minute > 10 minutes ELS > NS Individual training effects Male: ELS ExtControl > ExtTrained NS ExtControl > ExtTrained Female: ELS ExtControl = ExtTrained NS ExtControl > ExtTrained	2 minute > 10 minutes No effect of Stress Male > Female Individual training effects Male: ELS ExtControl > ExtTrained NS ExtControl = ExtTrained Female: ELS ExtControl = ExtTrained NS ExtControl = ExtTrained

Extinction recall	Males > female ExtControl > ExtTrained Males: ELS ExtControl > ExtTrained NS ExtControl = ExtTrained Female ELS ExtControl = ExtTrained NS ExtControl = ExtTrained	Males > female ExtControl > ExtTrained Males ELS ExtControl > ExtTrained NS ExtControl (trend >) ExtTrained Female ELS ExtControl = ExtTrained NS ExtControl (trend >) ExtTrained
Reminder test	Males > Females	No effects of Sex, Stress or extinction
Reminder recall	No effects of Sex Stress or extinction.	ELS > NS Males > Females
Longitudinal analysis	<u>Extinction control</u> Males > Females Time point	<u>Extinction Control</u> ELS > NS Males > Females Time point

	Extinction trained Time point	Extinction trained ELS > NS Males > Females Time point

3.4.1 Overview

This chapter revealed effects of sex and prior stress early in life on different stages of CFC, extinction, and long-term memory processes such as spontaneous recovery and reinstatement. Overall, animals which were exposed to foot shock freeze more following the shock than before the shock indicating the basic conditioning paradigm was leading to successful conditioning. Extinction training is dependent on shock intensity with animals becoming more resilient to extinction when given high shock intensity. Extinction recall was characterised by a complex interaction between sex and stress, where females show a lower level of freezing overall, while males that were exposed to ELS show stronger extinction recall than non-stressed animals. Reminder and reminder recall was characterised by primarily a sex effect with males freezing more than females at both time points.

3.4.2 Varying shock intensity changes freezing percentage across CFC.

Before discussing the experimental results in more depth, it is worth highlighting that two different levels of shock amplitude were administered to determine if different shock intensities would impact CFC and extinction. It has been previously shown that different shock intensities elicit a “dose dependant” effect on freezing (higher shock leads to more freezing) at various time points during CFC such as at recall and long-term memory tests [333, 334]. Secondly, it has been shown in previous work by Brydges et al 2014 [164] that females freeze less than males throughout CFM when given a 0.5mA shock. This low level of freezing is low enough to suggest that there may be a floor effect present, indeed this is a criticism that the authors of the work by Brydges et al [164] claim. To account for such potential floor effects, the higher shock intensity, 0.7mA was introduced. The main place in the current work where this was important was the extinction recall which, for females showed greatly reduced freezing

(likely approaching a floor effect). The starting hypothesis was that animals exposed to a higher shock intensity (higher current), would freeze more. Here, low, and high shock intensities were given at different times, with different cohorts and thus it is not suitable to inferentially, formally compare results from low to high. Nonetheless, throughout the CFM protocol animals freeze more when given the high shock intensity compared to the low shock intensity, as in line with the previous literature [333, 334]. Interestingly, for CFM recall, extinction training and extinction recall, the groups show a similar pattern between intensities (for example, ELS exposed animals freeze more than animals not exposed to ELS, and males freeze more than females) but the higher shock intensity are freezing more in general.

3.4.3 Rats exposed to a US freeze following CFM acquisition.

Animals exposed to a fearful stimulus (foot shock) successfully acquired contextual fear memory. Animals freeze more following foot shock than before the foot shock. Notably, animals exhibited increased freezing behaviour following foot shock exposure, with a distinctive pattern observed at lower shock intensities (0.5mA): males froze more than females, and stressed animals more than their non-stressed counterparts. This outcome, while initially unexpected, aligns with emerging research on the biological underpinnings of fear responses. Previous research such as that by Daviu et al [325] also shows research which supports this finding, namely, that females show less freezing behaviour at acquisition than males. One plausible explanation for these sex differences lies in the role of oestradiol, a sex-specific hormone. Barha, Dalton, and Galea's research [337] has shown that subcutaneous injections of oestradiol directly reduces freezing levels post shock, suggesting a hormone-driven modulation of fear memory acquisition. However, it is critical to note that their study employed a higher shock intensity (1mA) compared to the 0.5mA used in the current

research. Hence, there is likely to be differences between the work conducted by Barha et al[337] and the work conducted in this thesis, in that the shock intensity in the aforementioned work is much higher than that of the low shock utilized here (0.5mA in the current work and 1mA for that by Barha et al[337]) and indeed as described below a change in shock intensity influences how much both sex and stress have an effect on freezing immediately post shock.

The effects of ELS could be explained, plausibly, by changes in amygdala and hippocampal structures induced by the stressor. Alone the effects of ELS may be undetectable in adult life but when paired with an aversive stimulus during adulthood, interactions such as those proposed in the match mismatch hypothesis and allostatic hypothesis (described in section 1.10) could manifest. Changes in structures of the CNS, such as the prefrontal cortex, which interacts with both the hippocampal and amygdala systems, are sensitive to stress. Exposure to stress during a sensitive period may alter the structure or maturation of neuronal networks in these regions, as shown in Figure 7. This change could then lead to a heightened vulnerability in adulthood as the individual is primed to respond stronger to a stimulus later in life [338, 339]. Indeed, these are likely to be “dose dependant” and this is borne out in the current research where a higher shock intensity stops an effect of ELS being seen. The effects of stress priming are widely purported to have a neuro-inflammatory link [338]. This is described in more detail in Chapter 1 Subsection 1.17 and the neuro-immune involvement in the process of fear memory conditioning and extinction is a primary focus of Chapters 5 and 6.

When given a high shock intensity there was no difference in freezing between males or females or between ELS and NS animals. This is what was predicted and is in line with previous work [164, 330] showing that neither ELS, nor sex has an impact on

sensitivity to foot shock at acquisition. Despite the differences seen in the low shock intensity, examination of the data shows that all groups successfully acquired contextual fear conditioning with no groups showing major deficits or increased freezing.

3.4.4 Recall of CFM is stronger in males and those which had previously been exposed to ELS.

Animals which were exposed to ELS freeze more at CFM recall 48 hours following CFC using the lower shock intensity, than animals which were not exposed to ELS. This suggests that ELS could be either facilitating consolidation of CFM, facilitating the retrieval of CFM or a combination of both. Previous research has shown that different kinds of stressors can have an effect on memory in both animals [118, 340] and humans[341, 342] when the exposure to the stressor is during a consolidation window, reviewed further in a paper by Medina et al [343]. Also, research shows that being exposed to a stressor after the consolidation window can also impact on the level of freezing, however, this is more indicative of the stressor having an impact on the retrieval process, as the window for consolidation has already passed[344]. Elucidating the precise effects of ELS on this process becomes challenging as the nature of an ELS protocol is time locked (for example, here ELS was defined as PND 25 – PND27 only) and the neural mechanisms underlying CFM and extinction are significantly different[345].

These present results contrast with previous results by Brydges et al [164] who showed that males who experienced ELS froze less during CFM recall than animals which had experienced no stress. In the current work there was no interaction of ELS with sex (which is discussed in more detail below). ELS facilitated CFM recall which

was not seen by Brydges et al. There are a couple of potential reasons for this discrepancy. Firstly, the analysis period for recall that was used in the Brydges et al paper was 5 minutes which could be a long enough period for extinction to be occurring. In contrast the analysis for recall in the present study was only two minutes and thus extinction was unlikely to have occurred. Secondly, it is important to note, as is discussed extensively in Brydges et al[164], the timing of CFM recall is an essential factor in influencing the effects that ELS has on such behaviour. In the present work, the timing did differ to the protocol used by Brydges et al (24-hour recall for Brydges et al compared to 48-hour delay between CFM acquisition and recall for the current work). Although the temporal difference in this study is smaller than the 3-hour difference suggested by Brydges et al. (and used by Koseki et al. [346]), there is evidence that when animals are tested for CFM recall after more than 24 hours, the neural circuits involved differ from those engaged at the 24-hour mark. For example, this difference includes changes in the recruitment of the mPFC and in where the efferent connections of the PL cortex terminate [347]. Consequently, different memory processes, guided by activation of different brain circuits could explain the differences seen between the current work and that of Brydges et al [164]. Here when conditioned with 0.5mA ELS potentiates CFM recall.

There is some previous work which suggests CFM recall is facilitated by ELS. For example, Toda et al [348] shows that recall is facilitated 24 hours following CFM acquisition. Animals that were exposed to maternal separation early in life. Interestingly, there appears to be a stronger effect when analysed at 48 hours compared to the analysis they do at 24 hours. However, the statistics for this are not fully/accurately reported showing only that at 24 hours. Other work such as that by Diehl et al [349] shows a marked difference in CFM recall in terms of freezing between

animals which had been exposed to maternal separation compared to controls which had not. However, Kosten and Kim [166] show no effect of ELS on CFM recall, 24 hours following acquisition, when measured with freezing.

It can be hypothesised that being exposed to a stressor could predispose an animal to hypervigilance and to be primed for further defensive behaviours, this is the core reasoning in the allostatic hypothesis[179]. Consequently, in line with this literature and the theoretical hypothesis that could explain these results the current work in this chapter suggests that ELS facilitates CFM recall when previously exposed to a low intensity shock. With an increased foot shock intensity there could be a potential ceiling effect which could be reducing the effect of ELS in males (as can be seen by some animals showing 100% freezing in Figure 23). Despite the potential ceiling effect, in males exposed to high shock, the trend towards an effect of ELS is in the same direction as in low shock intensity. The results from animals exposed to a low intensity shock and the high intensity shock together suggest that ELS has a facilitating effect on CFM recall. This is potentially explained by the allostatic hypothesis [218] in that early life stress has a facilitating effect, sensitising the animal to show a stronger response in adult life.

Following the low shock intensity male animals freeze more than female animals suggesting that sex influences CFM recall. This is in congruence with a wealth of literature that suggests females show a weaker recall of CFM [113, 350-353] including that by Brydges et al [164]. Putative biological mechanisms which could explain these striking sex differences include the difference in circulating sex hormones[351, 354], sex dependant activation of hippocampal kinase signalling [352] and a difference in regulation of the HPA axis[325]. There are also potential behavioural differences in the response elicited following fear memory [106, 165, 335] as well as psychological

differences for example how much salience and attention, is placed onto contextual information[165]. Despite the literature suggesting an interaction of sex with regulation of the HPA axis, it is important to note that there was no interaction here between sex and ELS. This suggests that while both ELS and sex influence CFM recall at this shock intensity they do so in an independent manner.

Males freeze more than females at a high shock intensity suggesting that sex influences CFM recall and suggests that the modulatory effect of sex on CFM recall is apparent at different shock intensities. Like CFM acquisition, the pattern of freezing (group levels) is similar between low shock intensity and high shock intensity and while a direct comparison cannot be made, the level of freezing is higher in general in those exposed to the high shock intensity. Previous research has shown that CFC using a higher shock intensity increased freezing at recall [334]. However, this work uses only males and so the work in this chapter builds on this by showing that females also show a higher level of freezing with a higher foot shock. Finally, a ceiling effect could be blunting any difference between male ELS and male NS groups in the high shock group, this becomes even more apparent when considering that the effect is seen when the shock intensity is lower.

Females might just be failing to represent context as effectively as males. However, Tronson and Kiesier suggests in a recent review [355] that this may not be indicative of a deficit, but instead could be that females generalise more in conditions of ambiguity. This is not always a bad thing in ‘real life’ as environments associated with previous danger can predict future danger [355]. Females might also be instead expressing fear in a different way. It’s possible there could be no sex differences in the recall of CFC but instead freezing could be a poor measure of such recall. Understanding the relevance of such a possibility is an intriguing line of work, are these

sex differences due to differences in the processing of the memory, or just how they express the memory? Currently, it is difficult to answer this conundrum as both options are closely linked.

3.4.5 Extinction training is highly dependent on shock intensity.

After prolonged exposure to the context in which the animal underwent CFC a different memory engram forms (as described in Chapter 1 subsection 1.3), defined as extinction learning [31, 356]. In the current research the acquisition of extinction memory over time is indexed by a reduction of freezing from the beginning of the extinction training to the end point in both ELS and NS groups in males and the ELS group for females following the low shock intensity. Previous research by Trent et al [297] has shown that male non-stressed animals successfully extinguish during a 10 minute exposure. Importantly, the shock intensity used by Trent et al was 0.5mA, which is the same as low intensity shock in the current work. There was no analysis of female rats or exposure to ELS in this study. The learning of extinction has been shown in male and female non-stressed animals [286], but has not been examined in male and female animals exposed to ELS. Here there was an effect of ELS on freezing suggesting that stress potentiates freezing during extinction. This could be potentially due to those that were exposed to ELS having a stronger consolidation of the fear memory at recall, something which cannot be analysed independently of retrieval processes, in the current study. However, the group that did not show extinction, namely, females which were non-stressed have low levels of freezing throughout extinction training and this could therefore be indicative of a floor effect and not due to any effect of ELS exposure.

For animals exposed to high intensity shock during conditioning only male animals exposed to ELS extinction show evidence of extinction learning - evidenced by a

reduction in conditioned freezing in the last two min of the 10 min extinction session compared to the first two minutes. This was despite the increase in freezing for all groups compared to using the low intensity foot shock during CFC. The increased intensity appeared to attenuate extinction learning in both non-stressed males and females exposed to ELS. There are potential explanations for this. Firstly, males who have been exposed to ELS have already experienced a negative environment, consequently they have a resilience to future negative environments, which CFC could certainly be classified as (as noted previously foot shock has been used in some studies as a component of ELS[323]. In contrast, non-stressed males have not experienced this negative environment so have not had any chance to build this resilience. Put into context of this current work animals that have a mismatch fail to rapidly extinguish during CFM extinction training and this is only true when the second negative experience is of the higher intensity (low intensity shows no difference in stress condition). These results fit the proposed schema of the match-mismatch hypothesis [179]. But note this goes against the proposed schema of the allostatic hypothesis, as this suggests that the effects of stress compound and an early stress sensitises an individual to respond stronger to stress later in life[175].

Further previous work has examined sex differences in extinction learning [113] and shown that males show a higher level of freezing at the recall test than females, but then extinguish faster than females. Furthermore, in the previous work [113] it has been shown this is the case given a higher shock intensity (0.8mA) while not present following a moderate protocol (0.6mA). This finding has to be caveated by pointing out that this was done with repeated cued exposure sessions to elicit extinction training, the differences between cued and contextual conditioning, while converging on a final measure still require the activation of different pathways (see Chapter 1 subsection

1.5 for more detail) each of which could be influenced by ELS uniquely. Nonetheless, in the work presented by Clark et al [113], the extinction curve plateaus before dropping again to a floor level and at this point the males continue to extinguish. This is not the case when given the moderate protocol. It is likely that the female animals are freezing at floor levels during extinction as the increase in foot shock has not improved rate of extinction learning but instead has had a negative effect (the effect of extinction in females not exposed to stress has become non-significant). This is counter to what was predicted as it was hypothesised that a higher shock intensity would increase freezing at the initiation of extinction learning and would result in a higher baseline to see the effects of learnt extinction. A potential reason for this, however, could be that female non stressed animals when exposed to a higher intensity shock are still experiencing the higher intensity and are expressing fear to a higher magnitude but are in fact expressing fear in a different way to those exposed to the lower intensity shock.

3.4.6 Extinction recall is facilitated by ELS in males while females show extinction resistance.

For animals that were exposed to a low shock intensity, there was a sex dependant effect of ELS on extinction recall. In males, those that were exposed to ELS show less freezing after extinction training compared to those that did not experience extinction training. This suggests that male rats exposed to ELS have either successfully recalled the extinction memory which was previously learnt (as indexed by within session extinction; Figure 24,Table 3) which would have required successful consolidation of such a memory [89]. Or, that there is a stronger retrieval of extinction memory in this group compared to non-stressed males. This is challenging to unpick, extinction memory is often defined as a separate memory which requires acquisition, recall and

consolidation just as the association with the fear memory does. Therefore, the same issues apply. The metric which is used to index extinction memory recall relies on the strength of the extinction memory retrieval. Nevertheless, there is a difference between the extinction groups (extinction control and extinction training) that were exposed to ELS and non-stressed. This suggests that ELS is influencing one of these processes (enhancement of consolidation, enhanced retrieval). Here, however, this also becomes more complex as extinction memory is competing with the fear memory. In the male ELS group, which shows lower freezing in the extinction trained compared to the extinction control, this could be indicative of a weaker association between the US and the CS. However, this seems unlikely. There is little evidence that animals forget such an association across 6 days with many studies suggesting that forgetting occurs after several weeks[89].

Little research exists which is line with the present findings on extinction. Indeed, to the authors knowledge there is no research in rats which shows that exposure to a variable ELS confers improvement in extinction recall. Nonetheless, recently there has been an increase in literature suggesting that stress early in life can lead to resilience to various other kinds of behaviour. This relationship is described by Schmidt [179] as the match-mismatch hypothesis which is described in more detail in Chapter 1 subsection 1.10.2. Briefly, animals which have experienced a stressful environment early in life will be adapted to better deal with a stressful environment later in life. Experimentally, this has been shown by Santarelli et al [227]who show that deficits in various memory tasks (open field, forced swim, sociability tests and elevated plus) are strongest when there is a mismatch (ELS in early life, no stress in later life and no stress in early life, stress in later life). Essentially this is what was shown here and could suggest that in contrast to the vast literature that uses maternal separation as

the ELS protocol, when a variable stress protocol is used as the ELS a resilience phenotype emerges.

While the male group that was exposed to ELS shows interesting results that are suggestive of enhanced extinction memory recall, and putatively can be explained by a resilience phenotype as part of the match mismatch hypothesis, the non-stressed animals show behaviour that is indicative of a failure to recall extinction which was unexpected. In contrast, for females there is no effect of ELS on extinction. Neither ELS exposed females or females that were non stressed showed evidence of extinction recall. The lack of an extinction effect at extinction recall for non-stressed females is expected as this group showed a lack of within session extinction for extinction trained animals during extinction training therefore it could be postulated that animals either did not learn the extinction memory or showed such a low level that extinction learning hit a floor level. However, female animals exposed to ELS did show within session extinction but there was no effect of extinction training on the recall of extinction memory. This could be evidence that stress influences the recall of extinction memory, it could be that animals in this group are forgetting the extinction memory or that ELS weakens the context-shock association further between both extinction trained and extinction control. A more likely explanation is that female animals in this experiment are showing low levels of freezing during the first two minutes of recall and this level is low throughout further tests. This was a major reason for including a higher shock intensity which was the goal of aim 3.

There is an overarching theory that ELS in the form of maternal separation speeds up the development of certain brain circuitries, notably those related to fear conditioning and hypersensitivity to cues [50, 357]. Indeed, this could suggest why at least for males there is an improvement of extinction learning. The ELS speeds up development of

brain circuitries needed to learn extinction memories later in life. On the other hand, there is a wide body of literature that is in disagreement with the results which were described in this chapter, many studies are referenced in Maren and Holmes [31] and Izquierdo et al [358]. These reviews suggest that exposure to stress before CFC results in a resistance to extinction learning in mice. This is shown in an experiment which used a rat model[359], where chronic restraint stress inhibits extinction recall compared to animals which had not been exposed to restraint stress. These conflicting results must be caveated with some points explained below.

The time point in which the stress protocol occurs is highly critical. Hubel and Weisel [360] suggest that these critical periods are where brain formation is highly sensitive to the effects of stress. Brydges et al [96, 164] has suggested that stress occurring at this time point is highly implicated in disorders such as PTSD and studies suggest that childhood trauma is a major risk factor for the development of PTSD later in life[361]. However, the large majority of literature utilizes stressors that occur either before this time point [362] or much later (and just before the test for CFM extinction) [358, 359]. This makes it challenging to directly compare the results found in the current thesis to that of previous literature.

Much of the literature in the field also concerns itself only with male subjects [358, 363]. Therefore, while it is possible to compare the results in males presented above to the wide body of literature, it is more difficult to determine if the other results seen in the current work, namely, a lack of extinction recall in both ELS exposed and non-stressed female animals is a common finding across the literature. Males and females do show sex differences in terms of extinction learning and recall [364], the circuitries which are activated during these trials are also different across the sexes and there are sex differences in the prevalence of PTSD and anxiety disorders as described

previously[18, 365-367]. Here when both sexes are considered there is a strong sex specific effect of ELS on extinction recall. While this is driven by the fact that females show a lower level of freezing behaviour in general, (with the caveat of a good female specific measure of fear memory still not yet shown) despite stress or extinction it still highlights the importance of considering both sexes.

For animals that were exposed to the high shock intensity, there is still a sex dependant effect of ELS on extinction recall. The results are similar across the two intensities, there are some changes in trends towards effects, but the main result is that only male animals exposed to ELS show less freezing in those that were exposed to extinction training compared to those that did not experience extinction training. However, the overall levels of freezing appear higher (once again this cannot be compared directly as the different shock intensities were given to different cohorts that were not counterbalanced). Nonetheless, this suggests that successful extinction memory learning seen in male rats exposed to ELS occurs despite foot shock intensity level. This is somewhat in line with previous literature that suggests increasing shock intensity increases freezing levels at various points during fear conditioning [333, 334], however, no research has been conducted which examines the effect of shock intensity (or length of shock) on extinguishing contextual fear memory.

To further study the magnitude of differences seen in the current experiments an experiment where a direct comparison between the shock intensities, with the inclusion of ELS as a factor and between sexes, could be undertaken. Despite this, results over two shock intensities suggests males who have experienced ELS effectively recall extinction memory (through enhanced consolidation, or enhanced retrieval) compared to non-stressed males. This effect is resistant to increasing the shock intensity (perhaps these animals have a predisposed resilience to stress, which

a higher shock intensity would be). While males which were non-stressed fail to show extinction, although this is most likely explained by the lack of within session extinction seen during the extinction training (Table 3, Figure 24) or with the high shock the intensity of such a shock is impact.

When given the high shock intensity there is a sex difference with males freezing more than females in general. While females still don't show an effect of extinction in either animals exposed to ELS or non-stressed, the non-stressed animals' trend towards extinction. Once again there is still a potential floor effect seen. Some animals in the female non-stressed do not freeze at all throughout the extinction memory recall test. Giving a stronger stimulus may allow differences between the extinction control and extinction trained group to manifest while the current floor effect could be compressing this. Indeed, increasing the stimulus intensity, from low to high, elevates the level of freezing in females. If the increased freezing unveiled an effect of stress in females with a higher intensity stimulus it would suggest a direct interaction. Males show extinction in those which had been exposed to ELS while those not exposed fail to extinguish. Females show that those not exposed to stress extinguish while ELS attenuates extinction recall. However, an important consideration here is that increased intensity does not necessarily mean that the freezing level will increase at extinction recall. For non-stressed males in the low shock intensity, there is also a trend towards extinction. However, when the shock intensity is increased from 0.5mA to 0.7mA th e trend disappears. There is no sign of a ceiling effect here and the pattern between the extinction groups appears similar. Despite this, it can be seen in the data that freezing increases in females from 0.5mA to 0.7mA.

Most of the research suggests that exposure to stress, either early in life, chronically, or acutely impairs extinction memory recall [31, 96, 164, 208, 355]. This is not the

result that is seen in the current work. Either extinction memory recall was impaired in females for both those exposed to ELS or non-stressed or the effect showed a facilitation of extinction memory in males exposed to ELS compared to non-stressed animals. In contrast to the wealth of literature suggesting a negative effect of stress on extinction, there is very little primary research showing the opposite to be true. dDespite this, there are many studies suggesting that exposure to specific kinds of ELS (primarily intermittent separation) can confer resilience to later stress, can confer resistance to anxiety and improves arousal regulation [357, 368] Note that much of this work is done in monkeys and the negative effects of ELS on cognitive processes are seen to be evolutionarily conserved [369]. However, there are many more factors that are important to examine in more detail before making a conclusion. For example, the difference between an intermittent moderate stressor and a significant strong stressor appears to determine if such stressors lead to resilience and improve extinction memory retention or increases anxiety measures, respectively [370, 371]. it would be worth repeating this work to determine if different types of ELS indeed confer resilience to further stress, measures of anxiety and can facilitate extinction recall.

Animals were exposed to the conditioning context following at least 30 days to analyse the presence of spontaneous recovery [31]. Male animals freeze more than female animals in those which had been exposed to the lower intensity shock. Female animals are showing a pronounced floor effect with many animals showing no freezing throughout the test. Interestingly, in animals that were exposed to the high intensity shock the effect of sex is diminished. While not being able to compare directly across shock (for reasons described in 3.4.1) there does not seem to be a dose dependant increase in freezing percentage in males. Instead, this attenuation of sex differences is driven by females freezing more than the low shock intensity. Previous research has

shown that females are more susceptible to spontaneous recovery of fear memory than males [372, 373] which has been postulated to be due to a stronger resistance to extinction in females [113, 194].

Here however, this was not seen in the present work. There are some potential reasons for this. Firstly, the intensity and number of shocks differs across different studies. One used 3X 2s 0.75mA shocks [372]. Other studies have used multiple tone shock pairings for cued conditioning and see a substantial level of freezing in females [373]. Indeed, in the current work we can see that the level of freezing, when given just a single foot shock is approaching a floor level and therefore this could be masking effects of extinction and subsequent long-term tests of fear memory. It can be postulated that given a high enough shock intensity (or given more than one foot shock) females would show the increased spontaneous recovery that is seen in the studies mentioned above. However, increased shock intensity also increases the likelihood that animals will fail to extinguish [113]. Setting the correct parameters to analyse the correct fear memory process is something that must be considered carefully.

3.4.7 Reminder recall

Many of the seminal papers examining reinstatement of an extinguished fear memory such as that by Rescorla and Heth [109] and Halliday et al [374] use only male animals. In contrast another seminal paper by Bouton and Bolles [375] use females only. While this does give information about female reinstatement, the lack of males in this study precludes direct comparison between the sexes. In the present work males freeze more than females during the reminder recall test indicative of increased reinstatement. This is further evidenced by a lack of a sex effect on spontaneous recovery following a high shock intensity and suggests that this increased

reinstatement is not due to males freezing more pre-reminder. Further to this, animals which experienced ELS and were exposed to the higher shock intensity show stronger reinstatement than the NS group. Once again, the effects of stress were not present during the test for spontaneous recovery suggesting these effects are not driven by stronger recall of fear memory at the reminder test. There is no interaction between extinction condition and either of the above-mentioned factors (Sex, Stress) suggesting that both males and animals exposed to ELS show a stronger reinstatement regardless of extinction training. Work by Rescorla and Heth [109] has shown that the context is highly important as giving non-signalled shocks in a context unrelated to the extinction context fails to elicit reinstatement in the extinction context.

It is important to consider that CFC is a stressful procedure. Foot shock is itself given to induce stress [376]. Some experiments in contrast such as those by Luine [180], look at sex differences in the effects of chronic stress on tests of memory which are not stressful. It is important to consider the introduction of a secondary stressor considering the evidence presented supporting either the allostatic or match-mismatch hypothesis implies that results gleaned from tests inducing the second stressor will be dependent on a first stressor. Therefore, experiments looking at the effects of stress on non-stressful memory tests for example object recognition, radial arm and object location should be considered different to experiments looking at the effects of stress on stress inducing memory tests, for example CFC and water maze tests (see Harrison [377] which shows that the MWM induces stress, and note that a forced swim is used as a facet of the ELS protocol in the current work).

3.4.8 Criticisms and limitations

3.4.8.1 The methodological process of recording freezing.

The method of measuring freezing can be highly time consuming. Furthermore, across different studies some researchers measure continuous freezing, either manually via either a stopwatch or using software such as BORIS [378], while other researchers [96, 379] and including this work, take samples at specific time intervals. This can cause some discrepancies between results. Automation might be a way to reduce some bias as the accepted criteria for freezing will then be the same across experiments, laboratories and individual researchers. But, there are many challenges with this approach and such software can be highly expensive. For example, Ethovision XT [380] which a widely used behavioural analysis software can cost upwards of £4000. This is not true across all software platforms, for example Phobos [381] is a free MATLAB script which is designed specially to be used to analyse freezing behaviour in fear conditioning. Software suits based on python such as DeepLabCut [382] and EzTrack [383] can also be used to analyse freezing behaviour. Automation of freezing behaviour analysis is not completely devoid of caveats, however. Bias is still present in both the way individual parameters are set by the individual researchers and the way the data is analysed (there must be a cut off between movement due to respiration and movement that counts as non-freezing for example). It can be complex, and thus time consuming, to set up the pipeline to conduct these kinds of analysis (programmes such as Phobos require some MATLAB coding skills, and knowledge of video and audio codecs).

Also, setting up the software using older hardware, which was a factor in the current work, can be a challenge (high quality videos with a fine pixel resolution is often required). Finally, there is a large body of literature examining the correlation between

human freezing measurement and automated computer assisted analysis [384-386]. This is beyond the scope of the current work but is reviewed by Anagnostaras et al [384] in Table 1. The results are equivocal with some methods being comparable to human rating, while others are either not compared to a human rater or fit poorly. Nonetheless, once set up, automated methods of recording can be incredibly powerful and cheap. With the rapid development of machine learning algorithms, deep learning, and computer vision it is likely that the process of automating behavioural data analysis will continue to become more user friendly, will become better able to recognise subjects and be more able to handle noisy media from older hardware systems.

3.4.8.2 What constitutes chronic stress compared to acute stress, and where does mild stress become moderate or severe stress.

There is some debate as to what constitutes a chronic stressor, or an acute stressor and research has been conducted which suggests they have a different influence on the risk of developing disease later in life. Furthermore, the classification of mild/moderate/severe stress is different across difference sources. For example, as described above, many papers use maternal deprivation as their stressor, while here a different stressor was used. A previous paper [387] suggests that stressors which have a physical element to them could induce injury related physiological and neurochemical responses. Indeed, this could be a criticism of the current early life stress protocol as two of the facets of the stress protocol (forced swim and restraint) involve physical manipulation. However, it can be argued that this form of early life stressor is still very ecologically valid. Indeed, physical trauma early in life is cited to potentially increase risk of developing PTSD later in life[388].

3.4.8.3 It is important to note that the stage of oestrous was not controlled for in the current study.

As previously cited work [389] suggests, the stage of oestrous at which the female is in when exposed to a stressor can strongly affect how the brain reacts. Nonetheless, an important consideration here is that testing for oestrous stage is (while non-invasive, still) a stressful procedure that would induce a sex specific stress factor. Due to the nature of the procedure, it is impossible to administer an accurate control for males. This is an issue for analysing sex as a factor interacting with ELS and CFC. Previous work has shown that repeated tests using vaginal smears caused deficits in short term memory and long term memory during the sucrose preference test [390]. Interestingly, one group suggests that visual assessment, which is much less stressful, can be as accurate in determining oestrous cycle as vaginal smear [391]. However, this relies on the assessor being an expert and this technique further introduces issues of bias.

3.4.9 Conclusion.

The current work has shown that both sex and stress influence various parts of CFC, extinction learning and extinction recall. Males show a greater amount of freezing at CFM recall than females and those animals which had experienced ELS freeze more than non-stressed animals. This effect is present at both low and higher intensity foot shocks with a similar pattern of freezing behaviour being seen. During extinction recall, for males, animals exposed to ELS show potentially an increased extinction recall indexed by lower freezing suggesting a form of resilience is acquired during the stressor, as non-stressed animals here don’t show extinction. In females, neither stressed or non-stressed extinguish memory. The effects are generally the same

across shock intensities. However, there was still potentially a floor effect which could be probed further by giving a more intense foot shock to females.

The current work has repeated, in part, experiments conducted by Brydges et al [96, 164] some of the present findings are in line with these previous results while others conflict (for example the direction that ELS has on CFM recall), once again highlighting the complexity of extinction models. Future work should focus on elucidating brain regions which are changed depending on the exact facet of the CFC paradigm, for example how developmental reduction in amygdala, hippocampal or PFC volume due to ELS impact upon CFC and extinction memory. Freezing behaviour appears to be a simple metric to analyse but it is a challenging, time consuming and is suspect to various forms of bias, it is hoped that improvements in technology and consensus from researchers will lead to a better method to measure such behaviour. Finally, these results suggest that for treatment, which is still predominantly exposure therapy, the individual's sex and exposure to prior stressors must be considered. Indeed, here it appears that ELS might prove to improve the efficacy of exposure therapy, but only in males, if such a generalisation between extinction training in animal models and exposure therapy could be made. However, there are likely many more factors still to consider.

4 Chapter 4: The effects of early life stress on extinction memory measured via 22kHz ultrasonic vocalisation.

4.1 Introduction

The fear response is complex with verbal, somatic and motor responses noted. Classically fear memories are assessed in rodent models by looking at freezing behaviour in response to an aversive stimulus and indeed, here in the current thesis Chapter 3 was devoted to examining freezing as a metric to analyse contextual fear memory (CFM) acquisition, recall and extinction. Even though freezing behaviour is well documented to be an index of fear [165, 392-394], this measure may be measuring only one of many behavioural outputs associated with fear memory. Blanchard et al [395] has suggested that the nature of the behaviour is greatly influenced by the nature of the stressor/stimulus. For example, the response to predators, with rats primarily freezing and fleeing, is different to the response to a conspecific attack where rats will stand upright or lie on their back. These situations themselves are natural stimuli and elicit different responses compared to laboratory stressors for example eye puffs or foot shock. Many of these behaviours are conserved across different species [396] and the varying response due to varying stimulation suggested above highlights the importance of assessing a repertoire of behaviour. One important element of this repertoire of behavioural responses is the emission of ultrasonic vocalisations (USV). USV calls in rodents are anatomically separate from humans as they are produced through a whistle-like glottal space [397]. However, as

a behavioural metric Brudzynski suggests that USV calls have a human equivalent in the form of crying [398, 399]. The two primary categories of rat USV is those in the 22kHz and 55kHz range which are in response to stimuli which possess a negative or positive emotional valence respectively They occur in anticipation of events [400] and can be used to index associative learning and memory [166, 401, 402]. Together analysis of both freezing and USV provide a method to capture fear memory more accurately and subsequently its extinction.

4.1.1 The 22 kHz rodent response to fear.

Exposure to a noxious stimulus elicits a complex vocalisation pattern from rodents. Two, distinct responses can be detected; the audible portion (<20kHz) during and/or immediately after the stimulus, which is produced via the vocal cords, and the so-called vocalisation after discharge, which consists of ultrasonic (> 20kHz) calls. USV analysis in mice is complex with features such as the frequency slope properties, latency, volume, single call duration and whistle train frequency, well documented and the underlying behaviour eliciting such calls well understood [403-406]. In contrast, less work has been done to analyse vocalisations in the rat. Indeed, much of the literature [407] suggests rats primarily communicate in three main broad categories of call type, social 55kHz calls, 22kHz alarm calls and 30-40 kHz separation calls, the latter 30-40kHz calls being only present in juvenile rat pups[398] and potentially linked to maternal motivation [408].

In rats, 55 kHz calls are elicited during social play, and are described as a measure of joy or in response to appetitive stimuli. 22kHz calls are in instead shown to be emitted following aggression from other animals[409], in response to predators [395] and in response to lab stressors, and importantly following foot shock and fear conditioning [394, 410, 411]. 22kHz calls show a repetitive simple structure with a relatively long

duration (compared to 55kHz calls) and little frequency modulation (Figure 35 shows an example of this). The number of 22kHz calls emitted has been shown to be influenced by a variety of factors, for example: anxiety state [410], whether rats have been exposed to elements of juvenile stress [412] and intensity of foot shock [335] used during fear conditioning experiments. Intensity of foot shock had a major impact on the number of calls that are recorded at post shock. The data suggests that the most calls were seen between 0.8 and 1.1mA and that shock intensities at the 0.2–0.5mA range gave inconsistent results.

4.1.2 ELS effect on USV's

Much work has focused on the influence of maternal deprivation (which is often cited as a form of early life stress [315, 316]) on ultrasonic calls. As described above pup vocalisations are different to both the 55kHz appetitive calls and the 22kHz calls described above. These vocalisations may represent a different meaning, for example some work suggests that calls in this frequency range guide maternal behaviour[408] and in fact maternal deprivation can increase the level of these calls[413]. Nonetheless, these calls are rarely observed after pup stage and thus are not discussed in detail here. There is little work done which examines the effect of variable unpredictable stress on adult 22kHz USV during fear conditioning. One highly important study is that conducted by Yee et al [412]. They utilise a variable unpredictable stressor and examine the effect of this on CFC at the adulthood stage. It was found that exposure to early life stress caused an increase in the number of calls and the time spent calling on the day of fear conditioning however, they were unable to get conclusive results for post fear conditioning due to low numbers of calls.

4.1.3 Sex differences in ultrasonic vocalisation response to fear memories.

USVs have been used as a measure of fear conditioning and fear memory recall in both males and females. However, there are some differences, especially with regards to the stage of fear conditioning. For example, during cued CFM acquisition Laine et al [335] has shown that males call more than female rats. This is supported by work by Williadsen et al [402] who show both an increased number of calls as well as an increased number of callers compared to non-callers in male compared to female rats. Interestingly, Tyron et al [414] sees a sex difference in numbers of calls, with males calling more than females as above, but does not see a difference in caller/non-caller ratio. Tyron et al uses a 2-fold higher foot shock than Williadsen (0.5mA compared to 1mA respectively) and this might suggest that shock intensity mediates caller/non caller ratio (on an individual level) while not affecting the number of calls overall (the number of calls per caller does not necessarily increase). For recall and extinction animals call much less than during acquisition [335, 394, 402, 410] which makes conducting meaningful analysis on USV count difficult. Despite these challenges, some studies have managed to analyse these periods. For instance, Laine et al [335] demonstrate that males are more resistant to extinction than females when measuring 22kHz USVs. The information above suggests that sex differences are indeed apparent in 22kHz USV levels and are a useful fear associated measure to assess sex differences in CFC but may be somewhat limited in measuring extinction. There are sex differences in more complex analysis than solely vocalisation count, for example the caller/non-caller ratio seen by Williadsen et al [402]. Importantly, this research suggests that vocalisation can be measured in both males and females, therefore, this method could elucidate the effects of ELS on CFC and extinction which

was not possible by examining freezing (as in Chapter 3) due to potential floor effects. Consequently, including USV's as a measurement of fear behaviour will be a useful step in building an overall picture of fear responses.

4.1.4 Aims

USV calls in the 22kHz range are widely recognized as a primary response to noxious stimuli [335, 402, 410, 412]. The work in this chapter analysed these calls in the same rats, at identical time points during CFC, retrieval, and extinction, as those used to measure freezing behaviour in Chapter 3. Although previous research has predominantly utilised cued fear conditioning, the robustness of CFC for measuring USVs remains uncertain. However, based on trends in the literature [335, 402, 410] and prior findings by Graham and Kim [415], it is hypothesized that USV patterns will mirror freezing behaviour, with males emitting more calls than females and being exposed to ELS increasing the number of 22kHz calls following CFC. It is further expected that the number of USV calls will significantly decrease during recall, extinction training, and extinction recall, consistent with prior studies [335, 402, 410, 414].

Additionally, sex and ELS differences in USV are anticipated throughout various stages of CFC and extinction. Previous studies [166, 416] suggest that males typically vocalize more than females in response to noxious stimuli, a trend hypothesized here as well. Yee et al. [412] demonstrated that ELS increases USV calls following cued fear conditioning, but they did not explore extinction, a key focus of this work. Tryon et al [414] and Laine et al [335] have observed that males are more resistant to extinction than females, with vocalisation patterns likely reflecting this resistance. This study hypothesizes that USV patterns will align with freezing behaviour, though males may

exhibit greater resistance to extinction. The analysis may also reveal sex-specific effects of ELS in females. This leads to the following research aims:

Aim 1: Record and analyse 22kHz USV calls during contextual fear conditioning (CFC), retrieval, and extinction in the same rats used for freezing behaviour measurements in Chapter 3. It was hypothesized that USV calls will align with freezing behaviour, with males vocalizing more than females and ELS increasing 22kHz calls, particularly during CFC.

Aim 2: Investigate sex and ELS differences in USV during different stages of CFC and extinction. It was hypothesized that males will vocalize more than females throughout CFM acquisition and extinction, with ELS increasing USV calls. Additionally, it is expected that males will be more resistant to extinction compared to females, with vocalisation patterns reflecting these differences.

4.2 Methods

All animals used for ultrasonic vocalisation measurements are Lister hooded rats described in Chapter 2. The trials used in this chapter are the same as those used in Chapter 3 apart from the reminder and reminder recall sessions which were not analysed here. This was due to both the impact of the COVID-19 pandemic on restrictions to access behavioural laboratories and equipment availability where USV microphones were not available at these time points. Recordings of freezing behaviour and vocalisation were carried out in parallel.

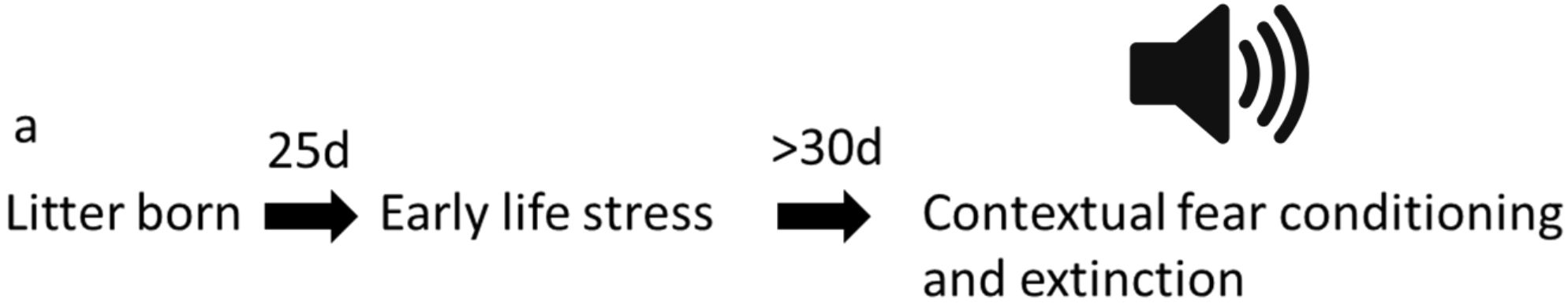


Figure 29: Schematic of behavioural paradigm. Animals were exposed to early life stress at PND-25-PND27 in the manner described in Chapter 2 subsection 2.2.2. Animals were split into two groups at extinction training: extinction control, which experienced a 2-minute session and extinction training which experienced a 10-minute session (Figure 15 shows this in more detail). All animals experienced CFC but animals did not experience the reminder trial as they had to be culled due to the COVID-19 pandemic or due to equipment availability. The speaker icon shows time points when vocalisations were recorded.

4.2.1 Ultrasonic vocalisations

4.2.1.1 Data acquisition

Ultrasonic vocalisations were collected using Avisoft-Bioacoustics microphones (CM16/CMPA) inputting into an UltraSoundGate 416H. Mics were positioned in the upper corner of the conditioning chambers facing into the chamber as is shown in Chapter 2 (Figure 16). The sampling rate was set as 4000 kbps. Recording range was set between 0 – 35 kHz. Duration of recordings matched the testing epoch of the behavioural assessment of CFC, extinction training and recall testing.

4.2.1.2 Audio pre-processing

Filters were applied using Audacity 2.4.2 [417]. A low pass noise filter was applied with a roll off of 48db cutting below 10 kHz and a high pass noise filter was applied with a 48db roll off, to filter out 30 kHz and above. All files were saved as WAV files for further processing.

To analyse only active sampling time periods (and not audio recordings of animals being taken out of or placed into the boxes) a custom script to crop the audio files was written in python. Sound files were cropped for the appropriate time before the ending of the experiment (2, 10 and 3 minutes, for extinction control, extinction training and CFM acquisition respectively). For the extinction training and the CFM acquisition where distinct time bins were analysed, the files were further divided. In brief, within the directory containing the 10-minute files, 5 more directories were created for 2-minute bins (similar to the 2-minute categories for the freezing analysis) of the 10-minute audio files. The pydub library [418] was used to open the original 10 minute file, segment into two minute sections and save the segmented files as .wav files. For the 3-minute CFM acquisition files two directories were created: one for the pre shock recording (of two minutes) and one (1 minute) for the post shock recording. Confirmation of accurate segmentation was examined manually with the software Audacity (version 2.4.2).

4.2.2 Analysis pipeline.

Analysis of vocalisation was conducted using Raven 1.5.0 (Version 1.6 existed at the writing of this thesis but did not allow use of the automated energy detector which is vital for the current pipeline). Parameters were trialled and validated manually through a trial-and-error basis where audio files were randomly selected, and the results of the detector were manually inspected and adjusted until the detector could include all calls

successfully while excluding noise. The parameters chosen are as follows(TARGET TAB:[minimum frequency:16000Hz], [Maximum Frequency:30000Hz], [minimum duration:0.50074S], [maximum duration: 2.50061S], [minimum separation:0.07987S] NOISE TAB:[minimum occupancy: 50%], [Return occupancy %: True], [SNR threshold: 8db above] [Block size: 10.00755S], [Hop Size: 3.00032S], [Percentile:10.0] Hz = Hertz, S = Seconds, db = Decibels). The count was validated with 10 randomly selected files where USVs were manually counted and compared to the automated band limited energy detector (data not shown). Once the parameters were confirmed manually, an R script was used with the package Rraven (https://github.com/maRce10/Rraven/tree/master) which allows R to access the raven package and warbleR (https://marce10.github.io/warbleR/) which includes functions related to USV and sound file processing. Briefly, the working directory is set, and the directories are loaded as lists. The files are then run through two primary functions: Automated raven detection and a count function. The automated raven detection function runs the USV detection algorithm within the raven software, the count function counts the number of calls in the recorded section.

22kHz vocalisations have a simpler structure compared to 55kHz calls. These 22kHz calls are typically long and maintain a steady frequency, except for a slight initial drop on the first call—starting at a higher pitch and quickly descending to the 22kHz frequency, which remains constant. These calls often occur in sequences, or "trains", where multiple calls are emitted with very brief pauses in between, followed by a longer pause before the next train begins (Figure 30). The primary goal of detection is to accurately count the number of individual calls, while filtering out noise and ensuring that each call is correctly identified as separate, even when the pauses between them are minimal.

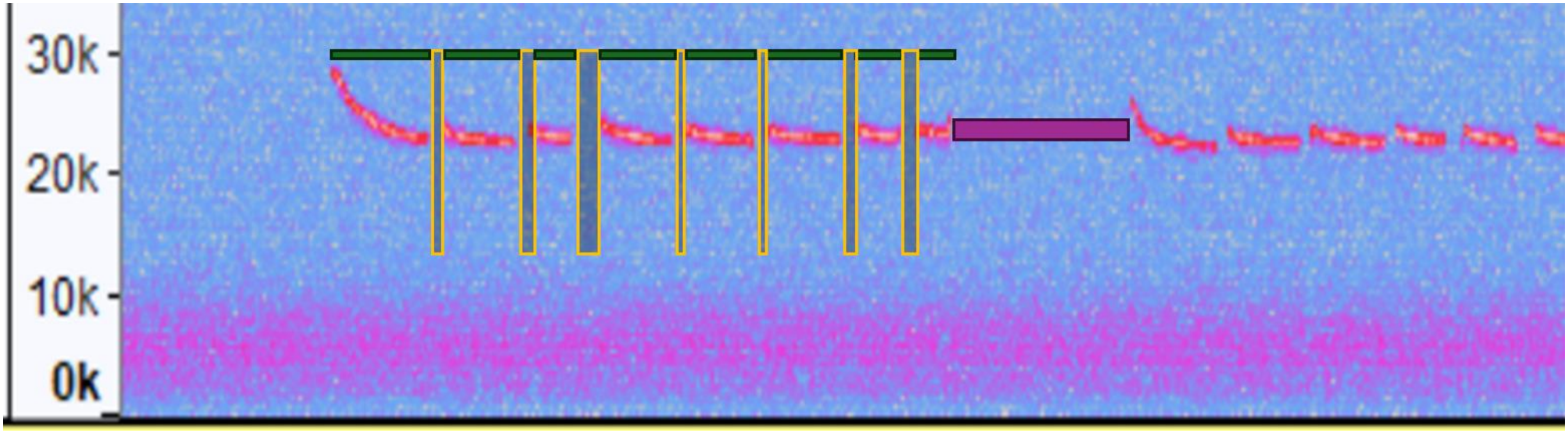


Figure 30: A representative sample of 22 kHz alarm calls from the current work visualised using Audacity software. Notice the inflection on the starting call which is different to the others. The rest of the structure does not differ greatly in frequency. Yellow squares show inter-call pauses, green bars show the call length, and the purple box is the between train pause. Scale shown in Hz. Blue is a weak signal with lighter colours showing stronger signal.

4.2.3 Statistical analysis

For the analysis of USV calls, both the number of calls and the caller/non caller ratio was analysed per sampling period. Visual analysis of distribution of number of calls showed a highly zero inflated data for post shock, extinction control and extinction recall. Thus, much of the USV data is highly zero inflated. To overcome the challenge of skewed data for standard parametric statistical analysis, two different hurdle models were employed. One, which uses the pscl library [419], was used for analysis which did not require repeated measures analysis (the CFM recall and the extinction recall). For the cases where repeated measures was needed (CFC and extinction training), the glmmTMB library [420] was used. Hurdle models are a type of regression model used to analyse count data, especially when there are excessive zeroes. It is important to note that while both the conditional and zero inflated model explain different elements of the data both should be considered together as the results of one model in the analysis will inform the meaning with regards to the other model in the analysis. They address the issue of excess zeros in two parts:

Zero-Hurdle Component: This models the probability that the count variable has a value of zero. It distinguishes between "true zeros" (e.g., situations where the animal never calls) and structural zeros (e.g., situations where the animal cannot call). This is modelled as a binary outcome using a probit/logit link function.

Positive Count Component: For counts greater than zero, the hurdle model estimates the distribution. Since zero values have been addressed in the zero-hurdle component, this part uses a truncated distribution starting from one. Mathematically this is shown in the equations below:

$$\Pr(x = 0) = \theta$$

$$\Pr(x \neq 0) = p_{x \neq 0}(x)$$

Animals can be a caller but emit either very few calls or many calls individually. For example, 5 animals could emit only 2 calls each thus there would be 10 total calls in that group. In contrast another group could have 2 callers that emit 5 calls. The same number of calls would be recorded, but the distribution of callers would be different.

4.3 Results

22 kHz USV analysis was not possible for all animals due to several factors including the COVID-19 pandemic and early inaccessibility of hardware. This primarily resulted in a lower number of animals for the low shock intensity experiment as this was the one which was affected most by the COVID-19 pandemic. In total 202 animals were assessed for 22 kHz USV counts.

4.3.1 Contextual fear conditioning

Low intensity shock: There is no effect of time point on 22 kHz vocalisation counts (Estimate (46,73) = -1.76 (z value = -0.56), $p > 0.05$). There is a sex by stress interaction effect on 22 kHz vocalisation counts (Estimate(46,73) = -2.459, (z value = -2.49), $p < 0.05$, hurdle model). Splitting the data into males and females and comparing stress effects shows that for males the effect of stress on 22 kHz USV counts trends towards an effect (Estimate (18, 25) = -1.553, (z value = -1.80) $p = 0.072$, hurdle model) with stressed males vocalising more than non-stressed males. For females there is no effect of stress on number of vocalisations (Estimate (28,45) = 0.96, (z value = 1.62) $p > 0.05$, hurdle model, Figure 31a).

Analysis of callers and non-callers showed a main effect of time point, with more callers compared to non-callers at post shock than at pre shock (Estimate (46,73) = 2.565, (z value = 2.97), $p < 0.05$, hurdle model). There was no interaction present between sex and stress (Estimate (46,73) =1.6487 (z value = -1.250) $p > 0.05$, hurdle model). There was no effect of sex (Estimate (46,73) =0.2513, (z value = 0.279), $p > 0.05$). There was no effect of stress (Estimate (46,73) = 1.4351, (z value = 1.745) $p > 0.05$, Figure 31b).

High intensity shock: There is a sex by timepoint interaction effect on the number of 22 kHz vocalisation counts (Estimate (132, 245) = -28, (z value = -44), $p < 0.05$, hurdle model, Figure 31c). There were no main effects of Timepoint (Estimate (132, 245) = -1.100, (z value = -0.27), $p > 0.05$, hurdle model), Sex (Estimate (132, 245) = 1.718, (z value = 0.52), $p > 0.05$, hurdle model) or Stress (Estimate (132, 245) = -0.490, (z value = -0.60), $p > 0.05$, hurdle model). Splitting the data into male and female reveals that for males there is no effect of timepoint on number of calls recorded (Estimate (66,121) = -1.57, (z value = 0.017), $p > 0.05$, hurdle model). However, for females, the number of callers was too low to reliably run the hurdle model and gather results for vocalisation counts. There was a sex by stress interaction (Estimate (132,245) = 0.482, (z value = 2.18) $p < 0.05$) effect on the number of calls recorded. Using the same split as above for males and females, for males there is no effect of stress (Estimate (66,121) = 0.011, (z value = 0.043), $p > 0.05$) for females again the number of callers was too low to reliably run the hurdle model.

There was a sex by time-point interaction influencing non-callers (Estimate (132,245) = 2.852, (z value = 3.95) $p <0.05$, hurdle model, Figure 31d). Males show more callers compared to non-callers at post shock than at pre shock (Estimate (66,121) = 25.86, (z value = 4.01) $p <0.05$, hurdle model). This is also true for females, there are more callers compared to non-callers at post shock than at pre shock (Estimate (66,121) = 2.186, (z value = 4.10), $p <0.05$, hurdle model). There was no effect of stress on caller/non-caller ratio (Estimate (132,245) = 0.11, (z value = 0.202), $p > 0.05$, hurdle model).

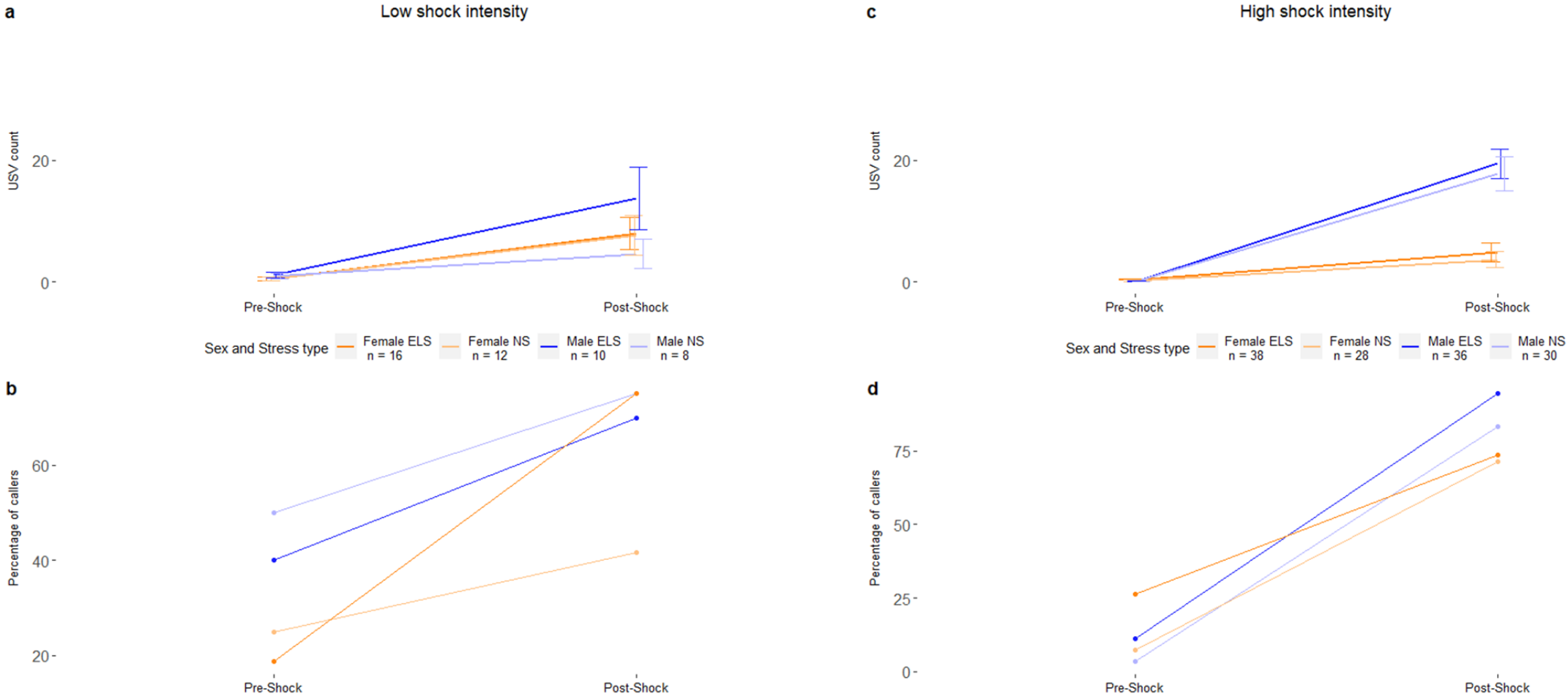


Figure 31: Rats vocalise in the 22kHz range more during the post shock period than pre-shock in a dose dependant way. 22 kHz USV counts and number of callers presented before (Pre-Shock) and after (Post-Shock) low intensity foot shock (a,b) or high intensity foot shock (c,d). Data are shown as means; error bars represent ± SEM. ELS = early life stress. NS = non-stressed.

4.3.2 CFM recall

Results here represent responses from the two-minute extinction control as well as the first two minutes from the 10-minute extinction group, which at this stage are not independent.

Low intensity shock: For animals which had experienced a low shock intensity during conditioning there is no interaction present between sex and stress (Estimate (8) = -0.656, (z value = -0.63), $p > 0.05$, hurdle model, Figure 32a) on number of 22kHz USV counts. There is a sex main effect on number of USV calls recorded with males calling more than females during CFM recall (Estimate (8) = 2.88, (z value = 3.03), $p < 0.05$, hurdle model). There was no effect of stress on 22 kHz USV count (Estimate (8) = 0.313, (z value = 0.304), $p > 0.05$, hurdle model).

There is a sex by stress interaction effect on the caller/non-caller ratio (Estimate (8) = -3.56, (-2.817), $p< 0.05$, hurdle mode, Figure 32b). Splitting the dataset into males and females shows that for males there is no effect of stress on callers/non-callers (Estimate (4) = -1.28, (z value = -1.55), $p > 0.05$, hurdle model) while for females those that experienced ELS show a lower percentage of callers compared to non-callers than for non-stressed females (Estimate (4) = -0.34, (z value = 2.39), $p < 0.05$, hurdle model).

High intensity shock: For animals which had experienced a high shock intensity at conditioning there is no interaction between sex and stress on 22 kHz USV count present (Estimate (8) = 0.175, (z value = 0.568), $p > 0.05$, hurdle model, Figure 32c). There is a sex difference with males calling more than females (Estimate (8) = 2.25, $p<0.05$, (z value = 2.957), hurdle model). There is no effect of stress (Estimate (8) = -0,.372, (z value = -1.125), $p > 0.05$, hurdle model).

The was no interaction between sex and stress on caller /non-caller ratio (Estimate (8) = 0.526, (z value = 0.568), $p > 0.05$, hurdle model, Figure 32d). There is a main effect of sex with males showing a higher percentage of callers to non-callers than females (Estimate (8) = 1.44, (z value = 2.957), $p< 0.05$, hurdle model). There are no main effects of stress (Estimate (8) = -0.511, (z value = -0.901), $p > 0.05$, hurdle model).

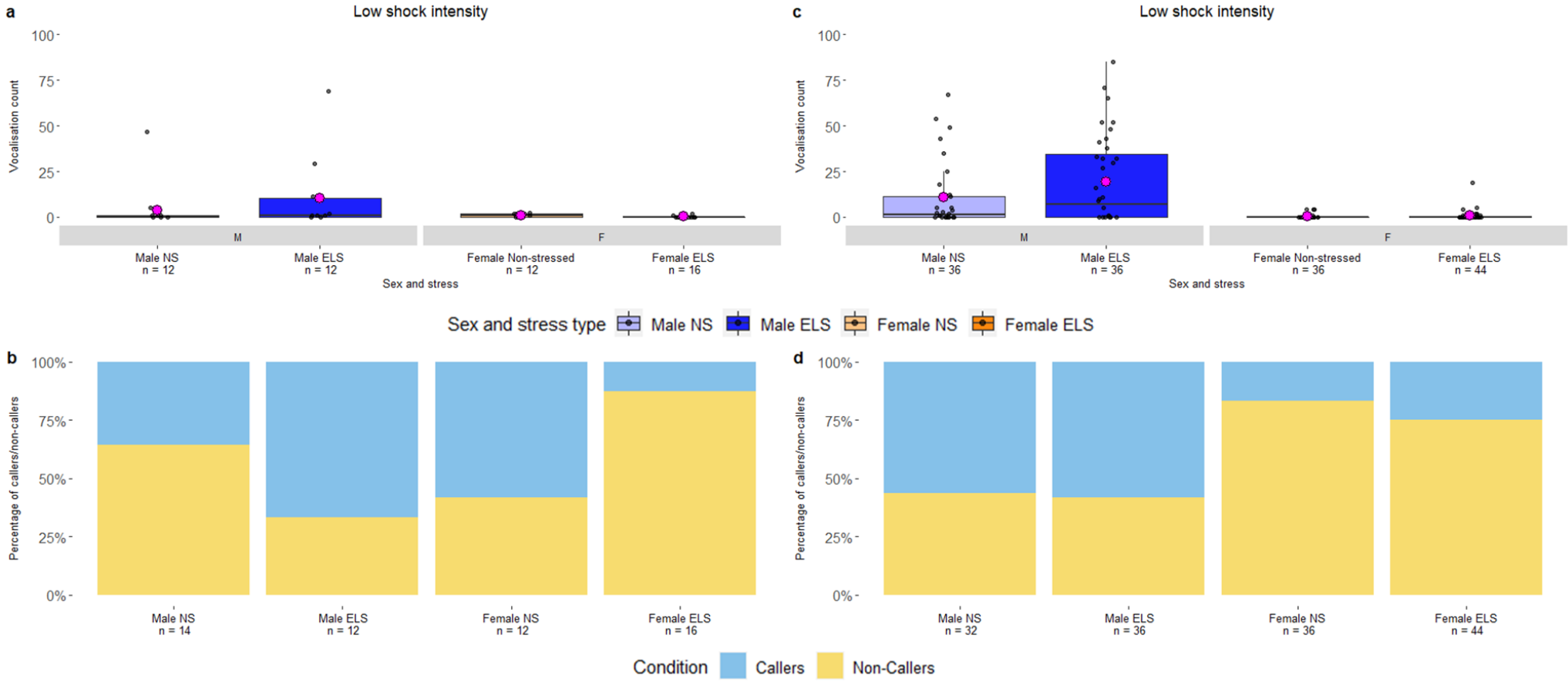


Figure 32: 22 kHz USV counts and percentage of non-callers to callers during the first two minutes of recall. Low intensity foot shock (a, b) and high intensity foot shock (c, d) are presented. (a,c) Horizontal lines show median with IQR as the hinges, the mean is shown as the solid magenta circle, whiskers show 1 SEM above and below. Black dots depict individual data-points. (b, d) The percentage of animals which don't call during the extinction training is shown in blue, the yellow is the percentage of animals which showed at least one USV during the training. Combined the stacked bars add to the full percentage. The number of animals is shown at the bottom of the figure as n

4.3.3 Extinction training.

Low intensity shock: There is no interaction effect of sex by stress on number of calls (Estimate (26, 111) = -17.54, (z value = -0.002) $p > 0.05$, Figure 33a). There was no effect of extinction training on number of calls (Estimate (26, 111) = 0.58, (z value = 0.10), $p > 0.05$) across all groups. There was no effect of sex present (Estimate (26, 111) = 0.27, (z value = 0.51), $p > 0.05$). There was no effect of stress on number of calls (Estimate (26, 111) = 0.41, (z value = 0.88), $p > 0.05$). Analysis of individual groups is presented in Table 12a. Males that were exposed to ELS call less at ten minutes than at two minutes. Females that were non-stressed also call more at ten minutes than at two minutes.

There was an effect of time point with an increase in non-callers at 10 minutes compared to two minutes (Estimate (26, 111) = -3.491 (z value = -3.119), $p < 0.05$, Figure 33b). Individual analyses of each group are presented in Table 12b, there are no effects of extinction training on the number of callers to non-callers in any individual group.

High intensity shock: There was no main effect of extinction training on number of calls across all groups (Estimate (70,331) = -0.14, (z value = -0.33), $p > 0.05$). There were no main effects of sex (Estimate (70,331) = 0.83, (z value = 1.466), $p > 0.05$, hurdle model, Figure 33c), or stress (Estimate (70, 331) = -0.76, (z value = -0.818), $p > 0.05$) on number of calls. Analysis of individual groups is presented in Table 12a. There were less calls following ten-minute extinction than at two minutes for males exposed to ELS. This was not shown for NS males. Females exposed to ELS show an increase in calls between the two minute and ten-minute extinction time points. This was not seen in the NS females. There was no interaction effect of sex by stress on number of calls (Estimate (70, 331) = 0.42, (z value = 0.408), $p > 0.05$).

There was an effect of time point on the caller/non-caller ratio with a decrease in callers compared to non-callers at 10 minutes compared to two minutes (Estimate (70,331) = -3.96, (z value = 0.58), $p > 0.05$, Figure 33d). There was an effect of sex on caller/non-caller ratio with more males having more callers to non-callers than females (Estimate (70, 331) = -3.88, (z value = -2.741), $p < 0.05$). There was no effect of stress on caller/non-caller ratio (Estimate (70,331) = -0.70, (z value = -0.53), $p > 0.05$). Individual analysis of each group is presented in Table 12b. Males who were exposed to ELS show a decrease in callers compared to non-callers at ten minutes compared to two minutes.

Table 12: Individual 22kHz USV count (a) and caller/ non-caller ratio (b) across extinction training analysed between the two minute and 10-minute bin. MCR = model cannot be run, there are too few callers to perform a conditional model. Green shows significant effects.

a

USV count	Low intensity	High Intensity
Male NS	MCR	Estimate (10) = 0.12, (z value = 0.69), p > 0.05.
Male ELS	Estimate (10) = 2.06, (z value = 5.43), p < 0.05.	Estimate (10) = 0.50, (z value = 2.81), p < 0.05.
Female NS	Estimate (10) = -1.79, (z value =3. 03), p < 0.05	MCR
Female ELS	Estimate (10) = 8.64, (z value = 0.07), p > 0.05	Estimate (10) = -2.90, (z value = -7.31) p < 0.05.

b

Caller/non-caller ratio	Low intensity	High intensity
Male NS	Estimate (10) = 18.57, (z value = 0.01), p > 0.05	Estimate (10) = 1.30, (z value = 1.54), p > 0.05
Male ELS	Estimate (10) = 1.61, (z value = 1.18), p > 0.05	Estimate (10) = 2.53, (z value = 2.84), p < 0.05
Female NS	Estimate (10) = 1.39, (z value = 1.13), p > 0.05	Estimate (10) = 1.87, (z value = 0.04), p > 0.05
Female ELS	Estimate (10) = 9.16, (z value = 0.67), p > 0.05	Estimate (10) = 1.30, (z value = 1.48), p > 0.05

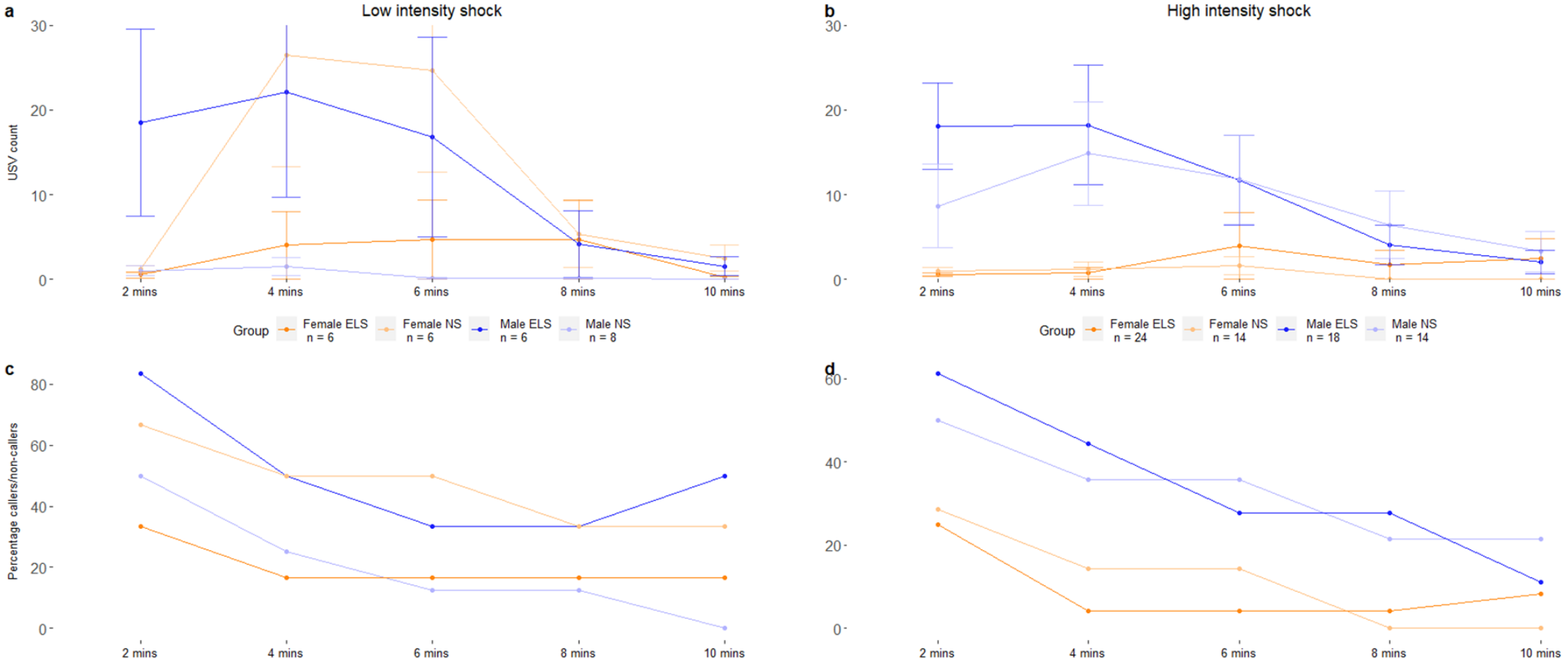


Figure 33: Number of 22kHz USV (a,c) and number of callers (b,d) across ten minutes of extinction training. In panels (a,c) the points show the mean and bars represent ± SEM Low shock intensity is shown on the left (a,b). High shock intensity is shown on the right (c,d). ELS = Early life stress.

4.3.4 Extinction recall

Low intensity shock: The level of 22 kHz USV becomes too low to conduct an overall hurdle model analysis with only 7 males and two females showing higher than 0 calls over the two minute sampling period (Figure 34a, b).Individual analysis of number of 22 kHz vocalisations between extinction conditions within each factor, sex and stress is presented in Table 13a. Despite the significant effect of males not exposed to ELS, where extinction control call more than extinction trained, here it can be seen that the number of calls here is very low and the effect likely due to a single outlier.

The number of callers/non-callers is presented in Table 13b. As for count there were not enough animals calling to conduct an overall hurdle model.

High intensity shock: For high shock, There was an effect of extinction condition (Estimate (137) = -0.68 (z value = -2.23), $p < 0.05$, hurdle model, Figure 34c) to probe this further individual analysis of number of 22 kHz USVs between extinction conditions within each factor, sex and stress is presented in Table 13a. There was no interaction between sex and stress (Estimate (137) = 4.85, (z value = 0.00), $p > 0.05$) on 22 kHz USV count and there was no effect of sex (Estimate (137) = 19.7, (z value = 0.002), $p > 0.05$) and no effect of stress (Estimate (137) = -5.40, (z value = 0.00), $p > 0.05$) on 22 kHz USV counts.

There was no interaction between sex and stress on the caller/ non caller ratio (Estimate (137) = 0.91, (z value = 0.60), $p > 0.05$). There was a main effect of sex on the caller / non-caller ratio during extinction recall (Estimate (137) = -3.54, (z value = -3.32), $p < 0.05$, Figure 34d) with males showing more callers, compared to non-callers, than females. There was no main effect of stress (Estimate (137) = -0.16, (z value = 0.00), $p > 0.05$) on the caller/non-caller ratio. Individual analysis of the caller/non caller

ratio between extinction conditions within each factor: sex and stress are presented in Table 13b. There are no effects of extinction across any of the factors.

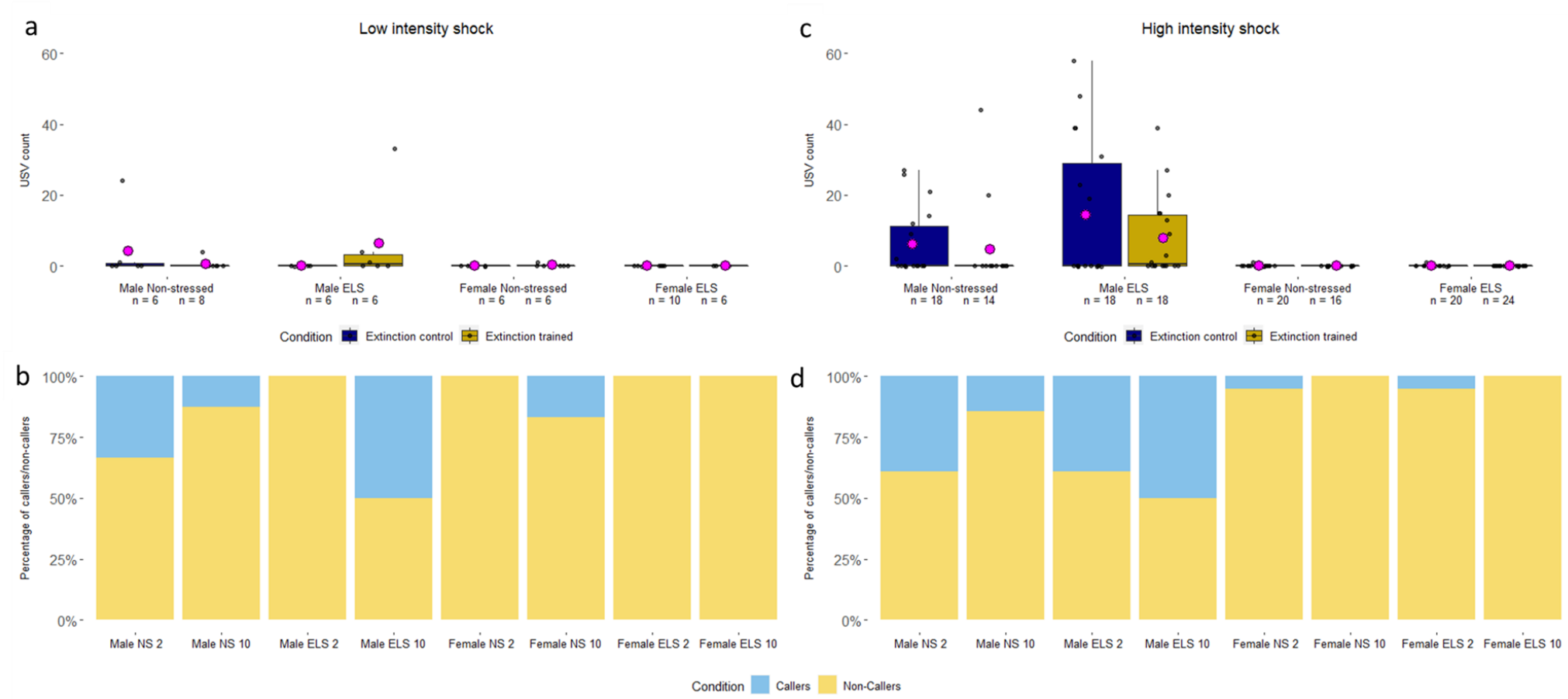


Figure 34: 22 kHz USV counts and percentage of non-callers to callers during extinction recall. Low intensity foot shock (a, b) and high intensity (c, d) foot shock are presented. Horizontal lines show median with IQR as the hinges, the mean is shown as the solid magenta circle, whiskers show 1 SEM above and below. Black dots depict individual data-points (a,c). The percentage of animals which don't call during the extinction training is shown in blue, the yellow is the percentage of animals which showed at least one USV during the training (b, d). Combined the stacked bars add to the full percentage. The number of animals is shown at the bottom of the figure as n.

Table 13: Conditional inferential analysis of extinction (control compared to extinction trained) on 22kHz USV count (a) and number of callers/non-callers (b). MCR = model could not be run

a

USV Count	Low intensity	High Intensity
Male NS	Estimate (4) = -1.16, (z value = -2.08), $p < 0.05$	Estimate (4) = 0.70, (z value = 4.47), $p < 0.05$
Male ELS	MCR	Estimate (4) = -0.84 (z value = -8.07), $p < 0.05$
Female NS	MCR	MCR
Female ELS	MCR	MCR

b

Caller/non-caller ratio	Low intensity	High Intensity
Male NS	Estimate (4) = -1.25, (z value = -0.91), $p > 0.05$	Estimate (4) = -1.34, (z value = -1.50), $p > 0.05$
Male ELS	Estimate (4) = 19.57, (z value = 0.003), $p > 0.05$	Estimate (4) = 0.45, (z value = 0.67), $p > 0.05$
Female NS	Estimate (4) = 18.96, (z value = -0.002), $p > 0.05$	Estimate (4) = -17.62, (z value = 0.002) $p > 0.05$
Female ELS	Estimate (4) = 0.00, (z value = 0), $p = 1$	Estimate (4) = -18.62, (z value = -0.002), $p > 0.05$

4.4 Discussion

4.4.1 Overview

22kHz USVs were successfully recorded in animals that were exposed to CFC, extinction training and extinction recall. These results are summarised in Table 14. While sex differences were present in both freezing (Chapter 3) and emission of 22kHz USV calls during CFC and extinction, the magnitude of difference was striking with USV calls. Males called more than females at every stage where there were enough calls to conduct analysis and at extinction calls the females were approaching floor levels. Levels of calls reduced from CFM acquisition to extinction recall which was predicted. Early life stress also influenced 22kHz vocalisations during CFC, recall and extinction. The effects of stress were sex dependant and effected by shock intensity. For CFM acquisition stress did not affect vocalisation count or the number of non-callers. However, for extinction training, males exposed to ELS extinguish at both low and high intensity shock, while for females at low intensity shock there is evidence that females not stressed call more while undergoing extinction training. However, this could be due to the low number of calls for females. Examining the number of callers found that males exposed to ELS showed a decrease in callers to non-callers following extinction training. For extinction at a low shock intensity 22 kHz USV count becomes too low to conduct meaningful analysis after extinction training. With a higher shock intensity, many female animals still do not call in the 22kHz range at extinction recall while males show evidence for extinction in vocalisation count with those exposed to extinction control vocalising more than those exposed to extinction trained.

Table 14: Effects of sex stress and extinction condition on 22kHz USV during CFC, recall, extinction training and extinction recall. M = Males, F = Females, Pre = Pre-shock, Post = Post shock, ELS = Early life stress, NS = non-stressed. 2 = two-minute extinction training timepoint, 10 = ten minute extinction training timepoint. ExtControl = Extinction control exposed, ExtTraining = Extinction trained.

Condition	**Low shock intensity**	**High shock intensity**
CFM acquisition	Vocalisation count Sex * Stress M ELS (trend >) NS F ELS = NS Caller / non-caller Post (callers) > Pre (callers) No other effects (sex, stress)	Vocalisation count Sex * Time point Post > Pre M > F ELS = NS Caller / non-caller Post (callers) > Pre (callers) No other effects (sex, stress)
Recall	Vocalisation count M > F Caller / non-caller Sex * Stress M ELS = NS F NS (callers) > ELS (callers)	Vocalisation count M > F Caller / non-caller M (caller) > F (caller)
Extinction	Vocalisation count Few females call. Individual analysis: Male ELS: 10 < 2	Vocalisation count Few females call. Individual analysis: Male ELS 10 < 2

	Female NS: 10 > 2 <u>Caller / non-caller</u> Overall, there was a decrease, but no individual groups show changes in caller/non-caller ratio during extinction training.	Female ELS: 10 > 2 <u>Caller / non-caller</u> Male ELS 10 > 2 non-callers
Extinction recall	Level of USV too low for meaningful analysis.	<u>Vocalisation count</u> M ELS ExtControl > ExtTraining NS ExtControl > ExtTraining <u>Caller / non-caller</u> No effects

4.4.2 Contextual fear conditioning

For animals exposed to the low shock intensity male animals that experienced ELS showed a trend towards a higher 22kHz USV count. This appears to be driven by the higher number of calls being emitted by the male ELS group (because male NS show a lower number of calls than the female groups). This suggests that ELS is facilitating 22kHz USV emission in response to fear following CFC but in a sex dependant manner. Interestingly there was no effect of either sex or stress on the caller/non-caller ratio. The findings are in line with previous research by Yee et al [412] who also found that using a 0.5mA shock for conditioning resulted in ELS exposed male animals (and not females) calling more. This suggests that it is the individual male animals exposed to ELS that are calling more, not that more of the animals in the ELS exposed group are becoming callers. In the current work when exposed to foot shock during conditioning, counts of vocalisation, in the 22kHz range is highly dependent on the intensity of the foot shock given. While it is not possible to compare the shock intensity directly, as the intensities varied across different cohorts, an increased intensity led to acquisition of CFM with USV as the measure. Furthermore, the different intensities elucidate different effects of sex dependant stress effects. This is different to freezing behaviour where all groups acquire CFM at both low and high shock intensity.

The results seen by Yee et al [412] appear much stronger when given the high shock intensity. Following exposure to 0.7mA foot shock there was an effect of sex with males calling more than females. There was still no effect of stress and there was no effect of either sex or stress on the caller/non-caller ratio. Another study which also used cued conditioning [414] and used 1mA as their stimulus intensity show that males call more than females during fear memory acquisition. Interestingly, these researchers also show no effect of sex on non-callers compared to callers on acquisition supporting

the observation that the individual male animals are becoming callers and not that each caller is emitting more vocalisations. This is important information to consider for further research, as many studies [410, 415, 421] only measure the number of calls and not the number of callers/non-callers. Analysing both gives a better understanding of how 22kHz USV emissions are emitted in response to fear conditioning. Nonetheless, the above results suggest that such an index of fear memory is sensitive to detecting sex differences in behavioural measures of fear which are present during CFC, especially at higher shock intensities.

4.4.3 CFM recall

Following CFC there is a sex effect on 22kHz vocalisations when testing recall of contextual fear memory. For low shock intensity sex differences were seen in rats trained with both high and low shock intensity with males calling more than females, with females exhibiting a floor effect. This is in line with work by Graham et al [415] which showed that males call more than females during recall in a CFC protocol. They also show that females at this stage show a diminished number of calls that are likely indicative of a floor effect. Despite not being able to conduct inferential analysis due to the shock intensities being over different cohorts the male animals appear to emit more calls when given the high shock intensity than low, at recall. This confirms other work by Laine et al [335] which shows males call more than females at recall in a conditioning shock intensity- dependant manner. For females there is not an increase in call number, when using the higher intensity shock and this is likely driven by a low number of calls at CFC. This is suggestive that females either are not becoming conditioned to the foot shock or are expressing the fear memory through a different behaviour. The former seems unlikely as Chapter 3 has shown that females freeze during recall after exposure to CFC and that these are the same females that were

recorded for vocalisation. Therefore, this suggests that females don't express conditioned fear vocalisation.

There is a sex by stress interaction in the caller / non caller ratio present which appears to be primarily driven by an unexpected increase in callers. Despite there being an effect of stress on the caller/non caller ratio in the females with non-stress calling more than stressed animals it is possible that this result is due to single bursts of USV noise being classed as vocalisations which is highlighted further in subsection 4.4.8.3. The measure of 22kHz USV however still proved to be a useful measure of conditioned fear response in males and when considering caller to non-caller ratio there was a sex effect that was dependant on the shock intensity.

There are relatively few papers which use 22 kHz USV's as an index of fear following CFC. It appears that the number of callers post CFC becomes too low to conduct meaningful analysis [412].There was no effect of ELS on recall in the current work. In the research by Kosten et al [166] there are differences in 22kHz USV counts at recall (24 hours following training) when given brief maternal separation (BS) compared to both control animals or those exposed to prolonged maternal separation (PS) with BS animals emitting less calls than the other two groups. However, maternal separation as used by Kosten et al. is a different stressor to the variable unpredictable stressor used in the current work. Research suggests that these different models mimic different forms/causes of stress and have different effects on behavioural expression later in life [422]. Furthermore, Yee et al [423] also showed during the beginning of extinction learning (an equivalent to our CFM recall) that there was no difference between sexes in terms of number of calls while there was a difference in latency to alarm. A lack of sex effect is in contrast to the consensus in the literature [335, 415] As the ELS protocol used by Yee et al [412] is very similar to the one used in the current

work another possible reason for these discrepancies in these studies could be the differences in the way USV's are measured (e.g. total time, number of calls, number of callers/non-callers, waveforms) and as there is very little research in this area currently it is challenging to come to conclusions about which effect is present. This highlights a pressing need to standardise analysis of vocalisations in fear conditioning going forward.

4.4.4 Extinction training

For animals that were exposed to the low intensity shock there were no effects of stress on vocalisation count and no interaction present. Analysis of each group for extinction individuals shows that males exposed to ELS show less vocalisations at the 10-minute than the 2-minute time point. There was an effect of extinction in the opposite direction to what was expected (10 minutes more calls than two minutes) in the female non-stressed group. In this dataset, the presence of a potential floor effect limits the range of possible values, as the number of calls cannot be negative. This restriction can lead to skewed data, particularly when dealing with small numbers. As a result, even a small number of calls can be significant, since they cannot be balanced out by negative values, unlike in a dataset where values both above and below the mean (like 4 and 6 around a mean of 5) can offset each other. There were no effects of extinction training on the caller/non-caller ratio in any of the individual groups (Male ELS, Male NS, Female ELS, and Female NS).

Interestingly, the number of calls increases at 4 minutes. An increase in calls shortly into extinction has been shown in previous work[400] where at two out of 21 sessions the number of calls increases sharply. This could be evidence of an extinction burst which has been described as a rapid increase in the behaviour before the decrease seen over time with extinction [424, 425]. The references here focus primarily on

addictive behaviour and aggression, less work has been conducted on the appearance of an extinction burst in CFC. It is interesting that this was seen in the number of 22kHz calls but not the number of callers. This could be evidence that the extinction burst behaviour is individually influenced by the extinction but that the group itself does not show an increase in number of animals expressing such a behaviour.

As described above, there are no current studies that could be found by the author that examine USV calls when using a CFC followed by recall which does not also use a tone as part of the paradigm. There are studies [335, 402, 414, 426] which have looked at extinction where the contextual element of the conditioning is tested in a cued design. Here the response to the cue is compared across different contexts. As has been described in detail in the neuroanatomy section of Chapter 1 subsection 1.5, cued memory trained in the presence of different contexts utilizes a similar but not identical pathway which recruits the amygdala and PFC but not the hippocampus which is primarily involved in the spatial processing of the contextual memory. One of the studies described above [426] show that males show a faster extinction to tone than to context in a cue/context hybrid model. Females however show similar rates of extinction in both. They suggest that there are no sex effects in regard to extinction learning in their paradigms but as described above, the level of calls at this stage is very low and finding differences is challenging at this point.

4.4.5 Extinction recall

For animals exposed to the low intensity shock there is a major floor effect across all of the groups. This suggests that either animals are showing a reduced number of 22kHz calls currently point or that the animals are expressing fear in a different way.

For the higher intensity shock more animals do call, but only males. It is still impossible to run the hurdle models on the female data suggesting that there are groups with only 0's. Interestingly, for males exposed to ELS, those which experienced extinction training call less than animals not undergoing extinction (2-minute extinction control. This suggests that stressed males are extinguishing and able to recall this extinction during the recall test. There is a significant difference between the extinction groups for males that were not exposed to ELS. However, this group did not show evidence of within session extinction and upon examination of the data it appears that the extinction trained group only show two animals above 0. This is likely indicative of a floor effect and caution should be taken when interpreting these results.

While there is some literature that is examining 22kHz calls as a measure of fear memory in long term memory retrieval there is a dearth in literature examining USV calls following extinction training. Here, using a high shock intensity at CFC (0.7mA) still results in calls at the extinction recall stage but only for a males exposed to ELS which suggests that despite seeing extinction, exposure to ELS is enhancing consolidation of CFM. This is supported by previous work [335, 415] showing that males extinguish cued fear conditioning more robustly than females. However, there are other papers which suggest that even with a higher shock intensity than the one used here for example 1.1mA ([410] the levels of vocalisation following extinction training decreases to a level that makes analysis very challenging and only by giving a cue alongside context will levels of 22kHz emission be above floor. There appears to be a plateau where giving a more intense stimulation does not elicit more calling as can be seen by Wohr et al [410]and Laine et al [335], both of which use a shock intensity over 1.0 mA. This work is novel in that it shows given a high enough shock intensity extinction of CFM learned through CFC is robust and resistant to effects of

ELS, although only as a measure for males. This measure is less sensitive than freezing behaviour because it relies on a higher shock intensity to see effects and rapidly plateaus with US's above 1.0 mA It is also less sensitive to the effects of ELS on CFM and extinction than freezing (Chapter 3).

4.4.5.1 Ultrasonic vocalisation as a social warning system.

Blanchard et al [395] suggests that USV calls act as a means to communicate to conspecifics about threat. Interestingly, sex differences seen in 22kHz USV emission depends strongly on whether other animals are present or not. Males call more in an artificial lab-based environment whereas females call more in a semi natural, social environment [427, 428]. Furthermore, this effect is complicated due to different USV responses at different times during CFC. For example, animals show different patterns of calling during CFM acquisition compared to the recall of such memories which is described in previous work [429] which has shown that a decrease in frequency modulated 55kHz calls is seen following foot shock acquisition and this decrease is reduced following extinction training. Interestingly, work by Karwicka et al [430] has shown that 22kHz calls, in response to a foot shock vary in length and are interspaced with 55kHz calls when the shocks are given in the presence of another animal. This suggests a mediation of the calls and a potential form of communication. Furthermore, playback of ultrasonic vocalisations can elicit a state of hypervigilance following fear conditioning which suggests that the call is a form of social communication[431]. This is further supported by work [432, 433] that shows exposure to an intruder animal elicits 22kHz and 55 kHz calls, highlighting the need for another rat to be present to elicit the more complex call pattern. Altogether, this research suggests that the USV response is complex and varies depending on the type of stressor given, alone, perhaps 22kHz may not be adequate to resolve female specific effects such as that

by exposure to ELS. But with a combined profile of 22 and 55 kHz, this may be more informative.

4.4.5.2 Freezing and vocalisation as measures of fear memory

Male and female rats react differently to a naturalistic or artificial environment [428], and this sex difference is potentiated further by the presence of other rats. Freezing represents a single behavioural strategy which serves to protect that individual. USV's could instead be a means to warn other animals, but also can be a means to perceive the intensity and distance of a threatening stimulus[395, 427]. A study conducted by Kim et al [434] has shown that playback of 22kHz alarm calls to rats elicits freezing suggesting that the two behaviours are linked. However, here in the current research we show that while there are sex differences in CFM recall which are apparent in both freezing and emission of 22kHz calls the sex differences for the 22kHz USV calling rate is stronger than that when analysing freezing. The current research suggests that freezing is a stronger CR than 22kHz USV's for females as emission of 22kHz USV calls are majorly reduced throughout the experiment, while freezing is present throughout (but reduced greatly at extinction recall). Nonetheless, it is not known if the females are instead expressing fear memory in a different way. For example previous work [392] examines multiple fear responses following CFC including, freezing, heart rate, ultrasonic vocalisations, defecation, body temperature, urination and locomotion measuring the "rapidity" of acquisition (measured as the onset of the behaviour at three successive test sessions; a rapid behaviour would be present in the first session while a less rapidly acquired fear behaviour response would show only in session three). It was shown that for certain fear responses such as a conditioned preference and freezing response, these are rapidly acquired, while behaviours such as ultrasonic vocalisation and defecation are less rapidly acquired. Unfortunately, the sex of the

animals in this study is not given and so there is no way to know if females or males would show any of these fear responses faster or slower than the other sex. This opens an interesting consideration: perhaps female animals would express fear memory through emission of 22kHz USV's, which Antoniadis and McDonald [392] suggests is acquired more slowly. However, the protocol used in the current experiment is designed to assess a rapid acquisition of fear memory [286]. Work which looks at recall of fear memory over multiple sessions (over multiple days as is done in work by Antoniadis and McDonald [392]) could potentially elucidate if females just slowly show emission of 22kHz USV or if they do in fact not emit these kinds of calls, and so either have a lower fear response, or are expressing fear as a different behaviour.

In response to predator exposure females emit more vocalisations than males. For example, when exposed to a cat, females vocalised more than their male counterparts[427]. The opposite is the case when the animals receive laboratory experimental stress such as a foot shock [166, 416]. The importance of the social nature of rats and thus the importance of the alarm call is a potential reason for these sex differences.

When comparing indices of fear behaviour freezing and emission of 22kHz vocalisations show some similarities. For example, with CFC the pattern between pre and post shock shows an increase in the index of fear (either freezing or vocalisation count) in post compared to pre shock across all groups, with the caveat that analysis of the number of callers/non-callers was required to see differences in vocalisation in the low shock intensity group. This suggests that when the intensity of the stimulus is low, animals express fear by both freezing and through the emission of 22kHz vocalisations. Stress did not influence either index during CFC. At the high intensity

shock when freezing was the index of fear there was no sex effects present, while for 22kHz vocalisation there is a sex dependant effect with males calling more than females. The reason why rats would either freeze or emit 22kHz USV's as a metric of fear behaviour is still unclear but research suggests that it has to do with signalling the intensity of the stimulus as well as to serve as a warning call to other animals [435, 436]. This is in contrast with freezing levels seen in Chapter 3, where the levels increase according to shock intensity, but there is no sex specific effect. This suggests that the calling response is more male specific and more strongly elicited with a more intense stimulus.

During recall of CFM there is a sex effect that is present when the index of fear is either freezing or vocalisation. Males express more freezing as well as higher counts of 22kHz vocalisations. This suggests that males have successfully acquired the CFM and are showing recall of such a memory during this test, as they are showing a high level of fear when indexed by two separate measures (freezing and 22kHz USV count). In contrast, females are showing fear behaviour only through freezing. It is possible that females rapidly stop expressing 22kHz when the US is not present, however USV counts are seen in females not exposed to stress. A possible explanation for the pattern of USV seen is that female animals are timing the USV burst to coincide with the prediction of the foot shock and could be a means of warning conspecifics. Indeed, this would be supported by work (described in more detail in subsection 4.4.5.1 suggesting that females call more as a warning signal to conspecifics (and the female animals would very likely have known another animal was in the room due to smell and sounds (described below in subsection 4.4.8.1)). However, it could also be possible that females are still expressing fear behaviour, just through a different metric. This is suggested to be a facet of the predatory eminence

continuum that Blanchard and Fanselow [437-439] have described in detail, whereby an increasing shock density leads to increasing expression of a fear index until the animal changes modes to another fear associated behaviour. Unfortunately, much of this work was conducted on males. Here, females are showing freezing at CFM recall while not showing vocalisation. However, there could be other indices of fear which have not been analysed that could elucidate fear behaviour in females. An example of this is darting, which was shown by Gruene et al [165], to be a response by females after cued fear conditioning. Importantly they suggest that darting is rarely seen in CFC. In the current work, when assessing freezing behaviour, very little darting behaviour was observed (data not shown). This is discussed further in Chapter 7 subsection 7.2. Other examples include slowed locomotion (but not freezing), defecation or fear induced analgesia which have been used as indices of fear previously[438]. Here, in the present work there were low responses of USV from females and therefore it becomes difficult to conduct meaningful analysis.

For extinction training and recall the only group that shows similarity is the male group exposed to ELS. Extinction learning within the training session appears present when both freezing and USV count are considered as metrics of fear behaviour. There does not appear to be an effect of ELS until extinction recall, on vocalisation. At extinction recall, in males exposed to ELS, those exposed to extinction training vocalise less than animals exposed to extinction control. This was not seen in the non-stressed animals. This effect was present for both low and high intensity shock. For female non-stressed animals there was an opposite effect of extinction training where animals exposed to extinction training call more than animals exposed to extinction control. Again, this was present for both low and high intensity shock. Nonetheless for female animals the levels are approaching floor levels and caution should be taken when

interpreting the results. However, especially for males the results for USV count during extinction recall does match with that seen in freezing behaviour, namely an effect of extinction training in the ELS exposed animals which is absent for non-stressed animals.

4.4.6 Comparison of shock intensity across sex

Shock intensity cannot be compared directly due to the differences in shock intensity being administered over different cohorts. Nonetheless, the higher number of calls in males at 0.7mA compared to 0.5mA suggests that shock intensity influences the amount of USV calls emitted at CFM acquisition but in a sex dependant manner. There is a wide body of literature that suggests that there are sex differences in the riskiness of the strategy employed by individuals in different conditions with males showing more risky strategies in situations than females (described above in subsection 4.4.5.1 [328, 440].

4.4.7 Comparison of vocalisation and freezing.

Generally, females vocalise less than males, and females also freeze less than males. There are some similarities in the results shown by the analysis of both freezing and 22kHz vocalisations. For example, being exposed to CFC increased the levels of both freezing and 22kHz calls. At recall, stress increases freezing and vocalisations in males, while in females, this effect is less apparent. However, for females, the level of vocalisations at recall is already low. During extinction training, the levels of both freezing and vocalisations follow an extinction curve, which does not show significant differences between sex or stress. Finally, while some interesting findings are observed in the analysis of freezing at extinction recall (for example the potential establishment of resilience in male animals exposed to ELS), this is not apparent in

the analysis of ultrasonic vocalisations. The level of vocalisations in females is at the floor for most groups, making analysis impossible in some comparisons. For males, the levels are also low; nonetheless, there are differences between the extinction control and extinction-trained groups in males exposed to high shock intensity. This suggests that, for males, the profile of fear memory extinction is expressed in both freezing behaviour and vocalisation in a similar way.

4.4.8 Limitations

4.4.8.1 The influence of, or lack thereof, social factors.

Previous research has shown that rats can communicate an aversive condition through vocalisations [434]. This is important to consider in relation to some potential hardware issues in the present study. Namely, the boxes are not completely soundproofed. No recordings of the outside of the boxes were taken but vocalisations at the audible frequency could be heard by the investigator during foot shock, and while the amplitude of the USV's is not as strong as the audible vocalisations (data not shown). Rat hearing is exceptionally sensitive across a wider frequency range than human perception [441] and so this is still a factor to consider. Interestingly, Wohr and Schwarting [421] suggests that there is no evidence that another rat calling at 22kHz in the vicinity of the rat being assessed, increases the rate of alarm calling. At every run animals were trained and tested in same group and sex pairs which would have reduced intergroup interference and variability. Furthermore, the "leaky" conditioning chamber represents a more ecologically valid situation as both humans and rats are social creatures [407, 434]. While attempting to completely soundproof the box might lead to a more controlled environment, allowing the rats to hear alarm calls might also be an interesting avenue of further research. However, these two approaches are tackling two separate research questions, the one done in the current study aimed to

carefully manipulate a relatively small number of variables to examine whether ELS has an effect on the encoding an extinction of CFM while the other approach would examine how social factors interplay within this question and would expand on the work presented above by Wohr and Schwarting [421].

4.4.8.2 The aversive 22kHz alarm call range vs the appetitive 55kHz USV range

A limitation of the current work is the limited frequency band that was analysed. Due to the nature of the protocol with noxious stimuli it was predicted that 22kHz calls would be the primary important measurement. To reduce the amount of data (and thus, the bandwidth usage) being transmitted through the system, two limits (~10kHz and 35kHz) were set on the frequency of the signals that were acquired. Unfortunately, this meant that calls in the 55kHz range which are indicative of an appetitive state, for example during play[442], mating[442] or in response to drugs of abuse [443, 444] were not stored. Interestingly calls which were above the 22kHz range were observed when looking at the live feed of the spectrogram during the pre-shock stage of CFM acquisition. Reyes et al.[429] has suggested that a decrease in 50kHz calls is indicative of anxiety like states. This further highlights how future work could aim to collect the entire spectrum of calls from 22kHz to 55kHz which could elucidate a behaviour underlying a lack of fear. Moreover, there are complex metrics in 55kHz which could be analysed. This is discussed further below.

4.4.8.3 A more complex profile of USV could be collected to make a richer analysis.

Investigation and analysis of rat vocalisations has lagged behind the analysis of mouse vocalisations. For analysis of mouse vocalisations, the pitch, the rate of change in pitch, the train length, the overall number, and amount of time calling have been considered. Recently, more detailed analysis of the vocalisations from rats have been

conducted with number of calls, duration, and structure of the waveform as factors for analysis [402, 410] and is further highlighted by the fact that analysis of callers/non-caller ratio suggests that measures other than counts might be useful. Previous work by Laine et al [335]found that when looking at the length of calls there were interactions between sex and intensity that were not present when examining just number of calls, or number of callers/non-callers In the present work USV length was not analysed. Laine et al also examined the shock calls, which are the calls which often span a very wide (and audible to humans) frequency range and found differences in shock intensity with higher shock intensities resulting in more audible calls. However there were no reported differences between males and females. Despite observing very interesting differences in the count and caller/non-caller data, there is still a plethora of data within these calls that have not been analysed (such as duration and waveform structure described above). An open source plugin for MATLAB, DeepSqueak [445] allows more complex waveform analysis for example assessing trills and syllable clusters. Through manual observation, and as is shown in Figure 35, the alarm calls emitted by rats, both in the present study, and in other work focusing on 22kHz calls, do not appear to be very complex, suggesting that in comparison to 55kHz vocalisations which show complex patterns, there is less information that can be gleaned from 22kHz calls. This plugin could also be particularly useful for analysing 55kHz calls. Inclusion of calls at the 55kHz frequency would likely benefit from analysis with a more complex programme such as DeepSqueak. Furthermore, DeepSqueak could pull out subtle changes in 22kHz that are hard to see such as length of calls and inter call intervals.

There may be an artificially high number of callers compared to the ground truth. This could be due to the call classifier not being a perfect detection of SNR. Although a subset of the files was checked for accuracy of the call counter pipeline, which was

high, this was not done for every file and a single erroneous call would have resulted in artificially labelling this noise as a call. While manually examining every single file could reduce this, there is still an issue of bias as to if a single 22kHz pulse should be included in the study or if it should be eliminated as noise.

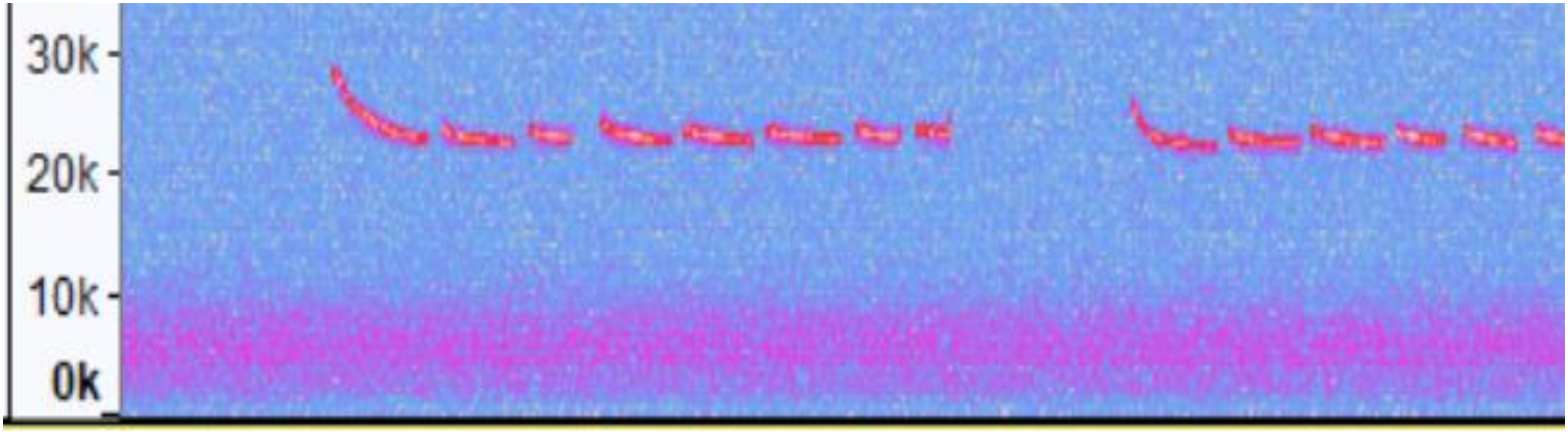


Figure 35: A representative sample of 22 kHz alarm calls from the current work visualised using Audacity software. Notice the inflection on the starting call which is different to the others. The rest of the structure does not differ greatly in frequency. Scale shown in Hz. Blue is a weak signal with lighter colours showing stronger signal.

4.4.8.4 The shock intensity may not be high enough to prevent floor effects.

Work by Wöhr et al [410]suggests that to get accurate USV recordings during fear conditioning a shock level of 0.8mA or higher is needed. Despite this the current work as well as others [335, 402] have shown that calls can be elicited as a response at 0.5mA and 0.7mA. Nonetheless, in the current work the shock intensity was not at what Wöhr et al suggest is the ceiling for USV calls. In the current work some analysis cannot be conducted due to the levels of USV being at floor level. While the level of calls seems to be greatly reduced in females at all post conditioning time points the calls for males suggests some influence of sex and extinction. Increasing the shock intensity could pull results up that are currently being compressed by a floor effect. However, as is suggested by Blanchard [439] different intensities of a stimulus can lead to different ways of expressing a responsive behaviour. Giving a very high shock intensity could mean that rats express fear in a different manner.

4.5 Conclusion

A very prominent sex difference in vocalisations during contextual fear memory conditioning at acquisition, recall, extinction training and extinction recall is shown in the current work. Males show more 22kHz USV calls than females. No animal expresses one single measure of fear and being able to collect a behavioural repertoire of responses in animals from laboratory tests will likely lead to more robust translatability. An analysis of both freezing and 22kHz USV has shown that there are more profound sex differences in USV count, but that there is concordance with male extinction training between USV count and freezing suggesting both measures are indexing a similar mechanism. This further highlights the importance of measuring multiple indices of fear response. There are various theories on the biological

mechanisms, environmental and social factors underpinning fear learning, extinction and the interplay between ELS and such memory processes such as a heightened resistance to extinction or resilience leading to improved extinction. One avenue of research is the topic of neuro-immunology and how this is involved in both, healthy physiology, and non-infection related pathology. This is the focus of the next two chapters.

5 Chapter 5: The effects of ELS and sex on the regulation of cytokines after CFM recall and extinction

5.1 Introduction

5.1.1 The neuroimmune mechanisms of PTSD

While the link between microglia and various cognitive process and emotional regulation has been shown in detail, less is known about the effects on contextual fear memory and extinction. The immune system has been postulated to be involved in PTSD onset following a traumatic event. There is abundant evidence demonstrating elevated levels of peripheral IL-6 [446-448] and TNF-α [449, 450] in veterans with PTSD. Despite this, central effects of these cytokines and the microglial involvement is less understood. The experiments presented in this chapter investigate the involvement of region-specific cytokine/chemokine protein expression following CFC and extinction that was conducted in Chapter 3 and Chapter 4. Microglia exert their effects both via production of signalling molecules such as cytokines and chemokines and through changes in their morphology (the focus of which is investigated in detail in Chapter 6).

There is growing evidence that there is an inflammatory profile present in individuals with psychiatric disease. For example a meta review by Costello et al suggests preliminary evidence that anxiety disorders such as GAD are associated with specific cytokine and chemokine profiles[69]. Studies also show that PTSD is linked to specific cytokines for example IL-6, IL-1β, and TNFα [62, 63]. This profile is characterised by an increase in pro-inflammatory cytokines such as CRP, IL-1 β, IL-6, IFN-γ and TNF-α and a reduction in anti-inflammatory cytokines such as IL-10 and IL-13. Some

studies suggest that an upregulation of the proinflammatory cytokines (and/or downregulation of anti-inflammatory cytokines) predicts disease, for example in the case of soldiers, where peripheral cytokine levels have been measured before and after combat deployment[451, 452]. Further work suggests that cytokine levels can be a biomarker for PTSD risk, with some chemokine protein levels such as CCL13 and CXCL6 showing higher levels in soldiers before developing PTSD (as a result of deployment to a warzone) as well as contributing to a prediction of severity in these individuals [453]. Other studies link PTSD with increased risk of autoimmune disease, suggesting the presence of a hyperactive immune system in these individuals [454].

Peripheral cytokines and central cytokines are linked in various ways.

5.1.2 Cytokine regulation in fear response and extinction.

Microglia have an essential role in learning and memory, the primary functional arms of activation that microglia use are changes in morphology (the primary focus of Chapter 6) and signalling through the modulation of cytokines/chemokines [455]. Cytokine concentration has been intimately linked with LTP, synaptic plasticity and memory maintenance as summarised in Figure 36. Previous work by Scholz et al [286] has looked at gene expression following the recall or extinction of contextual fear memory and shown that gene network clusters around immune system terms were changed between the recall and extinction samples. Those that were linked to recall were primarily the pro-inflammatory cytokines such as IL-1, IL – 6 and TNF-α. Those related to extinction were primarily linked to the NF genes, TGFβ, and PDGF tyrosine-kinase receptor activity. A wealth of information is now suggesting that besides microglia, neurons, and astrocytes both have receptors for and can express these cytokines which suggests that even subtle changes in concentration of these cytokines can impact on the CNS [456-458]. Furthermore, research has converged on several

cytokines, namely, TNF- α, IL-1 β and IL-6 that appear to be key targets in CFM recall while other cytokines such as TGF – β and TNF- α are likely key for extinction[247, 286]. Interestingly, TNF- α, which has been highly implicated in synaptic plasticity through the control of AMPA receptor trafficking [459] has been shown to be important for both CFM recall and extinction, highlighting the potential pleiotropic role for cytokines in fear memory.

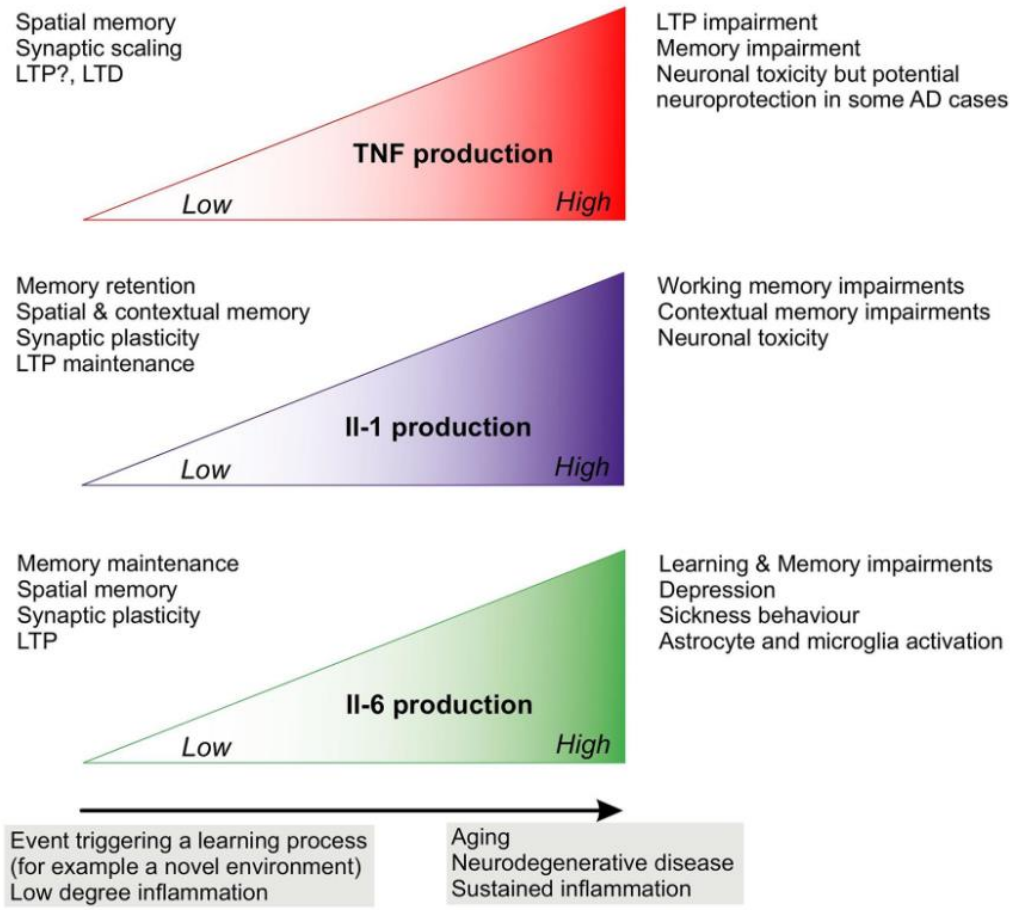


Figure 36: An overview of the canonical cytokines related to learning a memory. TNF-α at low levels has been strongly implicated in homeostatic plasticity related mechanisms, especially that via glial signalling[460]. IL-1 and IL-6 have both been implicated in disorders related to dysfunctional memory. Figure adapted from [247]

The role of such individual cytokines TNF- α, IL -1β and IL-6 in fear memory consolidation and extinction has been explored previously. IL-1β has been found to be upregulated after contextual fear conditioning (CFC) [285]. Furthermore, [461] found that IL-1β injection into the dorsal hippocampus, after training, reduces freezing following contextual fear conditioning when rats have been pre-exposed to a context. How modulation of learning and memory is induced by IL-1β expression is less well known but is potentially due to interaction with growth factor production such as NGF and BDNF both of which are important modulators of synaptic plasticity[462]. TNF- α RNA expression for example has been shown to be increased in microglia in animals which have undergone fear memory conditioning which is then reversed following extinction training[463]. Interestingly, they found that serum levels of TNF- α were not altered suggesting for fear conditioning and extinction, the cytokine expression (at least for TNF-α) was central and not peripheral. Other cytokines such as IL-6 have been shown to impair acquisition of fear memories but also the extinction of fear memories suggesting that IL-6 may play a role in the acquisition of memory, be it fear memory or the extinction memory [287, 464]. Cytokines may serve as autoregulators for microglia. Microglia respond to an unidentified target by releasing cytokines and chemokines [465]. These compounds amplify the reactions of other microglia, astrocytes, neurons, and influence the complement system as shown by Gomez-Abrolidas et al [466]. Reddaway has added depth to this understanding by employing an enhanced punch method, revealing localized alterations in the expression of complement factors [291].

5.1.3 Sex differences in cytokine profiles and the role in disease.

Recent work has begun to shed light on the complex interplay between sex, genetic factors, and environmental influences in increasing risk for disease such as PTSD. For

example, Lalonde et al [467] shows that pro-inflammatory cytokine protein concentration, in blood samples was significantly greater in men than women. Other work referenced in Chapter 1 subsection 1.1.5 highlights the importance of the neuroimmune system as a risk factor in PTSD.

Much work has suggested that there are distinct cytokine profiles between males and females during both development and at adulthood (as described further in Chapter 1 subsection 1.16). Work by Schwarz et al [264] characterised a profile of IL-1 family proteins, IL-10 and Toll-like receptor signalling proteins, that are upregulated in females of an adult age but not at a juvenile age. Furthermore, other work such as that by Berkiks et al [271], Loram et al [272]and Stephen et al [276] show sex specific responses to potentially pro-inflammatory stimuli. These sex differences are important to consider, especially the differences in sex seen at different developmental periods highlighted by Schwarz et al [264]. These cytokines can influence fear conditioning, fear memory and extinction learning (as described in detail in Chapter 1 subsection 1.18).

5.1.4 The effects of stress on cytokine profiles.

A wealth of information has shown that increases in stress has been shown to at first greatly increase the activation of central and peripheral immune cells, leading to an increase in pro-inflammatory cytokines [339], but then as the stress becomes more chronic it begins to lead to downregulation of the immune system, often described as an inverted U shape pattern [468]. Furthermore, work has shown that pro-inflammatory cytokine levels in the brain are upregulated, following psychological stressors [339] while other studies show that stress induced increases in glucocorticoids inhibit cytokine levels through inhibition of the NF-κB pathway and inhibition of the locus coeruleus [339].Chronicity of such a stressor impacts the response on the immune

system, with a reduction in cytokine production present more acutely. Chronic stress leads to a so called “HPA-axis fatigue” where the HPA axis is less able to suppress the immune system and pro-inflammatory effects become more present again[469]. Activation of the β-adrenergic receptors in the CNS increase levels of secreted pro-inflammatory cytokines [470]. Whether the stressor is acute or chronic[471], as well as the age at which the stressor occurs [184] appears to have a strong effect on activation. Interestingly, early life adversity has been shown to increase pro-inflammatory cytokines in humans and can lead to an increased risk of depression [472].

The effects of ELS has a further nuanced effect on the neuro-immune system, partly owing to the fact that development of the CNS is still ongoing and such mechanisms often require involvement of cytokines and the complement system [263, 466, 473]. An important meta review showed that increases in IL-6, IL-1β and TNF-α are present following ELS while an effect of ELS on anti-inflammatory cytokine production was absent Lumertz, Kestering-Ferreira [474]. The authors of this study suggest that the effects of ELS on cytokine concentration, that is present in adolescence, diminishes when the analysis is performed in adulthood, however, the effects of ELS on the immune system could be through an increased priming system which would mean that basal levels of cytokines would not be increased, but upon exposure to stimuli such as LPS injection, a psychological stressor there would be a faster or stronger response. Indeed, this is a concept explored in detail in by Danese and Lewis [475] who suggests that the effects of ELS, which include impacting on the developing immune system, remain hidden until there is a stimulus later in life. There is also some work which has shown that exposure to early life stress can influence cytokine expression, in particular IL-6, TNF-α and IL-1β (which were highlighted in the above

review) in adolescence and concordantly affect behaviour (such as, depressive behaviours, sensorimotor gating or Pre-Pulse inhibition) in adulthood [476-478].

5.1.5 Aims

The role of cytokines in CFC and extinction has begun to be explored in detail[286, 291]. The work by Scholz et al [286] has focused primarily on the role of differentially expressed genes highlighting upregulation of immune system related genes. Despite stress having a major role in the onset of PTSD and other anxiety disorders little work has focused on the neuroimmune system following ELS during the preadolescent period [184].

Here the focus of this chapter was to examine protein levels of cytokines/chemokines in areas of the hippocampus and mPFC which are known to be associated with CFC and extinction (see Chapter 1 subsection 1.5). The first part of the work was to conduct a partial replicate of the CFC and extinction training protocols in Chapter 3 and 4 to generate CNS samples, after recall or extinction for cytokine analysis. Sex differences in immune response to psychological stressors [479], response to trauma and during conditioning in animals models have been previously shown, often showing that females have more sensitivity to stressors than males, as well as the well-defined sex specific inflation of aetiology of anxiety disorders such as PTSD in females [19], and so both sexes will be included in the current experiments. The aims and hypotheses are as follows.

Aim 1: Investigate the differential immune responses, focusing on cytokine protein profiles, in the CA1 region of the hippocampus and IL subregion of the PFC in relation to stress and memory processes, with attention to sex differences. This study will

extend previous work by examining cytokine expression during CFC and extinction training in both males and females.

Aim 2: Test two contrasting hypotheses on cytokine response:

- Allostatic Hypothesis: ELS primes animals for an increased pro-inflammatory cytokine response in CA1 and IL mPFC, while non-stressed animals will show lower cytokine expression following CFC and recall.
- Match-Mismatch Hypothesis: A combination of stress and CFC might lead to a resilient immune response with reduced or unchanged cytokine levels in CA1, whereas non-stressed animals will show increased cytokine levels following CFC recall.

5.2 Method

5.2.1 Animals

Lister hooded rats (64 males and 56 females) housed in pairs or threes, housed on a reverse light cycle, as described in Chapter 2 were used for all experiments below.

5.2.2 Early life stress and contextual fear conditioning procedures.

ELS was conducted using the protocol described in Chapter 2. Briefly, the protocol was over three days where animals were exposed to a forced swim at PND 25, elevated platform at PND 26 and restraint stress at PND 27. Animals were then maintained until after PND 90. Animals underwent the same CFC protocol that is described in detail in Chapter 2 subsection. All animals were only exposed to the high shock intensity (0.7mA) during CFM acquisition that is described in Chapter 2 and 3. Two hours following extinction training or extinction control animals were sacrificed using a rising concentration of CO^2 and brains were collected. Brains were rapidly dissected out and snap frozen using dry ice. A schematic of this procedural workflow is shown in Figure 37.

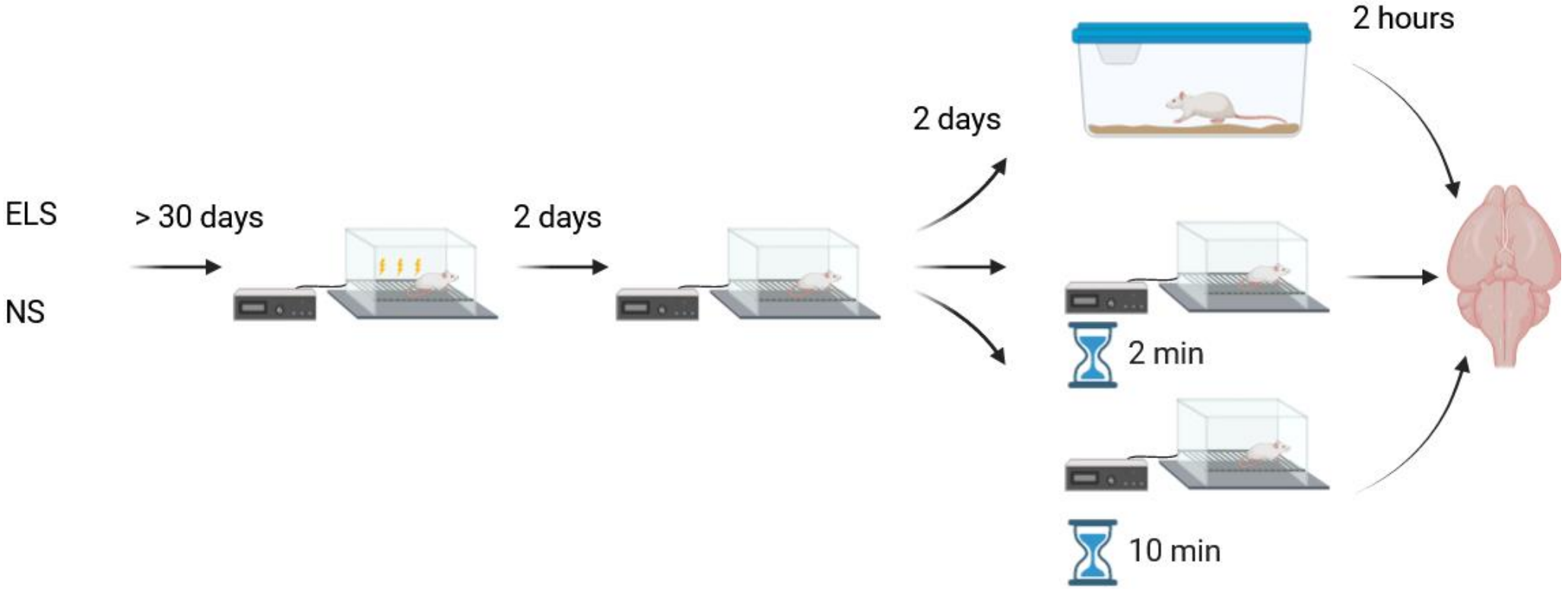


Figure 37:Behavioural schematic. Animals are exposed to either early life stress (ELS) or non-stress (NS). Animals are exposed to CFC and either extinction or recall (described in Chapter 2), a further group remains in the home cage and defined as no recall NR. Brains were collected two hours following extinction, recall or NR.

5.2.3 Brain Dissection and Punch Acquisition

Detailed descriptions of the tissue dissection and punch technique are given in Chapter 2 subsection 2.6. Briefly, brains from animals subjected to behavioural tests were dissected, focusing on a subset due to time constraints. Using a rodent brain matrix and coordinates from a stereotactic atlas, slices were taken to isolate areas of interest in the mPFC and hippocampus. After identifying specific brain structures, slices were flash frozen to a temperature where punches could be easily taken. Punches were taken from the PL cortex, IL cortex, and various hippocampus regions using the Palkovits punch technique, with only the IL cortex and CA1 regions analysed further. All punches were stored at -75°C.

5.2.4 Protein Extraction

Tissue punches were homogenised in ice-cold lysis buffer, dounced, and centrifuged. Protein concentrations were measured using a Pierce BCA assay.

5.2.5 Cytokine Analysis

Samples with high protein concentrations were diluted and all samples analysed using a multiplex bead array outsourced to Indoor Biotechnologies ltd. Further details are given in Chapter 2, subsection 2.6.4. Results, normalized to protein concentrations, were reported in picograms per sample.

Table 15: Mean protein concentrations from three triplicates, after dilution to optimal concentration required, for each sample processed. Condition refers to either the extinction control group (2) the extinction trained group (10) or the no recall group (NR). PFC - IL = infralimbic subregion of the pre-frontal cortex, HIP – CA1 = CA1 region of the hippocampus.

Sex	Stress	Condition	Brain region	Average protein concentration mg/ml
Female	ELS	2	HIP -CA1	0.72
Female	ELS	10	HIP -CA1	0.72
Female	ELS	NR	HIP -CA1	0.81
Female	NS	2	HIP -CA1	0.63
Female	NS	10	HIP -CA1	0.84
Female	NS	NR	HIP -CA1	0.73
Male	ELS	2	HIP -CA1	0.64
Male	ELS	10	HIP -CA1	0.64
Male	ELS	NR	HIP -CA1	0.66
Male	NS	2	HIP -CA1	0.79
Male	NS	10	HIP -CA1	0.56
Male	NS	NR	HIP -CA1	0.74
Female	ELS	2	PFC - IL	0.53
Female	ELS	10	PFC - IL	0.54
Female	ELS	NR	PFC - IL	0.71
Female	NS	2	PFC - IL	0.51
Female	NS	10	PFC - IL	0.56
Female	NS	NR	PFC - IL	0.49
Male	ELS	2	PFC - IL	0.63
Male	ELS	10	PFC - IL	0.63
Male	ELS	NR	PFC - IL	0.50
Male	NS	2	PFC - IL	0.37
Male	NS	10	PFC - IL	0.51
Male	NS	NR	PFC - IL	0.47

Table 16: List of detected analytes in multiplex assay and manufacturers (BIO-RAD) detection sensitivity (pg/ml)

Abbreviation	Full name	Manufacturer stated detection limit (pg/ml)
GM CSF	Granulocyte-macrophage colony-stimulating factor	0.6
GRO KC	Keratinocyte chemoattractant (KC)/ human growth-regulated oncogene (GRO)	0.6
IFN-γ	Interferon gamma	1
IL-10	Interleukin 10	5
IL-12	Interleukin 12	0.7
IL-13	Interleukin 13	0.9
IL-17A	Interleukin 17	0.1
IL-1α	Interleukin 1 alpha	1
IL-1β	Interleukin 1 beta	2
IL-4	Interleukin 4	1
IL-5	Interleukin 5	6
IL-6	Interleukin 6	10
IL-7	Interleukin 7	0.4
M-CSF	Macrophage colony stimulating factor	0.4
MCP-1	Monocyte chemoattractant protein 1	4
MIP1α	Macrophage Inflammatory Protein 1alpha	12
MIP3α	Macrophage Inflammatory Protein 3 alpha	0.7
RANTES	Regulated on activation, normal T cell expressed and secreted	3
TNF-α	Tumour necrosis factor alpha	3
VEGF	Vascular endothelial growth factor	0.3
IL18	Interleukin 18	4
IL-2	Interleukin 2	3

5.2.6 Statistical analysis.

Freezing behaviour

Behavioural data was analysed as described in Chapter 3. Freezing was measured for CFM acquisition, recall and extinction control or extinction training. For longitudinal analysis namely, acquisition and extinction training a general linear model was fit and for other analysis, namely CFM recall, ANOVA was fit.

Cytokine analysis

Data was acquired and collected, using Bio-Plex Manager software Bio-Plex SNP Manager data analysis utility, by Indoor Biotechnologies ltd (https://inbio.com). Reliability of the plate was assessed using a duplicate of one sample, coefficient of variation (CV) values (defined here below in Equation iii) that were less than 15% show reliable results.

$$CV = \left(\frac{Observed}{Expected}\right) * 100$$

Equation iii

Any values outside >+/-30%, were discarded as out of range. All targets besides IL-12 showed a high reliability. All statistical analysis was conducted using R running in R-studio 1.4.1. Statistical differences in protein and cytokine/chemokine concentration levels, between Sex, Stress and Condition factors, using a full factorial model, were analysed via ANOVA, post hoc testing was conducted with Tukey tests. All freezing analysis was conducted in the same way as described in Chapter 3.

5.3 Results

5.3.1.1 Freezing behaviour.

5.3.1.2 Contextual fear memory acquisition.

There was an effect of foot shock on freezing behaviour with animals freezing more during post shock than pre shock (Estimate = 65.8325, t (362) = 18.800, p <0.05, mixed linear model). There was a sex x stress x time-point interaction (Estimate = 14.4301, t (362) = 2.062, p <0.05, mixed linear model). Splitting the data into pre and post shock time points and fitting an ANOVA reveals that there was a sex difference in post shock freezing levels with males freezing more than females (Figure 38a, Table 17).

Table 17: Inferential analysis, via mixed linear model, of CFM acquisition. Pre and post are analysed separately. Green denotes a significant effect.

Factor	Pre	Post
Sex	F (1,181) = 0.001, p > 0.05	F (1,181) = 7.332, p < 0.05
Stress	F (1,181) = 0.208, p > 0.05	F (1,181) = 0.164, p > 0.05
Sex X Stress	F (1,181) = 0.542, p >0.05	F (1,181) = 3.766, p >0.05

5.3.1.3 Contextual fear memory recall following consolidation.

Stress and Sex influence freezing at recall following CFM consolidation (Figure 38b). There was no interaction effect of sex and stress on freezing at CFM recall (F (1,116) = 0.328, p > 0.05, ANOVA). There was a main effect of Sex (F (1,116) = 13.953, p < 0.01, ANOVA), with male animals freezing more following CFM consolidation than females. There was a main effect of Stress (F (1,116) = 6.100, p < 0.05, ANOVA) where animals which had experienced ELS showed greater levels of freezing behaviour than animals which did not.

5.3.1.4 Extinction training

There was no interaction between Sex and Stress (Estimate = -4.486, t (101.94) p > 0.05, mixed linear model). There was a main effect of extinction training (Estimate = -17.308, t (172) = -2.405, p < 0.05, mixed linear model, Figure 38c) with animals overall freezing less at the 10-minute recorded time point than the 2-minute time point. There was a main effect of Sex (Estimate = 21.013, t (101.94) = 2.139, p <0.05, mixed linear model) with males freezing more than females. There was no main effect of Stress (Estimate = 17.436, t (101.94) = 1.243, p > 0.05, mixed linear model). Individually, male non-stressed show an effect of extinction with animals freezing less at ten minutes compared to two minutes. Female animals exposed to ELS and male animals exposed to ELS did not show a decrease in freezing at ten minutes compared to two minutes (Table 18).

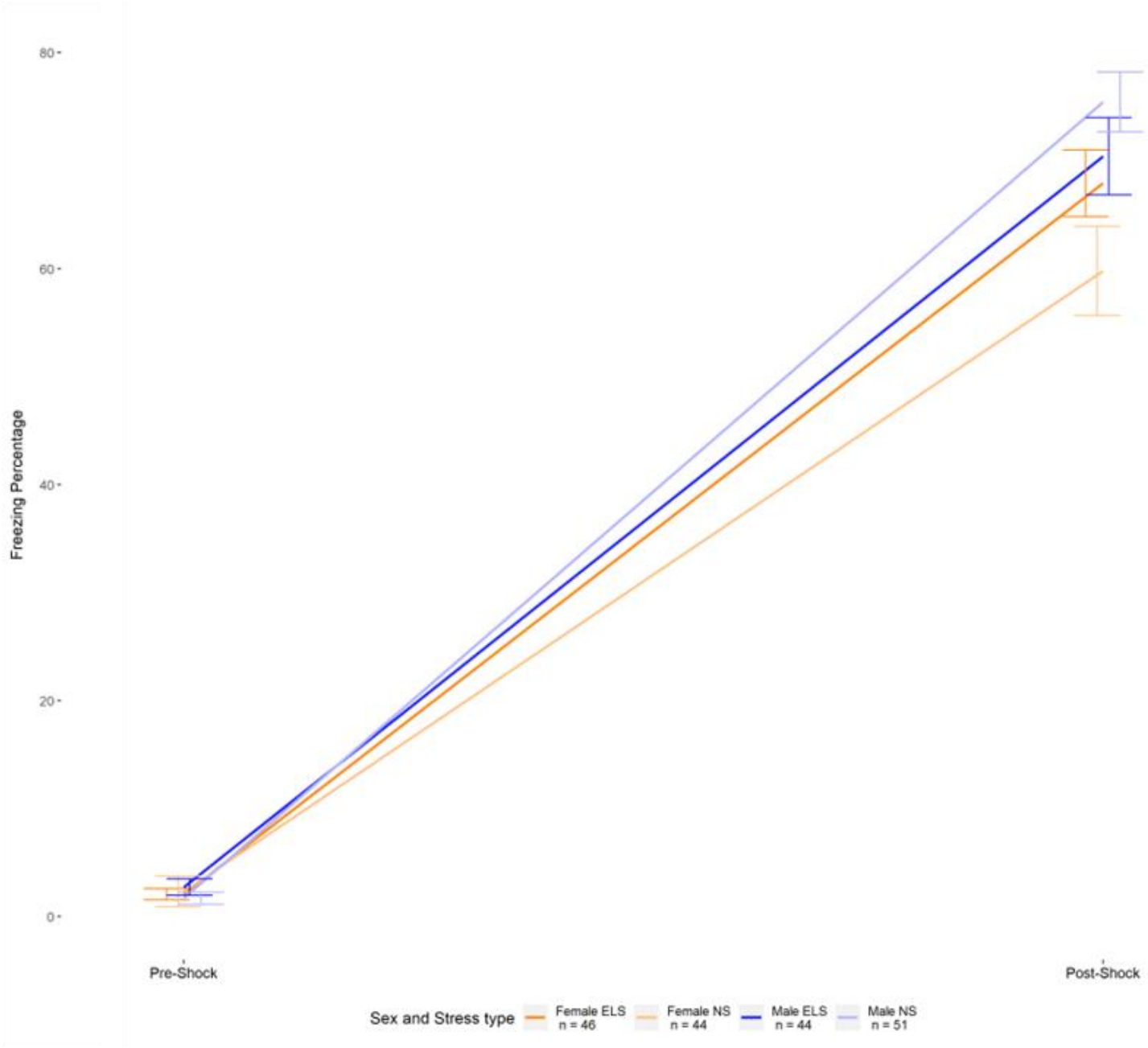
Table 18: Inferential analysis of freezing behaviour during extinction training. Green shows significant effects. Orange shows trend.

Group	Statistics
Male ELS	F (1,32) = 3.11, p = 0.087
Male NS	F (1, 22) = 8.40, p < 0.05
Female ELS	F (1, 24) = 2.72, p > 0.05
Female NS	F (1,8) = 0.37 p > 0.05

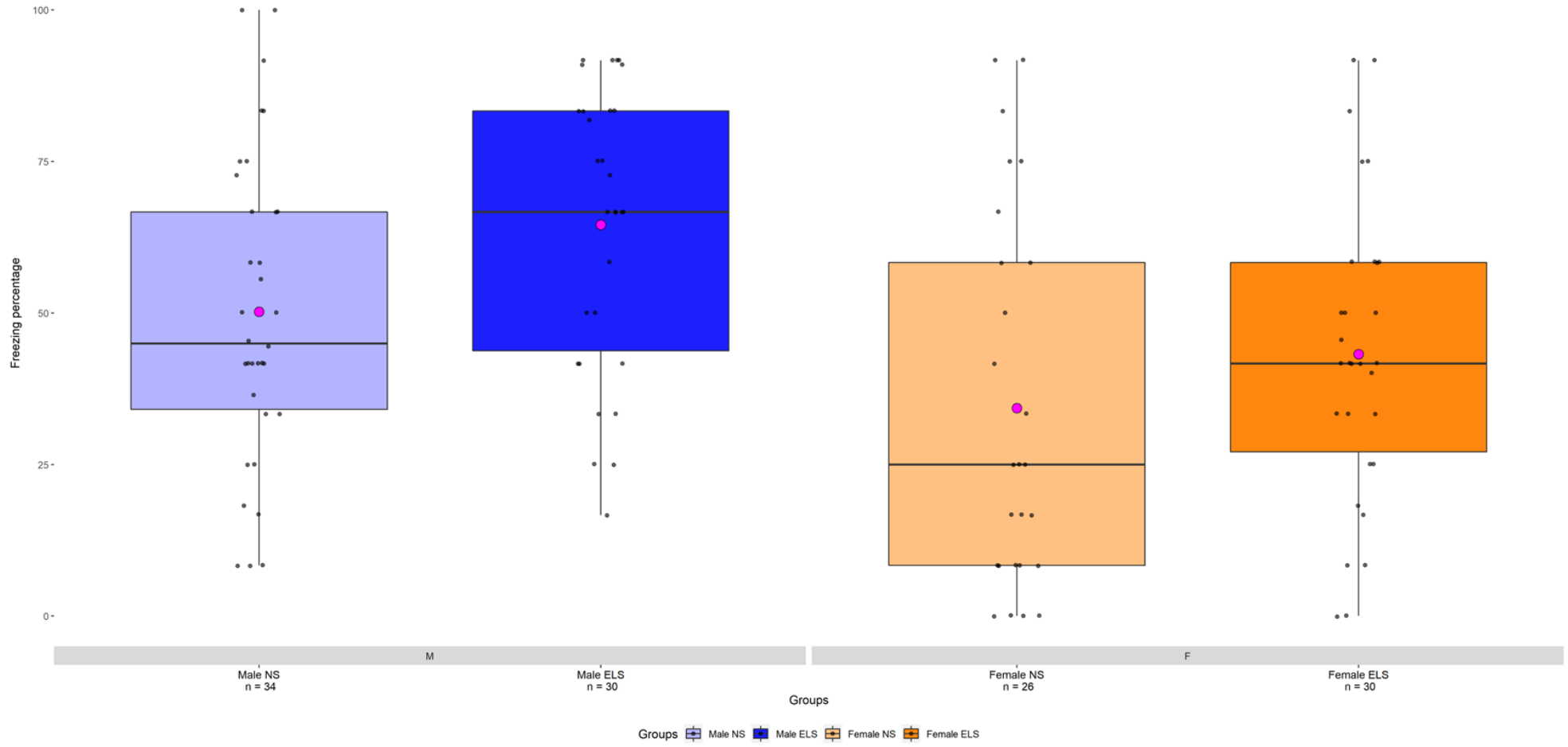
5.3.1.5 Behaviour overview:

Overall, animals freeze more after the shock, compared to pre shock during CFC. There is an effect of sex on post shock with males freezing more than females. At CFM recall, there was a main effect of sex and a main effect of stress, with animals that had experienced early life stress freezing more than animals that had not experienced stress, and males freezing more than females. During extinction training males not exposed to stress showed extinction although males exposed to ELS show a trend towards extinction.

a


80
60
40
20
0
Freezing Percentage
Pre-Shock
Post-Shock
Sex and Stress type
Female ELS n = 46
Female NS n = 44
Male ELS n = 44
Male NS n = 51

b


100
75
50
25
0
Freezing percentage
M
F
Male NS
n = 34
Male ELS
n = 30
Female NS
n = 26
Female ELS
n = 30
Groups
Groups
Male NS
Male ELS
Female NS
Female ELS

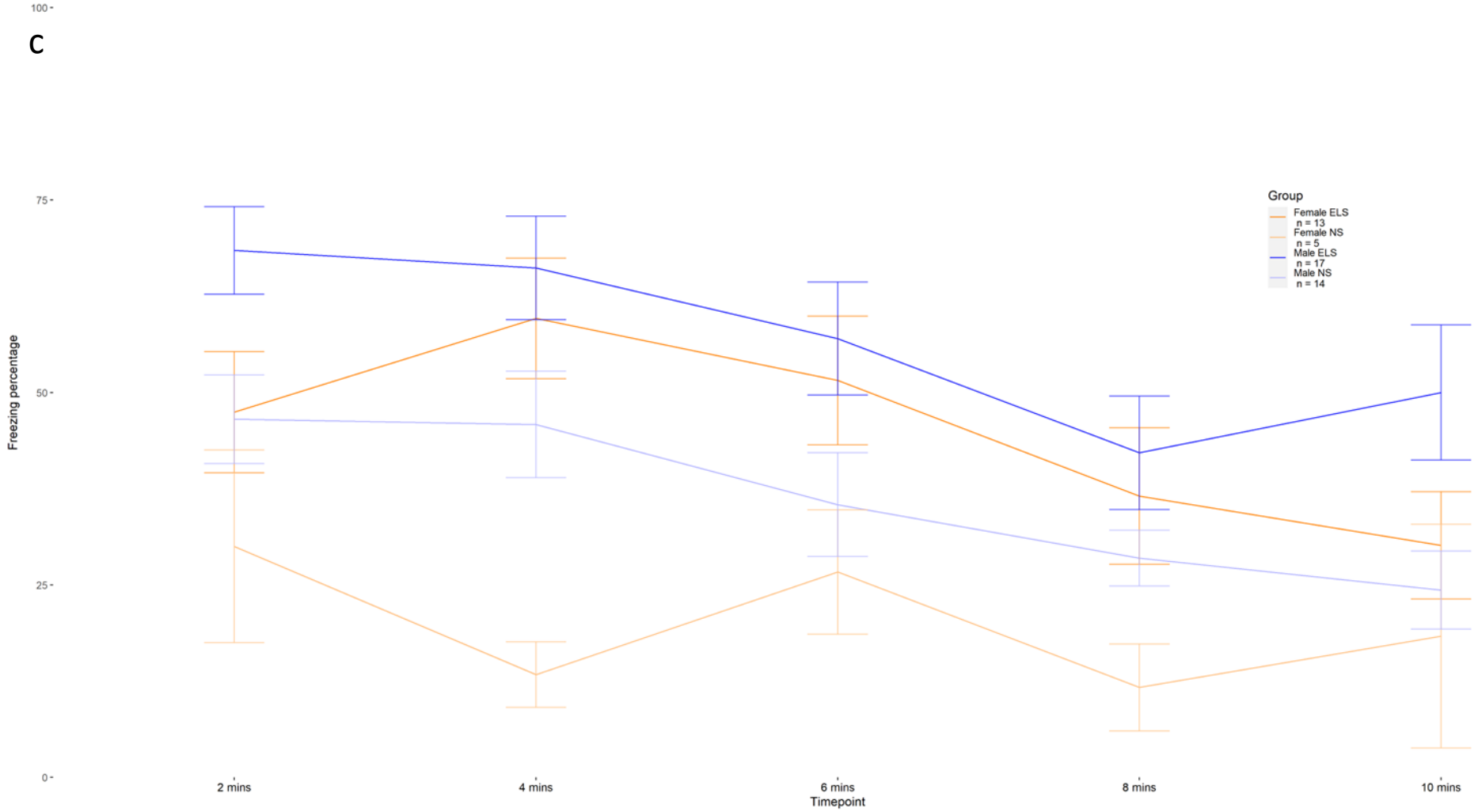

C
100
75
50
25
0
Freezing percentage
2 mins
4 mins
6 mins
8 mins
10 mins
Timepoint
Group
Female ELS
n = 13
Female NS
n = 5
Male ELS
n = 17
Male NS
n = 14

Figure 38: Behavioural analysis of CFM acquisition (a), recall (b) and extinction training (c) following exposure to early life stress (ELS) or no stress(NS). Males are shown in blue (dark = ELS, light = NS) and females in orange (dark = ELS, light = NS). Values at line ends (a,c) shown as means, error bars represent mean ± SEM. In bar and whisker blot (b). Black dots depict individual data-points. Horizontal lines show median with IQR as the hinges, top whisker = 1.5 * IQR up to maximum, bottom whisker = 1.5* IQR to minimum. Mean = solid magenta circle. Group numbers shown as n.

5.3.2 Cytokine analysis.

Number of animals per group is given in Table 19.

Table 19: Number of animals per group.

Sex	Stress	Condition	mPFC n	Hippocampus n
Female	ELS	10	3	3
Female	ELS	2	3	3
Female	ELS	NR	3	3
Female	NS	10	3	3
Female	NS	2	3	3
Female	NS	NR	4	4
Male	ELS	10	3	3
Male	ELS	2	3	3
Male	ELS	NR	3	3
Male	NS	10	3	3
Male	NS	2	3	3
Male	NS	NR	3	3

5.3.2.1 Overall protein levels.

There were no differences in raw protein concentration between levels of Sex (male or female), Stress (ELS or NS) or Condition (2 minute, 10 minute or NR, all $p > 0.05$, ANOVA) and there are no interactions present (Table 20, Figure 39).

Table 20: Inferential analysis of raw protein concentrations. There are no differences between any factor levels.

Factors	mPFC (IL)	Hippocampus (CA1)
Sex	$F(1,25) = 0.39$, $p > 0.05$	$F(1,25) = 3.87$, $p > 0.05$
Stress	$F(1,25) = 3.48$, $p > 0.05$	$F(1, 25) = 0.22$, $p > 0.05$
Condition	$F(2,25) = 0.25$, $p > 0.05$	$F(2, 25) = 0.48$, $p > 0.05$
Sex X Stress	$F(1,25) = 0.29$, $p > 0.05$	$F(1,25) = 0.96$, $p > 0.05$
Sex X Condition	$F(2,25) = 0.47$, $p > 0.05$	$F(2, 25) = 3.01$, $p > 0.05$
Stress X Condition	$F(2,25) = 0.23$, $p > 0.05$	$F(2,25) = 0.07$, $p > 0.05$

Sex X Stress X Condition	F (2,25) = 1.34, p > 0.05	F (2, 25) = 3.31, p > 0.05

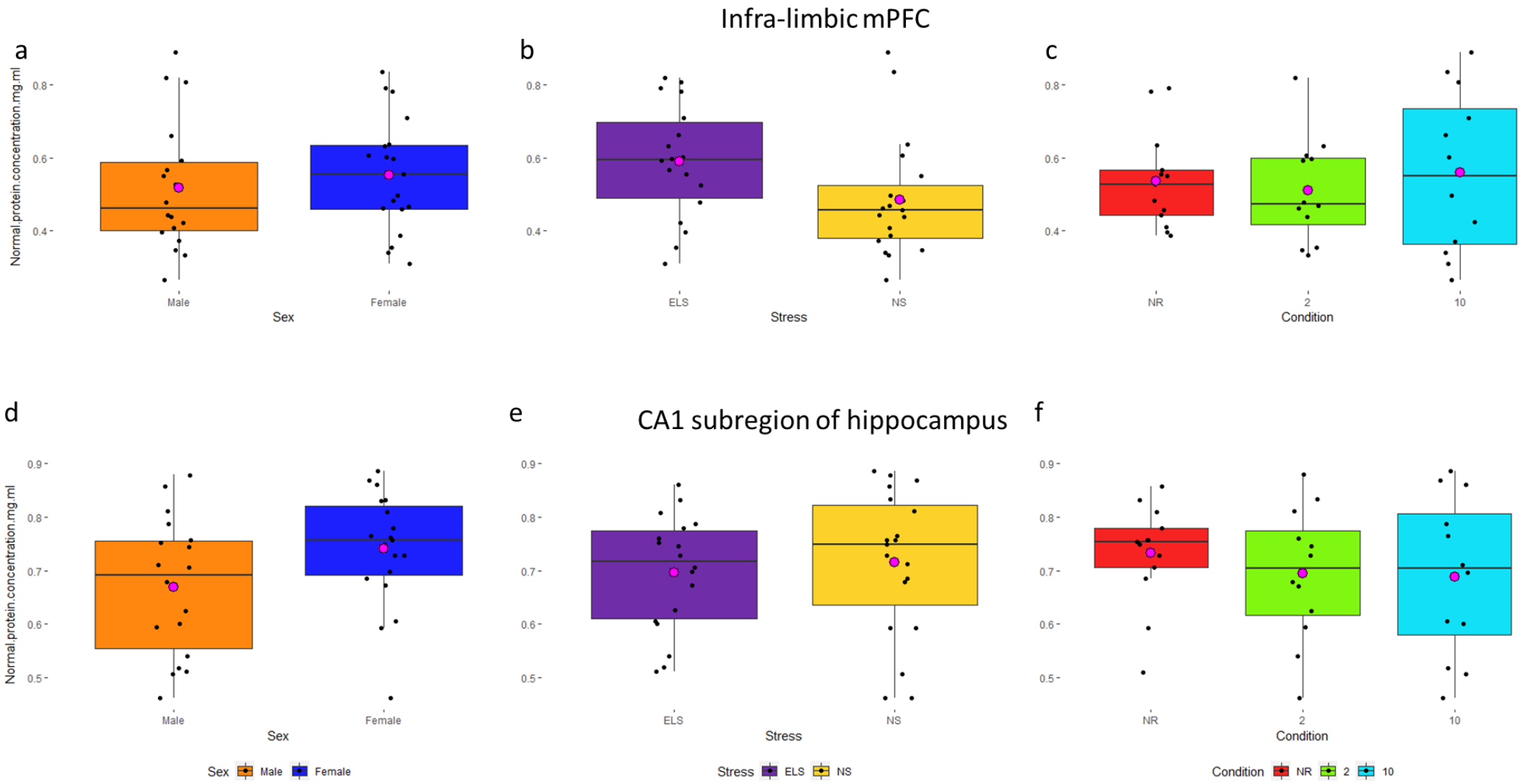


Figure 39:Protein concentration comparisons between either sex (male or female, shown in figures: a and d) stress (Early life stress, ELS; or non-stressed, NS; shown in figures: b and e) or condition (2-minute, 10 minute or no recall (NR) shown in figures: c and f). The top row (a,b,c) shows protein concentration levels in the infra-limbic (IL) area of the m PFC. The bottom row (d,e,f) shows protein concentration levels in the CA1 sub-area of the hippocampus Horizontal

lines show median with IQR as the hinges, the mean is shown as a solid magenta circle, top whisker shows up to 1.5 X IQR up to the maximum level bottom whisker shows down to 1.5* IQR. Infra-limbic = Infra-limbic subregion of the PFC. Black dots shows individual sample protein concentrations.

5.3.2.2 Cytokine concentrations in the in the CA1 subregion of the hippocampus.

There was an interaction between Sex, Stress and Condition for the following cytokines GM.CSF, IL-10, IL-12, IL-18, IL-1β, IL-5, IL-6, IL-7, MCP-1 (shown in Table 21 and Figure 42).

Figure 40: Cytokine concentration in the CA1 sub-region of the hippocampus across Sex, Stress and condition in groups that have a significant interaction. Sex and stress groups are shown on the x axis and condition is given as colour. Condition refers to the extinction condition that the animals undertook. NR is no recall (shown in red), 2 is extinction control (shown in green) and 10 is extinction trained (shown in blue). Faded dots depict individual data-points. Group numbers shown as n. Note: Y-axis scales vary between plots to optimize visibility of data distribution. Full names are presented in Table 16.

Chapter 5: Cytokine analysis.

Table 21: F values for inferential analysis of cytokines in CA1 sub-region of the hippocampus. Green cells show significant differences in extinction condition. Cells in green are $p < 0.05$. Cells in orange are trending towards a significant difference and p value is presented. Cells in white are $p > 0.05$. Cytokines that are highlighted in blue show either a significant main effect or significant interaction effect.

Cytokine	Differences in Sex df = (1,25)	Differences in Stress df = (1,25)	Differences in extinction condition df = (2,25)	Sex * Stress df = (1,25)	Sex * Condition df = (2,25)	Stress * Condition df(2,25)	Sex * Stress * Condition df = (2,25)
GM.CSF	0.85	0.22	0.14	0.00	0.47	0.13	4.17
GRO KC	1.17	0.00	3.94	0.08	0.48	0.30	2.34
IFN – γ	2.57	0.00	0.89	0.00	0.32	0.72	2.61
IL – 10	0.55	1.66	2.31	0.23	0.09	0.81	4.85
IL – 12	1.87	0.11	6.77	0.80	0.76	1.25	3.75
IL – 13	0.09	0.06	3.33, p = 0.052	0.19	1.26	0.15	1.30
IL - 17	3.38 p = 0.07	0.01	0.50	0.07	1.32	0.25	1.84
IL- 18	6.01	0.29	0.33	0.57	3.40	0.10	3.37
IL 1α	0.40	0.09	4.01	0.34	0.72	0.53	1.67
IL – 1β	0.60	1.29	0.14	0.00	2.13	0.05	7.18
IL -4	0.06	2.47	0.38	0.48	0.07	0.97	1.03
IL -5	0.47	0.09	7.00	2.28	2.53	0.91	4.26
IL – 6	3.02 p = 0.09	0.21	3.82	0.00	1.78	0.18	2.84, p = 0.07
IL -7	1.58	0.00	0.22	0.00	0.91	0.05	4.18

M CSF	1.74	0.61	4.03	0.00	0.93	0.31	2.03
MCP 1	2.60	0.12	0.71	0.30	1.21	0.08	3.02, p = 0.07
MIP 1 α	0.57	0.01	4.49	1.13	0.77	0.19	2.22
MIP 3 α	0.13	0.11	1.70	0.39	0.62	0.23	1.61
RANTES	1.86	0.00	3.12	0.09	0.95	0.68	1.48
TNF – α	0.59	2.93 p = 0.09	0.57	0.50	1.12	0.86	1.78
VEGF	1.26	0.01	2.35	0.05	0.84	0.21	1.17

Splitting the groups by sex and by stress and examining the effect of condition shows significant effects on a profile of cytokines for males exposed to ELS and females non-stressed, shown in Table 22. For males exposed to ELS multiple comparisons (with Tukey multiple comparison correction) find trends towards an effect for IL-12, with a trend between extinction trained and no-recall and extinction trained and extinction control. There was a lower concentration of IL-12 in the extinction trained group than the NR or extinction control group. There was a trend for an effect between extinction control and extinction trained for IL-6, there was a lower concentration of IL-6 in the extinction trained group compared to the extinction control group. There are no significant multiple comparison differences between condition for IL-10 concentration (Table 23).

Table 22: Independent analysis, with ANOVA comparison, per group of the effect of Condition.

Male ELS	Male NS	Female ELS	Female NS
IL -12	None	None	Il10 (trends)
IL 6			IL-1β (trends)
IL-10 (trends)			IL -5

Table 23: Multiple comparisons for groups with significant effects of condition on cytokine concentration. NR = no recall, 2 = Extinction control, 10 = Extinction training.

Group	M ELS			F NS		
Cytokine	IL-12	IL-6	IL-10	IL-10	IL-5	IL-1β
Multiple comparison	NR - 2	NR - 2	NR - 2	NR - 2	NR - 2	NR - 2
	NR - 10	NR - 10	NR - 10	NR - 10	NR - 10	NR - 10
	2-10	2-10	2-10	2-10	2-10	2-10

For females which were non-stressed multiple comparisons find trends towards an effect for IL-1β, with a trend towards a difference between the extinction control and

extinction trained groups with a lower concentration in the extinction trained group. There was a trend towards a difference between the extinction control and extinction trained group on IL-10 concentration level with extinction trained showing a lower level of concentration than extinction control. There is a significant effect of condition on concentration of IL-5 with the extinction control group showing a lower level of IL-5 concentration than the extinction trained group (Table 23).

There were main effects of extinction condition present in seven of the cytokines (GRO KC, IL – 12, IL – 1α, IL – 5, IL – 6, MCSF, MIP1α Figure 41a and in Table 21). For each of these the effect (following Tukey multiple comparison corrections) was consistently a difference between extinction control and extinction training with a higher concentration of cytokines in extinction control. Analysis of the interactions between Sex, stress and extinction for the other cytokines/chemokines did not show any significant effects besides the condition effects present in Table 21. For the other cytokines there were no effects of extinction condition, these are presented in Figure 41 and Table 21.

Only IL-18 showed a sex difference with males showing a higher concentration of IL-18 than females (Figure 42). There was no difference in cytokine concentration between extinction condition for GM CSF, IFN-y, IL-10, IL-17, IL-1b, IL-4, IL-7, MCP-1, MIP3-a, RANTES, TNF-a and VEGF (Table 24).

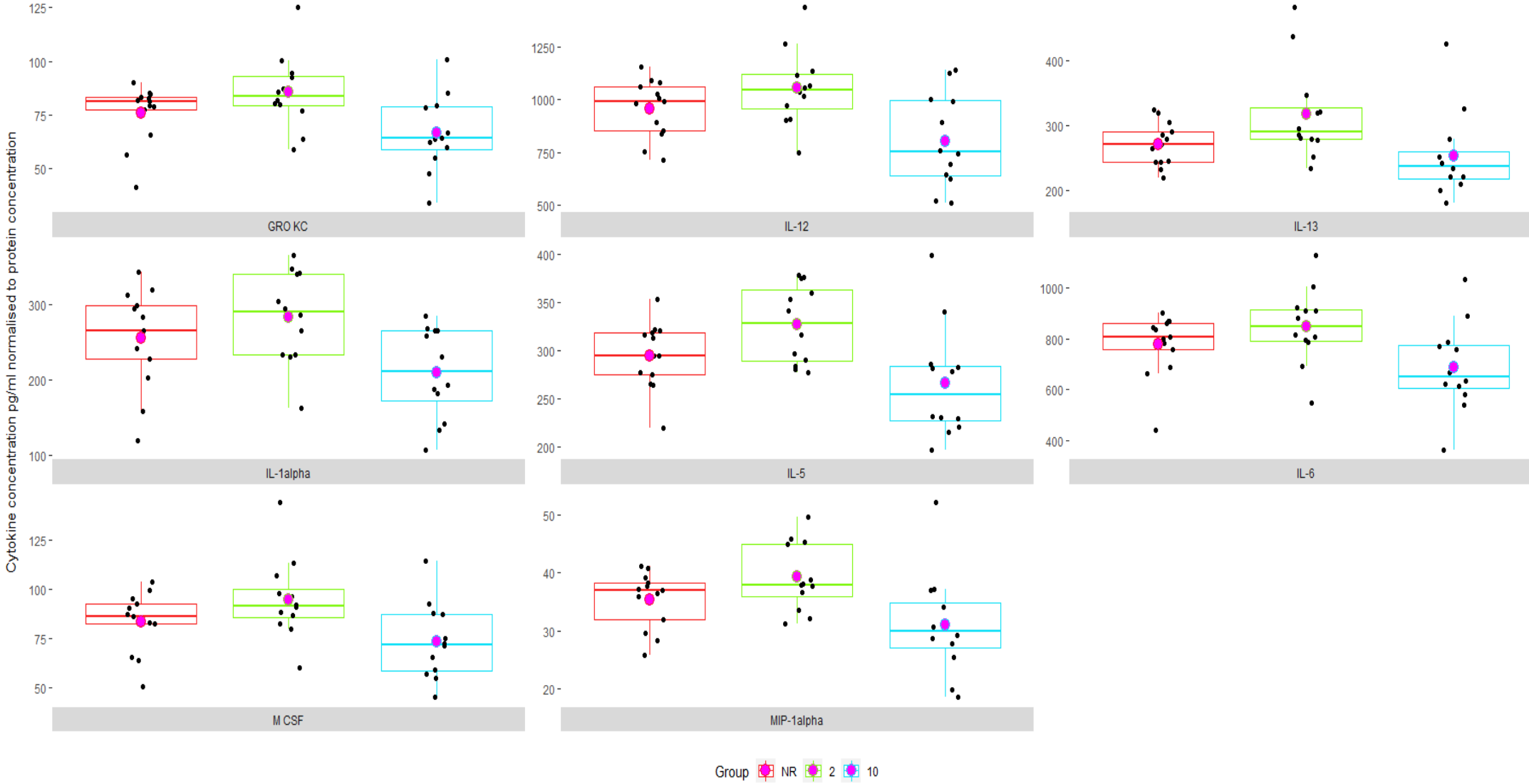
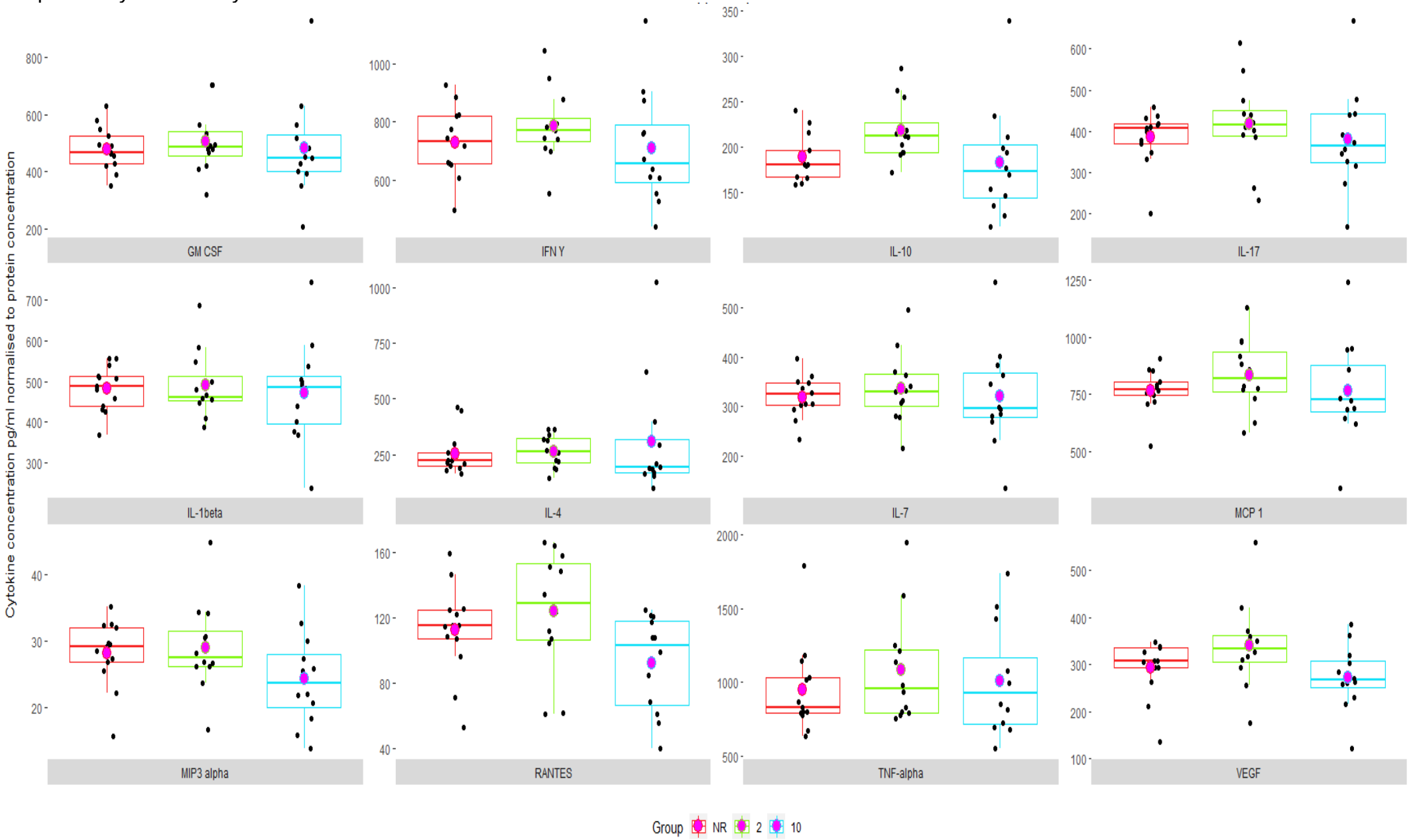


Figure 41: Cytokine concentration in the CA1 sub-region of the hippocampus. a) Cytokines which showed a significant difference between the extinction control (2) and the extinction training group(10). b) Cytokines which did not show a significant difference between the extinction control (2) and the extinction training group(10). Condition refers to the extinction condition that the animals undertook. NR is no recall (shown in red), 2 is extinction control (shown in green) and 10 is extinction trained (shown in blue). (Horizontal lines show median with IQR as the hinges, the mean is shown as a solid magenta circle, top whisker shows up

to 1.5 * IQR up to the maximum level bottom whisker shows down to 1.5* IQR). Black dots depict individual data-points. Group numbers shown as n. Note: Y-axis scales vary between plots to optimize visibility of data distribution. Full names are presented in Table 16.



continued from previous page (2 of 2)

Table 24: Post hoc analysis of significant results from Table 21.Green shows significant post hoc testing, orange shows trend. NR = no recall, 2 = extinction control, 10 = extinction trained

	Post hoc results		
Cytokine	NR - 2	2 - 10	NR -10
GRO KC	t (34) = -1.47, p > 0.05	t (34) = 2.87, p <0.05	t (34) = 1.46, p >0.05
IL - 12	t (34) = -1.34, p > 0.05	t (34) = 3.39, p <0.05	t (34) = 2.12, p > 0.05
IL – 1α	t (34) = -1.11, p >0.05	t (34) = 2.90, p <0.05	t (34) = 1.85, p >0.05
IL – 5	t (34) = -1.79, p >0.05	t (34) = 3.32, p <0.05	t (34) = 1.59, p >0.05
IL - 6	t (34) = -1.19, p >0.05	t (34) = 2.65, p <0.05	t (34) = 1.51, p >0.05
MCSF	t (34) = -1.56, p >0.05	t (34) = 2.84, p <0.05	t (34) = 1.35, p >0.05
MIP1α	t (34) = -1.49, p >0.05	t (34) = 3.04, p <0.05	t (34) = 1.60, p >0.05

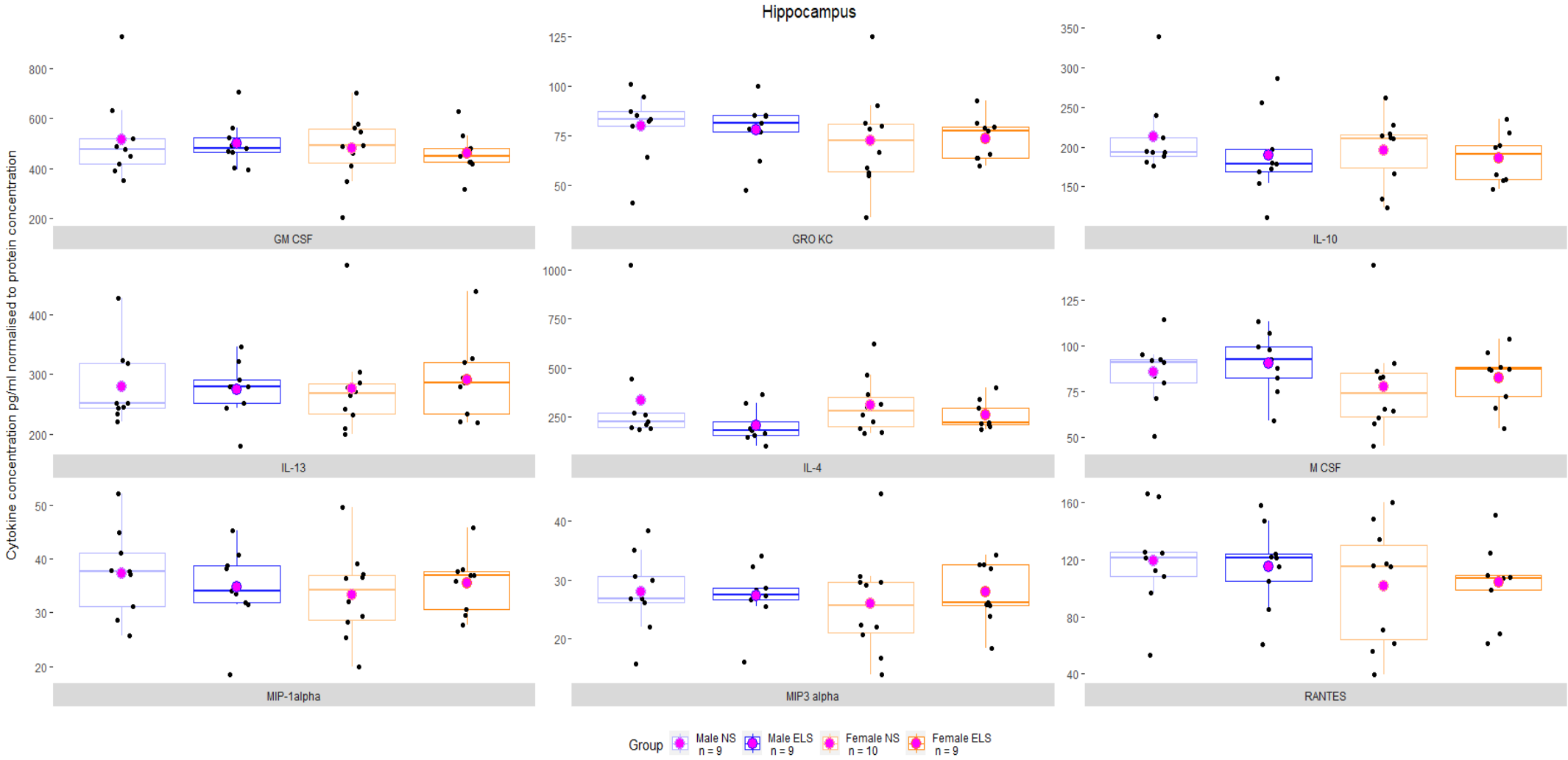
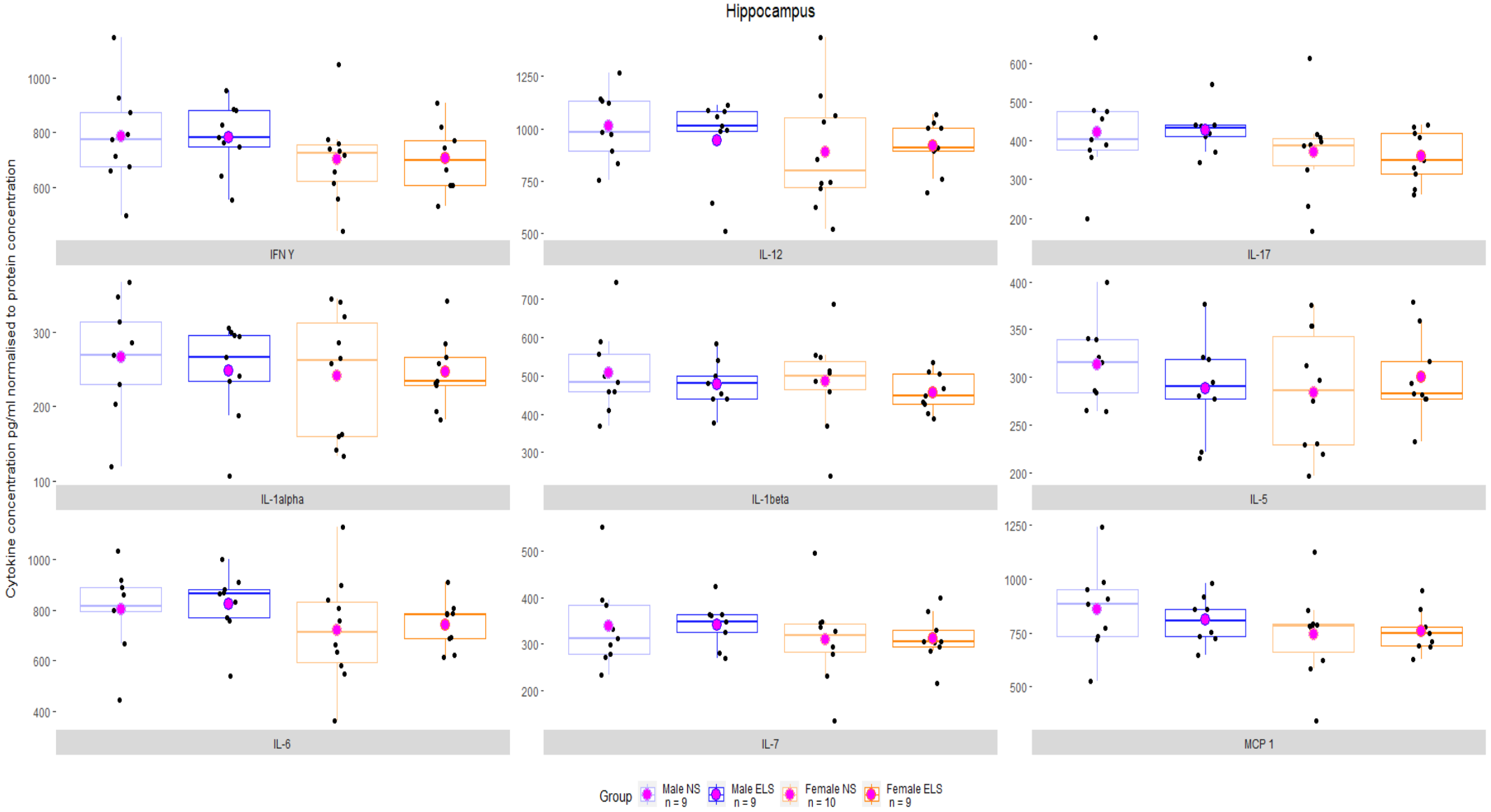
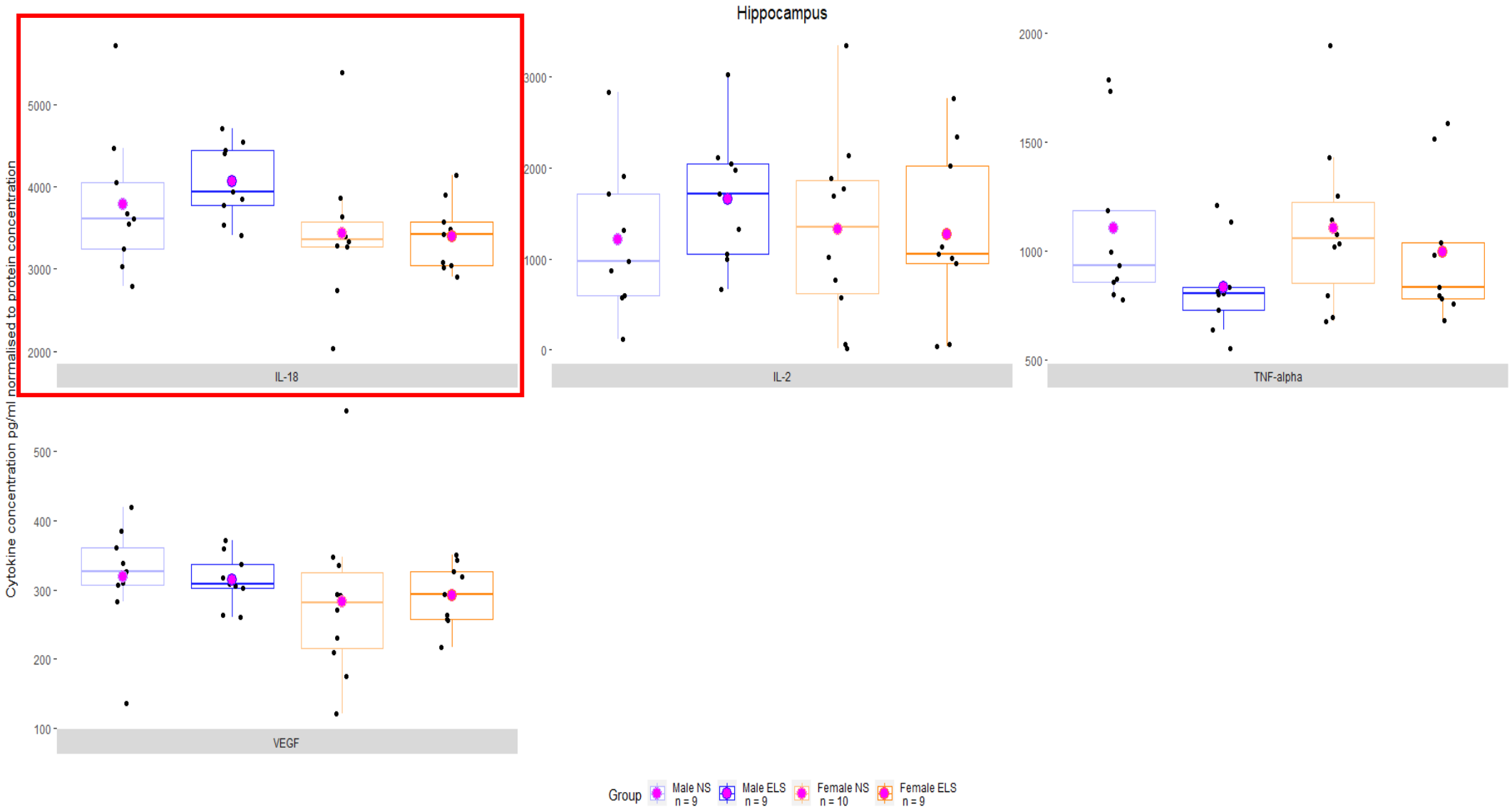


Figure 42 : Cytokines analysed in punches from the CA1 sub region of the hippocampus: Here extinction conditions (no recall, extinction control and extinction training) are combined. Males are shown in shades of blue with dark being ELS and light blue being non stressed (NS). Female animals are shown in shades of orange with dark orange being ELS and light orange being NS. IL-18 which is shown in the red box shows a significant difference between males and females. Animals which have undergone 2-minute non- extinction are shown in blue with animals which went through 10-minute extinction in yellow (Horizontal lines show median with IQR as the hinges, the mean is shown as a solid magenta circle, top whisker shows up to 1.5 * IQR up to the maximum level bottom whisker

shows down to 1.5* IQR). Black dots depict individual data-points. Group numbers shown as n. Note: Y-axis scales vary between plots to optimize visibility of data distribution.



continued from previous page (2 of 3)



continued from previous page (3 of 3)

5.3.2.3 Cytokine concentrations in the IL sub-region of the mPFC

There were no interactions between any of the factors (Sex, Stress or Condition) present on cytokine concentration in the IL–sub region of the mPFC (Figure 43, Table 25). There were also no main effects of Sex, Stress or Condition on any cytokine/chemokine concentrations in the IL-subregion of the mPFC (Figure 44, Table 25).

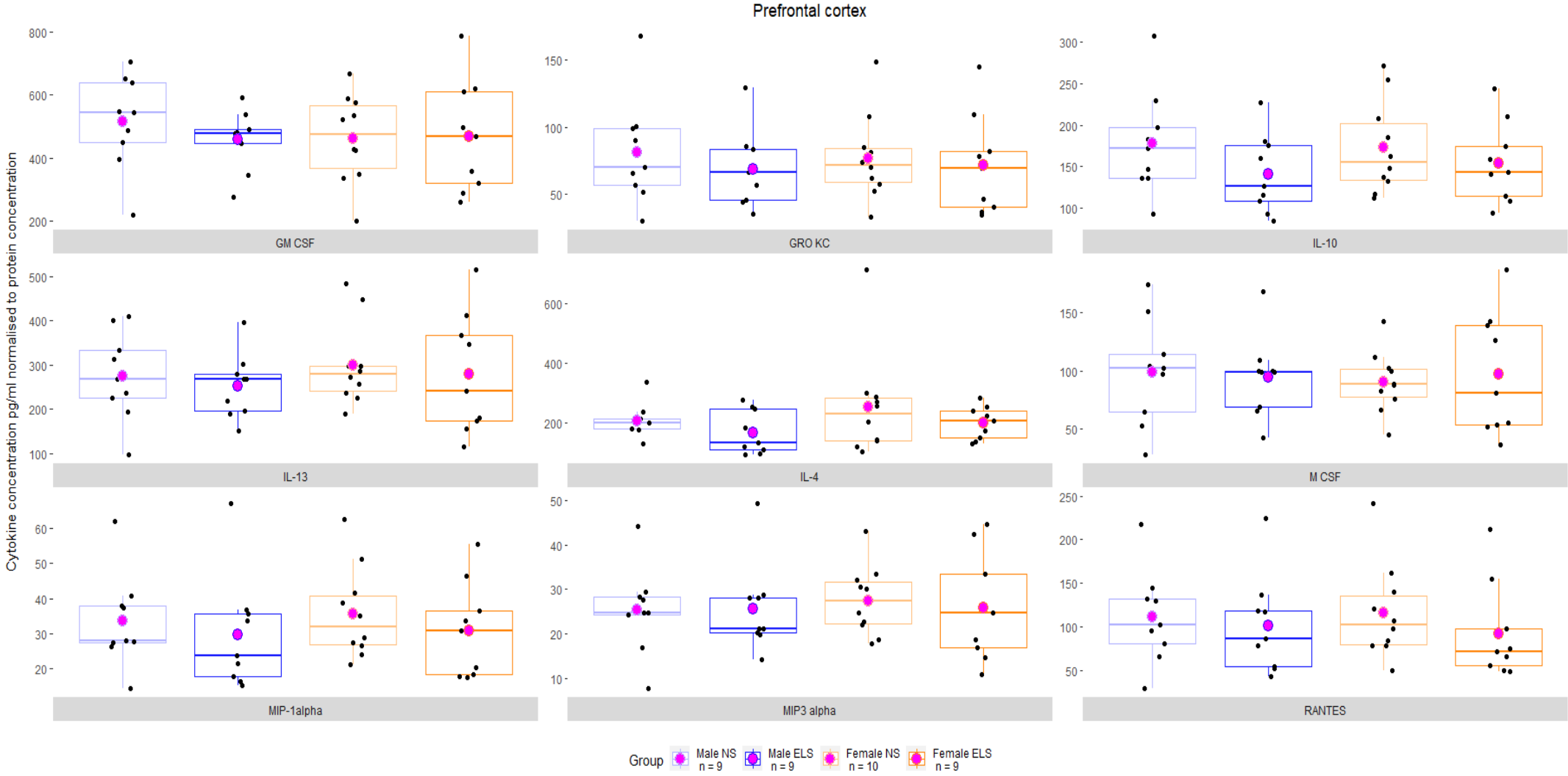
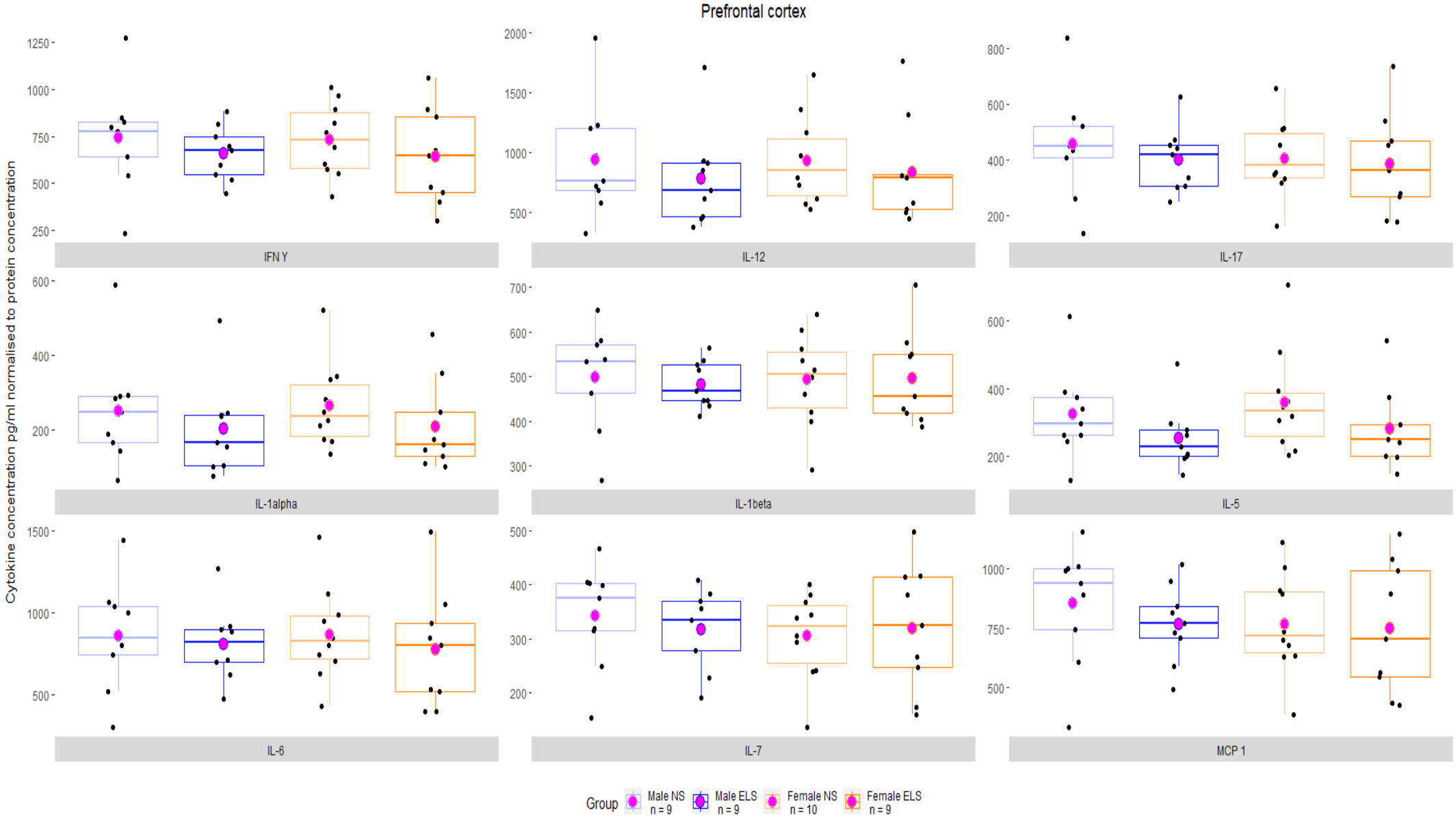
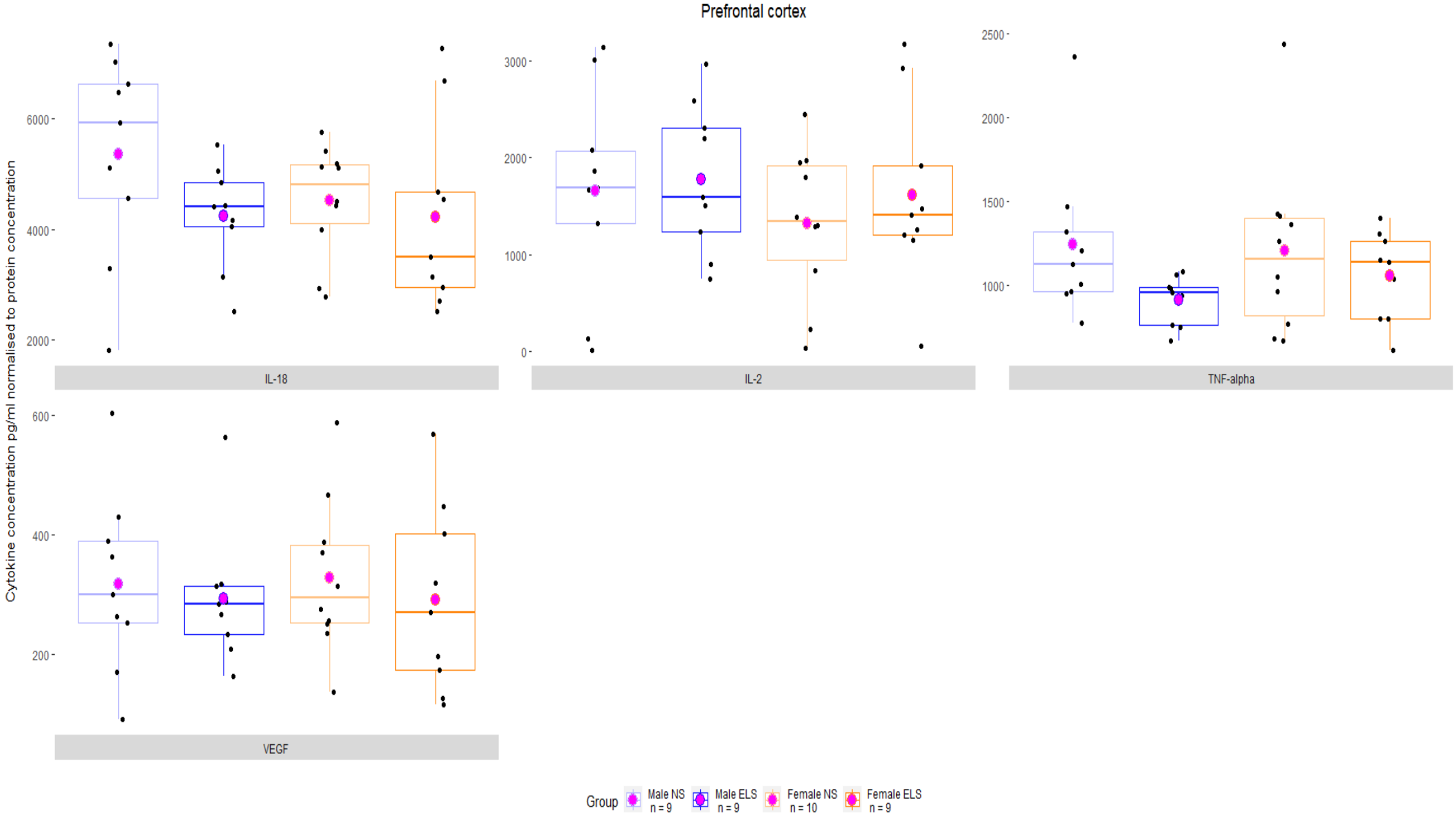


Figure 43 Cytokines concentrations analysed in punches from the infra-limbic sub region in the pre-frontal cortex (1 of 3 continued on next page). Here extinction conditions (no recall, extinction control and extinction training) are combined. Males are shown in shades of blue with dark being ELS and light blue being non stressed (NS). Female animals are shown in shades of orange with dark orange being ELS and light orange being NS. Animals which have undergone 2-minute non- extinction are shown in blue with animals which went through 10-minute extinction in yellow (Horizontal lines show median with IQR as the hinges, the mean is shown as a solid magenta circle, top whisker shows up to 1.5 * IQR up to the maximum level bottom whisker shows down to 1.5* IQR). Black dots

depict individual data-points. Group numbers shown as n. See Table 2 for full names to cytokine abbreviations Note: Y-axis scales vary between plots to optimize visibility of data distribution.



continued from previous page (2 of 3).



continued from previous page(3 of 3).

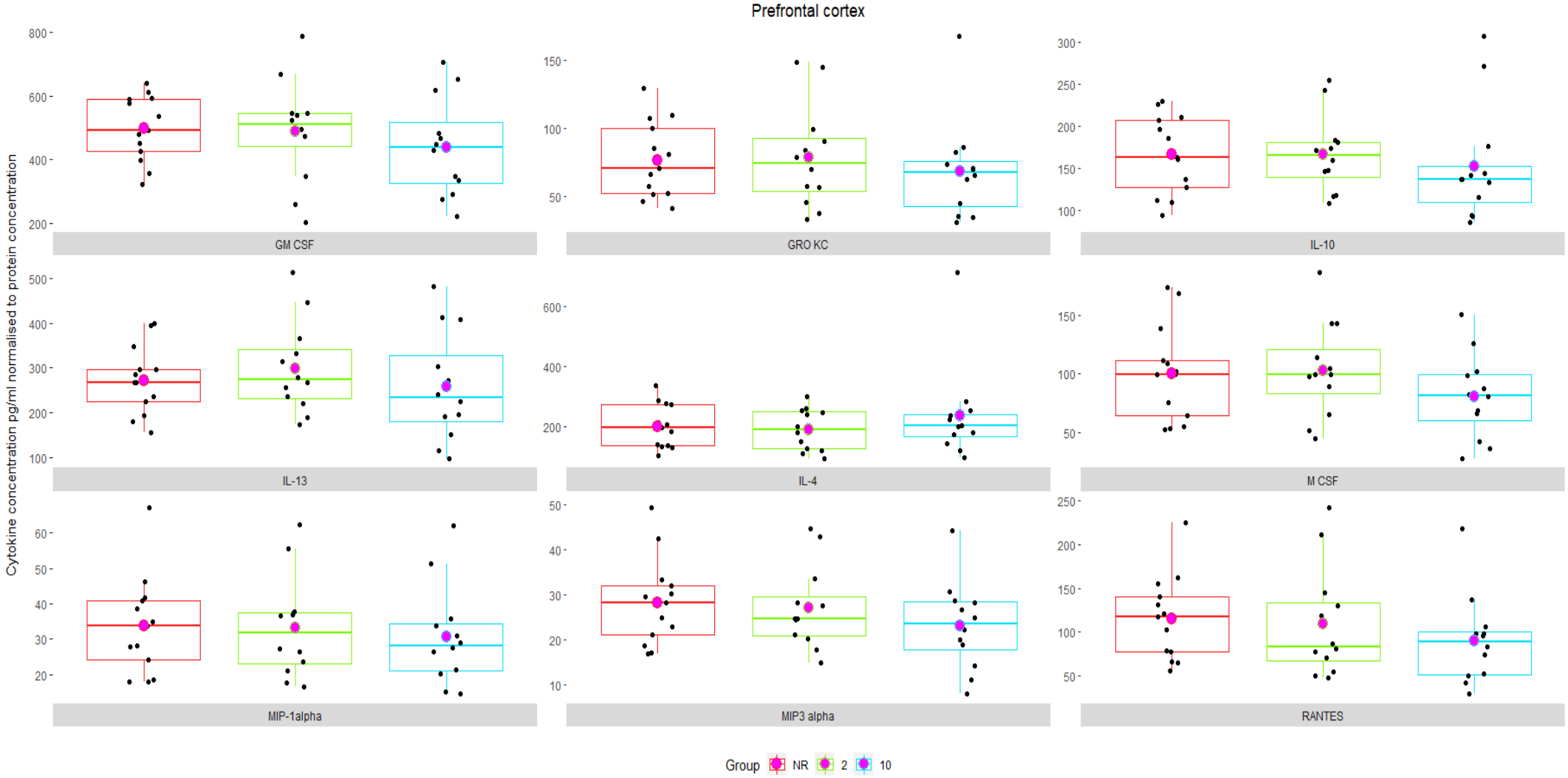
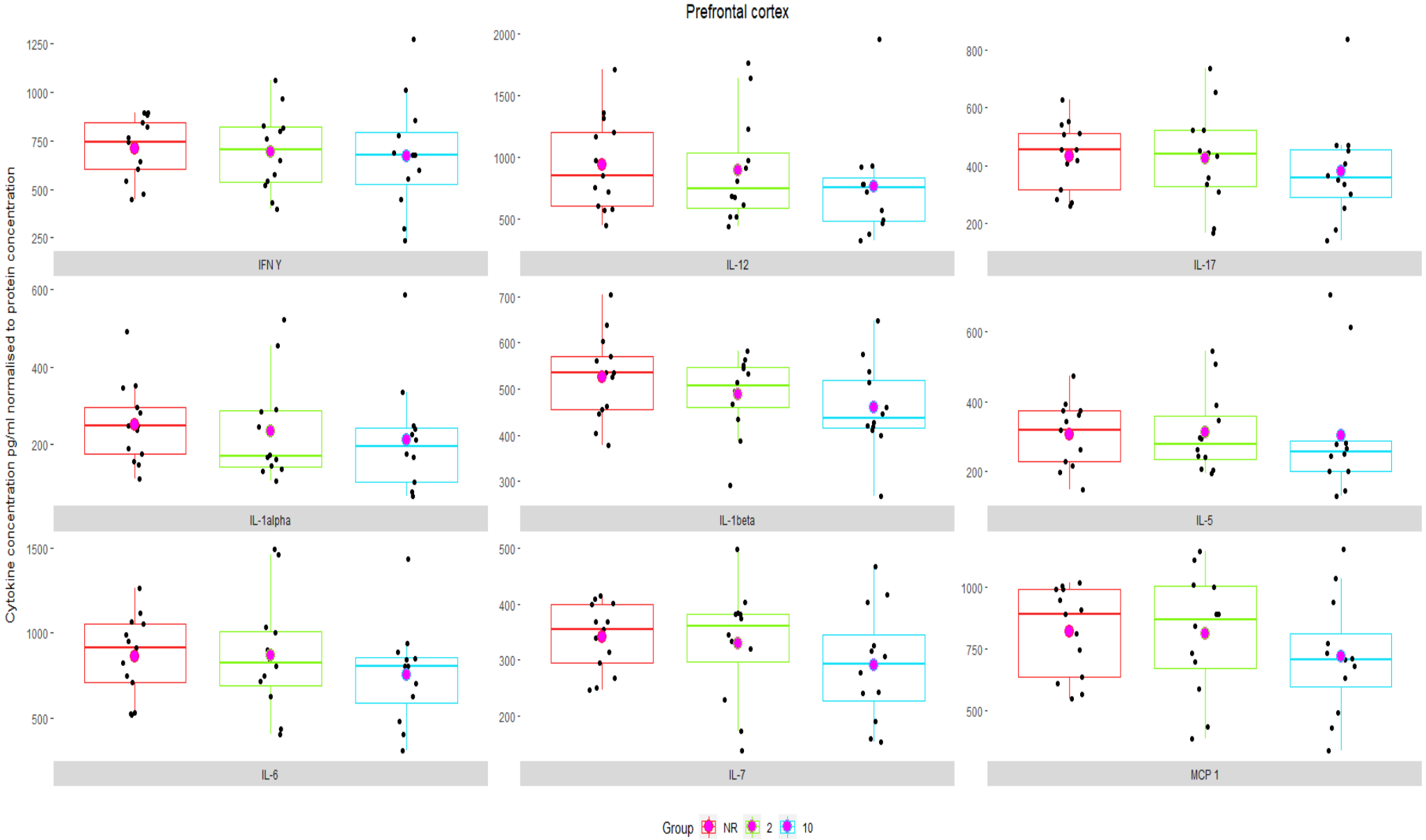
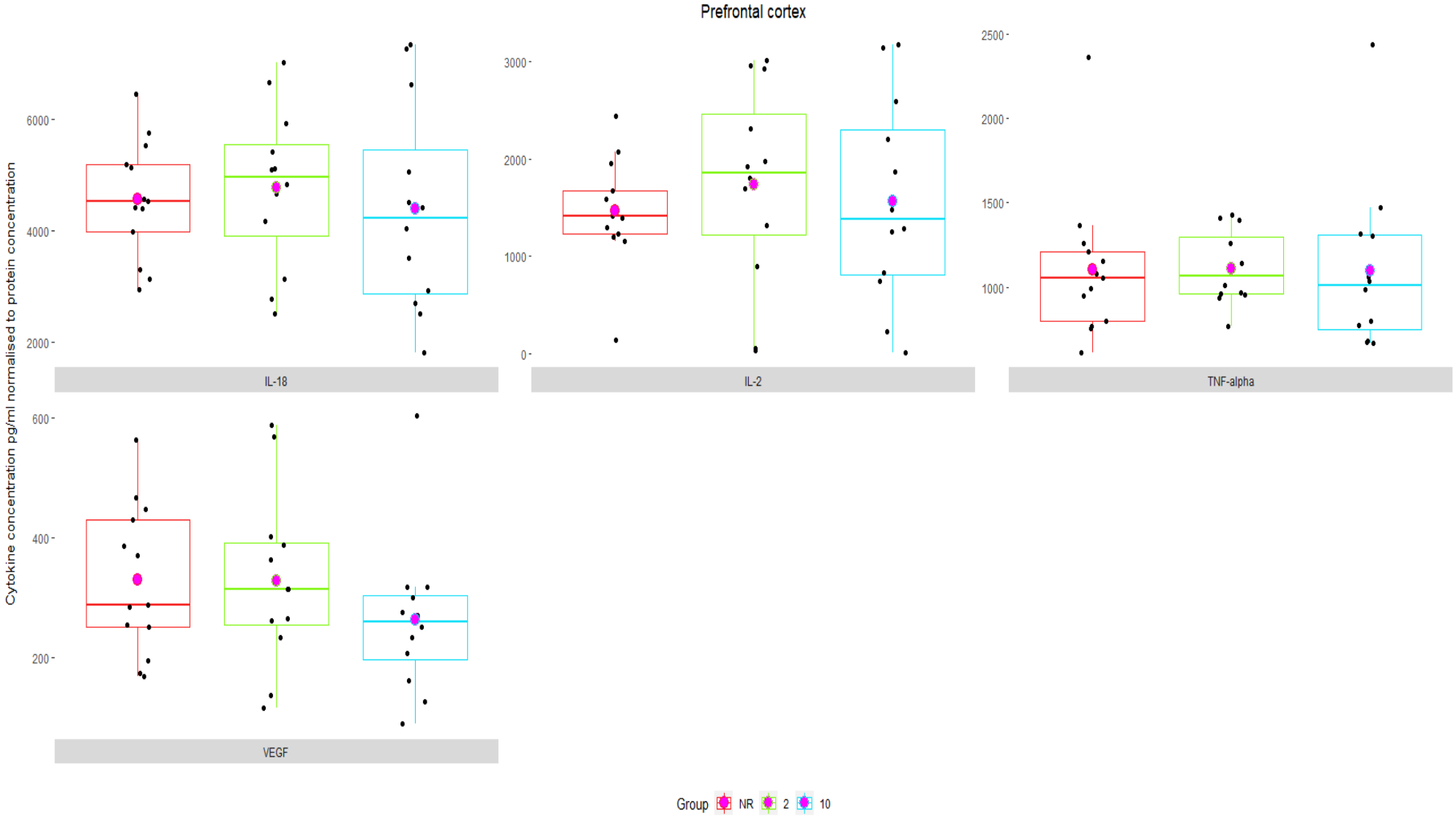


Figure 44: Cytokines analysed in punches from the infra-limbic sub region in the pre-frontal cortex: Here sex and stress factors are combined. Group refers to the extinction condition that the animals undertook. NR is no recall (shown in red), 2 is extinction control (shown in green) and 10 is extinction trained (shown in blue). (Horizontal lines show median with IQR as the hinges, the mean is shown as a solid magenta circle, top whisker shows up to 1.5 * IQR up to the maximum level bottom whisker shows down to 1.5* IQR). Black dots depict individual data-points. Group numbers shown as n. See Table 16 for full names to cytokine abbreviations. Note: Y-axis scales vary between plots to optimize visibility of data distribution."



Continued from previous page(2 of 3).



Continued from previous page (3 of 3).

Table 25: F values for inferential analysis of cytokines in IL sub-region of the mPFC. There are no effects and so all cells in white are $p > 0.05$.

Cytokine	Differences in Sex df = (1,25)	Differences in Stress df = (1,25)	Differences in extinction condition df = (2,25)	Sex * Stress df = (1,25)	Sex * Condition df = (2,25)	Stress * Condition df(2,25)	Sex * Stress * Condition df = (2,25)
GM.CSF	0.19	0.26	0.50	0.40	0.08	0.04	1.00
GRO KC	0.00	0.53	0.25	0.08	0.15	0.22	0.75
IFN – γ	0.03	1.21	0.07	0.00	0.03	0.23	0.76
IL – 10	0.057	2.039	0.211	0.235	0.789	0.513	0.233
IL – 12	0.037	0.677	0.501	0.060	0.208	0.140	0.646
IL – 13	0.496	0.357	0.449	0.001	0.866	0.079	0.659
IL - 17	0.346	0.438	0.336	0.116	0.082	0.154	0.992
IL- 18	0.666	1.883	0.186	0.637	0.008	0.078	1.359
IL 1α	0.062	1.346	0.238	0.002	0.354	0.409	0.325
IL – 1β	0.020	0.022	1.344	0.115	1.295	0.084	0.967
IL – 2	0.662	0.447	0.231	0.063	0.567	0.670	0.604
IL -4	1.222	1.668	0.586	0.046	1.576	0.317	0.144
IL -5	0.584	2.818	0.019	0.012	0.903	0.672	0.393
IL – 6	0.013	0.450	0.460	0.032	0.095	0.062	1.039

IL -7	0.318	0.019	0.866	0.415	0.003	0.071	1.242
M CSF	0.049	0.010	0.973	0.166	0.456	0.179	1.018
MCP 1	0.418	0.403	0.678	0.195	0.019	0.043	1.529
MIP 1 α	0.115	0.737	0.124	0.011	0.431	0.230	0.466
MIP 3 α	0.115	0.737	0.124	0.011	0.431	0.230	0.466
RANTES	0.006	0.723	0.567	0.110	0.174	0.236	0.598
TNF – α	0.176	3.031	0.007	0.449	1.357	0.500	0.069
VEGF	0.011	0.410	0.800	0.011	0.252	0.093	0.920

5.4 Discussion

5.4.1 Overview

The Palkovits punch technique allowed for the extraction of sufficient protein from two brain regions, the infralimbic (IL) sub-region of the medial pre-frontal cortex (mPFC) and the CA1 sub-region of the hippocampus, for a multiplex assay. This assay was used to investigate cytokine profiles in animals subjected to either CFM recall or extinction training. The findings revealed cytokine differences exclusively in the hippocampus's CA1 region, with no variations observed in the IL subregion of the mPFC. The impact of stress on cytokine levels was found to be sex dependent. In males that experienced early life stress (ELS), there was a notable trend suggesting that extinction training decreased the concentrations of IL12 and IL6, whereas non-stressed males showed no such effects. Among females, those not subjected to stress exhibited trends towards decreases in IL10 and IL1β levels due to the extinction training and a significant decrease in IL5 levels observed. Extinction-trained animals generally showed reduced cytokine concentrations compared to controls.

5.4.2 Male animals show extinction of contextual fear memory while female animals are resistant.

A partial repeat of the previous CFC experiments suggests that both male and female animals acquired and recalled CFM, however there was a sex effect here on CFM acquisition. There is a similarity however between the groups which suggests that animals are acquiring conditioned fear memory (CFM) in a comparable manner to Chapter 3. Males showed a higher level of CFM recall compared to females and animals which underwent ELS showed a higher level of CFM recall compared to non-stressed animals. This is in congruence with the results presented in Chapter 3.

In the extinction phase, females do not show significant extinction consistent with Chapter 3. This may indicate a floor level of freezing, as detailed in Chapter 3, subsection 3.4.2. In contrast, male animals display a pattern of extinction markedly different from that shown in Chapter 3, possibly due to methodological factors. Notably, the male ELS group that exhibited extinction in Chapter 3 only shows a trending reduction. There is an upward trend in the final 2 minutes, with some animals freezing throughout, influencing the percentage. The percentage of freezing demonstrates a consistent downward trend, suggesting animals are learning to extinguish CFM. While some results indicate trends rather than significant effects, the overall patterns align with that observed in previous chapters.

In summary animals exposed to CFC using the model above match previous behaviour closely or are different but in line with what we would have originally predicted.

5.4.3 There was a specific cytokine profile related to the effects of sex and extinction condition.

In animals exposed to extinction training compared to extinction control, a notable decrease in certain cytokines/chemokines was observed in the CA1 region of the hippocampus, while no significant changes were observed in the IL region of the mPFC. Specifically, cytokines GRO-KC, IL-12, IL-1a, IL-5, IL-6, MCSF, and MIP-1α were downregulated in the extinction trained group compared to the extinction control condition. This profile of cytokines will be referred to as the behavioural cytokine profile further below. Additionally, IL-18 exhibited a sex-specific effect, with males showing a higher concentration than females. Other cytokines/chemokines (IL-10, IL-1β, IL-7,

MCP-1) showed a trend towards interaction with sex, although these effects did not reach significance after applying multiple comparison corrections.

This distinct cytokine profile during extinction recall in the CA1 subregion of the hippocampus is of particular interest. Notably, many of these cytokines (IL-6, IL-1α, TNF-α, IL-1β) have canonically pro-inflammatory mechanisms [480]. The effects observed in the CNS are supported by literature demonstrating increased levels in the brain following injury[481], infection, and neurodegenerative pathology[482]. Furthermore, upregulation of these cytokines (IL-6, IL-1β and TNF- α) have been strongly implicated in an impairment of LTP and synaptic plasticity [247]

Previous work has shown that IL-6 is increased in people who are at greater risk of developing PTSD following a stressful condition[62]. Both IL-6 and TNF-α have been linked to PTSD [483] and dysfunctional extinction learning. Scholz et al [286] show that increased IL-6 is involved in CFM recall and not extinction. However, IL-6 has been shown to be required for hippocampal dependant memories and extinction memories have been shown to require the hippocampus. IL-6, in the CA1, trends towards being downregulated in the extinction trained compared to the extinction control group for stressed males. This could be suggesting a match phenotype, where exposure to a previous threat confers a beneficial protective effect when exposed to a threat later in life which is described in the Match mismatch hypothesis (Chapter 1 subsection 1.10.2). The resulting decrease in IL-6 seen in the stressed males could be inhibiting extinction as is seen in the behavioural results of this chapter.

The function in the CNS of many of the cytokines in the behavioural cytokine profile, especially during healthy physiology, is largely unknown. IL-5 has been poorly characterised in the brain, but peripheral levels have been linked to increased risk for

major depressive disorder [484] suggesting that the cytokine has some neuro-modulatory function. While the pattern of IL-5 is like other pro-inflammatory cytokines in this study, it is challenging to compare this to literature as very little has been done to examine this cytokine in the brain.

In the current work TNF-α regulation did not mirror the findings of Scholz et al [286]. during either recall or extinction, where it was found that there was an increased expression of TNF family genes in both (described in more detail in subsection 5.1.2). This discrepancy might stem from methodological differences, notably our focus on protein concentration levels as opposed to the emphasis on gene expression in work by Scholz et al [286]. It's important to note that this divergence does not imply a conflict with their results. Instead, it suggests, potentially, a variation in the time course of these molecular events as protein expression will not be temporally identical to mRNA expression.

For male animals that have experienced ELS, the level of IL-12 is decreased in those which have experienced extinction training compared to those which experienced extinction control. IL-12 is well defined as a pro-inflammatory cytokine which regulates production of IFN- γ and the maturation of T-cells [485]. While there is little work examining the effect of IL-12 in the brain, the effect in the periphery suggests that IL-12 will be pro-inflammatory in the CNS. However, it should be noted that the role of IL-12 in terms of autoimmune disease appears to be protective. For example work by Andreadou et al [235]finds that IL-12 is neuroprotective during experimental autoimmune encephalitis and attenuates neuroinflammation. While little research has been conducted which examines IL-12 response in the brain following extinction learning work by Boccia et al [486] and Scholz et al [286] shows that extinction learning results in reduction in NF-kB activity which influences many downstream cytokines (as

is described in further detail below in subsection 5.4.5) and may act to modulate IL-12.

Overexpression of IL-1β is linked to fear conditioning impairments but has also been shown to be essential for hippocampal memory processing and plasticity [230]. In the current work, for females that were non-stressed there was a decrease in the concentration of IL-1β in those which had undergone extinction training compared to those which had undergone extinction control. IL- β was downregulated, in animals which underwent extinction training compared to animals which underwent the extinction control. Work by Torrez Rodriguez et al [487] has shown that in females there is an increase in IL-1β following microglia depletion which is not present in males following microglia depletion. While this work focuses on microglia depletion, and thus likely tests a compensatory mechanism for IL-1β secretion, it does show that there are sex differences in IL-1β. These effects are also linked to the exposure to stress, as when microglia are isolated from the hippocampus, more IL-1 β is secreted in animals that were exposed to stress. This is somewhat in contrast to what is seen in the current work where IL-1β is decreased in females that were non-stressed, exposed to extinction training compared to extinction control. There are some methodological differences, however. Firstly, the animals were exposed to stress during adulthood. ELS has been shown to have a distinct neuro-immunological effect on development (as described in detail in Chapter 1 subsection 1.15). Stress during adulthood may lead to a different cytokine profile being elicited that is either more or less sensitive. Furthermore, the tissue in the study by Torres-Rodriguez et al [487] was collected 3 days post stressor and thus long-term effects of stress, which is what was done for the present work were not examined.

For IL-10 there was an effect in non-stressed females where animals exposed to extinction training show a lower concentration than those exposed to extinction control. The importance of IL-10 in learning and memory, especially in neuropsychiatric disease models is highlighted in work by Worthen et al [488] who shows that administration of IL-10 rescues learning, and memory deficits seen in a learned helplessness model of mice. Higher levels of IL-10 are seen correlated to lower levels of stress. Those which would be expected to have a lower overall level of stress (non-stressed compared to ELS) would be predicted to have higher levels of IL-10. However, when considering resilience, as is defined in the match mismatch hypothesis, exposure to a stressor, would then lead to a blunted and resilient response when exposed to a further stressor in life. This may be why a change in IL-10 is not observed in ELS animals but is seen in the non-stressed. This study may be capturing the anti-inflammatory side of the immune response which is working to maintain homeostasis (as is described in Chapter 1 subsection 1.7) following the pro-inflammatory cascade described above.

Examining the main effects of extinction condition reveals a specific cytokine profile. The concentration of this cytokine profile across all groups is decreased in animals which have experienced extinction training. This suggests a general decrease in cytokine concentration in animals which underwent extinction training.

5.4.4 Cytokine profile per animal.

An a-priori power analysis [489]indicates that detecting a moderate effect size (which is chosen due to the challenging nature of analysis cytokines described in section 5.4.7) would require an unfeasibly large sample size: approximately 420 samples. This high number is largely due to the inherent variability in cytokine expression and the large number of groups and conditions being analysed. The complexity increases

further when considering multiple cytokines across different conditions. Each additional cytokine and experimental group add to the number of comparisons, necessitating more stringent statistical corrections (e.g., Bonferroni correction) to control for Type I errors. These corrections, while necessary, further reduce the power of the study unless compensated by increasing the sample size.

One potential method to examine cytokine levels between conditions is to profile cytokines for each animal individually. This approach would help control for inherent differences in baseline cytokine levels, which might otherwise obscure true condition-related changes. To effectively implement this approach, a more complex model may be required, incorporating both fixed effects (such as the experimental conditions) and random effects (such as variability between animals). An example of this could be conducting a z-score analysis, where the differences between groups for each cytokine are expressed as dimensionless differences from a baseline group. This method accounts for cytokines with both high and low concentration levels, ensuring that the analysis corrects for variability across the entire range of cytokine concentrations.

Finally, there was a direct main effect of sex on IL-18 concentration in the CA1 region of the hippocampus, with males showing a higher concentration than females. IL-18 can be produced in the CNS and many neurons express receptors for the cytokine throughout the CNS, furthermore, work has shown that IL-18 has a role in mediating inhibition of LTP, potentially through action on NMDA receptors in the dentate gyrus[490, 491]. Interestingly, IL-18 has also been shown to inhibit secretion of CRH, and thus is suggested to have an antagonistic relationship with stress[492]. Finally, IL-18 is modulated by NLRP3 and the subsequent activation of the inflammasome mediated what is known as sterile inflammation (an inflammatory profile lacking PAMP

response[233]). This has been demonstrated in studies of human serum and brain tissue samples related to frontotemporal dementia [493]. While this pathology may induce a stronger inflammatory response—such as neuronal death leading to intracellular debris in the extracellular space acting as DAMPs—than stress or fear conditioning, it is crucial to consider this connection, especially since it can be mediated through NF-κB modulation which is described below.

5.4.5 The role of NF-kB as a mediator of cytokines and memory.

In the current study, NF-κB was not evaluated. Previous work has shown that inhibition of NF-kB led to increase extinction. This is supported by research which has demonstrated a decrease in NF-κB levels in the CA1 region two hours after recall, with a more pronounced effect observed following longer recall periods suggesting a time sensitive (for example, longer or shorter recall time, as extinction or recall respectively) role for NF-kB [286]. Many cytokines are regulated by NF-kB (see Figure 45). Scholz et al showed that there is a delicate balance and that levels of NF-kB are highly sensitive to recall and extinction. While our work did not assess NF-κB directly, Scholz et al.'s earlier study identified an effect on IL-6, a target downstream of NF-κB, indicating its potential involvement.

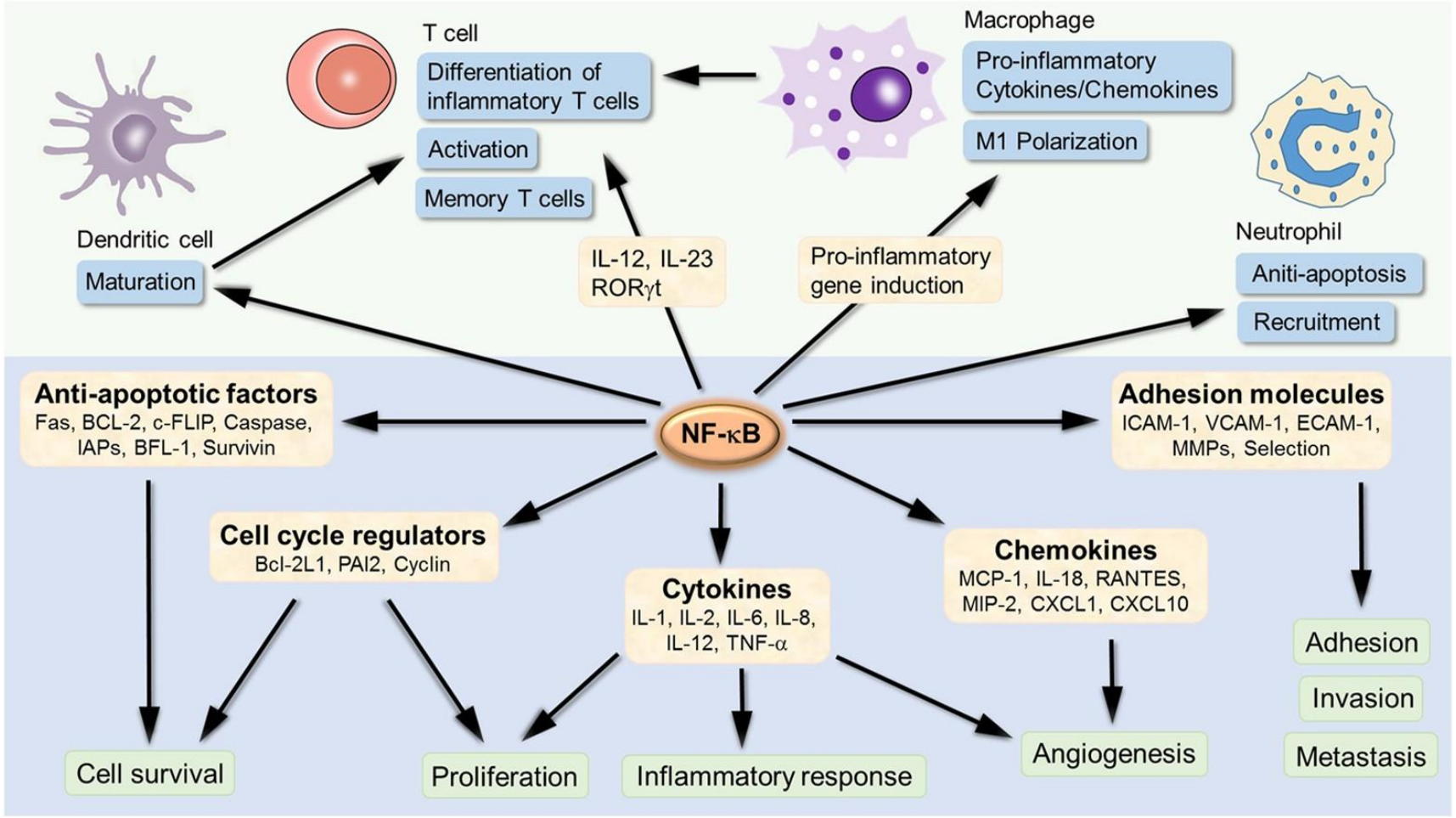


Figure 45: A complex overview of the downstream targets of NF-kB. While in this figure arrows are unidirectional many cytokines regulate other cytokines. Some downstream targets themselves regulate NF-kB. Figure adapted from Liu et al [494].

Here it is proposed that a “two hit” or allosteric model (which was described in greater detail in Chapter 1 subsection 1.10.1), consisting of ELS and later CFC may lead to a more increased cytokine concentrations in the brain. However, in the groups where we did see a difference this was because of extinction training. It could be the case that we see a downregulation in pro-inflammatory cytokines in the hippocampus, in response to extinction, compared to that seen in the extinction control (or recall) group.

5.4.6 The role of cytokines in PTSD

Previous work has shown that IL-6 is increased in people who are at greater risk of developing PTSD following a stressful condition[62]. Both IL-6 and TNF-α have been linked to PTSD [483] and dysfunctional extinction learning. There is much work examining peripheral cytokine levels at various stages of fear conditioning, trauma models, and in humans with PTSD[451-453]. Large trials have been conducted to look for biomarkers that could predict an increased risk to develop PTSD [446, 447]. Indeed, a meta review by Yang and Jiang[68] showed that IL-1β, IL-2, IL-6 IFN-γ, TNF-α, CRP, was higher, peripherally, in individuals with PTSD than a control population. An increase in cytokines such as IL-1β and TNF- α have also been shown to be increased in other disease such as depression [495]. Less work has been conducted to link together the increase in these peripheral cytokines to both a CNS mechanism, and any concurrent changes in cytokines in the CNS. Here we show that there is a distinct profile of cytokines in the CNS, that are downregulated in animals which underwent extinction training compared to extinction control and that this is sex and stress dependant.

5.4.7 Methodological technical issues and technical improvements.

A primary issue with the current study was its underpowered nature, which resulted from both time and financial constraints. Although this situation precluded a more comprehensive study, the ability to discern differences with a small sample size suggests that there is a significant effect size. This highlights the importance of examining cytokines in the processing of fear memory. However, future work should aim to increase the number of samples analysed.

A crucial element of cytokine/chemokine analysis which must be discussed is the challenging kinetics of cytokines/chemokines. Cytokine concentration levels (at least peripherally; little research exists examining cytokine pharmacokinetics in the CNS) can ramp up very quickly with changes being seen in as little time as 10 minutes [464]. These levels can then return to normal very quickly. In order to not cause excessive damage through increased inflammation, regulation of such systems requires fine temporal control with network changes occurring in hours[496]. This makes analysing cytokine levels tricky. The work presented here was a snapshot of a temporal window. It is likely that changing the time point for collection of samples would change the concentration of the cytokines and it is hard to know if they would go up or down without doing a time course analysis.

This is further compounded by the fact that different cytokines may have different effects temporally (some being pro-inflammatory at a specific stage of the inflammation cascade and anti-inflammatory at other stages for example) which is described well in the work by Callard George and Stark [497] where the authors suggest that cytokine profiles sometimes follow a nonlinear potentially chaotic profile, where small changes lead to large widespread changes rapidly. As an example of this as mentioned above, Scholz et al [273] and Barnes et al [272] have shown that the

levels of NF-κB is concomitant with recall and extinction with NF-κB activity preventing extinction. However, the downstream targets of NF- κB are many (see Figure 45 above for an example in the periphery). While many of these peripheral features are blunted or absent in the CNS, many are not, cytokines and chemokines are present and these themselves have effects on other cytokines, pathways as well as on NF-kB itself [498, 499]. This process is complex intertwined, studying one cytokine (or even a subset of cytokines) in isolation would be reductionist. It is likely that a finely balanced profile of cytokines is required for both the learning of extinction and the recall of CFM.

The reasons we did not see many significant differences as a direct main effect of ELS could be due to many reasons. Firstly, the time between the exposure to the ELS and the analysis of brain regions is over 30 days. This suggests that differences in cytokines between ELS exposed and non-exposed animals could have levelled out at this point, as cytokine responses are short lived [500] and even following a significant brain injury model (water percussion) when levels of TNF-α, IL-1 β and IL-6 are ramped up rapidly, they return to baseline within 48 hours[501]. A rapid increase in cytokine production would be controlled by a subsequent increase in anti-inflammation, to maintain homeostasis [496, 502].

Not all cytokines were detected; G-CSF levels were below detection range, and some, like MIP3-α and MIP1α, were near floor levels. Determining whether these cytokines are lowly expressed or if the assay lacks sensitivity remains challenging. The temperature of the brain (and thus the rigidity of the structure) the integrity of the structure and the size of the sub-region, all meant that some punches were susceptible to temperature fluctuations and variations in punch size. It could be envisioned that immediately following tissue punching the punches are lysed, divided into two samples and frozen. This would allow for protein concentration testing on one of the samples

while the other does not undergo the freeze thaw. This could reduce protein degradation and may be of use if analysis of targets with a smaller detection concentration was needed. Despite the limitation resulting in decreased cytokines such as G-CSF, MIP-3α and MIP-1α the concentration of most of the samples was sufficient for the current work.

5.4.8 Next steps and further experiments

One interesting experiment would be a long-time course experiment to longitudinally examine cytokine expression at different time points during CFC and extinction learning. Here, using a single snapshot in time there is a significant difference but by only taking a single point the results cannot tell us about temporal direction of the cytokines, if they are being upregulated or downregulated here. Figure 62 shows a theoretical experiment which would elucidate direction of cytokine change across different points of the CFC and extinction protocol.

The IL subregion of the mPFC was chosen for this current work due to its extensive involvement in regulating extinction memory (see Chapter 1 subsection 1.5.3). Here, we saw no differences in any cytokine concentration across any condition. This was surprising as a wealth of information has linked cytokines to extinction learning, [285, 286, 464, 487] although, these sources primarily focus on the hippocampus. It was expected that there would be a regulation of cytokines in this brain area. The PL subregion of the mPFC was not analysed here purely due to time and financial constraints, analysis of the PL region would be highly informative in elucidating if changes in cytokine concentration are absent from the mPFC in this experiment completely, or if there is a response in the PL subregion, especially in response to CFM recall (as described in Chapter 1 subsection).

Changes in the immune system underscore these behavioural induced changes further adding support that the modulation of the immune system can be used to modulate fear and extinction learning. One study by Barnes et al [285] has shown that injection of antisense nucleotides which knockdown specific transcription factors (BDNF or Zif268) impairs CFC consolidation or reconsolidation (which is defined as the same memory process as recall in the current work) depending on the transcription factor. Examining downstream mediated genes they found that interleukins related to the ones found modified in the current work were regulated such as IL-6, Cxcl1 and Il-1a. Functional networks found to be modified included those related to immune response, cell morphology and molecular transport adding support for a link between neuro-immune mechanisms, morphology (a key point for Chapter 6) and synaptic plasticity which is highly dependent on molecular transport mechanisms. Such brain region specific differences in cytokine expression are seen in the present work and suggests that cytokine/chemokine production, in response to different stimuli will differ across the regions and according to different states (as the mPFC and hippocampus regulate different facets of fear memory learning and extinction). A further step could be to examine how stress, interacts with the neuroimmune system to influence synaptic plasticity. The behavioural effects where stress impairs CFM recall or impairs CFC extinction have been shown [230, 293, 503] and mechanisms such as the increase in cytokines presented in the current work have been implicated, however, how this leads to regulation of synaptic plasticity is still relatively understudied. The importance of microglia (besides their role in producing cytokines) and the ability to perform synaptic pruning and trogocytosis (so called nibbling of a target without complete engulfment) forms the basis for Chapter 6.

There is a growing interest in exploring whether interventions that improve psychiatric symptoms, like CBT and especially exposure therapy for individuals suffering from PTSD, could also impact biological markers of inflammation, such as cytokine levels as these have been commonly associated with a risk for developing PTSD. While some work has been done to examine changes in peripheral cytokine levels following CBT on peripheral cytokines in humans with psychiatric disease[504] less has been done to examine the influence of cytokines on specific facets of PTSD. The work presented here suggests that targeting specific cytokines could lead to improved extinction memory especially in treatment where extinction memory is paramount such as exposure therapy [87-89]. This has been shown by both Scholz et al [286] and Barnes et al[285], but this work highlights that there is an important role of sex differences as well as an exposure to ELS. The therapeutic benefit of this type of treatment could consequently be highly dependent on both factors, which may interact (as they do in the current work). This could go some way to describing the issues with psychological treatment presented in Chapter 1.

5.5 Conclusions

This work adds to work by Reddaway [291], Scholz et al [286], and Barnes et al [285] which have thus far examined cytokine/chemokine RNA expression during CFC. Changes in predominantly pro-inflammatory cytokines are seen in the CA1 region of the hippocampus suggesting that the regulation of cytokines is not solely related to infection, brain injury or neurodegenerative disease, but also to modulation of behaviour, be that through alterations of synaptic plasticity, modulation of pruning via microglia or through another mechanism which has not yet been discovered.

6 Chapter 6: Microglia analysis through an advanced tool and behavioural applications.

6.1 Introduction

Although the function of microglia in response to explicit immune stimuli, such as infection and neoplasm is well-documented, their reaction to other aspects of physiological functioning including psychology and behaviour is not as thoroughly understood.

Work in the previous chapter has shown that a specific cytokine profile, including IL12, IL-6, IL-5 and IL-10, profile is modulated in the CA1 subregion of the hippocampus, following CFC and extinction and that this change is dependent on sex and exposure to ELS as an interaction. While these changes highlight the importance of the neuroimmune system in learning and memory, this is only one arm of the microglial response. Changes in morphology are also closely indicative of function and the ability of microglia to conduct synaptic pruning and trogocytosis is highly dependent on their morphology [263, 505, 506].

When exposed to a psychological event, infection related triggers such as damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs) are absent. Consequently, the changes in microglia might be more nuanced than that seen in a frank infection, TBI or stroke. Instead of undergoing dramatic transformations from a ramified, non-activated state to a more activated and amoeboid shape, they may exhibit more subtle adjustments in specific aspects of their morphology. For example, the morphology of microglia enables them to perform a wide

range of diverse functions, such as providing continuous surveillance of the local area for DAMPS or PAMPS [507] regulating synaptic pruning[505] and actively engulfing pathogens when in the amoeboid stage[508]. Research by Chaaya et al [509] shows that exposure to CFC alters microglia morphology in terms of branch length, tree complexity as well as measures of the cell body and the number of microglia (as indexed by IBA1 positivity) and shows that the microglia show shorter processes indicative of a more activated profile, indicative of a change in activation phenotype. Other work such as that by Wohleb et al [510], Kopp et al [511]and Tynan et al [512] show that microglia show a more activated profile after being exposed to stress or neuronal activity. Much of this work is reviewed in Calcia et al [513] and shows that being exposed to a psychological stressor (for example restraint or social defeat) increases activated microglia.

6.1.1 Perturbations in homeostasis including neuronal activity can influence morphology.

The morphological changes that occur have allowed researchers to loosely group microglia, in a specific state, based on a variety of features. The classes proposed most often in the literature are: Homeostatic, hyper-ramified, reactive, amoeboid and rod which are described in more detail in Chapter 1 subsection 1.13 and shown in Figure 9 [252, 514]. The ability to classify microglia into distinct states has been beneficial in allowing assessment, quantifiably, of, the effects of injury [515], synaptic remodelling during development[505] and in various models of neuropsychiatric disease such as schizophrenia [516] depression [517] and PTSD [518] on microglia morphology.

6.1.2 Sex differences in microglia following administration of LPS.

Previous work by Reddaway [291] suggested that a classification pipeline shows a drastically different percentage of activated/non-activated microglia between males and females. Females have a weaker response to the LPS stimulus. This is supported from literature showing sex differences in microglia morphology, especially in response to neuro-immune stimuli (including LPS[519]).

6.1.3 The current state of morphological analysis.

The challenges of analysing microglia morphology are many and many of the techniques that exist currently are fraught with challenging limitations such as being time consuming, and with elements of bias and complexity which are schematically shown in Figure 46.

6.1.3.1 Manual classification

Currently a simple and commonly used method of classifying microglia cells into different morphological categories is through manual classification of maximum intensity z-plane images [252]. A scorer classifies cells as being either: ramified, hyper-ramified, activated, amoeboid or rod-shaped following training where the scorer is shown many trainings, labelled, examples. This approach requires no specialist software however, is time consuming and can suffer from bias and is hampered by issues of subjectivity and poor reproducibility[252] because classification depends on personal choice and is not defined by cut off values (e.g. a certain branch length) this can lead to variability between different scorers.

6.1.3.2 Semi-automated methods

Semi-automated methods, which is where software assists in generating the trace or builds an imprecise trace which the user then modifies as needed, aim to overcome some of these biases[252]. The gold standard pieces of software for semi-automated

analysis of morphology are IMARIS (Oxford Instruments) and Neurolucida (MBF Bioscience) which can analyse more parameters but is still highly time consuming and is prohibitive to small labs due to very high costs[252]. There is also still some bias. For example, cells are manually selected and this can introduce bias as investigators may tend to select cells that are easier to trace[252]. While semi-quantitative methods provide good resolution and depth of analysis, they are challenging to scale and time consuming.

6.1.3.3 Fully automated methods

To address these challenges, the implementation of a fully automated system can mitigate the biases and time constraints inherent in these methods. 3DMorph is a project developed by York et al. [520] (https://github.com/ElisaYork/3DMorph) that enables high-throughput analysis of thousands of cells. The process involves several stages: A variant of Otsu's thresholding algorithm [521] is applied, which separates the image into foreground and background regions. Segmentation then separates the foreground into distinct microglia shapes. Finally skeletonization traces the endpoints to the soma centre via a random walker algorithm is described in York et al [520] and Reddaway et al [252] leads to the ability to analyse thousands of cells in a high throughput manner. The importance of these stages cannot be overstated, getting a good threshold value (a good threshold value accurately separates the background from the foreground, for example, the areas of interest to the background) is the difference between having good quality, accuracy skeletons and skeletons that are influenced by noise, background signal and under-/over-exposure (Reddaway, personal communication).

Very strong differences between microglia can be easily observed with manual classification. The difference between ramified and ameboid microglia is striking (see

Figure 9). However, when subtle differences are apparent, and seen across thousands of cells, the need to have an automatic, parameter-based method becomes apparent.

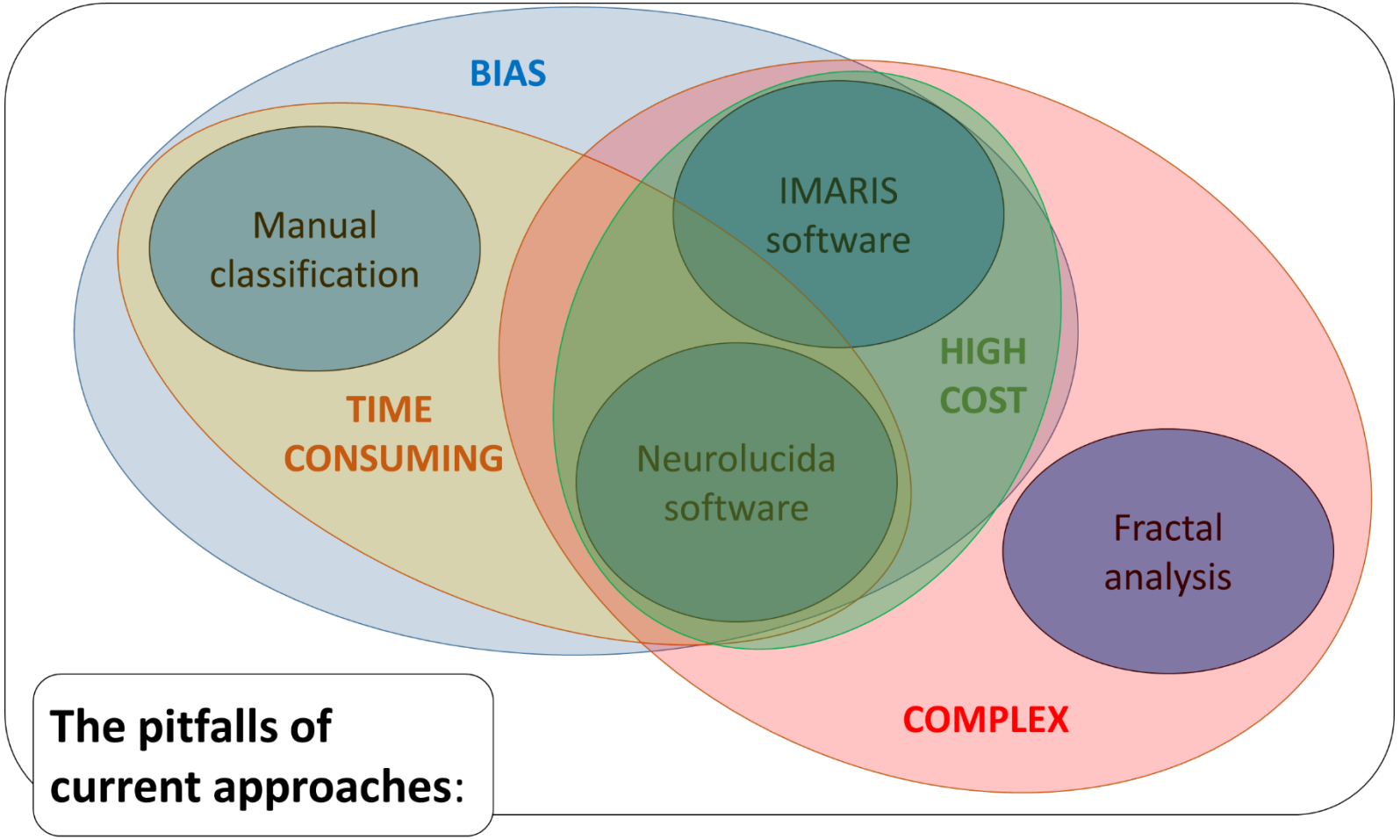


Figure 46: Challenges with current methods to analysis of microglia morphology. Complexity, cost, and the amount of time required to analyse cell morphology are non-trivial elements to consider when choosing a method.

Work by Reddaway injected animals with LPS or PBS intraperitoneally, to generate a scaled up version of 3DMorph which is able to analyse the morphology of thousands of microglia and classify them into either activated or non-activated with an acceptable level of accuracy [291]. Nonetheless, this work was conducted as a pilot study with a relatively low number of animals (4 males) and a simple, non-optimised random forest (RF) model.

6.1.4 Microglial morphology in response to stress.

One study by Li et al used a higher shock intensity than that used by Reddaway [522]. They observed microglia activation as evidenced through both morphological and gene expression profiling in both the PFC and the hippocampus. The amygdala was analysed and morphological changes in microglia were not observed [509, 518, 523].

One study by Maras et al [524] looked at the difference in microglia morphology in animals bred for specific temperamental phenotypes (low responders to novelty and utilizing a passive avoidance style vs high responders which are highly exploratory and utilize an active avoidance style). They suggest that the difference, between low and high responders to novelty, in terms of microglia morphology was seen in the territory area of ramified and reactive cells but not in amoeboid cells. Maternal deprivation, for 13 days from PND1, as a stressor also causes changes in morphology towards a more activated phenotype[525]. Another study by Sugama et al[526] has shown that microglia from animals restrained for up to 4 hours exhibit shorter processes and an enlarged soma; characteristics suggesting a more activated phenotype[252].

Furthermore, Gómez-González and Escobar's [527] work highlights region-specific alterations in microglial activity induced by prenatal stress procedures. Specifically,

there is an observed increase in microglia classified as ramified within the parietal, entorhinal, and frontal cortices, as well as in the basal ganglia, thalamus, medulla oblongata, and internal capsule. There was, a notable decrease in microglia classified as ameboid observed in the corpus callosum[527]. These findings underscore both the increased presence of ramified microglia in several key brain regions and the reduced ameboid microglia in the corpus callosum as concurrent outcomes following prenatal stress procedures and suggest that stress can alter the neuroimmune system, through microglial activation.

Contrastingly, existing research indicates that chronic stress impacts microglia morphology [269, 277, 512, 528], but little is known about the effects of ELS followed by additional manipulations on microglial morphology. This suggests that Reddaway's protocol might not have been intense enough to elicit significant microglia responses. Additionally, Reddaway focused on morphological changes post-extinction or recall, while the work in this chapter will examine these changes post-CFC.

6.1.5 Sex differences in microglial morphology.

Indeed, other work such as that by Bollinger et al [277] suggests that acute and chronic stress can have differential effects on microglia activation, with females showing more activation in those which are stressed compared to males. To the authors current knowledge, little work has been done to examine the effect of ELS on microglia morphology in animals following CFC. Examining the morphology of these subtypes which are predominantly ramified and activated classifying these cells using manual classification presents a challenge. Hinwood et al.[512, 528] have described the propensity of chronic stress to lead to hyper-ramification of microglia characterized by increased secondary, but not primary branching of microglial processes. The morphology is very complex and a subtle difference of around 100 μm^2 in area of

territory will be hard to see by eye. Research indicates the presence of subtle morphological changes in microglia sub-populations in models of schizophrenia[529], depression[517] and PTSD [530]. Given the complexity of discerning cell area differences between two ramified microglia, an automated method is more suitable for such detailed analysis.

Interestingly, a key research paper by Gildawi et al [519]suggests that there are sex dependant effects of a 2-hit adversity model on microglia morphology within the PFC. They find that both the adversity model (maternal separation) and LPS exposure cause changes in morphology. Nonetheless, this work used an infection model (LPS) that is an intensive stimulus which causes widespread microglia activation [291]. Less work has been done to examine the effects of multiple environmental/ behavioural stimuli on microglia morphology within the mPFC.

6.1.6 Aims

Prior findings in this thesis indicated that ELS affects memory consolidation in a sex-dependent manner, with corresponding changes in neuro-immune profiles, including specific cytokines[210, 277, 301, 512, 517, 531, 532]. It was hypothesized that ELS would prime microglia to respond more robustly to stress signals and adopt an activated morphology after CFM acquisition, despite limited research on ELS effects (see Catale et al. [29] Table 2). Animals were sacrificed one- or three-days post-exposure, as literature suggests morphological changes begin around 24 hours after environmental stressors, like foot shock, and subside by 3 days post-exposure [500, 509, 514, 524].

There are two primary goals of this chapter.

Chapter 6: Microglia analysis.

Aim 1: Build on Reddaway's work [522] by refining the characterisation of microglia activation states using immunohistochemistry with Iba1 antibody. The goal is to identify distinct morphological patterns in microglia between PBS-injected and LPS-injected animals, classifying them as PBS-like and non-PBS-like respectively to train a model for microglia detection.

Aim 2: Analyse a large-scale behavioural dataset to determine how microglia activation states change in response to ELS and CFC. The study will focus on the mPFC, hypothesising that ELS primes microglia to adopt an non-PBS-like morphology following CFM acquisition, particularly in a sex-dependent manner. While hippocampal tissue was also collected, analysis was focused on the mPFC due to methodological constraints.

6.2 Methods

6.2.1 Animals.

All animals used in the current chapter are Lister Hooded rats either ordered from Charles River (CR) or bred in house from breeding pairs supplied by CR. Animals were housed in pairs, trios or single housed. Animals that were single housed were males previously used for breeding, which could not be combined due to the high risk of fighting. Although single housing can be a source of stress [533], measures such as providing extra environmental enrichment were implemented to serve as a potential control to mitigate this stress. Animals had access to food and water ad libitum and were kept on a reverse light cycle where lights were on between the hours of 20:00 and 08:00. Two cohorts were used in the current chapter. The first cohort were assigned to be administered either lipopolysaccharide (LPS) or phosphate-buffered saline (PBS) these animals were sourced from Charles River. The second cohort, which consisted of animals bred in house, received CFC with or without early life stress (ELS).

6.2.2 Injection of LPS.

26 (16 male, 10 female) rats between 6 and 9 months old were given LPS (250 µg/kg, intra-peritoneally (IP) in 0.1 M PBS, E. col 0111:B4, InviboGen, California, USA) or PBS as control. Animals were closely monitored for signs of sickness or injection induced injury. No animals showed any signs of injection induced injury. Temperature recordings taken at 2 hours following injection and once again at 24 hours following injection. Animals injected with LPS showed signs of expected sickness behaviour described previously by Reddaway [291]. Briefly, animals showed lethargy, lack of grooming reduced locomotion, and decreased socialisation. Rats given just PBS

showed no sickness behaviour. At 24 hours following injection, animals were euthanized with overdose of pentobarbital (200 mg/ml Euthatal, Boehringer Ingelheim, Animal Health, Surrey, UK) administered via IP injection.

6.2.3 Early life stress and contextual fear conditioning experiment.

78 rats (40 female, 38 male) were assigned to either ELS (28 female, 17 male) or no stress controls (12 female, 21 male). Animals were exposed to ELS at PND25 – PND27 and using the same protocol (forced swim, elevated platform, and restraint) as described in detail in Chapter 2 subsection 2.2.2. Following ELS animals were maintained in their home cage until PND60+. Animals were given a 2 second 0.7mA foot shock inside conditioning chambers which is described in detail in Chapter 2. Brain tissue was collected at 1 day and 3 days after CFC as well as home cage controls which did not experience any conditioning. Due to time constraints only home cage controls and Day 1 was analysed.

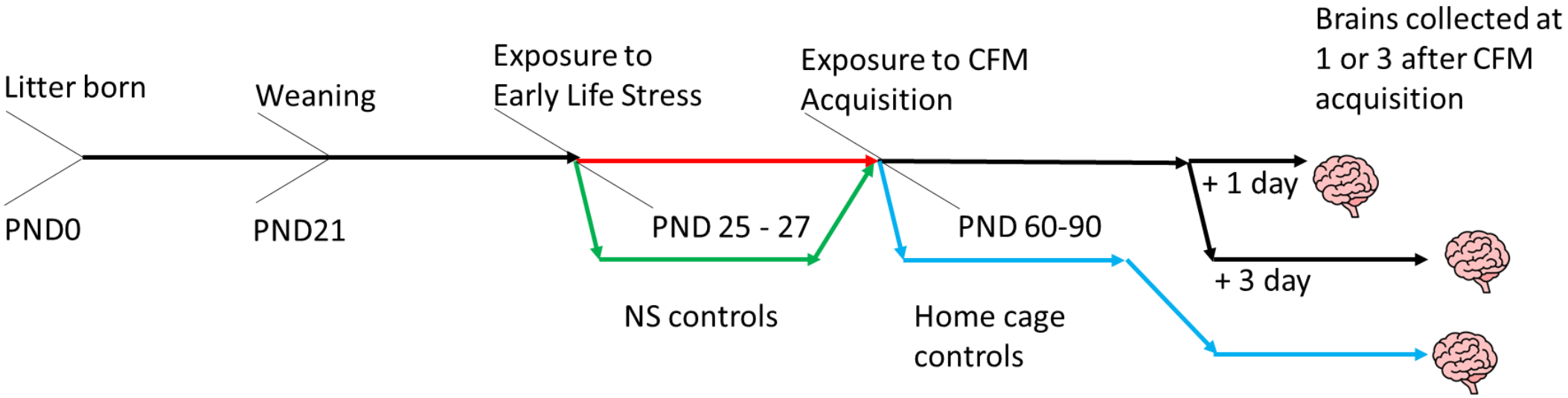


Figure 47: Rats underwent either early life stress (ELS, red line) or not (NS control condition, green line). Rats were then divided into a CFM acquisition group or assigned to be home cage controls (HCC, light blue line). For CFM acquisition rats were exposed to a 2 second, 0.7mA foot shock (US) 2 minutes after placement into the conditioning chamber. Brains were collected either 1 or 3 days after conditioning for the CFC group or 30 days after ELS or NS for the HCC group. PND = post-natal day.

6.2.4 Sample acquisition.

6.2.4.1 Perfusion

Animals were transcardially perfused with ice cold 0.1 M PBS solution followed by ice cold 4% PFA (in 0.1 M PBS) until the liver cleared and fixation tremors had ceased (approximately 8-10 min) as detailed in Chapter 2 subsection 2.2.2. Following perfusion animals were decapitated and brains removed and submerged in 4% PFA for 24 hrs at 4°C. Post-fixation brains were washed with 0.1 M PBS and transferred to 30% sucrose in 0.1 M PBS for cryoprotection. Post-cryoprotection brains were embedded in OCT compound (Scigen, California, USA) and stored at -75°C until required.

6.2.4.2 Sectioning

Brains were sliced coronally using a cryostat at a thickness of 40µm as described in Chapter 2 subsection 2.7.2. Coordinates were taken from the Paxinos and Watson Rat Brain Atlas [534] callosum (Bregma +4.20 mm to +2.28 mm) which contains both the PL and the IL.

6.2.4.3 Immunohistochemistry.

A 1/12 single series (where every 12th slice is taken) was removed from storage. Sections were washed using 0.1M PBS with agitation, at room temperature, three times for 10 minutes each. Sections were then blocked with NDS 1/10 and 1% v/v Triton X-100 for 2 hours, with agitation at room temperature. Sections were then incubated with anti-IBA1 primary antibodies (1/1000, Rabbit, Fujifilm Wako Chemicals, North Rhine-Westphalia, Germany) overnight at 4°C with agitation. Sections were then incubated for 2 hours with secondary antibodies (1/1000, Donkey, Alexa Fluor 488 polyclonal antibody, A-11094, Thermo-Fisher, Massachusetts, USA). Sections were washed with PBS for 10 minutes and counterstained with DAPI (0.1 ng/ml, 62248, Thermo-Fisher, Massachusetts, USA), sections were mounted on glass slides and cover slipped using either MOWIOL 4-88 (Merck & Co., New Jersey, USA) or Flurosave (Merck, 345789-20ML). Slides were stored at 4°C.

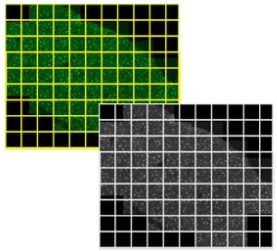
6.2.5 Sample analysis.

Modifications to the previous pipeline developed by Reddaway and me are shown in Figure 48. There were two major modifications to the pipeline. Firstly, porting the image dividing script which worked on FIJI (FIJI Is Just ImageJ) for the small subset of images produced by Reddaway to Python which could then run significantly faster and be used within the HPC. Secondly, the other modification was to convert the machine learning pipeline from R to python allowing access to the Sci-kit learn framework which made hyper-parameter tuning, SMOTE correction for unbalanced datasets and visualisation much simpler. Python contains many libraries set up to make machine learning workflows and allow direct comparisons of different models.

Imaging

Z stack imaging
Zeiss software

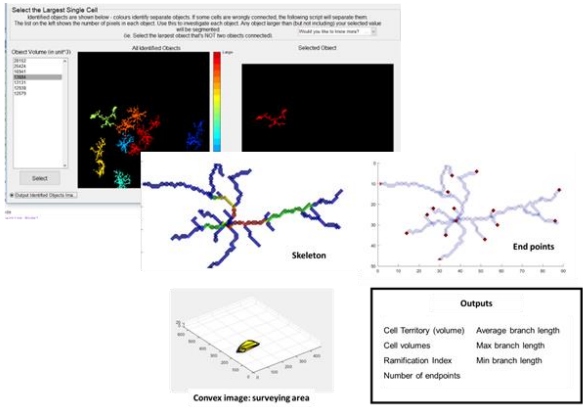
No major modifications



Pre-Processing

Image dividing
8-bit conversion
Python

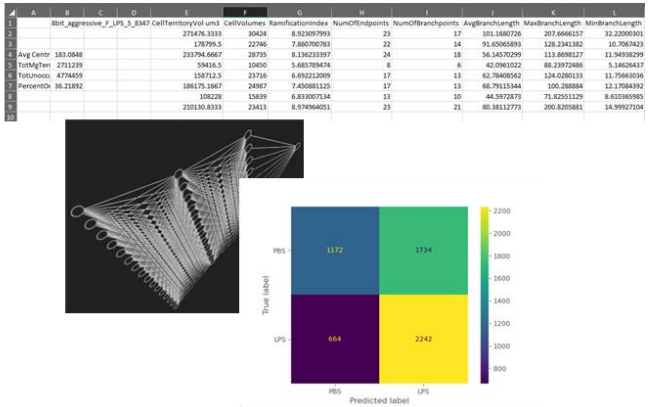
Converted from slow Image J process written by myself and Reddaway to python.



Segmentation and skeletonization

Skeletonization script 3DMorph
MATLAB
Dataset combination
R script provided by Clifton (reference in legend)

No major modifications



Machine learning pipeline

Preprocessing and model training
Python with sci-kit learn
Inferential statistics and visualisation
R

Major modifications from pipeline developed by Reddaway.

Figure 48: Schematic for analysis pipeline built from work by Reddaway[291]. The pipeline flows from imaging to the machine learning stage. Software is shown in Cyan. Reference for work by Clifton is found here: https://github.com/NiCl2.

6.2.5.1 Image acquisition and preprocessing.

Sections were scanned using a Zeiss Axioscan Z1. A template of the region of interest was defined and imaged. 20X images, acquired with 1µm Z-stacks, were taken for assessment. ROIs were selected to cover the desired brain region to image (Figure 49). Rectangular ROI's were selected to cover an approximate best fit of the region. Using curved ROI's, which fit the desired area better, greatly increased the imaging time. Up to 13 ROIs per animal, across multiple sections (up to 4 for mPFC (2 for PL and 2 for IL, and up to 10 for barrel cortex sections) were selected and assessed. Files were then converted from the CZI format (Carl Zeiss Imaging; the default for Zeiss software), automatically outputted by the slide scanner, into an OME-TIFF (open microscopy environment, tagged image file format) format for further processing. Only the images which show IBA1 staining (excluding DAPI) were selected.

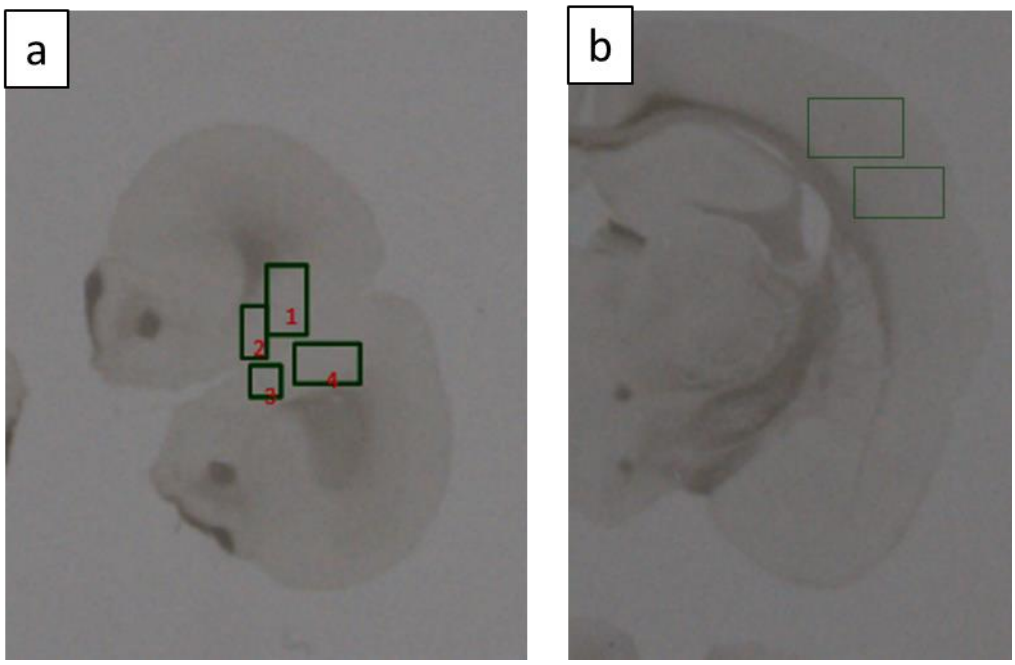


Figure 49: a, Example of ROI selection for mPFC. Boxes 1 and 4 cover the PL area and boxes 2 and 3 cover the IL area, b example of ROI selection for somatosensory barrel cortex (as the area curves around the lateral ventricle several boxes are used to cover the area).

The underlying MATLAB package 3DMorph written by York et al [520] (https://github.com/ElisaYork/3DMorph) was used as the basis for all morphometric analyses and adaptation to perform high throughput analysis is described below in section 6.2.6.

3DMorph was not able to run the segmentation function, on large images or anything higher than 8bit resolution. To overcome this, a custom python script was written which took OME-TIFFs and converted them to 8-bit as well as dividing the image into 500 x 500-pixel squares. The script calculates the number of whole squares that fit within the image. The incremental cropping of the image is repeated along the x-axis then down the y-axis until the entire image has been captured within these 500 x 500-pixel squares (Figure 50). The z-axis is conserved through the cropping process. When saved, images retain the original files names with the addition of a unique numerical identifier this process is defined in the thesis as image slicing.

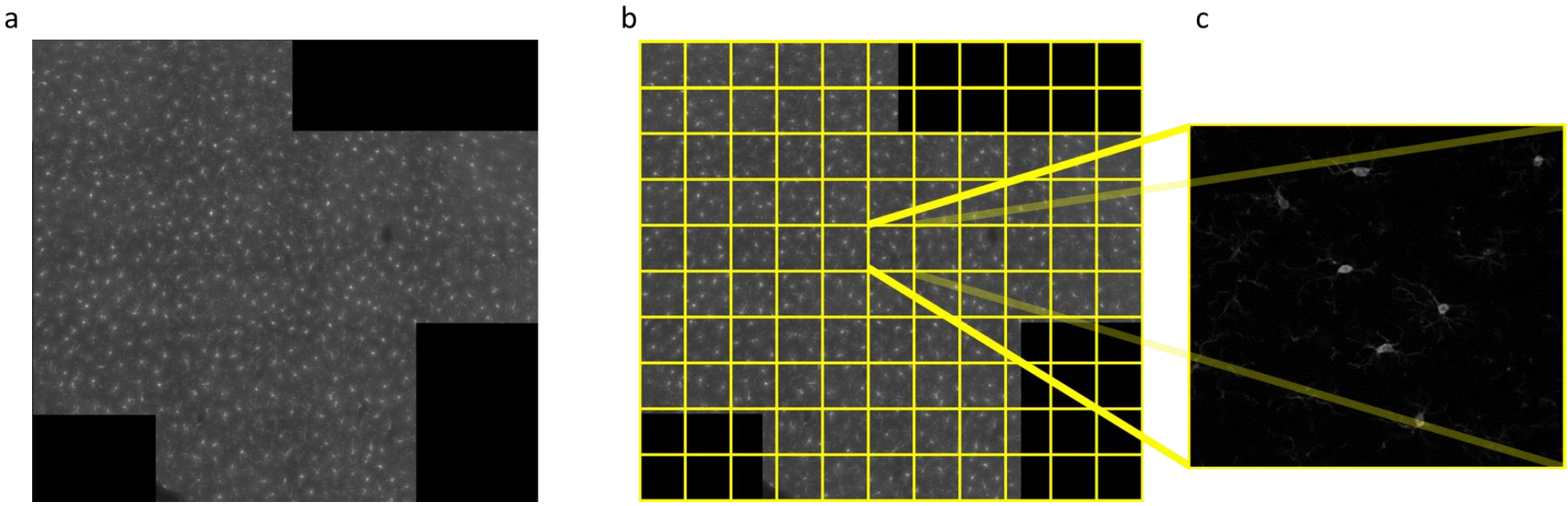


Figure 50:Representative image before image slicing using a python script (a), the image is divided into a set number of squares (the maximum that 500 X 500-pixel boxes can fit (b). A representative image post slicing which is ready to be input into 3DMorph (c). As no microglia will be present in cells that are completely black, e.g. bottom right of (b), these images were not processed.

6.2.6 High throughput analysis using NueMorph

To generate the threshold, cell cutoff and noise filter values an unedited version of the 3DMorph release was used where a researcher could adjust sliders to produce a .mat file containing the best parameters for use in automated analyses. The parameters file contains an optimal threshold value to input into a global Otsu thresholding [521] algorithm, as well as limits on the maximum and minimum size of microglia (to define objects which are too large to be segmented or objects too small to be combined), complexity of outputs (full branch structure or not, image output or not, parameter data or not) and whether to eliminate border crossing cells or not.

Large scale, high throughput generation of microglial morphometrics required the 3DMorph script to be run on Cardiff University's high-performance computing HPC cluster HAWK. This required the development of a modified, bash script, executable pipeline consisting of elements of the previous pre-processing pipeline, elements of 3DMorph, and the classification algorithms, here titled NueMorph. This required the adaptation of the segmentation and skeletonization script via removal of all graphical user interfaces (GUIs), inclusion of parallel processing at all stages of the pipeline due to the sheer number of cells to analyse, the introduction of a try loop to account for errors which would be thrown and thus halt the script due to either artifacts for example a folded, or damaged brain section, in the scanned image or a lack of cells in some images (eg in corner areas of the ROI's where cells were not present, for example the top right square in Figure 50b) and combining the (comma-separated value) CSV data files output from each image with a final CSV containing all of the parameters (Table 26) needed for further analysis, the script which combines CSV's was generously shared by Dr Nicholas Clifton (https://github.com/NiCl2/Morph3D).

6.2.7 Machine learning classification.

Cells that contained 0's on the continuous input parameters (cell territory volume, cell volume, average branch length, max branch length, min branch length) were eliminated as this is highly indicative of an artefact or noise: cells cannot have an area of 0 μm^2 and we did not expect amoeboid cells whereby branch length = 0.

Linear ML models (for example, logistic regression and SVC) require scaled data to generate accurate predictions [535] so the dataset was scaled using the StandardScaler function from Scikit learn. All parameters were scaled using the following equation:

$$z = (x - u) / s$$

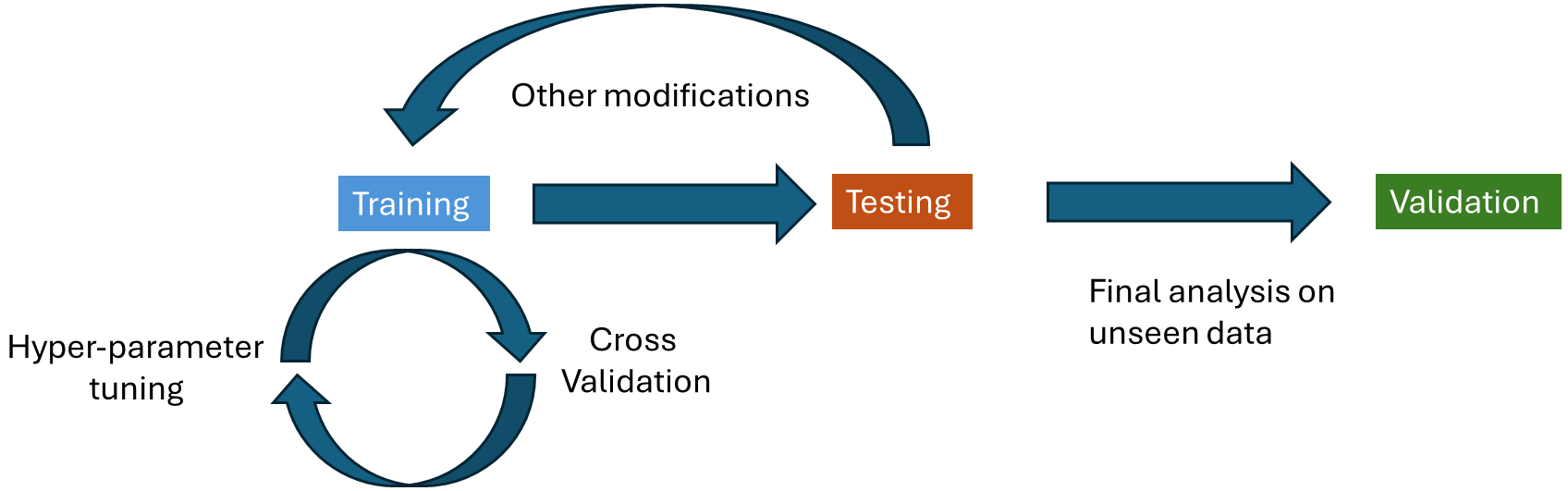
Equation iv

Where u is the mean, and s is the standard deviation of the training samples.

Any class imbalances (class is defined as non-PBS-like or PBS-like cells) were corrected for by Synthetic Minority Oversampling TEchnique (SMOTE) [536]. Briefly this is where samples in the minority class are synthesized using several neighbouring observations and the values are imputed to generate an observation. This is described in greater detail in Chapter 1 subsection 1.19.10.

The LPS/ PBS dataset is divided into a training, testing and validation set with percentage of cells set as 70/30 for the first split, giving a train and a test-validation set. The test validation set was then split 70/30 to give a test and validation set respectively. Cells were shuffled so that train, test and validation contained an equal split of LPS and PBS cells in each set, as part of a function from the Scikit learn library (train_test_split) [537]. For the microglia dataset taken following ELS and CFC there was no need to split the data (as the model had already been developed, trained, and tested) and so prediction was conducted on all cells. Numbers of cells is given in Figure 51.

a



b

Total cells before pre-processing: 49,789

Cells after split and pre-processing
Includes SMOTE, hence number goes up.

Train:19376
Test:5812
Validate:2492

Label	Training	Testing	Validation
PBS	9688	2906	1246
LPS	9688	2906	1246

Figure 51: (a) Overview of model workflow, other modifications include adjustment to cut off criteria during outlier removal and examining images that were rejected for artifacts (b) numbers of cells per condition after split.

For each cell, a set of parameters is generated these are defined in Table 26. The cutoff criteria for outliers were set as two times above or below the interquartile range. This would have removed cells that were biologically implausible such as an excessively large cell, or cells with an exceptionally small volume.

Table 26: Description of raw morphometric parameters from 3DMorph. A schematic of the parameters depicted with a (+) is shown in Figure 52.

Parameter metric (unit)	Description of metric
Cell territory volume (μm^3) (+)	A measurement of the convex hull of the endpoints. No erosion.
Cell volume (μm3) (+)	The volume of the cell following segmentation.
Ramification index	$\frac{\text{Cell territory volume}}{\text{Cell volume}}$
Number of endpoints	Number of termination points of skeleton image
Number of branch points	Number of points beginning at soma
Average branch length (μm)	Start point to endpoint
Max branch length (μm)	Maximum length of start point to endpoint
Min branch length (μm)	Minimum length of start point to endpoint

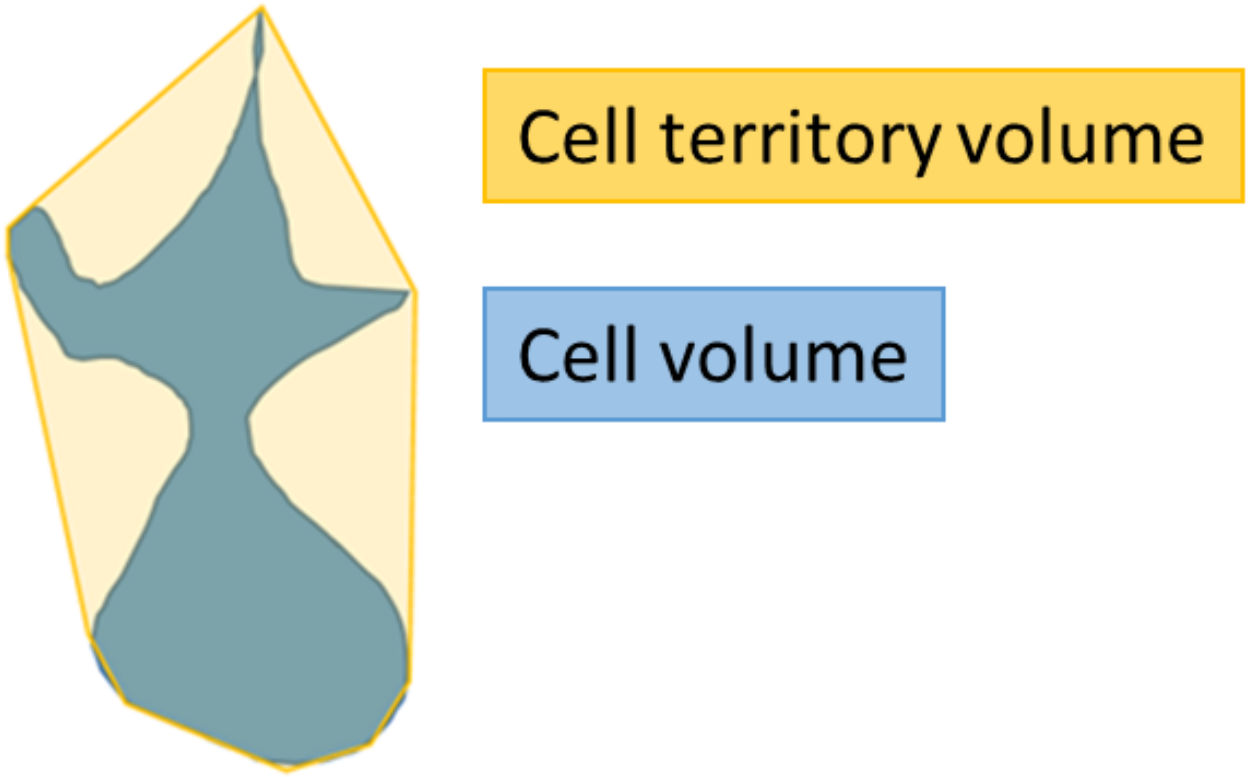


Figure 52: Schematic highlighting the difference between the cell volume and the cell territory volume. The cell is shown in 2D for simplicity, but the pipeline presented in this chapter works in 3D and thus volume instead of area is measured.

A selection of the models originally tested by Reddaway [291] has been adapted to more closely match the distinct characteristics of the LPS/PBS dataset, for example a mixture of discreet (branch points, number of endpoints) and continuous input (for example convex area) parameters are present in the current dataset and were not considered as important distinct factors by Reddaway[291]. A more detailed explanation of the models and why they are suitable candidates for the current work is give in Chapter 1 subsection1.16. The models assessed are as follows: Random Forest (RF), Support vector machine classifier (SVC), Gradient boosted [classifier] method (GBM), K nearest neighbours (KNN), Gaussian Naïve Bayes (GNB) and logistic regression. Throughout comparison cross validation (CV) was used with 10 splits and 3 repeats to reduce overfitting.

Sensitivity (Equation v) and specificity (Equation vi) were calculated to probe false negatives and false positives. To evaluate performance of the models the overall averaged (across cross validation splits) accuracy (Equation vii, which is a measure taking into account the two former measures) was computed with the cross_val_score function in Scikit Learn [537]. These were defined as follows:

Sensitivity (SN) is defined as:

$$SN = \frac{TP}{TP + FN}$$

Equation v

Specificity (SPC) is defined as:

$$SPC = \frac{TN}{FP + TN}$$

Equation vi

Accuracy (ACC) is defined as:

$$ACC = \frac{TP + TN}{TOTAL\ POP}$$

Equation vii

Where TP is the total positive rate, FP is the false positive rate, FN is the false negative rate and TN is the true negative rate. Total population (TOTAL POP) is the number samples analysed, SN is sensitivity, SPC is specificity and ACC is accuracy.

True positives are where the model correctly predicts the positive class. True negatives are where the model correctly predicts the negative class. False positives and false negatives are where the model makes an error in classifying the positive and negative class respectively.

Accuracy was chosen as the deciding metric as both resistance to false positives and false negatives was equally important. Furthermore, this was the metric that Reddaway [291] used and thus enabled direct comparison.

6.2.8 Model optimisation and performance metric validation.

Once the best performing models (as determined by the highest score for accuracy) were found the top three were considered for optimisation. Hyper-parameter tuning is searching for the best possible values for parameters which are not part of the input dataset, for example the depth and complexity of the trees in RF or the kernel type (in simple terms the shape of the transform function) in an SVM. Hyper-parameter tuning

has been shown to be highly effective in improving accuracy of SVC and RF models [538, 539]. Further details of hyper-parameter tuning are explained in Chapter 1 subsection 1.19.9. A parameter grid with every hyper-parameter permutation was tested on the top three performing models (Table 29) using randomised cross validated search using SciKit learns RandomisedCVsearch [537]. Cross validation prevents overfitting by using portions of the overall dataset (the training dataset) to train the model, this is explained further in Chapter 1 subsection 1.19.1. Grid search, which would analyse every possible iteration of the hyperparameters, was not used due to time constraints. The same CV metrics as described above (10 splits, 3 repeats) were used.–The best model was then chosen to conduct further predictive analysis. Predictions on the 10% left-out (not used to train the model) validation set were conducted, and accuracy was measured.

6.2.9 Analysis of microglia from animals exposed to ELS.

The best performing model was used to predict classes: non-PBS-like (activated), or PBS-like (non-activated), in the dataset generated from animals exposed to ELS and then CFC. Only a subset (23/78 rats consisting of 10 ELS, 13 NS) were processed and analysed due to time constraints.

A single value, per animal, per group (ELS/NS) for the proportion of total microglia non-PBS-like was calculated using Equation 6.1 as Reddaway previously described[252].

$$\%\ \text{of activation} = \frac{\text{number of non} - \text{PBS} - \text{like microglia}}{\text{total number of microglia}} \text{X } 100$$

Equation viii

To compare between groups the mean per animal is taken as a single datapoint and the groups are compared with ANOVA.

6.2.10 Software and inferential statistics

Code for the current project was written across MATLAB, and Python. Data visualisation and inferential analysis was conducted with R using RStudio (2023.06.0+421 "Mountain Hydrangea"). Analysis of differences between groups in the ELS/ NS and CFM acquisition experiments was conducted with linear mixed models. Post hoc testing was conducted using Tukey HSD testing.

6.3 Results

Images were acquired for the Barrel cortex without issues. For experiments which followed ELS and CFC there was a problem where microglia were not staining evenly through the slice. Individually, the quality of the stain in each microglia was good, but the stain was sparse throughout the slice (Figure 53).

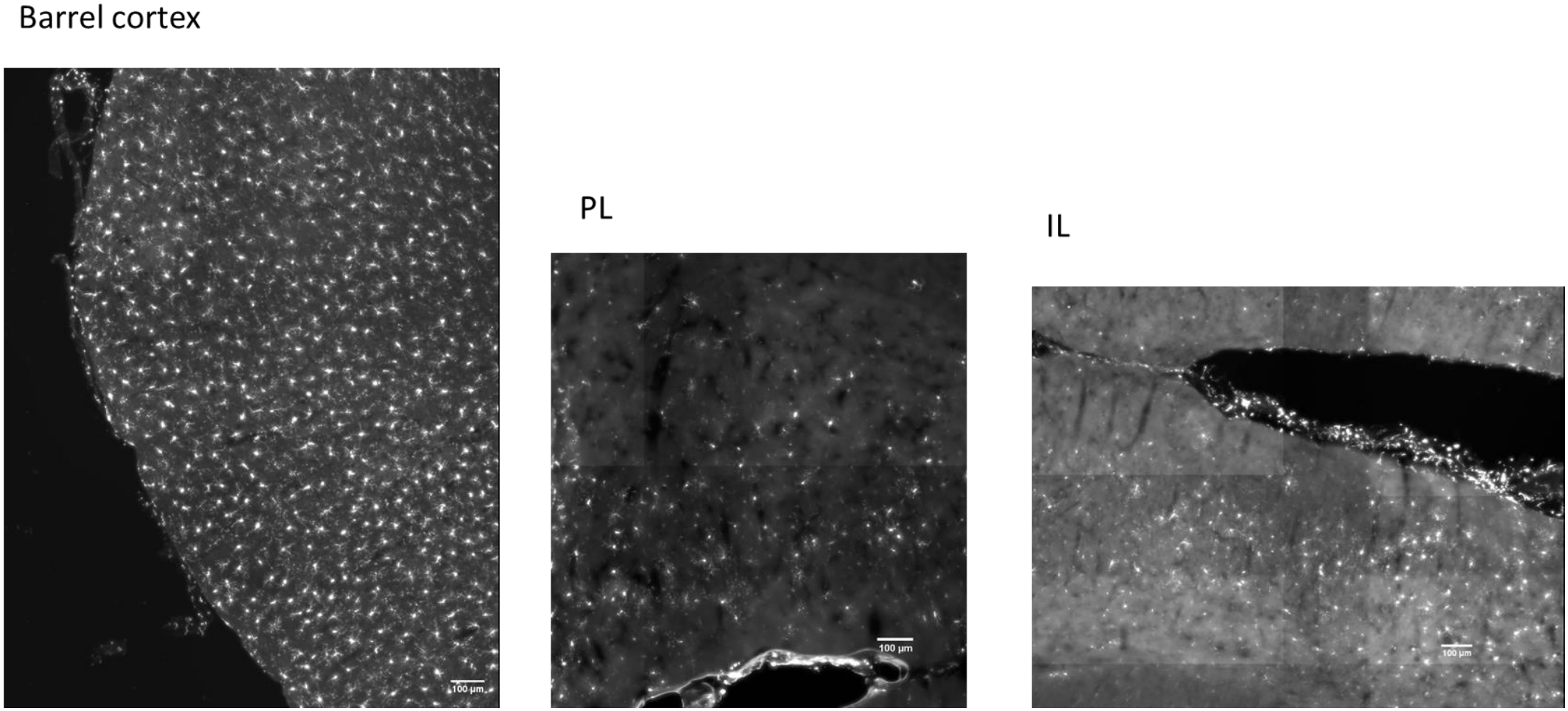


Figure 53: Staining in the barrel cortex was sufficient. Examples of the microglia staining artifacts are seen in both PL and IL mPFC and hippocampal sections (not shown, and were not acquired). Staining was better at the peripheral regions while central regions were affected by the artifact more.

6.3.1 Model training and testing.

To improve on previous work by Reddaway (aim 1) a larger dataset was generated from ten LPS (5 male, 5 female) and seven PBS (4 male, 3 female) injected animals. Brains were taken 24 hours following LPS or PBS injection. In total 49,789 microglia were analysed across the barrel and motor cortical areas in the brain in order to match areas analysed by Reddaway [291]. As suggested above, (section 6.1.2) when exposed to the same stressor/immune stimulus, females show less distinction between non-PBS-like and PBS-like microglia classes than males do (J. Reddaway, personal communication, 2023). To generate a dataset with the strongest binary separation between the LPS and the PBS dataset, males were used to develop the model this consisted of 24,615 cells.

Raw morphometric parameters for PBS-like and non-PBS-like cells in the barrel cortex are presented in Figure 54. In the mixed linear model analysis controlling for animal variability (random effect: individual animal), there were no significant differences in activation state across the individual morphometric parameters (Table 27).

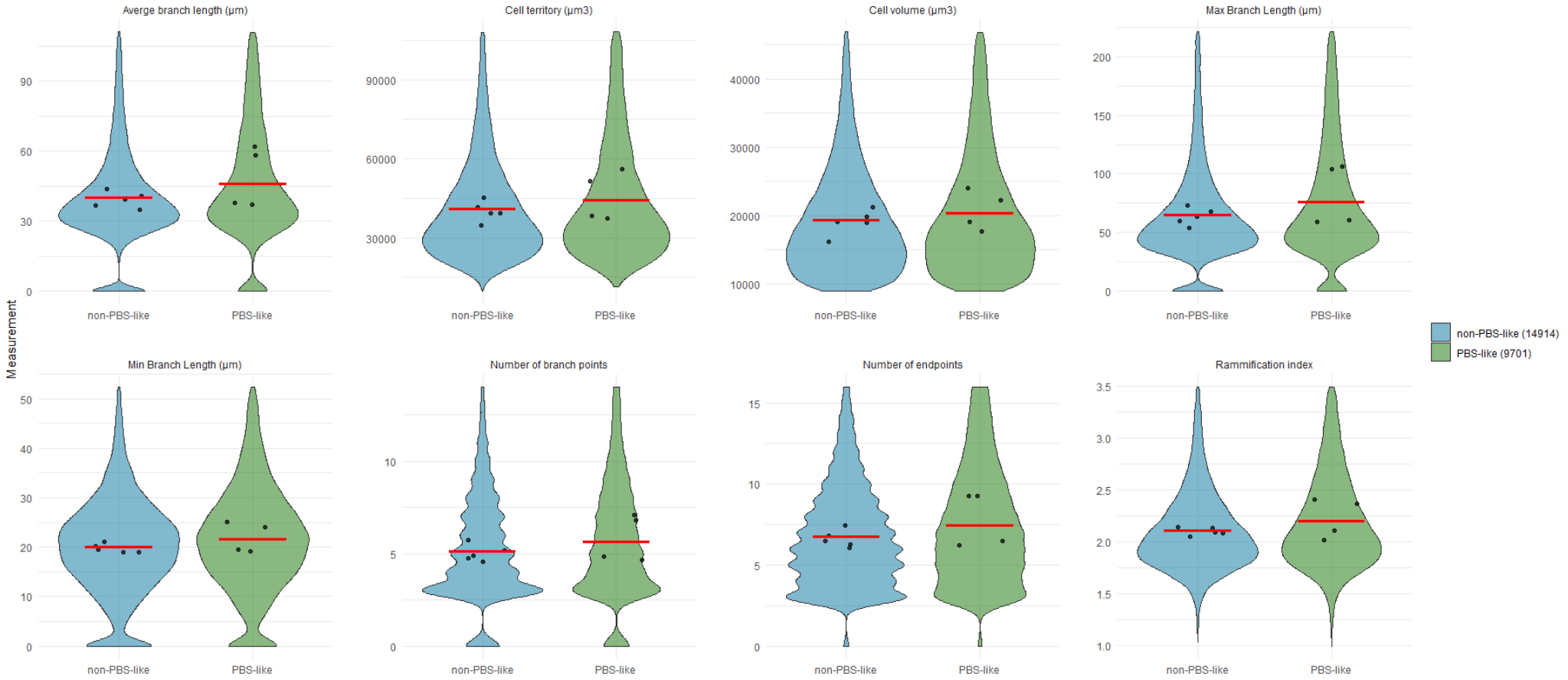


Figure 54: Changes in raw morphology following PBS or LPS treatment (to generate PBS-like or non-PBS-like datasets respectively). Red bar shows mean of all cells across all the animals for the LPS and PBS groups. Each point shows the mean cells per animal. The legend shows the number of total cells analysed across all animals.

Table 27: Summary of inferential analysis (via ANOVA) of differences observed in morphological parameters between animals treated with LPS or PBS.

Parameter	Test statistic
Average Branch length	Estimate = 9.61, t (6.994) = 1.592, p > 0.05
Cell territory	Estimate = 9.61, t (6.994) = 1.592, p > 0.05
Cell Volume	Estimate = 1701, t (7.007) = 1.082, p >0.05
Max Branch Length	Estimate = 18.960, t (6.944) = 1.588, p >0.05
Min Branch Length	Estimate = 2.288, t (6.985) = 1.608, p >0.05
Number of Branch points	Estimate = 0.831, t (6.999) = 1.375, p >0.05
Number of endpoints	Estimate = 1.196, t (6.996) = 1.51, p >0.05
Ramification index	Estimate = 0.124, t (6.994) = 1.434, p >0.05

6.3.2 Preprocessing

The pre-processing stages and segmentation and skeletonisation with 3DMorph resulted in generation of a dataset, from the LPS/PBS animals, with 49,789 microglia. There was a class imbalance present in the training dataset (9701/14914 for PBS-like to non-PBS-like, respectively). To correct for this, application of SMOTE described in detail in Chapter 1 subsection 1.19.10 was used to make up class values to 9688 for both. Cells that contained 0's on the continuous input parameters were eliminated for reasons described in subsection 6.2.7.

6.3.3 Model training and validation.

The previous work by Reddaway [291] showed that a non-weighted (RF) was the optimum classifier when compared to other models achieving an accuracy of 70%. Here a subset of the models used by Reddaway were included (RF, SVC, LR) as well as some others (logistic regression, K neighbours (KNN) and Gaussian naïve-Bayes (GNB)) were trained on the training dataset. A more detailed description of these models is presented in Chapter 1 subsection 1.19. Accuracy results from training are presented in Table 28.

Table 28: Accuracy of non-optimised models, determined on the test dataset following training.

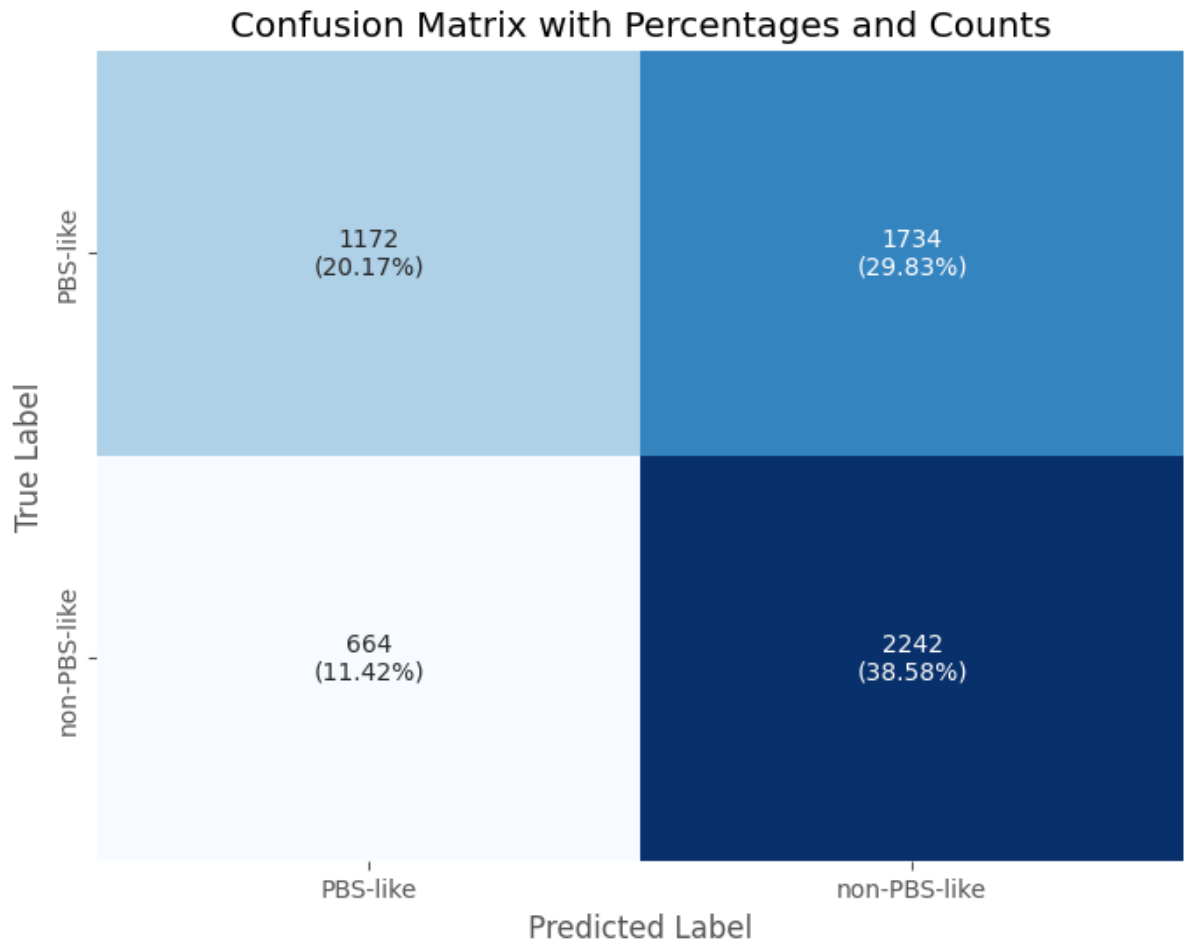
Model	Accuracy (standard deviation)
Random Forest	0.64(0.00)
Support Vector Machine	0.60(0.00)
K-nearest neighbours	0.58(0.00)
Gaussian Naïve Bayes	0.56(0.01)
Gradient boosted classifier	0.60(0.01)

Logistic regression	0.59(0.01)

The top 3 models were chosen for further optimisation using hyper-parameter tuning (Table 29). The highest overall accuracy was shown with the RF model, while SVC and GBM were not improved by hyperparameter tuning. Hyper-parameter tuning, which is described in further detail in Chapter 1 subsection 1.19.9 was able to produce a better accuracy result (64% to 66% accuracy). The final test (RF optimised and best performing) accuracy, on the validation dataset (completely unseen by the model) was: **0.59.** Figure 55 shows the corresponding confusion matrix displaying true positives and true negatives on the axis and false negatives and false positives off the axis. The model is showing a relatively high number of falsely classified non-PBS-like cells (which are PBS-like cells).

Table 29: Accuracy for models following hyper-parameter tuning using training data.

Model	Accuracy (Standard deviation)
Random forest optimised	0.66 (0.01)
SVC optimised	0.59 (0.00)
GBM optimised	0.59(0.04)



Sensitivity: 0.77

Specificity: 0.40

Figure 55: The confusion matrix for predictions, generated by hyper-parameter tuned RF model, on the test dataset. Top left shows the number of true PBS cells classified as PBS, bottom right shows the number of true LPS cells classified as LPS. Top right shows true PBS classified as LPS; bottom left shows true LPS classified as PBS. The heat map shows the increasing number from light blue being a low number of cells to dark blue which shows a high number of cells. The specificity and sensitivity values for the model are shown below the matrix. This visualisation helps in assessing the model's classification ability, where ideally, the true positives and true negatives should be high, and the false positives and false negatives should be low.

To ascertain whether the dataset was of adequate size—that is, to determine if adding more data would not enhance the model's accuracy—subsets of the training dataset ranging from 50% to 80%, in increments of 10%, were systematically analysed. The smallest subset showed no decrement in mean accuracy and increasing the subset size did not improve the model accuracy (Table 30). This suggests that the current training dataset is large enough as sub-setting does not lead to major decreases or variances in accuracy.

Table 30: Calculated accuracy, during testing, following subsets of the training data.

Subset data	Mean accuracy (standard deviation)
50%	0.66 (0.01)
60%	0.65 (0.01)
70%	0.66 (0.01)
80%	0.65 (0.01)
90%	0.65(0.01)

6.3.4 Effects of early life stress on contextual fear conditioning-induced microglial morphology changes.

6.3.4.1 Behavioural effects

Animals underwent a modified behavioural protocol to test the effects of ELS on CFC induced microglia morphology changes (Aim 2). Here animals underwent ELS as described in subsection 6.2.3. Animals then underwent CFC or were classed as home cage control. Brains were taken 1- or 3-days post CFC and in the same week for home cage controls.

There was an effect of foot shock (0.7 mA, 2s) on freezing behaviour with animals freezing more during post shock than pre shock (Estimate = 76.419, t (148) = 18.794, $p < 0.001$, linear mixed model). There were no interactions between Sex or Stress or interactions (Table 31). There was no effect of Sex (Estimate = 4.167, t (148) = 0.891, $p > 0.05$, linear mixed model, Figure 56). There was a main effect of Stress (Estimate = 11.111, t (148) = 2.117, $p < 0.05$, linear mixed model) with those which experienced ELS freezing more than non-stressed animals.

Table 31: Interaction terms for assessment of microglia as PBS-like or non-PBS like with corresponding inferential statistics.

Interaction term	Statistic (all with linear mixed model)
Sex X Stress	Estimate = 5.556, t (148) = 0.769, p > 0.05
Sex X Time point	Estimate = 4.199, t (148) = 0.639, p > 0.05
Stress X Time point	Estimate = 9.005, t (148) = 1.213, p > 0.05
Sex X Stress X Time point	Estimate = 4.699, t (148) = 1.213, p > 0.05

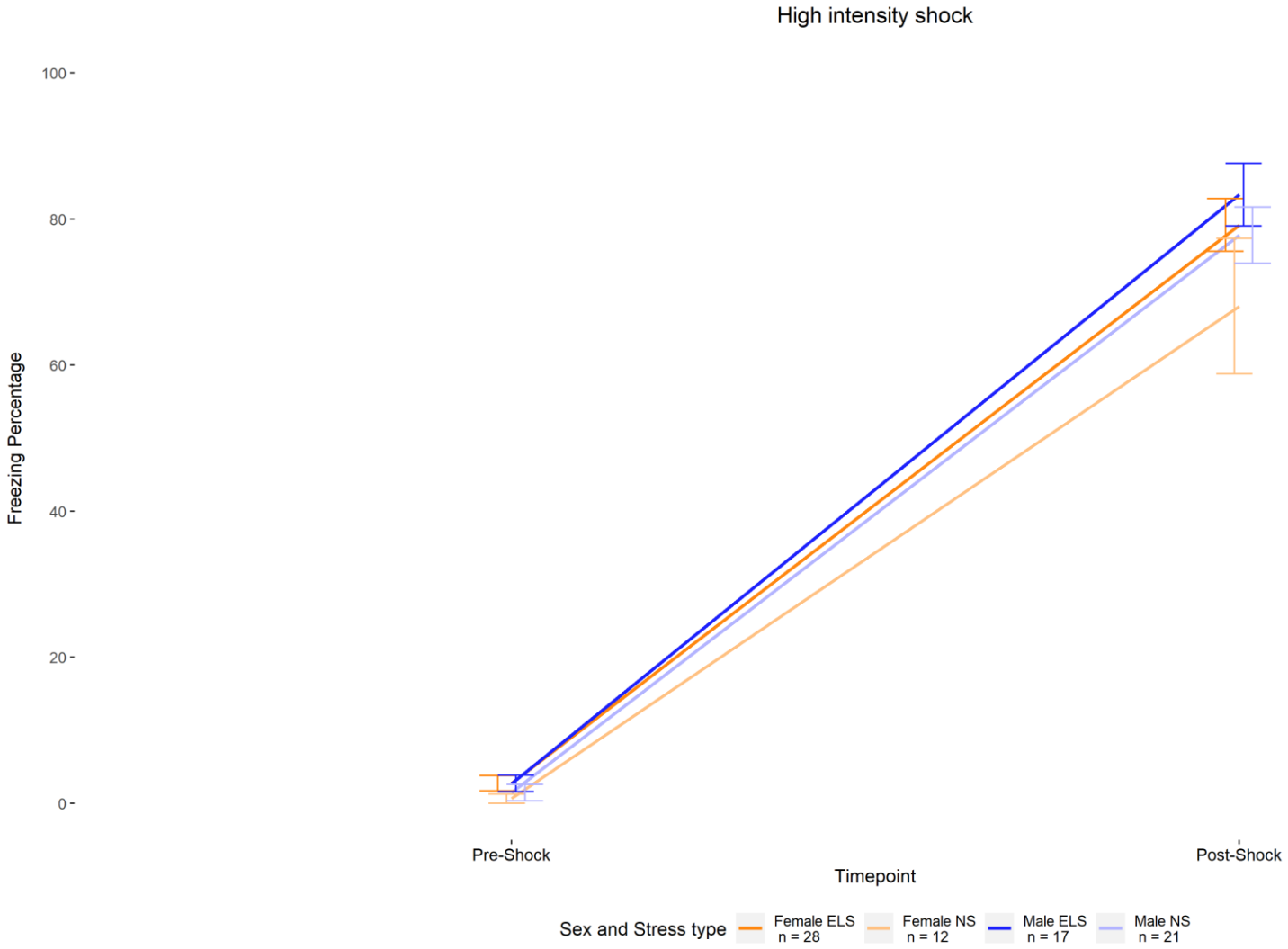


Figure 56: Males and females both in the ELS group and NS group show strong freezing following CFM acquisition. All rats were contextually fear conditioned with a scrambled foot shock (0.7mA, 2s) two minutes after placement in a novel context. All groups showed a higher level of freezing behaviour after the foot shock. ELS = Early life stress. NS = non-stressed. Results are shown as the mean ± SEM.

6.3.4.2 Microglia morphology classification

The NueMorph optimised RF classifier was trained on solely male microglia to show the strongest difference between the two classes as described in detail in section 6.1.2. Here, however, the percentage of activation can be assessed in both females and males. The PL and infra-limbic area of the PFC was assessed. A staining artifact was present in all images including mPFC and hippocampal images. Individual microglia were stained accurately, but there was a diffuse pattern, where relatively few of the microglia showed signal as seen in the PL and IL subregions of the mPFC in Figure 53. Due to these staining artifacts the hippocampus was not assessed but imaging of the PL and IL was possible. Home cage controls (defined below as HCC) and animals 1 day post shock were analysed. Animals 3 days post shock were collected but were not analysed here due to time constraints. Home cage control and if the animals experienced foot shock or not will be defined as Condition.

Sex effects: There was a Sex by Stress by Condition interaction in the PL subregion of the mPFC ($F(1,33) = 5.309$, $p < 0.05$, ANOVA). For the IL cortex there was an interaction between Sex and Stress $(1,35) = 13.020$, $p < 0.05$, ANOVA). To better analyse the effects of Stress males and females were analysed separately.

Male

Pre-limbic subregion of the mPFC

For the PL sub-region of the PFC, there was a main effect of Sex, with males showing a higher percentage of non-PBS-like microglia than females ($F(1,33) = 7.502$, $p < 0.05$, ANOVA).

There was an effect of Stress ($F(1,19) = 4.607$, $p < 0.05$, ANOVA, Figure 57) with ELS groups showing a higher percentage of non-PBS-like microglia than NS groups. There was a main effect of Condition ($F(1,19) = 4.771$, $p < 0.05$, ANOVA) with HCC animals showing more cells in the non-PBS-like class than those from CFC. There was a trend towards an interaction between Stress and Condition ($F(1,19) = 3.44$, $p > 0.05$, ANOVA). Post hoc analysis shows that this effect is driven by the difference between the HCC group and the CFC animals in the non-stressed group (14.23, $p < 0.05$, Tukey test) and the ELS-HCC group and the NS-CFC group (14.67, $p < 0.05$, Tukey test). Other effects are non-significant.

Infralimbic

For males there was a main effect of Stress ($F(1,20) = 11.869$, $p < 0.05$, ANOVA, Figure 57) with no main effect of condition ($F(1,20) = 11.869$, $p > 0.05$, ANOVA) and no interaction of Stress and Condition ($F(1,20) = 1.118$, $p > 0.05$, ANOVA) on the percentage of non-PBS-like microglia.

Female

Prelimbic

In the PL subregion there was a main effect of stress ($F(1,14) = 6.116$, $p < 0.05$, ANOVA) with stressed animals showing a lower percentage of non-PBS-like microglia than non-stressed animals. There was no effect of condition ($F(1,14) = 0.378$, $p < 0.05$, ANOVA) and no interaction effect between stress and Condition ($F(1,14) = 2.178$, $p > 0.05$, ANOVA).

Infralimbic

In the IL subregion, there was a trend towards a main effect of Stress with stressed animals showing a lower percentage of non-PBS-like microglia than non-stressed animals ($F(1,15) = 3.50$, $p = 0.0812$, ANOVA). There was no effect of Condition ($F(1, 15) = 0.16$, $p > 0.05$, ANOVA) and no interaction effect between Stress and Condition present ($F(1, 15) = 0.225$, $p > 0.05$, ANOVA).

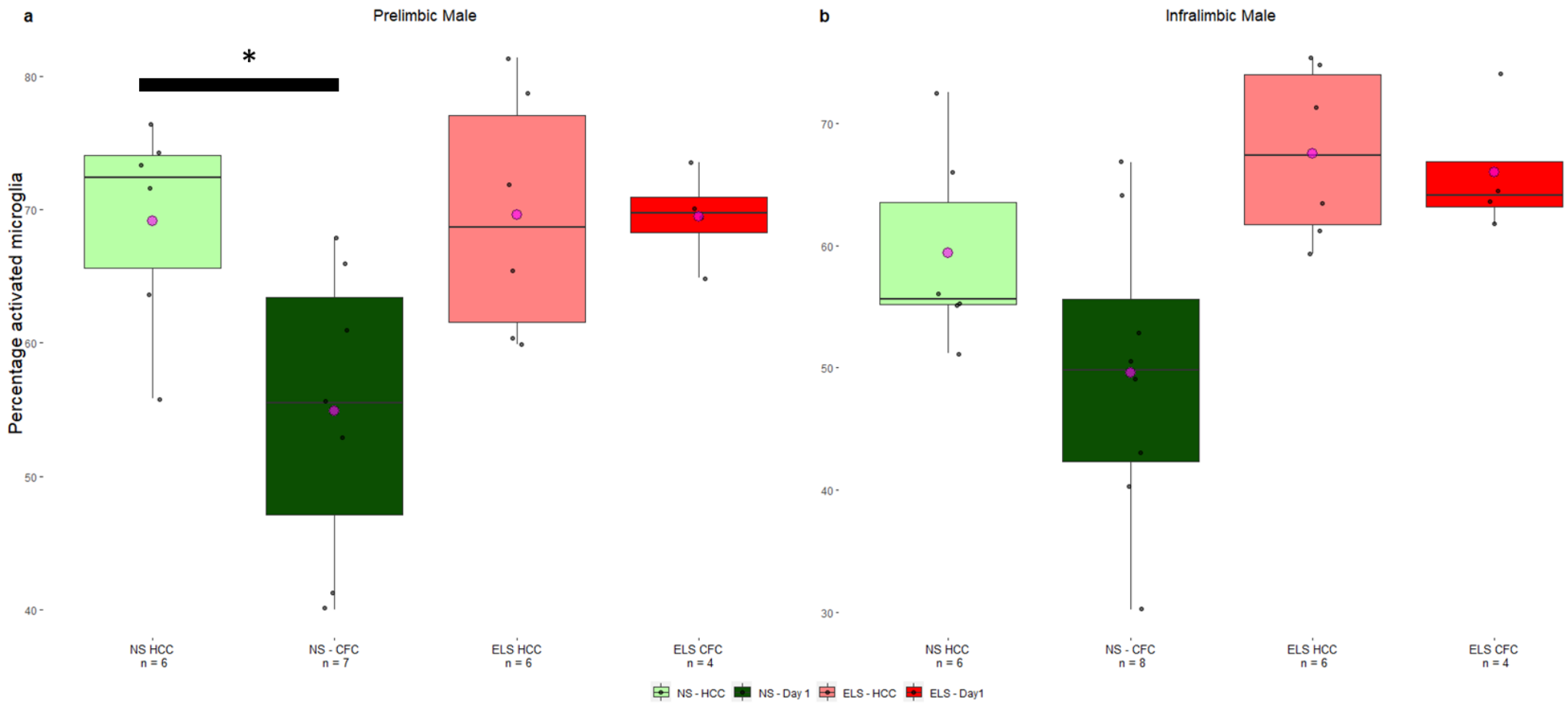


Figure 57: Percentage of non-PBS-like microglia in male rats as predicted by NueMorph pipeline in the pre-limbic (a) and the infra-limbic (b) cortex. Non-stressed animals are shown in shades of green, animals exposed to ELS shown in shades of red. Dark shades show animals exposed to CFC and light shades are animals used as home cage controls (HCC). Each data point represents the mean activation percentage of all microglia analysed within an individual animal. NS = non-stressed, HCC = home cage controls, ELS = early life stress, CFC = one day post CFC. Horizontal bar shows median. Pink circle shows the group mean. Sample size (n) beneath each condition. Solid bar with * shows significant difference.

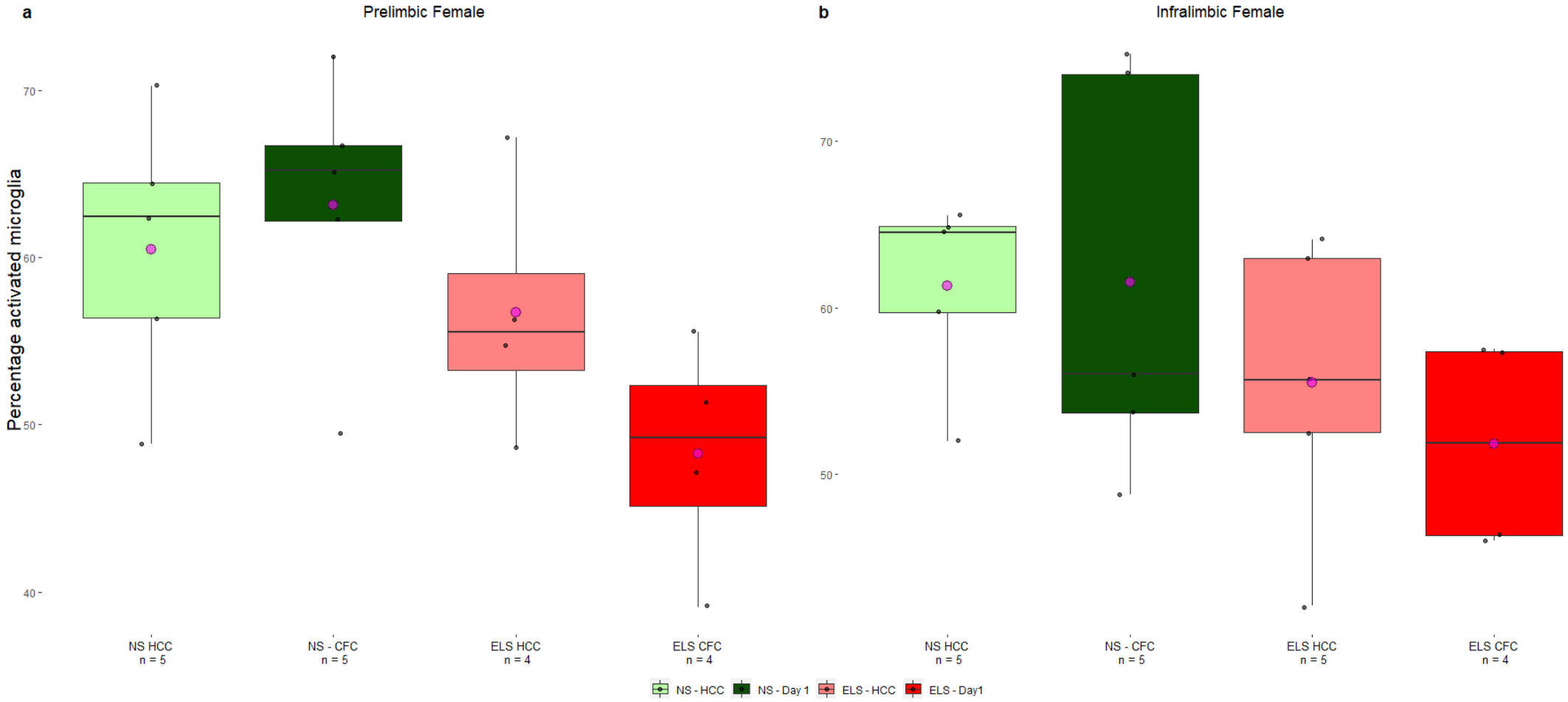


Figure 58: Percentage of non-PBS-like microglia in Female rats as predicted by NueMorph pipeline in the pre-limbic (a) and the infra-limbic (b) cortex. Non-stressed animals are shown in shades of green, animals exposed to ELS shown in shades of red. Dark shades show animals exposed to CFC and light shades are animals used as home cage controls (HCC). Each data point represents the mean activation percentage of all microglia analysed within an individual animal. NS = non-stressed, HCC = home cage controls, ELS = early life stress, CFC = one day post CFC. Horizontal bar shows median. Pink circle shows the group mean. Sample size (n) beneath each condition.

6.4 Discussion

This chapter focused on two primary objectives: generating a large dataset to test an enhanced version of Reddaway's pipeline [291]; and using this enhanced version to predict morphological activation, and generate a percentage of activation per group, in a modified ELS-CFC paradigm where animals were exposed to ELS or no stress, and at adulthood, brains were taken one day after CFC. The large dataset was created using animals injected with LPS or PBS, and Reddaway's pipeline, with the analysis originally written in R, was converted to Python to leverage advanced machine learning libraries. Random forest was found to be the best model as was seen in the work by Reddaway. Work was then conducted to examine how ELS and CFC influence the percentage of non-PBS-like microglia using the updated pipeline.

It was revealed that the impact of ELS on microglia activation is sex-specific, enhancing activation in males while reducing it in females. Interestingly, within males, an interaction between ELS and Condition nearly reached statistical significance, indicating that non-stressed controls may exhibit more activation than their stressed counterparts exposed to CFC. This chapter underscores the complex interplay between ELS, CFC, and microglia morphology, highlighting the nuanced effects of ELS on the neuro-immune system.

6.4.1 Comparing changes in microglia morphology in LPS injected and PBS injected animals.

The raw morphometric comparisons differed from that seen by Reddaway [291], the small magnitude of the difference between the LPS and PBS class suggests the need for a model with a better discriminative power. The updated pipeline presented here, provides additional support for the work presented by Reddaway, that skeletons of

microglia can be generated and parameters that are descriptive of functional activation state can be analysed. As is suggested by Reddaway[291] this method, with its high throughput nature allows for a superior numerical advantage compared to work in the current literature (see table 3 in Reddaway [252] for an overview of current numerical power in microglia analysis). The current pipeline, while not yet fully optimised, can analyse data from thousands to hundreds of thousands of cells, conferring more of a chance to capture these, rare, sparse changes. This gives the potential ability to see subtle changes that are brought on by behavioural stimulus and heralds a different way to look at microglia morphology.

Both in the thesis presented by Reddaway[291] and personal communication, it was found that sex had a major effect on the accuracy of the current model if cells from males and females were combined, with the model unable to accurately differentiate activation in females (data unpublished). Previous research such as that by Villa et al [540] supports this showing that female microglia have a weakened response to stressors. However, the effect appears to be area specific. Doyle et al [268] demonstrated that female microglia in the peri-aqueductal Gray (PAG) exhibit a significantly higher number of non-PBS-like cells compared to males following LPS administration. Similarly, research by Tsyglakova et al [269] revealed that in the dentate gyrus (DG) of the hippocampus, female microglia also show a greater number of activated microglia post-LPS administration.

Interestingly, much of the work which shows sex differences in microglia activation is focused on transcriptional changes[540], much less exists focusing on morphological differences, further highlighting the importance of tools which can rapidly analyse this facet of microglia. The first aim was to replicate the model that Reddaway developed which can accurately classify microglia cells as either non-PBS-like or PBS-like and

therefore to do this, the dataset with the most polarised differences between non-PBS-like and PBS-like (namely the male dataset, as females had a weaker polarisation) was used for building the model (both training and testing the model). Datasets from both males and females who were injected with either LPS or PBS were collected, but only males were used for this aim.

In order to see if the results presented by Reddaway [291] could be seen in a larger dataset, brain sections were imaged from LPS and PBS injected rats. When analysing morphology using single measures there is a significant overlap between the classes and therefore this suggests that differences between the cell classes solely by single morphological feature analysis is subtle and challenging to analyse with simple linear models as was done by Reddaway. Previous work shows that microglia morphology changes in response to LPS (of the same dosage) by only analysing raw morphometry[541-543]. Although issues with these papers include: use of cultures, and not sections from animals given a specific manipulation [541, 543]; and not giving the dosage in terms of concentration per kg [542], making it difficult to compare activation states with the current work. Nonetheless, microglia activation through morphology is confirmed by work conducted by Reddaway[291] who describes morphological differences which are expected in 3 of the morphological parameters. In contrast to these observations results gathered in the current chapter show that through morphological analysis there is very little difference between the groups in terms of animal compared to animal. However, the reason for this could be that analysis by Reddaway was done using t-tests on the entire cell population which could have inflated results through the effect of psudo-replication[544]. Analysis of the entire cell population (as was done by Reddaway) shows very highly significant p values (data not shown). Instead, analysis of microglia morphology between individual, LPS

and PBS, injected animals (with averaged cell values) show no differences in microglia morphology between LPS and PBS injected animals in any parameter. Despite this, it can be argued that compressing the data to just 5 points for LPS and 4 for PBS, which was what was done here for single morphometric analysis compresses any subtle differences between the two conditions. Consequently, a more complex statistical model might be needed, where the pseudo-replication that would be present analysing all of the cells as the condition, is controlled against the animals as a random factor, in order to further analyse this dataset taking into account all features that could be explaining the differences. The absence of observable differences in single morphological features between the two classes may be attributed to the stimulus intensity, with the LPS administered at a concentration of 250 μg/kg potentially eliciting only minimal morphological alterations. Consequently, the current study may lack the power to detect such subtle changes. Interestingly, by examining the direction of the means without doing inferential testing we can see that all the parameters show a decrease from PBS-like to non-PBS-like. This is in line with the work that Reddaway [291] showed.

6.4.2 Challenges with using machine learning to predict microglia activation class.

One issue with the earlier work by Reddaway was that there was an imbalance in the two classes of microglia during training the model. Previous literature suggests that this can produce a bias towards the majority class, which can produce skewed predictions on unseen data[545]. To correct for this, SMOTE sampling was to synthesize data points in the minority class to balance the data. This has been utilized previously, especially when data is low dimensional as is the case in the present work (8 dimensions [Cell territory volume, cell volume, ramification index, number of

endpoints, number of branch points, average branch length, max branch length, min branch length] compared to > 100 dimensions for genetic data input) and has been shown to improve accuracy in various ML models [546].

Reddaway was able to produce a RF classifier that was able to achieve a strong accuracy level for his dataset. Nonetheless, while the model was tested on a test dataset to derive accuracies following Platt scaling the model was not tested on a completely unseen dataset (here the validation dataset), therefore the ability of the model to classify microglia in a dataset that was not trained on remained unknown[291]. Since development of this model, further optimisation was conducted. As the dataset produced here was similar, but with different animals, the accuracy of unadjusted models was tested first. The RF showed the best accuracy at training, with SVC and GBM performing second best equally. This was expected as all three have shown suitability to cell morphology use cases [547-549]. The three (RF, SVC and GBM) were taken forward for hyper-parameter tuning, which was not done in work by Reddaway [291]. Overall, hyper-parameter tuning, where the best hyper-parameters are selected for the model through a randomised search method, was able to improve accuracy in classification (66% accuracy, compared to 64% for non-optimised).The likely reasons for this are described in Chapter 1 subsection 1.19.9. Nonetheless, this has potentially led to the model overfitting, where the model is very accurate in predicting the classes for the training data but shows a loss of accuracy when predicting on an unseen dataset (see Chapter 1 subsection 1.19.1 for a more detailed description). This can be seen by the number of cells inaccurately classified as non-PBS-like in the test dataset, here defined as false positives (if non-PBS-like is the positive class; Figure 55).

The results presented suggest that the current model can learn to discriminate microglia and outputs morphological processes, evidenced by a relatively high accuracy following optimisation (hyperparameter tuning, and correction for unbalanced classes using SMOTE) to improve the accuracy of the RF model. However, it appears that the discriminatory power of such a model is not strong enough to fully determine the difference between LPS and PBS cells when applied to an unseen dataset. The accuracy of the model is relatively high during training however when analysing the unseen test dataset, the accuracy falls away to 59% and the model appears to bias its classification towards non-PBS-like cells. The final accuracy level was unexpectedly lower than anticipated, diverging from 70% accuracy reported by Reddaway [291]. One potential reason is that Reddaway used cross validation to build the model, training the model on data and then testing on a left-out dataset, but then modified the result using Platt scaling and then did not test on an unseen dataset. The use of Platt scaling allowed for a probabilistic determination of the class (non-PBS-like or PBS-like) and the weight towards the class could be changed manually (for example to hold the model to only classify cells as non-PBS-like if 60% sure). However, there are some issues with this methodology. Firstly, Platt scaling assumes that probabilities can be gleaned from the data using a sigmoid function which gathered data in future experiments might not fit. Finally, Platt scaling was used to tune the model once complete, this was to overcome weaknesses in the random forest model used by Reddaway [291], in the current pipeline the random forest was optimised with hyperparameter tuning and SMOTE sampling. Nonetheless, Platt scaling was not included in the current pipeline and is something that could have been added as a method to optimise the model after testing. In the current work this was not included due to time issues. In the current work There was some issues, namely, that

optimisation might have caused some overfitting and resulting in a confusion matrix showing a high number of falsely LPS predicted cells. This was surprising as both the training and the test set had a substantial number of cells and cross validation was used throughout training to attempt to correct for overfitting. It may be that oversampling with SMOTE is generating cells that are not representative of the cells that they are to synthetically mimic. However dataset augmentation with SMOTE is a common feature of classification pipelines in microglia analysis, for example this is employed by Leyh et al when training their model[550] which was a reason for inclusion of this pre-processing step in the current work. A potential way to examine if an issue is present in the synthetically generated cells would be to examine those cells that have been synthetically generated and compare them to the cells that were acquired through imaging. Another potential suggestion is that the training dataset is too small to develop an accurate classifier, however, as is mentioned above and shown in Table 30 the accuracy for the model does not increase following successive increases in the dataset, suggesting that the size of the dataset is sufficient. Other more likely causes are that the quality of the skeletons, and therefore the quality of the features generated are not sufficient. This is discussed further below in subsection 6.4.5.1.

Two main papers which aim to classify microglia according to morphology are important to discuss. First, Leyh et al [550] have developed a segmentation pipeline that uses the VCC-16 [551] convolutional neural network (CNN) to classify cells into one of four classes (ramified, rod, activated and ameboid) This model is quite different to the one employed in the current work. Despite being able to classify cells into the four classes well and verifying this successfully with an ischemia animal model, the machine learning model they use has been shown to be what is often classed as a

“black box model”[552]. This is where the model is able to produce very good classifications but the ability to determine which features are the most important in explaining the data and what causes the model to shift towards a different classification becomes very opaque. CNN’s have been shown to pick up on very minute details, or features which are irrelevant which can hold very little biological relevance and use this to find patterns. For example a highly prominent case has shown that a CNN model trained to predict malignant skin cancer, could achieve a high level of accuracy, but the model was using the presence of a ruler (shown in cancer photos only) as the metric to classify as cancerous [553]. This can lead to erroneous predictions; instead, being able to classify cells in a biologically transparent manner allows an expert in the field to oversee the process and error check. Furthermore, being able to show that changes in biologically plausible parameters such as branch length, branch complexity or proximity to neural processes (something not done currently in this model) can occur following a stimulus is informative. This can elucidate mechanisms in the dynamics within microglial activity that can be modified and targeted in future work. Using a CNN which builds models from opaque parameters does not have this advantage.

The other is a paper by Choi et al [554] which, shows that an SVM model for classification is very effective in classifying microglia into one of 5 morphological classes (ramified, amoeboid, rod, activated and hyper-ramified). They present accuracy rates well above 80% with 4/5 classes being scored greater than 90% and ramified cells being classified 100% of the time. This accuracy rate is impressive and is considerably higher than the accuracy rates presented in the current work. However, examination of the images that the model is learning and predicting on (an example is shown in Figure 59a, which is in comparison with a representative image from the

current work Figure 59b) elucidates differences in complexity that could be underlying this difference in accuracy. Images in the current work are much higher resolution, with considerably more complex structures. The parameters that Choi et al [554] utilize, consist of Ferets maximum distance and Ferets Diameter ratio limited solely to the analysis of the soma. Furthermore, the authors limit their analysis to the cell body as shown in Figure 59a. Despite this, Choi et al have noted interesting characteristics of the cell body, for example the irregularity of the cell body (as a deviation from circularity) changes when comparing between ramified and activated (see the differences between the images in Figure 59a). This is something that could be included into the current pipeline to improve discriminatory power as the differences between these cells is subtle. So far, NueMorph has no method to examine soma characteristics.

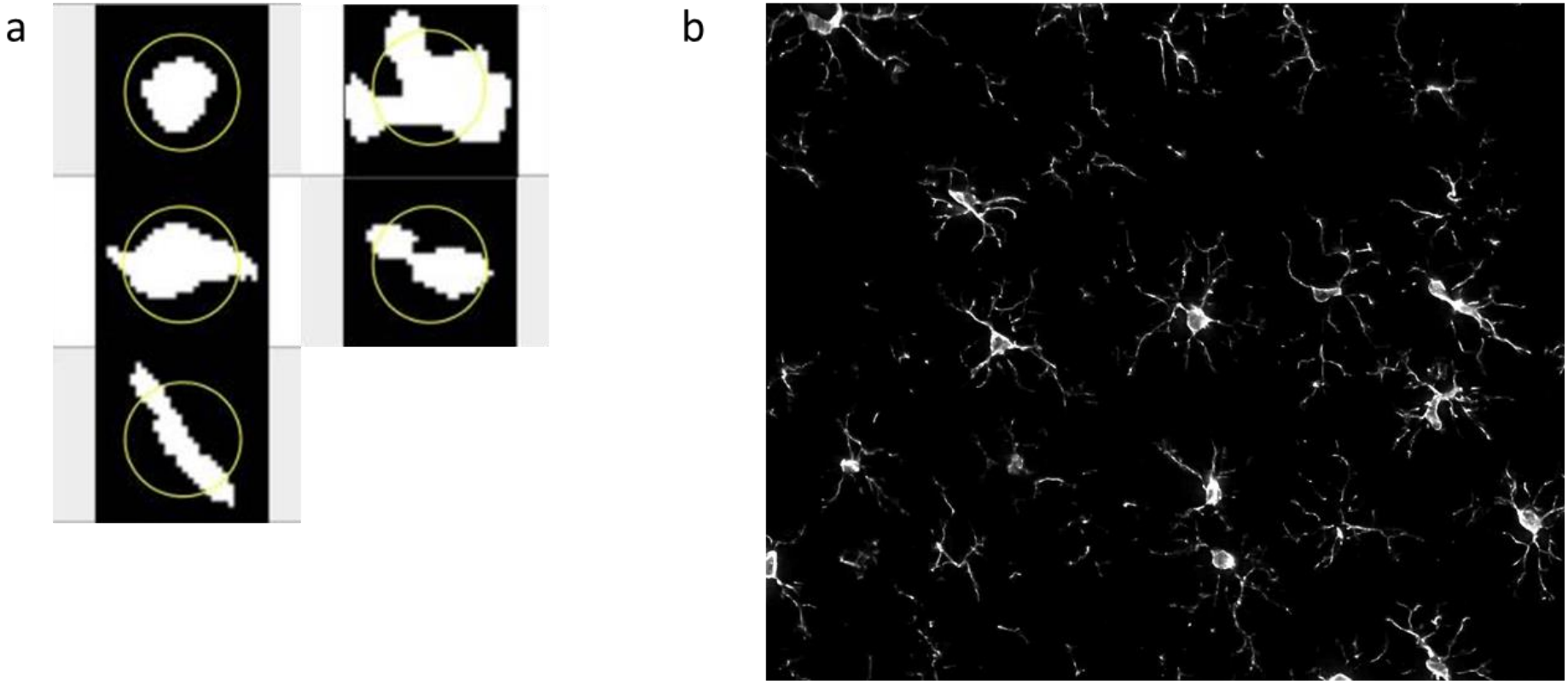


Figure 59 A visual comparison of the resolution between the cells (cell bodies) being classified by Choi et al (a) and the resolution of images of the microglia being classified in the current work (b). Note the substantial difference in complexity and resolution between the images.

6.4.3 The effects of ELS on microglia morphology following CFC

6.4.3.1 Behavioural overview

It is shown in the current work that animals freeze more following foot shock during CFM acquisition than pre-shock. This is in line with the work shown in Chapters 3 and 5 suggesting that animals acquired CFM. There was a significant effect of stress here which, at this shock intensity (0.7mA), differs from that seen in chapter 3. However, analysis of Figure 56 suggests that this could be driven by the lower level of freezing in females that were non stressed. This group is also that which has a lower n. All the other groups have a similar level of freezing therefore increasing the n for this group may reduce the difference between the stress groups. All groups still show an effect of foot shock on freezing with more freezing at post-shock than during pre-shock and there is no interaction with stress and the acquisition of the CFM.

Table 32: Percentage of non-PBS-like microglia compared across groups following early life stress (ELS) or non-stressed (NS) and one day post exposure to CFC or home cage controls (HCC).

Percentage of non-PBS-like microglia		
Sex effect		
Males > Females		
Males		
	PL	IL
Stress	ELS > NS	ELS > NS
Condition	HCC > CFC	NE
Interaction (Stress X condition)	Trend Post hoc NS HCC > NS CFC ELS HCC > NS CFC	NE
Females		
	PL	IL
Stress	ELS < NS	ELS < NS
Condition	NE	NE

Interaction (Stress X condition)	NE	NE

6.4.3.2 Changes in microglia morphology following ELS and CFC.

The interaction between ELS and CFC acquisition on microglia morphology is complex and sex dependant. A summary of the results is shown in Table 32.

Firstly, there is a main effect of sex with males showing a greater percentage of non-PBS-like microglia than females. As there was a clear sex effect the data was split into males and females for further analysis.

There is a sex dependant effect of ELS on microglia activation. Male animals that were exposed to ELS showing more non-PBS-like microglia than those that were not exposed to ELS. This was present in both the prelimbic cortex and the IL cortex. This is in line with the following work showing increases in microglia activation in the amygdala, hippocampus and mPFC. Firstly, Tynan et al[555] showed increased non-PBS-like microglia as defined as density of Iba1 positive cells, following chronic restraint. Further work by, Wohleb et al [510] showing that exposure to social defeat stress led to an increase in microglia displaying a morphological phenotype indicative of activation. More studies are examined in the review by Calcia et al [513] with the majority showing increased activation following various stressors. Further work, such as that by Bian et al [556], show increases in Iba1 positive cells (which they suggest to be indicative of increased activation) following repeated social distress, but without examining the morphology of these cells. In contrast female animals exposed to ELS there was an opposite effect, those exposed to ELS showed less non-PBS-like microglia than those which were not exposed to stress. Much research exists suggesting that stress can amplify immune responses (see Chapter 1 subsection 1.14).

The work presented in this chapter is in line with work by Bollinger [277] showing that females who were non-stressed showed a greater proportion of non-PBS-like (as

defined as primed to ramified) microglia relative to males. This could be indicative of a sex specific microglial priming, an effect that is seen in many scenarios where multiple stimulations (be that a traumatic insult or behavioural event) compound and the response to the second (or third and so on) insult is stronger than the first (see figure 3 in the review by Calcia [513]), which is occurring for males but not females. There is much research which suggests region specific differences in microglia morphology [557, 558], genetic profile [559] and protein expression[557]. Sex differences in microglia morphological phenotype is also well documented[264, 560-562]. Consequently, stress could be interacting with microglia, but due to the inherent sex differences present could result in sex specific effects. This has been shown in the previous chapter where cytokine expression following CFC and extinction differed in a sex and stress dependant manner and cytokines were regulated depending on the interaction between exposure to ELS and sex.

Work by Chaaya et al [509] has shown that animals exposed to CFC or unpaired fear conditioning (non-reinforced and non-overlapping tones given at random during the conditioning) show shorter processes (indicative of a more activated phenotype [252]) than control animals which had undergone no conditioning. This observation highlights the variability in microglial morphology, which appears to adapt based on the environmental conditions and specific stimuli encountered by the animal.

There is also an interaction, in males, between the stress and condition (home cage controls and animals exposed to foot shock) which is likely to be driven strongly by the low amount of non-PBS-like microglia in the non-stressed animals exposed to foot shock. Interestingly, in those not exposed to ELS, non-PBS like microglia appears higher in animals which had not experienced CFC than animals which have

experienced CFC. This effect was only a trend; however, it does suggest that activation is higher in animals not exposed to CFC.

This result is somewhat surprising as, following CFC, it was expected that there would be more non-PBS-like microglia present. Indeed, this is what was seen by Chaaya et al [509] who has shown that animals exposed to CFC or unpaired fear conditioning (non-reinforced and non-overlapping tones given at random during the conditioning) show shorter processes (indicative of a more activated phenotype [252]) than control animals which had undergone no conditioning.

However, if the purpose of microglia during memory formation and synaptic plasticity is to aid in synaptic pruning[505] and trogocytosis [563] a hyper-ramified morphology (with many end feet and processes) may be more beneficial for such a task. Little research exists according to the authors knowledge regarding the examination of hyper-rammified microglia regarding synaptic pruning and trogocytosis and is primarily focused on the retina or during non-stressed conditions [263, 505]. Two important papers however are those by Hinwood et al [512] which shows that following chronic restraint stress for 14 days, microglia adopt a more ramified morphology and that by Kopp et al [511] which shows that a similar chronic stress protocol results in an increase in microglia area which was not co-occurrent with increases in microglia count suggesting that the microglia that are present are “larger”. This evidence suggests that psychological stress does not always cause microglia to follow a “canonical” change in morphology from ramified to activated to ameboid. The change in morphology could be highly dependent on whether the stimulus is an immune related stimulus or a non-immune related stimulus and is highly pertinent to the current work where activation following an immune stimulus in animals is used to model activation following a psychosocial stimulus. Note, a ramified microglia responding to

an immune related stimulus, such as that seen by LPS injection[248, 564], stroke [565]or TBI [566] will not look different to a ramified microglia responding to a non-immune related stimulus as is shown in the work by Schaefer et al and Goyal et al [505, 563]. This suggests that the relationship between microglia morphology and their function may not be as clear cut as activated and non-activated having distinct roles. This is an important consideration as much research considers ramified microglia to be the resting microglia and a shift towards ameboid morphology to be indicative of microglia activation. For example Escoubas et al [567] shows that a subset of microglia requires type 1 interferon (IFN) to aid in neuronal remodelling. However, they classify microglia into ramified and phagocytosing. It would be interesting to consider that microglia in a ramified state can move towards an ameboid like structure or become more branchy in a hyper-ramified state.

The challenge of analysing microglial morphology is compounded further by temporal heterogeneity, where microglia are more responsive to stimulus at certain periods of life[560-562]. Seminal work by Thion et al [560, 568] and work by Grabert et al [569] and Lawson et al [557] shows that there is heterogeneity of microglia morphology with differences across cortical and thalamic regions which was shown by Lawson et al as well as differences between grey matter and white matter across brain regions and previous work [559, 568] showing temporal heterogeneity. In the context of the current work, the pre-pubertal sensitive period of development, described by Brydges et al [164] may be a point of increased microglia sensitivity for males, while for females this period of sensitivity could be characterised by a reduced microglia sensitivity. This does not mean that ELS exposure necessitates a global decrease or increase in microglia sensitivity across the whole CNS, but the work here suggests that microglia

in, specifically, the mPFC (both PL and IL) are primed to show increased activation in males compared to females.

The timepoint at which the brains are dissected is extremely important in determining activation of microglia [486, 570] as well as measuring the point when activation levels off and inactivation becomes dominant. Here we could be seeing a form of inactivation seen following a highly upregulated activation state following activation. While a potential temporal change in microglia has not been examined in detail following behavioural manipulations there has been an abundance of work examining microglia response, in a rapid temporal manner to injuries such as TBI. While the magnitude and nature of the stimulus differ from TBI to behavioural manipulations, the underlying biological processes and timelines of microglial response may share common features such as a shift in morphology (as described below) or changes in cytokine profiles [571] including many cytokines (Il-6, Il-1 β and TNF-α) analysed in Chapter 5. For example, previous work has shown that following TBI microglia exhibit a pro-inflammatory state followed by an anti-inflammatory state which can last for weeks [572]. However, interestingly they find that the rise in anti-inflammatory (M2) does not occur after a rise in pro-inflammatory (M1) microglia instead the M2 rise, and peak occurs during the rise of M1. The majority of analysis of time course tends to be at least a day following injury as shown by a meta review by Velayudhan et al [573]. However, work by McCabe [574] where the primary focus was to study ultrasound as a therapeutic mechanism for TBI found that IBA1 staining was increased as early as 2 hours post injury. This is important as it shows that microglia dynamics can be affected much earlier than 24 hours. Unfortunately, no analysis of activation state through morphology analysis was conducted, however the importance of this short period is relevant. This injury model, and others used for TBI is significantly more

intensive (the method was an open field blast injury) and different from behavioural manipulations such as the ELS and CFC paradigm used here. Therefore, while the rapid activation of microglia post-TBI provides valuable insights into the dynamic nature of microglia activation, the exact timelines and physiological processes may differ in the context of behavioural interventions. This gap highlights the need for focused research on microglial activation and inactivation in response to behavioural interventions. Further work which explores these temporal dynamics will potentially offer insights, revealing microglia's nuanced contributions to the effects of early life manipulations and neuroplasticity in fear conditioning and extinction.

Upregulation of microglia activation has been seen following stress (albeit after late timepoints (30 days later) compared to the current work) [301, 523] and it has been documented that activation of microglia is tightly, temporally controlled by complex feedback loops [502]. While the time point chosen was done so to be in line with other literature which has found changes in microglia morphology after foot shock [509, 518], the fact that the protocols seen in these papers differ in intensity (0.8 and 1mA) and length of stimulus (e.g. 10 seconds of foot shock for [518]) suggests that the time window to observe peak activation could have been at a different time period. In the present work there was an effect of ELS in the IL

In the IL, which has been suggested to be the homologue of the vmPFC in humans[104], there is also an effect of stress with animals which had been exposed to ELS showing more non-PBS-like microglia than PBS-like. Here, there was no effect of exposure to CFM acquisition. Much research suggests that activation in the IL is elicited during extinction learning [111, 115, 353, 356] not during fear conditioning, which this protocol was focused on. The increase in activation is still seen between

stress groups suggesting the effects from ELS affect both the PL and IL areas of the PFC.

6.4.4 Limitations and potential improvements

A major limiting factor of the current approach is that the training dataset will contain instances of the incorrect class. For example, the LPS dataset that the model uses to learn to classify cells as non-PBS-like, will not contain 100% non-PBS-like cells. Some of these cells will be non-activated and have differences in morphology which will produce errors. This is very apparent in the fact that there is a great overlap between the two classes in the dataset, something which was seen by Reddaway [291]. There are methods that could be used to counteract this, however. One of the most promising could be to use semi-supervised learning. This requires a small, manually, labelled dataset (which we currently have generated, data not shown) which can then be used to label the larger dataset. The larger the training, labelled, dataset, the better the training will be. However, gathering this kind of data is extremely time consuming and suffers from the same biases, which are present during manual classification[252]. To adopt this further a technique called active learning could be utilized which would require consistently adapting the model to more and more labelled data. Different labelled data from different laboratories could be pooled to improve accuracy, and analysed with ensemble models where a variety of models can take advantage of different datasets. This could greatly improve accuracy. Labelling data comes with some issues pertaining to manual classification which is described in detail in a review that examines some of the pitfalls of manual classification [252]. Nonetheless, properly labelled data would be a major step towards ameliorating the “impure dataset” problem.

Another issue is that the current model is highly sensitive to microscope artefacts and one that has been recently brought to attention is the fact that there is a substantial issue with out of focus fluorescent bleed through in the z plane images gathered. This can be seen in a 3D image showing the orthogonal view in Figure 60. As the fluorescence intensity of the cell body gets brighter, either through slice-to-slice variation in fluorescence intensity, or through biologically plausible expansion of the cell body as activation, the processes of the microglia become hidden within the noise. Characteristic hourglass shapes show that these images would be well suited to deconvolution (Figure 60). Work into this avenue is currently ongoing and is discussed further in section 6.4.5.1.

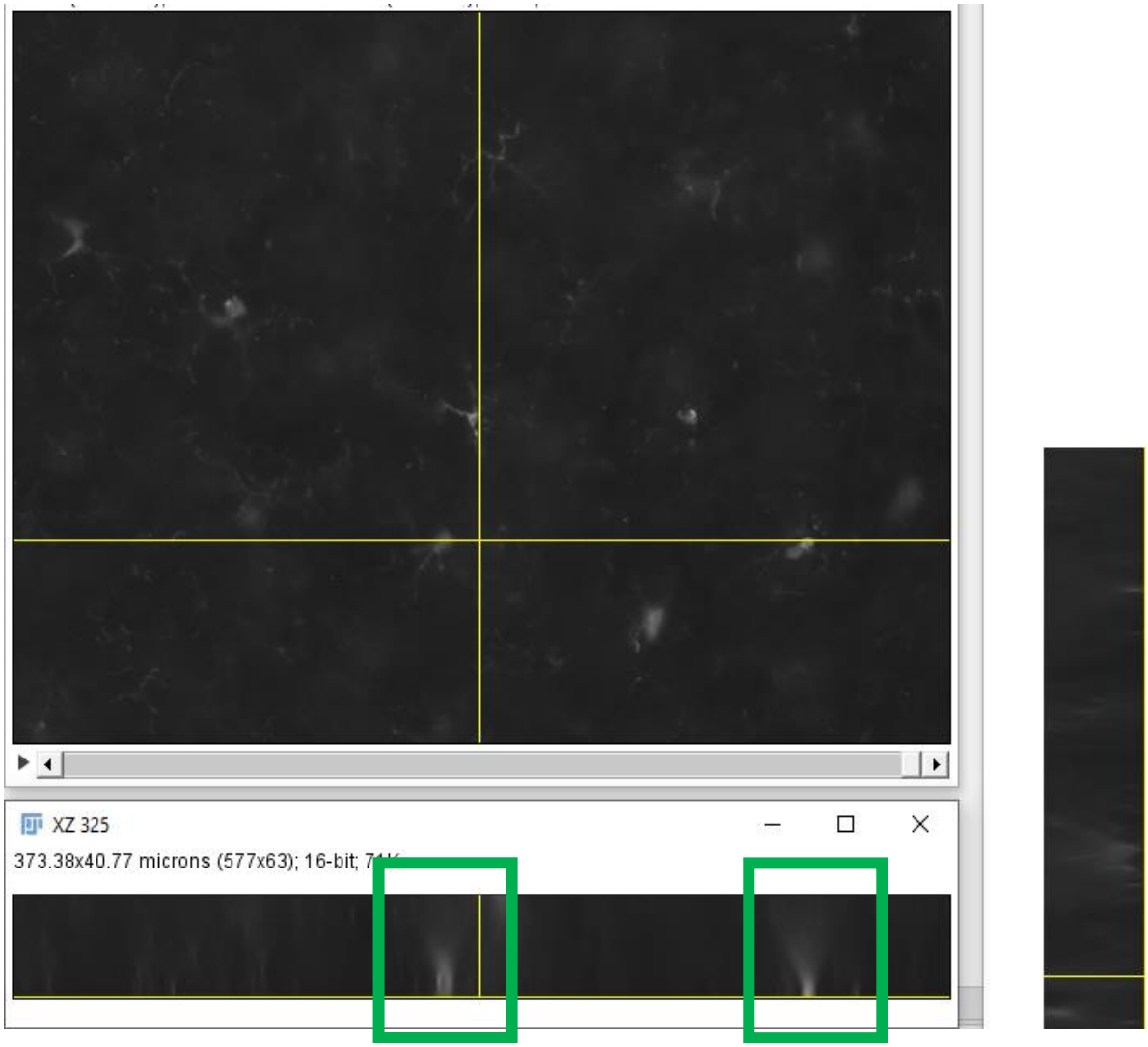


Figure 60 Example of fluorescent blur through microglia z stack images. Characteristic hourglass shape is seen when examining the Z domain. This is shown in green boxes.

A further challenge arises from the fact that this pipeline is constrained by a limited range of parameters; currently there are only eight parameters which the model can learn from. Other commonly used features in manual classification and semi-automated classification [252] could be added. For example, Sholl analysis is a method of classification that combines several simple features such as branch length, ramification index into measurements such as branching index and critical radius using concentric rings[550, 575]. These measures are important as they combine multiple features to produce a new feature (for example: branch length and complexity, combine to produce number of ring crossings). This becomes even more important when no single parameter can provide the discriminatory power needed in the experiments presented in this thesis, instead combining features into component features could be a way to improve discriminatory power. Another feature that has been shown in multiple papers to be an important morphological feature of microglia activation is soma size [550, 554, 576]. Choi et al [554] shows that they can classify simple images of microglia with a very high effectiveness, on soma alone. Leyh et al [550] also suggests that three soma parameters (area, perimeter and circularity) are useful in classifying microglia cells. These measurements could be combined with the current parameters to further improve discriminatory power.

6.4.5 Future work

6.4.5.1 Improvement of the model with Deconvolution pre-processing

Work being conducted currently (not shown) has shown that deconvolution with Huygens Essential software (Scientific Volume Imaging, The Netherlands, http://svi.nl) can eliminate the bleed through artefacts described above in 6.4.4. This allows better resolution of the fine processes in 3D as is shown in Figure 61a. This is also a potential

explanation for why the model is consistently bias towards non-PBS-like cells. Activation would cause a rounded shape more than one that is ramified. The shape of a cell that has intense z stack bleed through will also be of a rounded shape (Figure 61b). Once again, removal of this artefact should improve accuracy, as the skeletons measured will be closer to the ground truth.

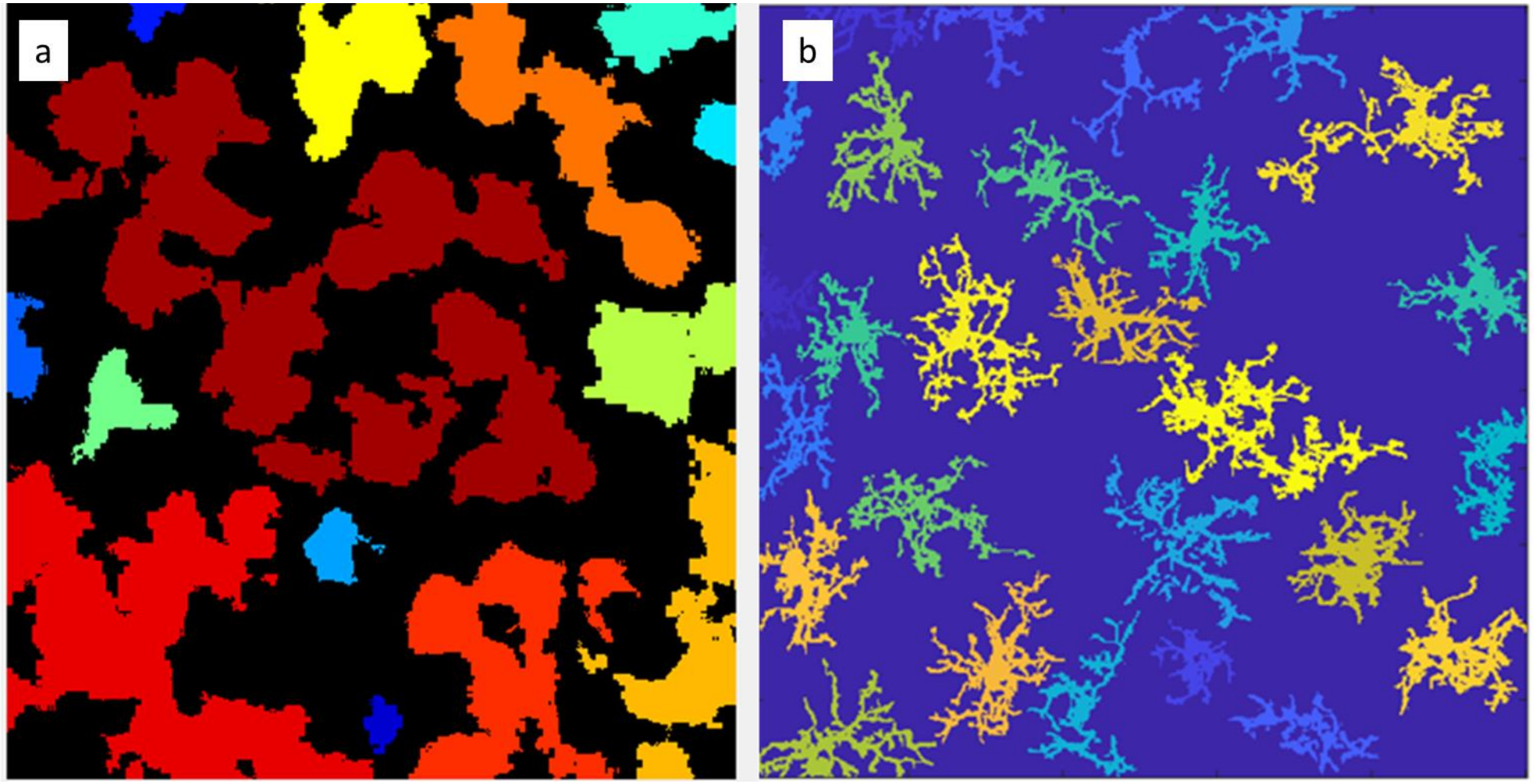


Figure 61: (a) Representative image showing a poor skeletonisation from the current pipeline. (b) Segmentation following deconvolution using Huygens software improves the current skeletons being generated.

As is stated in 6.1 in aim 2 hippocampal tissue was collected but could not be analysed due to methodological issues. Going forward it would be interesting to examine if the effects of ELS and CFC would result in a non-PBS-like profile of changes in morphology in the hippocampus, especially that towards hyper-ramification which has been described before as being observed following stress in the hippocampus of animals bred to respond more strongly to stress [577]. Furthermore, it would be interesting to examine the effects of ELS and CFC on amygdala microglia activation.

6.4.5.2 Addition of other parameters

The current model outputs parameters that have been demonstrated to account for microglial activation, a finding supported by Reddaway's preliminary analysis. Although these parameters can facilitate classification, there are concerns regarding the reliability of some features. For example, ramification index conflates two or more morphometrics into one meta-morphometric, with the goal of better summarising a cell’s morphology.

Other parameters, such as Sholl analysis and fractal analysis, are examples of microglial morphology measures, with Leyh et al. [550] providing a comprehensive list. Sholl analysis, prominently featured in their work, can be broken down into multiple components, as illustrated in sub-images O-R of Figure 3 in Leyh et al. [550]. This analysis has been widely used in studies examining microglial activation states following various traumas [578-580].

Cell centres are already recorded in the software and the skeleton is generated in 3D space with scaled branches. Determining crossing points of these processes through rings is already ongoing currently (data not shown).

An intriguing experimental plan would be to examine microglia activation following each element of the CFM protocol described in Chapter 3, following exposure or non-exposure to ELS. Here only the acquisition of CFM was assessed. To fully elucidate microglial response in the PL, IL and hippocampus (which was not looked at in the present research, due to technical issues but could be included in later experimental models) periods following CFM recall, CFM extinction training and CFM extinction recall would be informative. The IL has been intimately linked with extinction memory learning (and reviewed in Chapter 1 subsection 1.5.3).

6.5 Conclusion

The current work has presented a way to analyse many microglia in a high throughput method conferring the ability to analyse subtle changes in morphology brought upon by behavioural manipulations. Building on the pipeline first generated by Reddaway[291], the current work shows that changes in microglial activation can be measured after behavioural manipulations. Here the machine learning model was able to detect subtle changes in the microglia of animals exposed to ELS or not, following CFM acquisition. The metrics of model performance suggest that improvements must be made to further improve the classification of activation of microglia following behaviour. Improvements to the pipeline, such as better image processing (for example through deconvolution) and the inclusion of further parameters, could yield a tool that predicts activation state successfully through analysis of changes in morphology following behaviour.

7 Chapter 7: General Discussion

A major facet of PTSD is dysfunctional fear memory processing, this has been described in detail in Chapter 1 subsection 1.2. The over sensitivity to stimulus associated to the fear and the inability to extinguish fear memories is highly characteristic of the disorder. The disease is multifaceted with many environmental, genetic, and underlying biological mechanisms undoubtedly having a role, these are shown in Figure 1 and described in detail in Chapter 1 subsection 1.1. The following chapter aims to give an overview of how data gained from a model of early life stress (ELS) can influence these memory processes using a model of fear memory and extinction and how data gleaned show that differences in both cytokine concentration and microglia morphology is involved in regulating these memory systems.

Below are the principal findings for each thesis chapter:

Chapter 3: Behavioural analysis of ELS on CFC and extinction memory.

- The primary aims of this chapter were to replicate and expand on the work of Brydges et al [164] examining how early life stress (ELS) affects sex-dependent fear memory acquisition recall and extinction of fear memory in animals. The work presented here replicated the result of Brydges et al, which showed that females freeze less than males and that animals exposed to stress show a stronger recall than non-stressed animals.

- Animals acquire contextual fear memory. There is an effect of ELS and effect of sex on CFM acquisition at low shock, 0.5mA, which has been routinely used to generate long term fear memories but not at a high shock intensity of 0.7mA.

- ELS and sex affect CFM recall with males freezing more than females and animals exposed to ELS freezing more than non-stressed. This occurs following exposure to the low shock intensity, exposure to the high shock intensity reduces the effect of ELS. This is potentially due to CFM being enhanced using the higher shock intensity, thus occluding the difference seen when using the lower shock intensity. Females show very low levels of conditioned freezing at CFM recall.

- Extinction learning occurred in male animals regardless of stress and female non stressed animals following low shock intensity shock. With those following high intensity shock stressed males extinguish while non-stressed males do not. Females exposed to high shock show a lack of extinction learning. However, this could be due to a potential floor effect manifesting.

- During extinction recall males which have undergone ELS show evidence of learned extinction following exposure to low intensity shock. Those not exposed to ELS show resistance to extinction. In females, neither stressed or non-stressed show evidence of learned extinction of CFM. However, this could be due to such low levels of freezing seen in the females during CFM recall. As main effects, males show more conditioned freezing than females and animals

exposed to extinction control freeze more than those exposed to extinction training.

- At reminder training, for those exposed to a lower shock intensity which assesses spontaneous recovery shows that there are no differences between extinction condition for any group (male, female and ELS, non-stressed) suggesting no group showed stronger spontaneous recovery than another group. Nonetheless, numbers are reduced due to the required culling of a cohort of animals for the COVID-19 pandemic suggesting an issue of power. For animals which were exposed to the higher shock intensity, none of the subgroups, male/female, or ELS/non-stressed show evidence for extinction of fear memory indexed by lower conditioned freezing. Both results could be due to a floor effect seen at this time point.

- For reminder recall for the low shock intensity there is a sex by stress interaction. Nonetheless, there is evidence that a floor effect may be present and that these results may be driven by very low levels of freezing in the females. For both low and high intensity shock, there is no evidence of enhanced reminder recall for any extinction training group.

- In males, ELS enhances acquisition and recall of CFM and produces a resistance to between session extinction in males. This is dependent on the intensity of the stimulus and aligns with evidence that one facet of the clinical phenotype of PTSD is dysfunctional fear memory extinction. There was no clear evidence of spontaneous recovery or reminder effects, no sex effects, and no

ELS effects. The levels in these tests are generally low and suggest that the CFM is weak and potentially forgotten. A generally low female freezing response at extinction learning and recall suggests that another metric to index fear behaviour may be more appropriate to study female extinction learning and recall.

- These results suggest that sex and early life experiences play crucial roles in modulating fear memory processes, indicating a potential need for tailored approaches to therapeutic strategies for trauma-related disorders. Moreover, the lower freezing responses observed in female animals during CFM recall and extinction phases suggest the need for alternative metrics to better assess fear behaviour in female subjects, ensuring a more nuanced understanding of sex differences in fear conditioning and extinction.

Chapter 4: The effects of early life stress on extinction memory measured via 22kHz ultrasonic vocalisation.

- One of the main objectives of this chapter was to explore whether the emission of 22kHz USVs in males and females could serve as a useful behavioural alternative to measuring CFM and extinction. Results gleaned from Chapter 3 show that females were freezing at low levels following extinction recall and thus it was hypothesised that females may be showing a different behavioural response. The emission of 22kHz USV's for females however was low in this experiment suggesting that there still could be another metric that females are using to express fear memory.

- There are major sex differences at each stage of CFC and extinction learning in 22kHz ultrasonic vocalisations (USV). Males always emit more 22 kHz USV's than females. Analysis of 22 kHz USV count and the ratio of callers to non-callers elucidates different results throughout the experiment and each difference is described below.

- There are effects of sex and stress which interact during CFM acquisition. Males call more than females and ELS exposed animals call more than non-stressed. Furthermore, while the number of calls only increases following foot shock in high shock intensity during acquisition, the number of callers to non-callers increases in both shock intensities. Increasing the shock intensity reduces the effects of ELS on CFM acquisition which is in line with that seen with fear memory indexed by conditioned freezing suggesting that the effects of ELS are potentially sensitive to the intensity of the stimulus (with the caveat that this could not be tested directly as mentioned previously in subsection 3.4.2).

- During CFM recall males call more than females for those exposed to the low shock intensity. For females there are more callers than non-callers in those exposed to ELS compared to non-stressed animals. For animals exposed to the high shock intensity males call more than females. Analysis of the caller/non-caller ratio suggests male show more callers compared to non-callers but there is no effect of stress.

- During extinction learning, the level of 22kHz USV calls are very low. However, males which had experienced ELS show evidence of extinction. This was present for both the low and high shock intensities. Few studies have been identified that explore 22 kHz USV calls in CFC recall, without involving a tone in the paradigm. Although some studies suggest no sex differences in extinction learning, the low level of USV calls at this stage makes identifying such differences challenging.

- For extinction recall, during low shock intensity there were too few 22 kHz USV calls to make a genuine model. For high shock intensity, males show an effect of extinction training, with both stressed and non-stressed animals calling more in those exposed to extinction training than control.

- This chapter explores the use of 22kHz USVs in male and female rats as an alternative metric for measuring CFM and extinction, revealing significant sex differences and interactions between stress and shock intensity during various experimental stages. While males generally emit more 22kHz USVs across different conditions, the low emission rates in females suggest the need for alternative behavioural metrics to accurately assess their fear memory and responses to extinction training.

Chapter 5: The effects of ELS and sex on the regulation of cytokines after CFM recall and extinction.

- The primary aim of this Chapter was to examine the effect of ELS and sex on cytokine expression following CFM recall and extinction.

- CFM acquisition, using only 0.7mA shock intensity, assessed here was similar to that shown in Chapter 3. For CFM recall the results show congruence with chapter 3 in that males freeze more than females and animals exposed to ELS freeze more than animals not stressed. Animals underwent extinction learning and some differences were seen where male animals show extinction learning after being exposed to non-stress while stressed do not show extinction.

- Brains were collected 2 hours following extinction training or extinction control or those assigned to home cage controls. Before extinction recall brains were collected, sliced, and tissue punches were taken.

- In the CA1 sub region of the hippocampus there was a reduction in several cytokine/chemokine protein concentrations (GM.CSF, IL-10, IL-12, IL-18, IL-1β, IL-5, IL-6, IL-7, MCP-1), between extinction control and extinction recall, which have a range of roles downstream, from signalling pro-inflammation to anti-inflammation. Furthermore, there was an interaction of sex and ELS with male stressed animals and female non-stressed animals showing a difference in a select profile of cytokines (IL-6, IL-12, IL-10, IL-5 and IL-1β) between the extinction control and extinction trained groups.

- In the CA1 there was a sex difference in IL-18 with males having a higher concentration than females This result could direct future research into more detailed examinations of the mechanisms behind IL-18 modulation in stress responses. While there were no effects of ELS currently, it is important to consider that the current work was very likely underpowered and the sex

dependant effect of IL-18 suggest that this cytokine, and linked pro-inflammatory cytokines could be a target for further experimental work.

- There was no change in cytokine protein concentration, in the infra-limbic (IL) sub-region of the pre-frontal cortex (PFC), across either sex, stress or extinction groups.

- The work adds to previous literature, where previous work had primarily focused on mRNA levels, here the work has shown that a distinct immune protein profile is upregulated during CFM recall and extinction learning and subsequent recall. Cytokines such as IL-6 and IL-1β have been shown to be modulated following recall and extinction previously, and a similar pattern of cytokines were altered following extinction training, compared to extinction control in the current work.

Chapter 6: Microglia analysis through an advanced tool and behavioural applications.

- The primary goals of this chapter were to expand upon Reddaway's preliminary study by addressing its limitations in scope and by improving the model with hyper-parameter tuning, to better classify microglial activation states, hypothesizing distinct morphological patterns between PBS and LPS-injected animals. This model was then used to analyse a large-scale behavioural dataset to investigate how microglial activation states vary with exposure to ELS and conditioned fear, focusing on sex-dependent morphological changes

in the mPFC and hippocampus. Methodological effects precluded study of the hippocampus, but preliminary results suggest a sex and ELS interaction effect.

- A tool to detect changes in microglia morphology, from a ramified surveilling state (defined as PBS like) to a different state (defined as non-PBS-like) which was developed by Reddaway was optimised using hyper-parameter tuning and a larger, independent cohort of LPS or PBS injected animals was tested.

- Analysis of single parameters taken from raw morphometry analysis shows, non-significant, patterns in line with previous work by Reddaway (with the caveat that the statistics used by Reddaway likely cause type 1 errors) but highlights the inability to resolve significant differences using these measures independently and alone. The use of a machine learning method to incorporate all the parameters into a profile was tested further to glean differences.

- The random forest classifier emerged as the superior metric for classification, outperforming other classifiers like the support vector classifier and Gaussian Naive Bayes, as previously identified by Reddaway[291]. While optimization enhances the model's performance, it also raises concerns about overfitting, particularly when applied to unseen data. This overfitting suggests that although the model is highly effective for the specific dataset used in this study, its ability to generalize to other datasets, especially those involving microglia classification, may be limited. To mitigate this risk, further validation across diverse datasets could be conducted.

- The random forest classifier was found to be the best metric for classification, compared to other classifiers (such as support vector classifier and gaussian Naiive Bayes) as shown previously by Reddaway. Optimisation improves the model but potentially leads to overfitting when testing on unseen data. This suggests that the model is well suited to that specific dataset but may not be adequate at classifying microglia in other, non-seen datasets.

- Using this trained model the effect of ELS on microglia activation, either one day following CFC or not exposed to CFC, in the PL and IL sub-region of the mPFC shows a sex dependant effect.
    - There are more non-PBS like compared to PBS like microglia for males compared to females.
    - For males, those exposed to ELS showed more non-PBS-like microglia than those which were non-stressed. In females those that were non-stressed showed more non-PBS-like microglia than those exposed to ELS.
    - For males those exposed to CFM acquisition show more non-PBS-like microglia. The interaction present, between stress and condition, suggests this is due to the difference between those exposed to CFM acquisition and home cage controls in the non-stressed group.
    - PTSD is being seen as a neuroimmune disorder. This work suggests that with improvement of the model and a better classification pipeline microglia activation can be measured following behavioural conditions. This would be a powerful technique for investigating the neuro-immunological mechanisms underlying the dysfunctional fear memory

processing and subsequent issues with extinction seen in disorders such as PTSD.

7.1 Comparing single vs multiple measures in analysing responses to early life stress and fear conditioning

The goal of this thesis was not to analyse the role of ELS as a risk factor for PTSD, models of PTSD exist and the effects of ELS has been linked to higher risk for developing the disorder in humans[581, 582], however, the primary goal of this thesis was to examine the effects of ELS on acquisition, recall and extinction of contextual fear memories, a complex cognitive mechanism which can be dysfunctional in various disorders such as substance abuse[583], generalised anxiety disorder [87] and Schizophrenia [584] as well as PTSD [86, 89, 311]. Throughout this thesis the role of ELS on contextual fear conditioning manifests as an enhancement of acquisition and recall.

The adverse effects of ELS on brain development have been reported in great detail utilizing both animal models to probe specific facets of ELS (where the primary form of ELS is maternal deprivation) as well as through detailed longitudinal studies of human participants who had experienced childhood abuse [9, 211, 307]. Much of this work suggests that ELS has an adverse effect and negatively affects development. The work presented in this thesis suggests that ELS potentiates the recall of contextual fear memory, which in relation to psychiatric disease is seen as a detrimental aspect of the disorder. Interestingly, work by Lesuis et al [187] and by Brydges et al.[96, 164] show that ELS attenuates fear memory recall. However, some methodological differences are present. For example, Brydges et al [164] analyse recall over 5

minutes, which could have elicited some within session extinction. Furthermore, the method of ELS employed by Lesuis et al [187] is that of the limited nesting material. Previous work by Rocha et al [316] has shown that different methods of ELS (eg maternal separation, handling and limited bedding) can lead to different effects on both development of the CNS and different measures of fear conditioning, Morris water maze and novel object recognition, which is likely to elicit different effects on the developing CNS than the 3-day variable stressors employed in the current work.

It would be interesting to compare the effects of different ELS methods on freezing behaviour and 22kHz vocalisations. As suggested above, different methods of ELS can elicit different effects on CFM recall and extinction and it may be argued that the way that these methods differ (either by length, intensity, type) might be a reason why they confer either resilience or sensitivity to either recall or extinction of fear memory.

Another caveat in this research is how vulnerability and resistance are defined. For example, Lyons et al [1, 2] suggests that ELS can enhance arousal and awareness, which could be seen as a positive state, especially when attention or memory is the behaviour being tested. However, PTSD is often characterised as manifesting with symptoms such as hypervigilance [5]. In this case these increased symptoms would be seen as a negative. The individual differences between people highlights that the relationship between vigilance and hypervigilance, as well as the vulnerability and resistance to pathological hypervigilance will be different between people, but it does highlight a significant issue in the way these terms are described in the literature.

7.2 Sex differences in fear behaviour

There may be a sex difference in how animals express fear memory. Freezing may not be the only behaviour that the rats are exhibiting following CFM acquisition and

consolidation (and each stage following this). Another possibility is that females are showing a more active response to the fear memory, for example darting behaviour. Darting has been shown to be strongly associated with cued fear conditioning in females [165]. However, analysis of the current dataset shows no evidence of darting behaviour (data not shown, while assessing freezing the investigator saw negligible evidence of darting). It is possible that CFC does not generate the darting behaviour due to the context stimulus being constantly present as opposed to a cued stimulus which occurs for a brief time giving a precise time to dart. Further to this when a contextual task was employed by another laboratory [20] it was found that animals did not exhibit darting behaviour at any point during CFC.

A different form of locomotor response to fear may involve reduced or slowed movement which has been shown qualitatively as a measure for fear memory[585]. The current method of assessing freezing focused on specific time points to analyse this behaviour, which does not account for the periods between these time points. Analysing the entire locomotor activity of the animal over the entire trial could reveal different behavioural traits. However, it is crucial to establish a priori hypotheses for this kind of experiment to avoid "fishing" for results, given the rich behavioural repertoire that can be extracted from this type of data acquisition.

The metrics for analysis of female fear behaviour are still relatively unexplored. Here the work presented suggests that females are either resilient to extinction learning when exposed to a higher shock intensity or are expressing their fear memory in a different way (as described in Chapter 4 subsection 4.4). To explore this in more detail 22 kHz USV calls were analysed. However, this showed an even more pronounced sex effect where females approached floor levels rapidly following acquisition. Previous studies have indicated that there might be variations in both the production

and perception of 55 kHz USVs, which are generally recognised as appetitive calls and often described as 'chatter' [421, 442, 586]. These vocalisations may be emitted during experimental conditions, such as exposure to open field tests and can have an influence on behaviour when recordings are played back to the animals. These calls may reemerge after the process of extinction learning. Indeed, this 55kHz chatter was observed during recording of the pre shock time point during CFC and was not present at post shock (data not shown). Due to technical challenges and the need to prioritize bandwidth for capturing 22 kHz sounds—the primary call range for nociceptive stimuli—this data was not stored or analysed. Future experiments could examine this 55kHz chatter, especially in females, especially at extinction and extinction recall assessing if such calls are a different index of (extinguishing) fear memory. While some work exists looking at playback of 55kHz calls [431, 442] to influence CFC less exists examining the emission of 55kHz calls following extinction learning.

While freezing and measurement of USVs are commonly used to assess fear memory, other behavioural and physiological markers may offer a more comprehensive understanding of fear responses, particularly in the context of sex differences. For example, changes in heart rate and heart rate variability (HRV) could provide insights into the autonomic regulation of fear. Studies have shown that alterations in heart rate may reflect the intensity of the fear response, with HRV offering a measure of how the nervous system is modulating stress during and after conditioning [587] [588]. These metrics, especially since they can be measured in the home cage, combined with traditional behavioural measures, could help illuminate sex-specific differences in fear memory consolidation and extinction learning.

Similarly, rearing behaviour, often interpreted as exploratory or vigilance-related[589], could serve as an alternative index of fear response. Females, for instance, might

exhibit more rearing as a way of coping with fear. Tracking rearing across different stages of conditioning could reveal important sex-dependent behavioural strategies.

Grooming behaviour also represents a potentially valuable measure, as it is commonly associated with stress relief in rodents. In the current dataset, analysis of grooming patterns might reveal how animals, especially females, manage stress following fear conditioning. Increased grooming could indicate a shift towards more active coping mechanisms in females, where freezing is less pronounced.

Tronson and Keiser[355] suggest that, as of the time of writing, there are no female specific molecular mechanisms that explain facets of conditioning and memory. One can argue that the same holds true for behavioural analysis. There is very little work that exists which highlights the female response to fear conditioning. Those behaviours that are female centric such as darting are rare events compared to freezing which occurs more often [165]. However, the method of coping and subsequent response to PTSD is an intriguing line of research. Active mechanisms such as darting could be seen as protective to risk while passive behaviours such as freezing could be linked to greater risk. This suggests that the behavioural responses might be distinct and triggered by separate, specific mechanisms. According to the predatory imminence theory, proposed by Blanchard and Fanselow [437-439], increasing the intensity of a stimulus causes a rise in the expression of a fear index until the animal switches to a different fear-related behaviour. Other measures, including the diverse environmental factors presented in Figure 1, in Chapter 1, likely play an important role in how an individual responds to later trauma. How individual animals respond to an early life stressor could influence both the behavioural response as well as the threshold to change to another fear associated behaviour. In the current work, the response to the ELS was not analysed directly. Social interaction, aggressive

behaviours and play interactions following exposure to ELS could be predictive for deficits in CFC or extinction learning. A longitudinal study examining this could be informative of risk.

7.3 A link between neuroimmunology, stress and fear behaviour.

The work in this thesis adds to mounting evidence which underscores the intricate relationship between CFC and the neuroimmune system, delineating distinct neuro-immune profiles associated with fear memory recall and extinction. Key studies by Barnes et al [285], Scholz et al. [286], Reddaway [291], and Chaaya et al. [509] collectively suggest a connection between CFC processes and neuroimmune interactions. Notably, Barnes et al., along with Scholz et al. and Reddaway, highlight the pivotal role of cytokine signalling in the recall of contextual fear conditioning and how these are altered when undergoing extinction learning and recall. Concurrently, Chaaya et al. draws attention to the significance of microglia morphology in understanding these neuroimmune dynamics.

The research presented in this thesis builds upon these findings, demonstrating that both cytokine signalling and microglia morphology are associated with the process of CFC. It was observed that variations in cytokine levels are directly linked to recall of CFC and its extinction in a sex and stress dependant manner. This observation comes with the limitation that the analysis was constrained to a single temporal snapshot, as elaborated in Chapter 5, subsection 5.4.7. Additionally, it was noted that alterations in microglia morphology vary depending on sex and stress; however, the work in the current thesis did not specifically examine these changes in the context of memory recall and extinction. Instead, the focus was on CFM acquisition, which was conducted

as a preliminary exploratory experiment to investigate where the effects on microglia would be strongest (as defined as being the soonest after CFC).

This thesis serves as an exploratory investigation into the neuroimmune system's influence on CFC, laying the groundwork for future research. To further elucidate this complex relationship, a detailed experimental plan is proposed, as depicted in Figure 62. CNS areas detailed in this plan include the amygdala, hippocampus and subregions of the mPFC which have been shown to be intimately linked with regulation of fear memory processing as described in Chapter 1 subsection 1.5.

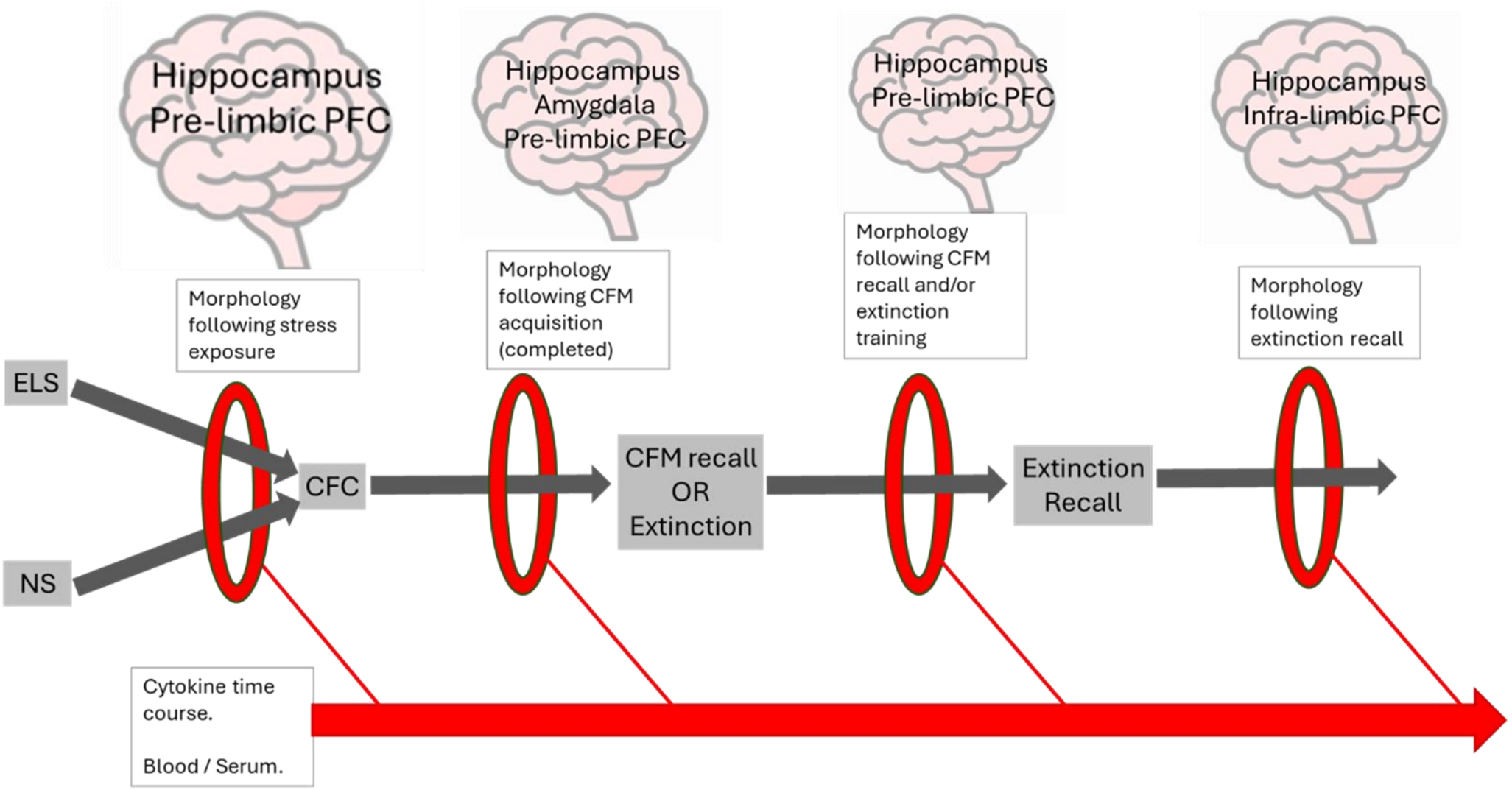


Figure 62: Theoretical, longitudinal, experimental plan to examine the effects of ELS on each element of CFC. Timed dissections at each point denoted by red rings would allow analysis of microglia morphology and cytokine protein expression. Sex differences, which have been shown in detail throughout this thesis, should be examined throughout and so both males and females should be included at each stage. Brain regions of potential interest are presented in the brain images.

Cytokines linked to fear memory and PTSD such as IL-6, IL-1β have been shown to be elevated peripherally in those with PTSD [62, 446, 590, 591]. To analyse the contribution of these cytokines and assess if they are upregulated following different stages of CFC a longitudinal blood/serum analysis would be useful. Importantly, peripheral cytokines do not necessary reflect central levels. The previous work examining peripheral cytokine expression in those with psychiatric disease likely indicates the HPA axis state, while the stress system and the neuroimmune system is highly linked as shown in Chapter 1 subsection 1.14, the regulation of cytokines centrally is likely to be local (due to the function of the BBB). Nonetheless, the fact that elevated cytokines are seen in those with PTSD this suggests the role of such peripheral cytokines and their role on CFC and extinction is of interest to examine. While this does come with some issues, such as the blood taking procedure itself causing stress, this kind of longitudinal analysis would potentially be able to capture the dynamic changes in cytokine expression and production across fear conditioning and extinction.

In the context of CFC and extinction learning, the analysis of cytokine protein expression and microglia morphology holds significant potential for advancing our understanding. A notable challenge in this research is the methodological limitation that precludes simultaneous analysis of cytokine protein concentrations (which necessitates snap freezing) and microglia morphology (which requires perfusion fixation with PFA). This would therefore require either splitting the brain and fixing one hemisphere while flash freezing the other (with the caveat that this can lead to further trauma from the slicing itself, important to consider when looking at immune markers) or using independent experiments. As mentioned previously, the timing of these dissection points is crucial as immune responses are rapidly up and downregulated.

Analysis of timepoints beyond extinction recall (not shown in the large prospective figure above), such as post reminder and reminder recall could elucidate how ELS modulates the role of the neuro-immune system in spontaneous recovery, generalisation, and reinstatement. All of which can be dysfunctional in PTSD [31]

7.4 Development of tools for further microglia analysis. Collaboration and utilization of advanced imaging techniques and ML methods.

The collaboration between computer science experts and neuroscience researchers has significantly enhanced the discipline of neuro-informatics (neuroscience combined with computational modelling), especially through the application of computer vision and deep learning in image recognition tasks. This has enhanced the precision and efficiency of biological research methodologies, leading to higher throughput analysis, and enabling a way to rapidly analyse complex biological images.

Recently, there has been an explosion of work utilizing convolutional neural networks (CNNs) for image recognition. The mechanisms of a CNN are beyond the scope of this thesis but in simple terms the algorithm looks for features in the image, when there are enough features analysed the results are pooled which allows the system to generalise across different examples of microglia cells (for example, the same microglia slightly rotated). For example, the implementation of a “you only look once” (YOLO) architecture has allowed rapid recognition of common objects (desk, chair, dog, human) from analysis of complex multisubject images [592]. Interestingly, this architecture has been utilized to segment microglia from brightfield images and achieve a high recall and precision score when comparing to ground truth [593]. Therefore, this model could be considered for accurate microglia segmentation and

classification. However, using a CNN to classify microglia classes can open up problems such as unexplainably of the model (see Chapter 6 subsection 6.4.4), indeed this is an issue present with the CNN classifier developed by Leyh et al [550].

Instead, the use of a CNN to threshold and segment images before running a script that can generate skeletons based on this processed image, and then analysing parameters taken from said skeleton could be a way to improve the quality of the data (Figure 63 shows a schematic example of this). Therefore, while the slices would still be stained and imaged as shown in this work, the CNN could more accurately segment the microglia and save the skeletons individually. These features could then be analysed with the downstream analysis pipeline and still maintain the explainability (for example the feature importance values) that are present in “shallow learning” such as a random forest.

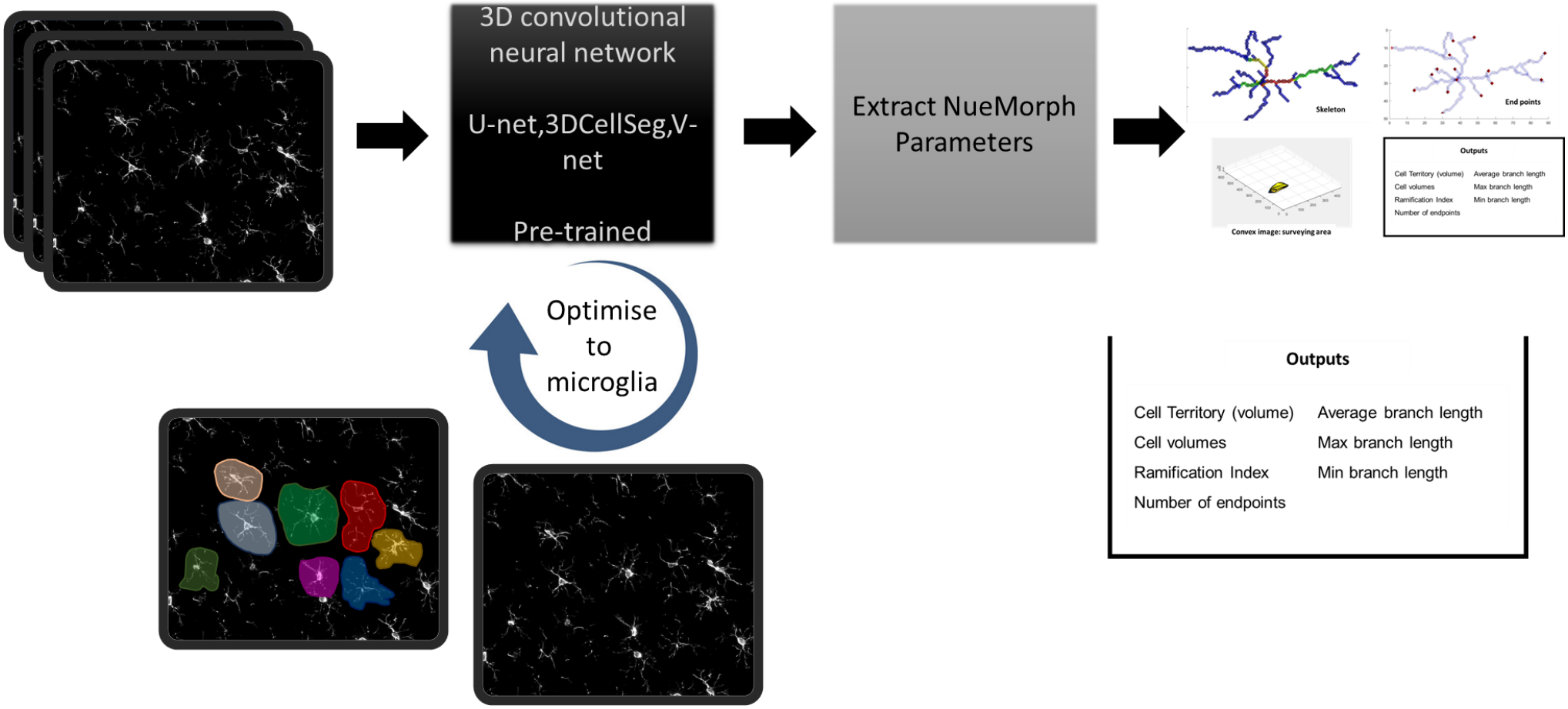


Figure 63: Using a CNN to generate better skeletons from the microglia means that the explainability of the model using Random Forest classification is still preserved but the fit of the skeletons is better, potentially resolving nuanced morphological changes.

As is presented in Chapter 6 subsection 6.4.4 there is a potential chance that the skeletonization script is not accurately following the path of the microglial processes. Nonetheless, the model is producing an accuracy level of 60% and can differentiate differences in animals exposed to ELS and CFC. This would be unexpected if the skeletonization was completely non-functional. Instead, potentially, the “bleed through” seen in some images might be a disparate artifact which is affecting some images but not all. If this is the case then the problem is adding noise, but not completely masking results.

Despite the challenges in accurately segmenting microglia due to their complex and atypical morphology, the use of CNNs for preprocessing tasks—such as image thresholding and segmentation before analysing biological parameters, presents a promising avenue for improving data quality. This approach maintains the relevance of biological parameters and allows researchers to trace back and identify the most crucial features used by the model. Thus, while leveraging the robust capabilities of CNNs for segmentation, it also ensures that the analysis remains grounded in biologically significant criteria.

The complexity of research, particularly in large-scale, high throughput imaging studies, has reached unprecedented levels. The key questions are not just about exploring behaviour or underlying biological mechanisms but is about developing the techniques to study such complex data. No single laboratory, regardless of its sophistication, can boast all the expertise and equipment necessary for such intricate, multidisciplinary, and large-scale research investigations. This reality suggests interdisciplinary collaboration is not just beneficial but essential.

For example, a single project where an entire brain is imaged (which would be highly informative for comparing network activation of microglia in different brain regions) can generate upwards of 100TB of imaging data [594]. The sheer volume presents a formidable challenge, not only in terms of storage, especially when such data must be stored for a lengthy period but also in pre-processing as well as consideration for statistical analysis. This scenario begs the question: How can we manage and make sense of this vast amount of information? The answer lies in the collaboration between neuroscientists, bioinformaticians and computer scientists. Fostering collaboration between these disciplines is crucial at every step of the research process. The critical article by Frégnac in Science [594] suggests that acquiring large scale data alone is not enough to answer complex questions, the implementation of sound theory from the conception of the experiment is important (Frégnac likens this to the way theoretical physicists are included in the design stage of large-scale particle, or astro-physics experiments). From the design phase, where experiments are conceptualized with data management in mind, to the final stages of data analysis, the interplay of these varied expertise leads to innovative solutions that no single field could achieve on its own. Challenges arose throughout the work to produce the results in Chapter 6 as described in Chapter 6 subsection 6.4.4, and discussion of results with individuals in other fields as mentioned above aided greatly in the advancement of the methodology.

There are many further examples of such successful collaborations. Some examples include the Blue Brain Project [595], the human connectome project [596], the human brain project and the Allen institutes Brain Atlas [597]. All of which bring together scientists from various disciplines to advance our understanding of the human brain. This initiative has led to breakthroughs in neuroscience, computing, and medicine,

demonstrating the power of collaborative innovation. While conducting the work in this thesis a notable point of challenge was a discrepancy between the definition of different microglia morphologies (as described in Reddaway et al [237]). This was compounded further by differences in how images are acquired (for example confocal vs widefield microscope) and how they pre-processed (any background subtraction, deconvolution, sharpening tools). Being able to build an online collaborative database of glial cells where different researchers with their own unique injury, infection and behavioural models could upload datasets could be an interesting project. There could be a set of guidelines to enforce standardisation would allow comparisons and could lead to the interdisciplinary work that is discussed above. Applying algorithms and pipelines such as the pipeline presented in Chapter 6 could lead to high throughput analysis of microglia (and other glial cells) across multiple conditions. This would be a highly ambitious project, and continuation from the current thesis but one that could yield important results.

7.5 Underlying biological mechanisms of sex differences

Despite a large body of literature which is showing major sex differences in behavioural measures of fear, there is less research which examines how factors such as underlying biological mechanisms as well as environmental factors can be driving these differences. This complexity is compounded by the fact that there are some factors influencing a behavioural measure of fear in the rat that will not be present (at least not as strongly) in humans, for example the influence of odour and olfactory processing of this stimulus. The reciprocal is also true with complex human social interactions not being present in a laboratory rat model. What is apparent across the different measures however is that there is a significant sex difference. This is further manifested through the sex differences in human psychiatric disease [1, 7, 14, 16, 28]

with PTSD being a prime example. Understanding these underlying mechanisms as well as choosing the correct behavioural index is vital for building towards a complete picture of the disease.

A further consideration is the highly female skewed sex prevalence for PTSD and both males and females experience different symptom clusters[179]. Taken together these strongly indicate a sex influence. Currently, the data presented here suggests that analysing female fear behaviour through either freezing or emission of 22kHz USV is imperfect, with floor levels observed after CFM acquisition. Despite the majority of pre-clinical behavioural research being concerned only with male subjects [180-183], there are a multitude of sex specific effects on cognitive behaviour such as fear conditioning and pattern separation[183], social interactions [181, 184] as well as how animals react to PTSD models such as predator exposure and variable prolonged stress [185]. However, another measure is needed to probe the effects of ELS on extinction further. This data also builds on the growing evidence base that the neuro-immune system is intimately linked, in a sex dependant manner, to fear memory. Moreover, it reinforces the hypothesis proposed by Wang et al [573] in their review titled 'Posttraumatic stress disorder: An Immunological Disorder?' that PTSD should indeed be considered a neuro-immunological disorder.

7.6 Concluding remarks

This work highlights an interesting interaction present between sex and the effects of ELS exposure on the learning and extinction of fear memory. The effects are better indexed depending upon the fear behaviour metric, namely freezing or emission of USV calls. The works suggests that a specific profile of cytokines is modulated differentially at either CFC or extinction learning and recall. The work presented here

also hints that microglia morphology can be altered following exposure to ELS and subsequent fear conditioning, in a sex specific way. This expands on the neuroimmune regulation of CFC and paves the way for examining more behavioural metrics of fear and anxiety as well as delving further into the role of the neuroimmune system in fear memory and extinction.

8 References:

1. Bisson, J.I., et al., *Post-traumatic stress disorder.* BMJ (Clinical research ed.), 2015. **351**: p. h6161-h6161.
2. Shalev, A., I. Liberzon, and C. Marmar, *Post-Traumatic Stress Disorder.* N Engl J Med, 2017. **376**(25): p. 2459-2469.
3. Davis, L.L., et al. *The Economic Burden of Posttraumatic Stress Disorder in the United States From a Societal Perspective*. The Journal of clinical psychiatry, 2022. **83**, 21m14116 DOI: 10.4088/jcp.21m14116.
4. Bothe, T., et al., *How expensive are post-traumatic stress disorders? Estimating incremental health care and economic costs on anonymised claims data.* The European Journal of Health Economics, 2020. **21**(6): p. 917-930.
5. McGowan, I., *The Economic Burden of PTSD. A brief review of salient literature.* International Journal of Psychiatry and Mental Health, 2019: p. 20-26.
6. Krystal, J.H., et al., *It Is Time to Address the Crisis in the Pharmacotherapy of Posttraumatic Stress Disorder: A Consensus Statement of the PTSD Psychopharmacology Working Group.* Biological Psychiatry, 2017. **82**(7): p. e51-e59.
7. Coventry, P.A., et al., *Psychological and pharmacological interventions for posttraumatic stress disorder and comorbid mental health problems following complex traumatic events: Systematic review and component network meta-analysis.* PLOS Medicine, 2020. **17**(8): p. e1003262.
8. Grasso, D.J., J.D. Ford, and M.J. Briggs-Gowan, *Early life trauma exposure and stress sensitivity in young children.* J Pediatr Psychol, 2013. **38**(1): p. 94-103.
9. Huh, H.J., et al., *Childhood trauma and adult interpersonal relationship problems in patients with depression and anxiety disorders.* Ann Gen Psychiatry, 2014. **13**: p. 26.
10. Sarkar, T., N. Patro, and I.K. Patro, *Cumulative multiple early life hits- a potent threat leading to neurological disorders.* Brain Res Bull, 2019. **147**: p. 58-68.
11. Zheng, K., et al., *Psychological resilience and daily stress mediate the effect of childhood trauma on depression.* Child Abuse & Neglect, 2022. **125**: p. 105485.
12. Lewis, C., et al., *Dropout from psychological therapies for post-traumatic stress disorder (PTSD) in adults: systematic review and meta-analysis.* Eur J Psychotraumatol, 2020. **11**(1): p. 1709709.
13. Roberts, N.P., et al., *Psychological interventions for post-traumatic stress disorder and comorbid substance use disorder: A systematic review and meta-analysis.* Clinical Psychology Review, 2015. **38**: p. 25-38.
14. Brady, K., et al., *Comorbidity of Psychiatric Disorders and Posttraumatic Stress Disorder.* The Journal of Clinical Psychiatry, 2000. **61**: p. 22-32.
15. Association, A.P., *Diagnostic and statistical manual of mental disorders : DSM-5-TR*. Fifth edition, text revision. ed. DSM-5-TR. 2022, Washington, DC: American Psychiatric Association Publishing.
16. Holmes, A. and N. Singewald, *Individual differences in recovery from traumatic fear.* Trends in Neurosciences, 2013. **36**(1): p. 23-31.
17. Breslau, N. and J.C. Anthony, *Gender differences in the sensitivity to posttraumatic stress disorder: An epidemiological study of urban young adults.* Journal of Abnormal Psychology, 2007. **116**(3): p. 607-611.

18. Kornfield, S.L., L. Hantsoo, and C.N. Epperson, *What Does Sex Have to Do with It? The Role of Sex as a Biological Variable in the Development of Posttraumatic Stress Disorder.* Curr Psychiatry Rep, 2018. **20**(6): p. 39.
19. Olff, M., *Sex and gender differences in post-traumatic stress disorder: an update.* Eur J Psychotraumatol, 2017. **8**(sup4).
20. Shalaby, R., et al., *Prevalence, Demographic, and Clinical Correlates of Likely PTSD in Subscribers of Text4Hope during the COVID-19 Pandemic.* Int J Environ Res Public Health, 2021. **18**(12).
21. Amstadter, A.B., et al., *Potentially traumatic event exposure, posttraumatic stress disorder, and Axis I and II comorbidity in a population-based study of Norwegian young adults.* Soc Psychiatry Psychiatr Epidemiol, 2013. **48**(2): p. 215-23.
22. Tang, B., et al., *A Meta-Analysis of Risk Factors for Post-Traumatic Stress Disorder (PTSD) in Adults and Children after Earthquakes.* Int J Environ Res Public Health, 2017. **14**(12).
23. Abedzadeh-Kalahroudi, M., E. Razi, and M. Sehat, *The relationship between socioeconomic status and trauma outcomes.* Journal of Public Health, 2018. **40**(4): p. e431-e439.
24. Wang, Y., et al., *Social support and posttraumatic stress disorder: A meta-analysis of longitudinal studies.* Clinical Psychology Review, 2021. **85**: p. 101998.
25. Ehlers, A., et al., *Cognitive therapy for post-traumatic stress disorder: development and evaluation.* Behaviour Research and Therapy, 2005. **43**(4): p. 413-431.
26. Nievergelt, C.M., et al., *International meta-analysis of PTSD genome-wide association studies identifies sex- and ancestry-specific genetic risk loci.* Nature Communications, 2019. **10**(1): p. 4558.
27. Agorastos, A., et al., *Developmental Trajectories of Early Life Stress and Trauma: A Narrative Review on Neurobiological Aspects Beyond Stress System Dysregulation.* Front Psychiatry, 2019. **10**: p. 118.
28. Tolin, D.F. and E.B. Foa, *Sex differences in trauma and posttraumatic stress disorder: a quantitative review of 25 years of research.* Psychol Bull, 2006. **132**(6): p. 959-92.
29. Kessler, R.C., et al., *Lifetime Prevalence and Age-of-Onset Distributions of DSM-IV Disorders in the National Comorbidity Survey Replication.* JAMA Psychiatry, 2005. **62**(6): p. 593-602.
30. Tortella-Feliu, M., et al., *Risk factors for posttraumatic stress disorder: An umbrella review of systematic reviews and meta-analyses.* Neuroscience & Biobehavioral Reviews, 2019. **107**: p. 154-165.
31. Maren, S. and A. Holmes, *Stress and Fear Extinction.* Neuropsychopharmacology, 2016. **41**(1): p. 58-79.
32. Koenen, K.C., et al., *Posttraumatic stress disorder in the World Mental Health Surveys.* Psychological Medicine, 2017. **47**(13): p. 2260-2274.
33. Seedat, S., D.J. Stein, and P.D. Carey, *Post-traumatic stress disorder in women: epidemiological and treatment issues.* CNS Drugs, 2005. **19**(5): p. 411-27.
34. Roeckner, A.R., et al., *Sex-dependent risk factors for PTSD: a prospective structural MRI study.* Neuropsychopharmacology, 2022. **47**(13): p. 2213-2220.
35. Hamner, M.B., J.P. Lorberbaum, and M.S. George, *Potential role of the anterior cingulate cortex in PTSD: Review and hypothesis.* Depression and Anxiety, 1999. **9**(1): p. 1-14.

36. Feng, P., et al., *Memory consolidation of fear conditioning: bi-stable amygdala connectivity with dorsal anterior cingulate and medial prefrontal cortex.* Soc Cogn Affect Neurosci, 2014. **9**(11): p. 1730-7.
37. Yehuda, R., S. Halligan, and R. Grossman, *Childhood trauma and risk for PTSD: Relationship to intergenerational effects of trauma, parental PTSD, and cortisol excretion.* Development and psychopathology, 2001. **13**: p. 733-53.
38. Yehuda, R., et al., *Enhanced sensitivity to glucocorticoids in peripheral mononuclear leukocytes in posttraumatic stress disorder.* Biological Psychiatry, 2004. **55**(11): p. 1110-1116.
39. Somvanshi, P.R., et al., *Role of enhanced glucocorticoid receptor sensitivity in inflammation in PTSD: insights from computational model for circadian-neuroendocrine-immune interactions.* American Journal of Physiology-Endocrinology and Metabolism, 2020. **319**(1): p. E48-E66.
40. McFarlane, A.C., et al., *Cortisol response to acute trauma and risk of posttraumatic stress disorder.* Psychoneuroendocrinology, 2011. **36**(5): p. 720-727.
41. Brenhouse, H.C., A. Danese, and R. Grassi-Oliveira, *Neuroimmune Impacts of Early-Life Stress on Development and Psychopathology.* Curr Top Behav Neurosci, 2019. **43**: p. 423-447.
42. Fuhrmann, D., L.J. Knoll, and S.-J. Blakemore, *Adolescence as a Sensitive Period of Brain Development.* Trends in Cognitive Sciences, 2015. **19**(10): p. 558-566.
43. Humphreys, K.L., et al., *Evidence for a sensitive period in the effects of early life stress on hippocampal volume.* Dev Sci, 2019. **22**(3): p. e12775.
44. Eiland, L. and R.D. Romeo, *Stress and the developing adolescent brain.* Neuroscience, 2013. **249**: p. 162-71.
45. Chen, Y., et al., *Early Life Stress Induces Different Behaviors in Adolescence and Adulthood May Related With Abnormal Medial Prefrontal Cortex Excitation/Inhibition Balance.* Frontiers in Neuroscience, 2022. **15**.
46. Guadagno, A., et al., *Effects of Early Life Stress on the Developing Basolateral Amygdala-Prefrontal Cortex Circuit: The Emerging Role of Local Inhibition and Perineuronal Nets.* Frontiers in Human Neuroscience, 2021. **15**.
47. VanTieghem, M.R. and N. Tottenham, *Neurobiological Programming of Early Life Stress: Functional Development of Amygdala-Prefrontal Circuitry and Vulnerability for Stress-Related Psychopathology.* Curr Top Behav Neurosci, 2018. **38**: p. 117-136.
48. Nanni, V., R. Uher, and A. Danese, *Childhood maltreatment predicts unfavorable course of illness and treatment outcome in depression: a meta-analysis.* Am J Psychiatry, 2012. **169**(2): p. 141-51.
49. Maercker, A., et al., *Age of traumatisation as a predictor of post-traumatic stress disorder or major depression in young women.* Br J Psychiatry, 2004. **184**: p. 482-7.
50. Bath, K.G., G. Manzano-Nieves, and H. Goodwill, *Early life stress accelerates behavioral and neural maturation of the hippocampus in male mice.* Hormones and Behavior, 2016. **82**: p. 64-71.
51. Wolf, E.J., et al., *A classical twin study of PTSD symptoms and resilience: Evidence for a single spectrum of vulnerability to traumatic stress.* Depression and Anxiety, 2018. **35**(2): p. 132-139.
52. Sartor, C.E., et al., *Common heritable contributions to low-risk trauma, high-risk trauma, posttraumatic stress disorder, and major depression.* Arch Gen Psychiatry, 2012. **69**(3): p. 293-9.

53. Maihofer, A.X., et al., *Rare copy number variation in posttraumatic stress disorder.* Mol Psychiatry, 2022. **27**(12): p. 5062-5069.
54. Pandey, S.C., et al., *Beta-adrenergic receptor subtypes in stress-induced behavioral depression.* Pharmacol Biochem Behav, 1995. **51**(2-3): p. 339-44.
55. MacCormack, J.K., et al., *β-Adrenergic Contributions to Emotion and Physiology During an Acute Psychosocial Stressor.* Psychosomatic Medicine, 2021. **83**(9).
56. Sarah, N.G., et al., *Impaired Contextual Modulation of Memories in PTSD: An fMRI and Psychophysiological Study of Extinction Retention and Fear Renewal.* The Journal of Neuroscience, 2014. **34**(40): p. 13435.
57. Binder, E.B., et al., *Association of FKBP5 polymorphisms and childhood abuse with risk of posttraumatic stress disorder symptoms in adults.* Jama, 2008. **299**(11): p. 1291-305.
58. Nievergelt, C.M., et al., *Discovery of 95 PTSD loci provides insight into genetic architecture and neurobiology of trauma and stress-related disorders.* medRxiv, 2023: p. 2023.08.31.23294915.
59. Uddin, M., et al., *Epigenetic and immune function profiles associated with posttraumatic stress disorder.* Proc Natl Acad Sci U S A, 2010. **107**(20): p. 9470-5.
60. Hodes, G.E., et al., *Neuroimmune mechanisms of depression.* Nat Neurosci, 2015. **18**(10): p. 1386-93.
61. Michopoulos, V., et al., *Inflammation in Fear- and Anxiety-Based Disorders: PTSD, GAD, and Beyond.* Neuropsychopharmacology, 2017. **42**(1): p. 254-270.
62. Passos, I.C., et al., *Inflammatory markers in post-traumatic stress disorder: a systematic review, meta-analysis, and meta-regression.* The Lancet Psychiatry, 2015. **2**(11): p. 1002-1012.
63. Katrinli, S., et al., *The role of the immune system in posttraumatic stress disorder.* Translational Psychiatry, 2022. **12**(1): p. 313.
64. Pavlov, V.A. and K.J. Tracey, *The vagus nerve and the inflammatory reflex--linking immunity and metabolism.* Nat Rev Endocrinol, 2012. **8**(12): p. 743-54.
65. Hart, B.L. and L.A. Hart, *Sickness Behavior in Animals: Implications for Health and Wellness☆*, in *Encyclopedia of Animal Behavior (Second Edition)*, J.C. Choe, Editor. 2019, Academic Press: Oxford. p. 171-175.
66. Yarlagadda, A., E. Alfson, and A.H. Clayton, *The blood brain barrier and the role of cytokines in neuropsychiatry.* Psychiatry (Edgmont), 2009. **6**(11): p. 18-22.
67. Wong, C.M., *Post-traumatic stress disorder: Advances in psychoneuroimmunology.* Psychiatric Clinics of North America, 2002. **25**(2): p. 369-383.
68. Yang, J.-J. and W. Jiang, *Immune biomarkers alterations in post-traumatic stress disorder: A systematic review and meta-analysis.* Journal of Affective Disorders, 2020. **268**: p. 39-46.
69. Costello, H., et al., *Systematic review and meta-analysis of the association between peripheral inflammatory cytokines and generalised anxiety disorder.* BMJ Open, 2019. **9**(7): p. e027925.
70. Eraly, S.A., et al., *Assessment of Plasma C-Reactive Protein as a Biomarker of Posttraumatic Stress Disorder Risk.* JAMA Psychiatry, 2014. **71**(4): p. 423-431.
71. Michopoulos, V., et al., *Association of CRP Genetic Variation and CRP Level With Elevated PTSD Symptoms and Physiological Responses in a Civilian Population With High Levels of Trauma.* The American journal of psychiatry., 2015. **172**(4): p. 353-362.

72. von Känel, R., et al., *Evidence for low-grade systemic proinflammatory activity in patients with posttraumatic stress disorder.* J Psychiatr Res, 2007. **41**(9): p. 744-52.
73. Tursich, M., et al., *Association of trauma exposure with proinflammatory activity: a transdiagnostic meta-analysis.* Translational Psychiatry, 2014. **4**(7): p. e413-e413.
74. Guo, M., et al., *Study on serum cytokine levels in posttraumatic stress disorder patients.* Asian Pacific Journal of Tropical Medicine, 2012. **5**(4): p. 323-325.
75. Plantinga, L., et al., *Association between posttraumatic stress disorder and inflammation: a twin study.* Brain Behav Immun, 2013. **30**: p. 125-32.
76. Rohleder, N., et al., *Hypocortisolism and increased glucocorticoid sensitivity of pro-Inflammatory cytokine production in Bosnian war refugees with posttraumatic stress disorder.* Biol Psychiatry, 2004. **55**(7): p. 745-51.
77. Gill, J., M. Vythilingam, and G.G. Page, *Low cortisol, high DHEA, and high levels of stimulated TNF-alpha, and IL-6 in women with PTSD.* J Trauma Stress, 2008. **21**(6): p. 530-9.
78. Boscarino, J.A. and J. Chang, *Higher abnormal leukocyte and lymphocyte counts 20 years after exposure to severe stress: research and clinical implications.* Psychosom Med, 1999. **61**(3): p. 378-86.
79. Sommershof, A., et al., *Substantial reduction of naïve and regulatory T cells following traumatic stress.* Brain Behav Immun, 2009. **23**(8): p. 1117-24.
80. Zhou, J., et al., *Dysregulation in microRNA expression is associated with alterations in immune functions in combat veterans with post-traumatic stress disorder.* PLoS One, 2014. **9**(4): p. e94075.
81. Darwin, C., J.T. Bonner, and R.M. May, *The Descent of Man, and Selection in Relation to Sex*. REV - Revised ed. 1981: Princeton University Press.
82. Wessa, M. and H. Flor, *Failure of extinction of fear responses in posttraumatic stress disorder: evidence from second-order conditioning.* Am J Psychiatry, 2007. **164**(11): p. 1684-92.
83. Mundy, E.A., et al., *Adult Anxiety Disorders in Relation to Trait Anxiety and Perceived Stress in Childhood.* Psychological Reports, 2015. **117**(2): p. 473-489.
84. Maren, S., K.L. Phan, and I. Liberzon, *The contextual brain: implications for fear conditioning, extinction and psychopathology.* Nature Reviews Neuroscience, 2013. **14**: p. 417.
85. Deslauriers, J., et al., *Current Status of Animal Models of Posttraumatic Stress Disorder: Behavioral and Biological Phenotypes, and Future Challenges in Improving Translation.* Biol Psychiatry, 2018. **83**(10): p. 895-907.
86. VanElzakker, M.B., et al., *From Pavlov to PTSD: the extinction of conditioned fear in rodents, humans, and anxiety disorders.* Neurobiol Learn Mem, 2014. **113**: p. 3-18.
87. Carpenter, J.K., M. Pinaire, and S.G. Hofmann, *From Extinction Learning to Anxiety Treatment: Mind the Gap.* Brain Sci, 2019. **9**(7).
88. Hoppen, T.H., et al., *Efficacy of psychological interventions for post-traumatic stress disorder in children and adolescents exposed to single versus multiple traumas: meta-analysis of randomised controlled trials.* Br J Psychiatry, 2023. **222**(5): p. 196-203.
89. Kida, S., *Reconsolidation/destabilization, extinction and forgetting of fear memory as therapeutic targets for PTSD.* Psychopharmacology, 2019. **236**(1): p. 49-57.
90. Adamec, R.E. and T. Shallow, *Lasting effects on rodent anxiety of a single exposure to a cat.* Physiol Behav, 1993. **54**(1): p. 101-9.

91. Albrechet-Souza, L. and N.W. Gilpin, *The predator odor avoidance model of post-traumatic stress disorder in rats.* Behav Pharmacol, 2019. **30**(2 and 3-Spec Issue): p. 105-114.
92. Masini, C.V., et al., *Non-associative defensive responses of rats to ferret odor.* Physiol Behav, 2006. **87**(1): p. 72-81.
93. Verbitsky, A., D. Dopfel, and N. Zhang, *Rodent models of post-traumatic stress disorder: behavioral assessment.* Translational psychiatry., 2020. **10**(1).
94. Driessen, M., et al., *Trauma and PTSD in Patients With Alcohol, Drug, or Dual Dependence: A Multi-Center Study.* Alcoholism: Clinical and Experimental Research, 2008. **32**(3): p. 481-488.
95. Kar, N. and B.K. Bastia, *Post-traumatic stress disorder, depression and generalised anxiety disorder in adolescents after a natural disaster: a study of comorbidity.* Clinical Practice and Epidemiology in Mental Health, 2006. **2**(1): p. 17.
96. Brydges, N.M., et al., *Sex specific effects of pre-pubertal stress on hippocampal neurogenesis and behaviour.* Translational psychiatry, 2018. **8**(1): p. 271-271.
97. Chen, C.M., et al., *Hippocampal lesions impair contextual fear conditioning in two strains of mice.* Behavioral neuroscience, 1996. **110 5**: p. 1177-80.
98. Conrad, C.D., et al., *Repeated restraint stress facilitates fear conditioning independently of causing hippocampal CA3 dendritic atrophy.* Behavioral Neuroscience, 1999. **113**(5): p. 902-913.
99. Johansen, J.P., et al., *Molecular mechanisms of fear learning and memory.* Cell, 2011. **147**(3): p. 509-24.
100. Broekman, B.F.P., M. Olff, and F. Boer, *The genetic background to PTSD.* Neuroscience & Biobehavioral Reviews, 2007. **31**(3): p. 348-362.
101. Tran, U.S., T.M. Glück, and B. Lueger-Schuster, *Influence of personal and environmental factors on mental health in a sample of Austrian survivors of World War II with regard to PTSD: is it resilience?* BMC Psychiatry, 2013. **13**(1): p. 47.
102. Shors, T.J. and L.D. Matzel, *Long-term potentiation: what's learning got to do with it?* Behav Brain Sci, 1997. **20**(4): p. 597-614; discussion 614-55.
103. Hall, J., K.L. Thomas, and B.J. Everitt, *Rapid and selective induction of BDNF expression in the hippocampus during contextual learning.* Nat Neurosci, 2000. **3**(6): p. 533-5.
104. Milad, M.R., S. Igoe, and S.P. Orr, *Fear Conditioning in Rodents and Humans*, in *Animal Models of Behavioral Analysis*, J. Raber, Editor. 2011, Humana Press: Totowa, NJ. p. 111-132.
105. Curzon, P., N.R. Rustay, and K.E. Browman, *Cued and Contextual Fear Conditioning for Rodents*, in *Methods of Behavior Analysis in Neuroscience*, J.J. Buccafusco, Editor. 2009, CRC Press/Taylor & Francis: Boca Raton (FL).
106. Fanselow, M., *Fanselow MS. Contextual fear, gestalt memories, and the hippocampus. Behav Brain Res 110: 73-81.* Behavioural brain research, 2000. **110**: p. 73-81.
107. Myers, K.M. and M. Davis, *Mechanisms of fear extinction.* Mol Psychiatry, 2007. **12**(2): p. 120-50.
108. Bouton, M.E., et al., *Renewal after the extinction of free operant behavior.* Learning & Behavior, 2011. **39**(1): p. 57-67.
109. Rescorla, R.A. and C.D. Heth, *Reinstatement of fear to an extinguished conditioned stimulus.* Journal of Experimental Psychology: Animal Behavior Processes, 1975. **1**(1): p. 88-96.

110. Luchkina, N.V. and V.Y. Bolshakov, *Mechanisms of fear learning and extinction: synaptic plasticity-fear memory connection.* Psychopharmacology (Berl), 2019. **236**(1): p. 163-182.
111. Quirk, G.J., R. Garcia, and F. Gonzalez-Lima, *Prefrontal mechanisms in extinction of conditioned fear.* Biol Psychiatry, 2006. **60**(4): p. 337-43.
112. Sierra-Mercado, D., N. Padilla-Coreano, and G.J. Quirk, *Dissociable roles of prelimbic and infralimbic cortices, ventral hippocampus, and basolateral amygdala in the expression and extinction of conditioned fear.* Neuropsychopharmacology, 2011. **36**(2): p. 529-38.
113. Clark, J.W., et al., *Sex differences in mouse models of fear inhibition: Fear extinction, safety learning, and fear-safety discrimination.* Br J Pharmacol, 2019. **176**(21): p. 4149-4158.
114. Yang, Q., et al., *Spontaneous recovery of reward memory through active forgetting of extinction memory.* Current Biology, 2023. **33**(5): p. 838-848.e3.
115. Bouton, M.E., *Context, time, and memory retrieval in the interference paradigms of Pavlovian learning.* Psychological Bulletin, 1993. **114**(1): p. 80-99.
116. Holmes, N.M., H.T. Leung, and R.F. Westbrook, *Counterconditioned fear responses exhibit greater renewal than extinguished fear responses.* Learning & memory (Cold Spring Harbor, N.Y.), 2016. **23**(4): p. 141-150.
117. Corcoran, K.A., et al., *Hippocampal inactivation disrupts the acquisition and contextual encoding of fear extinction.* J Neurosci, 2005. **25**(39): p. 8978-87.
118. Faucher, P., et al., *Acute pre-learning stress selectively impairs hippocampus-dependent fear memory consolidation: Behavioral and molecular evidence.* Neurobiology of Learning and Memory, 2022. **188**: p. 107585.
119. McNish, K.A., J.C. Gewirtz, and M. Davis, *Evidence of contextual fear after lesions of the hippocampus: a disruption of freezing but not fear-potentiated startle.* J Neurosci, 1997. **17**(23): p. 9353-60.
120. SAH, P., et al., *The Amygdaloid Complex: Anatomy and Physiology.* Physiological Reviews, 2003. **83**(3): p. 803-834.
121. Klüver, H. and P.C. Bucy, *"Psychic blindness" and other symptoms following bilateral temporal lobectomy in Rhesus monkeys.* American Journal of Physiology, 1937. **119**: p. 352-353.
122. Falls, W.A. and M. Davis, *Lesions of the Central Nucleus of the Amygdala Block Conditioned Excitation, but Not Conditioned Inhibition of Fear as Measured With the Fear-Potentiated Startle Effect.* Behavioral neuroscience, 1995. **109**(3): p. 379-387.
123. David, A.-F. and J.Q. Gregory, *Lesions of the Basal Amygdala Block Expression of Conditioned Fear But Not Extinction.* The Journal of Neuroscience, 2005. **25**(42): p. 9680.
124. Terburg, D., et al., *Hypervigilance for fear after basolateral amygdala damage in humans.* Translational Psychiatry, 2012. **2**(5): p. e115-e115.
125. Frick, A., et al., *Dopamine and fear memory formation in the human amygdala.* Molecular Psychiatry, 2022. **27**(3): p. 1704-1711.
126. Gale, G.D., et al., *Role of the basolateral amygdala in the storage of fear memories across the adult lifetime of rats.* J Neurosci, 2004. **24**(15): p. 3810-5.
127. Schafe, G.E. and J.E. LeDoux, *Memory consolidation of auditory pavlovian fear conditioning requires protein synthesis and protein kinase A in the amygdala.* J Neurosci, 2000. **20**(18): p. Rc96.

128. Schafe, G.E., et al., *Activation of ERK/MAP kinase in the amygdala is required for memory consolidation of pavlovian fear conditioning.* J Neurosci, 2000. **20**(21): p. 8177-87.
129. McKernan, M.G. and P. Shinnick-Gallagher, *Fear conditioning induces a lasting potentiation of synaptic currents in vitro.* Nature, 1997. **390**(6660): p. 607-11.
130. Mahan, A.L. and K.J. Ressler, *Fear conditioning, synaptic plasticity and the amygdala: implications for posttraumatic stress disorder.* Trends in Neurosciences, 2012. **35**(1): p. 24-35.
131. Fullana, M.A., et al., *Neural signatures of human fear conditioning: an updated and extended meta-analysis of fMRI studies.* Molecular Psychiatry, 2016. **21**(4): p. 500-508.
132. Mertens, G. and I.M. Engelhard, *A systematic review and meta-analysis of the evidence for unaware fear conditioning.* Neuroscience & Biobehavioral Reviews, 2020. **108**: p. 254-268.
133. Duits, P., et al., *UPDATED META-ANALYSIS OF CLASSICAL FEAR CONDITIONING IN THE ANXIETY DISORDERS.* Depression and Anxiety, 2015. **32**(4): p. 239-253.
134. Knierim, J.J., *The hippocampus.* Curr Biol, 2015. **25**(23): p. R1116-21.
135. Anagnostaras, S.G., G.D. Gale, and M.S. Fanselow, *Hippocampus and contextual fear conditioning: Recent controversies and advances.* Hippocampus, 2001. **11**(1): p. 8-17.
136. Fanselow, M.S. and H.W. Dong, *Are the dorsal and ventral hippocampus functionally distinct structures?* Neuron, 2010. **65**(1): p. 7-19.
137. Henke, P.G., *Hippocampal pathway to the amygdala and stress ulcer development.* Brain Res Bull, 1990. **25**(5): p. 691-5.
138. West, M.J. and H.J. Gundersen, *Unbiased stereological estimation of the number of neurons in the human hippocampus.* J Comp Neurol, 1990. **296**(1): p. 1-22.
139. Kim, W.B. and J.-H. Cho, *Encoding of contextual fear memory in hippocampal–amygdala circuit.* Nature Communications, 2020. **11**(1): p. 1382.
140. Hirsh, R., *The hippocampus and contextual retrieval of information from memory: A theory.* Behavioral Biology, 1974. **12**(4): p. 421-444.
141. Huerta, P.T., et al., *Formation of temporal memory requires NMDA receptors within CA1 pyramidal neurons.* Neuron, 2000. **25**(2): p. 473-80.
142. Bourtchuladze, R., et al., *Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein.* Cell, 1994. **79**(1): p. 59-68.
143. Liu, I.Y., et al., *Brain-derived neurotrophic factor plays a critical role in contextual fear conditioning.* J Neurosci, 2004. **24**(36): p. 7958-63.
144. Maren, S. and W. Holt, *The hippocampus and contextual memory retrieval in Pavlovian conditioning.* Behavioural Brain Research, 2000. **110**(1): p. 97-108.
145. Phillips, R.G. and J.E. LeDoux, *Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning.* Behav Neurosci, 1992. **106**(2): p. 274-85.
146. McNish, K.A., J.C. Gewirtz, and M. Davis, *Disruption of contextual freezing, but not contextual blocking of fear-potentiated startle, after lesions of the dorsal hippocampus.* Behavioral Neuroscience, 2000. **114**(1): p. 64-76.
147. Radulovic, J. and N.C. Tronson, *Molecular specificity of multiple hippocampal processes governing fear extinction.* Reviews in the Neurosciences, 2010. **21**(1): p. 1-17.
148. Herry, C., et al., *Switching on and off fear by distinct neuronal circuits.* Nature, 2008. **454**(7204): p. 600-606.

149. Daumas, S., et al., *Encoding, consolidation, and retrieval of contextual memory: differential involvement of dorsal CA3 and CA1 hippocampal subregions.* Learn Mem, 2005. **12**(4): p. 375-82.
150. Liu, X., et al., *Optogenetic stimulation of a hippocampal engram activates fear memory recall.* Nature, 2012. **484**(7394): p. 381-385.
151. Brown, V.J. and E.M. Bowman, *Rodent models of prefrontal cortical function.* Trends Neurosci, 2002. **25**(7): p. 340-3.
152. Rose, J.E. and C.N. Woolsey, *Structure and relations of limbic cortex and anterior thalamic nuclei in rabbit and cat.* J Comp Neurol, 1948. **89**(3): p. 279-347.
153. Laubach, M., et al., *What, If Anything, Is Rodent Prefrontal Cortex?* eNeuro, 2018. **5**(5).
154. Preuss, T.M., *Do Rats Have Prefrontal Cortex? The Rose-Woolsey-Akert Program Reconsidered.* Journal of Cognitive Neuroscience, 1995. **7**(1): p. 1-24.
155. Pelkey, K.A., et al., *Hippocampal GABAergic Inhibitory Interneurons.* Physiol Rev, 2017. **97**(4): p. 1619-1747.
156. Santini, E., G.J. Quirk, and J.T. Porter, *Fear conditioning and extinction differentially modify the intrinsic excitability of infralimbic neurons.* J Neurosci, 2008. **28**(15): p. 4028-36.
157. Herry, C. and R. Garcia, *Prefrontal cortex long-term potentiation, but not long-term depression, is associated with the maintenance of extinction of learned fear in mice.* J Neurosci, 2002. **22**(2): p. 577-83.
158. Laurent, V. and R.F. Westbrook, *Inactivation of the infralimbic but not the prelimbic cortex impairs consolidation and retrieval of fear extinction.* Learn Mem, 2009. **16**(9): p. 520-9.
159. Lonsdorf, T.B., et al., *Sex differences in conditioned stimulus discrimination during context-dependent fear learning and its retrieval in humans: the role of biological sex, contraceptives and menstrual cycle phases.* J Psychiatry Neurosci, 2015. **40**(6): p. 368-75.
160. Lebron-Milad, K., et al., *Sex differences in the neurobiology of fear conditioning and extinction: a preliminary fMRI study of shared sex differences with stress-arousal circuitry.* Biol Mood Anxiety Disord, 2012. **2**: p. 7.
161. Gillies, G.E. and S. McArthur, *Estrogen actions in the brain and the basis for differential action in men and women: a case for sex-specific medicines.* Pharmacol Rev, 2010. **62**(2): p. 155-98.
162. Zeidan, M.A., et al., *Estradiol Modulates Medial Prefrontal Cortex and Amygdala Activity During Fear Extinction in Women and Female Rats.* Biological Psychiatry, 2011. **70**(10): p. 920-927.
163. Ganella, D.E. and J.H. Kim, *Developmental rodent models of fear and anxiety: from neurobiology to pharmacology.* Br J Pharmacol, 2014. **171**(20): p. 4556-74.
164. Brydges, N.M., et al., *Prepubertal stress and hippocampal function: sex-specific effects.* Hippocampus, 2014. **24**(6): p. 684-92.
165. Gruene, T.M., et al., *Sexually divergent expression of active and passive conditioned fear responses in rats.* eLife, 2015. **4**: p. e11352.
166. Kosten, T.A., H.J. Lee, and J.J. Kim, *Early life stress impairs fear conditioning in adult male and female rats.* Brain Research, 2006. **1087**(1): p. 142-150.
167. Chrousos, G.P., *Stress and disorders of the stress system.* Nat Rev Endocrinol, 2009. **5**(7): p. 374-81.

168. Agorastos, A. and G.P. Chrousos, *The neuroendocrinology of stress: the stress-related continuum of chronic disease development.* Molecular Psychiatry, 2022. **27**(1): p. 502-513.
169. Thayer, J.F. and E. Sternberg, *Beyond heart rate variability: vagal regulation of allostatic systems.* Ann N Y Acad Sci, 2006. **1088**: p. 361-72.
170. Mridha, Z., et al., *Graded recruitment of pupil-linked neuromodulation by parametric stimulation of the vagus nerve.* Nature Communications, 2021. **12**(1): p. 1539.
171. Lerman, I., et al., *Non-invasive cervical vagus nerve stimulation effects on reaction time and valence image anticipation response.* Brain Stimulation, 2022. **15**(4): p. 946-956.
172. Joëls, M., H. Karst, and R.A. Sarabdjitsingh, *The stressed brain of humans and rodents.* Acta Physiol (Oxf), 2018. **223**(2): p. e13066.
173. Reul, J.M. and E.R. de Kloet, *Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation.* Endocrinology, 1985. **117**(6): p. 2505-11.
174. Flinn, M.V., et al., *Evolutionary functions of early social modulation of hypothalamic-pituitary-adrenal axis development in humans.* Neurosci Biobehav Rev, 2011. **35**(7): p. 1611-29.
175. McEwen, B.S., J.M. Weiss, and L.S. Schwartz, *Selective retention of corticosterone by limbic structures in rat brain.* Nature, 1968. **220**(5170): p. 911-2.
176. de Kloet, E.R., M.S. Oitzl, and M. Joëls, *Stress and cognition: are corticosteroids good or bad guys?* Trends in Neurosciences, 1999. **22**(10): p. 422-426.
177. Cordero, M.I., J.J. Merino, and C. Sandi, *Correlational relationship between shock intensity and corticosterone secretion on the establishment and subsequent expression of contextual fear conditioning.* Behav Neurosci, 1998. **112**(4): p. 885-91.
178. Korz, V. and J.U. Frey, *Stress-related modulation of hippocampal long-term potentiation in rats: Involvement of adrenal steroid receptors.* J Neurosci, 2003. **23**(19): p. 7281-7.
179. Schmidt, M.V., *Animal models for depression and the mismatch hypothesis of disease.* Psychoneuroendocrinology, 2011. **36**(3): p. 330-8.
180. Luine, V., *Sex Differences in Chronic Stress Effects on Memory in Rats.* Stress, 2002. **5**(3): p. 205-216.
181. Bowman, R.E., et al., *Aged rats: sex differences and responses to chronic stress.* Brain Res, 2006. **1126**(1): p. 156-66.
182. Bowman, R.E., et al., *Sex-dependent changes in anxiety, memory, and monoamines following one week of stress.* Physiol Behav, 2009. **97**(1): p. 21-9.
183. Luine, V.N., et al., *Chronic stress and neural function: accounting for sex and age.* J Neuroendocrinol, 2007. **19**(10): p. 743-51.
184. Reddaway, J. and N.M. Brydges, *Enduring neuroimmunological consequences of developmental experiences: From vulnerability to resilience.* Molecular and Cellular Neuroscience, 2020. **109**: p. 103567.
185. Segerstrom, S.C. and G.E. Miller, *Psychological Stress and the Human Immune System: A Meta-Analytic Study of 30 Years of Inquiry.* Psychological Bulletin, 2004. **130**(4): p. 601-630.
186. Vogel, S. and L. Schwabe, *Learning and memory under stress: implications for the classroom.* npj Science of Learning, 2016. **1**(1): p. 16011.

187. Lesuis, S.L., P.J. Lucassen, and H.J. Krugers, *Early life stress impairs fear memory and synaptic plasticity; a potential role for GluN2B.* Neuropharmacology, 2019. **149**: p. 195-203.
188. Teicher, M.H., et al., *The effects of childhood maltreatment on brain structure, function and connectivity.* Nature Reviews Neuroscience, 2016. **17**: p. 652.
189. Thome, J., et al., *Generalisation of fear in PTSD related to prolonged childhood maltreatment: an experimental study.* Psychological Medicine, 2018. **48**(13): p. 2223-2234.
190. Hoskin, R., M.D. Hunter, and P.W.R. Woodruff, *Stress improves selective attention towards emotionally neutral left ear stimuli.* Acta Psychologica, 2014. **151**: p. 214-221.
191. Kawahara, J., H. Sato, and I. Takenaka, *Does stress enhance or impair selective attention? The effects of stress and perceptual load on distractor interference.* Journal of Vision, 2011. **11**(11): p. 99-99.
192. Henckens, M.J.A.G., et al., *Stressed Memories: How Acute Stress Affects Memory Formation in Humans.* The Journal of Neuroscience, 2009. **29**(32): p. 10111-10119.
193. Rajbhandari, A.K., S.T. Gonzalez, and M.S. Fanselow, *Stress-Enhanced Fear Learning, a Robust Rodent Model of Post-Traumatic Stress Disorder.* J Vis Exp, 2018(140).
194. Baran, S.E., et al., *Chronic stress and sex differences on the recall of fear conditioning and extinction.* Neurobiology of Learning and Memory, 2009. **91**(3): p. 323-332.
195. Sandi, C., et al., *Effects of chronic stress on contextual fear conditioning and the hippocampal expression of the neural cell adhesion molecule, its polysialylation, and L1.* Neuroscience, 2001. **102**(2): p. 329-339.
196. Hill, M.N., et al., *Downregulation of endocannabinoid signaling in the hippocampus following chronic unpredictable stress.* Neuropsychopharmacology, 2005. **30**(3): p. 508-15.
197. Graybeal, C., C. Kiselycznyk, and A. Holmes, *Stress-induced impairments in prefrontal-mediated behaviors and the role of the N-methyl-D-aspartate receptor.* Neuroscience, 2012. **211**: p. 28-38.
198. Francis, D.D., et al., *Environmental enrichment reverses the effects of maternal separation on stress reactivity.* J Neurosci, 2002. **22**(18): p. 7840-3.
199. Youssef, M., et al., *Early life stress delays hippocampal development and diminishes the adult stem cell pool in mice.* Scientific Reports, 2019. **9**(1): p. 4120.
200. Ozturk, H., *Early Life Stress and its Impact on Brain Development: A Systematic Review.* Journal of Brain and Neurological Disorders, 2023. **5**(1).
201. Molet, J., et al., *Naturalistic rodent models of chronic early-life stress.* Dev Psychobiol, 2014. **56**(8): p. 1675-88.
202. Lajud, N., et al., *Periodic maternal separation decreases hippocampal neurogenesis without affecting basal corticosterone during the stress hyporesponsive period, but alters HPA axis and coping behavior in adulthood.* Psychoneuroendocrinology, 2012. **37**(3): p. 410-420.
203. Oreland, S., I. Nylander, and C. Pickering, *Prolonged maternal separation decreases granule cell number in the dentate gyrus of 3-week-old male rats.* Int J Dev Neurosci, 2010. **28**(2): p. 139-44.
204. Heine, V.M., et al., *Suppressed proliferation and apoptotic changes in the rat dentate gyrus after acute and chronic stress are reversible.* Eur J Neurosci, 2004. **19**(1): p. 131-44.

205. Chocyk, A., et al., *The effects of early-life adversity on fear memories in adolescent rats and their persistence into adulthood.* Behav Brain Res, 2014. **264**: p. 161-72.
206. Ohta, K.-i., et al., *The effects of early life stress on the excitatory/inhibitory balance of the medial prefrontal cortex.* Behavioural Brain Research, 2020. **379**: p. 112306.
207. Dennis, M., et al., *Age, plasticity, and homeostasis in childhood brain disorders.* Neuroscience & Biobehavioral Reviews, 2013. **37**(10, Part 2): p. 2760-2773.
208. Brydges, N.M., et al., *The effects of juvenile stress on anxiety, cognitive bias and decision making in adulthood: a rat model.* PLoS One, 2012. **7**(10): p. e48143.
209. Reemst, K., et al., *Early-life stress lastingly impacts microglial transcriptome and function under basal and immune-challenged conditions.* Translational Psychiatry, 2022. **12**(1): p. 507.
210. Delpech, J.-C., et al., *Early life stress perturbs the maturation of microglia in the developing hippocampus.* Brain, Behavior, and Immunity, 2016. **57**: p. 79-93.
211. Anda, R.F., et al., *The enduring effects of abuse and related adverse experiences in childhood.* European Archives of Psychiatry and Clinical Neuroscience, 2006. **256**(3): p. 174-186.
212. Felitti, V.J., et al., *Relationship of childhood abuse and household dysfunction to many of the leading causes of death in adults. The Adverse Childhood Experiences (ACE) Study.* Am J Prev Med, 1998. **14**(4): p. 245-58.
213. Rahman, M.F. and P.O. McGowan, *Cell-type-specific epigenetic effects of early life stress on the brain.* Translational Psychiatry, 2022. **12**(1): p. 326.
214. Lanius, R.A., et al., *Fear conditioning and early life vulnerabilities: two distinct pathways of emotional dysregulation and brain dysfunction in PTSD.* Eur J Psychotraumatol, 2010. **1**.
215. Bellavance, M.A. and S. Rivest, *The HPA - Immune Axis and the Immunomodulatory Actions of Glucocorticoids in the Brain.* Front Immunol, 2014. **5**: p. 136.
216. Brydges, N.M., C. Best, and K.L. Thomas, *Female HPA axis displays heightened sensitivity to pre-pubertal stress.* Stress, 2020. **23**(2): p. 190-200.
217. Essex, M.J., et al., *Influence of early life stress on later hypothalamic-pituitary-adrenal axis functioning and its covariation with mental health symptoms: a study of the allostatic process from childhood into adolescence.* Dev Psychopathol, 2011. **23**(4): p. 1039-58.
218. McEWEN, B.S., *Stress, Adaptation, and Disease: Allostasis and Allostatic Load.* Annals of the New York Academy of Sciences, 1998. **840**(1): p. 33-44.
219. DeRijk, R.H., *Single Nucleotide Polymorphisms Related to HPA Axis Reactivity.* Neuroimmunomodulation, 2009. **16**(5): p. 340-352.
220. Binder, E.B., et al., *Association of FKBP5 polymorphisms and childhood abuse with risk of posttraumatic stress disorder symptoms in adults.* Jama, 2008. **299**(11): p. 1291-1305.
221. van Rossum, E.F., et al., *Identification of the BclI polymorphism in the glucocorticoid receptor gene: association with sensitivity to glucocorticoids in vivo and body mass index.* Clin Endocrinol (Oxf), 2003. **59**(5): p. 585-92.
222. De Bellis, M.D., et al., *Neuropsychological findings in childhood neglect and their relationships to pediatric PTSD.* Journal of the International Neuropsychological Society, 2009. **15**(6): p. 868-878.

223. LeMoult, J., et al., *Meta-analysis: Exposure to Early Life Stress and Risk for Depression in Childhood and Adolescence.* J Am Acad Child Adolesc Psychiatry, 2020. **59**(7): p. 842-855.
224. Hostinar, C.E. and M.R. Gunnar, *Social Support Can Buffer Against Stress and Shape Brain Activity.* AJOB Neuroscience, 2015. **6**(3): p. 34-42.
225. Ding, Y., et al., *The Impact of Different Coping Styles on Psychological Distress during the COVID-19: The Mediating Role of Perceived Stress.* Int J Environ Res Public Health, 2021. **18**(20).
226. Long, B.C. and R.v. Stavel, *Effects of exercise training on anxiety: A meta-analysis.* Journal of Applied Sport Psychology, 1995. **7**(2): p. 167-189.
227. Santarelli, S., et al., *Evidence supporting the match/mismatch hypothesis of psychiatric disorders.* European Neuropsychopharmacology, 2014. **24**(6): p. 907-918.
228. Seery, M.D., et al., *An upside to adversity?: moderate cumulative lifetime adversity is associated with resilient responses in the face of controlled stressors.* Psychol Sci, 2013. **24**(7): p. 1181-9.
229. Ricon, T., et al., *Unpredictable chronic stress in juvenile or adult rats has opposite effects, respectively, promoting and impairing resilience.* Stress, 2012. **15**(1): p. 11-20.
230. Avital, A. and G. Richter-Levin, *Exposure to juvenile stress exacerbates the behavioural consequences of exposure to stress in the adult rat.* Int J Neuropsychopharmacol, 2005. **8**(2): p. 163-73.
231. Hoogland, M. and A. Ploeger, *Two Different Mismatches: Integrating the Developmental and the Evolutionary-Mismatch Hypothesis.* Perspectives on Psychological Science, 2022. **17**: p. 174569162210783.
232. Liu, J., et al., *Microglia: A Double-Edged Sword in Intracerebral Hemorrhage From Basic Mechanisms to Clinical Research.* Frontiers in Immunology, 2021. **12**.
233. Rock, K.L., et al., *The sterile inflammatory response.* Annu Rev Immunol, 2010. **28**: p. 321-42.
234. Galea, I., *The blood–brain barrier in systemic infection and inflammation.* Cellular & Molecular Immunology, 2021. **18**(11): p. 2489-2501.
235. Andreadou, M., et al., *IL-12 sensing in neurons induces neuroprotective CNS tissue adaptation and attenuates neuroinflammation in mice.* Nature Neuroscience, 2023. **26**(10): p. 1701-1712.
236. Sorrells, S.F. and R.M. Sapolsky, *An inflammatory review of glucocorticoid actions in the CNS.* Brain, Behavior, and Immunity, 2007. **21**(3): p. 259-272.
237. Webster, J.I., L. Tonelli, and E.M. Sternberg, *Neuroendocrine regulation of immunity.* Annu Rev Immunol, 2002. **20**: p. 125-63.
238. Chen, Y., et al., *Blood-brain barrier dysfunction and Alzheimer's disease: associations, pathogenic mechanisms, and therapeutic potential.* Front Aging Neurosci, 2023. **15**: p. 1258640.
239. Sofroniew, M.V., *Astrogliosis.* Cold Spring Harb Perspect Biol, 2014. **7**(2): p. a020420.
240. Lannes, N., et al., *Microglia at center stage: a comprehensive review about the versatile and unique residential macrophages of the central nervous system.* Oncotarget, 2017. **8**(69): p. 114393-114413.
241. Menassa, D.A. and D. Gomez-Nicola, *Microglial Dynamics During Human Brain Development.* Frontiers in Immunology, 2018. **9**.
242. Bachiller, S., et al., *Microglia in Neurological Diseases: A Road Map to Brain-Disease Dependent-Inflammatory Response.* Frontiers in Cellular Neuroscience, 2018. **12**.

243. Matcovitch-Natan, O., et al., *Microglia development follows a stepwise program to regulate brain homeostasis.* Science, 2016. **353**(6301): p. aad8670.
244. Paolicelli, R.C., et al., *Synaptic Pruning by Microglia Is Necessary for Normal Brain Development.* Science, 2011. **333**(6048): p. 1456-1458.
245. Cornell, J., et al., *Microglia regulation of synaptic plasticity and learning and memory.* Neural Regen Res, 2022. **17**(4): p. 705-716.
246. Pfeiffer, T., E. Avignone, and U.V. Nägerl, *Induction of hippocampal long-term potentiation increases the morphological dynamics of microglial processes and prolongs their contacts with dendritic spines.* Sci Rep, 2016. **6**: p. 32422.
247. Bourgognon, J.-M. and J. Cavanagh, *The role of cytokines in modulating learning and memory and brain plasticity.* Brain and Neuroscience Advances, 2020. **4**: p. 2398212820979802.
248. Lively, S. and L.C. Schlichter, *Microglia Responses to Pro-inflammatory Stimuli (LPS, IFNγ+TNFα) and Reprogramming by Resolving Cytokines (IL-4, IL-10).* Frontiers in Cellular Neuroscience, 2018. **12**.
249. Rock, R.B., et al., *Role of microglia in central nervous system infections.* Clin Microbiol Rev, 2004. **17**(4): p. 942-64, table of contents.
250. Yu, Z., et al., *Microglial production of TNF-alpha is a key element of sustained fear memory.* Brain, Behavior, and Immunity, 2017. **59**: p. 313-321.
251. Paolicelli, R.C., et al., *Microglia states and nomenclature: A field at its crossroads.* Neuron, 2022. **110**(21): p. 3458-3483.
252. Reddaway, J., et al., *Microglial morphometric analysis: so many options, so little consistency.* Frontiers in Neuroinformatics, 2023. **17**.
253. Eck, S.R. and D.A. Bangasser, *The effects of early life stress on motivated behaviors: A role for gonadal hormones.* Neuroscience & Biobehavioral Reviews, 2020. **119**: p. 86-100.
254. Deutsch, V., et al., *Sustained leukocyte count during rising cortisol level.* Acta Haematol, 2007. **118**(2): p. 73-6.
255. Morey, J.N., et al., *Current Directions in Stress and Human Immune Function.* Curr Opin Psychol, 2015. **5**: p. 13-17.
256. Dantzer, R., *Cytokine, sickness behavior, and depression.* Immunol Allergy Clin North Am, 2009. **29**(2): p. 247-64.
257. Selye, H., *The Stress of Life* 1976, New York: McGraw-Hill.
258. Radenbach, C., et al., *The interaction of acute and chronic stress impairs model-based behavioral control.* Psychoneuroendocrinology, 2015. **53**: p. 268-280.
259. Lin, Y., et al., *Sex Differences in the Effects of Acute and Chronic Stress and Recovery after Long-Term Stress on Stress-Related Brain Regions of Rats.* Cerebral Cortex, 2008. **19**(9): p. 1978-1989.
260. Hoeijmakers, L., P.J. Lucassen, and A. Korosi, *The interplay of early-life stress, nutrition, and immune activation programs adult hippocampal structure and function.* Frontiers in Molecular Neuroscience, 2015. **7**.
261. Simon, A.K., G.A. Hollander, and A. McMichael, *Evolution of the immune system in humans from infancy to old age.* Proc Biol Sci, 2015. **282**(1821): p. 20143085.
262. Hennessy, M.B., T. Deak, and P.A. Schiml-Webb, *Early attachment-figure separation and increased risk for later depression: potential mediation by proinflammatory processes.* Neurosci Biobehav Rev, 2010. **34**(6): p. 782-90.

263. Stevens, B., et al., *The classical complement cascade mediates CNS synapse elimination.* Cell, 2007. **131**(6): p. 1164-78.
264. Schwarz, J.M., P.W. Sholar, and S.D. Bilbo, *Sex differences in microglial colonization of the developing rat brain.* Journal of Neurochemistry, 2012. **120**(6): p. 948-963.
265. Frank, M.G., et al., *Microglia serve as a neuroimmune substrate for stress-induced potentiation of CNS pro-inflammatory cytokine responses.* Brain, Behavior, and Immunity, 2007. **21**(1): p. 47-59.
266. Knapp, L.T. and E. Klann, *Role of reactive oxygen species in hippocampal long-term potentiation: contributory or inhibitory?* J Neurosci Res, 2002. **70**(1): p. 1-7.
267. Bordeleau, M., et al., *Microglia along sex lines: From brain colonization, maturation and function, to implication in neurodevelopmental disorders.* Seminars in Cell & Developmental Biology, 2019. **94**: p. 152-163.
268. Doyle, H.H., et al., *Sex Differences in Microglia Activity within the Periaqueductal Gray of the Rat: A Potential Mechanism Driving the Dimorphic Effects of Morphine.* J Neurosci, 2017. **37**(12): p. 3202-3214.
269. Tsyglakova, M., et al., *Sex and region-specific effects of variable stress on microglia morphology.* Brain Behav Immun Health, 2021. **18**: p. 100378.
270. VanRyzin, J.W., et al., *Microglia and sexual differentiation of the developing brain: A focus on extrinsic factors.* Glia, 2019. **68**(6): p. 1100-1113.
271. Berkiks, I., et al., *The sex differences of the behavior response to early Life immune stimulation: Microglia and astrocytes involvement.* Physiology & Behavior, 2019. **199**: p. 386-394.
272. Loram, L.C., et al., *Sex and estradiol influence glial pro-inflammatory responses to lipopolysaccharide in rats.* Psychoneuroendocrinology, 2012. **37**(10): p. 1688-1699.
273. Weinhard, L., et al., *Sexual dimorphism of microglia and synapses during mouse postnatal development.* Dev Neurobiol, 2018. **78**(6): p. 618-626.
274. Kang, S.S., et al., *Microglial translational profiling reveals a convergent APOE pathway from aging, amyloid, and tau.* Journal of Experimental Medicine, 2018. **215**(9): p. 2235-2245.
275. Mangold, C.A., et al., *Sexually divergent induction of microglial-associated neuroinflammation with hippocampal aging.* Journal of Neuroinflammation, 2017. **14**(1): p. 141.
276. Stephen, T.L., et al., *APOE genotype and sex affect microglial interactions with plaques in Alzheimer's disease mice.* Acta Neuropathologica Communications, 2019. **7**(1): p. 82.
277. Bollinger, J.L., C.M. Bergeon Burns, and C.L. Wellman, *Differential effects of stress on microglial cell activation in male and female medial prefrontal cortex.* Brain Behav Immun, 2016. **52**: p. 88-97.
278. Xu, B., et al., *Microglia Activated by Excess Cortisol Induce HMGB1 Acetylation and Neuroinflammation in the Hippocampal DG Region of Mice Following Cold Exposure.* Biomolecules, 2019. **9**(9).
279. Weber, M.D., et al., *The Influence of Microglial Elimination and Repopulation on Stress Sensitization Induced by Repeated Social Defeat.* Biol Psychiatry, 2019. **85**(8): p. 667-678.
280. Helmy, A., et al., *Recombinant human interleukin-1 receptor antagonist promotes M1 microglia biased cytokines and chemokines following human traumatic brain injury.* J Cereb Blood Flow Metab, 2016. **36**(8): p. 1434-48.

281. Nie, X., et al., *The Innate Immune Receptors TLR2/4 Mediate Repeated Social Defeat Stress-Induced Social Avoidance through Prefrontal Microglial Activation.* Neuron, 2018. **99**(3): p. 464-479.e7.
282. Desplats, P., et al., *Microglial memory of early life stress and inflammation: Susceptibility to neurodegeneration in adulthood.* Neurosci Biobehav Rev, 2020. **117**: p. 232-242.
283. Basu, A., et al., *The type 1 interleukin-1 receptor is essential for the efficient activation of microglia and the induction of multiple proinflammatory mediators in response to brain injury.* J Neurosci, 2002. **22**(14): p. 6071-82.
284. Shaftel, S.S., W.S.T. Griffin, and M.K. O'Banion, *The role of interleukin-1 in neuroinflammation and Alzheimer disease: an evolving perspective.* Journal of Neuroinflammation, 2008. **5**(1): p. 7.
285. Barnes, P., A. Kirtley, and K.L. Thomas, *Quantitatively and qualitatively different cellular processes are engaged in CA1 during the consolidation and reconsolidation of contextual fear memory.* Hippocampus, 2012. **22**(2): p. 149-71.
286. Scholz, B., et al., *The Regulation of Cytokine Networks in Hippocampal CA1 Differentiates Extinction from Those Required for the Maintenance of Contextual Fear Memory after Recall.* PLoS One, 2016. **11**(5): p. e0153102.
287. Hao, Y., et al., *Intra-amygdala microinfusion of IL-6 impairs the auditory fear conditioning of rats via JAK/STAT activation.* Behav Brain Res, 2014. **275**: p. 88-95.
288. Hastie, T., R. Tibshirani, and J. Friedman, *Elements of Statistical Learning: Data Mining, Inference, and Prediction*. Second Edition ed. Springer Series in Statistics. 2009, New York: Springer.
289. Fawagreh, K., M.M. Gaber, and E. Elyan, *Random forests: from early developments to recent advancements.* Systems Science & Control Engineering, 2014. **2**(1): p. 602-609.
290. Geron, A., *Hands-On Machine Learning with Scikit-Learn, Keras, and TensorFlow: Concepts, Tools, and Techniques to Build Intelligent Systems*. 2019: O'Reilly Media, Inc.
291. Reddaway, J., *The role of the complement system in associative learning and risk for schizophrenia.* , in *School of Medicine*. 2022, Cardiff University.
292. Wang, L., *A primer on rodent identification methods.* Lab Animal, 2005. **34**(4): p. 64-67.
293. Brydges, N.M., et al., *Early life stress impairs social function through AVP-dependent mechanisms.* bioRxiv, 2019: p. 741702.
294. Tsoory, M.M., A. Guterman, and G. Richter-Levin, *"Juvenile stress" alters maturation-related changes in expression of the neural cell adhesion molecule L1 in the limbic system: Relevance for stress-related psychopathologies.* Journal of Neuroscience Research, 2010. **88**(2): p. 369-380.
295. Brydges, N.M., et al., *Early life stress produces compulsive-like, but not impulsive, behavior in females.* Behav Neurosci, 2015. **129**(3): p. 300-8.
296. Garner, J.P., *The significance of meaning: why do over 90% of behavioral neuroscience results fail to translate to humans, and what can we do to fix it?* Ilar j, 2014. **55**(3): p. 438-56.
297. Trent, S., et al., *Rescue of long-term memory after reconsolidation blockade.* Nature Communications, 2015. **6**(1): p. 7897.
298. Cai, W.H., et al., *Postreactivation glucocorticoids impair recall of established fear memory.* J Neurosci, 2006. **26**(37): p. 9560-6.

299. *Pain, Stress and Humane End Points*, in *Handbook of Laboratory Animal Management and Welfare*, S.L. Wolfensohn, M, Editor. 2003. p. 59-73.
300. Palkovits, M., *[23] Punch sampling biopsy technique*, in *Methods in Enzymology*. 1983, Academic Press. p. 368-376.
301. Smith, K.L., et al., *Microglial cell hyper-ramification and neuronal dendritic spine loss in the hippocampus and medial prefrontal cortex in a mouse model of PTSD.* Brain, Behavior, and Immunity, 2019. **80**: p. 889-899.
302. Famularo, R., R. Kinscherff, and T. Fenton, *Psychiatric diagnoses of maltreated children: preliminary findings.* J Am Acad Child Adolesc Psychiatry, 1992. **31**(5): p. 863-7.
303. Gotlib, I.H., et al., *Early Life Stress Predicts Depressive Symptoms in Adolescents During the COVID-19 Pandemic: The Mediating Role of Perceived Stress.* Frontiers in Psychology, 2021. **11**.
304. Bahari-Javan, S., et al., *HDAC1 links early life stress to schizophrenia-like phenotypes.* Proceedings of the National Academy of Sciences, 2017. **114**(23): p. E4686-E4694.
305. Popovic, D., et al., *Childhood Trauma in Schizophrenia: Current Findings and Research Perspectives.* Frontiers in neuroscience, 2019. **13**: p. 274-274.
306. Juruena, M.F., et al., *The Role of Early Life Stress in HPA Axis and Anxiety.* Adv Exp Med Biol, 2020. **1191**: p. 141-153.
307. Ho, G.W.K., et al., *Complex PTSD symptoms mediate the association between childhood trauma and physical health problems.* Journal of Psychosomatic Research, 2021. **142**: p. 110358.
308. Chen, C.V., et al., *Effects of trauma in adulthood and adolescence on fear extinction and extinction retention: Advancing animal models of posttraumatic stress disorder.* Frontiers in Behavioral Neuroscience, 2018. **12**.
309. Marin, M.-F., et al., *Skin Conductance Responses and Neural Activations During Fear Conditioning and Extinction Recall Across Anxiety Disorders.* JAMA Psychiatry, 2017. **74**(6): p. 622-631.
310. Holt, D.J., et al., *Failure of neural responses to safety cues in schizophrenia.* Arch Gen Psychiatry, 2012. **69**(9): p. 893-903.
311. Paredes, D. and D.A. Morilak, *A Rodent Model of Exposure Therapy: The Use of Fear Extinction as a Therapeutic Intervention for PTSD.* Frontiers in Behavioral Neuroscience, 2019. **13**.
312. Goswami, S., et al., *Animal models of post-traumatic stress disorder: face validity.* Front Neurosci, 2013. **7**: p. 89.
313. Lommen, M.J.J., et al., *Pre-trauma individual differences in extinction learning predict posttraumatic stress.* Behaviour Research and Therapy, 2013. **51**(2): p. 63-67.
314. Raio, C.M. and E.A. Phelps, *The influence of acute stress on the regulation of conditioned fear.* Neurobiology of stress, 2014. **1**: p. 134-146.
315. Wellman, C.L. and K.M. Moench, *Preclinical studies of stress, extinction, and prefrontal cortex: intriguing leads and pressing questions.* Psychopharmacology (Berl), 2019. **236**(1): p. 59-72.
316. Rocha, M., et al., *Deficits in hippocampal-dependent memory across different rodent models of early life stress: systematic review and meta-analysis.* Translational Psychiatry, 2021. **11**(1): p. 231.
317. Bali, A. and A.S. Jaggi, *Investigations in foot shock stress of variable intensity in mice: Adaptation and role of angiotensin II.* Eur J Pharmacol, 2015. **761**: p. 86-94.

318. Lugo, J.N., G.D. Smith, and A.J. Holley, *Trace fear conditioning in mice.* J Vis Exp, 2014(85).
319. Ghi, P., et al., *Stress and brain histaminergic system: Effects of weak electric foot-shock.* Pharmacology Biochemistry and Behavior, 1992. **41**(2): p. 317-320.
320. Maren, S., G. Aharonov, and M.S. Fanselow, *Neurotoxic lesions of the dorsal hippocampus and Pavlovian fear conditioning in rats.* Behavioural Brain Research, 1997. **88**(2): p. 261-274.
321. Kjelstrup, K.G., et al., *Reduced fear expression after lesions of the ventral hippocampus.* Proceedings of the National Academy of Sciences, 2002. **99**(16): p. 10825-10830.
322. Yavas, E., S. Gonzalez, and M.S. Fanselow, *Interactions between the hippocampus, prefrontal cortex, and amygdala support complex learning and memory [version 1; peer review: 3 approved].* F1000 research, 2019. **8**: p. 1292.
323. Brydges, N.M., et al., *Juvenile stress enhances anxiety and alters corticosteroid receptor expression in adulthood.* Brain Behav, 2014. **4**(1): p. 4-13.
324. Maren, S., B. De Oca, and M.S. Fanselow, *Sex differences in hippocampal long-term potentiation (LTP) and Pavlovian fear conditioning in rats: positive correlation between LTP and contextual learning.* Brain Research, 1994. **661**(1): p. 25-34.
325. Daviu, N., et al., *Sex differences in the behavioural and hypothalamic–pituitary–adrenal response to contextual fear conditioning in rats.* Hormones and Behavior, 2014. **66**(5): p. 713-723.
326. Voulo, M.E. and R.G. Parsons, *Response-specific sex difference in the retention of fear extinction.* Learning & memory., 2017. **24**(6): p. 245-251.
327. Blume, S.R., et al., *Sex- and Estrus-Dependent Differences in Rat Basolateral Amygdala.* J Neurosci, 2017. **37**(44): p. 10567-10586.
328. Keiser, A.A., et al., *Sex Differences in Context Fear Generalization and Recruitment of Hippocampus and Amygdala during Retrieval.* Neuropsychopharmacology, 2017. **42**(2): p. 397-407.
329. Trott, J.M., F.B. Krasne, and M.S. Fanselow, *Sex differences in contextual fear learning and generalization: a behavioral and computational analysis of hippocampal functioning.* Learn Mem, 2022. **29**(9): p. 283-296.
330. Manzano-Nieves, G., et al., *Early life stress impairs contextual threat expression in female, but not male, mice.* Behav Neurosci, 2018. **132**(4): p. 247-257.
331. Arain, M., et al., *Maturation of the adolescent brain.* Neuropsychiatric disease and treatment, 2013. **9**: p. 449-461.
332. Giustino, T.F. and S. Maren, *The Role of the Medial Prefrontal Cortex in the Conditioning and Extinction of Fear.* Frontiers in Behavioral Neuroscience, 2015. **9**.
333. Beatty, W.W. and R.G. Fessler, *Sex differences in sensitivity to electric shock in rats and hamsters.* Bulletin of the Psychonomic Society, 1977. **10**(3): p. 189-190.
334. Dos Santos Corrêa, M., et al., *Relationship between footshock intensity, post-training corticosterone release and contextual fear memory specificity over time.* Psychoneuroendocrinology, 2019. **110**: p. 104447.
335. Laine, M.A., et al., *Sounding the Alarm: Sex Differences in Rat Ultrasonic Vocalizations during Pavlovian Fear Conditioning and Extinction.* eNeuro, 2022. **9**(6).
336. Taitt, K.T. and L.V. Kendall, *Physiologic Stress of Ear Punch Identification Compared with Restraint Only in Mice.* J Am Assoc Lab Anim Sci, 2019. **58**(4): p. 438-442.

337. Barha, C.K., G.L. Dalton, and L.A. Galea, *Low doses of 17alpha-estradiol and 17beta-estradiol facilitate, whereas higher doses of estrone and 17alpha- and 17beta-estradiol impair, contextual fear conditioning in adult female rats.* Neuropsychopharmacology, 2010. **35**(2): p. 547-59.
338. Frank, M.G., et al., *Stress-induced neuroinflammatory priming: A liability factor in the etiology of psychiatric disorders.* Neurobiology of Stress, 2016. **4**: p. 62-70.
339. Johnson, J.D., et al., *Neuroendocrine Regulation of Brain Cytokines After Psychological Stress.* Journal of the Endocrine Society, 2019. **3**(7): p. 1302-1320.
340. Yang, C., et al., *Stress within a restricted time window selectively affects the persistence of long-term memory.* PLoS One, 2013. **8**(3): p. e59075.
341. Dolfen, N., et al., *Glucocorticoid response to stress induction prior to learning is negatively related to subsequent motor memory consolidation.* Neurobiol Learn Mem, 2019. **158**: p. 32-41.
342. Quaedflieg, C.W.E.M., et al., *Time dependent effects of stress prior to encoding on event-related potentials and 24h delayed retrieval.* Psychoneuroendocrinology, 2013. **38**(12): p. 3057-3069.
343. Medina, J.H., et al., *Do memories consolidate to persist or do they persist to consolidate?* Behav Brain Res, 2008. **192**(1): p. 61-9.
344. Sandi, C. and M.T. Pinelo-Nava, *Stress and memory: behavioral effects and neurobiological mechanisms.* Neural Plast, 2007. **2007**: p. 78970.
345. Gogolla, N., et al., *Perineuronal nets protect fear memories from erasure.* Science, 2009. **325**(5945): p. 1258-61.
346. Koseki, H., et al., *Alteration of synaptic transmission in the hippocampal-mPFC pathway during extinction trials of context-dependent fear memory in juvenile rat stress models.* Synapse, 2009. **63**(9): p. 805-13.
347. Do Monte, F.H., et al., *Retrieving fear memories, as time goes by….* Mol Psychiatry, 2016. **21**(8): p. 1027-36.
348. Toda, H., et al., *Maternal Separation Enhances Conditioned Fear and Decreases the mRNA Levels of the Neurotensin Receptor 1 Gene with Hypermethylation of This Gene in the Rat Amygdala.* PLOS ONE, 2014. **9**(5): p. e97421.
349. Diehl, L.A., et al., *Contextual Fear Conditioning in Maternal Separated Rats: The Amygdala as a Site for Alterations.* Neurochemical Research, 2014. **39**(2): p. 384-393.
350. Barker, J.M. and L.A.M. Galea, *Males show stronger contextual fear conditioning than females after context pre-exposure.* Physiology & Behavior, 2010. **99**(1): p. 82-90.
351. Anagnostaras, S.G., et al., *Testicular hormones do not regulate sexually dimorphic Pavlovian fear conditioning or perforant-path long-term potentiation in adult male rats.* Behavioural Brain Research, 1998. **92**(1): p. 1-9.
352. Gresack, J.E., et al., *Sex differences in contextual fear conditioning are associated with differential ventral hippocampal extracellular signal-regulated kinase activation.* Neuroscience, 2009. **159**(2): p. 451-467.
353. Farrell, M.R., et al., *Lesion of infralimbic cortex occludes stress effects on retrieval of extinction but not fear conditioning.* Neurobiology of Learning and Memory, 2010. **94**(2): p. 240-246.
354. Gupta, R.R., et al., *Estrogen modulates sexually dimorphic contextual fear conditioning and hippocampal long-term potentiation (LTP) in rats11Published on the World Wide Web on 1 December 2000.* Brain Research, 2001. **888**(2): p. 356-365.

355. Tronson, N.C. and A.A. Keiser, *A Dynamic Memory Systems Framework for Sex Differences in Fear Memory.* Trends Neurosci, 2019. **42**(10): p. 680-692.
356. Bouton, M.E., S. Maren, and G.P. McNally, *Behavioral and neurobiological mechanisms of pavlovian and instrumental extinction learning.* Physiological Reviews, 2021. **101**(2): p. 611-681.
357. Levine, S. and T. Mody, *The long-term psychobiological consequences of intermittent postnatal separation in the squirrel monkey.* Neurosci Biobehav Rev, 2003. **27**(1-2): p. 83-9.
358. Izquierdo, A., C.L. Wellman, and A. Holmes, *Brief uncontrollable stress causes dendritic retraction in infralimbic cortex and resistance to fear extinction in mice.* J Neurosci, 2006. **26**(21): p. 5733-8.
359. Miracle, A.D., et al., *Chronic stress impairs recall of extinction of conditioned fear.* Neurobiol Learn Mem, 2006. **85**(3): p. 213-8.
360. Hubel, D.H. and T.N. Wiesel, *The period of susceptibility to the physiological effects of unilateral eye closure in kittens.* J Physiol, 1970. **206**(2): p. 419-36.
361. McLaughlin, K.A., et al., *Mechanisms linking childhood trauma exposure and psychopathology: a transdiagnostic model of risk and resilience.* BMC Medicine, 2020. **18**(1): p. 96.
362. Fuentes, S., et al., *Early life stress in rats sex-dependently affects remote endocrine rather than behavioral consequences of adult exposure to contextual fear conditioning.* Hormones and Behavior, 2018. **103**: p. 7-18.
363. Remmes, J., et al., *Impact of Life History on Fear Memory and Extinction.* Front Behav Neurosci, 2016. **10**: p. 185.
364. Day, H.L.L. and C.W. Stevenson, *The neurobiological basis of sex differences in learned fear and its inhibition.* European Journal of Neuroscience, 2019.
365. Donner, N.C. and C.A. Lowry, *Sex differences in anxiety and emotional behavior.* Pflugers Arch, 2013. **465**(5): p. 601-26.
366. Kilpatrick, D.G., et al., *National estimates of exposure to traumatic events and PTSD prevalence using DSM-IV and DSM-5 criteria.* Journal of traumatic stress, 2013. **26**(5): p. 537-547.
367. Pooley, A.E., et al., *Sex differences in the traumatic stress response: PTSD symptoms in women recapitulated in female rats.* Biology of sex differences, 2018. **9**(1): p. 31-31.
368. Parker, K.J., et al., *Mild early life stress enhances prefrontal-dependent response inhibition in monkeys.* Biological Psychiatry, 2005. **57**(8): p. 848-855.
369. Murthy, S. and E. Gould, *Early Life Stress in Rodents: Animal Models of Illness or Resilience?* Frontiers in Behavioral Neuroscience, 2018. **12**.
370. Dettling, A.C., J. Feldon, and C.R. Pryce, *Repeated parental deprivation in the infant common marmoset (callithrix jacchus, primates) and analysis of its effects on early development.* Biological Psychiatry, 2002. **52**(11): p. 1037-1046.
371. Hinde, R.A. and Y. Spencer-Booth, *Effects of Brief Separation from Mother on Rhesus Monkeys.* Science, 1971. **173**(3992): p. 111-118.
372. Matsuda, S., et al., *Sex differences in fear extinction and involvements of extracellular signal-regulated kinase (ERK).* Neurobiology of Learning and Memory, 2015. **123**: p. 117-124.
373. Fenton, G.E., et al., *Sex differences in learned fear expression and extinction involve altered gamma oscillations in medial prefrontal cortex.* Neurobiology of Learning and Memory, 2016. **135**: p. 66-72.

374. Halladay, L.R., et al., *Reinstatement of extinguished fear by an unextinguished conditional stimulus.* Front Behav Neurosci, 2012. **6**: p. 18.
375. Bouton, M.E. and R.C. Bolles, *Contextual control of the extinction of conditioned fear.* Learning and Motivation, 1979. **10**(4): p. 445-466.
376. Hiraide, S., et al., *Possible modulation of the amygdala on metaplasticity deficits in the hippocampal CA1 field in early postnatally stressed rats.* J Pharmacol Sci, 2012. **119**(1): p. 64-72.
377. Harrison, F.E., A.H. Hosseini, and M.P. McDonald, *Endogenous anxiety and stress responses in water maze and Barnes maze spatial memory tasks.* Behav Brain Res, 2009. **198**(1): p. 247-51.
378. Friard, O. and M. Gamba, *BORIS: A free, versatile open-source event-logging software for video/audio coding and live observations.* Methods in Ecology and Evolution, 2016. **7**.
379. Tigaret, C.M., et al., *Neurotrophin receptor activation rescues cognitive and synaptic abnormalities caused by hemizygosity of the psychiatric risk gene Cacna1c.* Mol Psychiatry, 2021. **26**(6): p. 1748-1760.
380. Noldus, L.P.J.J., A.J. Spink, and R.A.J. Tegelenbosch, *EthoVision: A versatile video tracking system for automation of behavioral experiments.* Behavior Research Methods, Instruments, & Computers, 2001. **33**(3): p. 398-414.
381. Amorim, F.E., T.C. Moulin, and O.B. Amaral, *A Freely Available, Self-Calibrating Software for Automatic Measurement of Freezing Behavior.* Frontiers in Behavioral Neuroscience, 2019. **13**(205).
382. Mathis, A., et al., *DeepLabCut: markerless pose estimation of user-defined body parts with deep learning.* Nature Neuroscience, 2018. **21**(9): p. 1281-1289.
383. Pennington, Z.T., et al., *ezTrack—A Step-by-Step Guide to Behavior Tracking.* Current Protocols, 2021. **1**(10): p. e255.
384. Anagnostaras, S.G., et al., *Automated assessment of pavlovian conditioned freezing and shock reactivity in mice using the video freeze system.* Front Behav Neurosci, 2010. **4**.
385. Anagnostaras, S.G., et al., *Computer-assisted behavioral assessment of Pavlovian fear conditioning in mice.* Learn Mem, 2000. **7**(1): p. 58-72.
386. Marchand, A.R., D. Luck, and G. DiScala, *Evaluation of an improved automated analysis of freezing behaviour in rats and its use in trace fear conditioning.* Journal of Neuroscience Methods, 2003. **126**(2): p. 145-153.
387. Pugh, C.R., et al., *Role of interleukin-1 beta in impairment of contextual fear conditioning caused by social isolation.* Behavioural Brain Research, 1999. **106**(1): p. 109-118.
388. Margolin, G. and K.A. Vickerman, *Post-traumatic Stress in Children and Adolescents Exposed to Family Violence: I. Overview and Issues.* Professional psychology, research and practice, 2007. **38**(6): p. 613-619.
389. Shors, T.J., C. Chua, and J. Falduto, *Sex differences and opposite effects of stress on dendritic spine density in the male versus female hippocampus.* J Neurosci, 2001. **21**(16): p. 6292-7.
390. Varol, A.B., E.C. Esen, and E.E. Koçak, *Repeated Collection of Vaginal Smear Causes Stress in Mice.* Noro Psikiyatr Ars, 2022. **59**(4): p. 325-329.
391. Ajayi, A.F. and R.E. Akhigbe, *Staging of the estrous cycle and induction of estrus in experimental rodents: an update.* Fertility Research and Practice, 2020. **6**(1): p. 5.

392. Antoniadis, E.A. and R.J. McDonald, *Discriminative fear conditioning to context expressed by multiple measures of fear in the rat.* Behavioural brain research, 1999. **101**(1): p. 1-13.
393. Roelofs, K., *Freeze for action: neurobiological mechanisms in animal and human freezing.* Philosophical transactions of the Royal Society of London. Series B, Biological sciences, 2017. **372**(1718): p. 20160206.
394. Sakamoto, I.M., *Individual Differences in Ultrasonic Vocalizations and Freezing Behavior During Fear Learning and Extinction in Female Rats*. 2020.
395. Blanchard, R.J., et al., *Twenty-two kHz alarm cries to presentation of a predator, by laboratory rats living in visible burrow systems.* Physiology & Behavior, 1991. **50**(5): p. 967-972.
396. Darwin, C., P. Ekman, and P. Prodger, *The expression of the emotions in man and animals*. 3rd ed. ed. 1999, London: HarperCollins.
397. Lenell, C., et al., *Biological and Acoustic Sex Differences in Rat Ultrasonic Vocalization.* Brain Sci, 2021. **11**(4).
398. Brudzynski, S.M., P. Kehoe, and M. Callahan, *Sonographic structure of isolation-induced ultrasonic calls of rat pups.* Dev Psychobiol, 1999. **34**(3): p. 195-204.
399. Brudzynski, S.M., *Emission of 22 kHz vocalizations in rats as an evolutionary equivalent of human crying: Relationship to depression.* Behavioural Brain Research, 2019. **363**: p. 1-12.
400. Kikusui, T., et al., *Conditioned fear-related ultrasonic vocalizations are emitted as an emotional response.* J Vet Med Sci, 2003. **65**(12): p. 1299-305.
401. Dupin, M., et al., *New Insights from 22-kHz Ultrasonic Vocalizations to Characterize Fear Responses: Relationship with Respiration and Brain Oscillatory Dynamics.* eneuro, 2019. **6**(2): p. ENEURO.0065-19.2019.
402. Willadsen, M., et al., *Reduced emission of alarm 22-kHz ultrasonic vocalizations during fear conditioning in rats lacking the serotonin transporter.* Prog Neuropsychopharmacol Biol Psychiatry, 2021. **108**: p. 110072.
403. Castellucci, G.A., D. Calbick, and D. McCormick, *The temporal organization of mouse ultrasonic vocalizations.* PLoS One, 2018. **13**(10): p. e0199929.
404. Fischer, J. and K. Hammerschmidt, *Ultrasonic vocalizations in mouse models for speech and socio-cognitive disorders: insights into the evolution of vocal communication.* Genes Brain Behav, 2011. **10**(1): p. 17-27.
405. Vogel, A.P., A. Tsanas, and M.L. Scattoni, *Quantifying ultrasonic mouse vocalizations using acoustic analysis in a supervised statistical machine learning framework.* Scientific Reports, 2019. **9**(1): p. 8100.
406. Ruat, J., et al., *Why do mice squeak? Toward a better understanding of defensive vocalization.* iScience, 2022. **25**(7): p. 104657.
407. Schweinfurth, M.K., *The social life of Norway rats (Rattus norvegicus).* eLife, 2020. **9**: p. e54020.
408. D'Amato, F.R., et al., *Pups Call, Mothers Rush: Does Maternal Responsiveness Affect the Amount of Ultrasonic Vocalizations in Mouse Pups?* Behavior Genetics, 2005. **35**(1): p. 103-112.
409. Burgdorf, J., et al., *Ultrasonic vocalizations of rats (Rattus norvegicus) during mating, play, and aggression: Behavioral concomitants, relationship to reward, and self-administration of playback.* J Comp Psychol, 2008. **122**(4): p. 357-67.

410. Wöhr, M., A. Borta, and R.K.W. Schwarting, *Overt behavior and ultrasonic vocalization in a fear conditioning paradigm: A dose–response study in the rat.* Neurobiology of Learning and Memory, 2005. **84**(3): p. 228-240.
411. Wöhr, M., M.M. van Gaalen, and R.K.W. Schwarting, *Affective communication in rodents: serotonin and its modulating role in ultrasonic vocalizations.* Behavioural Pharmacology, 2015. **26**(6 Special Issue Pharmacological Approaches to the Study of Social Behaviour - Part 1: Reviews).
412. Yee, N., et al., *Juvenile stress potentiates aversive 22-kHz ultrasonic vocalizations and freezing during auditory fear conditioning in adult male rats.* Stress, 2012. **15**(5): p. 533-544.
413. Yin, X., et al., *Maternal Deprivation Influences Pup Ultrasonic Vocalizations of C57BL/6J Mice.* PLoS One, 2016. **11**(8): p. e0160409.
414. Tryon, S.C., et al., *ChAT::Cre transgenic rats show sex-dependent altered fear behaviors, ultrasonic vocalizations and cholinergic marker expression.* Genes, Brain and Behavior, 2023. **22**(1): p. e12837.
415. Graham, L.K., et al., *Strain and sex differences in fear conditioning: 22 kHz ultrasonic vocalizations and freezing in rats.* Psychology & Neuroscience, 2009. **2**.
416. Kosten, T.A., et al., *Sex-selective effects of neonatal isolation on fear conditioning and foot shock sensitivity.* Behav Brain Res, 2005. **157**(2): p. 235-44.
417. Audacity, *Audacity® software*. 1999-2021.
418. Robert, J., Webbie, M., & others, *Pydub*. 2018.
419. Jackman, S., et al. *Political Science Computational Laboratory (pscl)*. 2020; Available from: https://github.com/atahk/pscl.
420. Brooks, M., et al., *glmmTMB Balances Speed and Flexibility Among Packages for Zero-inflated Generalized Linear Mixed Modeling.* R Journal, 2017. **9**: p. 378-400.
421. Wöhr, M. and R.K.W. Schwarting, *Ultrasonic calling during fear conditioning in the rat: no evidence for an audience effect.* Animal Behaviour, 2008. **76**(3): p. 749-760.
422. Zhang, Y., et al., *Comparison of the chronic unpredictable mild stress and the maternal separation in mice postpartum depression modeling.* Biochemical and Biophysical Research Communications, 2022. **632**: p. 24-31.
423. Yee, N., et al., *Increased affective ultrasonic communication during fear learning in adult male rats exposed to maternal immune activation.* Journal of Psychiatric Research, 2012. **46**(9): p. 1199-1205.
424. Lerman, D.C., B.A. Iwata, and M.D. Wallace, *Side effects of extinction: prevalence of bursting and aggression during the treatment of self-injurious behavior.* J Appl Behav Anal, 1999. **32**(1): p. 1-8.
425. Leonardo, M., et al., *Intravenous fentanyl self-administration in male and female C57BL/6J and DBA/2J mice.* Sci Rep, 2023. **13**(1): p. 799.
426. Yoon, T. and J. Kim, *Strain and sex differences in fear conditioning: 22 kHz ultrasonic vocalizations and freezing in rats.* Psychology & Neuroscience, 2009. **2**.
427. Blanchard, R.J., et al., *Sex differences in the incidence and sonographic characteristics of antipredator ultrasonic cries in the laboratory rat (Rattus norvegicus).* Journal of Comparative Psychology, 1992. **106**: p. 270-277.
428. Inagaki, H., *Sex differences in ultrasonic vocal expression of negative emotional states in rats*, in *Handbook of ultrasonic vocalization: A window into the emotional brain.* 2018, Elsevier Academic Press: San Diego, CA, US. p. 337-344.

429. Reyes, K.-A.E., et al., *Rat ultrasonic vocalizations as an index of memory.* Neuroscience Letters, 2021. **741**: p. 135458.
430. Karwicka, W., et al., *Relaying Aversive Ultrasonic Alarm Calls Depends on Previous Experience. Empathy, Social Buffering, or Panic?* Brain Sciences, 2021. **11**(6): p. 759.
431. Olszyński, K.H., et al., *Increased Vocalization of Rats in Response to Ultrasonic Playback as a Sign of Hypervigilance Following Fear Conditioning.* Brain Sciences, 2021. **11**(8): p. 970.
432. Vivian, J.A. and K.A. Miczek, *Morphine attenuates ultrasonic vocalization during agonistic encounters in adult male rats.* Psychopharmacology, 1993. **111**(3): p. 367-375.
433. Vivian, J.A. and K.A. Miczek, *Diazepam and gepirone selectively attenuate either 20-32 or 32-64 kHz ultrasonic vocalizations during aggressive encounters.* Psychopharmacology, 1993. **112**(1): p. 66-73.
434. Kim, E.J., et al., *Social Transmission of Fear in Rats: The Role of 22-kHz Ultrasonic Distress Vocalization.* PLOS ONE, 2010. **5**(12): p. e15077.
435. Brudzynski, S.M., *Pharmacological and behavioral characteristics of 22 kHz alarm calls in rats.* Neurosci Biobehav Rev, 2001. **25**(7-8): p. 611-7.
436. Brudzynski, S.M., *Principles of rat communication: quantitative parameters of ultrasonic calls in rats.* Behav Genet, 2005. **35**(1): p. 85-92.
437. Fanselow, M.S., *The adaptive function of conditioned defensive behavior: An ecological approach to Pavlovian stimulus-substitution theory*, in *Ethoexperimental approaches to the study of behavior.* 1989, Kluwer Academic/Plenum Publishers: New York, NY, US. p. 151-166.
438. Fanselow, M.S., *Neural organization of the defensive behavior system responsible for fear.* Psychonomic Bulletin & Review, 1994. **1**(4): p. 429-438.
439. Blanchard, R.J., D.C. Blanchard, and K. Hori, *An ethoexperimental approach to the study of defense*, in *Ethoexperimental approaches to the study of behavior.* 1989, Kluwer Academic/Plenum Publishers: New York, NY, US. p. 114-136.
440. Bauer, E.P., *Sex differences in fear responses: Neural circuits.* Neuropharmacology, 2023. **222**: p. 109298.
441. Borg, E., *Auditory thresholds in rats of different age and strain. A behavioral and electrophysiological study.* Hearing Research, 1982. **8**(2): p. 101-115.
442. Demaestri, C., H.C. Brenhouse, and J.A. Honeycutt, *22 kHz and 55 kHz ultrasonic vocalizations differentially influence neural and behavioral outcomes: Implications for modeling anxiety via auditory stimuli in the rat.* Behav Brain Res, 2019. **360**: p. 134-145.
443. Ma, S.T., et al., *Repeated intravenous cocaine experience: development and escalation of pre-drug anticipatory 50-kHz ultrasonic vocalizations in rats.* Behav Brain Res, 2010. **212**(1): p. 109-14.
444. Mahler, S.V., et al., *A rodent "self-report" measure of methamphetamine craving? Rat ultrasonic vocalizations during methamphetamine self-administration, extinction, and reinstatement.* Behav Brain Res, 2013. **236**(1): p. 78-89.
445. Coffey, K.R., R.E. Marx, and J.F. Neumaier, *DeepSqueak: a deep learning-based system for detection and analysis of ultrasonic vocalizations.* Neuropsychopharmacology, 2019. **44**(5): p. 859-868.
446. Rodney, T., et al., *High IL-6 in military personnel relates to multiple traumatic brain injuries and post-traumatic stress disorder.* Behav Brain Res, 2020. **392**: p. 112715.

447. Butterick, T.A., et al., *Gulf War Illness-associated increases in blood levels of interleukin 6 and C-reactive protein: biomarker evidence of inflammation.* BMC Res Notes, 2019. **12**(1): p. 816.
448. Monsour, M., D.M. Croci, and S. Agazzi, *The role of IL-6 in TBI and PTSD, a potential therapeutic target?* Clinical Neurology and Neurosurgery, 2022. **218**: p. 107280.
449. Hussein, S., et al., *A Systematic Review of Tumor Necrosis Factor-α in Post-Traumatic Stress Disorder: Evidence from Human and Animal Studies.* Psychiatr Danub, 2017. **29**(4): p. 407-420.
450. Dib, P., et al., *TNF-Alpha as an Initiator of Allodynia and Anxiety-Like Behaviors in a Preclinical Model of PTSD and Comorbid Pain.* Front Psychiatry, 2021. **12**: p. 721999.
451. van Zuiden, M., et al., *Cytokine production by leukocytes of military personnel with depressive symptoms after deployment to a combat-zone: a prospective, longitudinal study.* PLoS One, 2011. **6**(12): p. e29142.
452. Smid, G.E., et al., *Cytokine production as a putative biological mechanism underlying stress sensitization in high combat exposed soldiers.* Psychoneuroendocrinology, 2015. **51**: p. 534-546.
453. Zhang, L., et al., *Potential chemokine biomarkers associated with PTSD onset, risk and resilience as well as stress responses in US military service members.* Translational Psychiatry, 2020. **10**(1): p. 31.
454. Bookwalter, D.B., et al., *Posttraumatic stress disorder and risk of selected autoimmune diseases among US military personnel.* BMC Psychiatry, 2020. **20**(1): p. 23.
455. Lenz, K.M. and L.H. Nelson, *Microglia and Beyond: Innate Immune Cells As Regulators of Brain Development and Behavioral Function.* Frontiers in Immunology, 2018. **9**.
456. Gadient, R.A. and U. Otten, *Identification of interleukin-6 (IL-6)-expressing neurons in the cerebellum and hippocampus of normal adult rats.* Neurosci Lett, 1994. **182**(2): p. 243-6.
457. Erta, M., A. Quintana, and J. Hidalgo, *Interleukin-6, a Major Cytokine in the Central Nervous System.* International Journal of Biological Sciences, 2012. **8**(9): p. 1254-1266.
458. Pearson, V.L., N.J. Rothwell, and S. Toulmond, *Excitotoxic brain damage in the rat induces interleukin-1beta protein in microglia and astrocytes: correlation with the progression of cell death.* Glia, 1999. **25**(4): p. 311-23.
459. Dalton, G.L., et al., *Disruption of AMPA Receptor Endocytosis Impairs the Extinction, but not Acquisition of Learned Fear.* Neuropsychopharmacology, 2008. **33**(10): p. 2416-2426.
460. Santello, M., P. Bezzi, and A. Volterra, *TNFα controls glutamatergic gliotransmission in the hippocampal dentate gyrus.* Neuron, 2011. **69**(5): p. 988-1001.
461. Barrientos, R.M., et al., *Memory for context is impaired by a post context exposure injection of interleukin-1 beta into dorsal hippocampus.* Behavioural Brain Research, 2002. **134**(1): p. 291-298.
462. Cunha, C., R. Brambilla, and K.L. Thomas, *A simple role for BDNF in learning and memory?* Front Mol Neurosci, 2010. **3**: p. 1.
463. Yu, Z., et al., *Microglial production of TNF-alpha is a key element of sustained fear memory.* Brain, behavior, and immunity., 2017. **59**: p. 313-321.
464. Young, M.B., et al., *A peripheral immune response to remembering trauma contributes to the maintenance of fear memory in mice.* Psychoneuroendocrinology, 2018. **94**: p. 143-151.

465. Hanisch, U.K., *Microglia as a source and target of cytokines.* Glia, 2002. **40**(2): p. 140-155.
466. Gomez-Arboledas, A., M.M. Acharya, and A.J. Tenner, *The Role of Complement in Synaptic Pruning and Neurodegeneration.* Immunotargets Ther, 2021. **10**: p. 373-386.
467. Lalonde, C.S., et al., *Sex Differences in Peritraumatic Inflammatory Cytokines and Steroid Hormones Contribute to Prospective Risk for Nonremitting Posttraumatic Stress Disorder.* Chronic Stress, 2021. **5**: p. 24705470211032208.
468. Calabrese, E.J., *Stress Biology and Hormesis: The Yerkes–Dodson Law in Psychology—A Special Case of the Hormesis Dose Response.* Critical Reviews in Toxicology, 2008. **38**(5): p. 453-462.
469. Tian, R., et al., *A possible change process of inflammatory cytokines in the prolonged chronic stress and its ultimate implications for health.* ScientificWorldJournal, 2014. **2014**: p. 780616.
470. Johnson, J.D., Z.R. Zimomra, and L.T. Stewart, *Beta-adrenergic receptor activation primes microglia cytokine production.* Journal of Neuroimmunology, 2013. **254**(1): p. 161-164.
471. Schramm, E. and A. Waisman, *Microglia as Central Protagonists in the Chronic Stress Response.* Neurol Neuroimmunol Neuroinflamm, 2022. **9**(6).
472. Hostinar, C.E., R. Nusslock, and G.E. Miller, *Future Directions in the Study of Early-Life Stress and Physical and Emotional Health: Implications of the Neuroimmune Network Hypothesis.* Journal of Clinical Child & Adolescent Psychology, 2018. **47**(1): p. 142-156.
473. Stephan, A.H., B.A. Barres, and B. Stevens, *The complement system: an unexpected role in synaptic pruning during development and disease.* Annu Rev Neurosci, 2012. **35**: p. 369-89.
474. Lumertz, F.S., et al., *Effects of early life stress on brain cytokines: A systematic review and meta-analysis of rodent studies.* Neuroscience & Biobehavioral Reviews, 2022. **139**: p. 104746.
475. Danese, A. and S. J Lewis, *Psychoneuroimmunology of Early-Life Stress: The Hidden Wounds of Childhood Trauma?* Neuropsychopharmacology, 2017. **42**(1): p. 99-114.
476. Wang, R., et al., *Dynamic Effects of Early Adolescent Stress on Depressive-Like Behaviors and Expression of Cytokines and JMJD3 in the Prefrontal Cortex and Hippocampus of Rats.* Front Psychiatry, 2018. **9**: p. 471.
477. Shortall, S.E., et al., *Characterization of Behavioral, Signaling and Cytokine Alterations in a Rat Neurodevelopmental Model for Schizophrenia, and Their Reversal by the 5-HT6 Receptor Antagonist SB-399885.* Molecular Neurobiology, 2018. **55**(9): p. 7413-7430.
478. Ko, C.Y. and Y.P. Liu, *Isolation rearing impaired sensorimotor gating but increased pro-inflammatory cytokines and disrupted metabolic parameters in both sexes of rats.* Psychoneuroendocrinology, 2015. **55**: p. 173-83.
479. Bekhbat, M. and G.N. Neigh, *Sex differences in the neuro-immune consequences of stress: Focus on depression and anxiety.* Brain Behav Immun, 2018. **67**: p. 1-12.
480. Kronfol, Z. and D.G. Remick, *Cytokines and the brain: implications for clinical psychiatry.* Am J Psychiatry, 2000. **157**(5): p. 683-94.
481. Malik, S., et al., *Inflammatory cytokines associated with mild traumatic brain injury and clinical outcomes: a systematic review and meta-analysis.* Front Neurol, 2023. **14**: p. 1123407.

482. Hofer, M., et al., *Cerebral expression of interleukin-12 induces neurological disease via differential pathways and recruits antigen-specific T cells in virus-infected mice.* Am J Pathol, 2004. **165**(3): p. 949-58.
483. Renner, V., et al., *Pro- and anti-inflammatory cytokines Interleukin-6 and Interleukin-10 predict therapy outcome of female patients with posttraumatic stress disorder.* Translational Psychiatry, 2022. **12**(1): p. 472.
484. Elomaa, A.-P., et al., *Elevated levels of serum IL-5 are associated with an increased likelihood of major depressive disorder.* BMC Psychiatry, 2012. **12**(1): p. 2.
485. Sun, L., et al., *Interleukin 12 (IL-12) family cytokines: Role in immune pathogenesis and treatment of CNS autoimmune disease.* Cytokine, 2015. **75**(2): p. 249-55.
486. Boccia, M., et al., *Activation of hippocampal nuclear factor-κB by retrieval is required for memory reconsolidation.* The Journal of neuroscience, 2007. **27**(49): p. 13436-13445.
487. Torres-Rodriguez, O., et al., *Sex-dependent effects of microglial reduction on impaired fear extinction induced by single prolonged stress.* Front Behav Neurosci, 2022. **16**: p. 1014767.
488. Worthen, R.J., et al., *Anti-inflammatory IL-10 administration rescues depression-associated learning and memory deficits in mice.* Journal of Neuroinflammation, 2020. **17**(1): p. 246.
489. Faul, F., et al., *G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences.* Behav Res Methods, 2007. **39**(2): p. 175-91.
490. Cumiskey, D., M. Pickering, and J.J. O'Connor, *Interleukin-18 mediated inhibition of LTP in the rat dentate gyrus is attenuated in the presence of mGluR antagonists.* Neurosci Lett, 2007. **412**(3): p. 206-10.
491. Cumiskey, D., et al., *A role for inflammatory mediators in the IL-18 mediated attenuation of LTP in the rat dentate gyrus.* Neuropharmacology, 2007. **52**(8): p. 1616-23.
492. Tringali, G., et al., *Interleukin-18 displays effects opposite to those of interleukin-1 in the regulation of neuroendocrine stress axis.* Journal of Neuroimmunology, 2005. **160**(1): p. 61-67.
493. Lok, H.C., et al., *Elevated GRO-α and IL-18 in serum and brain implicate the NLRP3 inflammasome in frontotemporal dementia.* Scientific Reports, 2023. **13**(1): p. 8942.
494. Liu, T., et al., *NF-κB signaling in inflammation.* Signal Transduct Target Ther, 2017. **2**: p. 17023-.
495. Maes, M., et al., *Increased plasma concentrations of interleukin-6, soluble interleukin-6, soluble interleukin-2 and transferrin receptor in major depression.* Journal of Affective Disorders, 1995. **34**(4): p. 301-309.
496. Dalgard, C.L., et al., *The cytokine temporal profile in rat cortex after controlled cortical impact.* Front Mol Neurosci, 2012. **5**: p. 6.
497. Callard, R., A.J. George, and J. Stark, *Cytokines, chaos, and complexity.* Immunity, 1999. **11**(5): p. 507-13.
498. De Simone, V., et al., *Th17-type cytokines, IL-6 and TNF-α synergistically activate STAT3 and NF-kB to promote colorectal cancer cell growth.* Oncogene, 2015. **34**(27): p. 3493-3503.
499. Strle, K., et al., *Interleukin-10 in the brain.* Crit Rev Immunol, 2001. **21**(5): p. 427-49.
500. Liu, C., et al., *Cytokines: From Clinical Significance to Quantification.* Advanced Science, 2021. **8**(15): p. 2004433.

501. Harting, M.T., et al., *Acute, regional inflammatory response after traumatic brain injury: Implications for cellular therapy.* Surgery, 2008. **144**(5): p. 803-813.
502. Saijo, K., A. Crotti, and C.K. Glass, *Regulation of microglia activation and deactivation by nuclear receptors.* Glia, 2013. **61**(1): p. 104-111.
503. Brydges, N.M., et al., *Childhood stress impairs social function through AVP-dependent mechanisms.* Translational Psychiatry, 2019. **9**(1): p. 330.
504. Santoft, F., et al., *Inflammatory cytokines in patients with common mental disorders treated with cognitive behavior therapy.* Brain Behav Immun Health, 2020. **3**: p. 100045.
505. Schafer, D.P., et al., *Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner.* Neuron, 2012. **74**(4): p. 691-705.
506. Frost, J.L. and D.P. Schafer, *Microglia: Architects of the Developing Nervous System.* Trends Cell Biol, 2016. **26**(8): p. 587-597.
507. Piccinini, A.M. and K.S. Midwood, *DAMPening inflammation by modulating TLR signalling.* Mediators Inflamm, 2010. **2010**.
508. Giulian, D., *Ameboid microglia as effectors of inflammation in the central nervous system.* J Neurosci Res, 1987. **18**(1): p. 155-71, 132-3.
509. Chaaya, N., et al., *Contextual Fear Conditioning Alter Microglia Number and Morphology in the Rat Dorsal Hippocampus.* Frontiers in Cellular Neuroscience, 2019. **13**(214).
510. Wohleb, E.S., et al., *β-Adrenergic Receptor Antagonism Prevents Anxiety-Like Behavior and Microglial Reactivity Induced by Repeated Social Defeat.* The Journal of Neuroscience, 2011. **31**(17): p. 6277-6288.
511. Kopp, B.L., D. Wick, and J.P. Herman, *Differential effects of homotypic vs. heterotypic chronic stress regimens on microglial activation in the prefrontal cortex.* Physiol Behav, 2013. **122**: p. 246-52.
512. Hinwood, M., et al., *Chronic stress induced remodeling of the prefrontal cortex: structural re-organization of microglia and the inhibitory effect of minocycline.* Cereb Cortex, 2013. **23**(8): p. 1784-97.
513. Calcia, M.A., et al., *Stress and neuroinflammation: a systematic review of the effects of stress on microglia and the implications for mental illness.* Psychopharmacology, 2016. **233**(9): p. 1637-1650.
514. Sierra, A., R.C. Paolicelli, and H. Kettenmann, *Cien Años de Microglía: Milestones in a Century of Microglial Research.* Trends Neurosci, 2019. **42**(11): p. 778-792.
515. Donat, C.K., et al., *Microglial Activation in Traumatic Brain Injury.* Front Aging Neurosci, 2017. **9**: p. 208.
516. Li, S., et al., *Ameliorative effects of Fingolimod (FTY720) on microglial activation and psychosis-related behavior in short term cuprizone exposed mice.* Molecular Brain, 2023. **16**(1): p. 59.
517. Hellwig, S., et al., *Altered microglia morphology and higher resilience to stress-induced depression-like behavior in CX3CR1-deficient mice.* Brain Behav Immun, 2016. **55**: p. 126-137.
518. Li, S., et al., *Microglial deletion and inhibition alleviate behavior of post-traumatic stress disorder in mice.* J Neuroinflammation, 2021. **18**(1): p. 7.
519. Gildawie, K.R., et al., *Sex differences in prefrontal cortex microglia morphology: Impact of a two-hit model of adversity throughout development.* Neurosci Lett, 2020. **738**: p. 135381.

520. York, E.M., et al., *3DMorph Automatic Analysis of Microglial Morphology in Three Dimensions from Ex Vivo and In Vivo Imaging.* eNeuro, 2018. **5**(6).
521. Otsu, N., *A Threshold Selection Method from Gray-Level Histograms.* IEEE transactions on systems, man, and cybernetics, 1979. **9**(1): p. 62-66.
522. Li, S., et al., *Microglial deletion and inhibition alleviate behavior of post-traumatic stress disorder in mice.* Journal of Neuroinflammation, 2021. **18**.
523. Sun, R., et al., *Hippocampal activation of microglia may underlie the shared neurobiology of comorbid posttraumatic stress disorder and chronic pain.* Mol Pain, 2016. **12**.
524. Maras, P.M., et al., *Differences in microglia morphological profiles reflect divergent emotional temperaments: insights from a selective breeding model.* Transl Psychiatry, 2022. **12**(1): p. 105.
525. Roque, A., A. Ochoa-Zarzosa, and L. Torner, *Maternal separation activates microglial cells and induces an inflammatory response in the hippocampus of male rat pups, independently of hypothalamic and peripheral cytokine levels.* Brain, Behavior, and Immunity, 2016. **55**: p. 39-48.
526. Sugama, S., et al., *Stress-induced microglial activation occurs through β-adrenergic receptor: noradrenaline as a key neurotransmitter in microglial activation.* Journal of Neuroinflammation, 2019. **16**(1): p. 266.
527. Gómez-González, B. and A. Escobar, *Prenatal stress alters microglial development and distribution in postnatal rat brain.* Acta Neuropathologica, 2010. **119**(3): p. 303-315.
528. Hinwood, M., et al., *Evidence that Microglia Mediate the Neurobiological Effects of Chronic Psychological Stress on the Medial Prefrontal Cortex.* Cerebral Cortex, 2012. **22**(6): p. 1442-1454.
529. Juckel, G., et al., *Microglial activation in a neuroinflammational animal model of schizophrenia — a pilot study.* Schizophrenia Research, 2011. **131**(1): p. 96-100.
530. Nahum, K., et al., *The Role of Microglia in the (Mal)adaptive Response to Traumatic Experience in an Animal Model of PTSD.* Int J Mol Sci, 2022. **23**(13).
531. Diz-Chaves, Y., et al., *Prenatal stress causes alterations in the morphology of microglia and the inflammatory response of the hippocampus of adult female mice.* Journal of Neuroinflammation, 2012. **9**(1): p. 71.
532. Kreisel, T., et al., *Dynamic microglial alterations underlie stress-induced depressive-like behavior and suppressed neurogenesis.* Molecular Psychiatry, 2014. **19**(6): p. 699-709.
533. Manouze, H., et al., *Effects of Single Cage Housing on Stress, Cognitive, and Seizure Parameters in the Rat and Mouse Pilocarpine Models of Epilepsy.* eNeuro, 2019. **6**(4).
534. Paxinos, G.W., C., *The Rat Brain in Stereotaxic Coordinates*. 1998, San Diego: Academic Press.
535. Géron, A.l., *Hands-on Machine Learning with Scikit-Learn, Keras, and TensorFlow : concepts, tools, and techniques to build intelligent systems*. Second edition. ed. 2019, Sebastopol, CA: O'Reilly.
536. Chawla, N., et al., *SMOTE: Synthetic Minority Over-sampling Technique.* J. Artif. Intell. Res. (JAIR), 2002. **16**: p. 321-357.
537. Pedregosa, F., et al., *Scikit-learn: Machine Learning in Python.* J. Mach. Learn. Res., 2011. **12**(null): p. 2825–2830.
538. Czarnecki, W.M., S. Podlewska, and A.J. Bojarski, *Robust optimization of SVM hyperparameters in the classification of bioactive compounds.* Journal of Cheminformatics, 2015. **7**(1): p. 38.

539. Probst, P., M.N. Wright, and A.-L. Boulesteix, *Hyperparameters and tuning strategies for random forest.* WIREs Data Mining and Knowledge Discovery, 2019. **9**(3): p. e1301.
540. Villa, A., et al., *Sex-Specific Features of Microglia from Adult Mice.* Cell Reports, 2018. **23**(12): p. 3501-3511.
541. abd-el-Basset, E. and S. Fedoroff, *Effect of bacterial wall lipopolysaccharide (LPS) on morphology, motility, and cytoskeletal organization of microglia in cultures.* J Neurosci Res, 1995. **41**(2): p. 222-37.
542. Hines, D.J., et al., *Prevention of LPS-induced microglia activation, cytokine production and sickness behavior with TLR4 receptor interfering peptides.* PLoS One, 2013. **8**(3): p. e60388.
543. Obuchowicz, E., et al., *Imipramine and fluoxetine inhibit LPS-induced activation and affect morphology of microglial cells in the rat glial culture.* Pharmacol Rep, 2014. **66**(1): p. 34-43.
544. Lazic, S.E., et al., *A Bayesian predictive approach for dealing with pseudoreplication.* Scientific Reports, 2020. **10**(1): p. 2366.
545. Bader-El-Den, M., E. Teitei, and T. Perry, *Biased Random Forest For Dealing With the Class Imbalance Problem.* IEEE Trans Neural Netw Learn Syst, 2019. **30**(7): p. 2163-2172.
546. Blagus, R. and L. Lusa, *SMOTE for high-dimensional class-imbalanced data.* BMC Bioinformatics, 2013. **14**(1): p. 106.
547. Habibzadeh, M., A. Krzyżak, and T. Fevens. *Comparative Study of Feature Selection for White Blood Cell Differential Counts in Low Resolution Images*. in *Artificial Neural Networks in Pattern Recognition*. 2014. Cham: Springer International Publishing.
548. Aliyu, H.A., et al. *Red Blood Cell Classification: Deep Learning Architecture Versus Support Vector Machine*. in *2018 2nd International Conference on BioSignal Analysis, Processing and Systems (ICBAPS)*. 2018.
549. Adeluwa, T., E. Kim, and J. Hur. *GBMPSO: Hybrid Gradient Boosting Machines with Particle Swarm Optimization in Cell Segmentation Data*. in *2021 IEEE Symposium Series on Computational Intelligence (SSCI)*. 2021.
550. Leyh, J., et al., *Classification of Microglial Morphological Phenotypes Using Machine Learning.* Frontiers in Cellular Neuroscience, 2021. **15**.
551. Simonyan, K. and A. Zisserman, *Very Deep Convolutional Networks for Large-Scale Image Recognition.* 2014.
552. Haar, L.V., T. Elvira, and O. Ochoa, *An analysis of explainability methods for convolutional neural networks.* Engineering Applications of Artificial Intelligence, 2023. **117**: p. 105606.
553. Narla, A., et al., *Automated Classification of Skin Lesions: From Pixels to Practice.* Journal of Investigative Dermatology, 2018. **138**(10): p. 2108-2110.
554. Choi, S., et al., *Automated characterisation of microglia in ageing mice using image processing and supervised machine learning algorithms.* Sci Rep, 2022. **12**(1): p. 1806.
555. Tynan, R.J., et al., *Chronic stress alters the density and morphology of microglia in a subset of stress-responsive brain regions.* Brain Behav Immun, 2010. **24**(7): p. 1058-68.
556. Bian, Y., et al., *Learning, memory, and glial cell changes following recovery from chronic unpredictable stress.* Brain Research Bulletin, 2012. **88**(5): p. 471-476.
557. Lawson, L.J., et al., *Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain.* Neuroscience, 1990. **39**(1): p. 151-170.

558. Verdonk, F., et al., *Phenotypic clustering: a novel method for microglial morphology analysis.* Journal of Neuroinflammation, 2016. **13**(1): p. 153.
559. Tan, Y.-L., Y. Yuan, and L. Tian, *Microglial regional heterogeneity and its role in the brain.* Molecular Psychiatry, 2020. **25**(2): p. 351-367.
560. Thion, M.S., et al., *Microbiome Influences Prenatal and Adult Microglia in a Sex-Specific Manner.* Cell, 2018. **172**(3): p. 500-516.e16.
561. Nelson, L.H., S. Warden, and K.M. Lenz, *Sex differences in microglial phagocytosis in the neonatal hippocampus.* Brain, Behavior, and Immunity, 2017. **64**: p. 11-22.
562. Bordt, E.A., A.M. Ceasrine, and S.D. Bilbo, *Microglia and sexual differentiation of the developing brain: A focus on ontogeny and intrinsic factors.* Glia, 2019. **68**(6): p. 1085-1099.
563. Goyal, M., et al., *Trogocytosis of neurons and glial cells by microglia in a healthy adult macaque retina.* Sci Rep, 2023. **13**(1): p. 633.
564. Hoogland, I.C.M., et al., *Systemic inflammation and microglial activation: systematic review of animal experiments.* Journal of neuroinflammation, 2015. **12**: p. 114-114.
565. Zhang, Y., et al., *Microglia: The Hub of Intercellular Communication in Ischemic Stroke.* Frontiers in Cellular Neuroscience, 2022. **16**.
566. Ziebell, J.M., H. Ray-Jones, and J. Lifshitz, *Nogo presence is inversely associated with shifts in cortical microglial morphology following experimental diffuse brain injury.* Neuroscience, 2017. **359**: p. 209-223.
567. Escoubas, C.C., et al., *Type-I-interferon-responsive microglia shape cortical development and behavior.* Cell, 2024. **187**(8): p. 1936-1954.e24.
568. Thion, M.S. and S. Garel, *On place and time: microglia in embryonic and perinatal brain development.* Current Opinion in Neurobiology, 2017. **47**: p. 121-130.
569. Grabert, K., et al., *Microglial brain region−dependent diversity and selective regional sensitivities to aging.* Nature Neuroscience, 2016. **19**(3): p. 504-516.
570. Stence, N., M. Waite, and M.E. Dailey, *Dynamics of microglial activation: A confocal time-lapse analysis in hippocampal slices.* Glia, 2001. **33**(3): p. 256-266.
571. Malik, S., et al., *Inflammatory cytokines associated with mild traumatic brain injury and clinical outcomes: a systematic review and meta-analysis.* Frontiers in Neurology, 2023. **14**.
572. Wang, G., et al., *Microglia/macrophage polarization dynamics in white matter after traumatic brain injury.* J Cereb Blood Flow Metab, 2013. **33**(12): p. 1864-74.
573. Velayudhan, P.S., et al., *Temporal patterns of microglial activation in white matter following experimental mild traumatic brain injury: a systematic literature review.* Acta Neuropathologica Communications, 2021. **9**(1): p. 197.
574. McCabe, J.T., et al., *Application of high-intensity focused ultrasound to the study of mild traumatic brain injury.* Ultrasound Med Biol, 2014. **40**(5): p. 965-78.
575. Chiu, K.B., et al., *A Method to Investigate Astrocyte and Microglial Morphological Changes in the Aging Brain of the Rhesus Macaque.* Methods Mol Biol, 2019. **1938**: p. 265-276.
576. Davis, B., et al., *Characterizing microglia activation: a spatial statistics approach to maximize information extraction OPEN.* Scientific Reports, 2017. **7**.
577. Maras, P.M., et al., *Differences in microglia morphological profiles reflect divergent emotional temperaments: insights from a selective breeding model.* Translational Psychiatry, 2022. **12**(1): p. 105.

578. Green, T.R.F., S.M. Murphy, and R.K. Rowe, *Comparisons of quantitative approaches for assessing microglial morphology reveal inconsistencies, ecological fallacy, and a need for standardization.* Scientific Reports, 2022. **12**(1): p. 18196.
579. Morrison, H.W. and J.A. Filosa, *A quantitative spatiotemporal analysis of microglia morphology during ischemic stroke and reperfusion.* Journal of Neuroinflammation, 2013. **10**(1): p. 782.
580. Migliarini, S., et al., *Microglia Morphological Changes in the Motor Cortex of hSOD1(G93A) Transgenic ALS Mice.* Brain Sci, 2021. **11**(6).
581. Targum, S.D. and C.B. Nemeroff, *The Effect of Early Life Stress on Adult Psychiatric Disorders.* Innov Clin Neurosci, 2019. **16**(1-2): p. 35-37.
582. Smith, K.E. and S.D. Pollak, *Early life stress and development: potential mechanisms for adverse outcomes.* Journal of Neurodevelopmental Disorders, 2020. **12**(1): p. 34.
583. Torregrossa, M.M. and J.R. Taylor, *Learning to forget: manipulating extinction and reconsolidation processes to treat addiction.* Psychopharmacology (Berl), 2013. **226**(4): p. 659-72.
584. Clifton, N., *The role of schizophrenia susceptibility genes in associative learning.*, in *School of Medicine*. 2016, Cardiff.
585. Markus, E.J. and M. Zecevic, *Sex differences and estrous cycle changes in hippocampus-dependent fear conditioning.* Psychobiology, 1997. **25**(3): p. 246-252.
586. Sadananda, M., M. Wöhr, and R.K. Schwarting, *Playback of 22-kHz and 50-kHz ultrasonic vocalizations induces differential c-fos expression in rat brain.* Neurosci Lett, 2008. **435**(1): p. 17-23.
587. Švorc, P., Jr., S. Grešová, and P. Švorc, *Heart rate variability in male rats.* Physiol Rep, 2023. **11**(18): p. e15827.
588. Park, S.E., et al., *Differential heart rate variability and physiological responses associated with accumulated short- and long-term stress in rodents.* Physiology & Behavior, 2017. **171**: p. 21-31.
589. Lezak, K.R., G. Missig, and W.A. Carlezon, Jr., *Behavioral methods to study anxiety in rodents.* Dialogues Clin Neurosci, 2017. **19**(2): p. 181-191.
590. Wang, Z., B. Caughron, and M.R.I. Young, *Posttraumatic Stress Disorder: An Immunological Disorder?* Frontiers in Psychiatry, 2017. **8**(222).
591. Wang, Z. and M.R.I. Young, *PTSD, a Disorder with an Immunological Component.* Frontiers in immunology, 2016. **7**: p. 219-219.
592. Redmon, J., et al., *You Only Look Once: Unified, Real-Time Object Detection*, in *2016 IEEE Conference on Computer Vision and Pattern Recognition (CVPR)*. 2016, IEEE Computer Society. p. 779-788.
593. Suleymanova, I., D. Bychkov, and J. Kopra, *A deep convolutional neural network for efficient microglia detection.* Scientific Reports, 2023. **13**(1): p. 11139.
594. Frégnac, Y., *Big data and the industrialization of neuroscience: A safe roadmap for understanding the brain?* Science, 2017. **358**(6362): p. 470-477.
595. Markram, H., *The blue brain project.* Nat Rev Neurosci, 2006. **7**(2): p. 153-60.
596. Leergaard, T., C. Hilgetag, and O. Sporns, *Mapping the Connectome: Multi-Level Analysis of Brain Connectivity.* Frontiers in Neuroinformatics, 2012. **6**.
597. Koch, C. and R.C. Reid, *Observatories of the mind.* Nature, 2012. **483**(7390): p. 397-398.