Comparative assessment of neurological vs metabolic allostasis as reflected in human skin fibroblasts

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Declaration

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Abstract

Incidence of mental health disorders are rising in modernity. Many mental health disorders share molecular and genetic overlap as well as having high incidence of comorbidities. Stress and the compounded effect of multiple low-grade stressors may be contributing to a relative increase in the pro-inflammatory and oxidative state seen in mental health disorders and other complex diseases. This leads to allostatic changes that potentially contribute to disease aetiology and progression. Allostasis is the process of homeostatic equilibrium under stress. Inflammation, which is often used to measure allostatic load, is potentially the incorrect measure as inflammation is transient and the damage ascribed to chronic inflammation is due to increases in reactive oxygen species (ROS) and decreases in antioxidant capacity. Post-traumatic stress disorder (PTSD) is a mental health disorder that is characterised by severe stressors and a maladaptive response to these stressors. Although the role of inflammation and oxidative stress have been implicated in the disease aetiology it is still a relatively neglected aspect of PTSD research. Furthermore, despite the high rate of comorbidities associated with PTSD there is still a lack of understanding in terms of the peripheral effects of PTSD. PTSD and potentially comorbid obesity, present ideal health paradigms to assess this relative neglect of allostatic changes, in particular those in the periphery, that may be contributing to disease outcome in PTSD. A novel therapeutic target, namely the trace amine system, is investigated as a potential anxiolytic in zebrafish larvae that could address allostatic changes of chronic diseases such as PTSD and obesity. Patient derived fibroblasts are used as model cell type to investigate potential functional changes in the periphery of PTSD patients as result of allostatic load. Finally, the potential for peripheral signalling to influence central function is explored in astrocytes, that represent the ideal candidate cell to investigate allostatic load in the context of mental health. Changes in peripheral calcium function and central redox function indicate the allostatic load of PTSD can modulate the chemiosmotic potential of cells through longitudinal shifts in the homeostatic set point. As a result, low grade cumulative stressors may be damaging to cellular function without activating endogenous defence mechanisms.

Uittreksel

Die voorkoms van geestesgesondheidsversteurings neem toe in moderniteit. Baie geestesgesondheidsversteurings deel molekulêre en genetiese oorvleueling sowel as 'n hoë voorkoms van komorbiditeite. Stres en die saamgestelde effek van verskeie laegraadse stressors kan bydra tot 'n relatiewe toename in die pro-inflammatoriese en oksidatiewe toestand wat in geestesgesondheidsversteurings en ander komplekse siektes gesien word. Dit lei tot allostatiese veranderinge wat potensieel bydra tot siekte-etiologie en progressie. Inflammasie, wat dikwels gebruik word om allostatiese lading te meet, is potensieel die verkeerde maatstaaf vir hierdie mate,

aangesien inflammasie verbygaande is en die skade wat aan chroniese inflammasie toegeskryf word, te wyte is aan toenames in ROS en afname in antioksidantkapasiteit. PTSV is 'n geestesgesondheidsversteuring wat gekenmerk word deur ernstige stressors en 'n wanadaptiewe reaksie op hierdie stressors. Alhoewel die rol van inflammasie en oksidatiewe stres by die siekte-etiologie van PTSV betrokke is, is dit steeds 'n relatief verwaarloosde aspek van PTSV-navorsing. Verder, ten spyte van die hoë koers as komorbiditeite wat verband hou met PTSV, is daar steeds 'n gebrek aan begrip in terme van die perifere effekte van PTSV. PTSV en potensieel komorbiede vetsug, bied ideale gesondheidsparadigmas aan om hierdie relatiewe verwaarlosing van allostatiese veranderinge te evalueer, veral dié in die periferie, wat kan bydra tot siekte-uitkoms in PTSV. 'n Nuwe terapeutiese teiken, naamlik die spooramienstelsel, word ondersoek as 'n potensiële angsverliggingsmiddel in sebravislarwes wat ook moontlik allostatiese veranderinge van chroniese siektes soos PTSV en vetsug kan aanspreek. Ten slotte word die potensiaal vir perifere sein om sentrale funksie te beïnvloed, in astrosiete ondersoek, wat die ideale kandidaatsel verteenwoordig om allostatiese lading in die konteks van geestesgesondheid te ondersoek. Veranderinge in perifere kalsiumfunksie en sentrale redoksfunksie dui aan dat die allostatiese lading van PTSV die chemiosmotiese potensiaal van selle kan moduleer deur longitudinale verskuiwings in die homeostatiese stelpunt.As gevolg hiervan kan laegraad kumulatiewe stressors skadelik wees vir sellulêre funksie sonder om endogene verdedigingsmeganismes te aktiveer.

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Contents

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List of Abbreviations

AADC – L-amino acid decarboxylase

ACTH – adreno-corticotropin releasing hormone

AD – Alzheimer's disease

AT – adipose tissue

AUC – area under the curve

BAT – brown adipose tissue

BBB – blood brain barrier

BDNF – brain-derived neurotropic factor

BMI – body mass index

Ca2+ – calcium

CAMKII – Ca2+/ calmodulin-dependent protein kinase 2

CAPS – Clinician Administered Posttraumatic Stress Disorder Scale

CaR – Ca2+ receptor

CBG – corticosteroid binding globulin

CICR – calcium induced calcium release

CORT – corticosterone

CREB – cAMP response element binding protein

CRH – corticotropin releasing hormone

CRP – C-reactive protein

CVD – cardiovascular disease

DA – dopamine

DMEM – Dulbecco's Modified Eagle Medium

DR – dopamine receptors

DSM–5 – The Diagnostic and Statistical Manual of Mental Disorders 5th edition

ECM – extracellular matrix

ELISA – Enzyme-linked immunosorbent assay

EndoMT – endothelial-to-mesenchymal transition

ER – endoplasmic reticulum

ERK – extracellularly responsive kinase

FAP – fibroblast activating protein

FBS – fetal bovine serum

FCM – fibroblasts conditioned media

FFA – free fatty acids

FGF-10 – fibroblast growth factor 10

FGF-2 – fibroblast growth factor 2

FKBP5 – FK506 binding protein 5

FMO – fluorochrome minus one

FNDC5 – fibronectin type III domain-containing protein 5

FPP – first principles of physiology

FRAP – ferric reducing antioxidant power

FSP1/S100A4 – fibroblast-specific protein 1

GC – glucocorticoid

GM-CSF – granulocyte macrophage colony stimulating factor

GPCR – G-protein coupled receptors

GR – glucocorticoid receptor

GWAS – genome wide association studies

H_2O_2 – hydrogen peroxide

hDF – human dermal fibroblasts

HPA axis – hypothalamic-pituitary-adrenal-axis

HPI-axis – hypothalamic–pituitary–interrenal-axis

IL1—β – Interleukin-1β

IL-6 – interleukin-6

In(1,4,5)P3R – Ins(1,4,5)P3 receptor

Ins (1,4,5)P3 – inositol 1,4,5-triphosphate

ITIMs – immunoreceptor tyrosine based inhibitory motifs

Kir – inwardly rectifying potassium channels

L-NAME – N(ω)-nitro-L-arginine methyl ester

LPL – lipoprotein lipase

LPS – lipopolysaccharides

MAO – monoamine oxidase

MAPK – mitogen-activated protein kinase

Mclk1 – a mitochondrial hydroxylase necessary for ubiquinone synthesis

MCP-1 – monocyte chemoattractant protein-1

MDA – Malondialdehyde

MDD – major depressive disorder

MEndoT – mesenchymal-to-endothelial transition

MMP – matrix metalloproteinase expression

m-OA – meta-octopamine

MR – mineralocorticoid receptor

m-TYR – meta-tyramine

NADPH – Nicotinamide adenine dinucleotide phosphate

NFκB – Nuclear factor kappa B

NMDA – glutamate receptor

NO – nitric oxide

NOX – NADPH oxidase

PB – permeabilization buffer

PBS – phosphate-buffered saline

PDT – population doubling time

PECAM/CD31 – platelet epithelial cell adhesion molecule

PKA – protein kinase A

PMN – polymorphonuclear leukocytes

p-OA – para-octopamine

PTSD – post-traumatic stress disorder

p-TYR – para-tyramine

ROC – receptor operated channels

ROS – reactive oxygen species

RT – room temperature

RYR – ryanodine receptor

SAPKs/JNKs – stress-activated protein kinases/c-Jun N-terminal kinases

SB – staining buffer

SERCA – sarco/endoplasmic reticulum Ca2+-ATPase

SGM – standard growth medium

SNc – substantia nigra pars compacta

SNP – single nucleotide polymorphism

SOD – superoxide dismutase

SSA – Sub-Saharan African

T1AM – 3-iodothyronamine

TA – trace amine

TAAR – trace amine associated receptor

TBARS – thiobarbituric acid reactive substances

TGF-β – transforming growth factor β

TIMP – Tissue inhibitors of metalloproteinase

TNFα – Tumor necrosis factor α

TRP – tryptamine

VOC – Voltage operated channels

WAT – white adipose tissue

WES – whole exome sequencing

XO – xanthine oxidase

α-SMA – α-smooth muscle actin

β-PEA – β-phenylethylamine

ΔMFI – mean fluorescent intensityPTSD

Chapter 1

Introduction

The 21st century has seen remarkable changes in society mediated through the advent of modern technology. This includes the comfort of motorised global travel, increased global food supply and variety, access to constant entertainment and novelty through the internet, and constant social connection through mobile communication. Together, this created a societal milieu that is constantly stimulating in some way, shape, or form. Whilst none of these improvements are inherently bad, it has led to a high paced stressful environment wherein poor lifestyle choices and anxiety are prominent. Given the contrast to our evolutionary history, the modern environment has the potential to promote chronic low-grade inflammation, oxidative stress, and maladaptive physiological responses. The readily available high calorie food stuffs, a sedentary lifestyle, increased workload, increasing exposure to potential toxins and a variety of other chronic stressors, have indeed been linked to heart disease and metabolic syndrome[1,2], various cancers[3], increased rates of autoimmune disease[4], and mental health disorders[5–7].

Taken together, the common denominator is an overall increase in relatively new stressors in the physical environment that has the potential to affect both psychological and physiological wellbeing by manifesting as micro-environmental stressors that disrupt homeostasis on a cellular level. The human body seems unable to counter this disruption, as evidenced by the high incidence of "accelerated ageing"-associated chronic disease[8–11]. Thus, we are currently in a situation where advances in medicine are assisting the global population to grow much older[12–14], but potentially without the ability to physically and/or mentally cope with the allostatic load of a longer lifespan in the modern era.

The global rise in mental health disorders[15] (particularly anxiety disorders, of which depression and post-traumatic stress disorder (PTSD) are high incidence examples) is likely – at least in part – due to this backdrop of allostatic predisposition to stress on a cellular level. The inclination to develop a mental health disorder is further exacerbated in some individuals due to their genetic architecture. Genome wide association studies (GWAS) suggests that there is a genetic basis for the propensity to develop mental health disorders in general, but is not necessarily indicative of any specific mental health disorder[16–18]. Instead, there are shared genetic overlaps in systemic physiological systems such as calcium signalling components, inflammatory complexes, cellular adhesion and structural proteins, as well as the expected aminergic signalling pathways that underlie common mental health disorders[19–21]. The interaction between psychological triggers, environmental factors, and the physiological responsiveness – or lack thereof – of an individual on a cellular level are therefore

likely contributors to the development of mental health disorders. Yet, very little emphasis is placed on systemic physiological consequences of these factors; as such, the peripheral outcome of mental illness has long been neglected[22].

From a patient management point of view, current approaches to pharmacological interventions for mental health disorders rely on treatments that are psychoactive, whereas the effects of mental illness on peripheral tissue and cellular dysfunction is largely ignored even though chronic co-morbidities manifesting in the periphery are highly prevalent in neurological conditions. Consideration of the peripheral, cellular profile associated with mental health disorders – and in particular those related to oxidative stress or damage- could lead to interventions aimed at addressing, or preventing, these pathologies in the periphery, which may either contribute to predisposition for mental health disorders or result from the disorder as secondary pathology[23–25]. Regardless of the direction of causality, which is still a point of contention, especially between different disciplines in the health sciences, and given the high incidence of co-morbidities in neurological conditions such as PTSD, alleviation of cellular pathology in the periphery will no doubt contribute significantly and positively to longer term prognosis of the patient.

The thesis that follows aimed to firstly evaluate the peripheral, cellular profile associated with chronic psychological stress (specifically PTSD), in comparison with a cellular profile characteristic of peripheral tissue manifestation of unhealthy lifestyle (specifically obesity). The main focus was on dysregulation of redox profile and calcium signalling. It further aimed to highlight the need for an approach to mental health that rethinks the current treatment focus on central intervention, with relative neglect of the peripheral compartment. Finally, it will re-introduce a system that has remained largely overlooked in the human physiology and medicine niches - the trace amine signalling system - which has strong links to both mental health and redox status, and which may be targeted to alleviate/prevent some of the cellular abnormalities characteristic of PTSD and/or obesity.

For the purpose of this thesis, PTSD was chosen as the specific mental health paradigm in which to assess neurological disorder-associated peripheral changes, because PTSD a) has clear diagnostic criteria[26], b) has a known inflammation-redox signature[27,28] and peripheral component through hypothalamic-pituitary-adrenal-(HPA) axis involvement[29], and c) is characterised by chronic psychological symptoms.[30,31] Primary dermal fibroblasts were selected as the cell type of choice in which to evaluate peripheral changes of PTSD, because a) fibroblasts are robust tissue resident cells which participate in immune actions, tissue maintenance and contribute to the inflammatory and redox status of peripheral tissues[32,33] and b) fibroblasts have niche-specific functions and are susceptible to functional change through cell-cell communication systems[34–36].

The thesis is structured as follows: Chapter 2 presents a general overview of the most relevant literature, including a) a basic overview of relevant signalling systems, b) a brief review of allostasis and c) a brief discussion of the potential role of trace amines at the interface of inflammatory and redox dysregulation, both centrally and peripherally. Here, potential shortcomings of the current conceptual framework will be discussed, followed by evidence to support a redox-based argument for PTSD susceptibility and disease progression. Furthermore, evidence will be provided that suggest a possible link between co-morbidities of PTSD and other mental health disorders to peripheral inflammation and increased oxidative stress, in comparison to obesity as one of the most pressing co-morbidities in modernity. The hypothesis statement is provided at the end of chapter 2. Chapter 3 provides data illustrating the peripheral profile of allostasis in PTSD and/or obesity as observed in fibroblasts. Chapter 4 describes a (biological) drug screen in zebrafish, which aimed to identify a trace amine intervention with anxiolytic effect. Chapter 5 describes a cell culture study in human astrocytes probing potential top-down and bottom-up communication in PTSD and obesity, between the brain (astrocytes) and the periphery (fibroblasts), with consideration of the effect of a candidate anxiolytic trace amine on redox profile. Chapter 6 will present a final synopsis and recommendations for future work.

Chapter 2

Literature review

2.1 Introduction

Stress and anxiety are some of the leading causes of illness in the modern era[37] and have many systemic effects that are still being elucidated. PTSD is a disorder that is rooted in the *stress-and-fear* response and is associated with the dysregulation of the hypothalamic-pituitary-adrenocortical (HPA) axis[29].

From a clinical perspective, although pharmacological intervention is a necessary component of psychiatric treatment by facilitating the psychological space for behavioural intervention, current treatments are ineffective in many cases, or cause side effects due to off-target effects of drugs designed primarily to target the brain on a neurotransmitter level[38–40]. The history of psychopharmacology illuminates this point; many of the important drug discoveries were serendipitous, but – even more telling – the development of certain compounds allowed psychiatrists to delineate between illnesses or even substantiate the existence of a new one, such as was the case with depression[41]. It is important to acknowledge that the success of psychiatric treatment requires a balance between the practical need for treating patient symptoms and the theoretical understanding of the underlying neurobiology. An important aspect that is generating more interest is the intersection of peripheral effects on physiology in the context of neurological disease.

The genetic commonalities that underlie mental health disorders highlight the underlying genetic architecture of mental health disorders, potentially pointing to a shared molecular mechanism that results in pathology[18]. For example, there are primary effectors of the state of “schizophrenia” that is characterized by a dopaminergic signalling dysregulation. However, there are also effects that are related to the dysfunction of more generic and systemic physiological systems, such as calcium channel function, mitochondrial function, intercellular adhesion, and inflammasome activation[42–44]. This holds true for other mental health disorders as well, such as bi-polar disorder, major depressive disorder (MDD), and anxiety disorders, that each have specific characteristic neurobiological states (dysregulated neurotransmitter signalling), but also share underlying dysfunctions in calcium channel function, mitochondrial function, redox dysregulation, intercellular adhesion and inflammasome activation[6,45,46].

While the existence and role of inflammation on physiological systems is accepted within the context of mental health[6,47], the aetiological role of chronic low-grade inflammation and accompanying increases in oxidative stress, is receiving relatively less attention and is definitely not being addressed

as part of patient management. Thus, the role that these low level, cumulative deviations from normality may play in both disease progression and risk for co-morbidities, requires more elucidation.

Before introducing my hypothesis on allostasis in this context, and the requirement to target the whole body (i.e., the periphery as well as the brain) to effect beneficial long-term prognosis in the context of mental health, I will first provide a brief overview of the physiological systems in which dysregulation underpins PTSD.

2.2 Stress: the progression from an acute to chronic condition

2.2.1 Acute stress

Stress is a physiological response that readies the body for action, both physically and mentally. A stressor may be a physical or psychological cue, but the physiological response is essentially conserved and has two main characteristics: resources are prioritised and mobilized for movement and mental acuity, and mechanisms for damage repair are initiated. This is captured by the "fight-or-flight" response, where the well-known action of adrenaline and cortisol mediate the short- and longer-term responses to acute stress[48]. The physiological response to transient fear or excitement is also a form of the acute stress response. When the acute stress response is activated, the nervous system processes sensory input and mobilizes the body for action. The effects of the nervous system, hormonal signalling, and cellular signalling all coalesce into actionable orders for the brain and organism to respond to its environment. Acute stress can thus be seen as a short term response to environmental cues designed to ready the organism for actions it needs to take to counteract threats to homeostatic balance[49].

Central to the acute stress response is the activation of the amygdala which is involved in fear, anxiety, and reactional behaviour[50,51]. The amygdala stimulates the release of corticotropin releasing hormone (CRH) from the hypothalamus which in turn activates the hypothalamic-pituitary-adrenocortical axis (HPA) and eventually leads to the increase in cortisol associated with stress[49]. This response is transient in nature, as the increase in cortisol levels act as a negative feedback mechanism that inhibits the release of more CRH from the hypothalamic neurons decreasing the release of cortisol and returning the whole state back to equilibrium, which is the basis for cortisol homeostasis. Another important aspect of acute stress that infers adaptation, is the link to memory and learning that goes along with the activation of the hippocampus and prefrontal cortex by the amygdala, HPA activity, and cortisol. The activation of the prefrontal cortex and hippocampus in turn activates neuroplasticity mechanisms that stimulate neurogenesis from stem cells in the hippocampus, as well as increase dendritic and synaptic growth - all of which increases learning and improves future

anticipation[52]. These neurological and physiological changes are brought on because of intracellular and epigenetic changes under the influence of increased CRH and cortisol signalling[49].

Cellular homeostatic mechanisms are of particular interest as they form the basis of a cell's ability to adapt to changes in its environment. Calcium signalling systems for instance are affected by acute stress, as glucocorticoid receptor activation induces a calcium flux as well as upregulating calcium channel expression[53]. Furthermore, acute restraint was associated with a significant increase in intracellular calcium in mice, modulated calcium receptor action, and increased spontaneous glutamate release from cerebrocortical synaptosomes[54]. The redox state of the cell is another component that can change in response to acute stress. For example, acute restraint stress in rodents is associated with increased lipid peroxidation, increases in hydrogen peroxide and superoxide anion concentrations, and decreased free-radical scavenging ability[55,56]. The fact that these adverse redox changes have also been reported in non-mammals – in four species of Japanese quail (*Coturnix japonica*)[57] – indicates the high species conservation of cellular redox balance as vital responder to cellular stress. Indeed, one of the few human studies of acute stress, using the Trier social stress test[58], reported increased ferric reducing antioxidant power (FRAP), which is a measure of antioxidant capacity, indicating that acute stress might cause shifts in antioxidant capacity as a counter-mechanism[59].

2.2.2 Chronic stress

Chronic stress typically results from an environment that is continuously challenged by an ongoing stressor(s) that keep reinitializing the stress response that normally is transient in nature, or due to an (inappropriate) exacerbated stress response, or most devastatingly a combination of a stressful environment coupled with a maladaptive stress response[60–62]. Although the stress response is transiently adaptive in the short term, the unresolved demands of chronic stress eventually result in a cumulative overload that evolves into a maladaptation.

While acute stress is transient and resolved through homeostatic feedback mechanisms, chronic psychological stress is entirely maladaptive. Although the cues and molecular pathways may be the same, an acute stress response almost invariably leads to dysfunction if repeatedly induced, even at low levels[63]. Indeed, a validated model of restraint stress in rodents has shown that mild chronic stressors can lead to low grade systemic inflammation both at in circulation and at tissue (liver) level[64]. Increased levels of oxidative stress, depletion of endogenous antioxidant mechanisms and a pro-oxidative state are well documented in chronic stress literature[65].

The cause of dysfunction under conditions of chronic stress is commonly accepted to be the allostatic load of repeated stress events that are compounded over time and thus overloading the homeostatic counter-mechanisms normally sufficient to deal with acute stressors[66]. A well-known example of

allostasis in the context of chronic stress is downregulation of glucocorticoid receptors in the chronic presence of elevated cortisol[29,66]. The resultant decreased efficacy of cortisol signalling then leads firstly to an increase in CRH release (due to the lack of negative feedback), which exacerbates the increase in cortisol secretion and eventually results in adrenal burnout, which is evident in depression. This glucocorticoid resistance and the resultant inability of cortisol to down-regulate the inflammatory response as a negative feedback mechanism, results in the relatively pro-inflammatory phenotype – and associated interlinked dysregulated redox profile – known as accelerated ageing, or allostasis[67,68].

Chronic stress can have severe effects on physiology and has been linked to several life-threatening conditions such as depression, cardiovascular disease (CVD), increased susceptibility to infectious diseases and cancer[69]. Individual differences in how the brain and body react to stress are being linked to accelerated aging and disease pathogenesis[65,70–72]. The overlap in conditions that have a component of chronic stress is centred around systemic pathways that have a connection to inflammation. They are also linked through mechanisms of energy metabolism and thus mitochondrial function. Mitochondria are the largest contributors to oxidative stress in cells, as one of the by-products of cellular respiration is reactive oxygen species (ROS), which can contribute to cellular degradation when chronically elevated beyond the countering capacity of endogenous antioxidant defences[73]. Increased ROS production is a hallmark of chronic stress disorders and is the primary effector thought to drive cellular and/or oxidative damage in disease aetiology and/or progression[74,75]. Much interest has been given to mitochondrial function, particularly in the context of disorders that have a stress component. However, the primacy of mitochondria in the general function of cells has led to an evolutionarily complex control mechanism that achieves a level of redundancy in the function of mitochondria that obfuscates potential mechanisms of dysfunction that may be at play. Mitochondria are mobile within the cell and migrate with precision to areas they are needed to facilitate a host of intra- and intercellular mechanisms[75]. Thus, dysregulation at this level is certain to result in a departure from normality.

Communication between various systems is predominantly achieved through mechanisms that affect calcium concentration within the cell[76] (Figure 2.1). The link between energy metabolism, inflammation and ROS production has a strong component of calcium signalling. Under conditions of chronic stress calcium signalling becomes dysfunctional, which has been cited as a potential mechanism or driver of mitochondrial dysfunction[75–77]. It is apparent that a deep and meaningful connection exists between the different components of cellular metabolism and respiration that are affected by dysregulation within chronic stress and inflammation. Molecular pathways such as genetic transcription pathways[78], metabolic signalling pathways[74,79], hormonal axis[80], brain signalling pathways[48], etc., can all be affected by chronic stress; prolonged activation of chronic stress pathways in turn leads to allostatic load. Allostasis has been identified as a (mal)adaptation of the body to

physiological and environmental cues, that changes internal states in an attempt to better adapt to these cues[81]. Over time, excessive allostatic cues brought about by chronic stress, increases the allostatic load on a system which has a capacity to adapt, but which gradually falls into a maladaptive physiological state[61].

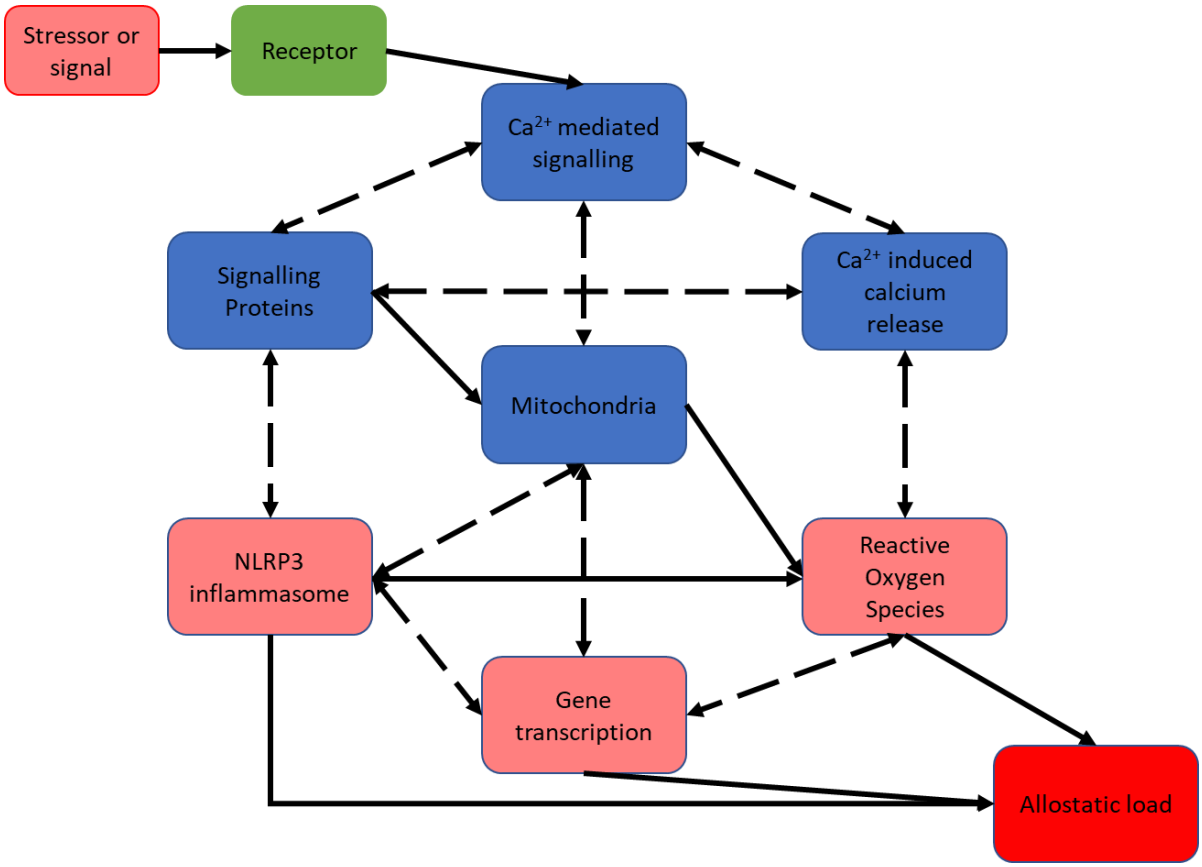


Figure 2.1. Schematic of the important longitudinal role players in allostatic load. Pink indicated the most direct contribution to allostatic load; blue is the most direct effectors of calcium signalling. Dashed lines indicate a two-way communication, whereas solid lines indicate a more unidirectional signalling.

2.2.3 Redox at the end of stress related dysfunction

ROS is a natural by-product of cellular respiration and is an important part of the redox signalling pathway. The main source of ROS in mammalian cells are mitochondria and the electron transport chain,[82] as well as NADPH (Nicotinamide adenine dinucleotide phosphate) oxidase (NOX)-mediated ROS release[83]. All alternate sources of ROS – such as xanthine oxidase (XO), lipo- and cyclo-oxygenase, cytochromes P450 (endoplasmic reticulum) and peroxisomes – have some endpoint in signalling metabolic need or activity, mostly through reversable protein modification via oxidation; however, high levels of ROS can cause irreversible protein modifications and cause permanent malfunction of proteins[84].

Although the role of oxidative stress and ROS has been of interest in the context of neurodegeneration[85], much less attention has been given to the contribution of redox in mental health disorders. Elevated ROS levels are associated with chronic stress, the activation of adaptive mechanisms, such as transcriptive changes, regulatory protein changes, and the change in mitochondrial regulation that have a profound effect on ROS production, which – as a result of oxidative damage - has the knock-on effect of increasing inflammation in a forward feeding loop[73].

Elevated ROS levels have been proposed to not always be detrimental, as *Mclk1* (a mitochondrial hydroxylase necessary for ubiquinone synthesis) (+/-) mutant mice – characterized by increased mitochondrial ROS production – show increased capacity for inflammatory reaction to pathogens and decreased susceptibility to tissue damage associated with inflammation in the short term[86]. It should be noted however that increased ROS levels do increase the inflammatory response of cells and will eventually lead to increased cytokine production and feed into the forward feeding loop mentioned above[87,88]. Thus, a different scenario may be evident over a longer time course, in these mutant mice.

Increased inflammation and ROS, as well as mitochondrial dysregulation, all influence calcium signalling and homeostasis[77,89–91]. This is a particularly complex system in which to address dysregulation, because calcium is firstly a ubiquitous signalling molecule used by a variety of inter- and intracellular proteins and organelles, making it difficult to tease apart differences in calcium function in complex systems. Secondly, it is not always clear whether the calcium dysregulation occurs prior to the dysfunction of the other systems. For instance, there are polymorphisms in the calcium signalling pathway, including genes coding for calcium channels and receptors, that suggest an *a priori* role for calcium in disease pathology[92]. Upregulation of inflammation has detrimental effects systemically, which may be ascribed largely to the production of increased products of respiration, that puts pressure on the mechanisms that are supposed to clear them. The antioxidant capacity of cells is energy dependent, which means a system that is overloaded, overworked, chronically active, or chronically stimulated, will have an increased energy demand, which over time diminishes the antioxidant capacity[91] and results in oxidative damage and cellular dysfunction.

In the next section, the profile of allostasis in the context of PTSD, will be presented.

2.3 Post traumatic stress disorder (PTSD)

PTSD is most readily characterised as an excessive fear and anxiety response following a trauma, with recurring intrusive thoughts related to the experience. The Diagnostic and Statistical Manual of Mental Disorders (5th ed.; DSM–5) diagnostic criterion for PTSD includes eight criteria for the diagnosis of PTSD, which include exposure to life-threatening events; re-experience of the event through memories, dreams, and dissociative states; distress at exposure to situations that resemble the

trauma; avoidance of trauma related events and experiences; alterations of mood and cognition; alterations in arousal and reactivity associated with the trauma. Within these criteria are subcategories that include variable symptoms, from the expected anxiety and fear responses, to anhedonia and dysphoric symptoms[93].

Distress experienced following trauma is highly variable. In many instances fear and anxiety is the context that most readily explains symptoms and the phenotypic clinical presentation. However, in some instances the clinical presentation is that of depressive behaviour such as anhedonia, externalizing aggressive behaviour, or even dissociative symptoms. Although the phenotype of clinical presentation may be variable, the constant that distinguishes PTSD from other trauma related disorders is the development of symptoms following exposure to one or more traumatic events, so that a clear primary, external, trigger for psychological pathology can be identified.

In the United States it was estimated that approximately 90% of individuals will be exposed to some kind of traumatic event during their lifetime with multiple exposures being the norm[94]. Lifetime PTSD prevalence was determined to be roughly 7%, with a definitive gender bias - US women seemed twice as likely to develop PTSD (9,7% vs. 3,6% for males)[95]. A recent meta-analysis of sub-Saharan African (SSA) countries had a pooled estimate prevalence rate for PTSD of 22%. This high value was due to the large portion of SSA countries in the analyses that had experienced war and armed conflict and who had a pooled prevalence rate for PTSD of 30%. The four non-exposed countries included South Africa, and their pooled prevalence for PTSD was 8%[96], which is in line with US statistics.

2.3.1 Physiological dysregulation in PTSD

From a physiological perspective, the classical fear and anxiety phenotype of PTSD is associated with increased activation of the amygdala, which accounts for the excessive fear response, as well as decreased activation of cortical neurons, which account for the intrusive thoughts and mood disturbances seen in PTSD[93,97,98]. Finally, the continuous, inappropriate activation of the HPA-axis is also a major contributor to the maladaptation to stress in PTSD[29].

HPA-axis over-activation has been thought to be the primary driver of PTSD symptomology in terms of the global effect of PTSD on physiology. The decrease in hippocampal volume found in chronic stress and PTSD cohorts has been interpreted as a decrease in the normal inhibitory function of the hippocampus on the HPA-axis[97]. This response should reflect initial findings in depression literature of increased cortisol levels in patients vs. controls which were attributed to increased HPA-axis activation[99]. Subsequently, MDD has been shown to have a more complex cortisol secretion pattern that is related to subtype and severity of depression[100]. In PTSD however, there has been some controversy in this context, as the expected elevated basal cortisol is often not found - instead,

evidence suggests that there is a glucocorticoid receptor (GR) sensitization to cortisol, which may explain the decreased levels often found in PTSD, in contrast to the expected higher levels normally found in conjunction with decreased hippocampal activity[101,102]. Given its metabolic role, cortisol levels may also not be the ideal measure for evaluating HPA-axis dysregulation or stress response in a cross-sectional study design, which may at least in part, contribute to the contradicting data reported in this context. However, assessment of cortisol may still have prognostic value. For example, in a recent study involving South African women with and without PTSD, there was a significant association between PTSD severity and hair cortisol concentration[24]. This suggests that in PTSD, a chronically exacerbated cortisol response occurs.

Cortisol, the primary glucocorticoid (GC) of the human body, binds to both GRs and mineralocorticoid receptors (MRs)[29]. Under conditions of minimal stress cortisol binds to MRs more readily due to their higher affinity for cortisol[103]. However, during an increased stress response - and thus cortisol release – the MR is saturated, leading to a greater extent of GR binding and activation by the abundant cortisol[29]. GRs are primarily cytosolic, where the main function is the mediation of slow genomic effects of GC. The GR form protein complexes that interact with cortisol within the cytosol forming homodimers that translocate to the nucleus and initiate the transcription of specific genes[104]. Interestingly, the sensitivity of the GR complex is altered depending on which chaperone molecules and co-chaperone molecules are present. In particular, the addition of FK506 binding protein 5 (FKBP5) lowers the affinity of the GR complex for cortisol, which has led to great interest in FKBP5 as a potential component and thus biomarker of GC sensitivity observed in PTSD[78,105]. Given the plasticity of cortisol and GR responses especially early in PTSD, FKBP5 may therefore be a more accurate marker of allostatic load (and thus longer-term prognosis) in this context.

GC dysregulation have been associated with increased oxidative stress and inflammation, due in part to the reciprocal relationship between the GR signalling and inflammation[31,106]. Oxidative stress is thought to be generally increased in response to both inflammation and elevated GC levels[106], however a more precise answer is a bidirectional causal relationship, both due to the reciprocal relationship mentioned above and the relationship between oxidative damage and inflammation[87]. Although there is a relative paucity in studies investigating ROS mechanisms in the context of PTSD, some studies have reported on relative levels of oxidants and antioxidants in PTSD vs controls. One investigation of patients who experienced the same traumatic event reported downregulated gene expression profiles for thioredoxin reductase and superoxide dismutase (SOD)[107] and another study reported elevated levels of lipid peroxidation and depletion of antioxidant enzymes in earthquake survivors with PTSD compared to survivors without PTSD[108]. Levels of antioxidant SOD and glutathione were also reported to be lower in Croatian war veterans with PTSD compared to veterans without PTSD[109]. Taken together these studies indicate an increase in the relative oxidative stress

levels of PTSD patients and/or a depletion in the mechanisms that clear oxidative factors. A lot more can be inferred about the deleterious effect of ROS through inflammatory based studies in PTSD patients and models[110–113], considering that ROS production is a large consequence of inflammation. This and the transient nature of the inflammatory signalling system makes redox signalling a better candidate for assessing allostatic load in PTSD.

2.3.2 Co-morbidities of PTSD

According to the DSM-5, individuals with PTSD are 80% more likely than others to have symptoms of at least one other mental health disorder[93]. Furthermore, evidence from association studies, animal models, GWAS findings, and clinical prevalence, suggest high incidence of "diseases of lifestyle" co-morbidities, such as obesity[114], chronic pain[115], inflammation[113,116], cardiac dysfunction[117], and potentially gut microbiome dysfunction[116]. Based on the evidence presented here there is a strong rationale for understanding co-morbidities of PTSD as a consequence of increased inflammatory phenotype (and thus oxidative stress/damage), which has been implicated in every instance of co-morbidity with PTSD, ranging from metabolic (gastrointestinal disease[118,119], obesity[119,120], cardiovascular disease[121]) to psychological (depression[122] and anxiety[123,124]) conditions.

Of particular interest is obesity, which is a global chronic health disorder and a potential confounder in the context of inflammation as a pathogenic driver of PTSD. Obesity has been well established to be associated with a pro-oxidant and pro-inflammatory phenotype. Interestingly there have been some studies that have found a paradoxical improvement in prognosis of cardiovascular disease in obese and overweight individuals[125,126]. The exact reason for this paradox is unknown, although some have suggested statistical and methodological error, such as incorrect BMI classification, are the main reasons for confusion. However, there may also be evidence of obesity subtypes that have different disease outcomes[127]. While this paradox still requires an explanation it does raise the question about the source of inflammation and oxidative stress and how this contributes to pathogenesis. Adipose tissue (AT) is considered an endocrine organ and two principle (and functionally different) types of AT are present in the human body, namely brown adipose tissue (BAT) and white adipose tissue (WAT). WAT in turn is subdivided into subcutaneous and visceral AT[127]. The composition and distribution of AT may be one of the reasons for the obesity paradox, as some individuals who are obese do not have metabolic syndrome and other normal weight individuals show symptoms of metabolic syndrome[128]. The existence of this paradox and the subsequent obesity phenotypes speak to a complex, but potentially independent mechanism of inflammation and oxidant homeostasis.

The main premise is that low-grade chronic activation of the HPA-axis, inflammation, or adipocyte signalling through adipokines, result in a feedback mechanism that increases CRH and cortisol levels, which increases the release of pro-inflammatory cytokines and adipokines. These in turn should decrease the release of CRH and cortisol, but due to the continuous activation there is an allostatic

modification of glucocorticoid receptor density and sensitivity, as well as an increase in corticosteroid binding globulin (CBG) and modulation of 11β-hydroxysteroid dehydrogenase, resulting in what is termed glucocorticoid resistance[106,129](Fig 2.2). Furthermore, free fatty acids (FFA) are natural uncouplers of mitochondria. Uncoupling in mitochondria is the process of proton leakage from the electron transport chain[76]. FFA increase uncoupling and uncoupling increases ROS production, which provides a direct mechanism by which obesity can disrupt the redox state of an individual. Increased overall ROS could also increase inflammation through upregulation of proinflammatory signalling pathways.

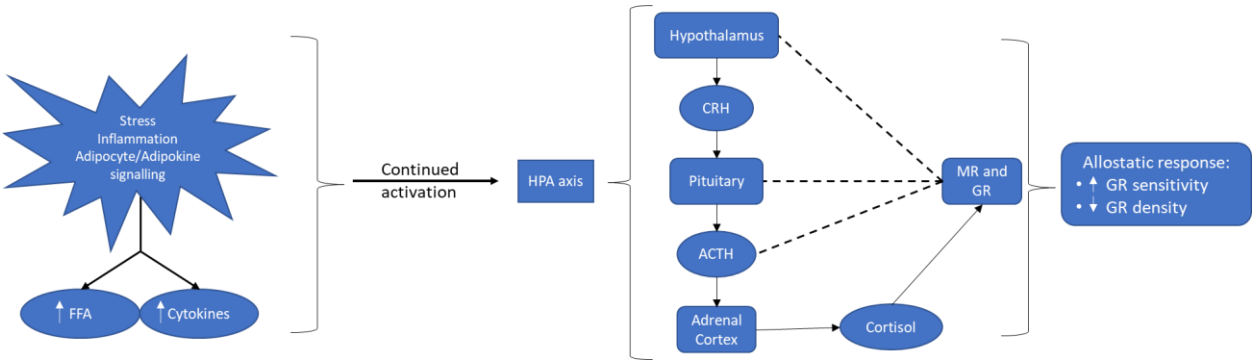


Figure 2.2. Complex interaction of obesity and stress pathways contributing to HPA-axis and glucocorticoid resistance. Dashed lines indicate negative feedback. Solid arrows indicate release, increase, or stimulation. FFA – free fatty acids; HPA-axis – hypothalamic pituitary adrenal axis; CRH – corticotropin releasing hormone; ACTH – adreno-corticotropin releasing hormone; MR – mineralocorticoid receptor; GR – glucocorticoid receptor.

2.3.3 Addressing the problem using the first principles of physiology

In an attempt to reconceptualise evolutionary theory from a cellular perspective, Torday and colleagues have argued for homeostasis as the mechanism of evolution[130–133]. The cellular approach and focus on homeostasis allow for first principles to emerge that may have predictive power for disease progression especially for chronic conditions[132]. The first principles that emerge from this reasoning are negentropy and chemiosmosis as the driving forces behind the homeostatic balance and evolution. Negentropy is the decrease in entropy that is characteristic of living systems - however, this process is energy dependent and primarily achieved in living systems through the process of chemiosmosis[131,134,135]. Collectively this could be termed the First Principles of Physiology (FPP) by which the integration of physiology on a systems level can be evaluated.

An approach using the FPP could be applied to redox signalling under chronic conditions. In chronic conditions the role of inflammation is normally the research focus for understanding dysfunction, while ROS is often merely considered an outcome, or symptom, of this dysfunction[124]. One challenge in understanding inflammatory signalling dynamics is the transient nature of inflammatory measures. The absolute levels of IL-1β, C-reactive protein (CRP), or NFκB may be measured as indicators of inflammation, but they must be interpreted along with the anti-inflammatory proteins and signals

responsible for inflammasome activation and the inflammatory signalling pathway. From a FPP perspective, a prediction can be made regarding the impact of chronic inflammatory signalling to change the chemiosmotic properties of the system over time, as well as impacting the negentropy of the system over time, all of which will result in a shift of the homeostatic balance, however the shift in homeostasis is more likely due to redox signalling than inflammatory signalling. In this context, measuring more stable homeostatic controlled outcomes, such as the oxidative state or signalling capability, become better proxies for the state of cell and/or system function.

From the cellular perspective the need to abstract and approximate function to measurable outcomes is necessary to untangle the complex nature of cellular function and how it contributes to disease, but from a systems level perspective, the abstracted version of disorders leaves only symptoms to measure and address instead of causes. Consideration of the FPP in combination with a single cell perspective of evolutionary pressures may provide novel insights into the dynamics at play in a complex disorder such as PTSD. The low-grade inflammation and increased ROS associated with PTSD serves to reset the homeostatic mechanisms that feed forward into pathology over time. Although the primary cause of the inflammatory shift can and must still be determined, the argument put forth in this thesis is one for immediate prominence of inflammatory mechanism, driven by changes in redox signalling, as the drivers of chronic symptoms of disease, if not the most important driver of all chronic conditions. Inflammation however is insufficient to explain the specificity of the aetiological progression towards manifestation of specific disorders, which means there must be other more specific factors that contribute to the aetiology of mental health disorders. For this reason, the focus in this thesis is redox signalling, which is active at low concentrations of ROS as vital to the homeostasis of metabolism, but also as the first signalling response to damage and potentially dysregulation[82,85]. PTSD is a good disease model in which to explore these distinctions because it has the underlying inflammatory and redox component as well as a specific trigger event, providing a model that has both chronic and acute aspects.

In summary, it is clear that the interlinked, self-perpetuating processes of inflammation and oxidative stress are central to the aetiology of complex neurological disorders such as PTSD. However, given the necessary plasticity and variability of these processes as part of one of the body's major regulatory systems, small allostatic changes are unlikely to be detectable in the "noise" of this variability, and perhaps even more difficult to target therapeutically. I have identified a number of more specific markers of allostasis to evaluate in the context of PTSD, and which may be assessed in the periphery. Using fibroblasts as representative peripheral cell, I will discuss these markers in the next section.

2.4 Peripheral profile of allostasis: identification of potential biomarkers

The effect of PTSD on cells in the periphery has only been focused on in immune cells[23,136,137], although there are lines of logical evidence from related research suggesting that complex disorders of the mind may affect both neuronal and somatic cells. Following on from the first principles of physiology (FPP), there should (at least) be a continuation of the physiological effects of PTSD related to the chemiosmotic and negentropic changes under chronic inflammation associated with PTSD[47] and (as a confounder) obesity[120].

In this section, I will provide a review of the literature which led me to select fibroblasts as proxy model for peripheral allostasis in PTSD (i.e., an allostatic stimulus of primarily neurological origin) in comparison to obesity (i.e., a peripheral allostatic stimulus).

2.4.1 Fibroblast characterization and heterogeneity

Fibroblasts are specialised cells of mesenchymal origin. They reside in skin, muscle, bone, and some specialised tissues such as the lungs, sex organs, heart, blood vessels, and the blood brain barrier - essentially in all mesenchymal tissues. Their primary function is the generation and maintenance of the extracellular matrix (ECM) and in this way, they play a critical role in how tissues are spatially arranged[34]. Apart from their primary function of ECM maintenance, fibroblasts also play an important role in cellular communication within the tissue they reside in and are therefore susceptible to pathological changes in the micro-environment. Not only do they communicate with native cells through direct cell-cell interaction, but also with more distant cells through paracrine signalling facilitated by cellular secretory products such as TGF- β, IL-6, and IL-8[138]. The signalling action of the surrounding native cells also affect the fibroblasts themselves - studies using co-culture of fibroblasts and keratinocytes have shown that keratinocyte derived IL-1 stimulated fibroblasts to produce fibroblast growth factor 10 (FGF-10), IL-6, and granulocyte macrophage colony stimulating factor (GM-CSF)[139].

Fibroblasts are characterized by the expression of markers such as α-smooth muscle actin (α-SMA) and vimentin[140]. There is still some difficulty in describing fibroblast-specific markers, as many of the classically considered fibroblast markers are either also found on other cell types, are not expressed on all fibroblast subtypes, or are markers for cellular differentiation in specific contexts[34,36,141]. Further complicating matters is the difference in cell surface expression markers for murine cells compared to human cells[36]. LeBleu et al (2020) has an excellent infographic depicting the overlap between cell surface markers for fibroblast and many other cell types such as neurons, immune cells, epithelial cells, adipocytes, and others[34]. They included the classic fibroblast markers such as vimentin and αSMA, but also list many other markers that have been used to classify fibroblasts such as PECAM/CD31 (platelet epithelial cell adhesion molecule), CD45, FSP1/S100A4 (fibroblast-specific protein 1), and FAP (fibroblast activating protein) among others. Despite the lack of fibroblasts

specificity, enrichment for these markers could well be indicative of fibroblasts[140] and modern protein expression techniques and profiles could be leveraged here. The complexity of fibroblast origin, molecular markers and function stem from heterogeneity of fibroblasts. Originally, fibroblasts were thought to be of a homogenous origin through embryonic development from the yolk sac and a homozygous function of ECM homeostasis[142]. However, more recently, the existence of fibroblasts and fibroblast-like cells that originate from epithelial-to-mesenchymal transition and other types of cell transitions (endothelial, adipocytes, and perivascular) has been reported[34,143]. Furthermore, these tissue-derived resident fibroblasts have heterogenous functions in the tissue niche they reside in. Although there is overlap in terms of their general function in inflammation activation and resolution, fibrosis, wound healing, angiogenesis, and cell-cell communication, there are differences between pathways activated, cell surface markers, and activation signals.

In terms of selection of fibroblast sampling, it is of importance to note than fibroblasts of the dermis – although readily accessible from dermal biopsies - are heterogenous in terms of function, morphology, and protein expression profiles. There is a distinct difference between fibroblast populations of the papillary dermis, the reticular dermis, as well as hair follicle fibroblasts. These include differences in collagen expression (both in expression levels of individual collagen types and in terms of relative distribution of collagen types), proliferation rate (faster for papillary fibroblasts), matrix metalloproteinase expression (MMP), α-smooth muscle actin (α-SMA), TGF- β expression and general cellular morphology[144–146]. These differences are thought to play an important role in the determination of fibroblast identity[36,147] and highlights the importance of standardisation of sampling procedure and biopsy site for this type of investigation.

Fibroblasts seem to have intrinsic mechanisms that determine their identity that is conditioned or set by the origin tissue factors, such as the ECM constituents, cell-cell signalling mechanisms, and soluble signalling mediators. This intrinsic mechanism is partially contributed to by the embryonic origin of the fibroblast and partially by the tissue and microenvironment that the fibroblast resides in[142]. For example, heterotopic transplantation experiments have shown that fibroblast identity is conserved through intrinsic mechanisms. Fibroblasts from human palms and soles were able to induce expression of keratin 9 in keratinocytes, whereas fibroblasts form non-palmoplantar sites were unable to induce said expression of keratin 9[148].

Although fibroblasts seem able to retain the function or molecular memory of its original tissue niche when cultured in isolation, it is ultimately the niche or the extrinsic environment of that fibroblast that governs the cellular identity (and function) of that fibroblast not the intrinsic function of fibroblasts themselves. Cell-cell contact and communication lead to specific secreted factors, signalling molecules, and proteins that make up the ECM and cellular environment. It has been shown that fibroblasts cultured in monolayer have different morphology and collagen expression levels than

fibroblasts in three-dimensional culture[149]. Interestingly, there seems to be a differential response to signalling factors such as TNF-α and IL1-β in fibroblasts cultured in monolayer vs fibroblasts cultured in three-dimensional culture[139,150], which also needs to be considered in study design.

There is some evidence that the functional heterogeneity and plasticity differences seen between fibroblasts of different origin, may function through epigenetic mechanisms and epigenetic memory[151,152]. This is interesting - considering the role that allostasis plays in the activation of epigenetic mechanisms - and may further support the use of fibroblasts as model tissue for investigation of global functional changes to physiological state under conditions of chronic psychological stress. Of particular relevance to the current topic, epigenesis is a consequence of allostatic mechanisms, and studies have shown that under certain conditions fibroblasts can be activated to perpetuate an inflammatory state even after the resolution of the primary stimulus[153].

2.4.2 Fibroblast function

From existing literature, the primary role of fibroblast in regulating ECM composition and maintenance has been well established. The role of fibrosis in disease is still under investigation but gaining momentum especially in the context of chronic inflammatory conditions such as cardiovascular conditions. For example, the increased inflammatory load in heart disease has been cited as a major role player in inducing increased fibrosis through collagen deposition changes associated with fibroblasts[143,154]. In the heart, the ratio of fibroblast to myoblast is higher than in any other organ, making it particularly susceptible to inflammatory changes and fibrosis. Critically, cardiac fibrosis can mean both an increase in ECM proteins and a disruption or qualitative change to composition of ECM proteins. The exact function of fibroblasts in the context of increased inflammation needs to be elucidated to make progress in untangling the nature of its role in disease context. Cardiac remodelling in rodent models have shown both negative effect of inflammatory cytokines in terms of increasing the overall fibrotic content of the ECM as well as changing the composition of the ECM. Changes in the composition of the ECM proteins can lead to deficits in crosslinking of collagen fibres in the ECM due to increases in MMP action and decreased TIMP function. Studies in cell lines have also presented compelling evidence suggesting that both inflammation and oxidative damage can increase fibrosis. Interfering RNA targeted at *Nox4* in rats inhibited TGF-β1 stimulated NADPH oxidase and alleviated subsequent fibrosis from activated kidney myofibroblasts[155]. Fibrocyte activation and recruitment is increased following increased redox signalling in bone and nephrotic cells[156,157]. Specifically relevant to MMP function, M2 macrophages have been shown to increase the expression of profibrotic factors from human dermal fibroblasts (hDF) such as increased collagen, increased cell proliferation of hDF, and decreased activity of MMP's[158]. Another study investigating cyclosporine effects on (cell type) in terms of MMP and TIMP, found both increases and decreases in subtypes of MMP and TIMP, which were dependant on

whether adult or neonatal cell lines were used[159]. These examples serve to illustrate the need to understand the binary inflammatory/anti-inflammatory classification of components like TIMP and MMP, as well as other classically inflammatory role players like TNF-α and IL-6, as being part of a holistic inflammasome function that is temporally and spatially resolved. This means that the function of a signalling protein or protein complex is subject to the context it functions in and that that context is generated through the complex interaction of the inflammasome and the cellular environment in the case of inflammation.

Despite the knowledge that obesity increases profibrotic signalling, there is a relative paucity of specific information pertaining to the role of fibroblasts in adipose tissue fibrosis[160]. Although the impact of obesity is acknowledged in many health contexts, primarily as a consequence of the increased oxidative stress and inflammatory load imposed on the physiological system, the specific contributions are still to be elucidated. This is perhaps due to the variety of signalling paradigms that intersect with inflammatory and oxidative stress pathways, making it difficult to tease apart specific dynamics from the more global effects. This is a particular issue for obesity, which is a quintessential systemic contributor to longer-term health outcomes. It is interesting that literature on the topic of the contribution of obesity to pathogenesis is limited to the general longitudinal effects of the proinflammatory and oxidative stress factors. More specific elucidation of obesity-linked effects, as well as potential heterogeneity in terms of responses effected by adipocytes from different tissue compartments, is critical to understanding the mechanism(s) by which obesity may affect fibroblasts more directly. Indeed, in a study of macrophage activation from M_0 to M_1 phenotype, it was found that adipose tissue macrophages showed none of the M_1 activation markers used in the study but did produce proinflammatory cytokines through a differential mechanism and/or different cell source[161].

2.4.3 Potential markers of allostasis in fibroblasts

Since there is no real consensus in literature about which markers are fibroblast specific, and since sample collection in the work presented here was standardised (i.e. negating the issue of niche heterogeneity), the approach for this review was not to evaluate fibroblast phenotypes, but rather to select markers expressed by fibroblasts, that are potentially modulated in PTSD and obesity and as such, may be used to reflect allostatic conditioning of non-neural tissue in the periphery.

Matrix metalloproteinases (MMPs) and Tissue inhibitors of metalloproteinases (TIMPs)

The ECM is made up of a multitude of molecules such as structural proteins (e.g., collagen and elastin), adhesion proteins (e.g., fibronectin), proteoglycans, and metalloproteases (e.g., MMP-3 and MMP-9)[162]. MMPs are a family of endopeptides that are responsible for the degradation and remodelling of the ECM. This family is made up of diverse membrane-type matrix metalloproteases such as collagenases, gelatinases, stromelysins, and matrilysins that each contributes to the

remodelling of a specific component of the ECM and other structural components. The functions of MMPs are inhibited by tissue inhibitors of MMPs (TIMPs), which are endogenous regulators of proteases. TIMPs inhibit MMPs through forming reversable stoichiometric complexes and show selectivity to specific MMPs and disintegrins[163].

The functions of MMPs and TIMPs are well described in the context of their primary roles in ECM regulation; however, the function and response of these elements are not yet well understood in other contexts. Inflammation has been shown to influence the function of both MMPs and TIMPs and this has been extended to include conditions of increased systemic inflammation and metabolic dysregulation such as obesity[164,165]. In general, the activity of both MMPs and TIMPs are increased in response to inflammation, where MMPs are activated in response to proinflammatory signalling by IL-1, IL-6 and TNF-α and TIMP activity is increased through the increase in MMP activity. Interestingly, the increase in MMP activity has an impact on BBB permeability[166] and could be one of the effector pathways for increased BBB permeability in response to inflammation. With regard to oxidative stress and MMP function, the general trend in literature is to ascribe increases in ROS as part of the inflammatory role of MMPs, however mitochondrial ROS may be implicated in the molecular control mechanisms of MMP function. For example, MMPs respond to mitogen-activated protein kinase (MAPK) signalling cascades (among others), of which some subtypes of MAPK are sensitive to ROS[167]. In rodent ventricular myocytes the stress-activated protein kinases/c-Jun N-terminal kinases (SAPKs/JNKs), the extracellularly responsive kinases (ERKs) and p38-MAPK were activated in response to oxidative stress[168]. Furthermore, the activity of human neutrophil collagenase was effectively increased by hypochlorous acid and hydrogen peroxide administration[169]. More recent work has reported MMP mediated neurotoxicity in zebrafish treated with high glucose levels, which is initiated by ROS induced MMP-13 activity[170].

A review of the specific function of each MMP and TIMP is beyond the scope of this thesis. However, MMP-3, MMP-9 and TIMP-2 are of particular relevance to the current topic and will be briefly introduced in the next sections.

MMP-3 plays a critical role in the maintenance of the ECM and is often cited along with MMP-9 as a major role player[171]. MMP-3 is thought to be a major contributor to the inflammatory dysregulation found in systemic inflammatory disorders such as atherosclerosis and arthritis, most likely due to the dysregulation of the ECM[172–174].

MMP-3 has also been reported to be increased under conditions of LPS-induced inflammation, suggestive of a role for MMP-3 in microbial or oxidative stress-related inflammatory events[175,176]. Of relevance to the context of PTSD, is the role that MMP-3 plays in the activation of microglia in response to inflammation. Microglial activation is implicated in PTSD pathogenesis, as microglial

function can alter fear memory circuitry through inflammatory signalling mediators such as TNF-α, and IL-1β, as well as neurotropic factors such as BDNF. Microglia are the main effectors of neuroinflammation; however it is still unknown how stress increases or decreases microglial inflammatory mediator release[110]. A recent study employing a murine model of PTSD showed an increase in microglial number and ratio to immune cells in response to foot-shock exposure, as well as increased microglial activity in the hippocampus, which may suggest an upregulation of MMP-3 in chronic severe stress conditions[177]. Indeed, data from animal models have demonstrated induction of some phenotypes of PTSD through LPS (and thus MMP-3 upregulation) mediated inflammation[178–180].

Furthermore, MMP-3 expression by endothelial cells in capillary blood vessels is capable of activating microglia in both an *in vitro* (BV2 cells) and *in vivo* (MMP-3 KO mice) setting[181]. Given the prominence of endothelial dysfunction not only in chronic conditions with an inflammatory component, but also in neurodegenerative conditions, MMP-3 is likely to be a valuable parameter in PTSD as well. In further support, MMP-3 also affects the permeability of the BBB: in MMP-3 KO vs WT mice, the upregulation of MMP-3 was associated with an increase in the permeability of the BBB[182]. BBB integrity and function has already been discussed as critical for normal brain and neuroinflammation, which may be dysregulated in PTSD. Taken together, MMP-3 is potentially modulated in PTSD and contributes to inflammatory mediated dynamics that contributes to pathogenesis.

MMP-9 is one of the most studied MMPs and apart from its function in ECM homeostasis, it also has an important function in the CNS where it plays a role in neurogenesis, axonal growth, and myelin formation[162]. MMP-9 expression is increased in response to inflammation and is linked to the increased permeability of the BBB[166]. MMP-9 is increasingly implicated in the disease progression of depression, highlighting the role of inflammation in depressive behaviour, and therefore also potentially in PTSD. In one study of recurring depression in young adults and older patients, there was a significant association between MMP-9 polymorphisms and disease outcome[183]. MMP-9 has also been implicated in the neurodegeneration associated with schizophrenia, bi-polar disorder, and major depressive disorder; however, there is still some scarcity in literature with regard to PTSD[184]. Nevertheless, in a recent study comparing MMP-9 mRNA levels in humans with PTSD and a rodent PTSD model (single foot-shock)[185] reported elevated MMP-9 mRNA after acute dexamethasone treatment, which decreased when PTSD patients were in partial remission. In the rodents however, elevated levels of prefrontal cortex MMP-9 mRNA were reported. This warrants more research to elucidate the potential of MMP-9 as disease marker in PTSD.

The prominent feature of MMP-9 in literature on other neurological conditions indicates the importance of also understanding the role of TIMPs (as the predominant inhibitors of MMP-9) in the context of inflammation as confounder in neurological pathology.

TIMP-2 also has affinity for MMP-9 but is less well researched in terms of its function, especially in relation to MMP-9. Recently however, a whole exome sequencing (WES) study found a rare *TIMP-2* variation in one family with history of schizophrenia[186], while a study on major depression in monozygotic twins with and without major depression (MD), showed enrichment in biological processes and cellular function for differentially methylated genes, one of which was *TIMP-2*[187].

In terms of a potential role for TIMPs in fibroblast functionality, very little is known apart from the prominent role for TIMPs in regulating the ECM changes associated with cancer and cancer metastasis. In general, the ratio of MMPs to TIMPs is an important factor for the maintenance of the ECM, because skewed MMP action would degrade the ECM or disrupt ECM architecture leading to deficits in inflammation response, cell to cell communication, and homeostatic imbalance[188,189]. In this context, TIMP-1 is the most studied TIMP, due to MMP-9 being its primary target. However, TIMP-2 has affinity for both MMP-3 and MMP-9 (albeit not to the same extent as TIMP-1 for MMP-9)[162], which potentially makes TIMP-2 a better choice for inclusion in research into neurological disorders.

Fibronectin type III domain-containing protein 5 (FNDC5) is a transmembrane protein that increases secretion of irisin, a cleaved component of FNDC5, in response to exercise (irisin is thought to induce the transition of white adipose tissue to the more metabolically favourably brown adipose tissue). Although the cleavage of irisin from full length FNDC5 is thought to be the primary way FNDC5 contributes to metabolism, the only evidence for this cleavage is from the single original study[190]. Subsequently, cleavage of some type was shown in mice, but the precise cleavage site and nature was not elucidated[191]. There is a difference between the cleavage or activation of FNDC5/irisin in muscle and in the brain both in humans and in rodent models and it is worth mentioning as well that with the original discovery of FNDC5, it was hypothesised that it may be a receptor for an unknown ligand[192,193]. Evidence of this is not yet available, however one recent study reported FNDC5/irisin to be present in multiple forms with distinct molecular weights, suggestive of alternative mechanisms of action for FNDC5/irisin[194]. Significantly reduced FNDC5/irisin levels were reported in the hippocampi of late-stage Alzheimer's disease (AD) patients (when compared to age matched controls) and in brain homogenates of AD model rodents[194]. This is suggestive of a protective effect of irisin in neurodegeneration.

There is to my knowledge no published data on the role of irisin/FNDC5 in PTSD, but there is an emerging role for FNDC5 in metabolism-related physiological functions. Dysregulation of FNDC5 has been found to lead to systemic metabolic imbalance that could lead to the onset of metabolic

disorders[195,196]. This links to the systemic feedback of increased inflammatory profile related to obesity. In line with this reasoning is the potential protective effect of FNDC5 in brain tissue against pathology, with the increased brain-derived neurotropic factor (BDNF) expression and protective effect in AD discussed above. Furthermore, there seems to be a role for FNDC5 in neural development, which may be unrelated to the synergy with BDNF; however, BDNF is also a major role player during neuronal development and is considered neuroprotective[197], which again speaks to a potential neuroprotective effect for FNDC5. FNDC5 is therefore a good target to assess in the context of PTSD and obesity as it spans the potential overlap between these two disorders in a manner that aligns with my central hypothesis of inflammation driven allostatic load contributing to pathogenesis in PTSD.

Platelet/Endothelial cell adhesion molecule-1 (PECAM-1, also CD31) is described as a differentiation marker for monocytes and platelets[198] and subsequently has emerged as an important marker for endothelial-to-mesenchymal transition (EndoMT) and mesenchymal-to-endothelial transition (MEndoT). Typically, in fibroblasts, which are mesenchymal type cells, there is a lack of CD31 expression at baseline (quiescent cells) but upon activation, fibroblasts show a shift towards increased αSMA and CD31 expression. The predominant context of research into CD31 is related to cardiovascular and cancer research, which is associated with the function of CD31 in epithelial adhesion and intracellular junctions, the EndMT, and inflammation in cancer conditions[199–203]. CD31/PECAM-1 also plays a critical role in leukocyte transmigration and endothelial junction integrity of the BBB[204,205]. In the context of mental health there is a paucity on information related to CD31 function, however the inflammatory and cell junction functions are interesting from a mental health perspective. A major contributor to brain health is the blood brain barrier (BBB) which has the critical role of allowing exchange between the peripheral blood supply and the brain. An increase in the permeability of the BBB has already been linked to adverse outcomes in several neurological conditions as well as being a consequence of increased peripheral inflammation and gut dysbiosis[206–210]. Interestingly, it is difficult to find any eligible studies that assess the BBB in the context of PTSD, however considering the evidence for the involvement of inflammation and oxidative stress in the pathogenesis of PTSD, it stands to reason that BBB permeability and function are likely to be altered in PTSD as well. Some review studies cite BBB permeability or disruption as a possible consequence of increased inflammation, oxidation, or cytokine signalling, but do not directly report BBB changes in PTSD[210,211]. The fact that CD31-CD31 interactions between endothelial cells are varied in response to different mechanostressors, and that CD31 immunoreceptor tyrosine based inhibitory motifs (ITIMs) are not phosphorylated under resting conditions,[203,212] suggests an active role for CD31 to respond to stress and inflammation. In one study of a predator-based model for PTSD in rodents, stressed hearts showed a decrease in CD31 expression interpreted as indicative of the EndoMT, which was contrary to the expected angiogenesis suggested by gene ontology analysis in the same study[213].

The importance of this study in the context of this thesis is the decrease in CD31 expression in response to a prolonged stress response. Taken together, the expression of CD31/PECAM-1 on endothelial and leukocyte cells is important for normal inflammatory responses as well as BBB function and a decrease in expression is seen under chronic stress conditions. Furthermore, CD31 is responsive to dynamic changes in homeostasis of inflammation and immune response, which could be critical in the pathogenesis of PTSD.

2.4.4 Fibroblasts as niche immune cells

An immunological function for fibroblasts is well established[32,138,214]. The ECM remodelling and fibrosis are both implicated in the immune response in terms of wound healing[143,215], trafficking of lymphocytes[216,217], and cell to cell cytokine signalling[138,218]. Mounting evidence in literature shows that beyond the ECM remodelling and fibrosis, fibroblasts also have an active role as immune regulators, which has been most studied in the context of cancer[153]. The question therefore is not whether fibroblasts have an immune function, but whether they should be considered as immune cells or – since tissue-specific resident immune cells already exist - immune supporting cells. This idea is further supported by the illustration of specialized fibroblasts that facilitate inflammatory signalling in the peripheral nervous system[219]. In rodent models of Schwann cell degeneration, fibroblasts of the endoneurium were shown to be activated by cytokine and chemokines released by defective Schwann cells[220]. These activated fibroblasts induce macrophage proliferation and contribute to the pathogenesis of neuropathy[219]. Our group and others have recently shown the brain to have a significant anti-inflammatory and antioxidant bias when compared to peripheral tissues[67,221,222]. Given this lower likelihood of traditional immune cell involvement in neuroinflammation, research into the roles of other candidate cells with immune modulatory functions – such as fibroblasts – in the context of neurological disorders such as PTSD, is clearly warranted. Furthermore, because of the immune and tissue remodelling related function of fibroblasts they are a good model cell in which to investigate allostatic changes, because they are sensitive to tissue homeostasis and thus facilitate allostatic changes.

Apart from these potential markers of PTSD-associated allostasis in the periphery, I have also identified cellular calcium dysregulation as potential sensitive marker of allostasis, given its tight regulation and direct link to redox status. In the next section, the most pertinent literature on calcium homeostasis will be reviewed.

2.5 Cellular calcium homeostasis

Calcium (Ca^{2+}) is an important component of the cellular signalling environment. A large range of effector complexes rely on Ca^{2+}-ion exchange and concentration changes to enable the biochemistry

of signal transduction[91]. This has led to the evolution of many different calcium channels and receptors as well as complex cellular machinery for storage and release of Ca^{2+}-ions[223]. Due to the importance of Ca^{2+} within signalling complexes, it is not surprising that several diseases have been linked to dysregulated cellular calcium dynamics[89,224–227]. First, I will provide an overview on the basics of calcium signalling, focussing on G-coupled protein receptors as example, with the understanding that there are other ways in which calcium is involved in maintenance of homeostasis. The details pertaining to the molecular function of calcium is important here to appreciate the level of complexity and the importance of redundancy involved in calcium signalling. I will then expand on calcium dysfunction within mental health and specifically in PTSD, followed by a discussion of the role of reactive oxygen species and inflammation with regard to calcium function in this context. Given the high global incidence of obesity – a condition similarly characterised by chronic inflammation and increased oxidative stress – this condition will be included in the discussion as a confounding factor.

2.5.1 Calcium homeostasis

Normal Ca^{2+} fluctuation is the result of interplay between reaction that increase Ca^{2+} influx and signalling, and reactions that decrease Ca^{2+} influx and signalling[228]. This is achieved through the actions of varied pumps, channels, buffers, and binding proteins that coordinate cellular effects of Ca^{2+}. Ca^{2+} sensing receptors are abundant within the extra- and intracellular environment, ensuring a very tightly controlled homeostatic balance between the influx of calcium from outside the cell, the efflux of calcium from Ca^{2+} stores within the cell (ER), and the pumps and exchangers that remove Ca^{2+} from the cytosol[229,230].

Influx of Ca^{2+} from outside the cell is mainly mediated through extracellular Ca^{2+} receptors (CaR) - the most studied types of Ca^{2+} receptors[229]. These receptors are G protein coupled receptors (Class C) that respond to ambient di- and polyvalent cations such as Mg^{2+}, Zn^{2+}, Gb^{3+}, spermidine, neomycin and spermine[228]. These receptors have also been found to be sensitive to L-amino acids and show high expression in both central and peripheral neurons and glial cells but seem to be concentrated in nerve terminals.

The main source of signalling Ca^{2+} comes from outside the cell, because of a large electrochemical gradient that exists across the plasma membrane. Voltage operated channels (VOC) are the best-known calcium channels and generate rapid Ca^{2+} fluxes that play a role in the control of fast cellular processes (such as exocytosis) during synaptic signalling[90]. Receptor operated channels (ROC) such as glutamate (NMDA) and dopamine receptors (DR), respond to external signals which stimulate the opening of the channel and influx of calcium, which in turn activates other signalling processes mainly through CICR (calcium induced calcium release) mechanisms[230]. Once an action potential has been initiated and Ca^{2+} channels opened, the CICR process mobilizes Ca^{2+} from internal stores which

are primarily located in the ER reticulum. Two receptors, Ins(1,4,5)P_3 receptor and ryanodine receptor (RYR), are responsible for the release of calcium form these stores and both of these receptors are Ca^{2+} sensitive, constituting a large portion of the CICR signal[231,232].

Intracellularly Ca^{2+} is regulated through an interplay between calcium sensing receptors located on the membrane of the cell and Ca^{2+} sensing receptors located on Ca^{2+} stores, which sense the Ca^{2+} concentration of the cytosol and allow Ca^{2+} entry from the extracellular environment or Ca^{2+} entry from stores, primarily the endoplasmic reticulum (ER)[90]. These stores are sensitive to change in cytosolic Ca^{2+} through the function of specific protein interactions, one of which is phospholipase C, inositol triphosphate (Ins (1,4,5)P_3) interaction which stimulates the release of Ca^{2+} from ER stores in response to low levels of Ca^{2+} in the cytoplasm.[233]

ER release of Ca^{2+} can be independent of membrane depolarization, where the ER can generate Ca^{2+} transients that increase cytosolic Ca^{2+} concentrations. The formation of these transients has been shown in neurons, however generally neuronal Ca^{2+} release form the ER follows depolarization, as these transient Ca^{2+} signals in the absence of membrane potential changes may decouple a neuron's Ca^{2+} homeostasis from synaptic signalling [91,234,235]. Furthermore, it is possible for these Ca^{2+} transients to be relayed to the nuclear envelope, where Ca^{2+} increase activates cAMP response element binding protein (CREB), which in turn can upregulate the transcription of certain genes, such as *BDNF*[234]. Although there is very little literature reporting on Ca^{2+} transients and PTSD directly, there is some emerging evidence that Ca^{2+} dynamics and homeostasis may be involved in PTSD pathogenesis, which is discussed in more detail in the next section.

Ca^{2+} signalling takes the form of an all or nothing reaction, but many components of Ca^{2+} signalling function differently at different concentrations of Ca^{2+} [228]. Subtypes of RYR and In(1,4,5)P_3R are activated at different concentrations for example, thus allowing for tighter regulation of Ca^{2+} levels within the cytosol at different time points[90,231,236]. Further mechanisms that help enforce all or nothing type response comes through the regulation of Ca^{2+} binding proteins such as calmodulin.

The release of Ca^{2+} from the ER and the tight regulation of Ca^{2+} homeostasis, creates pockets of high Ca^{2+} concentration near RYR and In(1,4,5)P_3R which is important for mitochondrial Ca^{2+} uptake[223]. Increase in Ca^{2+} uptake into the mitochondrial matrix increases the production of ATP and NADH. In this way there is a direct link to needed energy production during depolarization, however the potential of Ca^{2+} transients to increase the Ca^{2+} concentration in distal areas of the neuron would increase local ATP and NADH production in these mitochondria[91], perhaps even in the absence of direct depolarizing signals.

In short, Ca^{2+} is effectively the ion that enables most cells to be metabolically active. There are many ways that intracellular calcium is regulated which makes the tight regulation of Ca^{2+} concentration

have many redundancies that enable excitability or work even when some subsystems are malfunctioning. There is also a direct link between Ca^{2+} regulation and ROS production through the interactions of ER stores and receptors with mitochondria[73,231]. From a FPP perspective disruptions of Ca^{2+} signalling is potentially something that could increase the allostatic load and contribute to the pathophysiology of PTSD. The excitability of any cell determines its ability to function normally within the tissue it is situated in. Within a neural network, the dysfunction, or simply the potential change in signalling rhythm, may result in the entire network not functioning as expected. This type of dysfunction may have repercussions in many different aspects of mental health - indeed, calcium functioning have already been implicated in the aetiology of mental disorders[46,236]. For example, several subtypes of Ca^{2+} receptors with allelic variants have been implicated in Schizophrenia, MDD, ASD and BD[46]. The precise physiological consequences of specific single nucleotide polymorphisms (SNPs) are still being elucidated, but there is evidence for altered expression profiles in different brain regions as well as volumetric changes to brain regions such as the amygdala[237,238]. Ca^{2+} binding proteins (calmodulin, calreticulin, etc.) have been implicated in anxiety disorders, as well as evidence for dysregulation of inflammatory pathways[239].

The tight regulation of Ca^{2+} levels is what allows for many intracellular protein interactions to occur, by maintaining an ion rich and chemically stable environment. Fluctuation of Ca^{2+} levels is normal and can be varied, however only within a certain range (Extracellular free Ca^{2+} concentration: 1.2 m*M*; resting cytosolic free Ca^{2+} concentration: 100 n*M*)[91,230]. Any changes outside the normal homeostatic range could result in several undesirable states for normal cellular function. Neurons are susceptible to damage if the cellular environment changes with regard to pH, ionic content, inflammatory state, and reactive oxygen levels. Ca^{2+} not only directly affects the cytoplasmic state, but it also acts as a secondary messenger in the regulation of inflammation, redox status, and promotes gene transcription[223].

2.5.2 Calcium dysregulation in PTSD

Although calcium dynamics is incredibly important to normal physiological function, the practical difficulties of successfully measuring calcium signalling have limited the range of disease contexts in which calcium has been investigated. Furthermore, isolating specific calcium receptor and channel functions are practically difficult, limiting the types of inferences that can be made from the data. There are also a large variety of calcium related role players due to the aforementioned redundancies, which likely exist as result of the importance of calcium as a signalling molecule. Genome wide association studies (GWAS) data has suggested a role for a subset of calcium channels called *CACNAC* in the aetiology of several mental health disorders such as depression, schizophrenia, and PTSD. Indeed, a mouse model of heterozygous *cacnac1c* and conditional knockout mice, showed several aberrations of fear and memory circuits related to hippocampal and amygdala function similar

to those reported in human studies of PTSD, where amygdala and hippocampal function as well as fear circuitry has been implicated[240], suggesting an important role for calcium channels in PTSD[92]. Interestingly, the deficits exposed in this study suggested that the loss of calcium channels in dopamine receptor 1 expressing cells were associated with fear conditioning and extinction at remote time points[92]. Dopamine related dysfunction is a well-known component of several mental health disorders and is often the focus of drug-based interventions in mental health. However, it is interesting to note, that if studies like this hold true, the intervention on a dopamine level, or any neurotransmitter level, would be insufficient in addressing the causal issue at play. The neurotransmitter level may be too far down stream of the mechanism that dysfunctions and only truly reflects the effect of this deeper mechanism on neurotransmission. This is not trivial of course, because neurotransmitter levels determine a lot of behaviour and affect, but the critical point here is that changing the levels of neurotransmitters does not address the actual cause of the disorder, but merely alleviates the symptoms.

2.5.3 The calcium-redox link

Arguably one of the main aetiological causes of calcium dysregulation, is increased levels of reactive oxygen species (ROS)[89,241–243]. These by-products of cellular respiration mainly originate from complexes 1 and 3 of the mitochondrial electron transport chain. Several buffer mechanisms exist within the cell to mitigate the detrimental effects of ROS, such as superoxide dismutase (SOD), catalases, and peroxidases[91]. However, when ROS production exceed the capacity of these endogenous antioxidant defence systems, excess ROS can modulate the kinetics of several proteins through oxidation and glutathionylation of thiol groups[231]. Most pertinent to the current topic, proteins modulated by ROS include sarco/endoplasmic reticulum Ca2+-ATPase (SERCA), inositol 1,4,5-triphosphate receptor (Ins(1,4,5)P3R) and Ryanodine receptors (RyRs)[90,231], indicating that cytosolic Ca^{2+} concentration is susceptible to modulation by ROS. Furthermore, an increase in the Ca^{2+} concentration of the mitochondrial matrix itself increases the activation of key enzymes of the Krebs cycle - such as pyruvate dehydrogenase, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase - thereby increasing ATP and NADH production[244], which may exacerbate an already unfavourable redox status and cause more severe perturbations in terms of calcium regulation.

Indeed, in a murine model of PTSD (chronic restraint stress)[245], a relative increase in phosphorylated RyR type 2 (RyR2) was reported, which was ascribed to Protein kinase A (PKA) activation[246]. This led to destabilisation of the calcium channel complex, resulting in intracellular calcium leakage from the ER into the cytoplasm. In the cardiovascular literature, PKA is known to be activated by oxidative stress[247]. In addition, high levels of ROS in the heart have also been directly linked to Ca^{2+}/ calmodulin-dependent protein kinase 2 (CAMKII)-dependent phosphorylation of RyR2[242,248].

Furthermore, several studies in human and rodent models of PTSD confirms an unfavourable redox status[45,249,250]. Redox status can be assessed in several different ways by measuring the effects of increased ROS as evidenced by lipid peroxidation, and the effects of antioxidant systems such glutathione peroxidase and superoxide dismutase. Together, these studies confirm the role of oxidative stress as confounding factor in PTSD-associated Ca^{2+} dysregulation.

Similarly, evidence illustrating that ROS-mediated alterations in brain cells result in decreased cognitive function, have been provided. For example, studies in rodent PTSD models have implicated increased ROS in damage of neuronal tissue and inflammation,[251] as well as in disrupted neural signalling[252]. Impaired mitochondrial function has also been implicated in PTSD, where decreases in Bcl-2 proteins were associated with PTSD like symptoms of fear and anxiety in a murine model[253] . Important to the current context, Bcl-2 proteins are an integral part of the outer mitochondrial membrane, where they have an anti-apoptotic function in conditions of high ROS.[254] Interestingly, Bcl-2 expression can itself be modulated by ROS[255] and Ca2+ signalling[256].

2.5.4 Calcium dysregulation in Obesity

There is a well-established link between obesity, oxidative stress, and increased inflammation, suggesting that the autocrine action of adipose tissue and adipose associated cells are contributing to allostatic load. It is also established that obesity or metabolic changes are comorbid with several mental health disorders, in particular PTSD[114,116,257,25,258,259,260]. The precise causal nature between PTSD and obesity, if there is any, remains to be elucidated. However, the pro-inflammatory milieu seen in both disorders has direct and indirect impacts on HPA-axis function, sympathetic nervous system regulation, mitochondrial function, and metabolically active hormonal balance[116,261,262] - all of which amounts to secondary effects of inflammation altering the allostatic load in PTSD and obesity. Interestingly, in veterans with PTSD and metabolic syndrome, PTSD severity was found to predict increases in metabolic syndrome severity, but metabolic syndrome did not predict PTSD outcomes[263]

The ubiquity of calcium as a signalling molecule and essential mineral has led to a large body of research connected to calcium. However, relatively little research directly focus on calcium alterations in the context of obesity, despite the fact that increased inflammation and ROS production associated with obesity have the effect to dysregulate calcium signalling dynamics over time[264,265].

Glucocorticoid resistance is thought to play a prominent role in exacerbating the feedforward effect between inflammation, obesity, and calcium dysregulation[129,266]. The main premise is that the low-grade chronic activation of the HPA-axis, inflammation, or adipocyte signalling through adipokines, result in a feedback mechanism that increased CRH and cortisol levels, which increases the release of pro-inflammatory cytokines and adipokines. These in turn should decrease the release of CRH and cortisol, but due to the continuous activation there is an allostatic modification of glucocorticoid

receptor density and sensitivity, as well as an increase in corticosteroid binding globulin (CBG) and modulation of 11β-hydroxysteroid dehydrogenase, resulting in what is termed glucocorticoid resistance[106,129]. Cortisol plays a critical role in mobilizing energy and metabolic systems, which include increasing mitochondrial action and function. This translates to a mismatch in signalling paradigms between mechanisms to increase energy availability and mechanisms that attempt to mitigate the damage related to increased inflammation and subsequent ROS. Interestingly, glucocorticoids have been reported to translocate to mitochondria where they mediate calcium storage and mitochondrial gene expression[48,267].

From the literature, it is clear that calcium is such a ubiquitous and important aspect of cellular signalling dynamics, which makes it difficult to study it in systems other than isolated experimental setups in terms of specific channels or calcium dependant protein interactions. However, the global calcium state, concentration, and/or flux of calcium through the cell does give valuable insight into the state of the cell and potentially the tissue since calcium is involved in so many cellular processes.

In the next section, I shift the focus to potential interventions in PTSD and how redox-sensitive therapeutic approaches may affect allostasis.

2.6 Intervention: potential effect of anxiolytic signalling on allostasis

Given the challenges of patient management in chronic diseases such as PTSD, prevention or early intervention is a much-desired therapeutic approach after trauma exposure – not only to address the primary disease, but also to limit allostatic load which may increase risk for co-morbidities.

For the purpose of this thesis, I identified a signalling system that is functional at the interface of inflammation, redox and neuronal function – the trace amine system. This system has suffered relative neglect for the past few decades, largely due to the difficulty of accurate quantification of the trace levels at which signalling occurs. However, with advances in analytical equipment, research on trace amines and their associated receptors is gaining popularity. A comprehensive review on the pharmacology of trace amines – the most commonly accepted agonists of trace amine associated receptors (TAARs) – is beyond the scope of this thesis and can be accessed elsewhere[268–273]. I will however provide a brief description of the most relevant trace amines (TAs) for context.

2.6.1 Trace amines: an introduction

TAs have been implicated in neurologic and psychiatric disorders since the late 1970's[274–276] and share a similar structure to biogenic amine neurotransmitters such as adrenaline, noradrenaline, and dopamine. They are present in the central nervous system at trace levels[270], which is primarily an

attribute of their high turnover rate, short in vivo half-life (~30s)[277], and seeming lack of significant intracellular storage sites[272].

The classic trace amines relevant to my context are β-phenylethylamine (β-PEA), tryptamine (TRP), *para*-tyramine (p-TYR), *meta*-tyramine (m-TYR), *para*-octopamine (p-OA), and *meta*-octopamine (m-OA). TAs are continuously synthesized within neurons and glial cells through the function of L-amino acid decarboxylase (AADC) - specifically, β-PEA, m-TYR, and p-TYR are formed by decarboxylation of phenylalanine and tyrosine. Tyramines can also be converted to p-OA and m-OA by dopamine β-hydroxylase. Typically, TAs are degraded through the action of monoamine oxidases (MAO), although β-PEA may be a selective substrate for MAO-B[278]. Current evidence suggests that TAs are not stored in synaptic vesicles and their release is not dependant on depolarization. Rather, their mechanism for entry to have post synaptic effects seems to be by passive diffusion across the plasma membrane lipid bilayers[279]. Although there is some evidence to suggest that TYR may be released in an activity-dependant manner, as well as being stored in synaptic vesicles, it is unclear whether this is due to true storage and release of TYR, or some level of competition between TYR and biogenic amines for reuptake into storage vesicles[268,272]. The specifics of the molecular mechanisms surrounding TA storage and release may benefit from modern analytical tools and techniques to gain a better picture of TA dynamics *in vivo*.

In terms of the relevance of TAs to PTSD, I could find no published reports directly linking TAs and PTSD, although there is some suggestion that increased TA abundance may be implicated in depression[280]. Despite this lack of directly related data, studies not directly focussed on PTSD do provide information from which a likely scenario may be constructed. For example, early studies showed a clear relationship between biogenic amines and TA levels, where there seems to be an inter-regulation between TA's and monoamines as changes in monoamine activity affected TA levels and vice versa. For example, a decrease of TAs (in cell homogenates) as result of electrolytic damage resulted in increased dopamine (DA) release in the striatum [281,282], while an increase in TA levels (through experimental MAO-B inhibition), resulted in an increased response to dopamine[283] and dopamine agonists[284] - both suggestive of an outcome of increased dopaminergic signalling [285]. Interestingly, the vulnerability of dopaminergic neurons in the substantia nigra pars compacta (SNc) is related to both calcium channel composition[286] and availability of intracellular calcium binding proteins[224] – such as calmodulin – under conditions of calcium overload. Severely increased dopamine levels can contribute to Ca^{2+} overload[287], which may be under some regulatory control through TA signalling. However, without the knowledge of a bona fide TA receptor at the time (TAARs were only identified in 2003), the function of TA in the context of calcium dynamics could not be precisely determined. Nevertheless, this evidence suggest that TAs play a role in neuromodulation[270], although the precise nature and magnitude remains to be elucidated. Administration of supraphysiological

levels of TA (β-PEA and TYR) results in effects similar to methamphetamine administration[272], but the physiological role of TAs has been more difficult to elucidate, given the close relationship in synthesis of TAs vs. monoamines, as well as the generally low levels of TAs in the brain.

In addition, in the context of oxidative stress and inflammation, relatively higher doses of TAs are most definitely implicated as undesired contributors. Apart from their well-established direct implication in inflammatory outcome in the gut[288], three TAs – β-PEA, TYR and 3-iodothyronamine (T1AM) – have been linked to pro-inflammatory outcomes such as neutrophil chemotaxis and histamine release in vitro, which resulted from their binding to TAAR1 and TAAR2[289]. In the same study, EC50 values determined for β-PEA, TYR, and T1AM indicated that TA levels in the low nM range[270] were sufficient to trigger chemotactic migration of PMN cells[289]. Furthermore, IL-4 concentration increased in a TA-dose dependent manner when T cells were exposed to β-PEA, TYR, and T1AM and was attenuated with the addition of TAAR1 and TAAR2 specific siRNA[289]. With the discovery of TAAR1 expression in most immune relevant cells, more studies have reported a function for TAAR1 and by extension TAs in immune regulation[289–291]. Lastly, and most relevant to the current topic of allostasis, recent studies by our group in the context of irritable bowel syndrome, illustrated a major role of trace amines and their metabolites in redox status[292], with some acting as prooxidants, while others have antioxidant effects, in a manner highly dependent on concentration and availability of pathways responsible for TA metabolism.

Trace amines seem fairly promiscuous in terms of receptor binding, although a family of trace amine associated receptors (TAARs) located primarily in association with olfactory neurons, were discovered only relatively recently in mammals[293]. TAARs are G-protein coupled receptors (GPCR) class A (also rhodopsin-like) and are mainly expressed intracellularly[294]. 28 TAAR's have been described in literature, with wide variation across species in terms of number of genes and functional receptors[272]. Nine TAAR genes have been described in humans, of which three (*TAAR3*, *TAAR4*, and *TAAR7*) are pseudogenes[295], referring to a genomic sequence which is a truncated or non-functional version of a functional genes.[296] An exhaustive review of TAARs is outside of the current thesis scope – due to the extremely limited availability of reagents for visualisation and/or quantification, this was not a feasible experimental option. Rather, I will focus on the anxiolytic effects reported for specific trace amines, which – combined with redox-modulating properties - make them a potential therapeutic target in PTSD and PTSD-associated allostasis.

2.6.2 Anxiolytic trace amines and their potential to modulate allostatic load

Early studies on β-PEA showed strong anxiogenic effects of β-PEA on mice to the same order of known anxiogenics such as caffeine and pentylenetetrazol; however, these effects were seen in

response to extremely high concentrations of β-PEA (5000 or 10000 ng/g)[297] - several orders of magnitude higher than physiological concentrations (0.1-100 ng/g)[298,299]. In more recent studies, β-PEA concentrations at more physiologically relevant levels, reduced the effects of chronic exposure of rodents to corticosterone (CORT) – a well-known model for inducing depression behaviour in rodents – in cultured hippocampal neuronal cell lines through rescuing dendritic spine morphology and density as well as increasing excitatory synaptic activity, suggesting an anxiolytic effect for this TA[300]. Interestingly, CORT induction of depressive behaviour functions on the same neurobiological mechanisms that are thought to be active in PTSD aetiology, which is mediated through GC related dysfunction as well as glutamatergic signalling mechanisms[48,301]. Furthermore, β-PEA, TYR, and TRP were found to reduce perfusion pressure of rat isolated perfused mesenteric vascular beds through nitric oxide (NO) mediated vasodilation. Inhibition of NO synthase via N(ω)-nitro-L-arginine methyl ester (L-NAME) abolished the capacity for vasodilation by β-PEA, TYR, and TRP administration, suggesting that some TAAR mediated signalling occurs through redox messengers[302]. At high concentrations TYR is thought to sympathomimetically stimulate aminergic signalling (dopamine and glutamine), increase anxiogenic behaviour in animals, as well as induce ROS through monoamine oxidase (MAO)-A and -B degradation of TYR[303–305]. There is a relative paucity of information on TYR at physiologically relevant levels, however TYR is a preferred agonist for TAAR1[306] and therefor TAAR1 mediated anxiolytic effects are expected at lower concentrations. Furthermore, the inability of TYR to cross the blood brain barrier (BBB)[307,308] makes it likely that normal physiological function of TYR will be at low endogenous levels.

From these studies, specific trace amines may hold potential as anxiolytic treatments in PTSD, with the additional potential of reducing allostatic load as well.

Since zebrafish (ZF) larval models are increasingly being used for screening of potential anxiolytic drugs – and indeed also in this thesis – I will provide a brief background on the use of zebrafish in this context.

2.6.3 Zebrafish as tool in anxiety research

Animal models are an integral tool for modelling and understanding disease. The success of rodent models, especially in modelling complex psychiatric disorders such as ASD, bipolar and PTSD, has given access and tools for studying the pathophysiology of these types of disorders. Recently zebrafish (*Danio rerio*) have emerged as a cost-effective model organism for translational neuroscience[309]. There is overlap in the brain chemistry, immune function, and hormonal control of fear and anxiety pathways that make ZF well suited to study affective phenotypes[310]. ZF are particularly well suited to pharmacological intervention, display high sensitivity to acute and chronic stress, and are well suited for genetic modification[311–313]. The similarity between the HPA-axis and the ZF hypothalamic–pituitary–interrenal (HPI) axis - as well as the sensitivity to stress - have led to

development of ZF models that may be relevant for PTSD[314,315]. The fact that zebrafish larvae before the age of 5 days post-fertilisation are developed sufficiently to exhibit anxiety-like behaviour, while they are thought to not be developed far enough to experience pain or distress, makes the larval model an ideal, ethical alternative to rodent research models, especially in the context of anxiety disorders such as PTSD.

ZF show robust behavioural changes in response to certain environmental stimuli, which ranges from added substances in their housing water, to sounds and changes in lighting conditions, which has been used to great effect in neuropharmacological research[316]. In particular, light vs. dark preference and the response to exposure to alternating periods of darkness and bright light exposure, has been used extensively as model for anxiety in zebrafish larvae[316–318]. For example, in the light dark transition test, at onset of bright light exposure, ZF larvae freeze, followed by hyperlocomotion in the subsequent dark phase[317]. Both the freezing and sudden activity is an anxiety or fear response and can be modulated with pharmacological substances. Anxiolytics will decrease the level of hyperlocomotion in response to darkness while anxiogenic substances will increase the movement during the dark phase. In our lab we have shown this model to be an accurate model for probing anxiety-related neurological outcomes, for the rapid screening of anxiolytic substances[68]. Furthermore, this model is ideal for the anxiolytic screening of allostatic load induced by changes in redox levels.

2.7 Probing top-down and bottom-up allostatic modulation between the brain and the periphery

Characterisation of peripheral allostasis (e.g., in patient-derived fibroblasts) and identification of anxiolytic modalities (such as e.g., β-PEA or TYR) are important steps in medicine development for addressing PTSD-associated allostasis and risk for co-morbidities. Furthermore, although clinical studies cannot provide information on causality in terms of PTSD-associated neurological dysfunction and peripheral inflammatory and redox profile dysregulation, it is possible to probe these mechanisms of allostasis *in vitro* regardless of causal direction. The final experimental portion of this thesis will do exactly that, using commercially available human hippocampal astrocyte cultures. Therefore, in the last section of this literature review, I will provide some pertinent information on this cell type and its roles in redox regulation and other brain functions.

2.7.1 Astrocyte function

The primary function of astrocytes is the maintenance of the neural environment and direct modelling and remodelling of neuronal pathways[319–321]. Astrocytes also make up a large part of the neural connectome because they make connections with many different neurons and relay information as

well as coordinate signal transduction[322]. The role of astrocytes has been best described in the context of neural development and damage, as astrocytes are some of the easiest cells to culture and they can be differentiated into cell types that have aminergic signalling capacity and form functioning synapses[322–325]. There is a large body of literature surrounding astrocyte function; however, it is only in the last 10 years that the molecular tools started becoming available to describe the involvement of astrocytes in mental health disorders[326].

Astrocytes are classed as glial cells alongside microglia and oligodendrocytes and were originally thought to be support cells, responsible for providing neurotransmitter precursors and clearance, contributing to ion homeostasis, synaptogenesis, and degradation, as well as providing connection to vasculature[327,328]. Other important roles of astrocytes are the regulation of inflammatory events through cross talk with microglia[320], as well as the antioxidant capacity of astrocytes[73,327,329]. A number of subtypes of human astrocytes have been described to date and their distribution densities vary in different regions of the brain[321,329,330], suggesting functional differences between these subtypes. Indeed, the complexity of their anatomical distribution and function within the neural network is changing our understanding of astrocyte function. The two primary types of astrocytes are protoplasmic and fibrous astrocytes, which are biochemically distinct and found in grey and white matter of the brain respectively. Other subtypes include the Muller cells of the retina, the Bergman glia, perivascular glia, and velate astrocytes[321,329]. Astrocyte processes often surround synapses and protoplasmic astrocytes have the characteristic of having connection domains and strata such that one astrocyte is in contact with a distinct subset of neurons without overlapping with other astrocytes[331,332]. These connections between astrocytes and synapses have led to the tripartite synapse concept of one astrocyte, one presynaptic, and one postsynaptic neuron[333]. Fibrous astrocytes are more uniformly spread through white matter regions and have a large role in the spatial arrangement of neurons and synapses[328].

More recent development of novel technologies and techniques have contributed to a new understanding of the importance of astrocytes to brain function and development[334,335]. For example, established functions of astrocytes are their roles in ion homeostasis and neurotransmitter uptake. The close association of astrocytes with synapses are critical for ion homeostasis in the brain as astrocytes maintain a hyperpolarized state through inwardly rectifying potassium channels (Kir)[336]. High densities of the Kir4.1 channels are reported on astrocyte processes that are in close association with the synapses which allows for rapid uptake of K^+ ions from the synaptic cleft during neuronal activity[337]. Astrocytes are enriched for GABA and glutamate transporters, which allow for effective clearance of these neurotransmitters from the extracellular space[321]. By modulating the function of these transporters, astrocytes can influence synaptic signalling through spill over beyond the synapse, which in turn may alter the kinetics of excitatory post synaptic currents[321,338]. A further point to

consider is that the synaptic coverage of astrocytes varies by brain region and is also dynamically altered by a range of factors such as neuronal activity and homeostatic changes[321].

More directly relevant to the current topic, mounting evidence is suggesting astrocytes to have a heterogenous phenotype that is heavily dependent on the niche the astrocyte resides in (facilitated via feedback between neurons and astrocytes)[339,340], which is similar to the scenario seen in fibroblasts. This sensitivity and reactivity to their environments suggests than fibroblasts and astrocytes may be ideal cells with which to probe allostatic mechanisms in the periphery and centrally respectively.

2.7.2 Astrocytes as indicator of allostatic load

In the context of obesity, literature on the potential role of astrocytes is somewhat more abundant. Current literature implicates hypothalamic astrocytes in the pathogenesis of obesity through their role in nutrient sensing, hormone regulation, and neuronal crosstalk[341]. Several studies have determined that there is a bi-directional tuning of feeding circuits in rodents through astrocyte mediated activation and suppression of neuronal activity[342–344]. The tuning is thought to be affected by a range of potential factors such as diet, health, environmental factors, among others[341]. Astrocytes actively combat lipotoxicity through lipid uptake and storage via lipid transporters such as lipoprotein lipase (LPL), which when deleted in mice led to obesity and glucose intolerance in response to high fat diet[345]. In terms of a link to allostatic load, cultured astrocytes exhibiting this accumulation of lipid droplets in a fatty-acid rich environment, were shown to increase their secretion of proinflammatory markers TNFα, IL1—β, and MCP-1 [346]. Of further interest, studies have shown that these markers of inflammation are elevated in the hypothalamus before onset of significant weight gain, and in fact even within 24hrs of acute high fat diet exposure[347,348]. Although such an acute inflammatory response is transient and thought to be neuroprotective, it is clearly permanent in a state of continued exposure to a high fat diet [348]. To my knowledge, the effects of this allostatic load in the central compartment, has not been characterised in terms of redox outcome.

Turning attention to redox – and specifically ROS – as potential indicator of allostatic load in astrocytes, there is a well-established link between oxidative damage and mental health disorders in general[73,112,324], as well as specifically in PTSD[45,108,349]. The exact nature of the oxidative component is not known yet with respect to the direction of causality or the precise cellular machinery involved. In animal models of PTSD, the general consensus is that PTSD is characterised by an increased expression of oxidative stress markers and a decrease in antioxidant capacity[112]. For example, decreased activity of antioxidant enzymes such as superoxide dismutase (SOD) and increase in MDA levels and other prooxidant markers are also reported in several models of PTSD in rodents[27,350].

In the clinical psychiatry literature, the general consensus seems to be that PTSD increases oxidative stress and decreases antioxidant capacity as a secondary outcome of the primary neurological

dysregulation. This paradigm is largely based on associations between PTSD and adverse redox profile. For example, increased MDA levels have been correlated to higher depression and PTSD scores[351] and decreased antioxidant enzymatic activity was illustrated in combat exposed veterans[109]. In one study, earthquake survivors with PTSD showed higher MDA levels than non-exposed and exposed controls, which was interpreted as implicating PTSD and not stressor exposure as the mediating factor for the increase in MDA[108]. Thus, consideration is not given to a potential bidirectionality of the relationship, despite a lack of data supporting causality and despite the fact that genetic mutations in inflammatory genes such as NF-kB has been demonstrated in other neurological conditions, such as schizophrenia[352]. A recent meta-analysis of epigenome-wide association studies of PTSD found differentially methylated genes in oxidative and immune related pathways[353]. This clearly indicates a close association between PTSD and allostatic load, but what remains to be discovered is whether the ROS and inflammation also precede the PTSD diagnosis, as there is evidence that the state of PTSD itself induces ROS and inflammation[108]. Regardless of the direction of causality, this literature confirms that redox parameters may be accurate indicators of allostatic load in PTSD and obesity, although the exact profile of allostasis at the level of astrocytes, is still largely unexplored.

2.8 Conclusion and Hypothesis statement

A diverse range of topics were under review here, but everything is tied together by allostasis. Stress and stressors contribute to the allostatic load of cells through different mechanisms, but primarily through the increase in the oxidative state of cells. Although chronic inflammation is relatively well described in the context of PTSD, there is relative neglect of redox assessment, especially given that oxidative damage is directly responsible for cellular/protein damage and increasing allostatic load. This is one gap that this thesis is aimed at addressing, by utilizing a top-down cellular paradigm and a bottom-up cellular experimental model probing the communication between different compartments (peripheral vs central) in terms of allostatic load contributed by PTSD and obesity (as confounder).

From review of the literature, I hypothesised that allostatic load of PTSD will have a peripheral cellular signature and that it is distinct from chronic conditions also in the periphery such as obesity. Furthermore, I hypothesised that communication between central and peripheral compartments can contribute to neuronal allostatic load.

Chapter 3:

Fibroblast characterisation in the context of PTSD

3.1 Introduction

Very little emphasis has been placed on the peripheral effects of mental health disorders despite evidence for increased oxidative stress and inflammation associated with PTSD[111,113,263,354]. The allostatic load of PTSD inferred on peripheral cells could be contributing to the aetiology of PTSD and the comorbidities associated with PTSD[25]. Fibroblasts are the ideal cell type to probe the allostatic load experienced by peripheral cells due to the nature of fibroblasts cell to cell communication and role in inflammation and redox signalling[36,138,153,355]. In this chapter patient-derived fibroblasts were assessed for markers that show both functionality and potential for allostatic shift resulting from either obesity or PTSD, or both.

3.2 Materials and Methods

3.2.1 Ethics

Patient-dervived fibroblast were obtained from participants of the Shared Roots of Neuropsychiatric Disorders and Cardiovascular Disease Project[356] and ethical approval for use of these samples in a sub-study was granted through the Stellenbosch University Health Research Ethics Committee (HREC Reference number S20/01/024).

3.2.2 Patient characterisation

Participants were recruited for a larger study investigating the shared molecular roots of metabolic syndrome and anxiety disorders. Participants were assessed by clinical psychologists and psychiatrists for inclusion into the main study. A full list of measures can be found in the addendum (Addendum A). General health and cognitive screening were done, as well as the Hamilton Depression Scale. For PTSD specifically, the Clinician Administered Posttraumatic Stress Disorder Scale (CAPS) updated for the Diagnostic and Statistical Manual of Mental Disorders (5th ed.; DSM–5) was administered. The baseline measurements also included self-administered measures for PTSD symptoms and childhood trauma, as well as depression and anxiety. Physical and laboratory measures were taken, such as BMI, blood pressure, and physical measures. Laboratory samples included a dermal puncture from which primary fibroblasts were cultured and frozen down by trained haematologists.

From that cohort I selected n=40 patients for my study. These patients were selected based on their CAPS score and obesity levels (BMI), after which they were matched for age and other clinical factors (drug and alcohol use, smoking, other illnesses, comorbid mental health disorders) as best

possible. The groups were: **Control** (CAPS score < 20, BMI < 25); **PTSD** (CAPS score > 20, BMI < 25); **Obese** (CAPS score < 20, BMI > 30); **PTSD+Obese** (CAPS score > 20, BMI > 30). Equal numbers of males and females were selected. It is also important to note that even though the CAPS and BMI cut off values were definite, the samples chosen were at the more extreme ends unless no other matching sample was available. Initially, fibroblast samples from n=40 participants (n=10 per group) were obtained. However, some of the samples were contaminated during storage and were thus excluded (Fig 3.1). Another subset was simply non-viable in that there were no cells in the cryo-containers upon thawing (Fig 3.1). Mycoplasma testing was done on all viable samples and were found to be negative. This left a final n=23 (**Control**, n=4; **PTSD**, n=6; **Obese**, n=6; **PTSD+Obese**, n=7) for further analysis.

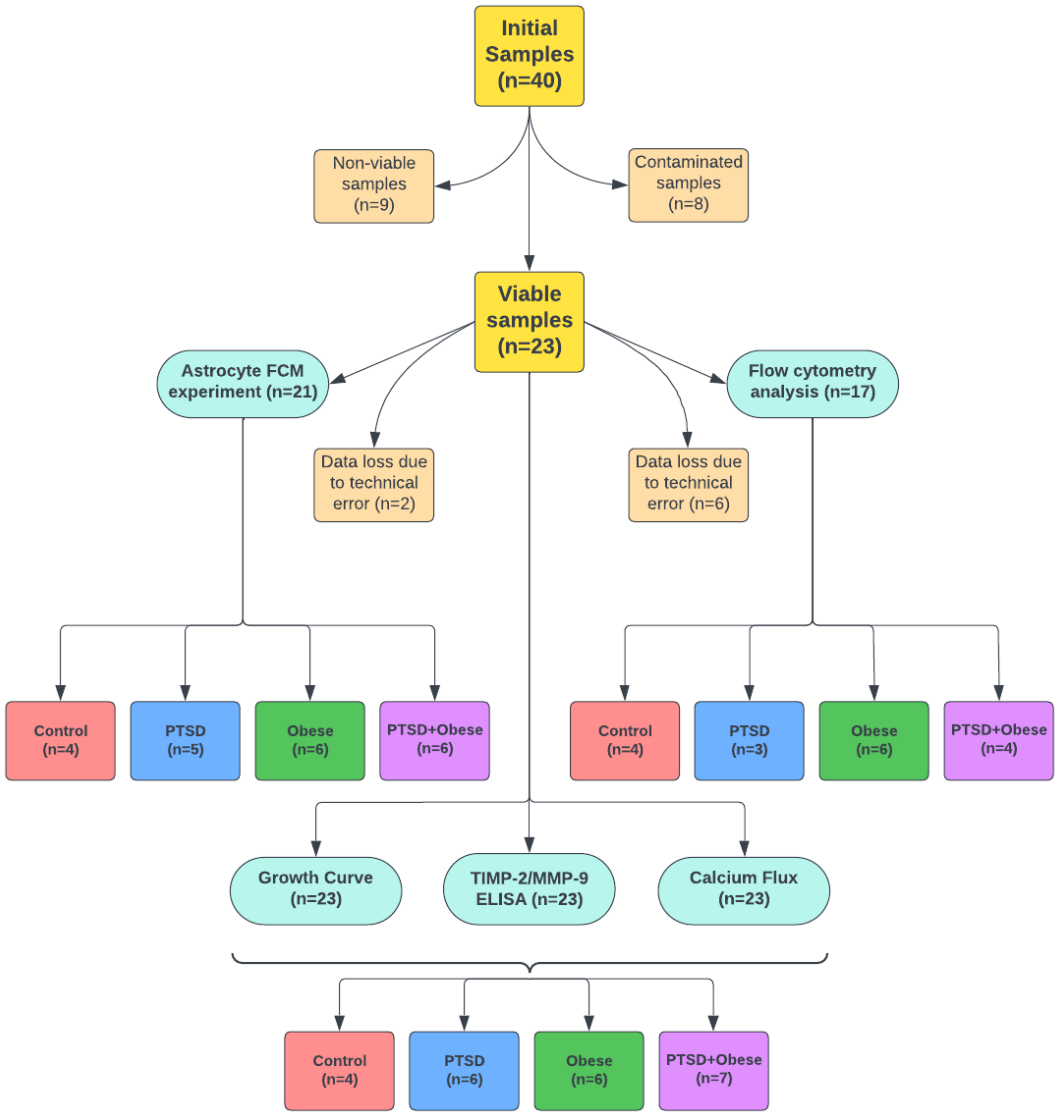


Figure 3.1 Flow diagram of patient inclusion. Initial samples from biobank storage n=40, however n=9 non-viable samples did not survive freeze/thaw and n=8 samples had underlying infection. A final n=23 viable samples were left for analysis. Technical data loss due to machine error and sample loss prevented an n=6 and n=2 samples from inclusion for the flow cytometry analysis and the astrocyte FCM experiment (Chapter 5). Final n for each patient group is also indicated for each experiment.

3.2.3 Fibroblast cell culture

Primary Fibroblasts were cultured in a standard growth medium (SGM) consisting of high-glucose Dulbeco's Modified Eagles Medium (DMEM) (Thermo Fisher Scientific, Gibco, Cat no. 11995040), 15% foetal bovine serum (FBS) (Merk Scientific, Sigma-Aldrich, Cat no. MFCD00132239) and 1% penicillin and streptomycin (Thermo Fisher Scientific, Gibco, Cat no. 15140122) and incubated at 5% CO2 in a humidified incubator. Normally the SGM for fibroblasts are recommended to have 10% FBS. However, for these fibroblasts, 15% FBS was added to compensate for time spent in cryogenic preservation.

Initially the fibroblasts were thawed into a T75 culture flask in 12ml SGM and allowed to adhere for 72hrs before the first media change. The initial seeding density at thawing (P0) is unknown, although the cell density at freeze was recorded as $1x10^6$ cells/ml. Some samples had difficulty adhering and were given an extra 96hrs to adhere. If there were still no adherent cells, the sample was removed from the T75 and centrifuged at 1500rpm for 10min. The resulting pellet was then resuspended in fresh SGM and reseeded into the T75 culture flask. SGM was changed every 72hrs until confluence was reached. The fibroblasts were then sub cultured – using TrypLE™ select enzyme (Thermo Fisher Scientific, Gibco, Cat.no. 12563029) into T175 culture flasks at a seeding density of 5000 cell/cm^2, as recommended for most fibroblast cell lines, to allow for greater number of cells at the next passage harvest (P1). P2 of the fibroblasts were plated into T75 culture flasks and images acquired every day for the duration of the growth period (Zeiss Primovert microscope using a 10x objective). Time of day for image acquisition was standardised to between 10 - 11:30 am.

3.2.4 Fibroblast morphology

The cultured fibroblasts in this study were morphologically normal. The morphology of fibroblasts in culture is fusiform and spindle shaped, with an oval nucleus, a large amount of rough endoplasmic reticulum and Golgi apparatus. This makes the cytosol appear granular or streaky under light microscopy.

Figure 3.2 shows representative fibroblast images for each group (Control-red, PTSD-blue, Obese-green, and PTSD+Obese-purple) at passage 1 and passage 2. The first image is 24hr after seeding (@5000cells/cm^2) and the second image is on day of harvest. Fibroblasts from all experimental groups appeared morphologically normal, with clear spindle shape and the granular appearance of large ER and Golgi apparatus. Images were captured at roughly the same time point each day (10:00 - 11:30).

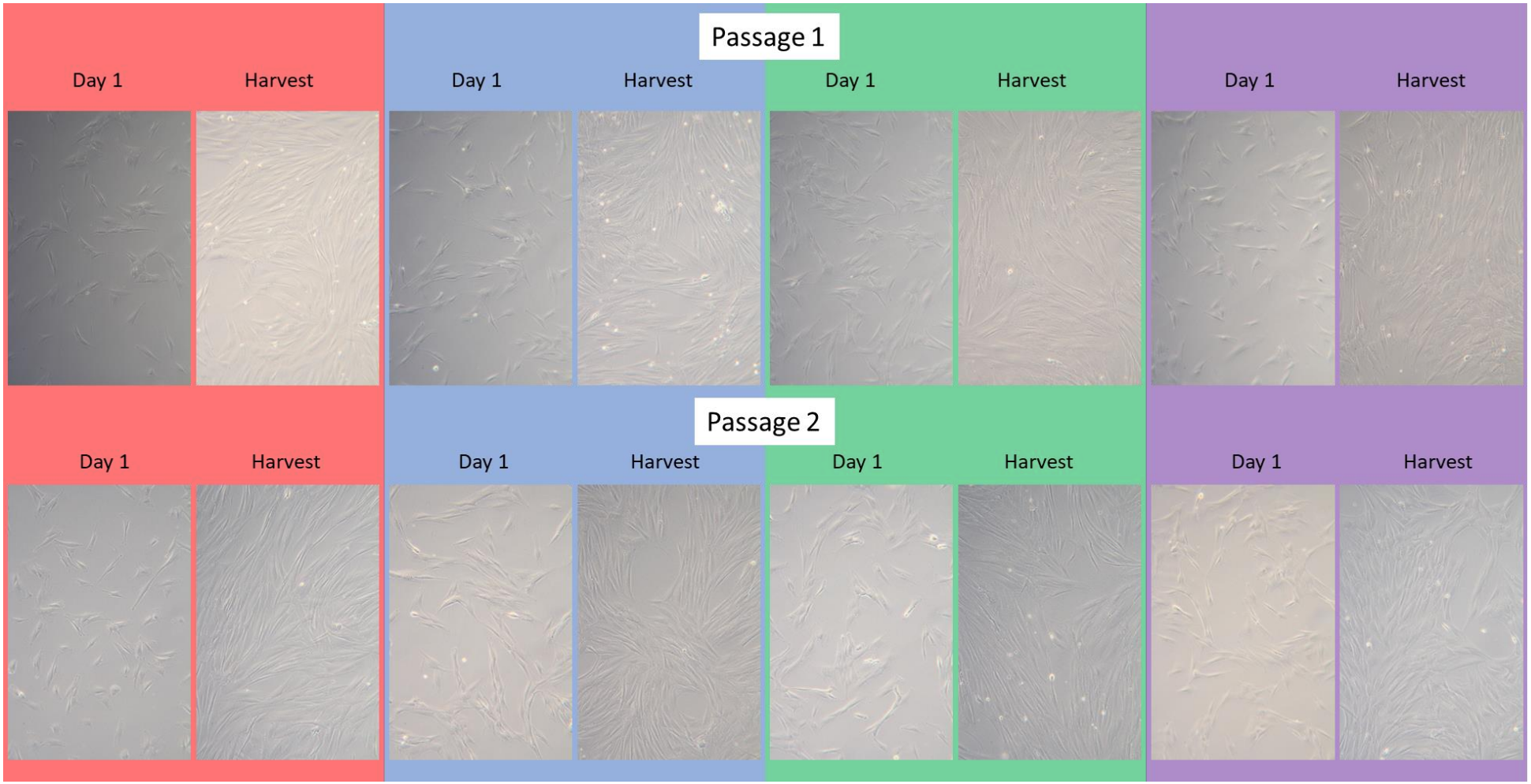


Figure 3.2. Fibroblast morphology. Representative fibroblasts images for Control (Red), PTSD (Blue), Obese (Green), and PTSD+Obese (Purple). 10x magnification on a Zeiss benchtop light microscope was used for image capture. Fibroblast show characteristic spindle shape morphology

3.2.5 Growth Curve

Growth curves were analysed based on a determination of confluency method adapted from Busschots et al (2015)[357]. This method had to be adapted for the imaging quality in this data set, by varying the set parameters of this method. ImageJ free open-source software was used in this method to give a quantitative indication of cell confluence. Although this technique and number are not a substitute for the researcher's estimation of confluence, it does provide objective standards to test one sample against the next, provided the sampling is consistent. Consistent sampling in this case would be the quality and consistency of the images taken on the light microscope (time of day and laboratory light conditions) as well as consistency of the analysis in ImageJ across the individual data points for each sample.

3.2.6 TIMP-2/MMP-9 ELISA

Enzyme-linked immunosorbent assay (ELISA) is an assay technique that allows for the detection of soluble factors. Supernatant of fibroblasts at passage 2 were collected and ELISA for TIMP-2 and MMP-9 was performed in accordance with the manufacturer's protocol. The supernatant was harvested at 60-70% confluence after a routine media refreshment step. ELISA kits were purchased form Elabscience and performed as per instructions. Briefly, standards were made and 100µL added to control wells with 100µL sample added to other wells and incubated for 90min @37°C. Liquids were removed from each well and 100µL of Biotinylated Detection Ab was added immediately and incubated for 1hour at @37°C. Wells were washed with wash buffer three times and 100µL of HRP conjugate working solution was added to each well and incubated for 30min @37°C. 50µL of substrate reagent was added to each well and incubated for 15min @37°C. Finally, 50µL of Stop Solution was added and the optical density of each well was measured by a microplate reader at 450nm.

3.2.7 Flow cytometric analysis of CD31, FNDC5, MMP-9 and MMP-3

Fibroblasts were dissociated using 5ml TrypLE express (GIBCO) and which was quenched with 5ml SGM and the samples were centrifuged at 1500rpm for 10 minutes. Samples were resuspended in staining buffer (SB – PBS containing 20% FBS) and split into sample FACS tubes for antibody staining. Samples are resuspended in 50µl of SB and incubated with the extracellular markers CD31 APC-Cy7 mouse anti-human (BD Pharmingen) and FNDC5/Irisin Alexa Fluor 405 mouse anti-human (R&D Systems) for 30min @ 4°C. Cells were then washed with 950µL SB and centrifuged for 10min at 250g and the supernatant is removed. Cells are resuspended in 250µL fixation/permeabilization solution (BD Biosciences) and incubated @4°C for 20min. 750µL permeabilization buffer (PB) was added and the cells were centrifuged for 10min at 250g, and the supernatant is removed. Cells are resuspended in 50µL of PB and incubated with the intracellular markers MMP-3 Alexa Fluor 647 mouse anti-human (R&D Systems) and MMP-9 FITC mouse anti-human (R&D Systems) for 30min

@4°C. Samples were washed with 950µL PB and centrifuged for 10min at 250g. Supernatant was removed, and samples were resuspended in 200µL SB. Data was acquired using a BD FACS Canto™ II instrument (FACSDiva Software, BD Biosciences). The staining index for each fluorochrome was determined by titration for the optimum concentration. Fluorochrome minus one (FMO) was also performed to compensate for potential fluorescent overlap using compensation beads (Comp beads, BD Biosciences). An unstained control for each daily sample run was included which was used to determine the optimal gating strategy. A minimum of 10000 events were recorded for each sample for analysis using Flow Jo 10.8.0 software (BD Biosciences).

On the last day of flow cytometry acquisition due to a technical error beyond my control the last batch of six anonymised samples were incorrectly labelled and I was forced to exclude those samples.

Figure 3.3 represents the gating strategy for the flowcytometric analysis. Forward and side scatter parameters were used to select for fibroblasts, then samples were gated according to CD31 positive or negative stain. Finally gates for MMP-9, MMP-3, and FNDC5 positive stain were selected.

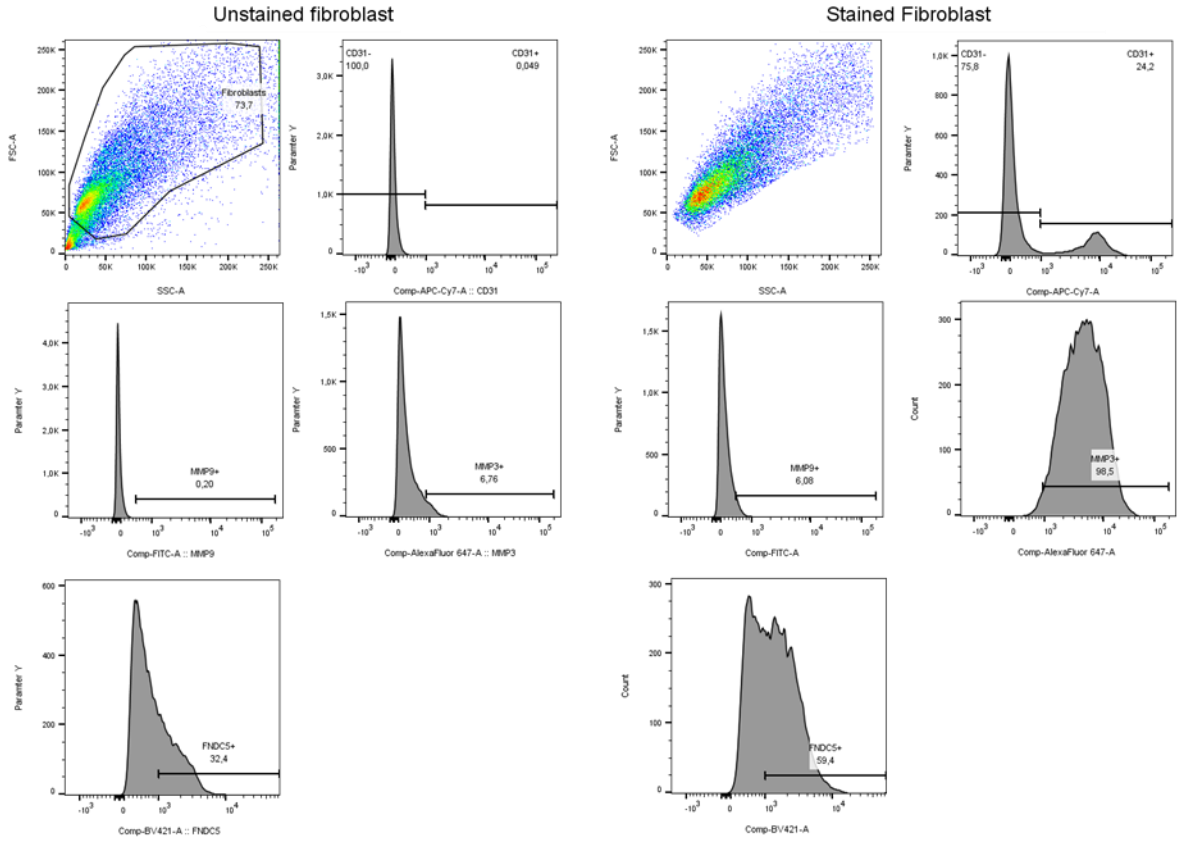


Figure 3.3 Representative gating strategy for fibroblast flow cytometry unstained and stained sample. Fibroblast were gated according to forward and side scatter.

3.2.8 Calcium flux assay

All calcium flux assays were run in P3, when an 8-well microscopy slide was seeded at 4000 cells/well, which is consistent with a seeding density of 5000 cells/cm2. The cells were cultured to between 70%-80% confluence (assessed visually) before the calcium flux assay was performed. Images were taken every day for the duration of each growth phase.

An optimized fluorometric assay was employed for global flux determination. The Fluo-4 calcium imaging kit (Thermo Fisher Scientific, Gibco; Cat# F10489) is a robust kit for gross calcium flux and includes a water-soluble probenecid that decreases baseline signal. The fluorescent excitation is at 488nm and exhibits a 100-fold increase in fluorescent intensity when bound to calcium. A proprietary Neuro Background Suppressor is added to reduce autofluorescence of growth media constituents. The resulting flux was analysed using a ZEISS technology light microscope and ZEISS ZEN 3.2 blue edition software.

Briefly, at 70-80% confluency, for each patient fibroblast sample, a DAPI nuclear stain was added, and the cells were incubated for 10min @ 37°C. Cells were washed with PBS and the Fluo-4 stain (Powerload concentrate, Fluo-4 AM 1000x, SGM, probenecid, and Neuro Background suppressor) added before incubation for another 15min @ 37°C and a further 30 min at room temperature (RT). The cells were then washed again with PBS and 50 µl of PBS was added to each well. The culture slides were then mounted on the fluorescent microscope stage and after 10 sec of baseline recording, 200 µl of live imaging solution was added to initiate the calcium flux. Flux duration was measured for 120 seconds.

3.2.9 Data analysis and statistics

Statistical analysis was done using GraphPad Prism (V9.1.1) and Statistica 14.0.0.15 (TIBCO Software inc.). Outliers were identified using the ROUT method (Q=1%), data was determined to be normally distributed or not using the Shapiro-Wilcox and Kolmogorov-Smirnov tests. For the calcium data alone, I had five metrics of calcium function that I compared with each other in the patient groups. Firstly, a test for normality and skewness, followed by a one-way ANOVA for differences between groups, Tukey correction for multiple testing. A two-way ANOVA for differences between male and females, group compared to group and significance was taken as $p < 0.05$. A Two-tailed T-test was used to compare the stratified data as PTSD vs Control and Obese vs Control.

The growth curve data was analysed in the log-growth phase from which the population doubling time (PDT) is derived by the equation PDT = (t2-t1)/3,32 * (logn2-logn1). This data was then used to determine the average PDT for each group measured is days, which was tested for normal distribution before a one-way ANOVA was performed to compare patient groups.

I also assessed the mole ratio of MMP-9 and TIMP-2. The function of MMPs and TIMPs are reciprocal and thus a ratio between the levels of TIMPs to MMPs can be a valuable indicator of the net outcome of this system. The levels of MMP-9 and TIMP-2 were both converted to ng/ml and the molar concentrations[358] were determined using the molecular weight of MMP-9 (92000da) and TIMP-2 (21000da) and then the ratio TIMP-2 to MMP-9 was taken.

The calcium flux data is continuous, and the raw data is expressed graphically. Based on the physiological understanding of calcium dynamics outlined in the introduction I could subdivide the flux data into several categories to be assessed statistically, such as the total duration of the flux (in sec) or the time to peak (in sec). For the purposes here I chose to subdivide the calcium data into the area under the curve (AUC) which represents the total flux capacity of the cell; the Peak area under the curve (Peak AUC) which also represents the total flux capacity, but due to only considering the peak flux, better reflects the difference between normal flux and disrupted flux; Peak Y (highest point of flux) representing the relative speed of calcium flux; peak duration (duration of peak AUC) which represents an indication of the balance between calcium entry and calcium sequestering; and finally recovery which represents the relative speed of recovery after flux.

3.3 Results

Preliminary statistical analysis was performed to ensure differences between the groups were due to CAPS and BMI and not age or sex. ANOVA and Two-way ANOVA were used to determine the difference between groups if any were present, where an ANOVA was used to detect differences between the different patient groups and a two-way ANOVA was used to detect differences between male and females within the different patient groups. For sex as confounder there was no significant difference between males (open circles) and females (filled circles) within the groups for CAPS scores (Figure 3.4). For BMI there was a significant difference between males and females ($p<0.001$) for the PTSD+Obese group only (Figure 3.5). Age showed no significant differences between the groups (Figure 3.6)

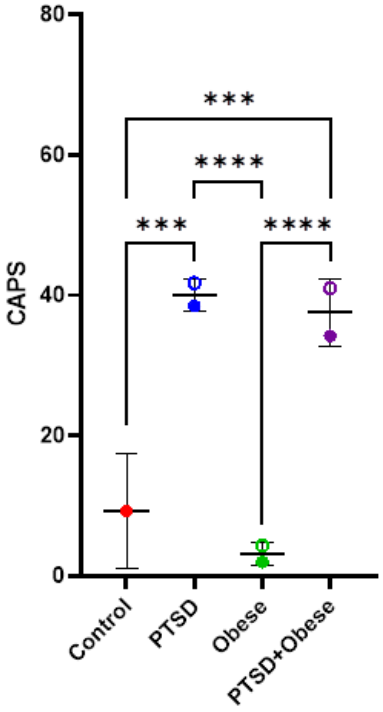


Figure 3.4 CAPS score Male and Female comparison. Solid circles indicate female mean and open circles indicate male mean. Control n=4 females; PTSD n=2 females and n=4 males; Obese n=3 females and n=3 males; PTSD+Obese n=5 females and n=2 males. Data presented as mean ±SD. Statistics: *** - p<0.001; **** - p<0.0001.

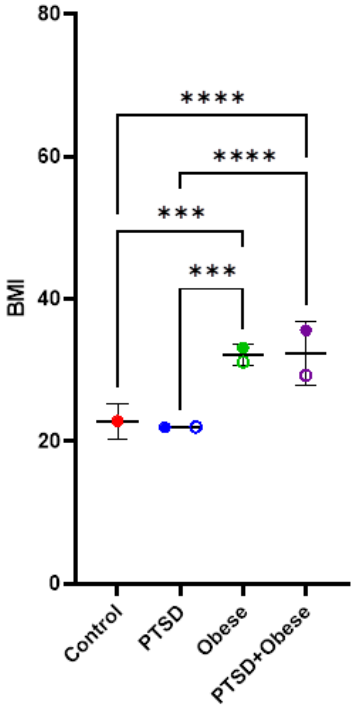


Figure 3.5 BMI Male and Female comparison. Solid circles indicate female mean and open circles indicate male mean. Control n=4 females; PTSD n=2 females and n=4 males; Obese n=3 females and n=3 males; PTSD+Obese n=5 females and n=2 males. Data presented as mean ±SD. Statistics: *** - p<0.001; **** - p<0.0001.

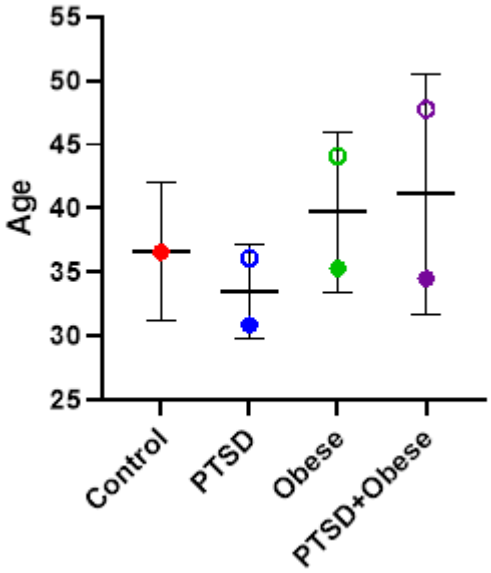


Figure 3.6 Age Male and Female comparison. No significant differences were detected. Control n=4 females; PTSD n=2 females and n=4 males; Obese n=3 females and n=3 males; PTSD+Obese n=5 females and n=2 males. Data presented as mean ±SD

In the control and the condition samples there were equal amounts of exposure to other environmental conditions such as smoking and alcohol use. The samples come from similar socio-economic and ethnic backgrounds. All the controls are also considered trauma exposed controls even if there is no formal trauma diagnosis, which is primarily due to the socio-economic realities of South Africa.

3.3.1 Growth rate

There were no significant differences in growth rate between the groups in either of passages 1 or 2 (Figure 3.7). Passage 2 doubling time seemed more variable, but overall, the growth rate of the fibroblast was normal and consistent across the groups.

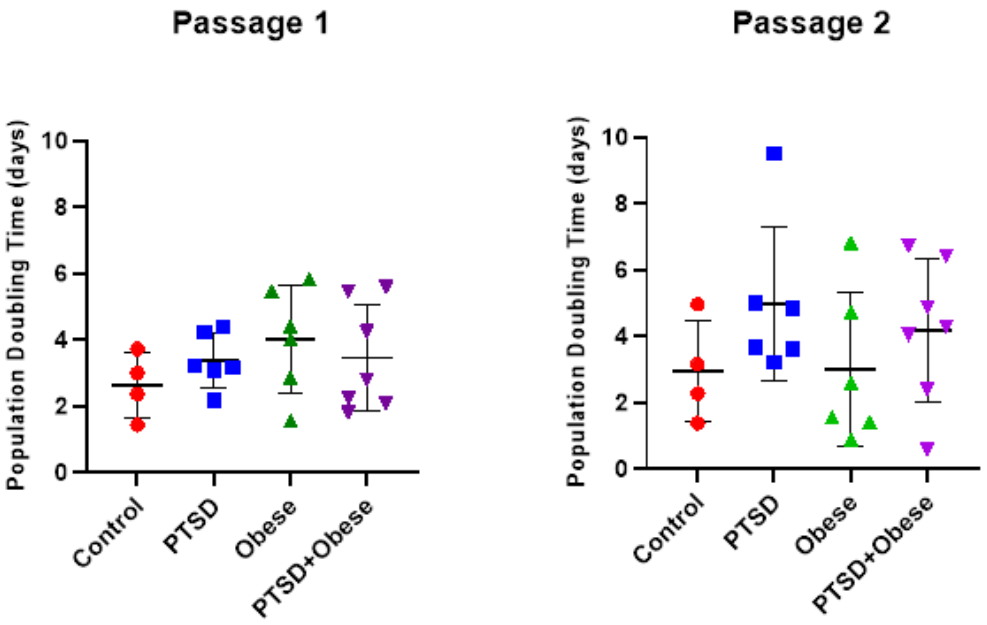


Figure 3.7 Population Doubling Time. Data presented as mean ±SD. Control n=4, PTSD n=6, Obese n=6, and PTSD+Obese n=7.

3.3.2 TIMP-2 and MMP-9 ELISA

Supernatant TIMP-2 levels did not differ between the groups, whilst the MMP-9 did appear to show differences between the groups none were significantly different and were highly variable (Fig 3.8 **A** and **B**). However, again, no significant differences between groups were observed. Furthermore, the ratioed TIMP-2/MMP-9 also showed no significant difference between the groups (Fig 3.8 **C**).

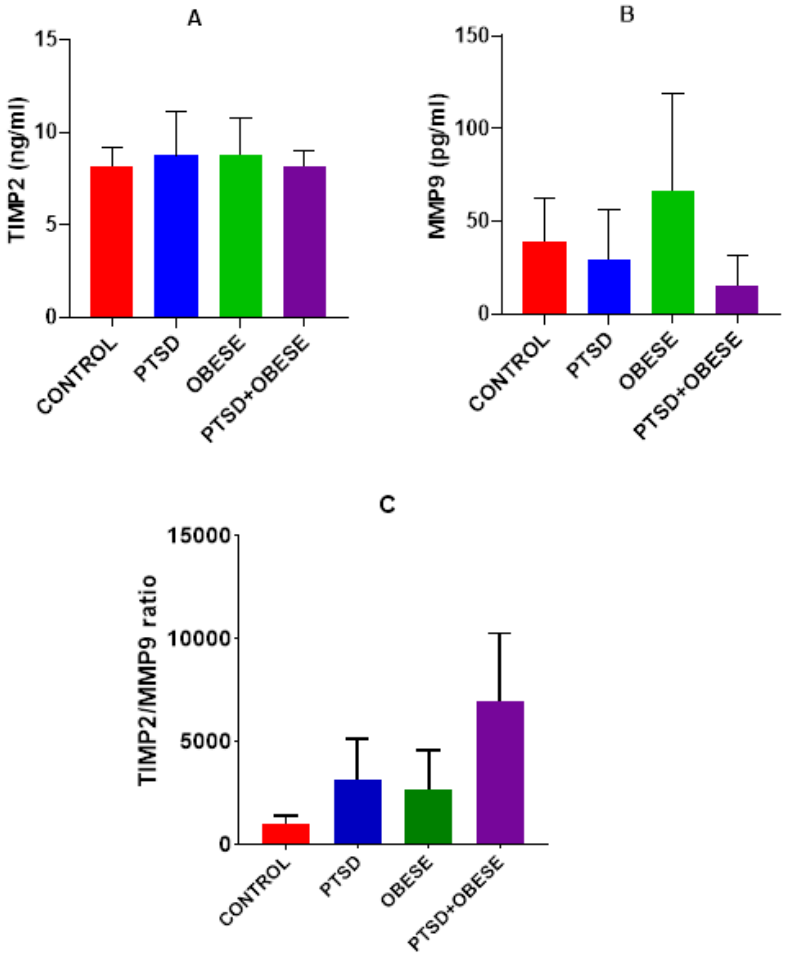


Figure 3.8 TIMP-2 and MMP-9 Elisa Graph A and B are the TIMP-2 and MMP-9 Elisa data respectively and graph C represents the molar ratio between TIMP-2/MMP-9. All data represented as mean and SD. Control n=3. PTSD n=5, Obese n=7, PTSD+Obese n=6.

3.3.3 Flow cytometric analysis of fibroblast function.

There were no significant differences between the group in terms of fibroblast FNDC5, MMP-9, or MMP-3 expression (Figure 3.9).

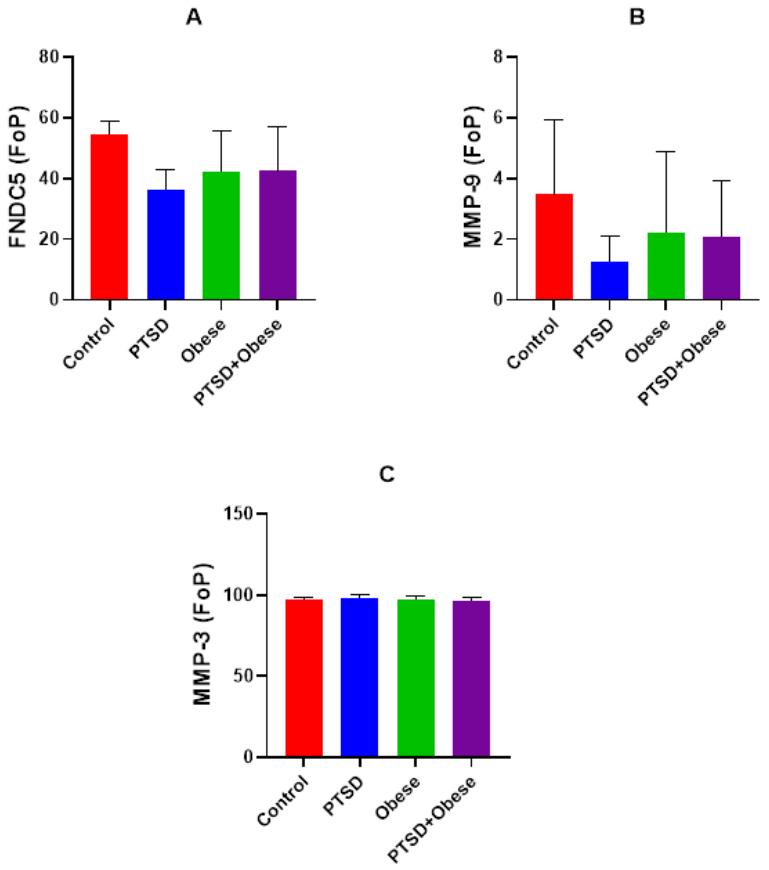


Figure 3.9 Frequency of Parent results of FNDC5 (**A**), MMP-9 (**B**), and MMP-3 (**C**). Data presented as mean ± SD. Control n=4, PTSD n=3, Obese n=6, PTSD+Obese n=4. FoP – Frequency of Parent.

CD31 populations were stratified into CD31+ and CD31- populations. Striking differences was evident in the PTSD group's fibroblasts, which exhibited a significantly higher proportion of the CD31-negative phenotype when compared to all other groups, although only reaching statistical significance vs. the control group ($p<0.05$). In support of an interpretation of PTSD having a different outcome than obesity in this context, the CD31+ PTSD population was also statistically significantly lower than both the control and the obese groups (Figure 3.10)

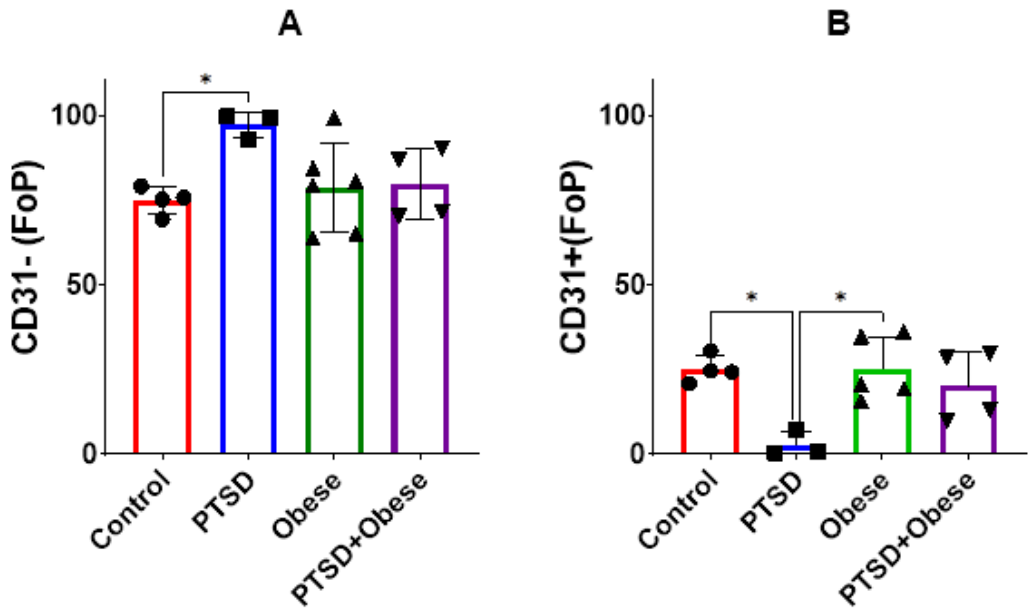


Figure 3.10 CD31 population Frequency of Parent. (* $p < 0.05$) Data presented as mean and SD. Control n=4, PTSD n=3, Obese n=6, PTSD+Obese n=4. FoP – Frequency of Parent.

3.3.4 Calcium Flux

The calcium flux for each patient sample, stratified by group, is presented in triplicate (Fig. 3.11). Some samples have curves missing due to cell loss in the microscopy imaging plate, which occurred during the staining procedure. As far as possible the assay was repeated to account for the loss, however due to sample limitations it wasn't possible for all individuals. The graphs are presented here to show firstly the variability between patients even within the same group, but also to show that repeated measures of the same individual show a high degree of similarity, indicating that the assay is robust and measures a physiologically relevant component of cell function.

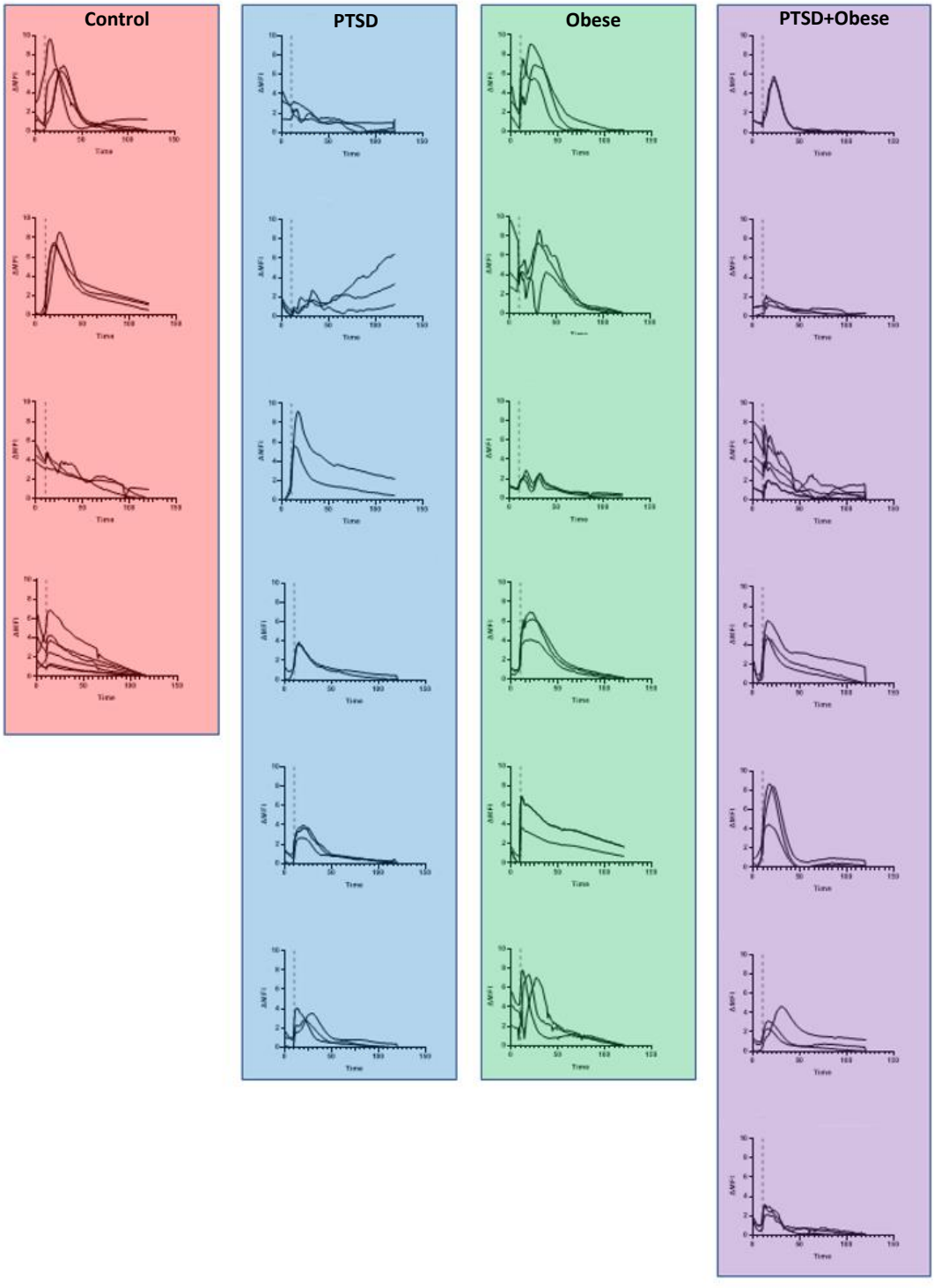


Figure 3.11 Fibroblast calcium flux. The calcium flux is measured as a change in mean fluorescent intensity (ΔMFI) and each repeat of every sample is graphed. Left to right: Red shading indicates Controls, Blue shading indicates PTSD, Green shading indicates Obese, and Purple shading indicates PTSD+Obese groups.

Numerical data for five calcium flux indicators are presented in Figure 3.12. When considering total area under the curve (AUC) for the calcium flux assessment, as indicator of the general magnitude of the response, there were no statistically significant differences between groups (Fig. 3.12 **A**), likely due to the relatively high variability between response trajectories from different patients.

For the AUCs for peak response however, ANOVA returned a main effect of group (p=0,02), with responses for both groups with a PTSD component lower than Control and Obese groups, although only reaching statistical significance when compared to the Obese group (Fig. 3.12 **B**).

For Peak Y – indicating relative speed of calcium flux – the fibroblasts showed a significant difference between the groups (ANOVA main effect of group, p= 0,0113), with the PTSD group exhibiting a significantly shorter Peak Y when compared to both Control and Obese (Fig. 3.12 **C**). A similar picture of shorter flux peak duration (Fig. 3.12 **D**), and shorter time to baseline (Fig. 3.12 **E**) was observed under basal conditions in the groups with a PTSD component, although it did not reach statistical significance.

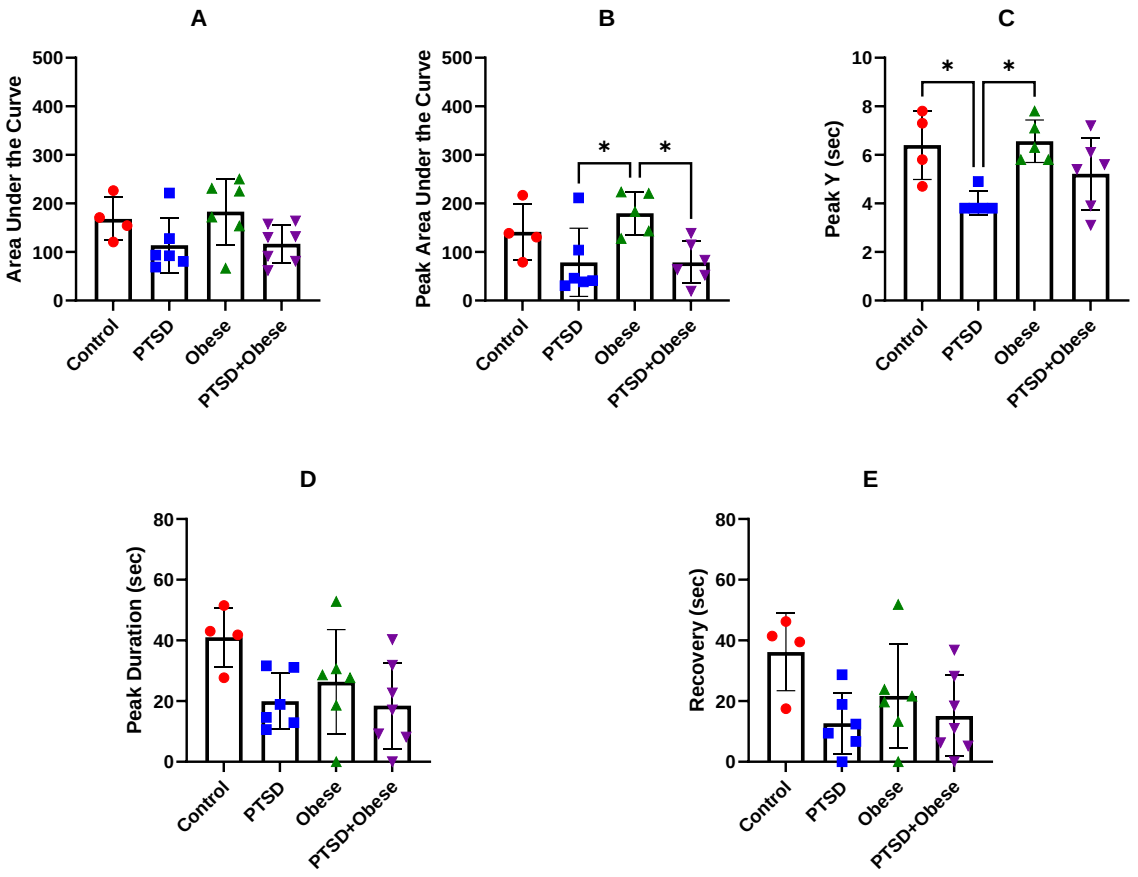


Figure 3.12 Calcium Flux Metrics. Calcium Flux was assessed statistically through five indicators namely Area Under the Curve (**A**), Peak Area Under the Curve (**B**), Peak Y (**C**), Duration (**D**), and Recovery (**E**) which are described earlier. Briefly, AUC is a representation of the total flux capacity of the cell, the Peak AUC is a representation of the acute flux capacity, Peak Y is an indication of speed of calcium influx, Peak Duration is an indication of rate of acute flux, and Recovery is a representation of rate of calcium sequestering. All data tested for normality and outliers were removed before an ANOVA with a Tukey's test for multiple correction was performed. Statistics: * - $p<0.05$.

Given the fact that humans without diagnosed anxiety disorders may also have some degree of anxiety (and thus elevated CAPS scores), and the fact that BMI also naturally distributes across a wide range in non-obese individuals, the decision was made to stratify the calcium flux data for the whole group

(n=23) according to CAPS (Fig. 3.13 Top row A-E) or BMI (Fig 3.13 Bottom row F-J) only. When stratified by CAPS score the Control and Obese groups are pooled together (n=10) since these groups had very low CAPS scores and are PTSD negative controls, while the PTSD and PTSD+Obese groups were pooled together (n=13) and has high CAPS scores and are PTSD positive. For stratification by BMI the Control and PTSD groups were pooled together (n=10) due to their low BMI scores and are thus obese negative controls, while the Obese and PTSD+Obese groups were pooled (n=13) due to their high BMI scores and are considered obese. Increased CAPS score was consistently associated with significantly lower values for all five calcium flux parameters assessed (Fig. 3.13 **A**-**E**), but BMI did not appear to have an effect on calcium flux parameters (Fig. 3.13 F-J).

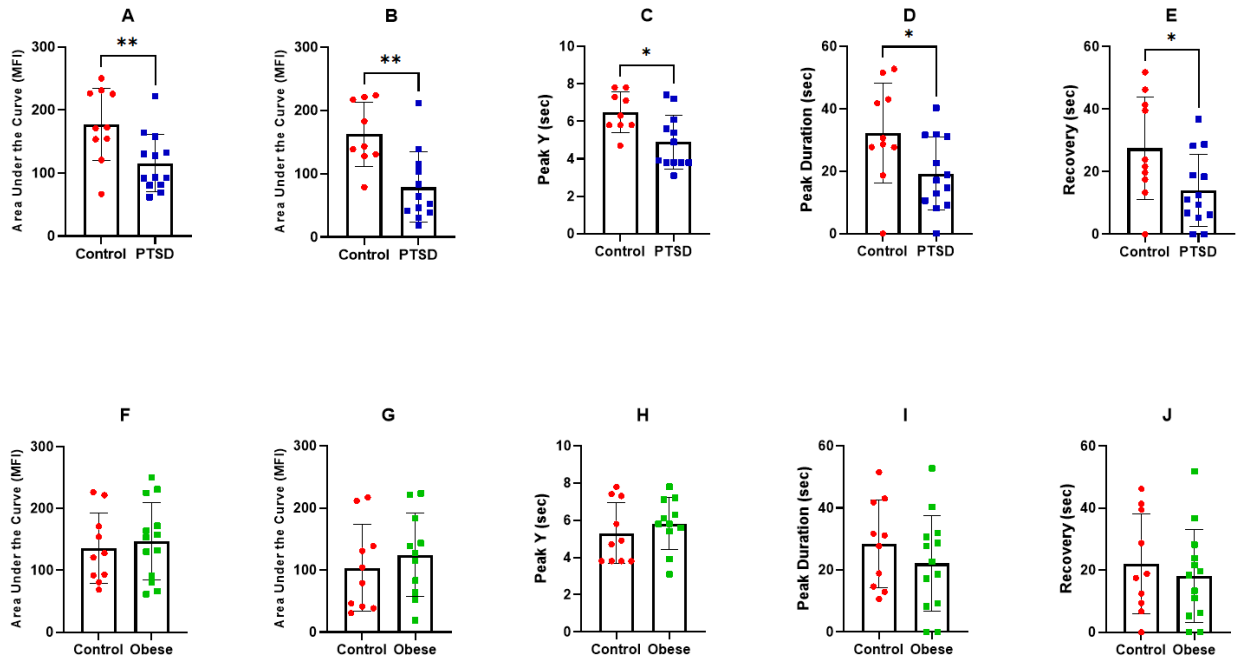


Figure 3.13 Whole population (n=23) stratified by CAPS score (Top row) and BMI (Bottom row) for all five calcium flux indicators namely Area Under the Curve (**A** and **F**), Peak Area Under the Curve (**B** and **G**), Peak Y (**C** and **H**), Duration (**D** and **I**), and Recovery (**E** and **J**). In the top row the Control (n=10) consisted of Control and Obese fibroblast data and PTSD (n=13) consisted of the PTSD and PTSD+Obese fibroblast data. In the bottom row the Control (n=10) consisted of Control and PTSD fibroblasts data and Obese (n=13) consisted of Obese and PTSD+Obese fibroblasts data. All data reported as mean ±SD and was tested for normality and outliers were removed before an ANOVA with a Tukey's test for multiple correction was performed. Statistics: * - p=0.05; ** - p=0.001.

3.4 Discussion

Fibroblasts are emerging as powerful and robust cells to use for transgenic and iPSC research. Much of our understanding of fibroblast function as homogenous, not differing across tissue niche or in function, was due to the use of fibroblast cell lines or fibroblast-like cell lines which recently has been challenged[36]. My data supports the interpretation of significant individual variability even at basal levels, for markers typically associated with fibroblast activation like CD31 and MMP9 as well as the

population doubling time. This is in accordance with the lack of conclusive data reported from *in vivo* human studies[204,212,359,360].

In terms of potential representation of disease states in patient-derived fibroblasts, the doubling time of the patient derived fibroblasts were not significantly affected by presence of PTSD and/or obesity (Fig. 3.2), which was somewhat surprising given the known higher turnover rate of cells at least in models of obesity[361–363]. This disparity could be interpreted in the light of my data as due to differences in allostatic load. In models of obesity the insult is large to elicit a definitive response, however the patient derived cohort is under allostatic pressure that firstly may be at a sub threshold level that does not affect cell growth rates, or there are endogenous mechanisms present *in vivo* that compensate through allostatic mechanisms to preserve cellular turnover rates. In my calculations, lag time was also included, so that values may err on the conservative side. Although some of the samples had to be resuspended after 72hrs due to a lack of adhesion, which could be indicative of mild cryogenic stress and may contribute to the variability of the data seen here, it did not have a significant effect on doubling times.

Further important information was gained from basal characterisation of patient-derived fibroblasts, and in particular in terms of CD31. Basal CD31 expression data suggest that the patient-derived fibroblasts may be exhibiting a different expression profile than the profile described in published literature, where data generation was mostly executed in immortalised cell lines (i.e., not primary cells). For example, published studies commonly report the use CD31 expression as marker for fibroblast activation[204,364,365]However, in the current study, I demonstrate a significant proportion of the cells in my cohort to exhibit CD31 expression in the absence of stimulation (Fig 3.10). In line with the high incidence of inflammatory stimuli in modern life, this data may suggest that even in the control cohort, potential effects of systemic inflammatory influences such as smoking, and alcohol use may have resulted in some degree of basal CD31 expression. This potential difference between immortalised and primary patient-derived cells warrants further investigation to inform on translation value of non-primary cell models.

To my knowledge, this is the first time that patient-derived fibroblasts representative of specific disease states was investigated in this manner. The more heterogenous patient primary fibroblast populations may thus have contributed to the overall variability of the results and decreased statistical power. However, this is not necessarily a limitation of the current study: an alternative interpretation may be that the similarity of cells across different disease states reflects the potent synergistic function between these different fibroblast subpopulations present within tissue niches, to maintain homeostasis. This has implication for the sensitivity of fibroblast culture models to interventions, as different subpopulations of fibroblasts may have opposing responses to an experimental stimulus, which may not be reflected in immortalised cell models. The fact that basal CD31 expression was

altered in PTSD, supports this interpretation. The use of primary cells – and with consideration of potentially confounding disease states - may therefore be a better option for future cell culture studies using fibroblasts specifically.

Despite the model differences, my CD31 data are in line with other reports[213] of decrease in fibroblast CD31 expression in PTSD. This may suggest that inflammatory dysregulation of PTSD is indeed reflected in fibroblast phenotype, especially when considering the role of CD31 as gatekeeper in inflammation. For example, during diapedesis, PECAM-1 (CD31) to PECAM-1 interactions facilitate the transmigration of leukocytes through the endothelial junctions and across the basal lamina. Furthermore, leukocytes that lack CD31 have been shown to get stuck in these junctions[366], exacerbating damaging effects of inflammation at the level of the endothelium. In the context of PTSD, endothelial damage, in combination with the known increased BBB permeability linked to decreased CD31 expression[205] , may pose a mechanism for the propagation of neuroinflammation and PTSD behavioural symptomology. Such a systemic decrease in CD31 expression and its potential harmful effects on endothelium could also link to the increased cardiovascular disease comorbidity found in PTSD[117]. Interestingly, the relative decrease in CD31 expression seen in PTSD was not evident in the Obese group. The fact that presence of obesity even seemed to somewhat counter the PTSD-associated decrease (in PTSD+Obese), underscores the importance of including obesity as a confounder in PTSD research.

From this data there seems to be no modulation of TIMP-2 or MMP-3 under conditions of PTSD and obesity. This was surprising as other authors have reported significant changes to the levels of MMP-3 measured under conditions of chronic stress[367]. Interestingly a cohort of human subjects with and without obesity showed an increase in both MMP-1 and MMP-2 in the obese and overweight groups but found no significant increases or decreases in MMP-9 and MMP-3 plasma levels[368]. Another study of mild chronic stress in rodents reported increased expression of MMP-9 and MMP-3 mRNA levels whilst reporting that the levels of TIMP-1 and TIMP-2 remained unchanged in response to the mild chronic stress[367]. Together, these studies highlight the complexity of MMP signalling, as well as suggesting these responses to be highly dependent on the disease context. From my data there is no clear effect of PTSD or obesity with relation to MMP or TIMP function in these patient derived fibroblasts, however there may be some effect with regard to MMP-9 function that a larger cohort may be able to elucidate.

One of the difficulties of research into calcium function is that it is so abundant and ubiquitously used that it has many different redundancies that ensure a tight homeostatic control, which means effect of disruption are difficult to determine at a subclinical level until there is catastrophic failure or disruption. One of the positives is that there is a lot of information on how these calcium mechanisms work and as such we are possibly in an ideal time to start refocussing on understanding the

fundamental aspects of cellular biology and the role, they may play in disease pathology. The CD31 data presented here indicates a rationale for cellular adhesion proteins and biomechanics to play a large role in the increased proinflammatory profile of systemic conditions such as obesity and PTSD and so too there is a rationale here that PTSD has a systemic effect by altering even the calcium dynamics of fibroblast, by decreasing the overall flux capacity of these cells. Electrophysiological methods have implicated calcium channel deficits in volumetric differences in the neocortex, hippocampus, and periaqueductal grey of *Cacna1c* knockout mice as well as increased anxiety[369]. These effects were reported to be due to decreased spontaneous Ca^{2+} activity in neural progenitors, which impacted brain development in these mice. In my data, there is a clear effect for PTSD on the calcium flux capacity of somatic cells. In Figure 3.13 when the data is stratified by CAPS score every calcium metric shows a significant deviation from the control. There is a general decrease in the flux capacity of the cells shown by a decrease in the AUC and peak AUC of the high CAPS samples (Fig 3.13 **A** and **B**). There is also a decrease in duration of the flux as measured by the duration of the peak. The faster time to peak as measured by the Peak Y and the faster recovery time combined with the shorter duration of the peak all indicate a reduced flux capacity for High CAPS fibroblasts (Figure 3.13 **C**, **D**, and **E**). When the data is stratified into the four main groups of the study there is still an effect for the PTSD group in the Peak AUC and Peak Y fibroblasts (Fig 3.12 **B** and **C**). Interestingly the Peak AUC was different for both the PTSD and the combined PTSD+Obese group compared to the Obese group and the Peak Y showed a faster peak for the PTSD compared to both the Obese and Control samples. Physiologically a larger Peak AUC combined with a faster Peak Y would indicate a rapid and large calcium flux, facilitated by CICR mechanisms such as ER calcium store release and calcium channel activation. The combination of a smaller Peak AUC and faster Peak Y in the PTSD fibroblasts indicates a lower calcium flux overall (this is visible in Fig 3.11, Blue panel), but the rapid Peak Y could indicate that there is continuous increased basal calcium signalling that reduces acute flux capacity. Increased ER calcium chaperone molecules in the amygdala of rats subjected to a single prolonged stress indicates upregulation of calcium regulation mechanisms in response to stress[233]. A similar upregulation of calcium chaperone proteins could explain decreased flux capacity in PTSD fibroblasts as a protective response to continued transient calcium signalling in PTSD. These data indicate that PTSD status may decrease calcium flux capacity of fibroblasts. Although the other metrics do not show any significant differences, there is a clear trend for the PTSD and PTSD+Obese groups to have a decreased flux capacity. A recent publication reported decreased cytosolic Ca^{2+} levels, measured by fluorescence, in fibroblasts isolated from Alzheimer's Disease (AD) patients compared to controls[355]. The authors also report a lack of studies investigating intracellular calcium handling in the context of AD, which, from my review of the literature, extends to other mental health disorders. My data thus adds to the relative paucity of information in this context, by clearly demonstrating an association between PTSD and relatively decreased calcium flux.

3.5 Conclusion

PTSD is significantly associated with a decrease in calcium flux capacity in patient fibroblasts. Although many studies use calcium flux as a measure for some cellular signalling capacity that is modulated under certain conditions, such as receptor knock-out or drug intervention, there are relatively few studies reporting on disease state and innate calcium flux potential, perhaps due to difficulties already mentioned. This is to my knowledge one of the first studies to report innate calcium flux differences between patients and controls in the context of PTSD. Further investigation into calcium flux dynamics of PTSD could expand on GWAS candidates like CACNA1C or other calcium channels and receptors of interest. It is not clear if the decrease in calcium flux capacity is related to an innate decrease in calcium handling or availablity, or if there is a increase in the demand for calcium signalling and a lack of capacity to meet this demand. Furthermore, there is at least some evidence that obesity decreases calcium flux capacity under increased work load, which underscores the need to control for obesity as confounder for PTSD related research.

My data thus far supports the idea that PTSD constitutes a prolonged systemic dysregulation that dysregulates homeostatic mechanisms over time resulting in increased allostatic load. Current treatment of PTSD fails to take into account the somatic consequences of PTSD[22] and pharmacological treatment of PTSD is sub optimal[370,371], suggesting that a novel approach to understanding PTSD as a systemic disorder could elucidate systemic treatment that better aligns with disease progression and outcomes. One such candidate is the trace aminergic system, specifically TAAR1, which has the potential to modulate aminergic signalling pathways, redox pathways, as well as neuroinflammatory pathways, which is discussed in the next section.

From a more practical perspective this data supports the use of patient-derived primary cells to elucidate more physiologically relevant aspects of disease progression. Although the sample sizes are difficult to increase given the time-consuming nature of culturing primary cells, there is still a strong rationale here for using these types of models to address some of the short comings of cell line based translational issues. The differential responses of these fibroblasts also cement the notion of *in vivo* heterogeneity as a major consideration when translating cell culture-based models of human disease.

Chapter 4:

Potential role of trace amines in decreasing allostatic load

4.1 Introduction

Trace amines (TAs) are a resurging area of interest in mental health disorders after the discovery of endogenous receptors, trace amine associated receptors (TAARs), of which TAAR1 is of particular interest[295,372]. Three TAs that preferentially agonise TAAR1, albeit at different binding affinities[306], has been chosen for screening as potential anxiolytic treatments using zebrafish larvae[68]. The anxiolytic potential and understanding of the potential physiological role of TAs and TAAR1 make TAs a viable treatment option for decreasing peripheral allostatic load. To that end the aim of this section is to assess the anxiolytic potential of three TAs, β-PEA, TRP, and TYR using a ZF larval model of locomotion.

4.2 Materials and Methods

4.2.1 Experimental Animals

ZF larvae at <5 days post fertilization was used to explore the anxiogenic and anxiolytic effects selected TAs. Wild type ZF larvae were raised in accordance with the Stellenbosch University Animal Research Ethics guidelines and all protocols were ethically cleared prior to experimentation (Ethics number ACU-2021-21995).

4.2.2 Trace amine exposure

β-phenylethylamine (β-PEA) (Torronto Research Chemicals: CatLog# P321335), tyramine (TYR) (Torronto Research Chemicals: CatLog# T898500), and tryptamine (TRP)(Torronto Research Chemicals: CatLog# T894600) at low (1,5625µM, 3,125µM, and 6,25µM), moderate (12µM, 25µM, and 50µM), and high (100µM, 200µM, and 300µM) doses were added to standard E3 media in a serial dilution and were administered to larvae for one hour (1hr) and continued for 24hr by immersion. Larvae were individually pipetted into 96 well chambers (n ellucidate = 12 at least per treatment group). The larvae were left to acclimatize for a period of at least 30 minutes after the pipetting to relay any acute stress effects of handling. The larvae were then treated with the TA for one hour and 24 hours before behavioural assessment.

4.2.3 Activity tracking

Daniovision hardware and Ethovision software (Noldus, Germany) were employed to track the movement of the ZF larvae during the behavioural assays at a capture rate of 25 frames per second. Experimental setup was as follows: baseline recording for 20min, followed by 1 min of exposure to

100% intensity white light, followed by 4 min of darkness (Fig4.1). Ethovison settings applied were as follows: a minimum movement distance of 0,2mm was set as threshold; time bins for data were set at 10-second intervals.

Figure 4.1 depicts the expected behavioural response expected in the anxiety model that was employed here (periodically Fig 4.1 will be shown again to assist the reader). Behaviour was assessed statistically at the following time points: Point A represents the mild increase in activity in response to movement of the larvae from the incubator to the activity tracker chamber at the start of the experiment. Point A was analysed for all TA, because it represents a milder anxiety response and although not as controlled as the light dark transitions between 96-well plates assessed, the magnitude of response is similar. (For this reason, all experimental groups are always represented on all plates, in cases where the use of multiple plates is required.) Point B was taken after larvae had settled down and returned to normal activity levels and before the light sequence was initiated. Basal activity was recorded for the last three minutes of this 20-minute period. (Point B). After 20min, bright white light (part of the equipment hardware) is switched on for 1 min at 100% intensity. Point CD represents the initial startle response to sudden light exposure, the magnitude which was quantified as the fold-change from C to D. Point E presents the lowest movement recorded in the 10 seconds immediately after the light was switched on, when the larvae exhibit a relative "freezing" behaviour. Finally, point F represents the peak hyperlocomotion response that ensues on switching off the light again. EF was again quantified as fold-change from E to F, to represent the extent of the hyperlocomotion response.

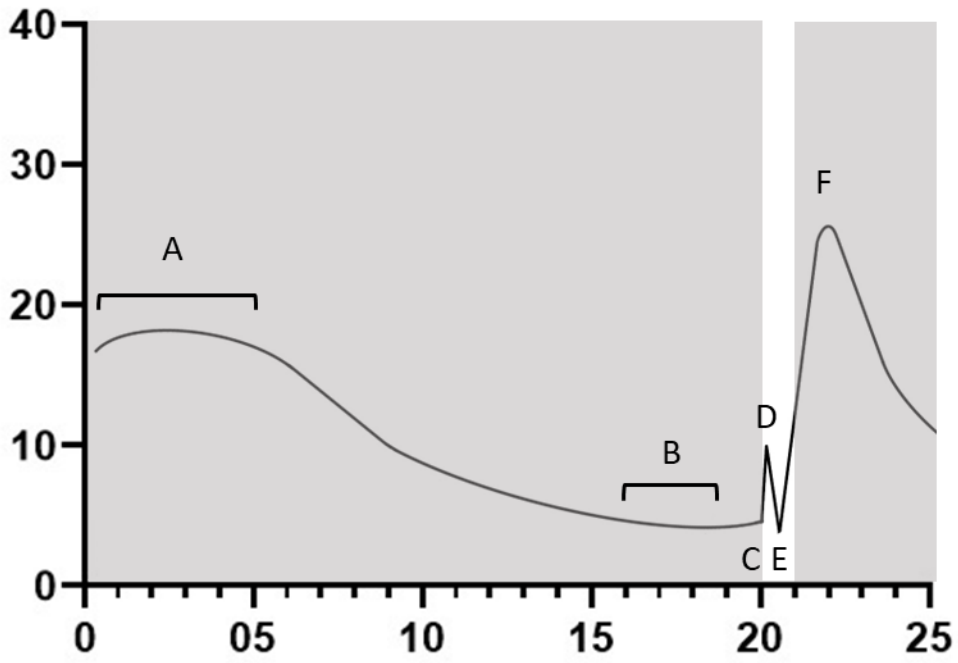


Figure 4.1 Representative image of the expected behavioural response in the anxiety model. A is the initial mild response to handling during the setup of the experiment. B is the baseline movement after an acclimatization period. C is the point where

the light is switched on. D represents the highest point of the startle response and is the average value taken for 10 sec after the light is switched on. E is the is the lowest value of the freeze response. F is the peak hyperactivity response in the dark period and is the average value taken for 10 sec after the light is switched off.

4.2.4 Statistical analysis

Data is reported as average total distance moved for each time bin ± SEM or ±SD. All data was checked for outliers using the ROUT method (Q=1%) and assessed for normality using the Shapiro-Wilk and Kolmogorov-Smirnov test. For all TA treatment assays, ANOVA was used to determine the main effect of treatment and a Tukey post hoc test to determine the significant difference between each group. In cases where data was not normally distributed, a Dunn's post hoc test was employed. Level of significance was set at $p < 0,05$.

Published data on ZF movement, and particularly light/dark transition models, use point F (Fig 4.1) or the difference (Δ Peak) between point E and point F (Fig 4.1) as the stress or anxiety response[309,317]. The Δ Peak was assessed here as well, however there were very little significant differences to report on β-PEA and TRP and as such, point **A** (Fig 4.1) was also assessed for all TA as a mild stress or anxiety response. This decision was also taken because allostasis happens in response to long term mild stressors, which makes data point **A** an appropriate choice. Data was assessed in 1-sec bins to increase the sensitivity of analyses. However, analysis at this level introduced many zero values for the parameter "distance moved", as zebrafish larvae are not continuously moving, which created large variability in data. Therefore, a portion of the data was analysed in 1 sec, 2sec, 3sec, 5sec, 10 sec, 20sec, 30sec, and 1 min bins to determine the binning strategy. The 10-sec bin was selected, as it was sufficiently sensitive to reflect the startle effect in all data sets, while allowing inclusion of sufficient data points for determination of change (Δ) and fold- change values.

For the more in-depth analysis of tyramine-associated responses, data point **B**, the startle effect (fold change from data point **C** to **D**), and ΔPeak (data point **F**-**E**) is additionally reported.

4.3 Results

Qualitatively, zebrafish larvae seemed to exhibit a dose-dependent treatment effect for all TAs assessed, on behaviour (level of activity), which differed between TAs, from a potentially hyperlocomotive (greater movement than control in response to light/dark transition) to hypolocomotive (less movement than control in response to light/dark transition) outcome.

For example, Fig 4.2.1 shows the behavioural response to exposure to different doses of β-PEA for both 1hr and 24hr exposure over the whole dose range. When considering behavioural response to the low doses, β-PEA seemed to have an initial hypolocomotion effect that returns to control levels fairly quickly. After 1hr exposure to the moderate doses of β-PEA (Fig 4.2.1 **A**), although there was a mild dampening of the hyperlocomotion response, a seemingly hypolocomotive response was seen even

before exposure to the bright light, as activity was increased relative to controls. However, this was transient, as the activity profile after 24hr exposure (Fig. 4.2.1 **B**) appeared similar to that seen after 1hr exposure. At 24hr low dose exposure (Fig. 4.2.1 **B**), the relative hypolocomotion effects of β-PEA were clearly visible across the data acquisition period for all doses. Together, these results clearly show that determination of appropriate dose is essential, to avoid crossing the threshold from a relative hypolocomotion to a hyperlocomotion outcome.

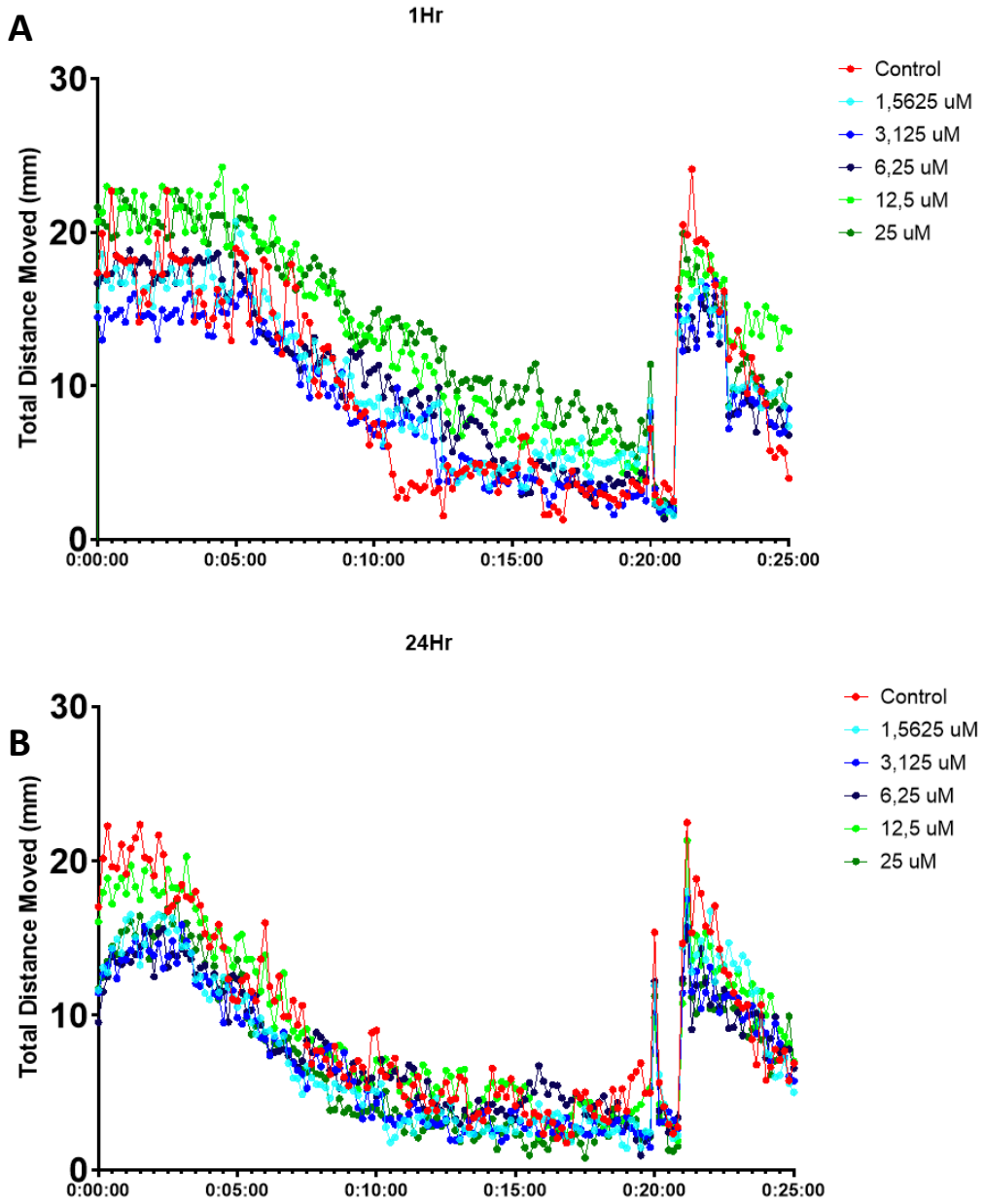


Figure 4.2.1 Effect of 1hr (A) and 24hr (B) exposure to different doses of β-PEA on zebrafish larval basal activity, as well as in response to a 1-min bright light stimulus administered at 20 min from start of experiment. - Data is presented as averages (n = 24 per treatment group) and in 10sec time bins. Error bars have been omitted for clarity

Figure 4.2.2 shows the dose response of TRP for both 1hr and 24hr exposure for all doses. Low dose exposure for 1hr seems to elicit a hypolocomotion response initially for all doses, while some evidence of hyperlocomotion is seen after zebrafish had settled, as well as during the dark phase following bright light exposure.

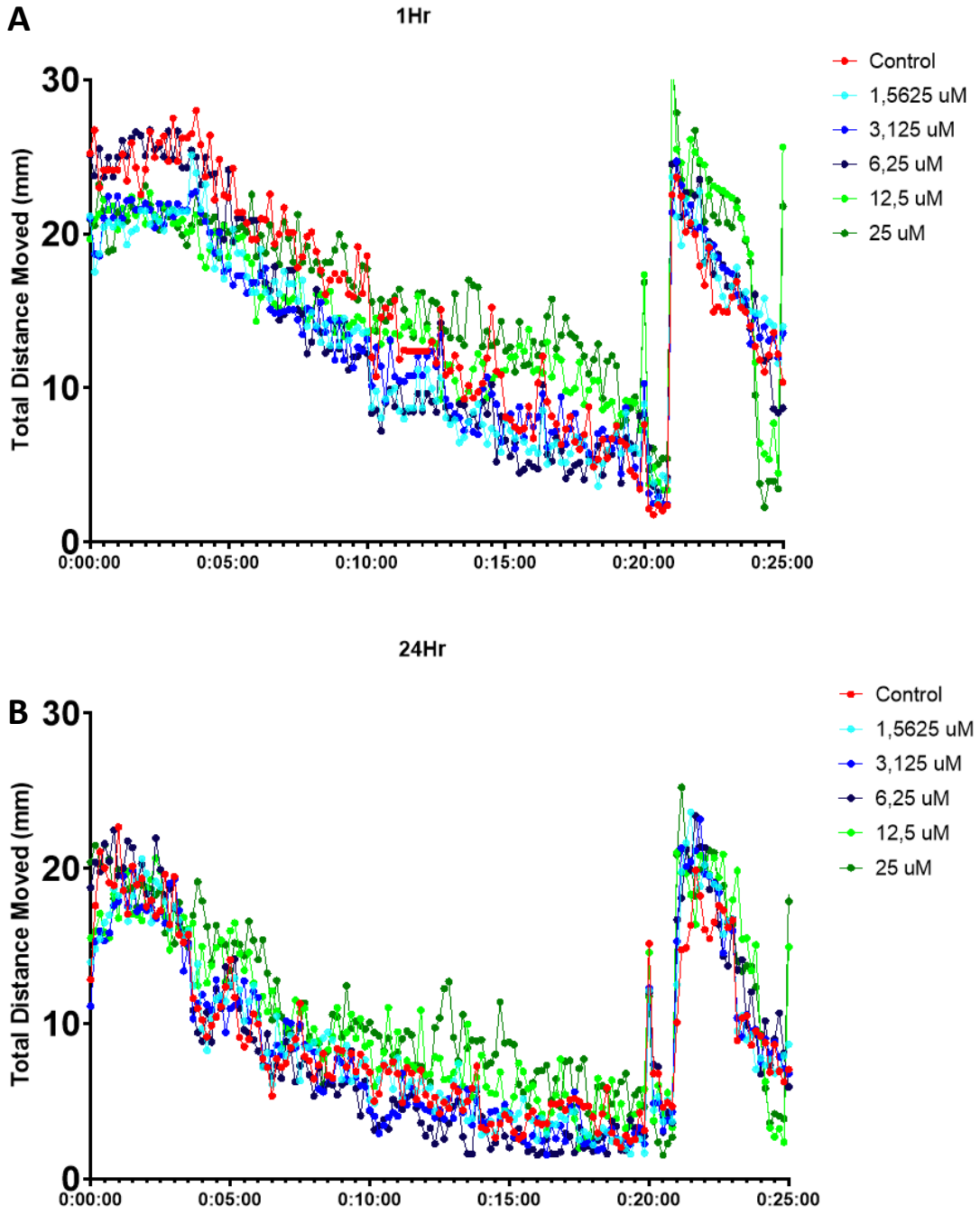


Figure 4.2.2 Effect of 1hr (A) and 24hr (B) exposure to different doses of TRP on zebrafish larval basal activity, as well as in response to a 1-min bright light stimulus administered at 20 min from start of experiment. - Data is presented as averages (n = 24 per treatment group) and in 10sec time bins. Error bars have been omitted for clarity

Figure 4.2.3 shows the dose response of TYR for both 1hr and 24hr exposure across all doses. Generally, TYR exhibited a similar mild inhibitory effect on locomotion as observed after β-PEA exposure, albeit perhaps somewhat milder for all doses except the 3,125uM dose which seemed to induce hypolocomotion throughout the experiment. Furthermore, this trend continued for the 24hr exposure, where even the light/dark anxiety stimulus showed a reduced hyperlocomotive response at the 3,125uM dose.

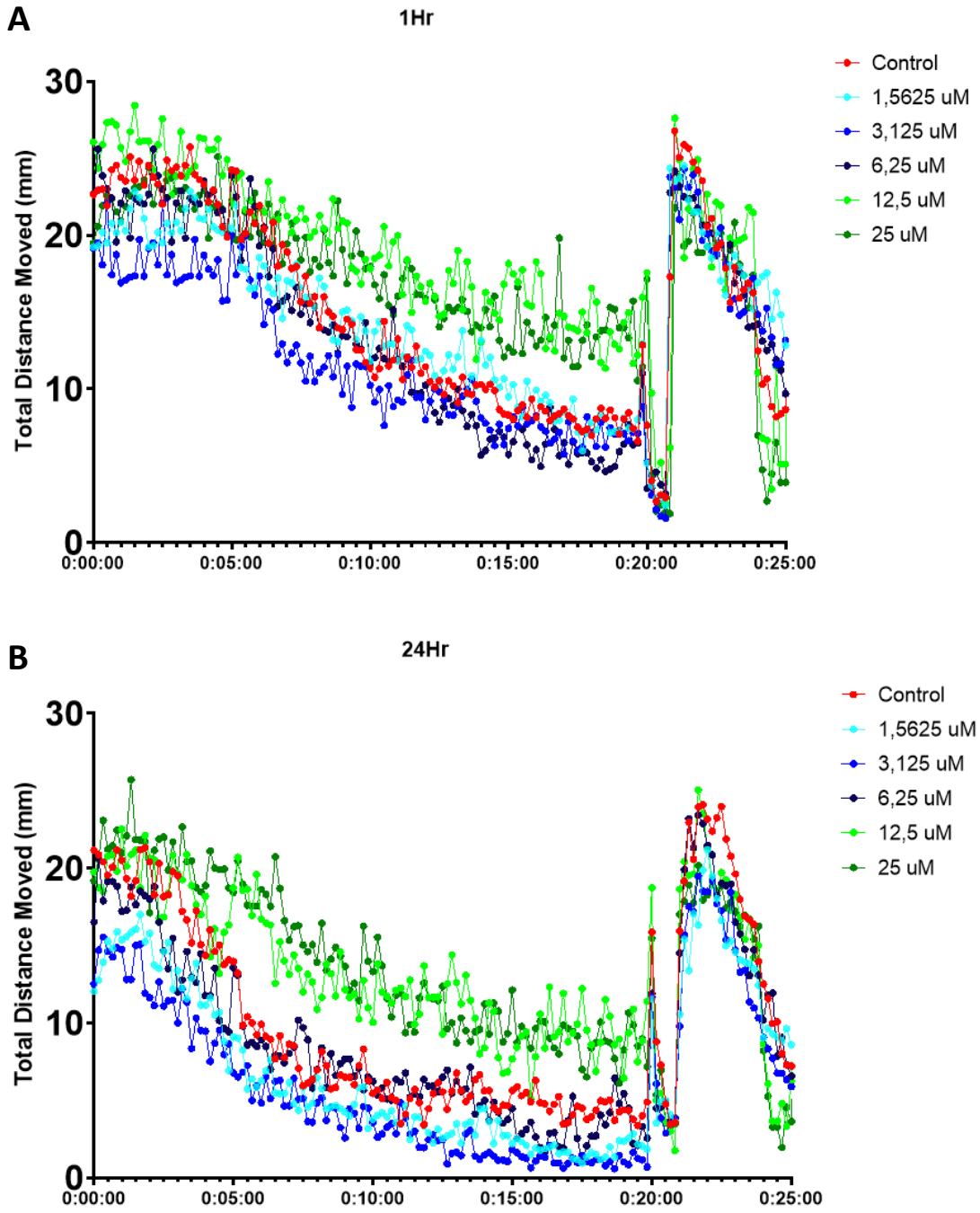


Figure 4.2.3 Effect of 1hr (A) and 24hr (B) exposure to different doses of TYR on zebrafish larval basal activity, as well as in response to a 1-min bright light stimulus administered at 20 min from start of experiment. - Data is presented as averages (n = 24 per treatment group) and in 10sec time bins. Error bars have been omitted for clarity

Quantitated behavioural data confirmed qualitative observations. When considering data point, A, at the start of the experiment, β-PEA appeared to have hypolocomotor effect at low doses in the qualitative assessment above (Fig 4.2.1), however only the 24hr treatment of 3,125uM was found to be significantly decreased compared to the controls (Fig 4.3 **B**)

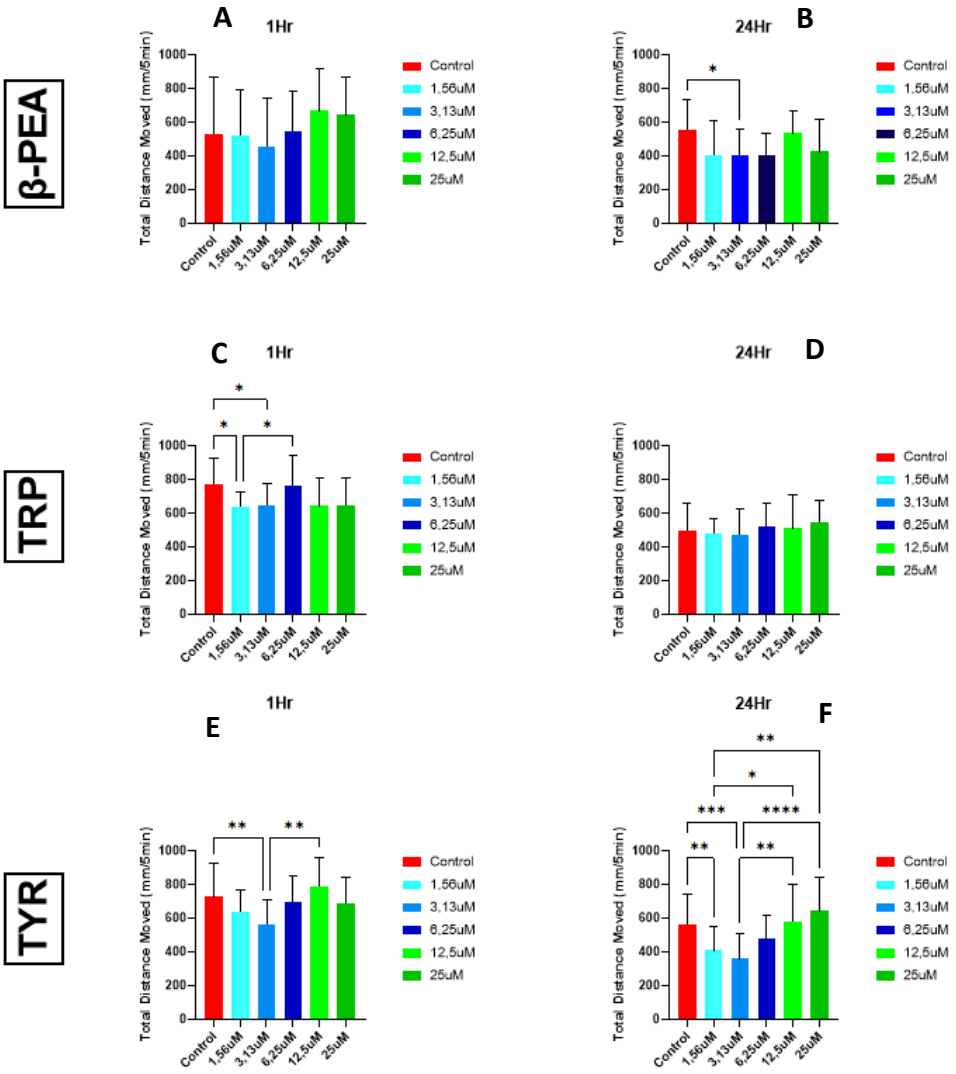
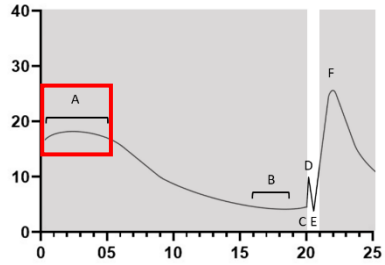


Figure 4.3 Total Distance Moved 0-5min Data point A after 1hr and 24hr exposure to different doses of β-PEA (**A** and **B**), TRP (**C** and **D**) and TYR (**E** and **F**). Data shown as mean ± SD, with n=24 (red and blue) and n=12 (green). Doses rounded to two decimals. Statistics: * - P<0.05, ** - P<0.01, *** - P<0.001, **** - P<0.0001.



1hr TRP treatment of 1,5625uM and 3,125uM significantly reduced locomotor activity compared to controls and interestingly the lowest dose also had significantly lower locomotor activity than the 6,25uM dose (Fig 4.3 **C**). This effect was not conserved for the 24hr treatment period (Fig 4.3 **D**).

Treatment with TYR significantly reduced locomotor activity at the 3,125uM dose compared to controls and the 12,5uM dose (Fig 4.3 **E**), with an interesting curve shape that decreases locomotor activity in a dose dependant manner that reverses to an increase in locomotor activity in a dose dependant manner. This same pattern is observed when treating for 24hr with TYR (Fig 4.3 **F**) with the low dose decreasing locomotor activity in a dose dependant manner and from the 6,25uM dose the locomotor activity increases in a dose dependant manner, potentially indicating some kind of threshold dose for TYR.

Figure 4.4 shows the basal activity (Data point **B**, Fig 4.1), startle effect (Fold change data point **C-D**), and Δ Peak for both the 1hr and 24hr durations treatment with TYR.

Data point **B** showed a steady decrease in basal activity levels in response to 1hr treatment of increasing (lower) doses of TYR, however the moderate doses increased activity when compared to controls and low doses (3,125uM and 6,25uM) (Fig 4.4, **A**). 24hr treatment showed significant hyperlocomotion compared to controls and the low doses, however the threshold for relative hypolocomotion to hyperlocomotion is at a dose lower that 6,125uM (Fig 4.4 **B**).

The startle response (fold change of **C** to **D,** Fig 4.1, reshown below) showed a significant decrease in response for 1hr treatment with 3,125uM and 12,5uM doses compared to controls (Fig 4.4 **C**). The 24hr treatment showed a significant increase in the startle response at the 3.125uM dose compared to the 25uM dose (Fig 4.4 **D**).

In the case of the ΔPeak for 1hr TYR treatment there were no significant differences between the groups, however there is a steady anxiolytic trend where the movement decreases with increased dosage up to a point. The 24hr low dose TYR group showed a significant decrease in the ΔPeak for the 1,5625µM dose when compared to both the control and the 6,25µM exposed larvae (Fig 4.4 **F**).

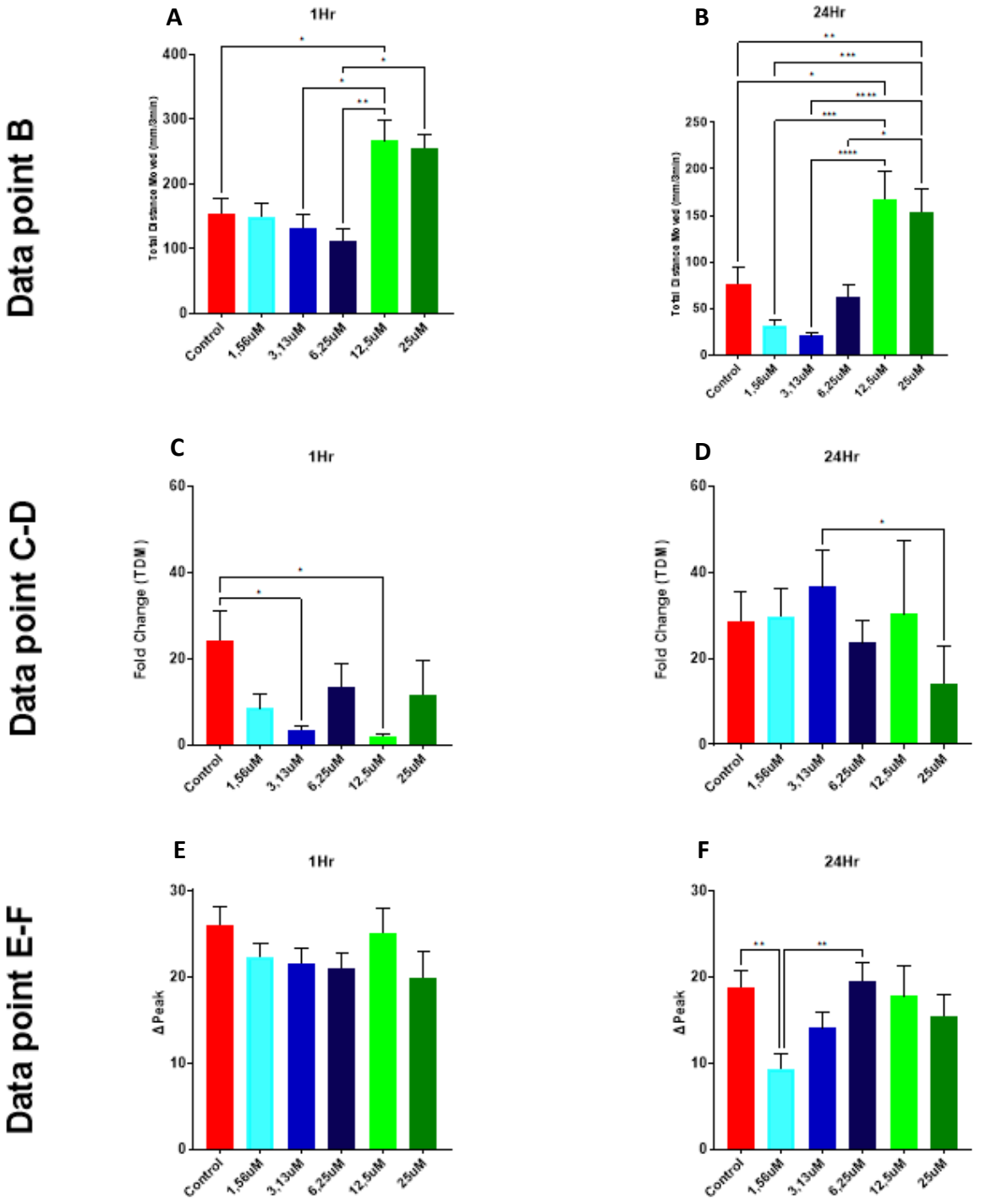
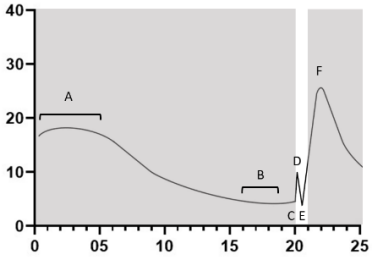


Figure 4.4 In depth TYR associated responses of Data point B, startle response, and ΔPeak (Fig 4.1, redisplayed below). Data point B represents the basal response after the ZF larvae have settled (panel **A** and **B**). Data point C-D represents the startle response measured as fold change in movement in response to bright light (panel **C** and **D**). Data point E-F, measured as the difference between F and E or ΔPeak (panel **E** and **F**). Data shown as mean ± SD, with n=24 (red and blue) and n=12 (green). Doses rounded to two decimals. Statistics: * - P<0.05, ** - P<0.01, *** - P<0.001, **** - P<0.0001.



4.4 Discussion

The data presented here suggest firstly that despite the anxiolytic properties reported for β-PEA[373,374], of the trace amines tested, β-PEA may not be the best candidate anxiolytic treatment. Secondly, TRP as a treatment, although inducing hypolocomotion – which is interpreted as an anxiolytic outcome - at low doses in response to a mild stress, was too variable at the other data points to be a viable candidate. However, TYR induced a consistent hypolocomotive response in ZF larvae across all the data points, indicating it has potential to modulate the effect of moderate and more severe stress. This made TYR the best choice moving forward as a potential treatment for modulating the allostatic load of PTSD.

In terms of the TA included in the current study, PEA is one of the most well studied TA to date and is one of the listed endogenous agonists of TAAR1[294,306,375]. Early work described PEA as an endogenous amphetamine[376] and indeed amphetamines and phenethylamine can have the same effect in the central nervous system in particular with regard to dopamine transporter function[377–379]. Early attempts at PEA administration showed amphetamine like effects and although oral ingestion is not efficient due to the function of MOA, PEA was still self-administered in animal models of addiction[285]. In the current study, ZF larvae exposed to lower doses of PEA, showed evidence of the generally accepted anxiolytic effect of PEA[373,374] firstly at the start of the experiment, where they exhibited a milder hyperlocomotion in response to a mild disturbance in their environment, as well as at the end of exposure to bright light, when they again had a dampened hyperlocomotion response to the anxiety-provoking bright light. It is perhaps important to firstly explain the interpretation of changes in locomotion in this model. When combining treatment by immersion and the ZF light-dark transition assay as employed here, a treatment dose-dependent decreased locomotion (e.g. at 1.56 and 3.13 uM), followed by normalisation (e.g. as seen at 12.5uM) and second depression phase (as seen at 25uM) is indicative of firstly an anxiolytic outcome, followed by avoidance behaviour (i.e. zebrafish senses too much treatment in their environment and increases movement in an attempt to escape the offending stimulus) and finally a toxicity outcome[309,313,315,317]. Considering the relatively short half-life of most TAs, this data highlights the necessity for fine control of dose, should PEA be developed as potential anxiolytic.

Tryptamine is a relatively new compound to investigate for neuromodulation properties. Tryptamine has been generating more interest in recent years for its shared biosynthesis and structure to serotonin, melatonin, dimethyltryptamine, and psilocybin[380–383]. These compounds have effects on dopaminergic, serotonergic, and glutamatergic systems and are of interest for their neuromodulartory effect. TRP is a tryptophan derivative and has structural homology to serotonin[384]. There is evidence that TRP is a weak or partial agonist of TAAR1 and that many of the neuromodulation effects of TRP is mediated

through 5HT receptors[385] and is likely the explanation for competing with other amines such as serotonin for reuptake. Interestingly TRP is an endogenous ligand of the $5HT_{2A}$ and $5HT_{1B}$ receptors that are both expressed in immune cells, suggesting a potential immune related function for TRP[385]. Despite this literature, suggesting TRP as candidate to exert anxiolytic effect, TRP exhibited the least impressive anxiolytic profile of the three TAs assessed. This is potentially due to the extremely short *in vivo* half-life of TRP (0.24 nmol/g/h)[277], and potential for TRP to compete with other biogenic amines for reuptake and sequestering in axon vesicles, which could mean *in vivo* clearance mechanisms may be too effective[269,270]. Furthermore, as with PEA, higher doses seemed to elicit a toxic outcome, suggesting that similar caution should apply when considering developing TRP as therapeutic in the context of anxiety and/or PTSD. Interestingly the 6,25uM dose and the controls for the 1hr both showed increased locomotor response in comparison to the lowest dose and the 24hr treatment, although not significant, did show the same trend with increased locomotion around the 6.25uM dose. This threshold effect at 6.25uM is potentially indicative of a differential signalling mechanism at low dose which must be considered when designing experiments, especially in the light of TRP potentially signalling through multiple agonistic or partial agonist interactions with other types of aminergic receptors as mentioned above.

TYR showed a consistent anxiolytic effect at lower doses, which was carried through from the 1hr exposure to the 24hr exposure. This likely indicates a different receptor binding affinity for TYR in comparison to PEA and different receptor type to TRP, which may speak to a differential preference for signalling through TAAR1, as TYR is currently classed as the most potent TAAR1 agonist of the endogenous TAs[306]. PEA as the most studied TA was the logical starting point but subsequently has been shown to have effects that are mediated through non TAAR1 related pathway such as TAAR2 and may have a higher affinity for TAAR2 than TAAR1[306]. TRP has the same difficulty of non-specific binding and does not primarily signal through TAAR1, with a much higher affinity for TAAR2 and other non-TAAR receptors such as the 5HT receptor family. More work is needed to understand the effects of dose in the context of TYR, and TAs in general, but the fact that TYR has only one known endogenous receptor, TAAR1, may already shed some light on the data seen here.

4.5 Conclusion

In summary, the main purpose of this experiment was to determine effective ranges for TA dosing of ZF. Data seems to clearly point toward TYR as the best candidate among the TAs I have assessed. Of interest, TYR does not cross the blood-brain-barrier[308]. The effects of TYR in the brain can at this stage be ascribed to de novo synthesis by the same biochemical pathways that synthesize dopamine and serotonin through the action of AADCs and other enzymes[272]. In the ZF model, it is not clear yet if TYR can cross the ZF BBB, which is established from 4dpf[386]. However, the effects of TYR can

still be explained by sympathomimetic peripheral effects or increase in other intermediary substrates or secondary signalling mechanisms such as norepinephrine transporter and α2-adrenoceptor function[387]. TAAR1 heterodimerizes with G-coupled protein receptors[273,375] and although I could find no direct reference to all the G-coupled receptors TAAR1 can bind, the general biochemistry of TAAR1 related signalling allows for heterodimerization with potentially any G-coupled protein. Thus, apart from the more desirable anxiolytic profile of TYR illustrated in the current study, these mechanistic studies suggest that TYR is also likely the best candidate of the three TAs assessed, to modify a primary function in the brain that is augmented through signalling from peripheral activation of TAAR1[288]. Importantly, the dose or rather the physiological concentrations of TYR function is important to consider. Most studies to date use supraphysiological levels of TYR and at high doses there are some potentially detrimental effects such as the increase in inflammation and ROS through adrenoceptor signalling[387,388] and MAO breakdown of TYR[305]. However, through the function of TAAR1, which has been associated with improved immune response and improved anxiolytic effectiveness[389–392], TYR signalling at physiological levels could improve outcomes for peripheral allostasis.

In the next chapter I will use a bottom-up cell signalling model to probe potential effects of disease-associated peripheral allostatic load pressure on the central compartment, and the role of TYR as potential therapeutic in this context.

Chapter 5

Probing bottom-up communication of allostatic load

5.1 Introduction

Our group has previously demonstrated a peripheral inflammatory profile both in human subjects exposed to trauma and those with state anxiety, as evidenced by a relatively pro-inflammatory plasma cytokine profile and increases in GR receptor expression levels in leukocytes[393]. Similarly, obesity is known to result from a similar undesired redox and inflammation profile. This data establishes the association between the two conditions and unfavourable redox. My fibroblast data supports a preliminary interpretation of undesired peripheral adverse oxidative stress in PTSD and obesity. Furthermore, obesity has been suggested to signal from the periphery to cause central changes, such as microglial proliferation[394]. Thus, while causality of inflammation and oxidative stress in PTSD is still debated, there is clear evidence that there is definitely signalling from peripheral sites to cells within the brain.

The niche filled by fibroblasts is very similar to that of astrocytes and histologically they are very difficult to tell apart under a microscope. Since I have shown in previous sections that the patient-derived somatic cells show differences in calcium handling between control and PTSD groups and may have potential differences under obesity, it is likely that the proteins, chemoattractant, signalling molecules, and other cellular constituents would be present in the supernatant of fibroblast cultures and would potentially mimic the cellular environment of peripheral cells with an obesity and/or PTSD signature. A key aspect of fibroblasts which is vital to understanding the fibroblast literature, is that fibroblasts are in a continuous signalling feedback loop with the other host tissue and play a significant role in the maintenance of the host tissue[36,138,218]. The reported fibroblast heterogeneity[34,153,339] could be due to the feedback between fibroblasts and other host tissues, causing fibroblasts themselves to also change to fit their niche. This would be a physiological basis for allostasis at a tissue level playing a role in chronic disease.

Therefore, given the relative lack of data on the role of astrocytes in PTSD and obesity, the aim of this experiment was to investigate whether peripheral cellular (fibroblast) redox dysregulation associated with PTSD and/or obesity, may impact on the redox state of astrocytes, as indicator of allostatic load. A secondary aim was to assess the potential capacity of the anxiolytic trace amine tyramine, to modulate these potential changes in the redox status of astrocytes. In order to facilitate this aim, experiments were conducted both in the absence and presence of LPS, which is a known oxidative stressor.

5.2 Materials and Methods

5.2.1 Preparation of conditioned media from primary human fibroblasts affected by PTSD and/or obesity

Ethical considerations, patient recruitment, and tissue culture protocol can be found in chapter 3 (Section 3.2 Materials and Methods).

Using the same fibroblast samples used in chapter 3, conditioned media was prepared between passage 2-3. Fibroblasts were refreshed at ~60% confluence and the conditioned media harvested 24hr later. The fibroblast conditioned media (FCM) was stored in opaque containers at -80°C.

5.2.2 Maintenance of astrocyte culture

Human Hippocampal astrocytes (ScienceCell™, Cat.No.1830, Lot. No. 5557) were cultured as recommended by the supplier. Initial astrocytes, at a seeding density of $5x10^5$ cells/ml, were plated into a T75 cell culture flask, pre-coated with 2 µg/cm^2 poly-L-lysine 24hrs prior to plating. Cells were split into two T75 culture flasks once the astrocytes reached 80-90% confluence.

5.2.3 Experimental intervention protocol

When astrocyte cultures reached confluence, the astrocytes were plated into a 24well cell culture plate at a seeding density of 5000 cells/cm^3 and allowed to adhere and grow for 3 days. FCM was added to each well containing astrocytes along with a 1% N2 supplement (ThermoFisher Scientific Cat.No.17502048). In addition, astrocytes were exposed to either 0 mM (control), 3,125 mM (low dose) or 100 mM (high dose) of tyramine, in the absence or presence of LPS (10 ng/ml) (Sigma-Aldrich LPS from *E.coli* O111.B4 Cat.no. L4391). A media control, containing the growth media used for preparing the FCM (which included 1% N2 Supplement) was also added to a 24well culturing plate of astrocytes, in the assay without added LPS, to exclude differences in growth media as potential confounder. Two repeats for each patient across exposure conditions.

Astrocytes were cultured overnight (24 hrs) with the FCM, before the supernatant was removed and cells were frozen in 300 µl PBS at -80°C. Cell homogenate were prepared the next day for redox assays by scraping cells from each well and transferring to 1 ml Eppendorf tubes.

5.2.4 Sample analysis

5.2.4.1 Redox assays

Oxidative stress profile was assessed using three assays measuring oxidative damage, total antioxidant capacity and reactive oxygen species in cell homogenates. Thiobarbituric acid reactive substances (TBARS) assay measures the lipid peroxidation product malondialdehyde (MDA) which is an indicator of oxidative damage. The Trolox Equivalent Antioxidant Capacity (TEAC) assay measures

the total antioxidant capacity against a known antioxidant equivalent. Finally, the H_2O_2 concentration was determined using the Hydrogen Peroxide (H_2O_2) Assay Kit (Elabscience, E-BC-K102) as a measure of the reactive oxygen species concentration in the cell homogenates.

5.2.5 Statistical and Data analysis

Statistical analysis was carried out using GraphPad Prism (V9.1.1). All data was screened for outliers using the ROUT method (Q=1%) and an ANOVA was performed for the H_2O_2 and the TE data between the different groups.

5.3 Results

The media control and treatment control (FCM) data are shown below (Fig 5.1), clearly indicating higher H_2O_2 levels for the FCM compared to the media controls (Fig. 5.1A), but no significant difference between conditions for the antioxidant capacity (Figure 5.1 **B**), suggesting that the FCM alone did not activate endogenous antioxidant mechanisms.

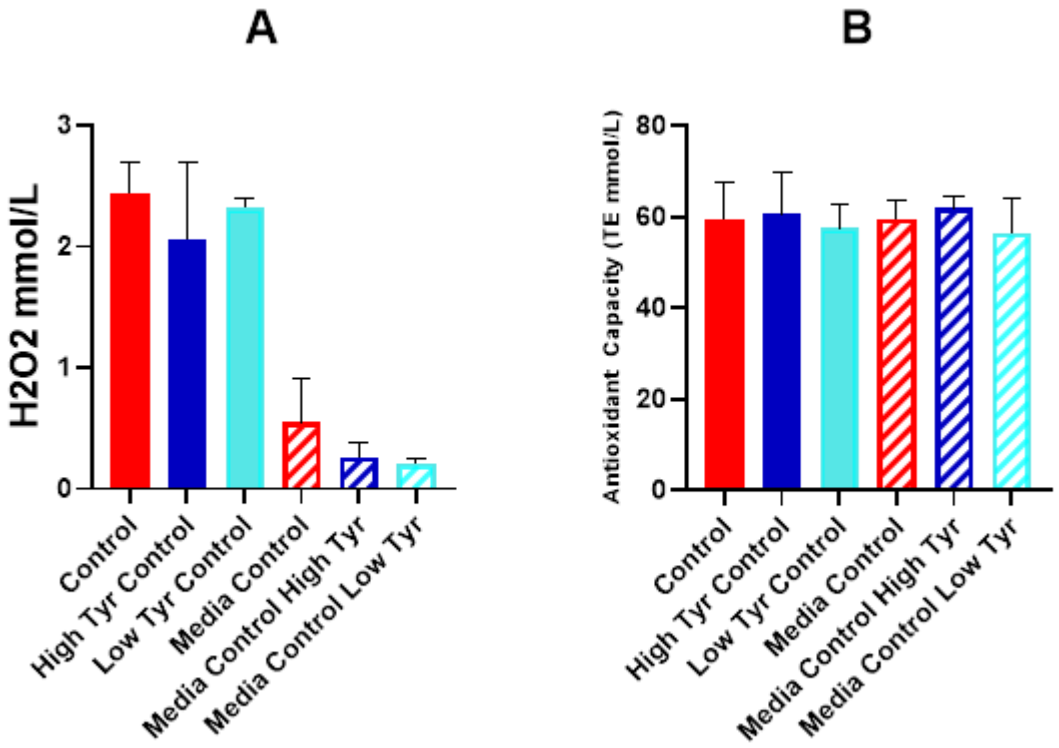


Figure 5.1 All control (FCM) and media control samples (LPS samples excluded). **A** represents the hydrogen peroxide production, significance is not indicated on the graph, but all media control differed from control data ($p<0.00001$). **B** represents antioxidant capacity measured as Trolox equivalence. Data expressed as mean ±SD.

In terms of H_2O_2 levels (the accepted proxy for ROS levels) in patient-specific groups, there was no main effect for treatment of astrocytes with either LPS (Fig. 5.2, bottom row) or tyramine (Fig 5.2,

high dose in middle and low dose in right column). Mean Obese H_2O_2 levels were consistently highest of all groups for all conditions, although the increase when compared to control only reached statistical significance in the Low dose TYR treatment group (Fig 5.2 **C**). There were also significantly higher levels of H_2O_2 in the Obese group compared to the PTSD+Obese group for all treatment groups, although this difference was only statistically significant in cells also supplemented with tyramine (Figure 5.2 **B**, **C**, **E**, and **F**).

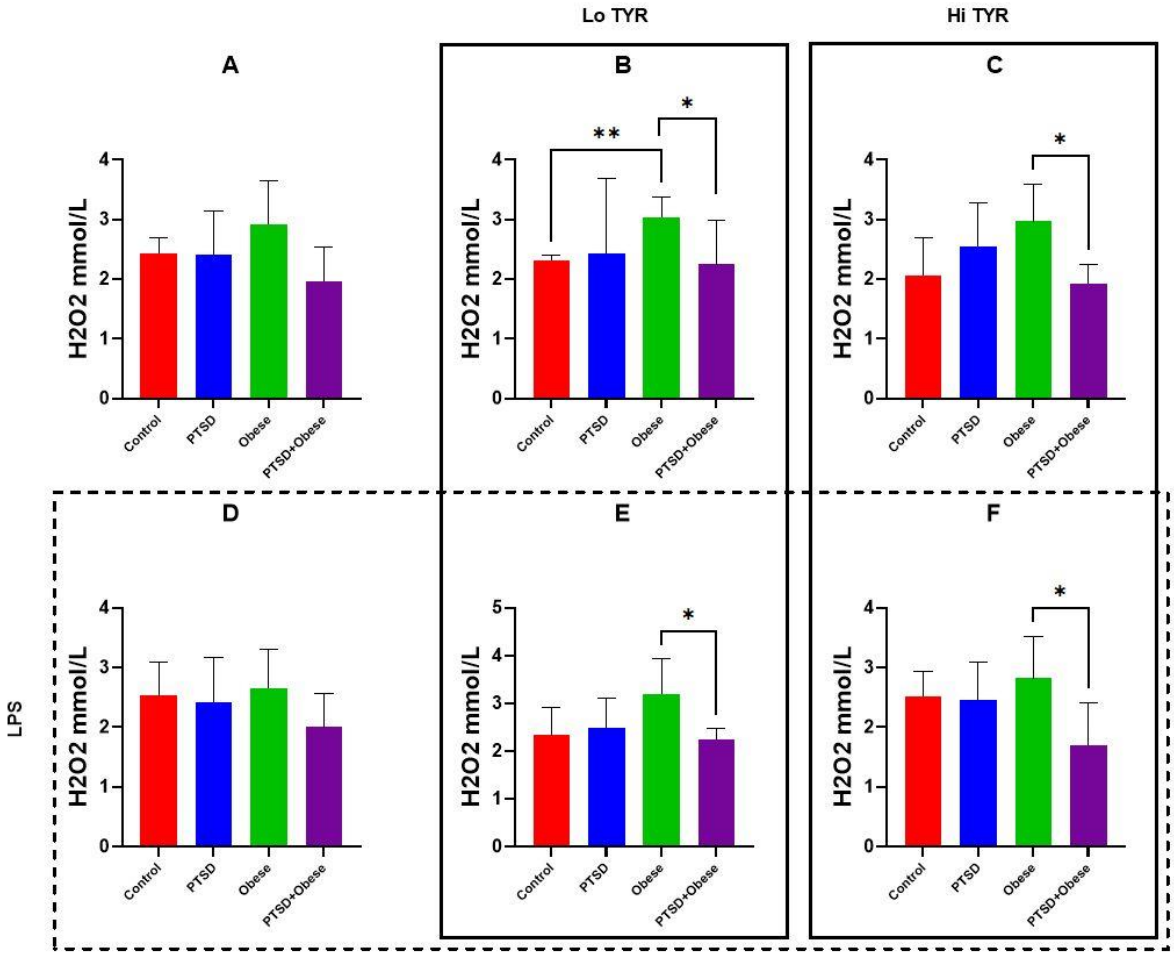


Figure 5.2 Hydrogen peroxide levels for all participant groups in response to fibroblast conditioned media, in the absence (A-C) or presence of added LPS (D-F). Furthermore, cultures were exposed to either low (B and E) or high (C and F) dose TYR. Data expressed as mean ±SD with Control n=4, PTSD n=5, Obese n=6, and PTSD+Obese n=6. LPS – lipopolysaccharide. Statistics: * - 0.05; ** - 0.001; *** - 0.0001.

In terms of total antioxidant capacity, there was no significant effect of either treatment or experimental group (Figure 5.3). In the MDA assay, no sample had detectable MDA levels, suggesting minimal to no oxidative damage.

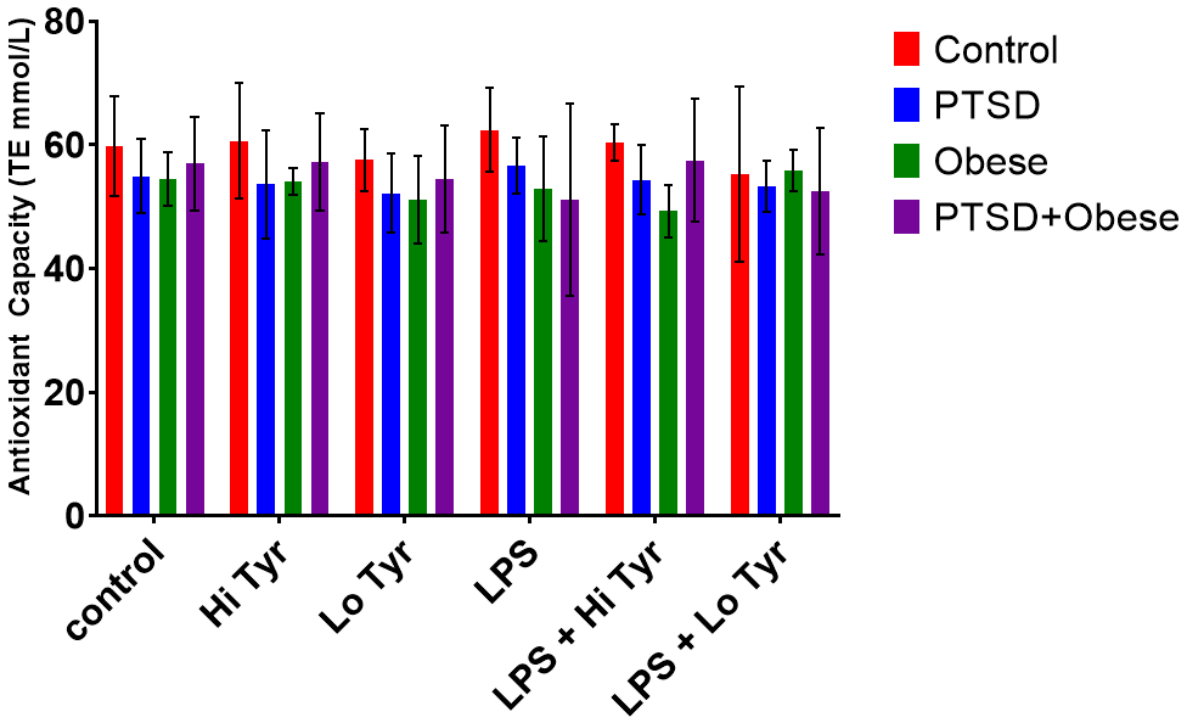


Figure 5.3 Antioxidant capacity measured in TE for each patient group treated with tyramine, LPS, and LPS+tyramine in combination. Data expressed as mean ±SD with Control n=4, PTSD n=5, Obese n=6, and PTSD+Obese n=6. Hi Tyr – High Tyramine dose 100µM; Lo Tyr – Low Tyramine dose 3,125µM, LPS – lipopolysaccharide.

To further explore the relationship between the antioxidant capacity and the H_2O_2 levels, the data was ratioed as Antioxidant capacity/H_2O_2 levels (Figure 5.4). On average, a consistent trend across all treatment groups was that antioxidant capacity relative to the ROS stressor was slightly reduced in PTSD, with a greater reduction in the obese group. Interestingly, in the PTSD+Obese groups, this decrease was ameliorated, suggesting a relatively higher antioxidant capacity. Although this latter difference only reached statistical significance in the presence of high dose TYR (Fig 5.4E), the fact that these group differences were consistently found across treatment groups, may have biological significance.

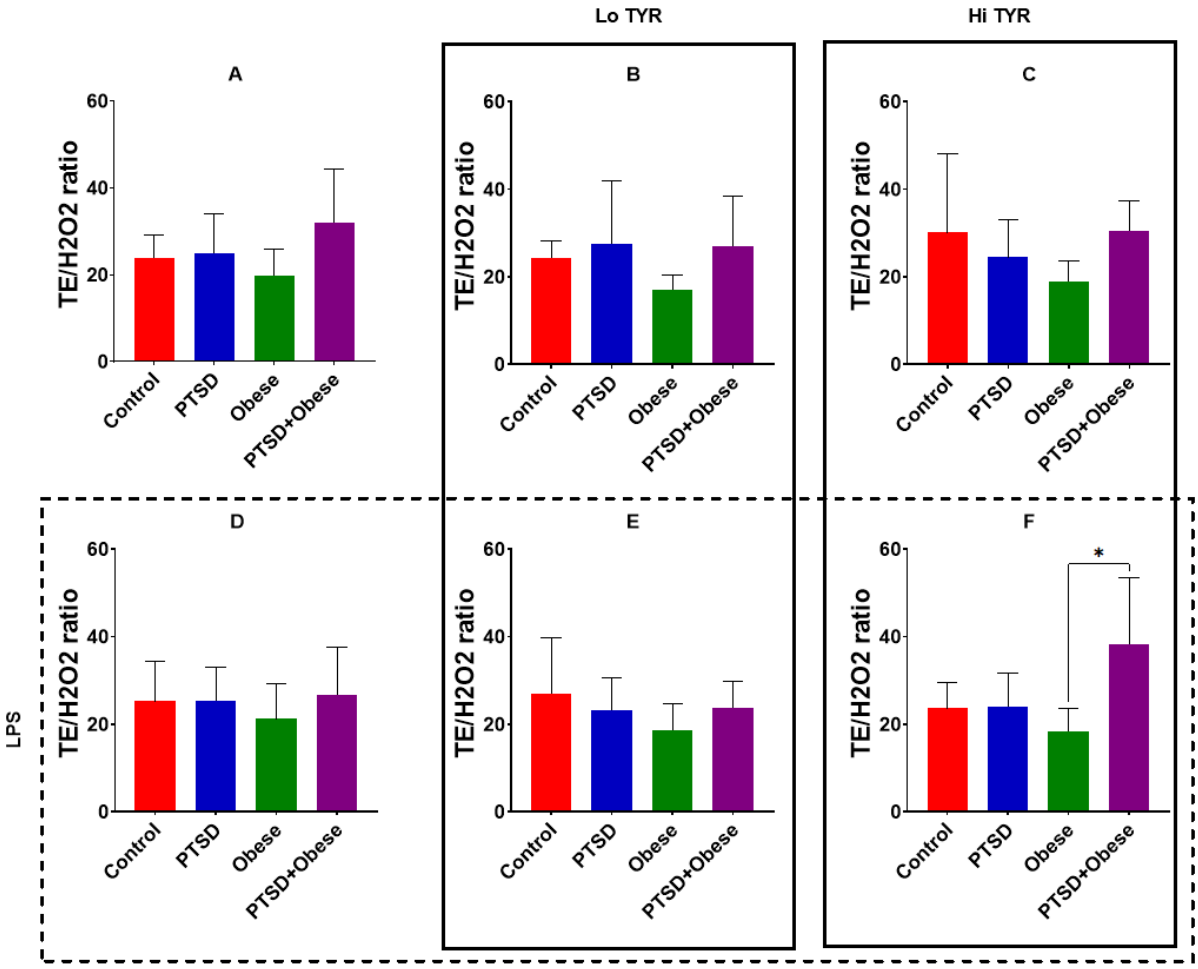


Figure 5.4. Ratio of Antioxidant Capacity to Hydrogen peroxide for all participant groups in response to fibroblast conditioned media, in the absence (A-C) or presence of added LPS (D-F). Furthermore, cultures were exposed to either low (B and E) or high (C and F) dose TYR. Data expressed as mean ±SD with Control n=4, PTSD n=5, Obese n=6, and PTSD+Obese n=6. LPS – lipopolysaccharide. Statistics: * - 0.05; ** - 0.001; *** - 0.0001.

5.4 Discussion

The aim of this experiment was to determine if PTSD- and/or obesity-associated dysregulation in fibroblast signalling may affect redox status – as indicator of allostatic load – in human hippocampal astrocytes. Current data illustrate the following novel contributions in the context of allostasis: a) fibroblast media components has the capacity to significantly affect astrocyte ROS levels and antioxidant capacity, b) TYR seems to act as an antioxidant, but with risk of prooxidant effect when present in abundance, c) the obesity signature in fibroblasts contributed more significantly to ROS levels in astrocytes than a PTSD signature, but antioxidant defences were only activated when the two were both present.

At first glance, the idea of directly exposing astrocytes to a cocktail of secretory products from cells situated distantly in the periphery – and importantly, across the blood brain-barrier - may seem

unphysiological. However, this intervention is not as drastic as it may seem; when considering the control group only, it is evident that although astrocytes responded to the constituents in FCM by increasing ROS production, this stimulus seemed insufficient in severity to activate antioxidant mechanisms in astrocytes, suggesting that the FCM components did not induce a significant cellular stress in the astrocytes. It is difficult to conclusively interpret whether the levels of ROS measured in the current study constitutes a high or low ROS response, as disparate astrocyte ROS levels have been reported in literature[86,247,251,395,396]. Furthermore, since ROS has many functions as a signaling molecule in different spatial compartments of the cell, ROS homeostasis is tightly regulated by specific mechanisms - not unlike the tight control known to exist for cellular calcium levels. It therefore seems most likely that changes in ROS levels – especially in the absence of overt cell damage as seen in terms of the MDA levels in the current study – in the relatively short cell culture protocol duration, would be subtle.

In my opinion, this relatively robust resistance to *ex vivo* modulation speak to the significant capacity of astrocytes to mitigate the effects of potential cellular stressors, which may make these cells an ideal cell type for modelling of allostasis. The fact that these cells showed similar low responsiveness to LPS as well as TYR, further supports this interpretation. This relative robustness of astrocytes has been demonstrated before. For example, in two parallel inflammation-focused experiments in our group, astrocytes were found to be significantly more resistant to activation by LPS than macrophages[397,398]. Specifically, astrocytes responded very mildly to administration of 20 μg/ml LPS, while a much lower dose of 50 ng/ml administered to macrophages using an identical protocol, elicited a significant cytokine response. Although astrocyte responses to TAs have not been reported in the context of redox, it is feasible that astrocytes may be resistant to TA load as well, considering that astrocytes are major contributors to aminergic biosynthesis and thus have the molecular tools to clear increased TA concentrations[399].

A consistent pattern is observed across all treatment (TYR and LPS) groups, when considering the H_2O_2 data, whereby Obesity-conditioning effected a somewhat larger ROS accumulation than PTSD. Interestingly, when Obesity and PTSD conditioning is superimposed (i.e., present and co-morbidities), signalling from fibroblasts seemed to *decrease* ROS accumulation. No data exist with which to contextualise this finding. However, when interpreted together with the antioxidant capacity data, expressed as ratio of antioxidant data:H_2O_2 concentration, the most likely scenario is indeed the phenomenon of allostasis; it seems that the subtle increases in ROS resulting from either PTSD or Obesity alone, is not severe enough to push ROS production over a threshold where antioxidant defences would be activated. Only when PTSD and Obesity effects are cumulatively present, is this achieved, resulting in the lower ROS levels, and the more favourable antioxidant capacity:H_2O_2 ratio observed. In terms of cell culture, my study is in line with literature standard in terms of sample size

for *ex* vivo and cell culture-based studies. Rather, the fact that statistical significance is only achieved in some instances, should be interpreted in the light of the small effect size that is allostasis. In terms of clinical relevance, my data may suggest that the failure to activate endogenous antioxidant defences to the (largely undetected) gradual, low grade deviation from normalcy, may underpin morbidity risk in PTSD and Obesity.

Although there is a relative paucity of literature in this context, some support for this interpretation does exist in the obesity literature. For example, a study of central adiposity in males showed a compensatory increase in H_2O_2 catalyse in response to accumulation of H_2O_2 in central adipose tissue. Although the main aim of this study was to investigate the effects of increased oxidative stress on insulin resistance it also provides valuable insight into the potential compensatory mechanisms that are switched on in response to cellular stress resulting from obesity. Although other studies also exist that illustrate changes that oxidative stress can bring about in the cellular context[74], the predominant context for these studies are adipose tissues themselves, which have specialized systems in place for lipid and oxidative interactions. To my knowledge there are far fewer[28,31,122] studies considering these effects in the context of obesity and PTSD as factors, with no conclusive insights[108]. I could also not find any studies that looked at potential mechanisms that are activated to deal with increased oxidative stress in the context of PTSD. There still exists a large gap in the literature pertaining to understanding the complex interactions of these mechanisms. A further complication is the fact that the long-term low grade inflammatory consequences of mental health disorders is known to be a factor[47,63], but the nature and specific cellular outcomes are still unclear in humans.

Despite this lack of mechanistic insight, evidence of allostasis in PTSD is emerging in the clinical literature, with some studies reporting on the cumulative allostatic load, including markers of ROS, in mental health disorders and PTSD specifically. One such study, published in 2021, attempted to gauge the allostatic load of French soldiers with PTSD, anxiety, or depression as well as those without pathology in an attempt to match allostatic load with psychological profiles of clinical and potentially subclinical PTSD, anxiety, or depression[400]. Sub-depressive patients had low blood BDNF values, while anxious subjects had no detectable biological changes. The traumatized subjects were characterized by chronic stress and had high ROS and cortisol levels in early morning urine samples. Of particular relevance is the fact that individuals with a combination of anxiety and trauma, presented with a trend toward increased cortisol excretion but showed normal free radical secretion, which is in line with the lower ROS levels in the current study, when endogenous counter mechanisms were triggered.

From the broad literature I have consulted, ROS is emerging as clear driver of allostasis, but the majority of published studies on allostasis in the context of mental health disorders, have not considered ROS as part of allostatic load panels except indirectly through inflammatory markers[401–

[403]. However, there are studies that have started to show the regulatory function of ROS through protein modification[85]. ROS could be a primordial signalling mechanism that has been adapted so well that the effects could not be teased apart until enough evidence had accrued. Indeed, a recent review excellently demonstrates the accrued evidence for ROS as a "pleiotropic" signalling mechanism on par with calcium signalling in terms of versatility and homeostatic control[404]. Given this relatively recent understanding of ROS as such an important signalling system (of course evidence has existed for years, but only recently has the general consensus begun to shift) it is imperative to not only include ROS in allostatic models but pay attention to the primacy of ROS as regulator of allostatic mechanisms.

Lastly, in terms of the role of TYR, current data seem to indicate that the administration of TYR constituted another allostatic load, rather than a therapeutic effect. However, this does not exclude TYR as potential therapeutic option – rather, my data highlights the importance of dose optimisation. Given the complexity of the protocol employed in terms of number of treatments, the decision was made to not a dose range of TYR, especially since I was interested in assessing the dose shown to have anxiolytic effect. In hindsight, a better approach may have been to forfeit the LPS condition to rather include a range of TYR doses. Recent work by our group (L Pretorius, PhD thesis, 2022) has demonstrated – albeit in another disease context – that the effect of TYR is highly dependent on its timeous metabolism, as well as other modulating factors such as oestrogen levels. These "synergistic" role players would have been present in the zebrafish model, but not the cell culture model, which may explain the adverse outcome in terms of TYR in the astrocyte culture.

Taken together, my data is in line with the sparse studies on allostasis and redox in PTSD and Obesity, but much exploration of mechanisms at play is required before firm interpretations can be made in the context of bi-directional allostatic signalling effects in PTSD (or obesity). Finally, in the context of allostasis research, data clearly highlights several limitations for cell culture models, suggesting that identification of a suitable model as research tool, should receive priority going forward.

Chapter 6

Synthesis

Given the small measurable effect size of allostasis, especially in the short time span of most highly standardized research protocols, it is not unexpected that little progress has been made in this context. From my review of the literature which spanned several regulatory system and disease contexts, it is evident that a marker for allostasis has not been identified despite the fact that some attempts have been made. Current parameters used to measure allostasis in literature[401–403] are variable and therefore non-viable as they don't contribute to a functional characterisation of allostatic load. Also, ROS has been completely neglected as a potential marker for allostasis despite its central role in stress-related health outcomes. This thesis contributes to this relative gap in knowledge in more than one way.

Firstly, I have demonstrated the value of using ROS and calcium changes (Chapter 3) as more sensitive indicators of allostasis, by demonstrating that PTSD has a different allostatic signature in the periphery than obesity. Calcium signaling is one of the primary mechanisms that drive cellular function, and its importance is highlighted in literature. The shift in calcium signaling seen in PTSD fibroblasts points at a disruption that will influence the chemiosmotic potential of many important systems, such as mitochondrial function, protein synthesis and folding, receptor signaling, secondary cellular communication, etc. The role of fibroblast in their native cell niche is also important to consider if their calcium flux is perturbed, which would have a knock-on effect on the tissue ability to heal, mount an inflammatory response, and maintain homeostasis. In PTSD, the resulting neurochemistry of increased dopamine and altered glutamatergic signalling, as well as increased inflammation can be interpreted as a response to increased low grade ROS, which has been shown to be able to affect all these systems[45,404,405]. The calcium dysregulation will also contribute to the dysfunction in PTSD with the same rationale as there is a strong reciprocal relationship between ROS and calcium[231,264] as well as the importance of calcium as a signalling molecule means any disruption would affect many physiological systems.

Most of the functional markers assessed in the patient fibroblasts were normal, indicating that the cells were healthy to a degree. The allostatic signature of PTSD however was detectable in the calcium signalling function, where the decrease in flux capacity is an indication of increased workload for PTSD samples. Variants in calcium genes have been implicated in mental health disorders[92,287,406] and perhaps here as well a predisposition to increased allostasis through upregulated calcium signalling contributes to PTSD aetiology. A future study could assess unique calcium polymorphisms in patient's vs controls or assess epigenetic and translational differences for calcium function. The

lack of CD31 expression in PTSD fibroblasts could also point to a compensatory mechanism attempting to decrease the infiltration of immune cells into central compartments or decrease immune response through CD31 mechanisms[203]. A potentially allostatic response to increased low grade inflammation. The patient derived fibroblasts were also able to increase the oxidant load and antioxidant capacity of astrocytes through secreted effectors, as there was no such increase in the media controls. This speaks not only to the capacity of peripheral signalling to impact the allostatic load of central compartments, but also to the base allostatic load experienced by even control patients.

In chapter 5, despite general limitations of cell culture models in this context, I validated the use of astrocytes as model cell in the context of allostasis. The conditioned media used here, which represents the potential disease conditioning of PTSD and Obesity, did not significantly change the outcomes for astrocytes when compared to the controls. However, the cumulative load of the disease states combined with a known oxidant stressor (LPS[407,408]), was enough to illicit an antioxidant effect. Therefore, the combination of the calcium flux alterations and the cumulative redox effect, indicate a state where low grade stress related changes, do alter the physiological functions of cells. If these changes are low enough, they will not activate endogenous mechanisms of defense, however there will still be detrimental effects within microdomains of the cell. This is the mechanism that will drive allostatic changes causing the reset of the homeostatic norm in cells. Only once there is cumulative defects will the endogenous defenses, such as antioxidant systems, respond, but by that time the system may be too dysfunctional to adequately mount a defense. This mechanism not only applies to the aetiology of chronic diseases, but also has explanatory power for the high levels of systemic comorbidities of chronic and mental health disorders. Future studies could use a proteomics or metabolomics approach to determine the driving factors of the FCM contribution to allostatic load. Proteomic assessment of astrocyte function under the cumulative stressors could also elucidate more specific redox and calcium related markers for allostatic changes.

A parallel can also be drawn between the primacy of calcium signaling and ROS as signaling molecules. At low levels the signaling function of ROS is also an important messenger and has impact on protein function and synthesis, mitochondrial function, receptor signaling, among others. This indicates the importance of ROS as an additional driver of the chemiosmotic potential of cells.

Although allostasis has been used in a more clinical setting, focusing on patient markers to indicate the cumulative stressors faced by patients, these markers are, at a cellular level, too variable to be useful as true biomarkers, which translates to interventions that are not timely enough, because the clinical measures of allostasis are forms of dysfunction driven by already pathological states. It is therefore imperative to address the role of allostasis in appropriate models that can probe the more subtle and longitudinal effects of allostasis. In the context of PTSD-associated allostasis, the use of astrocytes is likely the most appropriate in terms of cell-based models, since these cells function in a

niche that is responsible for the homeostasis of neuronal systems and therefor does have the cellular mechanisms to respond to allostatic changes. Furthermore, astrocytes are robust enough to handle acute insults of potentially damaging events, particularly that of increased inflammation and ROS. Given the relative neglect of ROS as a marker for allostasis, an astrocyte model for allostatic load would be the most appropriate for this cumulative damage type investigation. However, given the many limitations demonstrated in this model as well, especially in terms of potential therapeutic modulation, the search for an appropriate model is still ongoing and the first obstacle to overcome in allostasis research.

In terms of practical limitations, this thesis was impacted by the COVID-19 pandemic, which severely limited reagent availability and delivery times. It would have been beneficial to be able to repeat the calcium flux experiment on the fibroblasts (Chapter 3) using tyramine as a potential modulator of calcium signalling as well as assessing the calcium flux of the astrocytes treated with FCM (Chapter 5). Unfortunately, a limit in terms of the number of samples the available Fluo-4 calcium imaging kit (ThermoFisher; Cat# F10489) could analyse prevented that from being included here. Another limitation was the inability to characterise the constituents of the FCM, which was omitted firstly due to unavailability of reagents, but later due to time constraints. In the final experiment it would have been beneficial to expand the redox markers assessed, both for ROS markers and antioxidant capacity. This was not possible due to the large number of fibroblast samples I managed to culture – a triplicate number of astrocyte samples to correspond to each patient fibroblast sample meant a large number of astrocytes were also cultured, which limited the amount of specialized astrocyte media for the experimental protocol, which had to include media controls. I could have expanded the fibroblast and astrocyte lines further using in house media that could have expanded the astrocyte line normally. However, not only would that have been a confounder in terms of culturing media used, but a repeat of the cell culture protocol was not feasible, due to time and financial constraints.

Going forward, the question to answer foremost, is what model to use to investigate allostasis in the context of complex disorders. Given the high variance of biologically relevant markers used in other allostatic measurements, such as cortisol or IL-6 levels, a more stable longitudinal measure is needed. This thesis makes the case that ROS mechanisms and calcium signalling mechanisms are good candidates to assess allostatic load or shift. Furthermore, the clearance mechanisms of ROS are well understood, meaning that translation from *in vitro* to *in vivo* work may be easier. Using *in vivo* models is more appropriate, because whole animals have intact physiological systems that allow for the interaction of relevant endogenous regulatory mechanisms, as well as accurate simulation of pharmacokinetic and pharmacodynamic influences of and on therapeutic interventions. In this thesis this is evident from the zebrafish larval response to TYR, which was largely anxiolytic compared to the astrocyte cell line in which TYR was a stressor, potentially due to lack of additional clearance

mechanisms present in the animals. The difficulty however is that there is very little animal work accurately probing the links to predisposition to PTSD, an area where a focus on allostasis through redox signalling could elucidate this link. I propose modelling PTSD with consideration of allostatic predisposition in rodents. Although the nature of this predisposition is not clear, several neurological conditions, has been linked in GWAS data to overexpression of NF-kB. Thus, employing a PTSD model such as maternal separation in a NF-kB knock-in rodent model, may provide some versatility in terms of elucidation of top-down and bottom-up communication of allostasis modulation, as well as activation of endogenous counter mechanisms.

Lastly, TA based preventative treatment in the context of allostatic load in the periphery of whole organisms, is in my opinion still a viable option, given the zebrafish results and the fact that no toxicity or adverse outcome was observed at the doses used here. Given the parallel between ROS and calcium, and the development of optogenetics in zebrafish as powerful tool with which to study calcium signalling[409,410], a feasible approach may be to investigate redox and calcium modulation in zebrafish, where these allostatic changes may be correlated with behavioural outcome. Once the most appropriate research model has been identified, future studies would need to focus on the correct dosage range for anxiolytic effects as well as fully elucidate the role of receptor binding affinities and TAAR1 activation effects.

In conclusion, despite limitations, this thesis was able to test and accept the majority of posed hypotheses, thereby contributing to our understanding of allostasis in PTSD, as well as obesity, and highlighting ROS accumulation and calcium flux as potential indicators of allostasis, given a suitable model context. However, given the relatively recent, still emerging, recognition of allostasis as factor in clinical prognosis in PTSD, several avenues for future research have been identified that remains unexplored.

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Addendum

Addendum A

List of Shared Roots clinical measures

Baseline

Clinician administered

Cross-cutting measures

Demographic questionnaire	To gather information on age, gender, education, employment, income, marital status and ethnicity
General medical questionnaire	To assess personal psychiatric and medical history (including age of illness onset), family history of medical and psychiatric illness, substance use, record of previous and concomitant medication.
Female medical questionnaire	To obtain an obstetric and gynaecological history in females
MINI International Neuropsychiatric Interview (MINI) version 6.0	This is a clinician administered, structured diagnostic interview for major psychiatric disorders based on DSM-IV diagnostic criteria
the Hamilton Depression Scale (HAMD-D)	Clinical assessment of depression
Clinical Global Impression (CGI)	A global impression of severity
Social and Occupational Functioning Assessment Scale (SOFAS)	To assess functional impairment due to medical or psychiatric disorders
The Montreal Cognitive Assessment (MOCA)	Cognitive screening
WHO STEPwise approach to surveillance (STEPS)	The WHO STEPS will be used to assess for risk factors for cardiovascular and metabolic disease. The WHO STEPS gather information on Tobacco use, Alcohol consumption, Diet, Physical Activity and History of Hypertension and Diabetes.
24 hour dietary recall	Nutritional assessment
Indicator food list	Dietary intake of foods
Indicator food list - Caffeine	Dietary intake of caffeine

Cohort specific measures

PTSD

Clinician Administered Posttraumatic Stress Disorder Scale (CAPS) updated for DSM 5	To diagnose and determine severity of PTSD.

Self-administered measures

Cross-cutting measures

Events Checklist for DSM-5 (LEC-5)	To assess for a history of traumatic events
PTSD Checklist for DSM-5 (PCL-5)	To screen for PTSD symptoms
Childhood Trauma Questionnaire (CTQ)	A measure of childhood trauma
A timeline	To follow-back of other lifetime traumatic event exposures
Spielberger State-Trait Anxiety Inventory	To evaluate for the presence of anxiety
The Perceived Stress Scale (PSS)	To assess self-perceived stress
Connor-Davidson Resilience Scale (CD-RISC)	To assess resilience

Pittsburgh Sleep Quality Index (PSQI)	To assess subjective sleep quality
Multidimensional Scale of Perceived Social Support (MSPSS)	To assess social support
The Beck Cognitive Insight Scale (BCIS)	To evaluate insight
The World Health Organisation WHOQOL-BREF Quality of Life Scale	To assess functioning and quality of life
The Sheehan Disability Scale (SDS)	To assess for disability
The Edinburgh Handedness Questionnaire	Scores are computed to indicate whether participants are left-handed, right-handed or ambidextrous.

Physical and laboratory measures

Physical measures

- Weight
- Length
- Waist circumference
- Hip circumference
- Neck circumference
- Blood pressure
- Pulse

Laboratory measures

- C-reactive protein and HS-CRP
- Fasting HGT
- HbA1c
- Fasting lipogram (total cholesterol, HDL chol, LDL chol, triglycerides)

Other laboratory measures

- DNA
- RNA
- Plasma and serum biomarkers
- Hair cortisol
- Gut micrbiome
- Dermal fibroblast culture

Addendum B

Patient Fibroblasts Ethics approval S20/01/024

Addendum C

Zebrafish Larva Ethics approval ACU-2021-21995

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Approval Letter
Amendment

11/09/2020

Project ID: 13340

Ethics Reference No: S20/01/024 (PhD)

Project Title: Characterising physiological, psychobiological, and psychopathological profiles of stress

Dear Mr Rohan Benecke

Your amendment request dated 13/04/2020 12:07 refers.

The Health Research Ethics Committee (HREC) reviewed and approved the amended documentation through an expedited review process.

The following amendment was reviewed and approved:

1. Protocol Amendment #1 dated, 10 April 2020
    - Changes to the protocol are aligned with the originally approved study (as well as the original Shared roots study from which samples were obtained) and does not change the risk profile.

Where to submit any documentation

Kindly note that the HREC uses an electronic ethics review management system, *Infonetica*, to manage ethics applications and ethics review process. To submit any documentation to HREC, please click on the following link: https://applyethics.sun.ac.za.

Please remember to use your project ID 13340 and ethics reference number S20/01/024 (PhD) on any documents or correspondence with the HREC concerning your research protocol.

Yours sincerely,

Mrs. Brightness Nxumalo
Coordinator: Health Research Ethics Committee 2

National Health Research Ethics Council (NHREC) Registration Number:

REC-130408-012 (HREC1)•REC-230208-010 (HREC2)

Federal Wide Assurance Number: 00001372
Office of Human Research Protections (OHRP) Institutional Review Board (IRB) Number:
IRB0005240 (HREC1)•IRB0005239 (HREC2)

The Health Research Ethics Committee (HREC) complies with the SA National Health Act No. 61 of 2003 as it pertains to health research. The HREC abides by the ethical norms and principles for research, established by the *World Medical Association (2013). Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects;the South African Department of Health (2006). Guidelines for Good Practice in the Conduct of Clinical Trials with Human Participants in South Africa (2nd edition)*; *as well as the Department of Health (2015). Ethics in Health Research: Principles, Processes and Structures (2nd edition).*

The Health Research Ethics Committee reviews research involving human subjects conducted or supported by the Department of Health and Human Services, or other federal departments or agencies that apply the Federal Policy for the Protection of Human Subjects to such research (United States Code of Federal Regulations Title 45 Part 46); and/or clinical investigations regulated by the Food and Drug Administration (FDA) of the Department of Health and Human Services.

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Animal Tissue Use Approval

06/05/2021

PI: Prof C Smith

REC: ACU Reference #: ACU-2021-21995

Title: Use of zebrafish larval model for drug and toxicity screening

Dear Prof C Smith

Your Notification with reference number #ACU-2021-21995, was reviewed on 28 April 2021 by the Research Ethics Committee: Animal Care and Use via committee review procedures and was approved. Please note that this clearance is valid for a period of five years. A new application must be submitted when the source of the material changes.

Applicants are reminded that they are expected to comply with accepted standards for the use of animals in research and teaching as reflected in the South African National Standards 10386: 2008. The SANS 10386: 2008 document is available on the Division for Research Developments website www.sun.ac.za/research.

As provided for in the Veterinary and Para-Veterinary Professions Act, 1982. It is the principal investigator's responsibility to ensure that all study participants are registered with or have been authorised by the South African Veterinary Council (SAVC) to perform the procedures on animals, or will be performing the procedures under the direct and continuous supervision of a SAVC-registered veterinary professional or SAVC-registered para-veterinary professional, who are acting within the scope of practice for their profession.

Please remember to use your REC: ACU reference number: # ACU-2021-21995 on any documents or correspondence with the REC: ACU concerning your research protocol.

If you have any questions or need further help, please contact the REC: ACU office at 021 808 9003.

Visit the Division for Research Developments website www.sun.ac.za/research for documentation on REC: ACU policy and procedures.

Sincerely,

Mr Winston Beukes

Coordinator: Research Ethics (Animal Care and Use)