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Evaluation of Hippocampal Allostatic Load-Associated Factors in Animal Models of Post-Traumatic Stress Disorder: Relevance to Human PTSD

Dennis Parker Kelley

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EVALUATION OF HIPPOCAMPAL ALLOSTATIC LOAD-ASSOCIATED FACTORS IN ANIMAL MODELS OF POST-TRAUMATIC STRESS DISORDER: RELEVANCE TO HUMAN PTSD

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillments of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Comparative Biomedical Sciences

by
Dennis Parker Kelley
B.S., B.A., Louisiana State University, 2014
May 2022
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I must not fear.
Fear is the mind-killer.
Fear is the little-death that brings total obliteration
I will face my fear.
--Frank Herbert
Dune
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<td>ATP</td>
<td>Adenine triphosphate</td>
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<td>ACTH</td>
<td>Adrenocorticotropin releasing hormone</td>
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<td>PKB</td>
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<td>CVD</td>
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<td>CNS</td>
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<td>CHOL</td>
<td>Cholesterol</td>
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<td>CRS</td>
<td>Chronic restraint stress</td>
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<td>CUMS</td>
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<td>CAPS</td>
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<td>CP</td>
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<td>Cornu Ammonis 1/2/3/4</td>
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<td>CRH</td>
<td>Corticotropin releasing hormone</td>
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<td>Cortisol/ Corticosterone</td>
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<td>DAMPS</td>
<td>Damage associated molecular patterns</td>
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<td>DHEA-S</td>
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<td>DG</td>
<td>Dentate gyrus of the hippocampus</td>
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<td>Department of Defense</td>
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<td>DSM-V</td>
<td>Diagnostic statistical manual of psychiatric disease 5</td>
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<td>DEG</td>
<td>Differentially expressed genes</td>
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<td>DMT</td>
<td>N,N-Dimethyltryptamine</td>
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<td>Dopamine</td>
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<td>dHPC</td>
<td>Dorsal hippocampus</td>
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<tr>
<td>dmIns</td>
<td>Dorsal mid insula</td>
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<td>dpIns</td>
<td>Dorsal posterior insula</td>
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<td>Doublecortin</td>
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<td>Emotional Processing Theory</td>
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<td>Endoplasmic reticulum</td>
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<td>eLF2</td>
<td>Eukaryotic elongation factor 2</td>
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<td>ET</td>
<td>Exposure therapy</td>
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<td>Acronym</td>
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<td>EMDR</td>
<td>Eye Movement Desensitization and Reprocessing therapy</td>
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<td>fMRI</td>
<td>Functional magnetic resonance imaging</td>
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<tr>
<td>GCR</td>
<td>Glucocorticoid receptor</td>
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<td>GRE</td>
<td>Glucocorticoid response elements</td>
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<td>G-6-Pase</td>
<td>Glucose-6-phosphatase</td>
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<td>Guanine diphosphate</td>
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<td>Guanine triphosphate</td>
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<td>HDL</td>
<td>High density lipoprotein cholesterol</td>
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<td>Hippocampus</td>
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<td>HPA axis</td>
<td>Hypothalamic-pituitary-adrenal axis</td>
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<td>IRS1</td>
<td>Insulin receptor substrate 1</td>
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<tr>
<td>IGF1</td>
<td>Insulin-like growth factor 1</td>
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<tr>
<td>IGF1R</td>
<td>Insulin-like growth factor 1 receptor</td>
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<tr>
<td>IGF2</td>
<td>Insulin-like growth factor 2</td>
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<td>IGF2R</td>
<td>Insulin-like growth factor 2 receptor</td>
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<tr>
<td>ISR</td>
<td>Integrated stress response</td>
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<tr>
<td>IL-1β</td>
<td>Interleukin 1β</td>
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<td>IFS</td>
<td>Internal Family Systems therapy</td>
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<tr>
<td>LA</td>
<td>Lateral amygdala</td>
</tr>
<tr>
<td>LEC</td>
<td>Lateral entorhinal cortex</td>
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<tr>
<td>LC-MS</td>
<td>Liquid chromatography tandem mass spectrometry</td>
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<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>LTD</td>
<td>Long-term depression</td>
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<td>LTP</td>
<td>Long-term potentiation</td>
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<td>LDL</td>
<td>Low density lipoprotein cholesterol</td>
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<td>MEC</td>
<td>Medial entorhinal cortex</td>
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<td>MTL</td>
<td>Medial temporal lobe</td>
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<td>MetS</td>
<td>Metabolic syndrome</td>
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<td>Met</td>
<td>Methionine</td>
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<tr>
<td>MAP2</td>
<td>Microtubule associated protein 2</td>
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<tr>
<td>aMCC</td>
<td>Mid anterior cingulate cortex</td>
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<tr>
<td>MCR</td>
<td>Mineralocorticoid receptor</td>
</tr>
<tr>
<td>NALP3</td>
<td>NLR family pyrin domain containing 3</td>
</tr>
<tr>
<td>NAv</td>
<td>Non-avoiders</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
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<tr>
<td>Nf-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
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<tr>
<td>OXS</td>
<td>Oxidative stress</td>
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<tr>
<td>PhC</td>
<td>Parahippocampal gyrus</td>
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<td>PVN</td>
<td>Paraventricular nucleus of the hypothalamus</td>
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<td>PRR</td>
<td>Pattern recognition receptors</td>
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<tr>
<td>PS</td>
<td>Pattern separation</td>
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<tr>
<td>PERCK</td>
<td>Phosphoenolpyruvate carboxykinase</td>
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<td>PTSD</td>
<td>Post-traumatic stress disorder</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PrC</td>
<td>Postrhinal cortex</td>
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<tr>
<td>PE/PSS</td>
<td>Predator exposure + psychosocial stress</td>
</tr>
<tr>
<td>PSS</td>
<td>Predator scent stress</td>
</tr>
<tr>
<td>PFC</td>
<td>Prefrontal cortex</td>
</tr>
<tr>
<td>pgACC</td>
<td>Pregenual anterior cingulate cortex</td>
</tr>
<tr>
<td>PDH</td>
<td>Pyruvate dehydrogenase</td>
</tr>
<tr>
<td>PDK4</td>
<td>Pyruvate dehydrogenase kinase 4</td>
</tr>
<tr>
<td>FADH2</td>
<td>Reduced flavin adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>Reduced nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SSRI</td>
<td>Selective serotonin reuptake inhibitor</td>
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<tr>
<td>5-HT</td>
<td>Serotonin</td>
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<tr>
<td>SERT</td>
<td>Serotonin transporter</td>
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<tr>
<td>SES</td>
<td>Socioeconomic status</td>
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<tr>
<td>AUG</td>
<td>Start codon</td>
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<tr>
<td>StAR</td>
<td>Steroidogenic acute regulatory protein</td>
</tr>
<tr>
<td>sgACC</td>
<td>Subgenual anterior cingulate cortex</td>
</tr>
<tr>
<td>TC</td>
<td>Tertiary complex</td>
</tr>
<tr>
<td>TLR4</td>
<td>Toll-like receptor 4</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
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<tr>
<td>TCA</td>
<td>Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TG</td>
<td>Triglycerides</td>
</tr>
<tr>
<td>US</td>
<td>Unconditioned stimulus</td>
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<tr>
<td>Abbreviation</td>
<td>Term</td>
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<tr>
<td>vHPC</td>
<td>Ventral hippocampus</td>
</tr>
<tr>
<td>vmPFC</td>
<td>Ventromedial prefrontal cortex</td>
</tr>
<tr>
<td>WGCNA</td>
<td>Weighted gene co-expression network analysis</td>
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ABSTRACT

Post-traumatic stress disorder (PTSD) is associated with elevated allostatic load, nearly double the risk for metabolic syndrome, reduced hippocampal volume, and contextual memory processing deficits. Emerging evidence suggests that these stress effects may predispose individuals to the development of PTSD, and there is a known relationship between chronic stress and metabolic dysfunction. In this work, we utilized two rat models of PTSD to explore these connections. We used an acute predator odor stressor to investigate the relationship between PTSD-like behaviors and mitochondrial dysfunction in the hippocampus of rats, and we observed that conditioned place avoidance was associated with reduced mitochondrial spare capacity and ROS production. In a separate multiple stressor + chronic psychosocial stress model, we observed increased cholesterol, triglycerides, and ROS production, fundamental allostatic load factors, in the hippocampus. In the same model, we also tested an experimental 5-HT$_{2A}$ agonist and known psychoplastogen and observed that it reduced ROS production in the cortex and hippocampus and normalized gene expression overlapping with human PTSD. Here, we show an association between mitochondrial dysfunction in the brain, the development of PTSD-like behaviors, and the induction of known allostatic load factors in the rat hippocampus after predator exposure + chronic psychosocial stress. Together these data support emerging models that suggest metabolic and allostatic characteristics of PTSD are related to functional changes in the hippocampus.
CHAPTER 1. INTRODUCTION

1.1. This work

The following section will provide important background information necessary for understanding the connections between the three experiments contained within this dissertation and human post-traumatic stress disorder (PTSD). I will describe core concepts including stress, homoeostasis, hormesis, allostasis, and allostatic load (AL) and provide a brief literature review of stress induced alterations to the hypothalamic-adrenal-pituitary (HPA) axis, glucocorticoids (GCC), insulin signaling, systemic energetic substrate availability, ROS production, inflammation, and how each of these factors is mitochondrially regulated. I will discuss the role of hippocampal (HPC) function in PTSD and how AL factors can impair basic HPC processes, like pattern separation and contextual processing, which can subsequently lead to dysregulated allostatic processing and metabolism.

In Experiment 1, I show that AL and metabolic factors are associated with the development of a conditioned place avoidance phenotype (CPA) and that mitochondria play a central role in this association. In Experiment 2, I show that traumatic + chronic stress leads to alterations in AL factors in the HPC that parallel peripheral changes in PTSD and that despite previous reports of HPC dysfunction in this model, stressed animals did exhibit adaptive physiological shifts that likely prevented catastrophic damage. Finally, in Experiment 3, I report that N,N- dimethyltryptamine (DMT) and pharmahuasca normalize reactive oxygen species (ROS) production and rescue aberrant gene expression that overlaps with human PTSD, which in addition to the present literature, suggests that 5HT2A agonist psychedelics exhibit a unique capacity to
normalize both psychological and physiological aberrations in human PTSD and represent a viable path forward.

1.2. What is stress?

The word “stress” was first used to describe personal hardship and affliction around the middle of the last millennia, but had no psychological implications at that time. In the 19th century, the term “stress” was adapted for use in physics and engineering to describe the interaction between a force and the counter resistance to that force.¹ “Stress” was later incorporated into the medical lexicon by Hans Selye, who defined it as the “non-specific response of the body to any demand.”¹ Selye went so far as to eschew the study of specific signs and symptoms of illness, instead focusing on universal causes and responses to disease. This approach was far ahead of its time, in stark contrast to the emerging views of 20th century medicine, which focused closely on specific signs and symptoms of disease. Selye’s work anticipated the emergence of late 20th century/ early 21st century concepts like allostatic load (AL), which elaborates and quantifies the multi-level and multi-systemic contributors to chronic stress-associated disease states.²

Allostasis is defined as an organism’s ability to maintain physiological stability through change.³⁻⁶ Bruce McEwen and Eliot Stellar introduced the concept of AL in 1993⁷ as the cumulative impact of ordinary daily life experiences, major life events, and deleterious behaviors on physiology.²,³,⁸ The existing physiological context of such events and the genetic background of the individual likewise play a role in the effects of stress on an organism. This impact can be expressed as a cumulative index calculated from a set of biomarkers including: body mass index, waist-hip ratio, high density
lipoprotein (HDL) and low density lipoprotein (LDL) cholesterol, triglycerides (TG),
glycosylated hemoglobin, plasma C-reactive protein (CRP), fibrinogen, serum measures
of Interleukin-6 (IL-6), soluble adhesion molecules E-selectin, intracellular adhesion
molecule-1, resting systolic and diastolic blood pressure, urinary epinephrine,
norepinephrine, cortisol, and serum dehydroepiandrosterone sulfate (DHEA-S).\textsuperscript{9,10} AL is
predictive of all-cause mortality, cognitive deficits, and cardiovascular (CVD) risk and is
associated with metabolic syndrome (MetS).\textsuperscript{10} Additional important molecular, cellular,
and systemic mediators of AL are still under consideration and case based
computational modeling has exhibited 4 clinical profiles of AL: 1) Healthy, which is
linked to lower health risks 2) Proinflammatory profile, which is linked to hypertension
and diabetes, 3) Low stress hormones, which is linked to CVD, diabetes, TIA/ stroke,
and circulation issues, 4) High stress hormones, which are linked to CVD and
hypertension.\textsuperscript{11} Additional experimental molecular and cellular factors including
mitochondrial function, reactive oxygen species (ROS) production, antioxidant pathway
expression, sensitivity to glucocorticoids (GCC), and insulin signaling, and systemic
factors including additional basal and stimulated innate immune factors, glucose level,
brain lipid profile, hypothalamic-pituitary-adrenal axis (HPA axis) sensitivity, heart rate
variability, and vagal nerve tone also contribute to CVD, hypertension, diabetes, stroke,
and stress induced cognitive deficits, and may therefore also contribute to AL.\textsuperscript{12-22}

The stress response is present across all biological levels from the cell to the
whole organism and exists to maintain energy homeostasis under increased resource
utilization. Its secondary purpose is to prevent damage associated with stress-induced
increased metabolism. Bruce McEwen and Martin Picard divide the stress response
into: 1) good stress, 2) tolerable stress, and 3) toxic stress. The stress response exerts biphasic effects on biology dependent upon intensity relative to endogenous buffering systems, defined as hormesis. The stress response follows an inverted U-shaped curve such that acute or less intense stressors produce beneficial adaptation up to the top of the curve (good stress). When stress exceeds endogenous buffering capacity and moves down the other side of the curve, the system is in allostatic overload (AOL) and stress induced damage can accrue. However, when this state is transient and supported by sufficient external and internal support and coping mechanisms, damage is minimized (tolerable stress). Chronic unpredictable AOL leads to individuals feeling overwhelmed by the demands of everyday life and deleterious physiological and behavioral consequences (toxic stress). However, both psychological and physiological adaptation can develop through which the system can reach a new allostatic balance with exogenous stress factors. This can occur biologically through the establishment of new homeostatic set points, which can be generally beneficial or beneficial only in the domain most impacted by stress, but deleterious in other domains. It can also occur through cognitive coping strategies or alterations to the interpretive framework of the self and world.
Example of AOL

The homeostatic balance that underpins AOL is directly analogous to intra and intercellular oxidative balance. Basic cellular and systemic activities like mitochondrial oxidative phosphorylation (OXPHOS) and immune activation lead to the production of reactive oxygen species (ROS). However, both of these processes can be modulated by stress and stress hormones, like glucocorticoids (GCC), which acutely increase ROS production.\textsuperscript{25,26} When the stressor is mild and the stress response itself is acute, ROS production is controlled through endogenous antioxidant systems and does not cause the accumulation of cellular damage. Oxidative stress (OXS) is a state defined by the imbalance of ROS production and endogenous antioxidant systems.\textsuperscript{27} When stress is chronic and/or extreme, ROS production can become chronically increased\textsuperscript{12} and in excess of endogenous antioxidant production, leads to lipid peroxidation, cellular damage, and innate immune activation, which leads to further ROS production, and in the worst cases, to apoptosis, programmed cell death.\textsuperscript{12,25-33} OXS is therefore a major example of toxic stress.

Biological organisms are not a simple composite of separate systems, so interdependence of all organismic stress response systems is a fundamental underpinning of the notion of stress itself and is the core insight of Hans Selye’s work and the modern concept of AL. Due to the number of AL factors, diversity of experience, genetic background, socioeconomic status (SES), and previous experiences of stress, the stress response and the manifestation of AL across individuals is highly heterogeneous. Therefore, such a vast array of distinct AL factors can exert bidirectional influence on one another and predispose an organism to so many disease
states due to their interdependent relationship to every other system. This point is underlined by the 4 distinct clinical profiles of AL mentioned at the beginning of this section.

Anticipatory allostatic regulation

Homeostasis is defined as the maintenance of the physiological parameters essential for life. The most common view of homeostasis is that it is purely reactive such that factors essential for life, like pH, oxygen tension, glucose levels, and body temperature for homeotherms, have strict set points that cells and biological systems maintain in direct response to perturbations. Attempts have been made to develop homeostasis into a concept that covers adaptation as well as the basic setpoints of life, but in general these attempts have produced more confusion than clarification. Homeostasis is defined as the maintenance of those factors essential for life, therefore homeostatic overload would imply the end of life, which would be more properly defined as homeostatic failure. Alternatively, AOL implies the accumulation of stress-induced damage, but does not imply the death of the organism.

This underlines the need for the separate concept of allostasis for anticipatory adaptive changes to future energy demands. Allostasis can apply to situations as broad as the adaptive changes a cow must undergo in order to lactate, preparatory metabolic changes that occur when a rodent experiences a sensory cue that predicts a predator encounter, or to the anticipatory release of cortisol to public speaking that a human may experience immediately prior to their talk, a psychosocial stressor. Control of metabolism and energy production are central to allostasis. Stress requires substantial utilization of cellular energy stores and the primary purpose of the stress response is to
mobilize cellular energy stores in anticipation of increased energy demands.\textsuperscript{36-38}

Allostasis is regulated across myriad integrative molecular, cellular, physiological, and neural information hubs. I will briefly review the central nervous system (CNS) mechanism underpinning allostatic processing in the next section and the central systemic regulator of allostasis during the stress response in the following one, the mitochondria.

\textit{What are the neural networks contributing to allostatic processing?}

Allostasis is essentially the anticipatory balancing of energy requirements with energy stores and inputs, which is highly dependent upon the current state of the system. Lisa Feldman Barrett and colleagues established two interconnected brain networks that orchestrate these processes through elaborate tracing experiments in Rhesus monkeys and confirmed these data through two independent functional magnetic resonance imaging (fMRI) studies in humans\textsuperscript{4,39,40}. The first brain network centers on the primary interoceptive cortex, which consists of the dorsal mid insula (dmlns) and the dorsal posterior insula (dplns) and receives direct afferent inputs from the vagus nerve and small diameter C and A\textdelta fibers from the viscera, and direct and indirect inputs from brain stem and hypothalamic nuclei, which provide sensory data on the state of the system. Afferent information about the current state of the system only provides half of the information required for allostatic control however. Predicted future energetic demands are crucial for CNS control of allostasis. This information is supplied from memory and therefore depends on the hippocampus (HPC). The present affective state also feeds into this calculation. According to control theory, any system that regulates a second system must contain a model of that system. The second brain network
identified by Barrett and colleagues centers on the granular and dysgranular visceromotor cortex, which includes the subgenual, pregenual, and mid anterior cingulate cortex, (sgACC, pgACC, aMCC). These visceromotor regions collaborate with dorsomedial and ventromedial prefrontal cortex (dmPFC/ vmPFC), sourcing information from long term memory to produce internal models of an organisms metabolic landscape through active inference.\textsuperscript{41} This model is constantly updated in real time by prediction errors stemming from primary interoceptive cortex. According to Lisa Barrett and Anil Seth, affect is literally the real time experience of our internal predictive models of endogenous energy balance.\textsuperscript{4,41} Our feelings about self and world are therefore internal predictions about the metabolic implications of the present relationship between self and world.

\begin{quote}
I predict myself, therefore I am
--Anil Seth
\textit{Being You}\textsuperscript{42}
\end{quote}

1.3. Cellular energetics and mitochondrial control of allostasis

A man never steps in the same river twice, for it is not the same river and he is not the same man
-- Heraclitus\textsuperscript{43}

\textit{Energy flow in biological systems}

Life is defined by energy flow within an autopoietic system.\textsuperscript{44} A fresh cadaver differs from a living being only by its lack of animation through self-sustaining energy flow.\textsuperscript{45} Energy is the capacity to do work and therefore mediates the production of change within a biological system and living organisms are maintained through constant change. Life is movement, flow, and change. The lack of change is stagnation and death. Even the maintenance of homeostasis, a steady state required for life, is
dependent upon change because the world changes and the maintenance of life is therefore the ability of an organism to dance in step with the world as it moves.

In animals, energy is obtained from and stored in chemical bonds.\textsuperscript{46} Broadly, we utilize energy stored in the chemical bonds of macronutrients, like carbohydrates, fats, and protein, to produce adenine triphosphate (ATP), the primary energetic currency of the cell.\textsuperscript{47} Glucose can be used directly, but triglycerides (TG) and amino acids can undergo glucogenesis in the liver to be converted to glucose, stored as glycogen, or TG can be converted to ketones, which can be used directly. Glycolysis produces ATP and other energy rich substrates like reduced nicotinamide adenine dinucleotide (NADH) or reduced flavin adenine dinucleotide (FADH2). The end product of glycolysis is pyruvate, which is enzymatically converted to acetyl-COA to enter the tricarboxylic acid cycle (TCA) to produce more NADH. Ketones and lactate also enter the TCA as acetyl-COA to produce NADH.\textsuperscript{48}

NADH and FADH\textsubscript{2} then enter the mitochondria and provide the electrons and hydrogen protons (H\textsuperscript{+}) required for ATP synthesis. The mitochondria have 4 complexes that participate in the establishment of a proton gradient between the mitochondrial matrix and the intermembrane space. Complex 1-3 oxidizes NADH or FADH\textsubscript{2}, pumping their protons into the intermembrane space and using electron carriers to move their electrons between complexes. Complex 4 pumps the greatest number of H\textsuperscript{+} across into the intermembrane space using oxygen as the final oxidizer and electron acceptor. Finally, ATP synthase uses the potential energy in the proton gradient to phosphorylate adenine diphosphate (ADP) into ATP.\textsuperscript{49} Other sources of ATP production are also present in biological systems, like anaerobic metabolism that produces lactate as a
byproduct and secondary energy source, but for brevity and relevance, I will not
describe those systems here.

_The mitochondria as an endocrine organelle regulating systemic energy states_

Cellular energy homeostasis is dependent upon the availability of sufficient energy
substrates to meet ATP requirements. Stress, by definition requires energy.\(^2,6,50\) Energy
is often required for the actual physical response to the stressor i.e. movement of large
muscles, but also for the requisite changes in physiology required to adapt to the
stressor i.e. energy for metabolism, enzymatic action, active transport, phosphorylation,
transcription, protein synthesis, cell repair, etc. This is even more pronounced the brain,
which uses a disproportionate share of energy in the body due to the large ATP
requirement for maintaining electrochemical gradients in neurons.\(^51\) Energetic substrate
availability to different tissues is regulated by stress hormones, which modulate insulin
and insulin like growth factor (IGF1/2) receptor (IGF1/2R) activity and expression and
subsequently the activity and expression levels of glucose transporters (Glut 1-5).
Stress hormones likewise modulate glucogenesis in the liver and transporters for other
energetic substrates, like ketones and lactate.\(^52\)

The anticipation of increased energy demand activates the hypothalamic-
pituitary-adrenal (HPA) axis to release glucocorticoids, which increases organismic
energetic substrate availability.\(^52\) Parvocellular cells in the paraventricular nucleus
(PVN) of the hypothalamus releases corticotrophin releasing hormone (CRH) into the
median eminence, which is transported to the anterior pituitary through the hypophyseal
portal system, which then synthesizes and releasees adrenocorticotropin releasing
hormone (ACTH) into the blood stream. ACTH is transported to the adrenal cortex of
the adrenal gland and stimulates the synthesis of cortisol or corticosterone (CORT) from cholesterol (CHOL), which is released into systemic circulation and crosses the blood brain barrier.\textsuperscript{53} CORT then provides negative feedback to the pituitary and hypothalamus to reduce CRH and ACTH production. The adrenal medulla produces catecholamines, like epinephrine and norepinephrine (NE) that likewise play a major role in the stress response.\textsuperscript{54}

Mitochondria are the site of all steroid hormone synthesis. GCC are produced within the mitochondria of cells of the zona fasciculata of the adrenal cortex.\textsuperscript{45} CHOL is imported through mitochondrial membranes by steroidogenic acute regulatory protein (StAR),\textsuperscript{55} the rate limiting step for steroid synthesis, which is activated by ACTH.\textsuperscript{56} CHOL is transformed into pregnanolone by cytochrome p450 side chain cleavage enzyme (P450\text{scC}). Pregnanolone is exported to the endoplasmic reticulum (ER), where additional enzymes mediate the conversion of pregnanolone to deoxycorticosterone in rodents or 11-deoxycortisol in primates. These metabolites are then exported to the mitochondrial matrix, where a final reaction catalyzed by 11β-hydroxylase (11βH) produces cortisol in primates or corticosterone in rodents.\textsuperscript{57} While the mechanisms of release from the mitochondria remain understudied, its commonly believed that due to its lipophilic nature, CORT then diffuses across the membrane and into systemic circulation through its concentration gradient.\textsuperscript{21,45}

GCC like CORT in particular modulate the availability of energy substrates to different tissues in the body. Two major mechanisms mediate this function of GCC. The first is an overall elevation of systemic energy substrates through direct action on the liver to reduce glucose utilization and increase glucose output. GCC do not modulate
the insulin receptors in the liver, but reduce glucose utilization through increased pyruvate dehydrogenase kinase 4 (PDK4), which inhibits pyruvate dehydrogenase (PDH). Glucocorticoids induce increased gluconeogenesis through increasing the activity of phosphoenolpyruvate carboxykinase (PERCK) and glucose-6-phosphatase (G-6-Pase). GCC also lead to increased lipogenesis and other alterations to fatty acid metabolism and export, but the mechanisms are less well understood. The second major mechanism is reducing glucose uptake in skeletal muscle and adipose tissue through lowering insulin stimulated GLUT4 translocation through reduced phosphorylation of AKT/protein kinase B (PKB). Together this leads to increased circulating energetic substrates and therefore a higher availability of them to the brain, exhibiting a fundamental characteristic of the stress response.

In the brain, short term GCC exposure increases glucose utilization, but long term exposure causes insulin resistance through the desensitization or downregulation of insulin receptors and/or glucose transporters. Short term exposure to insulin or CORT increases neuronal excitability, but also increases propensity for excitotoxicity. Insulin resistance in the hippocampus is induced both by CORT and chronic restraint stress (CRS) through decreased phosphorylation of insulin receptor substrate 1 (IRS1), the primary signaling target of the insulin receptor, and is associated with functional deficits in the HPC. Other studies report that CORT induces reduced transcript expression of insulin and insulin receptor genes, which also correlate with HPC deficits or reduced translocation of glucose transporters, like Glut4, therefore reducing glucose uptake, similar to the effects observed in the periphery.
Basal effects of GCC are mediated by the mineralocorticoid receptor (MCR), while stress induced effects of CORT are mediated by the glucocorticoid receptors (GCR). GCR exert both genomic and non-genomic effects. GCR in the cytosol bind to GCC and are translocated either to the nucleus, the canonical pathway, or to the mitochondria in a chaperone specific fashion. The GCR binds to glucocorticoid response elements (GRE) to exert genomic effects. Nuclear GCR also regulate gene transcription through protein-protein interactions with nuclear regulatory proteins. The GCR binds to the mitochondrial outer membrane, inner membrane and mitochondrial matrix, and modulates mitochondrial gene expression and function in all cells. GCC modulate insulin signaling through the GCR and insulin signaling modulates HPA axis function, exhibiting a secondary indirect feedback loop along the HPA axis.

Bruce McEwen, Martin Picard, and coauthors therefore defined CORT and other mitochondrially produced steroid hormones as “mitokines,” hormones produced by the mitochondria that regulate the metabolic physiology of stress. They make this claim based on 4 elements that connect the mitochondria to stress: (1) Energy is required for the stress response across all levels of biological organization. (2) Stress hormones like GCC are produced and metabolized by the mitochondria. (3) Mitochondria are sensitive to stress hormones and other neuroendocrine and metabolic mediators of stress. (4) Physiological and behavioral responses to stress are altered by manipulations of the mitochondria.

Adrenal mitochondria communicate directly to all mitochondria in the body through GCC and the GCR and therefore orchestrate whole organism mitochondrial function during stress. They control whole organism energetic substrate availability by
controlling glucogenesis, lipogenesis, and substrate export from the liver, and access to the available substrates through tissue specific modulation of insulin signaling, which controls substrate uptake. Mitokines also control their own production through direct and indirect modulation of the HPA axis through direct negative feedback and insulin signaling respectively. Therefore the mitokine system regulates all organismic ATP production under stress, even that from glycolysis due to the regulation of glycolytic substrates. These features place the mitochondria at the center of whole organism cellular energetic allostasis during stress and therefore the whole organism stress response.\textsuperscript{45}

Surprisingly, Picard and McEwen further propose that adaptation to stress requires the transduction of environmental stress signals to short and long term transcriptional and translational regulation. Regulation of the transcriptome, the translatome, and the proteome are metabolically expensive and would be expected to be regulated by mitochondrial metabolic signals.\textsuperscript{71} The energetic environment and the cellular transcriptional landscape are coupled through mitochondrial metabolic intermediates and byproducts, like Acetyl CoA, NAD+, succinate, α-ketoglutarate, and ROS, that reach the nucleus and modulate gene expression through interactions with chromatin, genes, and regulatory proteins.\textsuperscript{45,73} Mitochondria can relocate to the nucleus during hypoxia and generate an oxidative environment through ROS production, which leads to hypoxia-inducible factor 1α activation and gene transcription.\textsuperscript{74} ROS also modulate broad organismal energetic homeostasis\textsuperscript{75} and control important physiological systems, most importantly for our purposes, neurogenesis in the dentate gyrus (DG) of the HPC,\textsuperscript{76,77} which is critical for pattern separation (PS) or PS-like processes.\textsuperscript{78,79}
Metabolic control of neurogenesis through ROS is consistent with the idea that ROS acts as a metabolic signal for cellular energy status because neurogenesis in the adult brain comes at substantial metabolic cost. Finally, general cellular stress including redox balance modulate the integrated stress response (ISR) that controls global protein translation through altering the cellular concentration of a tertiary complex (TC) including eLF2, guanine triphosphate (GTP), and Met-tRNA\textsubscript{i}. The TC is required for the initiation of transcription of AUG-initiated reading frames in the cell’s transcriptome. Recognition of the AUG codon triggers GTP hydrolysis, which results in the TC releasing Met-tRNA\textsubscript{i} to the ribosomal p site. The remaining eIF2-GDP then dissociate and the ribosomal complex is formed and protein synthesis occurs.\textsuperscript{80}

Endogenous ROS are deleterious and must be balanced with endogenous antioxidants and other oxidant defense systems in order to act as signaling molecules without causing widespread cellular damage. When ROS exceeds endogenous buffering capacity, cellular damage accumulates and damage associated molecular patterns (DAMPs) are recognized by pattern recognition receptors (PRR), like TLR4, which activate inflammatory pathways, like Nf-κB and the NALP3 inflammasome, which produce inflammatory mediators, like IL-1β. Cytokines like IL-1β modulate brain function, particularly in the HPC.\textsuperscript{81-83} Collectively, these intersecting narratives place the mitochondria at the center of stress-induced organismic cellular energy allostasis and subsequently, regulatory control of energy expensive cellular processes involved in adaptation, growth, and development. However, the flip side of that coin is that the mitochondria are also the center of the deleterious effects of stress when AL shifts into AOL and toxic stress develops.
1.4. Trauma, PTSD, and the Hippocampus

Trauma is a psychic wound that hardens you psychologically that then interferes with your ability to grow and develop. It pains you and now you're acting out of pain. It induces fear and now you're acting out of fear. Trauma is not what happens to you, it's what happens inside you as a result of what happened to you.

--Dr. Gabor Maté

*Interview with Human Window* 84

*Trauma changes everything, from mind to metabolism.* In a single ineffable moment or a prolonged cloud of ineffable occurrences, lives are shifted into a new state of being. Despite the typical intensity of traumatic events, individuals are commonly unaware that this shift has taken place until days, weeks, months, or even years later when the implications of the event or events fully emerge in the psyche and behavior. This point is highlighted in the diagnostic statistical manual of psychiatric disorders 5 (DSM-V) delayed specification for post-traumatic stress disorder (PTSD) in which the full diagnostic criteria of PTSD are not reached until over 6 months post traumatic event. The DSM-V requires that a diagnosis of PTSD be associated with “a stressor”: “death, threatened death, actual or threatened serious injury, or actual or threatened sexual violence.” 85 There must be at least one intrusion symptom including intrusive memories, nightmares, flashbacks, emotional distress or physical reactivity after considering trauma-associated memories. There must be at least one avoidance symptom, either of trauma related feelings or external reminders, and at least two negative alterations in cognition and mood which can include deficits in trauma-associated memories, overall negative views of the self and others, negative affect, anhedonia and feeling isolated or disconnected to others. There must be at least two alterations in arousal and reactivity including hyperarousal, hypervigilance, aggression, exaggerated startle response, irritability, reckless behavior, difficulty concentrating, or difficulty sleeping. Lastly, these
symptoms must persist for at least one month and cause distress and/or functional impairment. In addition to delayed specification PTSD mentioned previously, the DSM also considers a dissociative specification of PTSD in which the classic PTSD symptoms are combined with depersonalization and derealization symptoms. PTSD is also associated with oxidative stress, metabolic syndrome, and allostatic load factors mentioned in section 1.

Despite the DSM-V defining a traumatic event as a “stressor,” trauma is distinct from chronic or even intense acute “stress.” The major difference is the extent to which a trauma is dependent upon interpretive context. Both stressors and trauma induce reactive homeostatic and adaptive allostatic responses that alter an organism’s internal landscape in order to cope. Stress can lead to adaptation in the physiological and cognitive domains, but stress-induced alterations to affective and cognitive predictive processing (adaptation) are limited to coping strategies. Trauma also alters the physiological stress response systems in similar ways, but according to the cognitive worldview model of PTSD, its defining characteristic is fundamental changes to the highest level cognitive models of the self and world, a violation of meaning. A traumatic event is therefore necessarily associated with an interpretive framework of self and world, implying that trauma and adaptation to trauma is fundamentally interpretive.

People generally don’t suffer high rates of PTSD after natural disasters. Instead, people suffer from PTSD after moral atrocities. Soldiers who’ve endured the depraved world of combat experience their own symptoms. Trauma is an expulsive cataclysm of the soul. --David Brooks

Only ~30% of individuals exposed to a potentially traumatic event go on to develop PTSD. This proportion depends on numerous AL associated factors across various populations,\textsuperscript{94-96} as well as the type of trauma experienced. Exposure to natural disasters only causes PTSD in ~10% of those exposed, while rape and physical assault causes PTSD at a rate of ~50%.\textsuperscript{94-97} This variability exemplifies the interpretive nature of the kinds of trauma that lead to PTSD compared to even very intense stressors. In the wake of major earthquakes, fires, hurricanes, tornados, or tsunamis, hundreds of thousands or millions of people can be exposed to potentially traumatic levels of stress. People experience mass death and destruction, resource scarcity, and substantial physical insecurity. However, relatively few people go on to develop PTSD after such events compared to war, physical assault, or rape. In fact, people often report that such experiences are the most meaningful of their lives. Natural disasters do not typically shatter people’s identity or undercut basic feelings of safety in the world independent of the disaster. Alternatively, individuals are often involved in the creation of meaning with their communities in the aftermath of such events, as described in “A Paradise Built in Hell” by Rebecca Solnit.\textsuperscript{98}

Many symptoms of PTSD are adaptive within the context in which the trauma occurred, but not in other contexts. For instance, hyperawareness, hypervigilance, and avoidance of trauma-associated stimuli, and hyperarousal symptoms may protect an individual from further traumatic events during war, or from further hardship in a circumstance of domestic abuse. Feelings of anger and revenge may likewise be adaptive in war or to escape from an abuser, but hyperarousal, avoidance, and outbursts of intense anger during civilian life may lead to deteriorating social
relationships. These responses are adaptative in the context in which they developed, but are highly maladaptive in social contexts. This idea is highlighted in a 2016 review by David Diamond and Philip Zoladz. The PTSD phenotype may therefore have provided substantial evolutionary advantage to human ancestors. After trauma, we observe at least two major phenotypes, those that develop PTSD after 30 days and those that do not. The PTSD phenotype becomes more affectively specialized for events similar to the domain in which the trauma occurred while the non-PTSD or “resilient” phenotype remains more cognitively generalized, but may still exhibit behavioral and physiological adaptation. This concept explains the conservation of multiple adaptive phenotypes across many species through evolutionary time.

Adaptive phenotypes may be categorized by primary diagnosis (PTSD+ vs PTSD-), but also exhibit individual and trauma-specific features. These specific features may differ in the cognitive domain (What specific PTSD symptoms emerge and when/how?) and in the biological domain (Which phenotype of AL emerges?) as outlined in section 1.1.

Stress is anything that adds to the total metabolic requirements of an organism. AOL and toxic stress develop when a stressor exceeds the endogenous stress buffering capacity of the organism. Both trauma and PTSD are associated with stress. A trauma is an event that defies existing meaning systems, particularly those that impact an individual’s view of self and world. PTSD develops when an individual chronically struggles to integrate that event with their understandings of self and world. Trauma and PTSD are particularly impactful on AL because allostasis and therefore AL at the CNS level is based on predicted future energy expenditure, which can become dysregulated due to the high level of cognitive uncertainty that trauma produces, a major contributor
to AL. This may explain the relationship between PTSD and other disorders associated with elevated AL. The PTSD phenotype is an adaptive response to the high degree of cognitive uncertainty induced by a chronic cognitively unresolvable traumatic event and consists of adaptive specialization to trauma-associated contexts.

Animal models of stress and trauma

Animal models used for studying stress and chronic stress have emerged, which are commonly used to study aspects of depression and other stress-associated psychiatric disorders. The major model systems used in biomedical research are chronic restraint stress (CRS) for 6h/21 day,\textsuperscript{101,102} and chronic unpredictable mild stress (CUMS) for up to 6 weeks.\textsuperscript{103} Both systems induce a behavioral phenotype defined by reduced sucrose preference, reduced preference for female urine in males, reduced open arm time in the elevated plus maze (EPM), and increased immobility in the forced swim and tail suspension tests.\textsuperscript{103} CUMS is likewise associated with decreased HPC neurogenesis and atrophy of CA1 and CA3 apical dendrites,\textsuperscript{104} atrophy of cortical and limbic brain regions,\textsuperscript{105} serotonergic sensitization, overactivation of the noradrenergic system,\textsuperscript{106} increased inflammatory cytokines in the HPC,\textsuperscript{107,108} HPC microglial activation,\textsuperscript{109} and HPA axis dysregulation.\textsuperscript{110} Likewise, chronic stress leads to cognitive, affective, and memory impairments\textsuperscript{111} in humans and is associated with systemic inflammation and the development of numerous associated conditions including hypertension, type 2 diabetes, irritable bowel disease, gastric ulcers, Parkinson’s disease, Alzheimer’s disease, and cancer.\textsuperscript{112} However, acute stress is alternatively associated with increased neurogenesis in the dentate gyrus (DG) of the HPC.\textsuperscript{113}
Animals are not likely capable of the kinds of interpretive narrative construction necessary for the development of PTSD. However, there has been a substantial effort to develop animal models for the study of PTSD that model specific features of the disorder, like avoidance behavior, GCC abnormalities, or fear memory generalization. A useful animal model for the study of PTSD should exhibit face validity (symptomology of PTSD), construct validity (etiology), and predictive validity (treatment response), in addition to individual variability in response to the stressor independent of stressor intensity. Rachel Yehuda suggested 5 specific criteria that should be fulfilled for a model of PTSD to be useful: 1) brief stressors should be able to induce biological and behavioral sequelae of PTSD, 2) the stressor produces intensity-dependent PTSD-like sequelae, 3) biological alterations secondary to the stressor should persist or become more pronounced over time, 4) biobehavioral alterations to the stressor should exhibit the possibility of bidirectional changes, and 5) stress responses should exhibit inter-individual variability. Whitaker et al. (2014) proposed a 6th criterion, the ability to produce co-morbid conditions associated with PTSD, like increased nociception or escalation of alcohol self-administration.

Two important ethologically based models that have emerged are predator exposure + psychosocial stress models (PE/PSS) and predator scent stress models (PSS). The most common PE/PSS models were generated and standardized by a group in Israel led by Hagit Cohen and Joseph Zohar and by David Diamond at the University of South Florida. Predator scent stress models (PSS), have been developed by many groups, but a particularly useful version was developed by Nick Gilpin’s lab at LSU, who paired PSS with conditioned place avoidance (CPA).
In this work, we utilized Nick Gilpin’s PSS model, which indexes for avoidance (or lack thereof) of a predator odor-paired chamber to investigate individual differences in adaptive phenotypes and stress responses. We also used David Diamond’s PE/PSS model, which produces a single phenotype characterized by features of PTSD progression, to investigate stress and trauma-induced biological adaptations to trauma + chronic stress. I will therefore focus on these two models in particular in the remainder of this section.

The Diamond model utilizes Sprague Dawley rats and two separate hour long predator exposures on day 1 and 11 of a 30 day psychosocial stress regiment. One exposure takes place during the day and one at night. Daily PSS consists of chronic social instability due to daily cage rotation in which animals get different cage mates each day and only experience the same cage mate up to 3 times per experiment. Predator exposure can be paired with trace fear conditioning paradigms such that a tone (US) is played immediately prior to animals being restrained and placed into the predator exposure chamber. Later, the US alone is played while the animal is inside of a fear conditioning chamber and freezing behavior is measured. Behavioral testing usually occurs after the 30 day stress period and cage rotation usually continues through behavioral testing, but many different iterations of this and similar models have been published.\textsuperscript{116,118,124,126-130} This model is associated with increased reactive oxygen species (ROS) production and inflammation (NALP3, IL-1\(\beta\), TLR4)\textsuperscript{12,131} in the prefrontal cortex (PFC), HPC, blood, and adrenal glands.\textsuperscript{12} Dr. Joseph Francis’ lab also measured reduced serotonin (5-HT) and increased norepinephrine (NE) in the PFC and HPC, and SSRI (sertraline) treatment normalized 5-HT levels, but also further increased NE.\textsuperscript{14,130}
This model is also associated with reduced levels of IGF1R in the PFC and HPC and alterations to pre and post synaptic protein expression.\textsuperscript{131}

The Gilpin model uses Wistar rats and only one 15 min bobcat urine exposure as the stressor. 15 minutes of predator urine stress is sufficient to induce a long term traumatic memory in a subpopulation of rats exemplified by chronic conditioned place avoidance (CPA). This allows for the mechanistic study of CPA, a rodent analogue of avoidance behavior in PTSD with minimal experimental noise. Rats are exposed to three chambers of an apparatus designed for CPA for 5 minutes on day 1 of a 5 day protocol. Time spent in each chamber is measured and the chamber most different from the others in time spent is removed for that animal to eliminate bias. On day 2, animals are exposed to the two remaining chambers for 5 minutes. On day 3, animals are exposed to only one chamber without predator odor for 15 minutes. On day 4, animals are exposed to predator odor in the remaining chamber for 15 min. On day 5, animals are exposed to both chambers again and indexed for CPA of the odor associated chamber. Time spent in each chamber is recorded and subtracted from time spent in that chamber at baseline to remove baseline bias. “Avoiders” (Av) are defined as those animals that spend > 10 seconds less time in the odor chamber than their baseline and “non-avoiders” (NAv) are those that do exhibit show CPA.\textsuperscript{114} All stressed animals exhibit reduced open arm time in the EPM and females exhibit abnormalities in endocannabinoid signaling in the amygdala.\textsuperscript{132} Av also exhibit attenuated ACTH and CORT production immediately after odor stress and escalating alcohol consumption, two features of human PTSD.\textsuperscript{114}

\textit{Contextual Processing and Hippocampal Function in the Stress Response}
There now exist 5 major theoretical frameworks that attempt to explain PTSD symptoms from a neuroscientific perspective: 1. disruption in fear learning/ extinction, 2. disruption of executive function/ emotional regulation, 3. exaggerated threat detection, 4. disruption of contextual processing (CP), and 5. disruption of hippocampally-dependent associative learning.\textsuperscript{133}

One of the most useful and well-established of these models is the contextual processing (CP) model. According to the CP framework, PTSD is associated with cognitive inflexibility resulting from deficits in CP. The function of CP is allowing for flexible, yet situation-specific information encoding, representation, retrieval and most importantly, discrimination. This allows organisms to carry out contextually appropriate behaviors and suppress inappropriate ones. CP allows organisms to filter affective responses to cues associated with danger in one context, but that are not applicable to the current context. PTSD patients show impaired fear extinction recall, the inability to use safe context to reduce the physiological response to a danger cue, and more surprisingly, impaired fear renewal, the inability to use a previously dangerous context to predict a present danger cue, indicating general contextual memory deficits in PTSD beyond deficits in fear extinction.\textsuperscript{134-137} The medial temporal lobe (MTL) and HPC are required for contextual processing and consequently regulate brain control of allostasis through generating, retrieving, and discriminating the explicit cognitive representations of the present context and its relationship to relevant past contexts.

While each has shortcomings, 1-3 particularly fail to explain non-cued intrusive and re-experiencing symptoms, sleep abnormalities and nightmares, smaller hippocampal volume, and emotional numbing. Number 2, but not 1 and 3 explain the
high prevalence of addiction associated with PTSD and the high likelihood of persons with PTSD to retraumatize themselves through reckless behavior. A disruption of CP or, more generally, associative learning explain all of these symptom clusters and physical abnormalities, in addition to all of the criteria for a PTSD diagnosis. A major advance made by the associative learning hypothesis is the integration of a wide range of literature on memory deficits in PTSD i.e. contextual processing, episodic memory, autobiographical memory, and emotional working memory under a single framework.

The most well established function of the HPC in humans is to form explicit episodic memories about the self and world. This was first elucidated by the famous case study of patient HM, who following bilateral removal of the HPC due to epilepsy, developed complete anterograde amnesia and substantial retrograde amnesia for events that occurred in the months and years prior to surgery. Further investigations in humans and animal models established that the hippocampus maps physical space and cognitive space, including abstract value space and its relation to physical spaces. Investigators have also exhibited place cells in the HPC that map onto grid cells in the entorhinal cortex (EC) as a cognitive graph, providing a mechanism of spatial mapping. More recently, schema cells were observed in the macaque HPC and concept cells in the human HPC, collectively extending the mechanism underlying spatial mapping to the complete construction of episodic memories across all cognitive domains.

The HPC has three cell layers, the outer molecular layer, the inner pyramidal cell layer, and the inner stratum oriens. Its major functional regions are Cornu Ammonis 1
(CA1), CA3 and the dentate gyrus (DG). A fourth region, CA2 lies between CA1 and CA3, but exhibits a cytoarchitecture similar to CA3 and is rarely discussed due to its small size. Some investigators also consider CA4 a distinct region, which forms the intersection of CA3 and the DG. Information flows into the HPC from the entorhinal cortex (EC). The EC is a five layered paleocortical structure and has two major relevant divisions. The lateral entorhinal cortex (LEC) receives input from the temporal (ventral) stream through the postrhinal cortex (PrC) and processes information about individual objects. The medial entorhinal cortex (MEC) receives input from the parietal stream through the parahippocampal gyrus (PhC) and processes spatial and contextual information. The three major HPC regions form the trisynaptic loop and most information flows from EC-DG-CA3-CA1-EC. The DG is the primary input region of the HPC and receives afferent projections from layer 2 of the EC carrying multisensory information, the perforant pathway. The DG is one of few sites of neurogenesis in the adult brain and has three layers that differ slightly from the hippocampus proper: the molecular layer, the middle granule layer, and an inner polymorphic layer, the major difference being that the middle layer contains granule cells rather than pyramidal cells. Granule cells project mossy fibers to CA3 pyramidal neurons and CA3 also receives direct input from the EC layer 2. CA3 has recurrent collaterals back to itself (recurrent collaterals) and projects to CA1 through Schaffer collaterals. CA1 also receives direct input from EC layer 3 and is the primary output region of the HPC, projecting to the subiculum and to EC layer 4 and 5. The function of the HPC depends on its inputs (MEC or LEC), but also its dorsal-ventral axis. The posterior hippocampus (dHPC in
rodents) predominantly processes spatial and contextual information, while the anterior (vHPC in rodents) predominantly processes affective information.\textsuperscript{156}

There are three major levels of biological memory: 1) cellular memory, 2) intercellular memory, and 3) neural circuit level memory. I will only discuss episodic memory here, a form of neural circuit level memory. There are three stages of episodic memory construction. The first is encoding in which the firing patterns of neurons in the HPC are entrained to incoming highly processed sensory stimuli. Two major information streams flow into the HPC, one that brings highly processed “object” information into the HPC through the LEC and, one that brings highly processed “context” information from the MEC. In both cases, this information is held in memory through the protein synthesis independent adjustment of HPC firing patterns around the trisynaptic circuit of the HPC, which integrates object, spatial, affective, and contextual information into a cognitive map of the world and events, which constitute an engram.\textsuperscript{147-149,151,157} The second stage is memory consolidation and is protein synthesis dependent. Consolidation is thought to take place during light slow wave sleep when the neurons that constitute an episodic memory in the HPC exhibit synchronous activity through long range connections with cortical neurons in the MTL and PFC where long term memory tracers are stored.\textsuperscript{158} Memory retrieval is the process of recollecting stored memories and requires the HPC and MTL for some time (days-weeks) after the memory, but not for long term memories.\textsuperscript{158} Memory extinction is the process of generating a new memory that inhibits a response associated with an existing one such that the existing memory is not erased, but its impact is modulated. Extinction does require the HPC. When memories are retrieved, they again become liable to editing. Memory reconsolidation is the process of
moving retrieved memories back to long term memory, stabilizing them, and also requires the HPC and protein synthesis.159

Pattern separation (PS) is a computational process hypothesized to take place in the DG-CA3 (CA4) circuit of the HPC through which incoming inputs are orthogonalized into more distinct representations through sparse coding or another similar mechanism. Mounting evidence also suggests a role for neurogenesis in this process.78,79,160,161 There is some debate as to whether a process computationally defined as PS actually takes place in the HPC and if it actually involves new born neurons,79 but most, although not all investigators162 agree that this circuit is involved in the orthogonalization of distinct engrams, whether that be objects (LEC-DG), contexts (MEG-DG in the dHPC) or affective information (EC-DG in the vHPC).155

The HPC exhibits relatively higher expression of the GCR compared to the rest of the brain and is subsequently particularly sensitive to both the salutary and deleterious effects of stress.25,70,163-165 GCC acutely enhance HPC dependent affective memory consolidation and extinction, but reduces memory retrieval, which may or may not require the HPC.159 In humans, PS is enhanced by acute stress-induced cortisol elevation during memory consolidation.166 However, the timing of GCC on HPC contextual memory processing also matters. Acute GCC 90 minutes prior to encoding reduce memory retrieval 24 hours later, but delayed GCC 210 min before encoding enhance retrieval 24 hours later.167 In general, the effects of GCC on HPC memory exhibits an inverted U shaped curve such that basal effects driven by the MCR are permissive for important cellular functions, moderate levels driven by both the MCR and GCR enhance memory processing, and excessive levels produce deleterious effects.168
The effects of GCC on memory are blocked by GCR antagonists and do not occur when the adrenergic system is not simultaneously activated. Beta adrenergic receptor antagonists block the beneficial effect of GCC, and yohimbine, a stimulator of norepinephrine (NE) release, facilitates the memory enhancing effects of GCC on non-affective memories, indicating a collaborative effect of GCC and catecholamines, like NE. Alternatively, high levels or chronic GCC or chronic stress exhibit broad deleterious effects on HPC structure and function. Acute stress increases proliferation of HPC progenitor cells and astrogliogenesis, while suppressing neurogenesis into microtubule associated protein 2 (MAP2) and doublecortin (Dcx)-positive neurons, but chronic stress or GCC reduce HPC cell proliferation and neurogenesis without increasing astrogliogenesis, and causes retraction of CA3 pyramidal cell dendrites and deficits to HPC synaptic function. In general, acute stress or GCC exposure enhances glutamatergic signaling and LTP, while chronic stress or GCC suppress glutamatergic signaling and LTP and enhance long term depression (LTD), a major link between the chronicity of GCC and HPC function. Chronic GCC are also linked to increased ROS production and inflammation in the brain, which also modulate HPC function. IL-1β and other cytokines impair neurogenesis, LTP, context discrimination, and PS.

In general, contextual memory processing enhances all other forms of memory, but context-dependent effects on memory are minimized in circumstances where context is suppressed. The most replicated neuroanatomical correlate to PTSD is reduced HPC volume and the effect may be primarily driven by reduced posterior HPC volume, which processes contextual information. Smaller HPC volume predicts
vulnerability to psychological trauma\textsuperscript{182} and is associated with overgeneralization of negative contexts.\textsuperscript{137,183,184} HPC subregion analysis indicates that this effect may primarily driven by reduced DG-CA4 volume, which is negatively correlated with PTSD symptoms.\textsuperscript{183,185} Furthermore, greater DG volume is a resilience factor for trauma-related PTSD symptoms.\textsuperscript{186} line with this, PTSD patients exhibit broad contextual memory processing deficits\textsuperscript{135} and HPC pattern separation has recently been linked to fear generalization, a common feature of PTSD.\textsuperscript{187}

1.5. Treatments and Experimental Therapeutics

Current FDA approved treatments for PTSD are not aligned with our modern understanding of the disorder. Emerging evidence suggests that PTSD is best described as a memory processing disorder, although there is currently debate as to range of memory processes that are disturbed.\textsuperscript{133} There is now a rich literature on contextual memory deficits in PTSD,\textsuperscript{136,139,188,189} episodic memory deficits,\textsuperscript{140,141} and autobiographical memory deficits.\textsuperscript{141,142} PTSD is additionally associated with a tendency to overgeneralize memories, especially those associated with fear.\textsuperscript{190,191} A critical point is that while certain memory deficits, like fear generalization are fear or trauma related, whereas most forms of memory deficit observed in PTSD are general. PTSD can be caused by a wide range of events ranging from accidents and the observation of a serious trauma occurring to another person, events that cause only about 8\% to 10\% of people who experience them to develop PTSD, to combat, childhood trauma, and rape, which cause between 35\% and 50\% of individuals who experience them to develop PTSD.\textsuperscript{85,117,192,193} The types of traumatic incidents that tend to cause high rates of PTSD are those that disrupt belief systems and/or future goals. The cognitive worldview model of PTSD is able to explain
48% of the variability in PTSD symptomology on the clinician administered PTSD scale (CAPS-V) by measuring trauma related belief and goal violations, and their impact on negative views of self and world. But 52% of CAPS-V variance isn’t explained, indicating that factors like genetics, epigenetics and other physiological factors also play a role.

The first line treatments for PTSD are selective serotonin reuptake inhibitors (SSRI) and psychotherapy. Two major frameworks are currently being utilized in trauma-focused forms of psychotherapy. These emphasize the importance of exposure-based extinction therapy or traumatic memory integration.

Exposure therapy (ET) is a preferred first line treatment for PTSD by the department of defense (DOD) and exhibits substantial efficacy in reducing symptoms of PTSD. ET is one of only a handful of trauma-focused forms of psychotherapy currently being utilized for PTSD. The rationale for ET is based on the well-established process of memory reconsolidation and fear extinction explained in the previous section. The mechanisms of memory consolidation and reconsolidation have been well articulated in rodents, but there still exists an ongoing debate on these processes in humans. In rodents, when a previously consolidated memory is reactivated, it again becomes liable for editing. At this point, the engram cannot be deleted, but additional engrams can become associated with the memory structure, influencing its impact on physiology and behavior. Emotional processing theory (EPT) proposes that the emotion of fear is dependent on cognitive fear memory representations that link information about the stimuli, the fear response, and its meaning. EPT suggests that PTSD emerges from erroneous associations to the fear memory that do not actually predict the dangerous
stimuli. Therefore, due to memory reconsolidation, EPT suggests that recovery from PTSD can be achieved through the activation of the fear memory in a safe context.\textsuperscript{194} Similarly, in fear conditioning paradigms in rodents, a conditioned fear response to an unconditioned stimulus (US) can be extinguished through repeated exposure to the US without pairing it to the conditioned stimulus (CS). Mechanistic studies suggest that this process involves the generation of a new engram that inhibits the aversive response to the original fear memory.\textsuperscript{158} The important causal factors at play in this model are 1) the specific aberrant associative features of the traumatic memory and what they are associated with 2) the emotional/ affective reaction of the patient to those features and 3) the ability of an individual to reconsolidate and/or generate new engrams capable of inhibiting the fear response to the original traumatic memory. As mentioned in the previous section, this process is dependent on the HPC.

Trauma memory integration based therapies do not deny the aforementioned mechanistic basis of ET, but instead view ET based explanations as incomplete and ET as insufficient for recovery for many patients. ET exhibits a large effect size (total: $d=1.19$, completers analysis: 2.1), indicating clinically significant changes, but like most therapies for PTSD the dropout rate is typically >20\%,\textsuperscript{195,196} leaving 1/5 of participants untreated. Furthermore, ET is not as useful for C-PTSD or aspects of traumatic experiences that are not amenable to chronic re-exposure to a discrete trigger, sexual trauma for instance. Trauma-focused integration-based therapies include internal family systems (IFS) therapy and advanced integrative therapy (AIT), among others. These therapeutic strategies emphasize the importance of integrating a traumatic memory with a person’s existing models of self and world. The cognitive worldview model of PTSD
suggests that events that lead to PTSD are those most likely to precipitate negative views of self and world. Integrative therapies for PTSD therefore focus on integrating traumatic memories with a patient’s views of self and world in order to resolve those features of the traumatic event that lead to negative views of self and world. Under this framework, the important causal factors at play are: 1) the type of trauma experienced, 2) memory performance-mediated cognitive flexibility and the underlying neuroplastic capacity of the trauma exposed individual (molecular/physiological factors), 3) the interaction of the type of trauma an individual is exposed to and their existing belief systems. These factors could theoretically combine to produce a measure of the difficulty one may experience in integrating a traumatic memory(s) into one’s representations of self and world. Factors 1 and 3 are impossible to ethically control, but factor 2 appears malleable; memory performance can be manipulated to facilitate traumatic memory processing and integration. While the mechanisms underpinning integrative therapies have not and likely cannot be established in animal models like ET, the HPC is almost definitely required.

Another trauma-focused therapeutic strategy that depends on memory processing is eye movement desensitization and reprocessing (EMDR) therapy. The mechanisms underpinning EMDR are still speculative and I won’t go into them here, but evidence for its efficacy is mounting. While mechanistic explanations are still preliminary, initial explanations suggest that memory processing is its fundamental feature.¹⁹⁷

Memory processing and integration models are in contrast to models that emphasize simpler known physiological processes like fear extinction and executive processing deficits. These simpler models explain some aspects of PTSD, but do not explain the whole range of PTSD-associated symptoms and comorbidities. While trauma-
focused forms of psychotherapy have emerged and ET has been endorsed by the DOD, no currently FDA approved therapeutic strategy for PTSD addresses any PTSD specific criterion.

SSRI’s exhibit some efficacy, with sertraline and paroxetine among the most useful. SSRIs may achieve full remission for roughly 1/3 of patients (CAPS V score <50), but the factors governing their efficacy in PTSD remain uncertain and > 50 percent of patients continue to have PTSD even after treatment. Interestingly, the effectiveness of SSRIs may be directly inhibited by the heightened inflammatory milieu that accompanies PTSD. For example, the inflammatory cytokines, IL-1β and TNF-α may directly counteract SSRIs by accelerating the activity of the SERT, the 5-HT transporter. Psychotherapy is typically more effective than SSRI’s alone and larger effects have been observed with both therapies in combination. But even in combination, >50 % of patients continue to carry a PTSD diagnosis post treatment. SSRI’s have been shown to enhance fear extinction in animal models and exposure therapy and cognitive behavioral therapy (CBT) in small human clinical trials, but effect sizes are small and this combination therapy does not represent a viable way forward. Numerous other non-FDA approved drugs are also commonly used off label for PTSD. These include histone deacylase (HADC) inhibitors like valproic acid, atypical antidepressants like trazodone, and noradrenergic drugs like prazosin and propranolol. Many of these off label drugs exhibit some efficacy alone, but exhibit the greatest effects when paired with psychotherapeutic tools, for example, treatment immediately following trauma memory reactivation. Like SSRI’s, these drugs are effective for many individuals, but do not likely represent a viable path forward for full remission of PTSD in most patients.
There is currently a large scale research effort to produce pharmacotherapeutics that facilitate fear extinction, but while this is a step in the right direction, it does not actually target the process which are most explanatory of PTSD symptomology, associative and/or contextual memory processing and the impact of a traumatic memory on views of self and world. MDMA (methylenedioxy-methamphetamine) assisted therapy is currently in phase 3 clinical trials and exhibits remission of PTSD for >60% of participants, nearly doubling the efficacy of current FDA approved combination therapies, but this still leaves >30% of treated individuals with PTSD and furthermore, MDMA therapy is not yet available to the general population. Thus far, MDMA therapy is the only form of pharmacologically assisted therapy to show long term promise for PTSD, but it may only represent a single version of this type of therapeutic, one based on the enhancement of plasticity and critical period reopening through increased acute monoamine and hormonal signaling, predominantly 5-HT, DA, NE, and oxytocin. Alternatively, 5-HT$_{2A}$ agonist psychedelics also induce plasticity and reopen critical periods for reward learning (Nardou, Dolen unpublished), but do not substantially modulate monoamine reward pathways. Furthermore, these compounds may have neuroprotective properties and reduce inflammation and oxidative stress, possibly counteracting systemic allostatic load factors observed in PTSD. The failure to design treatments for PTSD that target fundamental causal disease features creates a massive barrier to progress in this field. Novel treatment strategies employed from this point forward should pair a psychoplastogen with trauma focused forms of psychotherapy in order to facilitate the integration of traumatic memories into the self-structure and/or trauma memory reconsolidation and fear extinction.
1.6. The Work Herein

Collectively, these sections provide the background and context for understanding not only the information provided in the following chapters themselves, but their important relevance to the stress and PTSD literature. Briefly, the overall conceptual background of this project is to test aspects of the following framework:

The fundamental purposes of the stress response are to provide energetic resources to the brain and heart during metabolic stress and to protect against the resulting deleterious effects of stress. These effects are regulated by the mitokine system, which regulates HPA axis function and cellular metabolism in the whole organism during stress. Metabolic stress and allostatic load factors directly contribute to HPC dysfunction due to its susceptibility to GCC and other stress factors. HPC dysfunction leads to contextual memory processing deficits and contributes the fundamental cognitive features of PTSD. It also leads to dysregulated allostatic processing through deficient contextual processing and the inability to regulate CNS allostasis. The main overarching idea is that the stress response is a metabolic process and the major currency of this economy is energetic substrate availability. In the following experiments I performed measures across the center of these proposed dependent factors from the mitochondrial level to the level of AL factors and HPC function and behavior, however it was beyond the scope of this work to explore the proposed HPA axis and GCC related underpinnings, although some have been previously elaborated by the Gilpin lab. I was also unable to explore the proposed higher level implications of the observed alterations to HPC function on allostatic processing, although it was important to review here for the purpose of illustrating the
importance of HPC function for both the cognitive and behavioral features of PTSD, which may be mediated by CNS allostatic processing, which generates affect.\textsuperscript{4,39,40} Affect modulates all behavior and metabolic output, forming a functional loop between the mitokine stress response system, which controls cellular metabolism organism-wide, and the CNS allostatic processing network that controls behavior and subsequent metabolic output. Furthermore, affect is dysregulated in PTSD (negative alterations to cognition and mood).

In the following chapters, I show in Experiment 1 that the avoidance of a predator odor-associated context is associated with distinct metabolic features in the HPC related to allostatic load, and that ROS production correlates with HPC PS performance. In this experiment, we exposed Wistar rats to predator odor (i.e., bobcat urine) once, then measured avoidance, as well as the association between avoidance other stress-related outcomes. This exhibits a relationship between metabolic factors and phenotypes that express CPA. In Experiment 2, I show that two incidents of traumatic stress + daily psychosocial stress lead to increased allostatic load factors in the HPC that parallel changes observed in humans in the periphery, exhibiting that trauma + chronic stress can lead to upregulated AL in the HPC. In one case, ROS is associated with the stress-induced conditioned place avoidance phenotype, a model for studying avoidance in PTSD; in the other we show that predator exposure and chronic psychosocial stress induces substantially increased AL factors in the HPC. In Experiment 3, I show that 5-HT2A agonist experimental therapeutics can normalize ROS production and gene expression overlapping with human PTSD in the PE/PSS
model, exhibiting a viable path forward in treating PTSD in light of the emerging model of PTSD as a memory and metabolic disorder associated with increased AL.

1.7. Notes


Schoenfeld, T. J., McCausland, H. C., Morris, H. D., Padmanaban, V. & Cameron, H. A. Stress and Loss of Adult Neurogenesis Differentially Reduce


CHAPTER 2. CONDITIONED PLACE AVOIDANCE OF A BOBCAT URINE ASSOCIATED SPATIAL CONTEXT IS ASSOCIATED WITH A DISTINCT HIPPOCAMPAL PHENOTYPE

2.1. Introduction

The hippocampus (HPC) and surrounding areas of the medial temporal lobe (MTL) map contextualize cognitive space across many domains.1,2 Space and time are primary delimiters of event representations,2 but additional factors derived from all sensory domains participate in the contextual differentiation of spaces and events.3,4 Contextual differentiation depends on pattern separation (PS) through which multisensory inputs from the entorhinal cortex (EC) to the dentate gyrus (DG)-CA3 circuit are orthogonalized into more distinct representations by granule cells through sparse coding.5-10

Acute stress increases pattern separation (PS) performance in humans and this effect correlates with stress induced cortisol production.11 However, PS is impaired by intense or chronic stressors, thereby exhibiting an inverted U shaped effect of stress on HPC PS.12 Stress-induced deficits in PS may partially mediate MTL memory deficits and contribute to fear generalization5,6,11 and avoidance behavior13 in neuropsychiatric disorders like Schizophrenia14,15 Major Depressive Disorder (MDD)15-17 and Post Traumatic Stress Disorder (PTSD).10 Chronic stress or glucocorticoids have deleterious effects on broad HPC structure and function, particularly neurogenesis in the DG,18 the retraction of CA3 pyramidal cell dendrites,19 and in numerous behavioral memory paradigms in humans and rodents.20-22 Many of these effects may be regulated by glucocorticoid receptor (GCR)-mediated modulation of cellular metabolic processes and their downstream effects on factors underpinning HPC network function, like neurogenesis in the DG and HPC mean firing rate setpoints.23
Stress and CORT modulate insulin signaling in both the brain and periphery. Chronic stress drives insulin resistance, but even acute stressors produce substantial alterations to brain insulin signaling. Insulin signals through the insulin receptor (IR) to upregulate glucose transporters (Glut1-4) in astrocytes and neurons and insulin-like peptides (IGF1/2) signal through IGF1R/2R to modulate the activity of glucose transporters. Collectively, this process tightly controls substrate availability to the TCA cycle and mitochondrial oxidative phosphorylation, therefore controlling mitochondrial respiration particularly maximal respiration and spare capacity. IR/IGF1/2R additionally control broad cellular processes, including protein synthesis, which is required for long term memory (LTM) through numerous signaling pathways including mTOR and Akt. For example, IGF2 controls adult neurogenesis in the DG of the HPC through Akt signaling.

Stress also exerts inverted U shaped effects on mitochondrial structure and function. Both acute and chronic stressors can produce long term transcriptional reprogramming of mitochondria, impacting cellular energy production in the brain. Acute stress can damage mitochondria, but can also enhance certain mitochondrial functions. The mitochondria is the primary cellular source of reactive oxygen species (ROS), although cytosolic sources of ROS also contribute, especially during proinflammatory states. ROS must be balanced with endogenous antioxidant pathway activity to prevent the development of oxidative stress, which leads to cellular damage and immune activation through pattern recognition receptors (PRRs), and further ROS production. However, ROS also acts as a signaling molecule and cellular
ROS status is the lynchpin of adult neurogenesis in the DG,\textsuperscript{59} an important mediator of PS,\textsuperscript{60,61} and this process is controlled by the mitochondria.\textsuperscript{62}

The DSM-V criterion for PTSD requires that an individual be exposed to a trauma and exhibit symptoms from four symptom categories, hyperarousal symptoms, avoidance symptoms, negative alterations to cognition and mood, and re-experiencing symptoms.\textsuperscript{63} Avoidance of specific contexts in which a trauma took place is mediated by contextual cognitive representations and the discrimination between these contexts and other similar contexts likely requires PS.\textsuperscript{5,6,64} In this work, we exposed Wistar rats to bobcat urine on day 4 of a 5 day conditioned place avoidance (CPA) paradigm and measured dorsal hippocampal (dHPC) pattern separation using the object pattern separation (OPS) task. In this model, we have previously exhibited GCC abnormalities; specifically attenuated CORT production immediately after the stressor in animals that develop CPA, Avoiders (Av), compared to those that do not, Non-Avoiders (NAv).\textsuperscript{65-69} In another chronic stress model, predator exposure + psychosocial stress, we observed increased ROS production and inflammation in stressed animals compared to controls\textsuperscript{70} and other labs have exhibited HPC-mediated cognitive deficits in novel object recognition and the radial arm maze in the same model.\textsuperscript{71} Based on these previous studies, we hypothesized that stress would exhibit deleterious effects on OPS performance, but that Av would exhibit reduced PS performance compared to NAv and exhibit increased ROS production and mitochondrial gene transcripts measured by Electron Paramagnetic Resonance Spectroscopy (EPR) and RNAseq respectively. Further, we hypothesized that Av animals would exhibit increased mitochondrial basal respiration, spare capacity, maximum respiratory capacity, and proton leak measured
by Seahorse Extracellular Flux analysis (SEFA). We also hypothesized that these animals would exhibit reduced growth factor pathway expression and reduced synaptic associated transcripts measured by RNaseq. We therefore investigated the effects of predator urine exposure on PS 16 days after the stressor and the relationship between conditioned place avoidance (CPA), PS, HPC gene expression, mitochondrial function and ROS production. 16 days was chosen in order to measure stable steady state changes that persist long after traumatic stress, rather than acute effects. Roughly 30% of male rats developed CPA for the urine associated chamber, a similar proportion to individuals exposed to trauma that develop PTSD in humans. Overall, we observed a deleterious effect of stress on OPS performance, but contrary to our hypothesis NAv did not learn on any day of the OPS test, while Av learned on day 5 (24 cm) and exhibited >2X the effect size of NAv across all 5 days. Av also exhibited lower ROS production and lower mitochondrial maximal respiration and spare capacity compared to unstressed Controls, while NAv were highly variable. WGCNA analysis exhibited a negative correlation between avoidance and insulin and growth factor related pathways and a positive correlation between avoidance and mitochondrial oxidative phosphorylation and ribosomal gene transcripts.
2.2. Results

Experiment 1. Conditioned Place Avoidance (CPA): Predator odor stress causes persistent CPA in a subset (~1/3) of rats

Rats were indexed for conditioned place avoidance (CPA) 24 hours after predator odor stress. (Figure 2.1.) As we have previously published,\textsuperscript{65,66,68} Avoider animals spend significantly less time in the odor associated chamber, relative to their own baseline,
than Non-Avoiders (two tailed t test: t=6.245, df=14, p <0.0001). We also previously showed that the Avoider and Non-Avoider phenotypes are persistent and stable over time.69

Object pattern separation (OPS): Av exhibit pattern separation on day 5, low variability. NAv exhibit high variability and no pattern separation on any day

A two way RM ANOVA with the Geisser-Greenhouse correction and the Bonferroni post hoc test was used to calculate within group comparisons, comparing DI on each successive day to the population DI on day 1. We observed a significant effect of time (F(2.265, 47.57)= 10.29, p< 0.0001), but no significant effect of group (F(2, 21)= 0.4385, p= 0.6508), subject (F(21, 84)= 0.7693, p= 0.7475), or a Time x Group interaction (F(8, 84)= 0.9851, p= 0.4535). Bonferroni’s post hoc test revealed that the control group did not exhibit learning at day 2 (6 cm) (p > 0.999, SE = 0.070) but did exhibit robust learning at day 3 (12 cm) (p= 0.0188, SE= 0.038), day 4 (18 cm) (p= 0.0460, SE = 0.0727), and day 5 (24 cm) (p= 0.0422, SE = 0.095). NAv did not exhibit learning at any day; day 2 (6 cm) (p= 0.8784, SE = 0.059), day 3 (12 cm) (p> 0.999, SE = 0.066), day 4 (18 cm) (p> 0.999, SE = .103) or day 5 (24 cm) (p= 0.061, SE = 0.093). Av did not exhibit learning on day 2 (6 cm) (p > 0.999, SE = 0.081), day 3 (12 cm) (p> 0.999, SE = 0.067), or day 4 (18 cm) (p = 0.516, SE = 0.082), but did exhibit learning on day 5 (24 cm) (p= 0.0154, SE = 0.072). Overall, there was a clear effect of stress such that unlike unstressed Controls, neither Av or NAv groups learned on days 3 and 4 despite NAv exhibiting higher statistical power than the control group. Unstressed controls and Av learned on day 5 despite the Av group necessarily being relatively underpowered due to the nature of the model (only ~1/3 become Av). (Figure 2. 2.a.)
This model produces differential group sizes for NAv and Av, therefore we calculated effect size for each within-group comparison to compare the results of each group independently of statistical power. Controls exhibited an effect size (f) of 0.88. The combined NAv, Av group (stress) exhibited an effect size (f) of 0.55, while NAv exhibited an effect size of 0.42 and Av exhibited an effect size of 0.99, more than twice that of the NAv group. This difference in effect size was primarily driven by 4/10 NAv animals that showed a near zero or below zero DI on day 5. In contrast, the other 6 NAv showed robust learning on that day. All 6 Av animals exhibited positive DI scores on day 5 indicating consistent learning across all Avoiders. (Figure 2. 2.b.)

In order to perform a direct statistical comparison between NAv and Av, we calculated slope as a measure of rate of learning for each animal across all 5 days, using the population baseline as the day 1 score. Due to a trend towards significant differences in variance (p=0.10) between groups, we used an unpaired two tailed t test with Welch’s correction. We observed a trend towards differences between Av and NAv (t= 1.770, df = 13.44, p=0.099). Despite the lack of significant effect between groups, the slope analysis reaffirms what we observed above. Using the arbitrary cut-off of a slope of 0.04 as an indication of “learning”, 60% of Nav learned across all 5 days, but 40% of that group did not. Two animals were marginal around this threshold. In total, 6/10 Nav showed a lower slope than all Avoiders, while 1 NAv exhibited a higher slope than all Av, exhibiting the high level of variance in the NAv group. (Figure 2. 2.c.)
Figure 2.2. Object Pattern Separation (OPS) performance across all groups, effects of predator urine exposure on Electron Paramagnetic Resonance Spectroscopy (EPR) measure of reactive oxygen species (ROS), and correlation to OPS. a) Discrimination Index (DI) calculated for OPS performance relative to the population baseline for all groups. 2 way within groups RM ANOVA and Bonferroni post hoc test reveal that control animals exhibited robust learning on day 3 -12 cm (p=.0188), day 4 - 18cm (p=.0460), and day 5 - 24 cm (p=.0422). b) Effect size (f) calculated for a one way ANOVA within groups for all groups. Control (f=.88), Stress (f=.55), Non-Avoider (NAv) (f=.42), Avoider (Av) (f=.99). c) Welsh’s two tailed t-test revealed a trend towards increased rate of learning in Av vs NAv (t= 1.770, df= 13.44, p=.0994). NAv exhibited double the standard deviation of Av (.57 vs .25 respectively). d) Welch’s two tailed t-test revealed that Av exhibited significantly reduced ROS production relative to NAv (t=2.138, df = 21.83, p=.0439). NAv exhibited nearly double the SD of Av (725 vs 386 respectively) e) EPR data exhibits a significant correlation with the slope of the DI index for predator urine exposed animals (r^2= 4.35, p=.0054). NAv are represented in blue, Av in red.
**Electron Paramagnetic Resonance Spectroscopy (EPR):** Av exhibit lower ROS production compared to NAv

We compared ROS production between the two phenotypes of animals exposed to predator urine. Due to a trend towards different variances between groups (F= 3.528, DFn= 14, Dfd= 8, p= 0.078), we implemented a two tailed t-test with Welch’s correction. Av exhibited significantly lower ROS production than NAv (t= 2.138, df= 21.83, p= 0.0439). (Figure 2. 2.d.) Controls were not considered in this analysis because our hypothesis was based on differences between stressed animals, not unstressed controls. However, no differences were observed between either group and control animals, which were always observed between Av and NAv. (Figure A .3.d.)

**ROS production correlates with OPS slope**

In order to determine the role of cellular ROS status in OPS performance in predator urine exposed animals, we performed simple linear regression between these two endpoints across both NAv and Av groups. This analysis revealed a significant relationship between OPS performance and ROS such that those animals with lower ROS exhibited greater performance in OPS (F= 10.80, DFn= 1, DFd= 14, R^2 = 0.436, p= 0.0054). (Figure 2. 2.e.)

**Seahorse Extracellular Flux Analysis (SEFA): Reduced maximal respiration and significant group effect in spare capacity in Av vs Control**

In order to determine if bobcat urine exposure causes differences in mito respiration at 16 days post stress, we performed SEFA in ex vivo punches of the CA3-DG circuit in the dHPC. Due to a higher degree of variance in NAv animals, we did not assume equal variance and used the Welsh’s one way ANOVA for these comparisons. Welsh’s one way ANOVA did not exhibit any significant effects of group in basal respiration (W=
0.5235, 2, 20.36, p= 0.60), ATP production (W=2.553, 2.0, 14.24, p= 0.1128), non-mitochondrial respiration (W= 0.1468, 2.0, 17.69, p= 0.8678), or proton leak (W= 0.0396, 2.0, 19.85, p=0.9612). However, we did observe significant effects of group in maximal respiration (W= 4.461, 2.0, 11.56, p=0.0367) and Dunnett’s T3 multiple comparisons test revealed no differences between control and NAv (t= 0.592, 12.99, p= 0.9) or NAv and Av (t= 0.1.674, 11.65, p= 0.252), but we did observe a marginally significant difference between Av and control (t=2.867, 8.62, p= 0.0515). In spare capacity, we also observed a significant effect of group using Welsh’s ANOVA (W= 4.763, 2.0, 10.12, p= 0.0348). Using Dunnett’s T3 test, we did not observe differences between control and NAv (t= 0.0678, 12.45, p= 09.8672) or between NAv and Av (t= 1.857, 10.22, p= 0.2391), but we did observe a strong trend between control and Av (t= 2.815, 6.551, p= 0.0696). (Figure 2.3.)
Figure 2.3. Seahorse extracellular flux analysis (SEFA) for control, non-avoider (NAv) and Avoider (Av) animals. One-way Welsh’s ANOVA exhibited an effect of group for a) maximal respiration ($p=.037$) and b) spare capacity ($p=.035$), but no significant differences of group for c) basal respiration ($p=.60$), d) ATP production ($p=.11$), e) Non-mitochondrial respiration ($p=.86$), or f) proton leak ($p=.96$). Dunnett’s multiple comparisons test revealed a significant reduction in Av vs control in maximal respiration ($p=.05$) and a trend for spare capacity ($p=.07$).
Experiment 2.

RNA sequencing

Avoiders exhibit increased mitochondrial transcripts and oxidative phosphorylation pathways, reduced insulin and growth factor related transcripts and pathways, and reduced synaptic transcripts and pathways in the dorsal hippocampus

We performed whole transcriptome RNA sequencing on a separate group of animals in order to investigate the potential molecular underpinnings of differences in dHPC mediated CPA and OPS performance. DEG analysis revealed that Av and NAv cluster separately and controls cluster in the middle, with only a single overlap between NAv and control. (Figure 2. 4.a.) We performed Gene Ontology (GO) Molecular Function pathway analysis to investigate differences in DEGs between NAv and Av groups, which revealed that the electron transfer activity GO pathway was observed to be lower in NAv vs Av animals in addition to the structural constituent of ribosome GO pathway. Passive transmembrane transfer activity and growth factor binding GO pathways were observed to be significantly upregulated in NAv vs Av. The growth factor binding GO pathway contained the IGF1R, IGF2R, and numerous other transcripts related to insulin signaling. Collectively, these data indicate that differences in insulin and insulin like growth factor (IGF) related signaling and alterations to Mito gene expression and function may partially mediate the differences in dHPC mediated CPA and OPS performance. (Figure 2.4.) Furthermore, we searched the DEG data for antioxidant related genes and observed that Gpx4, Sirt2, and Txn1, all potent antioxidant associated genes, were all upregulated in Av compared to NAv. (Figure A. 3. a, b, c.)
Caption on following page
Figure 2.4. a) Heatplot of DEG’s between Control, Non-Avoider (NAv) and Avoider (Av) groups. b) Venn diagram of overlapping measured gene transcripts between all three groups. c) Gene ontology (GO) Molecular Function pathway analysis of DEGs between NAv and Av groups exhibit reduced mitochondrial electron transport related DEGs in NAv vs Av and increased passive transporter activity and growth factor binding in NAv vs Av.

**Weighted Gene Co-expression Network Analysis**

Figure 2.5. WGCNA analysis of identified two modules associated with the avoidance phenotype. a) Genes in the indianred4 module, n = 4 mice/group were significantly upregulated in Non-avoiders (NAv) compared to Avoiders (Av) (p<0.05, t-test). b) Overrepresentation analysis of genes within the indianred4 module. c) Top 30 gene correlations within the indianred4 module. Size of the circle represents the number of connections and the thickness of connections represents weight of correlation. d) Genes in the magenta module were significantly upregulated in Av compared to NAv (p < 0.05, t-test, n=3-4). e) Overrepresentation analysis of genes in the magenta module. f) Top 30 correlations of genes in the magenta module.
**Weighted Gene Co-expression Network Analysis (WGCNA)**

We performed WGCNA analysis to identify gene networks and cellular pathways that were dysregulated in avoiders and non-avoiders. WGCNA analysis of Av and NAvs identified a total of 23 modules. (Figure A. 1.a.) We next performed module-trait correlations using time spent in the predator odor-paired compartment as the trait of interest. We found two modules that were significantly correlated with the avoidance phenotype: the magenta module which was negatively correlated and the indianred4 module which was positively correlated with time spent in the predator odor chamber. (Figure A. 1.b) The magenta and indianred4 modules were highly enriched for differentially expressed genes further underscoring the importance of these modules in (data not shown). The indianred4 module contained 3041 genes. Genes in this module were significantly downregulated in Av compared to NAv. (Figure 2. 5.a.) Cell-type specific enrichment analysis of the indinared4 module revealed that the module was highly enriched for genes expressed in neurons, newly formed and myelinating oligodendrocytes and oligodendrocyte precursors (OPC). (Figure A. 2.a.) We next performed overrepresentation analysis of genes in this module. Insulin signaling and Axon guidance were the top two pathways enriched in this module. (Figure 2. 5.b.) 42 genes related to the insulin signaling pathway were detected in this module and include Akt3, Foxo1, InsR, Irs2, and GSK3b. Insulin growth factor receptor genes IGF1R and IGF2R were also detected in this module. 59 genes in the axon guidance pathway were detected and included genes such as Sema5a, Nrp1, Robo1, Plxnc1, and Ephb1-4. Cytoscape was used to identify the top 30 most connected genes in this module. (Figure 2. 5.c.) Candidate hub genes with high connectivity scores and high differential
expression included the chaperone Suppressor of Ty6 homolog 6H (Supt6h), SET binding factor 1(Sbf1) which is a member of the protein tyrosine phosphatase family, and the microtubule associated protein Map1b, and the cytoskeletal protein Spectrin B1(Sptbn1).

There were 2,949 genes in the Magenta module. Genes in this module were significantly upregulated in Av compared to NAv (Figure 2. 5.d.) Cell-type enrichment analysis did not reveal enrichment of any particular cell-type (Figure A. 2.b.) Pathway analysis of genes in the magenta module revealed genes related to oxidative phosphorylation, electron transport chain, and cytoplasmic ribosomal proteins as the top pathways. (Figure 2. 5.e.) Genes in the oxidative phosphorylation pathway (70 genes) included several NADH:Ubiquinone oxidoreductase subunits including NDufa11, 12, and 13, Ubiquinol-Cytochrome C Rductase, Complex III Subunit XI (Uqcr11), and Glutathione peroxidase 4 (Gpx4). Network visualization of the top 30 most connected genes in the module is shown. (Figure 2.5.e.) Candidate hub genes with high connectivity scores and high differential expression included Gpx4, NDufa12, and purkinje cell protein 4-like protein 1(Pcp4l1).

2.3. Discussion

This stress model produces two groups of animals, avoiders (Av) and non-avoiders (NAv), that differ on the degree of avoidance (or lack thereof) of a predator odor-paired chamber. In this study we observed 38% Av and 62% NAv. (Figure 2. 1.c.) Our a priori hypothesis was that stress would have deleterious effects on OPS performance, but that Av would be more susceptible to the effects of stress and exhibit worse performance than Nav. “Deleterious effects” and “poorer performance” are defined as
one group exhibiting a significantly lower DI index on a testing day than another or as one group exhibiting significant performance on a testing day, while the other group did not. Our hypothesis was based on the fact that PTSD patients exhibit persistent avoidance of contexts previously associated with threat, but that no longer predict a genuine threat. PTSD patients also exhibit reduced contextual memory performance compared to trauma-exposed individuals who don’t develop PTSD, which is suspected to play a role in avoidance. Stress-exposed animals did not exhibit significant OPS performance on days 3 and 4, while unstressed controls did. However, Av animals did exhibit significant PS on day 5 and had the highest effect size of any group, while NAv did not. The NAv cohort further divided into two subgroups with 6/10 animals exhibiting slopes above .04 and 4/10 well below that arbitrary threshold. There are two possibilities regarding PS performance (slope) for each group: Av/ NAv that exhibit PS (AvPS+, NAvPS+) or subthreshold PS (AvPS-, NAvPS-) on day 5. We observed 100% AvPS+, but we observed 60% NAvPS+ and 40% NAvPS-. The observed 100% AvPS+ supports the idea that PS and/or similar or overlapping HPC mechanisms are necessary for CPA, but due to the observed 60% NAvPS+, these data do not indicate that PS is sufficient for CPA. The 60/40 split in the NAv group suggests that the NAvPS- group cannot perform consistent PS, even 16 days following odor stress, while NAvPS+ retain this ability, but still do not exhibit CPA. These data support our hypothesis that stress would reduce OPS performance compared to unstressed controls, but not our hypothesis that Av would exhibit lower OPS performance relative to NAv. The null hypothesis also cannot be confirmed because the opposite was true; Av exhibited superior OPS performance on day 5 compared to NAv. Despite the clinical connection
between contextual memory deficits and avoidance, 100% of Av exhibit good PS at 24 cm (day 5) and a high slope across all 5 days. 60% of NAv also exhibited good PS on day 5 and a slope of >.04, while 40% of NAv did not. Therefore, PS may be necessary, but not sufficient for CPA in this model.

Within the NAv group, the 60/40 split raises the following questions:

1. Do NAvPS+ animals not develop CPA because they were able to extinguish or reframe the odor associated negative valence due to its absence on the day that animals are indexed? This is what we would have expected had Av not exhibited PS. This would be an equally adaptive evolutionary strategy as the avoidance phenotype, possibly allowing for access to resources unavailable to Av in the wild. This is unlikely however. NAvPS+ did exhibit PS deficits on day 3 and 4, just like Av, and therefore have not exhibited greater PS performance that would explain such a large difference in PS ability. Alternatively, based on previous work in our lab, lower activation of circuits within the central nucleus of the Amygdala (CeA), possibly through interactions of corticotropin releasing factor (CRF) with CRFR1, may underpin the lack of CPA in NAvPS+, despite intact PS at 24 cm.77

2. Do NAvPS- animals not develop CPA because they lack the PS mediated capacity to discriminate and/or due to the aforementioned amygdalar mechanisms? The best explanation of these data are that both Av and NAvPS+ exhibit good enough PS to distinguish between the neutral and odor contexts, while NAvPS- may not. Therefore in NAvPS+, the main driver of CPA in this model is likely to be the CeA. Alternatively, in NAvPS-, it is unclear if their lack of CPA is due to stress-induced deficits in PS and/or due to reduced CeA activity.77
PTSD is associated with abnormalities in HPA axis function. PTSD patients exhibit lower corticosterone (CORT) in the hours after trauma,\textsuperscript{75,78-81} higher hypothalamic pituitary adrenal (HPA) axis sensitivity,\textsuperscript{82} and show chronically lower levels of CORT in urine and blood,\textsuperscript{75,79} but possibly higher levels in hair.\textsuperscript{83} PTSD is also associated with alterations to the GCR-associated chaperones SKA-2\textsuperscript{84,85} and FKBP5.\textsuperscript{86,87} In this model, the Gilpin lab exhibited lower ACTH and CORT expression in Av compared to NAv immediately after stress and systemic treatment with CORT prior to stress reduced the magnitude of CPA and the percent of animals classified as Avoiders.\textsuperscript{65,88} No differences were observed in GCR expression in the paraventricular nucleus (PVN) of the hypothalamus, the central amygdala (CeA), or the ventral hippocampus (vHPC). However, Av exhibited decreased FKBP5 and SRC-1 expression in the PVN and CeA and increased SRC-1 expression in the vHPC.\textsuperscript{88} The stress-induced effects of CORT are mediated though the cytosolic glucocorticoid receptor (GCR), which exerts genomic effects through chaperone-mediated translocation to the nucleus and non-genomic effects through direct interactions with mitochondrial proteins on the outer and inner membranes and the mitochondrial matrix.\textsuperscript{51} Chronic stress induces mitochondrial damage and reduced mitochondrial function in the brain\textsuperscript{89} and periphery,\textsuperscript{90} but can also induce increased mitochondrial density at synapses\textsuperscript{50} and increased mitochondrial biogenesis and gene expression in Leydig cells.\textsuperscript{52} Some of these effects are mediated by direct interactions between the glucocorticoid receptor (GCR) and mitochondria, altering mt-DNA gene expression.\textsuperscript{51,91} Alternatively, genomic effects of the GCR likely mediate the expression of mitochondrial genes housed in the nucleus.\textsuperscript{92} Mitochondrial oxidative phosphorylation is the primary cellular source of
reactive oxygen species (ROS), but cytosolic processes like NADPH oxidase also contribute to total cellular ROS. Mitochondrial ROS has deleterious effects on HPC contextual memory processesing and modulates gene expression, cell signaling, and neurogenesis through numerous mechanisms. GCC increase ROS production and accumulation in the HPC and exert complex effects on mitochondrial function through the GCR.

Our a priori hypothesis was that Av would exhibit elevated ROS production compared to NAv in addition to elevated mitochondrial oxygen consumption rate during basal respiration, maximum respiration, spare capacity, ATP production, and proton leak. This hypothesis originated in our previous work exhibiting elevated ROS production in another model of PTSD that utilized two one hour long predator exposures and 30 days of daily psychosocial stress (PE/PSS). In that model, we did not use cut off behavioral criterion to index animals by behavioral phenotype as we did here. The data observed here did not support our hypothesis, nor can we affirm the null hypothesis. Like the OPS data, we observed the opposite of our predicted effect. Av exhibited reduced ROS production compared to NAv. We also observed a significant effect of group in SEFA for mitochondrial maximum respiration and spare capacity, with post hoc tests exhibiting lower max respiration and a trend towards lower spare capacity in Av vs control. In SEFA and our EPR measure of ROS, NAv again exhibited very high variability compared to Av. All other parameters measured by SEFA did not exhibit any significant differences. (Figure 2. 3.)

Stress acutely reduces insulin expression, but drives up blood concentrations of glucose through the GCR and adrenergic mechanisms, which itself drives insulin
expression. Reports of stress and glucocorticoids on insulin signaling in the brain are mixed, but suffer from a lack of consistent investigation of related endpoints in the same model systems. Chronic stress or CORT exposure drives insulin resistance in the hippocampus, which is associated with cognitive dysfunction. Our lab exhibited that predator + psychosocial stress (PE/PSS) induces downregulations in IGF1R in rats and other groups have exhibited reduced IGF2 after stress. Exogenous intranasal insulin improves cognition in humans and exogenous IGF2 improves cognition in rats. Furthermore, IGF2 gene expression was upregulated in the dIPFC in human PTSD and in the PFC of the PE/PSS model. Treatment of PE/PSS rats with N,N dimethyltryptamine, a psychedelic drug with antidepressant and psychoplastogenic properties, reduced IGF2 transcript expression in association with reduced ROS production and other salutatory effects in overlapping genes between human PTSD and the PE/PSS model. Finally, IGF2 treatment promotes neuroprotection, neuroplasticity, and recovery from glucocorticoid induced oxidative insult, exhibiting the link between these processes.

WGCNA analysis also identified insulin signaling as a biological process that was disrupted in Avs. Genes in the insulin signaling pathway were enriched in the indianred4 module which was positively correlated with the avoidance phenotype. Insulin signaling genes in this module were all downregulated in Avs compared to NAvs. In addition to IGF1/2R, the indianred4 module contained 42 additional genes in the insulin signaling pathway. Based on the well-established relationship between CORT expression and insulin signaling, and our lab’s previous work exhibiting lower stress induced CORT expression and elevated SRC-1 expression in the vHPC of Av animals, we propose that
reduced insulin signaling pathway gene expression in Av animals is driven by the known differences in HPA axis and GCR signaling in Av. Another module that was significantly positively correlated with time spent in the odor-paired compartment was the Magenta module. Genes in this module were significantly upregulated in AVs compared to NAvs. Overrepresentation analysis revealed cytosolic ribosomal proteins and the mitochondrial electron transport chain as the top two most enriched pathways in this module. Typically, insulin signaling upregulates protein translation and ribosomal proteins, so the contrasting changes observed here between lower expression of transcripts for insulin/IGF1/2R signaling and elevated ribosomal gene expression highlight the complex relationship between stress, insulin signaling, and the mechanisms of protein translation. WGCNA analysis also found several candidate hub genes in both these modules whose expression was highly correlated with other genes in the module and were also significantly differentially expressed between Av and NAv. Future experiments will test the hypothesis that these hub genes drive the development of the Av/NAv phenotype after stress exposure.

100% of Av exhibit good PS performance, while only 60% of NAv do, which we deemed NAvPS+. Av animals exhibited lower ROS production compared to NAv at 17 days post stress and ROS production was negatively correlated with OPS performance (p=.0054) across both groups. NAvPS+ therefore tended to exhibit lower ROS production than NAvPS-. (Figure 2. 2.) These data support the idea that animals with lower ROS production may exhibit a higher level of neurogenesis, which underpins better contextual discrimination, likely through PS or PS-like processes. Since mitochondria are the primary cellular source of ROS, we performed SEFA to measure
OCR across 6 different parameters of mitochondrial function. SEFA did not exhibit significant differences in most mitochondrial parameters, but we did observe significant group effects in maximal respiration and spare capacity, which were lower in Av compared to control, while NAv were highly variable. Neither of these processes typically account for ROS production in most cell types, but neurons only use 6% of their reserve respiratory capacity at rest, but utilize up to 80% when firing. In highly active neurons, it’s possible that reduced spare capacity has a substantial negative impact on total respiratory activity and leads to reduced ROS production. This may confer a protective effect by reducing maximal ROS production in circumstances of increased mitochondrial load, for example, during stress or memory consolidation. Both spare capacity and maximal respiration are predominantly modulated by insulin and IGF1/2 signaling, which upregulate energetic substrate transporters on astrocytes and neurons, controlling substrate availability to the mitochondria. Lower max respiration and spare capacity in Av may therefore be driven by the lower expression of insulin and IGF signaling pathways, which were negatively correlated with CPA odor time in the WGCNA analysis. (Figure 2.4., 2.5.) HPC network function is dependent upon the establishment of neuronal mean firing rates (MFR). MFRs are metabolically controlled and are regulated by changes in mitochondrial spare respiratory capacity, providing a potential direct link between these metabolic measures: lower insulin signaling, ROS, maximum respiration, and spare capacity, and the OPS measure of dHPC mediated PS performance, and possibly CPA.

On the other hand, WGCNA and candidate gene expression analysis revealed higher mitochondrial electron transport chain gene expression in Av compared to NAv.
rats. This appears inconsistent with the lack of changes in basal respiration and ATP production, and reductions in maximal respiration and spare capacity. Increased transcript expression may lead to increased mitochondrial biogenesis, therefore one explanation is that increased transcript levels are a cellular response to mitochondrial dysfunction and/or the metabolic consequences of the observed changes in gene expression for insulin and IGF1/2 signaling and reduced spare and maximal respiratory capacity. We also observed increased ribosomal transcript and pathway expression in Av compared to NAv that correlated with increased mitochondrial transcript expression and CPA odor time in the WGCNA analysis. (Figure 2.5.) One would expect higher ribosomal transcript expression to lead to higher protein synthesis. Protein synthesis is driven by insulin and IGF1/2 pathways, in addition to other growth factor pathways, which activate mTOR-CREB to initiate transcription and translation. Each of these steps exhibited downregulated gene expression in this study. The increased mitochondrial and ribosomal gene expression may be compensatory actions to prime cells to more quickly upregulate protein translation upon even low level stimulation by persistently downregulated growth factor signaling in Av. Protein synthesis is metabolically expensive and in circumstances of intense stress and/or limited resources, it can be beneficial for cells to limit protein synthesis, instead favoring repair, rather than replacement of damaged proteins, which confers a protective effect.\textsuperscript{114} For example, calorie restriction causes a reduction in protein synthesis, favoring repair, but also reduces oxidative stress, while maintaining ATP output.\textsuperscript{115}

DEG analysis exhibited that the antioxidant genes Gpx4, Sirt2, and Txn1 were upregulated in Av compared to NAv (Figure A.3.a, b, c.) with unstressed controls.
consistently exhibiting expression between both groups. WGCNA analysis also exhibited that Gpx4 was a top hub gene in the Magenta module that positively correlated with CPA. (Figure 2. 5.f.) CORT reduces antioxidant gene expression\textsuperscript{116} and Av exhibit lower CORT expression immediately after stress exposure compared to NAv. However, since CORT production and GCR signaling were not measured in this study and have not been measured longitudinally between NAv and Av in this model, we cannot be certain as to the connection or time course of CORT and antioxidant gene expression at this time. We also did not examine antioxidant genes at the protein level and therefore this possible connection should be considered cautiously. Overall, the most parsimonious explanation of these and the preexisting data is that differences in the HPA axis response between Av and NAv during CPA initiate changes in HPC metabolism that exert downstream effects on HPC function. Whitaker 2016 observed
reduced Src-1 expression in the HPC 12 days after stress, exhibiting long term alterations in HPC GCC signaling. Because GCC regulate insulin signaling, we propose that the previously observed differences in GCC signaling mediate the observed

![Diagram of the stress response system](image-url)

Figure 2.6. Schematic of multisystem differences between Avoiders (Av) and Non-Avoiders (NAv). Black arrows indicate stimulation. The blunted red line indicates inhibition. Square ended solid large red arrow indicates previously measured changes in Av. Tapered red arrows indicate change in Av in electron paramagnetic resonance spectroscopy and seahorse extracellular flux analysis functional measures. Solid red arrows with faded ends indicate bidirectional changes between Av and NAv observed in RNA sequencing DEG or WGCNA analysis. Dashed red arrows indicate bidirectional changes between groups that appear inconsistent with functional data and are likely compensatory responses to acute stress effects. Image created with BioRender.com
reductions in insulin, IGF1/2, and mTOR signaling in Av, which limit substrate availability to the mitochondria and subsequently reduce maximum mitochondrial respiration and spare respiratory capacity. We propose that during elevated activity, reduced spare capacity can lead to reduced ROS production in Av compared to NAv animals, a potential protective effect. Both ROS production and mitochondrial spare capacity modulate HPC function and we propose that these factors mediate differences in HPC OPS performance in this study, providing the link between these metabolic endpoints, HPC function, OPS performance, and CPA. (Figure 2.6.) In regards to the presented RNAseq data, it should be noted that we did not confirm protein expression or functional mediation for any of these targets and they should therefore be understood as evidence-based future directions, not confirmed mechanisms.

Two important themes that emerged from this work are 1. Av consistently exhibited lower variability than NAv in every measure except the CPA measure itself. 2. Av and NAv consistently exhibited bi-directional expression relative to control across numerous measures including OPS effect size (Figure 2.2), ROS production (Figure A.3), and the best example, the DEG and WGCNA analysis of the RNA sequencing data. (Figure 2.4, 2.) In each of these cases, the only observed statistical differences were between Av and NAv and neither group was different than controls, but numerically, Av and NAv exhibited divergent expression relative to unstressed controls. This observation was not observed in SEFA however, where only Av exhibited changes. These two themes emerge across behavioral (OPS), functional (EPR), and molecular (RNAseq) levels of biological organization and in two independent groups of rats.
Together these data clearly show that Av and NAv rats exhibit distinct HPC metabolic phenotypes. (Figure 2. 6.)

These data and our previous work collectively suggest that Av exhibit a phenotype characterized by CPA, lower CORT immediately after stress and higher SRC1 expression in the HPC, lower ROS production, maximal respiration, and spare capacity, higher antioxidant gene expression, and lower insulin/IGF and growth factor signaling. This characterizes a state of reduced maximal energetic metabolism and oxidative damage, especially in highly active neurons, which occurs during stress. It is not possible to perform these measures *in vivo* during stress however, so it remains speculative if these observed changes actually alter HPC function during stress. Reduced peak HPC metabolism may support the protection of the DG-CA3 circuit in the dHPC during stress-induced elevated activity. This protection could preserve the functional capacity of the HPC to implement PS or PS-like activity in the face of intense stress. NAv_{PS+} might also exhibit a HPC phenotype that is resilient to the effects of stress, possibly through the protective effects of elevated GCC. Due to the preservation of PS in this group, the simplest explanation is that NAv_{PS+} do not develop CPA due to non-HPC mechanisms. The molecular, mitochondrial, and ROS related effects observed in Av may be one alternative adaptive biological strategy to retain HPC PS performance in the face of intense stress through other mechanisms aside from elevated CORT expression. 40% of NAv (NAv_{PS-}) exhibit a complete loss of PS performance, which is possibly linked to stress and/or CORT-induced toxic stress in the HPC. Future investigations should confirm and examine the two NAv subgroups to determine why elevated CORT is associated with protective effects in 60% of animals,
while being associated with deleterious effects in 40%. The further biological implications of these two biological strategies (Av, NAv_\text{PS}^+) for retaining moderate PS capacity after intense stress have yet to be elaborated, but future investigations in this area may lead to major breakthroughs in the field of adaptative stress response mechanisms and their impact on memory function.

This study has several major limitations. The first is that it was only performed in males and should be repeated in females. The second is the lack of protein measures for mitochondria, insulin signaling, and antioxidant related genes and pathways we measured by RNAseq. The third major limitation is the design such that animals used for E2 cannot be correlated with OPS performance because OPS was only performed in E1. We used WGCNA to perform correlational analysis between pathways and CPA performance, which suggests that insulin signaling and mitochondrial gene expression may be drivers of CPA, but we cannot directly connect this to OPS performance in the same animals. Another related limitation is that we cannot correlate the RNAseq data to either the EPR ROS data or the SEFA data for the same reasons. These two design aspects limit our ability to truly mine the data for all possible relationships. A final major limitation of this study is that the OPS measure in E1, but not in E2 may limit the relevance of the RNAseq data to the behavioral, mitochondrial and ROS measures in E2. While the two groups were sacrificed at roughly the same time point, (day 17 for E1, day 16 for E2) it’s possible that the 5 prior days of OPS learning and testing altered mitochondrial function and ROS production differently in Av and NAv and the relative differences in these parameters between the groups was altered. However, in both the RNAseq data and in the measures that were observed to significantly differ between
those groups in G1, the same pattern emerged: Av were relatively tightly clustered and NAv exhibited high variance. (Figure 2. 2-2. 5.) Despite these limitations, this study does provide a strong basis for the continued investigation of the linked effects of CORT, insulin signaling, mitochondrial function, dHPC PS, and CPA secondary to traumatic stress. Future investigations should include direct measures of CORT over time, protein measures, measures of DG neurogenesis, direct measures glycolytic function, which is available on newer SEFA machines and protocols, as recently published,117 and in vivo electrophysiology to confirm the impact of these metabolic effects on HPC function.

2.4. Methods

Animals

36 male specific pathogen free Wistar rats of 56-63 days old were ordered from Charles River, Raleigh NC to be delivered in groups of 12 separated by three weeks for the first two orders and two weeks for the last order. There was an identified environmental interference in the first group of 12 rats and control animals did not exhibit OPS. Those animals were not included in the OPS analysis, but their brain samples were used to bolster the n sizes of the EPR and Seahorse data. Animals were housed in a humidity- and temperature-controlled vivarium on a 12/12 light/dark cycle in which the lights go off at 8 am. All rats were pair housed and were acclimated for one week prior to the start of experiments and were handled daily for the 5 days prior to beginning the CPA protocol. All behavior was performed during the dark cycle between 8 am and 1 pm. All procedures were approved by the Institutional Animal Care and Use Committee at
Louisiana State University Health Sciences Center and were in accordance with National Institute of Health Guidelines.

**Conditioned place avoidance (CPA)**

As previously described,77 rats underwent a 5 day predator odor conditioned place avoidance protocol. On day 1, rats were allowed access to three chambers differing by floor (tactile) and wall (visual) cues for 5 minutes. For each animal, the chamber in which they spent the most deviant time relative to the other chambers was removed. On day 2, animals were exposed to the remaining two empty chambers for 5 minutes. Rats were then assigned to either control or stress groups such that the more preferred and less preferred chambers from the day 2 data were balanced. On day 3, rats were exposed to one chamber without odor for 15 minutes. On day 4, rats were exposed to the opposite chamber paired with bobcat urine for 15 minutes. On day 5, animals were allowed to explore both chambers for 5 minutes and time spent in each chamber was quantified and animals were indexed for CPA. Rats that exhibited a >10 second decrease in time spend in the odor-associated context were classified as avoiders, while animals that did not cross this threshold were classified as non-avoiders.

**Object pattern separation (OPS)**

Rats were tested across 5 days for performance in the OPS measure, which consisted of one 5 minute learning trial and an hour later, one 5 minute testing trial per day. In the learning trial two objects were placed in the center of the open field. In the testing trial, the moving object was moved 6 cm farther than the previous day in the same direction. On day 1, the testing trail consisted of two objects in the same location as on the learning trial and therefore constitutes a baseline measure with no reflection of PS
performance; therefore we used the population baseline as the day 1 score for all animals in all groups in order to eliminate the effect of highly variable baseline scores on the statistical analysis. The learning trial was the same on all 5 days. Two different sets of objects were alternated on each successive day to prevent adaptation to object novelty. Discrimination index (DI) was calculated based on time spent between the stationary and moving object on each day.

*Tissue extraction for Seahorse and EPR*

Rats were sacrificed under isoflurane anesthesia and 200 μm-thick coronal sections containing the dorsal hippocampus were collected using a vibratome (Leica VT1200S, Nussloch, Germany). Sections were incubated in oxygenated, chilled aCSF (120 mM NaCl, 3.5 mM KCl, 1.3 mM CaCl2, 1 mM MgCl2, 0.4 mM KH2PO4, 5 mM HEPES, and 10 mM D-glucose; pH 7.4) prior to punching. The left dorsal hippocampus was extracted and placed in a seahorse assay buffer published by Underwood et al. 2020 (aCSF, 0.6 mM pyruvate, 4 mg/ml of BSA). After slices were incubated in ACSF for 1 hour, the left dHPC DG-CA3 was punched with a 17 gauge spinal needle were placed directly into the seahorse plate, which was stored in a small Styrofoam ice container while half submerged in ice until all sections were punched. Once all sections were punched, samples were transported to the Katakam lab and Seahorse was performed. All remaining left HPC tissue not used for Seahorse was placed directly into aCSF described below in a 24 well plate. The entire right hemisphere was flash frozen whole and stored for future analysis.

*Superoxide and ROS measurement in hippocampal tissue sections:*
Superoxide anion levels in the hippocampal tissue sections were measured using electron spin spectrometry (ESR) using spin trap, 1-Hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine (CMH).\textsuperscript{118} Freshly isolated tissue sections were in placed in twenty four-well plates containing aCSF on ice (one section/well representing single rat). Iron-chelating agents, Deferoxamine methanesulfonate (DF, 25 µmol/l) and Diethyldithiocarbamic acid (DETC, 2.5 µmol/l) were added to the ice-cold modified Krebs-HEPES (KH) buffer (NaCl, 99.0 mmol/l; KCl, 4.69 mmol/l ; CaCl2, 2.5 mmol/l ; MgSO4, 1.2 mmol/l; NaHCO3, 25 mmol/l ; KH2PO4, 1.03 mmol/l ; HEPES sodium salt; 20.0 mmol/l, D-Glucose; 5.6 mmol/l. pH 7.4). KH buffer containing chelating agents was deoxygenated by bubbling using dry nitrogen gas in ice cold conditions for twenty minutes. Later, the superoxide spin trap, 1-hydroxy-3-methoxycarbonyl -2, 2, 5, 5-tetramethylpyrrolidine (CMH, 200 µmol/l) was added to the KH buffer. aCSF was removed from the wells and 500 µl of ice-cold KH buffer containing spin trap was added to the tissue sections followed by fine mincing of tissue using scissors for five minutes. Later, the tissue was incubated at 37oC for an hour (5% CO2). After the incubation, tissue along with the KH buffer is immediately transferred to syringe (1 ml) and snap frozen in liquid nitrogen. Samples were stored in -80oC until the analysis. KH buffer without tissue sections and processed similar to the samples was taken as the background control. During ESR measurements, frozen samples placed in liquid nitrogen and transferred to finger Dewar vessel and readings were taken using EMX ESR eScan Benchtop spectrometer (Bruker, Germany). ESR settings include; field sweep, 80 G; microwave frequency, 9.39 GHz; microwave power, 2 mW; modulation amplitude, 5 G; conversion time, 327.68 ms; time constant, 5242.88 ms; 512 points resolution and receiver gain,
1\times10^4. Three amplitude readings were taken for each sample from the respective ESR spectra. The readings with baseline errors were corrected using WinEPR Processing software (Bruker, Germany). Later, samples were spun down and the KH buffer was discarded and the tissue was digested with NP 40 buffer (Thermo Fisher Scientific, Waltham, MA) and quantified for protein using BCA assay (Thermo Fisher Scientific, Waltham, MA). Measurements were normalized to protein and averaged measurements were reported as arbitrary units. DF, DETC and CMH were from Noxygen Science Transfer & Diagnostics GmbH, Germany.

**Measurement of Mitochondrial Respiration in DG-CA3 Dorsal Hippocampus Punches using Seahorse Analyzer:**

Mitochondrial respiratory characteristics were measured in the hippocampal tissue punches by mitostress assay using Seahorse XFe24 analyzer (Agilent Technologies, Santa Clara, CA) (PMID: 32764697, PMID: 30074132). 200ul of aCSF containing pyruvate (0.6 mmol/l sodium pyruvate & 4mg/ml BSA, pH 7.4) was taken into each well of XF24 islet capture microplate (Agilent Technologies, Santa Clara, CA). One tissue punch was placed into the depressions of each well and followed by placing the islet screens over the tissue sections. Sections were incubated at 37°C in non-CO2 incubator for an hour. Triplicate wells were used for each tissue sample. Mitostress drugs oligomycin, FCCP and antamycin A/rotenone 10X stocks were prepared in the aCSF containing pyruvate and BSA, later loaded into the respective injection ports of the cartridge (saturated overnight with the XF calibrant). After the cartridge calibration, islets capture plates containing tissue punches were placed in the analyzer and the oxygen consumption rates (OCR) were measured. Basal OCR were measured (3 reads) followed by OCR after successive injections of oligomycin (25 µmol/l, 4 reads), FCCP (7.5 µmol/l,
4 reads) and antamycin A/rotenone (5 µmol/l/10 µmol/l, 4 reads). Each OCR reading cycle include mixing (2 minutes), wait (1 minute) and measurement (2 minutes). Maximal OCR readings were taken into consideration for analysis for basal and FCCP (out of 3 reads), whereas the lowest OCR readings were considered for the oligomycin and the antamycin A/rotenone (out of 4 reads). Data was analyzed using MS Excel and parameters including basal, maximal respirations were calculated along with the spare respiratory capacity, ATP production, proton leak and the non-mitochondrial respiration. Measurements were averaged for the triplicates and normalized to mean basal readings from the control rat tissues punches in the single experiment or in the same day experiments. Final statistical analysis was performed in GraphPad PRISM.

Illumina RNA sequencing

Whole dorsal hippocampal tissue was placed in Trizol and stored at -80 °C until RNA extraction. Total RNA was extracted using a Qiagen Lipid Tissue Mini Kit Cat. No. 74804, and two aliquots were prepared, one for RNA sequencing, and one for real time RT-PCR confirmation of those data. RNA quality and quantity were measured by Nanodrop Spectrophotometer ND-1000 UV/Vis and by nucleotide fragment analysis on an Advanced Analytical Fragment Analyzer. RNA quality number in all samples was ≥ 6.8. 3-4 samples per group were chosen based on optimal RNA quality and shipped to Novogene corporation at UC Davis for RNA sequencing on the Illumina platform. cDNA library construction was performed with 1 ug of RNA using NEBNext® Ultra 2 RNA Library Prep Kit for Illumina® (cat NEB #E7775, New England Biolabs, Ipswich, MA, USA) according to the manufacturer’s protocol. Novogene enriched the mRNA using oligo (DT) beads and followed this with two rounds of purification and fragmented randomly by
adding fragmentation buffer. The first strand of cDNA was synthesized using random hexamers primer. Next, dNTPs, RNase H, DNA polymerase 1, and a custom second strand synthesis buffer (Illumina) were added to produce the second strand (ds-cDNA). Subsequently, terminal repair, poly-adenylation and sequencing adaptor ligation were performed, followed by size selection and PCR enrichment. This resulted in 250-350 bp insert libraries, which were quantified using a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Walkham, MA, USA) and quantitative PCR. An Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) was used to determine size distribution. Sequencing was performed on an Illumina NovaSeq 6000 Platform (Illumina, San Diego, CA, USA) using a paired-end 150 run (2x150 bases) Q30≥80% at 20M raw reads/sample. Alignment was performed using hisat2, v2.1.

**DEG and Gene Set Enrichment Analysis (GSEA)**

HTSeq gene counts obtained from Novogene were used to perform tests in DESeq2 v1.29.6. Prior to running tests for differential gene expression, we corrected unwanted variation (noise stemming from technical and biological factors unmodelled in the experiment) using RUVseq to increase power and biological insight specific to the experimental design. Briefly, after normalizing the counts (upper quantile) using the RUVr function we estimated the variables that capture the unwanted variation by factor analysis on deviance residuals after a running a regression of the counts on the covariates of interest. Those variables were included in the design formula in DESeq2. For the tests run in DESeq2 we used un-normalized HTSeq counts after filtering genes with less than 10 reads in at least 3 samples. Statistical significance was set at FDR<0.1 (Benjamini-Hochberg (BH) adjusted p value). For clustering analysis (PCA and unsupervised
hierarchical clustering heatmaps), we used the variance stabilized RUVseq corrected counts. Variance stabilization was performed with the functions in the vsn package \textsuperscript{124,125} embedded in DESeq2. Hierarchical clustering was performed and plotted using the functions in the ComplexHeatmap package \textsuperscript{126}. Briefly, we calculated the Euclidean distances and used Ward’s linkage to obtain the clusters. Gene Set Enrichment Analysis (GSEA) was performed for the genes that passed the FDR threshold in pairwise comparisons with gene ranks calculated from shrunken log fold2 changes multiplied by the FDR\textsuperscript{127} in the WebGestalt \textsuperscript{128} web application using the Gene Ontology (GO) and KEGG pathway functional databases.

*Weighted Gene Co-expression Network Analysis (WGCNA)*

WGCNA was used to identify groups of co-expressing genes in Av (n =3) and NAv (n =4) samples.\textsuperscript{129} WGCNA inputs were log2-transformed normalized counts for each sample, excluding genes containing <5 counts in any of the samples. WGCNA was performed using the R package.\textsuperscript{129} The general framework of WGCNA has been previously described.\textsuperscript{129} Briefly, we constructed a signed adjacency matrix by calculating Pearson correlations for all pairs of genes. To emphasize strong correlations on an exponential scale, we raised the adjacency to power $\beta$. We chose a power of $\beta = 18$ for so the resulting networks exhibited approximate scale-free topology. To identify gene modules, all genes were hierarchically clustered based on connection strength determined using a topological overlap dissimilarity calculation. Resulting gene dendrograms were used for module detection using the dynamic tree cut method (minimum module size = 100). To determine module-trait relationships, Pearson correlations were calculated for module eigengene expression with time spent in the
odor compartment. To functionally characterize modules, all genes belonging to a module were submitted to Enrichr, WebGestalt, or Wikipathways for pathway analysis. Module visualizations were created with the top 50 edges (based on topological overlap connectivity) of each module using Cytoscape.\textsuperscript{130} We identified hub genes for each module by examining intramodular connectivity and differential gene expression.

Statistics for OPS, EPR, and Seahorse
Graphpad Prism 9.0 was used for all statistical comparisons for these measures. We observed significantly higher standard deviation in the NAv group in nearly all cases and therefore we corrected for this in all comparisons. For OPS, a two way RM ANOVA with the Geisser-Greenhouse correction and the Bonferroni post hoc test was used to calculate within group comparisons, comparing discrimination index (DI) on each successive day to the population DI on day 1. For EPR, a two tailed t test with Welsh's correction was used. For SEFA, Welsh’s one way ANOVA was used and Dunnett’s T3 test was used for post hoc comparisons.

2.5. Notes


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CHAPTER 3. PREDATOR EXPOSURE AND PSYCHOSOCIAL STRESS IS ASSOCIATED WITH ALTERED OXIDATIVE AND LIPID HOMEOSTASIS IN THE HIPPOCAMPUS

3.1. Introduction

The brain is roughly 50% lipids by dry mass. Maintaining lipid and cholesterol (CHOL) homeostasis in the brain is critical for the maintenance of cell membranes, ion homeostasis, and cell signaling. Stress has long been known to increase serum cholesterol levels. CHOL concentrations in neuronal membranes control growth factor signaling through allosteric alterations to TrkB receptors (BDNFR), modulating GPCR signaling, and may modulate the action of antidepressants (AD). Furthermore, CHOL is a precursor to neuronally produced neurosteroids, which modulate GABAergic neurotransmission and Astrocyte derived CHOL contributes both directly and indirectly to synaptogenesis. CHOL is also a significant constituent of synaptic vesicles and plays a role in organizing lipid rafts at pre and post-synaptic densities, making CHOL and lipid pathways essential modulators of all neurotransmission, and therefore hippocampal (HPC) function. Other lipids likewise modulate synaptic function and are modulated by stress. Phosphatidylcholine (PC) concentrations modulate acetylcholine synthesis and increase DAG generation in the context of oxidative stress in synaptosomes and ether PC species are reduced in the HPC by chronic unpredictable mild stress. Myelin sheaths are produced by oligodendrocytes and constitute 80% of brain cholesterol. Stress and glucocorticoids are now known to increase oligodendrogenesis and myelin formation in the HPC, a region that is not typically myelinated and increased myelin was also recently observed in the brains of PTSD, but not mTBI participants.
Post-Traumatic Stress Disorder (PTSD), Mild Traumatic Brain Injury (mTBI), and depression are also associated with alterations to lipid species in plasma. One study using Raman imaging indicates that animal models of TBI are associated with alterations to lipid concentrations in the brain over time, particularly elevated CHOL. Elevated reactive oxygen species (ROS) production can lead to oxidative stress, a characteristic feature of a numerous diseases associated with elevated allostatic load including TBI, major depressive disorder (MDD), metabolic syndrome, diabetes, hypertension, Lupas, and PTSD, among other associated conditions. Each of these conditions is also associated with alterations in peripheral lipid homeostasis, particularly increased LDL CHOL and Triglycerides (TG) and reductions of many phospholipid species, like phosphatidylcholine (PC). Chronic or traumatic stress may therefore disturb broad organismic molecular and lipid homeostasis and whole organism allostasis. Many of these effects have been studied in animal models, but very few investigations have focused on stress-induced alterations to lipid species in the brain of models of traumatic stress.

LC-MS is the gold standard technique for quantitative measurements of lipid species in biological tissue. However, classical lipidome analysis results in the loss of spatial information due to the requirements of tissue homogenization and lipid extraction, an important limitation of this technique. Lipid concentrations modulate synaptic function, and spatial localization may be critical for the identification of brain circuit-specific alterations to lipid species. The ability to derive spatial information on lipid concentrations in the brain in animal models would surpass currently available methods and may improve our ability to understand and treat psychiatric diseases.
associated with stress. Raman spectroscopy to study lipids has attracted particular attention in the field because of the strong Raman scattering of lipids provided by long nonpolar acyl chains in their structure. Lipids have Raman bands in both the fingerprint (400-1800 cm\(^{-1}\)) and higher wavenumber group frequency regions (2800–3800 cm\(^{-1}\)). The most typical characteristics of lipids originate from their hydrocarbon chains, which manifest themselves in 1200-1050 cm\(^{-1}\) (C-C stretch), 1250-1300 cm\(^{-1}\) (CH\(_3\) scissor and twist), and 1400-1500 cm\(^{-1}\) (CH\(_2\) scissor and twist) ranges. At higher wavenumbers, strong Raman bands appear in the 2800-3100 cm\(^{-1}\) region assigned to C-H stretching of lipids.

Based on these factors, we previously utilized Raman to investigate lipid alterations in a predator exposure and psychosocial stress model (PE/PSS) of PTSD and confirmed these results with MALDI imaging. We found that PE/PSS is associated with broad alterations to lipid homeostasis in the paraventricular thalamus (PVT), notably increased CHOL and Cholesterol Esters (ChE) and modifications to several phospholipid species.

In this study, we hypothesized that the PE/PSS model would be associated with increased CHOL and ChE concentrations and reduced PC, and distinct lipid profiles measured by principle components analysis (PCA) in the HPC. First, we confirmed the PE/PSS phenotype by measuring a previously published endpoint in this model, ROS production in the prefrontal cortex (PFC) and HPC, which was upregulated after PE/PSS. Next, we implemented a dual forward/reverse approach to validate our technique by making a hypothesis, followed by testing it with Raman imaging, and then validating those results with data using an LC-MS-based lipidomic
analysis and vice versa. We first utilized Raman to image and quantify CHOL and ChE concentrations in formalin-fixed coronal sections of the dorsal hippocampus (dHPC). We subsequently performed LC-MS-based lipidomics and confirmed elevations of three ChE species, in addition to acquiring data on hundreds of lipid species. We next performed PCA of the Raman and LC-MS data sets. We identified top targets from the LC-MS data through a bioinformatics analysis and quantified alterations to both individual lipid species and lipid classes. We then reversed the direction of our validation procedure by generating three hypotheses from top biomarkers that emerged from lipidomics and testing them with Raman. We hypothesized that phosphatidylcholine (PC) and phosphatidylserine (PS) would be downregulated, and triglyceride (TG) would be upregulated. We were able to reject the null hypothesis for all three species. For further validation, we chose two “control” lipids that were not identified as top targets in the lipidomic analysis, one target that was differentially expressed in the LC-MS data, cardiolipin (CL), and one that was not, phosphatidylinositol (PI). Neither target exhibited similar alterations between Raman and LC-MS. We subsequently measured mRNA expression of the major functional components of brain lipid homeostasis by qRT-PCR. We hypothesized that we would observe increased ApoE, Srebf2, and Cyp27a1 expression and reduced Srebf1. We rejected the null hypothesis for ApoE, Cyp27a1, and Srebf1, but not Srebf2.

To provide further insight into the implications of elevated ROS in this model, we quantified malondialdehyde (MDA) concentrations in the HPC. We observed decreased MDA in PE/PSS, which significantly correlated to reduced total lipid species (other than CHOL, ChE, and TG) measured by LC-MS. Furthermore, Raman analysis exhibited an
increased intensity of peak at 1660 cm$^{-1}$, in PE/PSS animals, which indicates increased docosahexaenoic acid (DHA), a lipid species that exhibits protective effects against oxidative stress. Together, these data suggest a remodeling of lipid and oxidative molecular homeostasis in the HPC after PE/PSS. To our knowledge, this is the first time that Raman spectroscopy was used to measure lipid and CHOL species in the HPC of an animal model of traumatic stress and confirmed those data with a quantitative gold standard technique, LC-MS.

3.2 Results

*Raman: PE/PSS altered the lipid profile of the dHPC*

Figure 3.1.b shows the comparison of representative Raman spectra of PE/PSS and control brain tissue. The Raman features associated with lipids (1270, 1446, 1660 cm$^{-1}$) were intensified in the PE/PSS samples compared to the control. The intensity of the peak associated with phenylalanine (1004 cm$^{-1}$) did not change for PE/PSS and control.
The peak at 1660 cm\(^{-1}\) is attributed to \(\omega-3\) fatty acid (docosahexanoic acid) in the brain (arising from sterol C=C stretching vibration).\(^{48-50}\)
The Raman band at 1446 cm\(^{-1}\) originates from the acyl group (CH\(_2\) or CH\(_3\) scissoring vibration). The peak at 1270 cm\(^{-1}\) is due to \(=\text{CH}\) in-plane deformation arising from unsaturated acid chains mainly in PC and PE of the brain. \(^{40}\) The Raman band at 832 cm\(^{-1}\) (responsible for Proline) intensified in PE/PSS compared to the control. Figure 3.1.c shows the score plot using the first two principal components (PC1 and PC2) based on 15 representative Raman spectra from each group (PE/PSS (pink) and control (blue)). The score plot clustered these two groups distinctly with positive PC1 values for PE/PSS and negative PC1 for Raman spectra obtained from the control groups. Figure 3.1.d and 1e show the cholesterol distribution in the HPC of control and PE/PSS samples, respectively. The Raman maps (blue = low; red=high) clearly show increased cholesterol in PE/PSS sample compared to the control. The corresponding weighted mean values for CHOL were calculated from the Raman maps of the dHPC (Figure 3.1.h) and subregions of the dHPC. Figure 3.1.i (CA1), Figure. 3.1.j (CA3), and Figure. 3.1.k (DG) indicate a widespread increase in CHOL that is independent of HPC circuit. Tracings of HPC subregions used for dHPC and subregion-specific quantification of CHOL are provided in Figure B. 1.i,j.

**PE/PSS is associated with increased ROS production in the PFC and HPC**

We previously published that ROS production is increased in the PFC and HPC of this PE/PSS model \(^{51}\), which has emerged as a characteristic biomarker in this model system. We once again measured elevated ROS production in this study, confirming the phenotype. An unpaired two-tailed t-test revealed that compared to control, PE/PSS animals exhibited increased ROS production in the PFC (n=6-8/ group, t=6.200, df=12, p<.0001) and HPC (n=7-8/group, t=4.559, df=13, p=.0005). (Figure 3. 1.f, g)
Lipidomics: PE/PSS altered the lipid profile of the HPC measured by LC-MS

We extracted lipids from whole HPC tissue from control and PE/PSS animals and performed LC-MS-based lipidomics on the lipid extracts. In total, 774 individual lipid species were detected from 32 total classes of lipids. Phospholipids constituted a total of 72.75% of total measured lipids, with PC species alone accounting for 49.15%. All data analysis was performed on mass spectrum intensity data normalized by the mass of the sample. (Table B. 1)
Data was analyzed in two ways. 1) Bioinformatics analysis to identify the top 15 individual lipid species biomarkers between control and PE/PSS. This analysis was performed on all 774 individual lipid species. 2) Analysis of all 33 lipid classes: each lipid species was grouped and considered as part of its lipid class. The bioinformatics analysis exhibited 15 top individual lipid species between PE/PSS and control. This included 7 downregulated PC species (PC424pH, PC428H, PC4410H, PC405eH, PC363H, PC182p/242H, and PC60/180H), 3 upregulated TG species (TG181/182/182N, TG160/171/204N, TG160/160/226N), 1 downregulated PS species (PS414-H), 1 downregulated PG species (PG225/226-H), 1 downregulated PE species (PE180p/160H), and 2 downregulated dMePE species (dMePE344p-H, dMePE160/160-H). (Figure 3. 2.a-c.)

**Bioinformatics analysis:**
Figure 3.2.a represents the partial least squares-discriminant analysis (PLS-DA) results for PE/PSS and control. PLS-DA revealed two distinct clusters with significantly differentially expressed metabolites shown on the Variable Importance for Projection (VIP) plot. (Figure 3. 2.b.) This indicates that these two group of samples exhibit distinct expression patterns of the measured metabolites. The obtained PLS-DA model was validated by seven-fold Cross-Validation predictive residual (CV-ANOVA) and response permutation with 900 random re-classifications (random assignment of class labels to The permutation test revealed the model overfits due to the low amount of replicates vs. a large number of detected metabolites. “Overfit” means that the predictability is not excellent, but that will not substantially impact the presented results. The VIP plot in Figure 3.2.b shows the important lipid species identified by PLS-DA in descending order
of importance. The graph represents the relative contribution of lipid species to the variance between the PE/PSS and control samples. Sample A is PE/PSS, and sample B is the control. A high VIP score indicates a greater contribution of the lipid species to the group separation. The red and green boxes on the right indicate whether the lipid concentration is increased (red) or decreased (green) in the tissue of the PE/PSS (red) vs. control (green) samples. The discriminating metabolites toward the clustering in the PLS-DA model were tested by regression coefficient plot with 95% jack-knifed confidence intervals where metabolites with VIP values exceeding 1.6 were selected as metabolite cut-off. Four compounds from the VIP plot were tested by the FDR (false discovery rate) method. Three compounds (which have three highest VIP scores) were found with the lowest FDR value of 6.7% \( q = 0.067 \): PC (42:4p), PC (42:8), TG (18:1/18:2/18:2). Further, Receiver Operating Characteristic (ROC) curve analysis was used to evaluate the performance of the selected compounds. The higher values (close to 1) of AUC, the better confidence of biomarker compounds. We found PC (42:4p)H, and PC (42:8)H with AUC of 1.0, and TG (18:1/18:2/18:2) with AUC of 0.944, validating the importance of these three compounds. Between-group comparisons of these compounds are shown in Figure 3.2.h-j (red is PE/PSS, green is control).

*Analysis of lipid classes and validation of Raman with LC-MS:*
Analysis of alterations to lipid classes was performed by summing all species within a class within each animal and comparing the group means of between PE/PSS and control with a t-test. Overall, phospholipid species consisted of 15/33 total measured lipid species and tended to be downregulated. In total, eight lipid species showed significant differences between groups. Only two lipid species were significantly upregulated, ChE and TG, and six downregulated, including the phospholipids PC, PA, PG, and dMePE, in addition to CL and GM1. (Figure 3.2.c.) The corresponding Raman map for control and PE/PSS showing the distribution of cholesterol palmitate in the HPC.
are presented in Figure 3.2.d,e respectively. The corresponding quantification of cholesterol palmitate averaged from the Raman maps of each animal in the dHC region are shown in Figure 3.2.f,g. The results in Figure 3.2.f show that there is a significant increase of cholesterol palmitate in the dHC region of the brain in the PE/PSS group compared to the control group. LC-MS exhibited a significant 45% increase of ChE species (t=3.034, df=10, p=.0126). Results shown in Figure 3.2.g therefore, validate the Raman map results. The Raman map in Figures 3.3.a,b and the corresponding Raman image analysis (Figure 3.3.c), as well as LC-MS results (Figure 3.3.d) of TG, showed similar changes to cholesterol with an increased level in PE/PSS compared to the control group. The LC-MS data exhibited a significant 24% increase of TG species (t=2.843, df=10, p.0175). However, Phosphatidylcholine (PC) (Figure 3.3.e-h) and Phosphatidylserine (PS) (Figure 3.3.i-l) were reduced in the dHPC region of the PE/PSS group compared to the control group. We observed a significant 44% decrease in PC species (t=2.476, df=10, p=.0328), but only a trend toward the reduction in PS (t=2.077, df=10, p=.0645) measured by LC-MS, providing validation for PC and near validation for PS. We also observed a significant 53% decrease in CL species (t=2.4, df=10, p= .0373), the opposite of the Raman results, and no significant difference in PI (t=1.171, df= 10, p= .2686), all analyzed by a two-tailed unpaired t-test. (Figure A. a-h.) Overall, we were able to validate ChE, PC, PS, and TG, endpoints chosen from past data and Raman (ChE), and the VIP plot, but not CL and PI, targets not chosen from existing data. All of the above Raman images were constructed using the DCLS method. We observed trends in the LC-MS data towards a reduction in total lipid species, not including CHOL, ChE, or TG (p=.069) (Figure 3. 4.a) and total phospholipids (p=.06)
We also observed a significant reduction when considering only the downregulated lipid species \( (t = 2.266, \text{df} = 10, p = .047) \). These data support the Raman data exhibiting increased CHOL, which was not quantified in LC-MS, although we did measure and validate increased ChE. The LC-MS data was normalized by sample mass, therefore, we should expect to observe a proportional downregulation of other lipid species in LC-MS to the observed upregulation in CHOL in Raman. This provides additional validation of our Raman results for CHOL.

**PE/PSS is associated with reduced MDA concentrations in the HPC, significant correlation with reduced measured lipids (not including CHOL, ChE, or TG)**

Malondialdehyde (MDA) concentrations were measured using an MDA assay. Variance differed between groups \( (f = 65.76, 5, 6, p < .0001) \), and therefore Welch’s t-test was used for the analysis. MDA concentrations were reduced in PE/PSS compared to control \( (t = 2.686, \text{df} = 5.13, p = .0424) \). Reduced MDA concentrations were significantly negatively correlated with total measured lipids (Not including CHOL, ChE, or TG) \( (N = 11, R^2 = .3725, p = .0461) \), suggesting that reduced total lipids may partially mediate reduced MDA concentrations despite, or in response to, elevated ROS production.

**PE/PSS is associated with increased ApoE and Cyp27a1 expression, and reduced expression of SREBF1**

We performed qRT-PCR to examine differential expression of the following genes within the whole HPC of PE/PSS versus control rats: Abca1, Hmgcr, ApoE, Cyp46a1, Srebf2, Srebf1, Cyp27a1, Lrp1, Lxrb, and Ldlr. Of these main transcriptional drivers of CNS cholesterol and lipid maintenance, only ApoE \( (t = 3.339, \text{df} = 13, P = 0.0053) \), Srebf1 \( (t = 2.490, \text{df} = 12, P = 0.0284) \), and Cyp27a1 \( (t = 2.283, \text{df} = 12, P = 0.0414) \) showed
significant differences in mRNA expression between PE/PSS and control groups using an unpaired t-test. (Figure 3. 4.e-i.) There was a marked 44% increase in the expression of ApoE, the gene responsible for the transfer of cholesterol between cells, in the PE/PSS group versus control. CYP27a1 also exhibited an approximate 37% increase in expression in the HPC of the PE/PSS group versus control. However, there was a 53% decrease in the expression of Srebf1 compared to the control. All other genes assessed did not show statistically significant differences. (Figure B. 1.i-o.)

3.3. Discussion

Figure 3.4. a) Malondialdehyde (MDA) concentration in whole HPC tissue is reduced in PE/PSS vs control. b) Sum of total measured lipids not including cholesterol esters (ChE) or triglycerides (TG) between groups measured by LC-MS. c) Sum of all downregulated lipid species between groups exhibits significant reduction in these lipid species in PE/PSS d) Linear regression analysis of TML - (ChE, TG) vs MDA concentrations exhibits significant positive relationship between MDA and TML. e-i) PCR measure of relative mRNA abundance between control and PE/PSS. e) PCR measure of ApoE mRNA expression exhibits significantly greater ApoE in PE/PSS vs control. f) PCR measure of CYP27A1 mRNA expression exhibits significantly greater CYP27A1 expression in PE/PSS vs control. g) PCR measure of CYP46A1 expression does not exhibit significant differences between groups. h) PCR measure of Srebf1 exhibits significantly lower expression in PE/PSS vs control. i) PCR measure of Srebf2 exhibits no difference in expression between PE/PSS and control.
This study and emerging evidence from other labs suggests that lipid and CHOL homeostasis are altered by stress and glucocorticoids (GCC) and play a role in stress-induced disease states. The E4 polymorphism at the ApoE gene, which codes for a CHOL transporter, may be related to PTSD symptom severity, particularly intrusion and re-experiencing symptoms, which likely involve the HPC pattern separation function and fear generalization. APOE4 also confers an increased risk of late-onset Alzheimer’s disease and is associated with memory impairments. In line with this, numerous lipid species including CHOL, ceramides, spingolipids, PC, PI, and PS are implicated in neural function and alterations to lipid homeostasis are associated with cognitive and memory impairment and neurodegenerative disease.

Chronic stress leads to HPC memory processing deficits and chronically elevated glucocorticoids (GCC)/elevated HPA axis/GCC sensitivity, reactive oxygen and nitrogen species (ROS/RNS), and inflammation, all of which have been observed in this model. Excess GCC, ROS, or inflammation can lead to reduced neurogenesis and neuroplasticity and a greater propensity for excitotoxicity in vitro. Short-term glutamatergic excitotoxicity reduces cholesterol (CHOL) concentrations, but chronic excitotoxicity increases it. ROS/oxidative stress, inflammation, and glucocorticoids may therefore mediate stress-induced functional alterations in the HPC and MTL through modulations to cholesterol and lipid homeostasis.

CHOL cannot cross blood brain barrier, therefore all CNS CHOL is synthesized locally from acetyl-CoA into 3-hydroxyl-3-methylglutaryl-coenzyme A (HMG-CoA) through HMG-CoA-synthetase and HMG-CoA reductase. The standard model is that CHOL is synthesized in astrocytes and transported to neurons by apolipoproteins.
like ApoE \(^{75}\), but recent evidence also suggests that CHOL can be synthesized in neurons through distinct pathways \(^{76,77}\). Likewise, most circulating ApoE is synthesized in the liver, but ApoE found in the CNS is synthesized locally. \(^{78}\)

CHOL homeostasis is a major regulator of cell and organelle membrane fluidity and therefore modulates membrane receptor organization and the propensity for receptor dimerization and oligomerization, with important implications for cell signaling and neurotransmission. \(^{4,5}\) One of the primary receptors studied in this respect is the serotonin 5-HT\(_{1A}\)R, which contains a CHOL recognition/interaction amino acid consensus (CRAC) motif. \(^{79}\) This receptor plays a major role in brain development and psychiatric disease, including depression, PTSD, and general anxiety disorder (GAD), for which the major FDA-approved therapeutics, tricyclic antidepressants (TCA), and selective serotonin reuptake inhibitors (SSRIs) acutely increase serotonin (5-HT) levels. We have previously shown that 5-HT levels are reduced in the PFC and HPC of this PE/PSS model and are subsequently increased by Sertraline (SSRI) treatment. \(^{80}\) SSRIs block SERT (Slc6a4), the 5-HT transporter, acutely increasing extracellular 5-HT levels, but in humans, their AD effects require up to two weeks to take effect. \(^{81}\) The canonical view is that their antidepressant (AD) effects are mediated by increased activation of cortical 5-HT\(_{1A}\)R and delayed neurochemical adaptations, like the desensitization of the 5-HT\(_{1A}\)R autoreceptor in the Raphe Nucleus to acutely increased 5-HT levels. \(^{82,83}\) However, more recent work suggests a critical role of BDNF-TrkB signaling downstream of 5-HT signaling. \(^{84,85}\) 5-HT\(_{1A}\)R is a G protein-coupled receptor that undergoes clathrin-mediated endocytosis upon stimulation and traffics along the endosomal recycling pathway. Upon Statin-induced CHOL depletion, the endocytic
pathway shifts towards caveolin mediated endocytosis and a greater number of 5-HT\textsubscript{1A}R are rerouted towards lysosomal degradation.\textsuperscript{3} 5-HT\textsubscript{1A}R also exhibits increased CHOL-dependent conformational plasticity, increasing its propensity to dimerize with other 5-HT\textsubscript{1A}Rs and potentially with other receptor types\textsuperscript{4} with important effects on serotonergic neurotransmission. Furthermore, other 5-HT receptor subtypes, like the 5-HT\textsubscript{2A}R, also dimerize to other GPCRs, and this process may likewise be mediated by CHOL concentrations in the cell membrane.\textsuperscript{86}

AD effects may also be dependent on TrkB activation downstream of serotonin receptors or glutamatergic NMDAR.\textsuperscript{85} However, in contrast to this view, recent work has exhibited that TCA and SSRI AD and the recently FDA approved fast-acting AD Ketamine bind directly to TrkB receptors\textsuperscript{87}, receptor tyrosine kinases that mediate brain-derived neurotropic factor (BDNF) signaling, which is a critical mediator of activity-dependent neuronal plasticity\textsuperscript{88,89} and the effects of all known AD drugs.\textsuperscript{85} Casarotto and colleagues exhibited that mutation of the TrkB AD-binding motif impaired behavioral, cellular, and plasticity-promoting effects of AD in vivo and in vitro.\textsuperscript{87} BDNF signaling increases membrane CHOL concentrations\textsuperscript{90,91}, and CHOL concentrations tune TrkB signaling. The TrkB transmembrane domain (TMD) senses changes in membrane CHOL levels through a CRAC domain. Casarotto and colleagues depleted CHOL and observed reduced TrkB signaling; however, further increases in membrane CHOL concentrations beyond control levels also reduced TrkB signaling, exhibiting the bi-directional effects of CHOL levels on BDNF signaling. These effects were additionally rescued by AD treatment, exhibiting the complex relationships between CHOL, BDNF-TrkB signaling and AD treatment.
Here, we observed increased ROS production, increased CHOL, ChE, and TG, reduced phospholipids, including PC, PS, and PG, and reduced MDA concentrations, a marker of oxidative stress stemming from oxidized polyunsaturated fatty acids. MDA concentrations were correlated with reduced total lipid species (not including CHOL, ChE, and TG). Using Raman, we also observed an increased peak at 1660 cm$^{-1}$ in PE/PSS vs. control, which indicates the increased presence of DHA, a highly protective lipid species against oxidative stress. The shift observed here towards relatively increased CHOL/ChE and TG and lower levels of other lipids, predominantly phospholipids, which correlate with reduced MDA concentrations, may indicate a homeostatic response by cells in the HPC to reduce their propensity for OXS. It was unexpected to observe reduced MDA in this model despite increased ROS production, but this fact exemplifies the adaptive capacity of organisms to function across a broad range of homeostatic setpoints. This point is further supported by increased DHA concentrations in PE/PSS animals, suggesting a shift towards lipid species that are resistant to OXS.

Finally, we investigated possible molecular mechanisms underpinning alterations to lipid homeostasis in this model. We sought to provide initial data in this area by performing qRT-PCR for ten important mediators of lipid synthesis, transport, or metabolism. We hypothesized that ApoE, Cyp27a1, and Srebf2 would be upregulated in PE/PSS and that Srebf1 would be down-regulated. We observed upregulated CHOL and therefore we expected increased CHOL synthesis (Srebf2), transport (ApoE), and metabolism (Cyp27a1). We rejected the null hypothesis for ApoE and Cyp27a1, but not Srebf2. (Figure 3. 4.) We also investigated six other transcripts associated with CHOL
signaling or transport, but did not observe significant differences in any of those genes. (Figure B. 1.i-o.)

ApoE is unable to cross the BBB\textsuperscript{94} and is primarily generated by astrocytes in the CNS. ApoE is a major regulator of intracellular lipid homeostasis\textsuperscript{95} and exhibited increased expression in the PE/PSS group compared to the control. Increased ApoE expression is required for the transfer of cholesterol between CNS cell types to meet cellular demands for repair in the presence of cellular stress\textsuperscript{96}. ROS and OXS is have a substantial impact on cellular viability of all neural cell types, but oligodendrocytes, cells that form myelin-sheaths and contribute up to 80% of the brain’s total cholesterol are particularly sensitive.\textsuperscript{20,75,97-99} Stress and glucocorticoids increase oligodendrogenesis in the HPC\textsuperscript{18} and whole brain myelin concentrations are increased in human PTSD.\textsuperscript{19-21} Therefore, increased myelination of the HPC may contribute to the observed increased cholesterol here. ApoE plays a significant role in CNS response to injury\textsuperscript{100} and stress\textsuperscript{101}. ApoE also participates in the regulation of hippocampal neurogenesis and synaptogenesis.\textsuperscript{102,103} It’s unknown if the increased ApoE expression and CHOL concentrations observed here play a role in regulating neurogenesis, oligodendrogenesis, or allostatic responses to stress, but it likely impacts all three and future investigations should focus on the role of ApoE expression and CHOL concentrations on these factors.

Srebf1 mediates the synthesis of monounsaturated and polyunsaturated fatty acids (MUFAs/PUFAs) in astrocytes, which commonly shuttle fatty acids to neurons, and is particularly significant in neurite outgrowth and synaptic transmission. Srebf2 mediates cholesterol synthesis.\textsuperscript{104} In this study, we observed decreased Srebf1 and no
significant change in Srebf2. Lower expression of Srebf1 is consistent with our observed
trend towards a decrease in overall lipids not including CHOL, ChE, and TG. The lack of
the expected increase in Srebf2 expression may indicate that increases in CHOL
synthesis occurred earlier in the stress protocol from predator exposure on day 1 and
11 and were not sustained by psychosocial stress alone. Alternatively, there could be
celling effects in the upregulation of CHOL by stress that occurred earlier in the protocol
and while CHOL is still elevated, upregulated CHOL synthesis has since normalized.
Specific investigations of this time course would be required to confirm this idea.

This study exhibits the efficacy of Raman to image numerous lipid species \textit{in situ}
with confirmation by the gold standard, LC-MS. However, two of our “control” targets
that were not either hypothesis driven from previous work, or among the top 15 targets
that emerged from the LC-MS data, PI and PC, did not match the Raman data
directionally. CL exhibited significant differences in both measures, but differed
directionally, while PI exhibited a significant increase when measured by Raman, but no
change when measured by LC-MS. (Figure B. 1.a-h.) This could result from the fact that
the lipid standards used for Raman were not representative of all lipid species
measured by LC-MS. Individual variation within lipid species was expected and
divergent expression of individual species was observed in some cases, although in
most cases there were few divergently expressed individual lipid species within a lipid
class. This discrepancy between the techniques could also result from the fact that
Raman was only performed on \textit{in situ} dHPC slices, while LC-MS was performed on
extracted lipids from whole HPC tissue, including the ventral HPC. It’s possible that PI is
indeed increased in the dHPC, but not the ventral or that CL was divergently expressed
across the D-V axis of the HPC. The decision to use whole HPC tissue for LC-MS was made to increase tissue availability such that aliquots of the same tissue could be used across LC-MS, qRT-PCR, and MDA assays. Divergent results between techniques could also exemplify limitations associated with using Raman to measure CL or PI species specifically and/or further standardization required for such measurements in fixed brain tissue. Another major limitation of this study was that we did not perform behavioral analysis on these animals to measure endpoints related to HPC function, nor did we experimentally modulate CHOL or other lipid levels to determine their effects on behavior. Future investigations should pair lipid analysis techniques, like Raman with behavioral analysis of HPC function, like object pattern separation, and modulate HPC cholesterol levels with statin drugs with and without PE/PSS to determine the causal impact of stress-induced CHOL and lipid concentrations on HPC function. We also did not directly measure oxidized lipid species. An MDA assay is only an index of oxidized PUFAs and is subject to off target effects. Direct measurements can be performed by LC-MS and such measures will be required for a complete evaluation of stress induced changes to oxidative and lipid homeostasis in this model system. Finally, we did not measure protein expression of lipid-related gene transcripts, antioxidant gene or protein expression, or Pla2, a gene implicated in inflammation that modulates lipid expression. Future investigations should measure protein expression of these targets to confirm expression changes at the functional level and measuring antioxidant protein expression will be required for a full evaluation of oxidative stress in this model. Biologically, this study exhibits alterations to primary peripheral drivers of allostatic load (CHOL, TG, ROS, lipid metabolic pathway gene expression, and oxidative stress (MDA)
in the HPC after PE/PSS, a model of PTSD. We observed that despite increased ROS and the typical sequela that follow, increased CHOL and TG, we did not observe increased MDA, a marker of OXS. Further, we did observe increased expression of DHA, a highly neuroprotective PUFA against OXS. Together, these data exemplify potential lipid metabolism-mediated mechanisms of biological adaption to increased ROS, preventing the development of allostatic overload exemplified by markers of OXS like MDA.

3.4. Materials and methods

Animals

Sprague Dawley rats were ordered from Charles River and were between 125 and 150 days old upon emerging from quarantine. Two independent cohorts of animals were used for these experiments. One cohort n=10/ group was split into two groups such that n=2-3/ group was used for Raman and n=7/ group was used for EPR, PCR, and LC-MS. A second independent cohort of rats were only used to confirm the Raman data (n=2-3/ group).

PE/PSS model

Animals were pseudo-randomly assigned into either control or PE/PSS groups based on baseline body weight. PE/PSS rats began the stress regiment the following day. PE/PSS induction was performed as previously described. Briefly, rats were placed into plexiglass cylinders, which were rubbed with cat food, and placed into a large metal cage where they were exposed to a live cat on day 1 and 11 of a 30 day stress regiment (PE). Every day from day 1-30 also required daily psychosocial stress (PSS), which consisted of cage rotation such that each stressed animal is paired with every other
stressed animal before getting paired with a familiar rat again. Rats only experienced
the same cage mate up to 3x per experiment.

*Electron Paramagnetic Resonance Spectroscopy (EPR)*

We measured ROS production by Electron Paramagnetic Resonance Spectroscopy
(EPR) as previously published by our lab. 45,46 A separate aliquot from the same tissue
homogenate was used for EPR, qRT-PCR, and the MDA assay.

*Raman spectroscopy*

*Sample preparation*

Animals were sacrificed on day 31 of the protocol. Animals were transcardially perfused
with phosphate buffered saline (PBS) perfusion buffer until the fluid ran clear, and
subsequently with 250 ml of 4% paraformaldehyde (PFA). Brains were then removed
and incubated with 15% sucrose in PFA for 24 hours and then 30% sucrose in PFA until
the brain sank to the bottom of the solution. Brains were then stored at 4C until they
were sectioned on a cryostat at 50 um, which were then stored in 12 well plates at 4C
until Raman was performed.

*Raman imaging:*

Raman spectra were obtained with a Renishaw inVia Reflex Raman Spectroscope. The
laser excitation wavelength of 785 nm, acquisition time of 20 s, and laser power of 30
mW were used. For the Raman map, static mode with a center wavenumber of 1200
cm\(^{-1}\), with acquisition time of 4 s was used. Streamline Mapping (Y\text{bin}=20) mode of the
Renishaw WiRE 4.4 software were used for fast image acquisition. The total number of
spectra in each brain tissue slice were ~ 10,000. The preprocessing, and principal
component analysis (PCA) of spectra were performed in Origin 2018 (OriginLab,
Northampton, MA). For the preprocessing of the spectral data, background fluorescence
removal, min-max normalization, smoothing using Savitzky-Golay method were done.
The Raman map used direct classical least squares analysis (DCLS) method to analyze
and to generate the lipid distributions. The Raman images were analyzed further using
ImageJ 1.8 software.

**LC-MS lipidomics:**

**Sample preparation at LSU-SVM**

Animals were sacrificed on day 31 of the protocol. Upon sacrifice, the whole brain was
removed in under 60 seconds and placed directly onto a cold plastic plate buried in ice.
The whole HPC was immediately dissected by hand, hand homogenized on ice, and
aliquoted for EPR, LC-MS, and qRT-PCR. For LC-MS, small aliquots of each sample
were immediately flash frozen in aluminum foil. For tissue extraction, samples were
weighed, recorded, and sonicated on ice until homogenous. The sample was
transferred to an O-ring sealed screwed vial and 1 mL of solvent (chloroform: methanol
(2:1 v/v)) was added. The samples were then shipped overnight on dry ice from the LSU
School of Veterinary Medicine to the University of Illinois Urbana Champaign for
lipidomic analysis by Dr. Zhang.

**Lipidomics, MS and data analysis at UIC**

Samples were first sonicated with a Model Q700 QSonica sonicator equipped with an
Oasis 180 Chiller (4 °C; amplitude, 95; process, 5 min; pulse on 30 s; plus off 55 s),
centrifuged at 14,800 r.p.m. for 10 min at 4 °C, then 50 µl of the extract supernatant was
spiked with 2 µl, 50 µg ml⁻¹ internal standard mixture (Cer 18:1/12:0; PC 12:0/12:0; PE
14:0/14:0; PG 14:0/14:0; PS 14:0/14:0, etc). The samples were then analyzed using the
Thermo Q-Exactive MS system in the Metabolomics Laboratory of the Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign. Xcalibur 3.0.63 was used for data acquisition and analysis. A Dionex Ultimate 3000 series HPLC system (Thermo) was used, and LC separation was performed on a Thermo Accucore C18 column (2.1 × 150 mm, 2.6 μm) with mobile phase A (60% acetonitrile: 40% H2O with 10 mM ammonium formate and 0.1% formic acid) and mobile phase B (90% isopropanol: 10% acetonitrile with 10 mM ammonium formate and 0.1% formic acid) and a flow rate of 0.4 ml min⁻¹. The linear gradient was as follows: 0 min, 70% A; 4 min, 55% A; 12 min, 35% A; 18 min, 15% A; 20–25 min, 0% A; 26–33 min, 70% A. The autosampler was set to 15 °C and the column was kept at 45 °C. The injection volume was 10 μl. Mass spectra were acquired under both positive (sheath gas flow rate, 50; auxiliary gas flow rate, 13; sweep gas flow rate, 3; spray voltage, 3.5 kV; capillary temperature, 263 °C; auxiliary gas heater temperature, 425 °C) and negative (sheath gas flow rate, 50; auxiliary gas flow rate, 13; sweep gas flow rate, 3; spray voltage, −2.5 kV; capillary temperature, 263 °C; auxiliary gas heater temperature, 425 °C) electrospray ionization. The full scan mass spectrum resolution was set to 70,000 with a scan range of m/z ~230–1,600 and the automatic gain control (AGC) target was 1E6 with a maximum injection time of 200 ms. For MS/MS scans, the mass spectrum resolution was set to 17,500 and the AGC target was 5E4 with a maximum injection time of 50 ms. The loop count was 10. The isolation window was 1.0 m/z with normalized collision energy (NCE) of 25 and 30 eV. LipidSearch (v.4.1.30, Thermo) was used for data analysis and lipid identification. Lipid signal responses were normalized to the corresponding internal standard signal response. For those lipid classes without
corresponding internal standard, positive lipid ion signals were normalized with the signal of internal standard Cer 18:1/12:0 and negative ion signals were normalized with the signal of internal standard PG 14:0/14:0. The percentage of lipid classes within a sample was calculated by adding that of each of the individual molecular species quantified within a specific lipid class, and the relative abundance was represented by the mean percentage of three replicates for each group of samples. Abundance data was subsequently normalized by the mass of the sample.

Relative Quantification of Gene Expression by Real-Time PCR

Whole hippocampal tissue was placed in TRIzol reagent according to manufacturer’s instruction (Life Technologies) and stored at -80 °C until RNA extraction. Total RNA was extracted using a Qiagen Lipid Tissue Mini Kit Cat. No. 74804, and aliquots per gene were prepared for real time RT-PCR confirmation of those data. RNA quality and quantity were measured by Nanodrop Spectrophotometer ND-1000 UV/Vis. Real-time RT-PCR (n=7-8) was used to confirm the mRNA levels of differentially expressed genes explained by RNA sequencing in the hippocampus (Abc1, Hmgcr, Cyp46al, Cyp27a1, Srebf1, Srebf2, ApoE, LxrB, Lrp1, and Ldlr). All 10 genes were run on at least 3 replicates across 2 PCR runs, totaling 6 runs/gene/sample. Reverse transcription of RNA was performed by using primers generated by PrimerQuest Tool (Integrated DNA Technologies). Total RNA extraction was performed as aforementioned. cDNA synthesis was performed using a Bio-Rad iScript cDNA synthesis kit (Catalog # 1708891) as previously reported (Sriramula et al., 2008). Gene expression was calculated by ΔΔCT and was normalized to GAPDH mRNA levels. These data are
presented as fold change of the gene of interest relative to control animals. Table of primers (Table B. 1.).

*Malondialdehyde (MDA) assay*

Tissue samples of the whole HPC were flash frozen immediately upon extraction and stored in aluminum foil at -80 Celsius. Upon removal, tissue samples were homogenated in RIPA buffer, aliquoted, and frozen once more at -80 until the assay was performed. A lipid peroxidation malondialdehyde (MDA) assay kit was purchased from Abcam (ab118970) and utilized for MDA measurements per the manufacturer’s instructions.

3.5. Notes


Wilson, C. B. *et al.* Inflammation and oxidative stress are elevated in the brain, blood, and adrenal glands during the progression of post-traumatic stress disorder in a predator exposure animal model. **8**, e76146 (2013).

Clement, A. B. *et al.* Adaptation of neuronal cells to chronic oxidative stress is associated with altered cholesterol and sphingolipid homeostasis and lysosomal...


CHAPTER 4. PHARMAHUASCA AND DMT RESCUE ROS PRODUCTION AND DIFFERENTIALLY EXPRESSED GENES OBSERVED AFTER PREDATOR AND PSYCHOSOCIAL STRESS: RELEVANCE TO HUMAN PTSD

4.1. Introduction

Post-traumatic stress disorder (PTSD) is a trauma and stressor-related psychiatric syndrome characterized by intrusive thoughts and memories, hyperarousal, reexperiencing symptoms, and negative alterations to cognition and mood. PTSD is also linked to psychological and physiological comorbidities, including major depressive disorder (MDD), anxiety disorders, substance use disorder, type 2 diabetes, cardiovascular disease, inflammatory bowel disease, and chronic kidney disease. Each of these conditions, including PTSD, is associated with elevated oxidative stress (OXS) and sterile inflammation (INF). Elevated OXS and INF are thought to arise from PTSD-related chronic stress, and enigmatically, comorbid inflammatory conditions and/or genetics that predispose the individual to reduced antioxidant capacity and increased INF may predispose individuals to developing PTSD. PTSD is also associated with altered cortical and hippocampal volume, and cognitive deficits, which may preexist and play a role in PTSD development. Chronic cases of PTSD can lead to neuroprogression in which patients experience further memory impairments and cognitive deficits. PTSD is therefore bidirectionally associated with increased whole organism allostatic load, a characterization shared with major depression (MDD).

Yehuda et al. suggested that investigating stress induced alterations to the molecular landscape of the brain between human PTSD and animal models through RNA sequencing will be pivotal for the optimal validation of animal models and exploring...
the effects of experimental therapeutics on stress related gene expression. While individual features of PTSD have been explored in animal models, to our knowledge, there is no available direct comparison between human brain RNA sequencing data and RNA sequencing data from an animal model of traumatic stress.

In this regard, our lab previously demonstrated elevated central and peripheral INF and reactive oxygen species (ROS) production \(^{20}\) and altered neurotransmitters \(^{21}\) in a predator exposure/psychosocial stress (PE/PSS) model of traumatic stress in rats, which was originally established and validated in David Diamond's lab. The only FDA approved pharmacotherapies for PTSD are the SSRIs sertraline and paroxetine. Our lab demonstrated in this model that sertraline treatment reduced inflammation in the prefrontal cortex (PFC) and hippocampus (HC) and increased 5-HT levels. \(^{22}\) However, in practice, treatment with SSRIs for PTSD is insufficient \(^{23,24}\) and novel efficacious strategies will likely require atypical pharmacology and treatment approaches. \(^{25,26}\)

\(N,N\)-dimethyltryptamine (DMT), a serotonergic psychedelic 5-HT\(_{2A}\)/5-HT\(_{1A}\) agonist, has recently gained attention as an experimental therapeutic for PTSD, MDD, and anxiety disorders. \(^{27-32}\) DMT is one component of a traditional plant-based tisane used by indigenous peoples of the Amazon basin. It is commonly called “Ayahuasca” by Westerners and is essentially a combination of two plants: (1) *Psychotria viridis* (Chacruna) containing DMT and (2) *Banisteriopsis cappi* containing the monoamine oxidase inhibitors (MAOIs) harmine (HA), harmaline (HL) and tetrahydroharmaline (THH), which also acts as a weak SSRI. \(^{33}\) Pharmahuasca is a pharmaceutical combination of two components of Ayahuasca, DMT and HL, or DMT and another MAOI. \(^{34}\) DMT is not orally active at standard doses due to its rapid metabolism by
monoamine oxidase (MAO) enzymes, but when paired with MAOIs like HL, oral DMT causes an intense psychedelic experience lasting 4 to 6 hours. DMT and all three major β-carbolines in B. cappi, HA, HL and THH induce neurogenesis in vitro and DMT does so in vivo. Likewise, DMT has been deemed a psychoplastogen for its ability to induce mTOR-dependent neuroplasticity in vitro. Ayahuasca is the most researched formulation of DMT for the treatment of psychiatric illnesses, where it has been studied in Peru, Brazil and Canada. Ayahuasca treatment in humans is associated with immunomodulatory effects and reduced peripheral blood C-reactive protein levels, which correlate with its antidepressant effects. DMT also exhibits anti-inflammatory and tissue protective effects in vitro and in animal models.

To further validate the stress model, we performed total RNA sequencing in the PFC of control animals and those subjected to the PE/PSS model, which was previously behaviorally validated in our lab and others. We compared our differentially expressed genes (DEGs) with DEGs from a recently published human data set from the dorsolateral prefrontal cortex (dLPFC) of PTSD vs control post mortem brains using a hypergeometric test. We observed 20 overlapping genes between the two data sets and 17 are directionally consistent.

We hypothesized that DMT and pharmahuasca would reduce ROS production in the PFC and hippocampus (HC) and inflammatory gene expression in the PFC. Additionally, we hypothesized that DMT and pharmahuasca would rescue the expression of DEGs overlapping with the human data set and induce the expression of genes and pathways associated with neuroplasticity. To test this hypothesis, we administered DMT (2 mg/kg IP), HL (1.5 mg/kg IP), and both in combination
(pharmahuasca) every other day for 5 days to animals who just completed the 30 day PE/PSS regimen. (Figure. 4.1.a.) We measured ROS production in the PFC by electroparamagnetic resonance spectroscopy (EPR) and performed RNA sequencing on total PFC RNA. We performed pathway analysis on DEG data by Gene Ontology (GO), KEGG, and Ingenuity Pathway Analysis (IPA). Based on the RNAseq data, we hypothesized that DMT and HL would both exhibit affinity and efficacy at the human 5HT₂AR. To test this, we performed in vitro assays to measure the affinity and efficacy of DMT, HL, and 5-HT at the human 5HT₂AR. To our knowledge, we are the first to directly compare RNA sequencing data sets from the brain between human PTSD and a stress model and are the first to show that DMT and pharmahuasca reduce ROS production in the PFC and HC. We also show that DMT, HL and pharmahuasca normalize 9, 12, and 14 overlapping DEGs between human PTSD and our PE/PSS model respectively, and modulate the expression of genes and pathways associated with ROS production, inflammation, growth factor signaling, neurotransmission, and neuroplasticity. Finally, we show that 5-HT and DMT, but not HL show affinity and efficacy at the human 5-HT₂AR.

4.2 Results

Relevance of the rat model to the human PTSD dlPFC transcriptomic profile
PE/PSS alters gene expression in the PFC of rats
The PE/PSS model was created to induce a preclinical PTSD-like phenotype that recapitulates some features of human PTSD. (Figure 4.1.a.) We observed 193 annotated genes (230 total identified genes) out of 17,573 (1%) total identified genes that significantly differed between PE/PSS and control (FDR<.1). (Figure. 4.1.b.) This gene set comprises genes related to inflammation, growth factor signaling, and neurotransmission. First, we found an upregulation of interleukin-1 receptor, type I (Il1r1) in PE/PSS compared to control. (Figure. 4.1 b,d,e.) While no lab has previously reported this target in a PTSD model, it corresponds with our prior functional validation at the protein level of the upregulation of the NLRP3 inflammasome and its product, interleukin 1 beta (IL-1β), a pro-inflammatory cytokine and ligand of IL-1R1. 20 Taken together, the previous functional and the current transcriptomic profiling support the fact that neuroinflammatory signaling is elevated in this PE/PSS model. Second, we observed alterations to growth factor expression including the upregulation of insulin-like growth factor 2 (Igf2) and insulin-like growth factor-binding protein 2 (Igfbp2) in PE/PSS compared to control. Third, we observed an alteration to GABA signaling, an effect also observed in humans with PTSD, although we only observed one overlapping GABAergic DEG between the two data sets (SST). 59 We found a 2.97-fold upregulation
Figure 4.2. a) Heat plot exhibiting log fold changes (LFC) of overlapping differentially expressed genes (DEGs) between rat PFC predator exposure + psychosocial stress (PE/PSS) vs control and human dPFC PTSD vs control, and the observed modulations of treatments in rats vs PE/PSS. All values are statistically significant DEGs. X indicates no significant alteration by treatment b) Bar plot of overlapping DEG LFCs between human and rat data sets and effects of treatment in rats vs PE/PSS c) GABA receptors and transporters graphed across all groups in rats. d) Glutamatergic DEGs graphed across all groups in rats e) Serotonin receptors and Beta arrestin 1 graphed across all groups in rats f) Cytokines and pattern recognition receptors graphed across all groups in rats
(Slc6a13) (Figure 4. 1.b), a GABA transporter typically expressed at low levels in the brain but that is transcriptionally induced after PE/PSS. Gabrg1, a subunit of the GABAAR linked to alcoholism was downregulated in PE/PSS vs control. Somatostatin (Sst) was also downregulated in PE/PSS. Together, these findings may indicate a shift in the excitatory/inhibitory balance in the PFC.

PTSD alters the transcriptome in the dlPFC in humans: analysis of overlap with the PE/PSS model

Girgenti et al. observed 393 total DEGs in the dlPFC between combined male and female control and PTSD post mortem brain samples (FDR<0.05). We performed a hypergeometric (one sided Fishers exact test) to compare these two data sets, which revealed a significant enrichment of PE/PSS DEGs in the human data set (P<0.0001, Odds Ratio= 5.080, 95% C.I.= 3.190 to 8.163). (Figure 4. 1.c.) Among the 20 overlapping genes, 17 genes were modulated in the same direction in both studies. In total the 20 overlapping genes significantly correlated between both studies (Spearman’s r= .5414, p=.0137). (Figure 4. 1.e.) The 20 DEGs modulated in both studies were: ANXA2, BMP6, COL1A1, COL3A1, FOXO3, GRM2, IFITM2, IGF2, IGFBP2, IL1R1, NID1, RESP18, SLC13A4, SLC36A1, SLC6A20, SRSF6, SST, TAGLN, TGFBR3 and VIM. (Figure. 4. 2.a.) Important targets for our analysis are in italics and represent pathways associated with IGF signaling (IGF2, IGFBP2), inflammation (IL1R1) and GABAergic neurotransmission (SST). Its noteworthy that IL-1β, the ligand for IL-1R1 was also increased in the human data set, which we previously observed at the transcriptional and protein levels in the PFC of this model. 

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Figure 4.3: Ingenuity pathway analysis schematic of transcriptional changes in the IPA GABA receptor signaling pathway. a) Predator exposure + psychosocial stress (PE/PSS) + Vehicle vs control animals. b) PE/PSS + DMT vs PE/PSS + Vehicle. Red/Green indicate up/down regulated genes respectively, Red + Green indicates uncertainty in the direction of abundance and white indicates parts of the pathway that do not significantly differ between groups.
Effects of treatment

DMT alters gene expression in the PFC of PE/PSS rats and reverses 9 DEGs observed in PE/PSS animals and human PTSD

DMT treatment in PE/PSS rats altered the expression of 3869 identified genes out of 17,802 total genes measured in this comparison, or 22%. (Figure C. 1,2.) Overall, DMT reversed the expression changes of 9 genes modulated in both human PTSD and the PE/PSS model including ANXA2, BMP6, COL3A1, IGF2, IL1R1, SLC6A20, SRSF6, SST and VIM. (Figure 4. 1.d,e, Figure 4. 2.a,b.) DMT reversed PE/PSS-induced changes to GAT2, increased the expression of Slc32a1, a vesicular GABA transporter downregulated in human PTSD. 59 It upregulated Gabbr1, Gabbr2, subunits of the Gi coupled GABA_B receptors that mediate the effects of gamma-hydroxybutyric acid (GHB), upregulated Gabbr1, reduced Gabrg1 and Gabrg2 and reduced Gabra1 and Gabra2, subunits of the GABA_AR that are upregulated in human PTSD. 59 DMT upregulated mRNA expression of GAD1 and GAD2, enzymes that metabolize glutamate to GABA, the latter of which is downregulated in the dlPFC in human PTSD. 59 DMT increased the expression of Aldh9a1, an enzyme that metabolizes GABA into GHB, and Aldh5a1, a mitochondrial enzyme that metabolizes GABA into succinate, an input into the citrate cycle. (Figure 4. 2.c, Figure 4. 3.) DMT reversed PE/PSS induced changes to Sst (Figure C. 5.a) and upregulated its receptors Sstr1, Sstr2, and Sstr4.
PE/PSS animals exposed to three doses of DMT (2mg/kg IP) exhibited reduced expression of genes involved in oxidative phosphorylation in the mitochondria. GO and KEGG analysis support the downregulation of mitochondrial pathways. (Figure C. 6.a, 7.a.) DMT modulated the NRF2-Mediated Oxidative Stress Response IPA pathway. (Figure C. 8.) DMT downregulated Nrf2 and upregulated Keap1, its molecular inhibitor. The transcriptional changes observed herein support the idea that DMT treatment
reduces oxidative stress in PE/PSS. DMT upregulated genes associated with glutamatergic neurotransmission (Figure 4.2.d.) and neuroplasticity including the presynaptic genes synapsin (Syn2, Syn3) and synaptotagmin (Syt1), the postsynaptic genes for NMDAR (Grin1), AMPA (Gria1), ephrin-B (Efnb2) and its receptor, EPHB (Ephb3, Ephb6). DMT significantly altered the expression of G proteins (Gαs, Gαi, and G0αq) (Figure C.9), G protein-coupled receptors (GPCRs) including 5-HT1αR (Htr1a) and 5-HT1βR (Htr1b) (Figure 4.2.e), among others, and their effectors. In line with the fact that DMT reduced ROS production and oxidative stress response pathways, DMT also modulated the expression of many cytokines and pattern recognition receptors (PRR). (Figure 4.2.f.) DMT upregulated Lrp8 and growth factor receptors (GFR), like Ntrk2 and Ntrk3 and downstream effectors responsible for new protein synthesis including mTORC1, mTORC2, and CREB binding protein (CBP/Crebbp).

**HL alters gene expression in the PFC of PE/PSS rats and reverses 12 DEGs observed in PE/PSS animals and human PTSD**

Surprisingly, HL elicited the greatest total number of significant transcriptional changes compared to PE/PSS + vehicle (5279/17769 or 30%). (Figure C.3.) Overall Harmaline reversed expression changes in 12 genes that differed in both human PTSD and in the PE/PSS model including ANXA2, BMP6, COL3A1, IFITM2, IGF2, IGFBP2, IL1R1, SLC13A4, SLC6A20, SRSF6, SST, and VIM. (Figure 4.2.a, b.) HL modulated the expression of over a dozen genes involved in mitochondrial oxidative phosphorylation including downregulating ND1, ND2, ND3, ND4, ND5, COX1, COX2, COX3 and upregulating ATP5D and ATP5A1, among others, and modulated GO, KEGG and IPA mitochondrial pathways. (Figure 4.4.a, Figure C.6.c, 7b.) HL upregulated Sst, Sstr1, Sstr2, Sstr3 and Sstr4. HL upregulated the IPA Synaptogenesis Pathway, indicating
neuroplasticity-inducing effects. HL had the most substantial effects on the ephrin system, modulating both EphA and EphB and their ligands EfnA and EfnB. HL modulated G proteins (Gαs, Gαi, and Gαq) (Figure C. 10.) and various GPCRs and their effectors.

Pharmahuasca alters gene expression in the PFC of PE/PSS rats and reverses the expression of 14 DEGs observed in PE/PSS animals and human PTSD

The effects of pharmahuasca were similar to those of each component alone but were also unique in several ways. (Figure C. 2.) Pharmahuasca significantly modulated the expression of 2,969 genes out of 17690 detected (17%). (Figure C. 4.) Unlike the other treatments pharmahuasca did not upregulate Sst (Figure C. 5.a); however it did upregulate the somatostatin receptors Sstr1, Sstr3, and Sstr4. Pharmahuasca was also the only treatment to reduce the expression of Indolethylamine-N-methyltransferase (Inmt). (Figure C. 5.c.) Pharmahuasca reversed expression changes in 14 overlapping DEGs between human PTSD and our PE/PSS model: ANXA2, BMP6, COL1A1, COL3A1, IFITM2, IGF2, IGFBP2, IL1R1, NID1, SLC13A4, SLC6A20, SRSF6, TAGLN, and VIM. (Figure 4.2.a, b.)

Like the other treatments, pharmahuasca altered the expression of transcripts involved in GABA signaling (Figure 4. 2.c.), and upregulated mGluR5 (Grm5) among several other glutamatergic targets (Figure. 2d.) Pharmahuasca, like DMT and HL, also downregulated Nrf2 and upregulated Keap1. Pharmahuasca, like harmaline downregulated the IPA Oxidative Phosphorylation signaling pathway (Figure 4. 4.a.), but unlike harmaline, did not upregulate any mitochondrial transcripts. (Figure 4. 4.b.) In line with this, pharmahuasca downregulated numerous cytokines and PRRs (Figure 4. 2.f, Figure 4. 5.), and was the only treatment to downregulate the IPA Role of Nitric
Oxide and Reactive Oxygen Species in Macrophages pathway and NF-κB2 (Nfkb2), a major transcription factor for inflammatory cytokines. (Figure 4.5., Figure C. 5.e.) These effects are supported by the downregulation of the GO Biological Process Pathways for Innate Immunity (Figure C. 6.f.) and the IPA Pathway for Neuroinflammation. (Figure C. 11.) Furthermore, pharmahuasca exhibited the greatest effects on reducing ROS production in the PFC and HC measured by EPR spectroscopy (Figure 4.6.a, b.), a functional validation of the reductions in ROS related IPA pathways mentioned previously.

Pharmahuasca increased the expression of the Igf2r, had the greatest effect on Igf2, and was the only treatment to normalize Igfbp2 levels. (Figure 4.6.c, d, g, h.) Pharmahuasca additionally modulated the expression of Gαq, Gai, and Gαq linked GPCRs and their effectors, insulin receptor signaling, and like DMT, mTORC1 and mTORC2 signaling. Further, pharmahuasca upregulated the IPA pathway for CREB signaling. (Figure C. 12.) Pharmahuasca upregulated transcription of the IPA Synaptogenesis pathway including ephrin signaling (Figure C. 13) and the IPA Long Term Potentiation pathway (Figure C. 14), supporting our hypothesis that this compound would induce the transcriptional signature of neuroplasticity. Pharmahuasca upregulated the IPA Opioid Signaling Pathway and was the only treatment to upregulate both the delta opioid receptor (Oprd1) and the receptor for nociception (Oprl1) (Figure C. 15.) Collectively these data support the psychoplastic effects of DMT in the cortex elucidated by David Olson’s lab,\textsuperscript{39-41,61} among others.\textsuperscript{61}

*PE/PSS increases ROS production in the PFC and HC, effects that are reversed with DMT and pharmahuasca treatment*
We previously demonstrated elevated ROS production in the HC, PFC, and adrenal glands of the PE/PSS rat model of PTSD. In this study, we quantified ROS production in the PFC and HC in control and PE/PSS rats and PE/PSS rats treated with either vehicle, DMT, HL, or pharmahuasca. (Figure 4.6.a, b.) Differences in ROS production in the PFC were calculated by the nonparametric Kruskal-Wallis test (24.56, n=5-7/group, p<.0001). Multiple comparisons revealed significant differences between control and PE/PSS in the PFC (n=7, 0.003040 ± .00018 µM/mg protein/ min, mean rank = 4.714, n=5, 0.012580 ± .000981 µM/mg protein/ min, mean rank = 28.00, respectively, mean rank diff = -23.29, p<.0001, q<.0001), and between PE/PSS and PE/PSS + DMT (n=7, 0.005376 ± .000558 µM/mg protein/ min, mean rank = 17, mean rank diff = 11, p=.0520, q=.0364), and PE/PSS and PE/PSS + pharmahuasca (DMT + HL) (n=7, .004264 ± .000359 µM/mg protein/ min, mean rank = 12.86, mean rank diff = 15.14, p=.0075, q=.0079), but not between PE/PSS and PE/PSS + HL (n=7, .018306 ± .005556 µM/mg protein/ min, mean rank = 25.57, mean rank diff = 2.429, p= .6680, q = .3507) (Figure. 6a.) Differences in ROS production in the HC were also observed (13.59, n= 5-7/group, p=.0087).
Figure 4.5. Effects of predator exposure + psychosocial stress (PE/PSS) + phamahuasca vs PE/PSS + Vehicle on the IPA Role of Nitric Oxide and Reactive Oxygen Species in Macrophages signaling pathway. Red/Green indicate up/down regulated genes respectively, Red + Green indicates uncertainty in the direction of abundance and white indicates parts of the pathway that do not significantly differ between groups.
Multiple comparisons revealed significant differences between control and PE/PSS (n=6, 0.005692 ± 0.000284 µM/mg protein/min, n=5, 0.016768 ± 0.003941 µM/mg protein/min respectively, mean rank = 12.33, mean rank 2 = 25.20, mean rank diff = -12.87, p=0.0194, q = 0.0077), between PE/PSS and PE/PSS + DMT (n=7, 0.007091 ± 0.001310 µM/mg protein/min, mean rank = 13.00, mean rank diff = 12.2, p=0.0219, q = 0.0077), PE/PSS + pharmahuasca (n=7, 0.005785 ± 0.000924 µM/mg protein/min, mean rank = 9.714, mean rank diff = 15.49, p=0.0036, q = 0.0038), but not between PE/PSS and PE/PSS + HL (n=6, 0.020614 ± 0.007326 µM/mg protein/min, mean rank = 22.83, mean rank diff = 2.367, p=0.6673, q = 0.1752). (Figure 4. 6.b.) HL actually increased ROS production in 3/7 animals in the PFC and 2/6 animals in the HC. (Figure 4. 6.a, b, Figure C. 16. a-d.), while no increases in ROS production were observed with either of the other treatments.

In vitro analysis

*HL does not exhibit affinity or efficacy at the human 5-HT\textsubscript{2A}R in HEK cells*

Based on the high degree of overlap in the transcriptional effects of DMT and HL, particularly on genes related to neuroplasticity, we next sought to determine if, like DMT, HL exhibits appreciable affinity or efficacy at the 5-HT\textsubscript{2A}R. HL did not exhibit substantibinding (K\textsubscript{i} >10,000 nM) or calcium flux (~7% 5-HT E\textsubscript{MAX}) downstream of 5-HT\textsubscript{2A}R activation. On the other hand, the K\textsubscript{i} of DMT for the human 5-HT\textsubscript{2A}R was 511 nM and the E\textsubscript{MAX} & EC\textsubscript{50} for activating calcium flux were 95% 5-HT and 19 nM, respectively. (Figure 4.7.)
Caption on next page
4.3. Discussion

We report that [1] PE/PSS is associated with 17 concordant overlapping DEGs between the PFC of the PE/SS model and DEGs reported in the dIPFC of human PTSD post mortem tissue samples. [2] DMT rescued 9, HL rescued 12, and pharmahuasca rescued 14 overlapping DEGs. [3] PE/PSS is associated with elevated ROS production and inflammatory gene transcription (Il1r1), confirmation of our previous work, 20 and novel changes to growth factor expression (Igf2, Igfbp2) and GABAergic neurotransmission (Slc6a13, Gabrg1, Sst). [4] Pharmahuasca and DMT reduced ROS production in the PFC and HC and HL exhibited highly variable effects on ROS production. [5] All treatments altered the NRF2 pathway and mitochondrial oxidative phosphorylation pathways. Pharmahuasca reduced transcription of the NADPH oxidase
All treatments reduced \( \text{Il1r1} \) transcription in addition to numerous other inflammatory cytokines. Pharmahuasca downregulated NF-\( \kappa \beta \) (\( Nfk\beta 2 \)), a transcription factor for inflammatory cytokines. Pharmahuasca reduced the GO Innate Immune pathway. [6] All treatments reduced \( l1r1 \) transcription in addition to numerous other inflammatory cytokines. Pharmahuasca downregulated NF-\( \kappa \beta \) (\( Nfk\beta 2 \)), a transcription factor for inflammatory cytokines. Pharmahuasca reduced the GO Innate Immune pathway.

<table>
<thead>
<tr>
<th>Drug</th>
<th>h5-HT(_{2\alpha})</th>
<th>r5-HT(_{2\alpha})</th>
<th>h5-HT(_{1\alpha})</th>
<th>r5-HT(_{1\alpha})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[^{[3]}\text{H}\text{Kaytanserin}]</td>
<td>[^{[3]}\text{H}\text{Kaytanserin}]</td>
<td>[^{[3]}\text{H}\text{8OH-\text{DPAT}}]</td>
<td>[^{[3]}\text{H}\text{8OH-\text{DPAT}}]</td>
</tr>
<tr>
<td>5-HT</td>
<td>187 ± 51</td>
<td>614 ± 43</td>
<td>2.67 ± 0.33</td>
<td>2.2 ± 0.5</td>
</tr>
<tr>
<td>DMT</td>
<td>511 ± 67</td>
<td>660 ± 30</td>
<td>450 ± 150</td>
<td>200 ± 10</td>
</tr>
<tr>
<td>Harmaline</td>
<td>&gt; 10,000</td>
<td>7790 ± 1200</td>
<td>ND</td>
<td>&gt; 10,000</td>
</tr>
</tbody>
</table>

Figure 4.7. a) Binding affinities for 5-HT, DMT, and harmaline at human and rat 5-HT2AR and 5-HT1AR (ND: No data available). b) Competition binding isotherm for 50HT at human 5-HT2AR. c) Competition binding isotherm for DMT at human 5-HT2AR. d) Competition binding isotherm for harmaline at human 5-HT2AR. e-f) Comparison of DMT and harmaline relative to serotonin (5-HT) in the human 5-HT2AR G\( \alpha \)q-dependent calcium flux assay. f) In vitro analysis of binding affinity and G\( \alpha \)q-dependent canonical signaling at the human 5-HT2AR for 5-HT, DMT, and harmaline.
Pathway and the IPA Neuroinflammation Pathway, confirming that pharmahuasca reduced the transcription of inflammatory pathways. [7] All treatments downregulated \( lgf2 \) and upregulated \( lgf2r \). Pharmahuasca normalized \( lgfbp2 \). [8] All treatments normalized GAT2 (\( Slc6a13 \)) and modulated numerous other GABA signaling related transcripts. DMT and harmaline rescued somatostatin (\( Sst \)) and all treatments upregulated Sst receptors. [9] All treatments upregulated genes associated with neuroplasticity; DMT and pharmahuasca upregulated the IPA mTOR signaling pathway, and pharmahuasca upregulated the CREB pathway. [10] DMT and 5-HT, but not HL bind to the 5-HT\(_2\)AR and provoke \( Ca^{2+} \) release in HEK cells.

Based on previous work 20 and our initial data (Figure C. 16.a, b.), we hypothesized that PE/PSS animals would exhibit a transcriptional signature of elevated ROS that would be attenuated by treatment, but we did not observe any direct transcriptional links to elevated ROS production between PE/PSS and control. Therefore, the mechanism underpinning the increase in ROS in PE/PSS is likely functional and/or post-transcriptional. Increased ROS production may result from elevated neuroimmune signaling, which is supported by this study (\( Il1r1 \)) and our previous work (NALP3, IL-1\( \beta \), TLR4). 20,52 Elevated ROS in PE/PSS could also emerge from the disinhibition of prefrontal networks caused by altered GABAergic signaling (upregulation of GAT, downregulation of \( Sst \)), increased \( lgf2 \) signaling, reduced \( IGF1R \),52 increased norepinephrine, decreased serotonin21 and/or increased levels of corticosterone observed previously could also contribute.20,21 Collectively, these effects could lead to elevated neural activity and subsequently to elevated mitochondrial activity.
without necessarily leading to increased mitochondria and a measurable transcriptional effect.

Effects on ROS pathways were clearer in treated animals and we have elucidated three mechanisms by which these treatments could modify ROS production. [1] All three treatments altered the IPA NRF2-Mediated Oxidative Stress Response pathway, downregulating Nrf2 and upregulating its modulator and ROS sensor, Keap1. Keap1 senses oxidative stress through cytosine residues. In response to stress, a molecular mechanism within Keap1 allows Nrf2 to escape ubiquitination and translocate to the nucleus where it activates antioxidant responses. (Figure C. 8.) [2] Pharmahuasca had the greatest effect on ROS and the IPA Production of Nitric Oxide and ROS in Macrophages pathway, leading to the downregulation of CYBA (p22phox), CYBB (gp91phox), p47phox, and Rap1, all components of NADPH oxidase 2 (NOX2). (Figure. 5.) NOX2 produces superoxide in microglia and is known to exacerbate traumatic brain injury. ⁶² DMT did not impact either CYBA or CYBB, HL reduced CYBB expression, and all three treatments modulated the expression of Rap1. Importantly, these data suggest a special effect of pharmahuasca. [3] All three treatments reduced the expression of mitochondrial transcripts, as evidenced by DMT and HL downregulating pathways for respiratory chain and NADH dehydrogenase. (Figure C. 6. a, c, 4.7.a, b.) and both HL and pharmahuasca downregulating the IPA Oxidative Phosphorylation Pathway (Figure 4.4.), albeit somewhat differently. HL downregulated components of complex I, the greatest source of mitochondrial ROS, and complexes III, IV and V, but also upregulated genes in each. Pharmahuasca downregulated many of the genes that HL did, in addition to others, but did not upregulate any mitochondrial
transcripts. (Figure 4.4.) Taken together, these data support a multifaceted interpretation of the effects of DMT, HL, and pharmahuasca on brain ROS production with all three treatments impacting the three pathways differently.

It is well understood that there exists a reciprocal relationship between ROS and inflammation such that ROS can lead to OXS, leading to cell damage, activation of DAMPS, and subsequent inflammation. Inflammation can then lead to the production of additional ROS, (superoxide excreted from macrophages and microglia) through NADPH oxidase 2.63,64

PE/PSS was associated with increased expression of \textit{Il1r1}, the receptor for IL-1\(\beta\), a pro-inflammatory cytokine that we previously established is increased at the protein level in the PFC in this model. Both genes were likewise upregulated at the transcriptional level in human PTSD.59 \textit{Il1r1} was significantly reduced by all three treatments, supporting the anti-inflammatory effect of these compounds. Treatments reduced expression of \textit{Il1\alpha}, \textit{Tlr4}, \textit{Tlr6}, \textit{Tlr7}, \textit{Ifngr1}, and a host of different cytokines to different extents, depending on the treatment. (Figure 4.2.f.) However, only pharmahuasca significantly reduced the expression of NF-\(\kappa\)\(\beta\)2 (\textit{Nfkb2}), a functional analogue of NF-\(\kappa\)\(\beta\), which was reduced by 5-methoxy-DMT in human brain organoids.65

In accord with this, only pharmahuasca downregulated the GO Biological Process Innate Immunity Pathway (Figure C. 6.f.) and the IPA Neuroinflammation Pathway. (Figure C.11.) Together, these results suggest that DMT and HL have modest anti-inflammatory effects in the brain at the dosing regimen implemented here, but stronger effects were observed in combination as pharmahuasca. This may be a result of the
short half-life of DMT in the rat brain (6.5 ± .7 minutes at 3.2 mg/kg – 12.5 ± .9 minutes at 10 mg/kg), that is extended in pharmahuasca through MAO inhibition by HL.

It is noteworthy however that in a rat model of allergic asthma, DMT and 5-methoxy-DMT did not exhibit anti-inflammatory or protective effects, while other psychedelic 5-HT₂A agonist, including tryptamine derivatives, like psilocin (4-HO-DMT), did. Yet, both DMT compounds were given intranasally at low doses (0.5 mg/kg), without an MAOI. All other existing studies in the DMT literature used, at minimum, 1 mg/kg IP as a “microdose” and up to 10 mg/kg as a “high dose.” The asthma study (.5 mg/kg IN), this work (2 mg/kg IP), and recent work studying repeated so-called “microdoses” of DMT (1 mg/kg IP), all gave multiple doses over time. The primary metabolic enzyme for DMT and 5-MEO-DMT, MAO, is present in the nasal mucosa and in conjunction with the low dose, may have prevented sufficient DMT from reaching the lung. Furthermore, the anti-inflammatory and tissue protective activity of DMT may be mediated by the Sigma-1 receptor, which exhibits lower expression in alveolar cells compared to neurons. While interesting, the asthma study does not represent conclusive evidence against the anti-inflammatory activity of DMT in all tissues, and at all doses, dosing regimens, and experimental conditions.

We observed increased expression of Igf2 and Igfbp2 mRNA in the PFC of PE/PSS animals compared to control and in human PTSD. Igf2 is a paternally linked gene that may play a role in the intergenerational effects of trauma and the effects of maternal stress on offspring. IGF2 may mediate memory deficits and HPA axis alterations across numerous psychiatric diseases including PTSD.
The literature on IGF2 in the brain is focused on the HC, where acute stress sharply increases IGF2 expression and mediates fear memory acquisition, but chronic stress reduces it and it mediates depressive behaviors. It is unknown how chronic stress impacts IGF2 expression in the PFC. IGF2 is produced by the leptomeninges, endothelial cells, choroid plexus, stem cells, and progenitor cells of the hippocampal subgranular zone (SGZ), and bathes the brain through the ventricular system.

Typically, astrocytes, microglia, and neurons do not express IGF2 mRNA, but it can be

Figure 4.8. Effects of PE/PSS + DMT vs PE/PSS + Vehicle on the IPA mTOR signaling pathway. Red/Green indicate up/down regulated genes respectively, Red and Green together indicate divergent expression of subunits, and white indicates parts of the pathway that do not significantly differ between groups.
induced in human astrocytes and microglia by IL-1β and LPS, respectively. Additionally, it is induced by oxidized low density lipoprotein (OX-LDL) in macrophages. This PE/PSS model is characterized by chronically elevated IL-1β and ROS, which can lead to oxidized lipid species. IL-1β and ROS are therefore excellent candidates for the induction of IGF2 expression. DMT, HL, and pharmahuasca reduced Igf2 transcription and increased transcription of the Igf2r, Insr, and Igf1r. Pharmahuasca had the greatest effects on ROS production, innate immune pathways, and Igf2, and is the only treatment to normalize Igfbp2 expression. (Figure 4.6.c, d, g, h.) Taken together, it is plausible that the elevated Igf2 expression in this model is linked to higher baseline ROS and IL-1 signaling and that these treatments attenuate ectopic and/or elevated Igf2 expression through the modulation of these factors.

Psychedelics, including DMT, induce 5-HT2A-R-dependent structural neuroplasticity, comparable to the recently FDA-approved antidepressant NMDAR antagonist, ketamine. In vitro experiments with lysergic acid diethylamide (LSD) and ketamine suggest that there are two requisite phases for the induction of neuroplasticity, an initial “stimulation” phase and a secondary “growth” phase. Existing evidence suggests that serotonergic psychedelics activate the 5-HT2A-R through unique functional selectivity, which leads to the selective recruitment of GPCR effectors. (Figure C. 9, 10.) and β-arrestin 1,2, depending on the compound. This leads to the stimulation phase through increased AMPAR (Gria1) activation, likely through elevated calcium signaling downstream of Gαq, triggering brain-derived neurotropic factor (BDNF) release and the initial induction of a plastic state through TrkB receptors. Subsequently, the growth phase is initiated through activation of mTOR complex 1
and/or 2 (mTORC1, mTORC2), which we observed to be transcriptionally upregulated by DMT and pharmahuasca (Figure 4.8.), leading to CREB pathway activation, which we also observed to be upregulated by Pharmahuasca. CREB activation leads to the transcription of genes required for synaptogenesis, including ephrin and its receptors EphB and EphA. (Figure C. 13.) Collectively, these factors underpin long term potentiation (LTP), a critical mechanism for cognitive flexibility and learning also observed to be transcriptionally upregulated by pharmahuasca in this study. (Figure C. 14.) We observed similar molecular patterns at the transcriptional level after all three treatments in the current study, but particularly with pharmahuasca, which induced the expression of pre and post synaptic genes involved in synaptogenesis, mTOR1/2, and CREB signaling IPA pathways. These data therefore support the work of David Olson's lab, among others who have exhibited the plasticity inducing effects of serotonergic psychedelics and ketamine in the cortex.

Finally, we hypothesized that the overlapping effects of both compounds are mediated through agonist activity at the 5-HT2AR. To test this, we used a surrogate cellular model to determine the binding affinity and Ca2+ signaling pathway activation at the human 5-HT2AR for DMT and HL. We confirmed the null hypothesis that HL does not exhibit substantial binding affinity (Figure 4. 7. a-d.) or functional activity at the human 5-HT2AR. (Figure 4. 7. e, f.) These data suggest that the observed overlap in the transcriptional effects is convergent through 5-HT2AR agonism and MAOI activity, rather than direct activity at the 5-HT2AR by HL. However, HL likely increased the concentration of 5-HT, which may have acted through the 5-HT2AR or other 5-HTRs to induce the overlapping effects. In fact, both HL and DMT upregulated the IPA Gaq
pathway (Figure C. 9, C. 10.), but HL altered the expression of more genes overall than DMT. HL may therefore exert effects on the terminal domains of broad neuromodulator systems (MAOI), compared to the selective effects of 5-HT2AR/5-HT1AR agonism. (Figure 4. 7.) These data are not conclusive however; cell types and biomolecular context (i.e., effector expression, membrane lipid composition, receptor ensemble energy landscape) in which the competition binding and signaling assays were performed may impact these values. It is possible that DMT and HL bind with altered affinity and/or produce distinct functional changes in a state of chronic inflammation, oxidative stress, or at a different local pH compared to a “normal” individual.

Taken together, this work highlights the capacity of 5-HT2AR agonist psychedelics, like DMT and pharmahuasca to reduce allostatic load and facilitate recovery from traumatic stress through four mechanisms: [1] normalizing ROS production, [2] normalizing inflammatory markers, [3] modulating growth factors, neurotransmitter signaling, and gene expression, and subsequently [4] modulating neuroplasticity. Each of these effects could be driven by 5-HT2AR agonism, but could also be enhanced by concurrent sigma-1 receptor agonism by DMT. (Figure C. 20.)

The purpose of this study was to examine biological endpoints related to PE/PSS. Therefore, a major limitation of this work is that it was beyond the scope to investigate the PTSD-like behaviors previously measured in our lab and others; however some have already been examined for DMT in rodents and Ayahuasca in humans. To address this shortcoming, we chose to compare our DEGs to those observed in human PTSD to identify overlapping DEGs, as these molecular
alterations are likely to have direct relevance to PTSD. The human study used combined male and female data and these data were adjusted for the effect of sex. Since the human data were already adjusted, we used their combined dataset of males and females for our comparison to male rats. However, this decision carries a limitation in the ability to determine sex specific differences in gene expression in PTSD and their overlap with the PE/PSS model. To address this, future experiments should be carried out using different groups of male and female rats and specific comparisons to transcriptomic data from men and women with PTSD. It was also beyond our scope to investigate specific GABAergic, serotonergic, glutamatergic or immune related alterations in protein expression. This is a major limitation of this study and the RNAseq results should be considered with caution in all cases in which we do not indicate that protein expression or other functional validations have taken place in this study or by other labs. Furthermore, cell type specific changes in brain mRNA expression were also beyond our scope. The former should be addressed by LC-MS based proteomics paired with immunohistochemistry for specific targets and the latter can theoretically be addressed in our data set using computational methods, given access to single cell sequencing data from the PFC, or by single cell RNA sequencing. Disentangling the exact mechanisms of action of these compounds will require the replication of this work utilizing 5-HT$_{2A}$R, 5-HT$_{1A}$R, and sigma-1R selective antagonists. A final limitation of this study is that it was beyond our scope to replicate these experiments in unstressed animals. Future work should include both stressed and stress naïve animals to determine if the effects of these treatments differ between PE/PSS and naïve animals.
4.4. Materials and Methods:

*Ethics statement:* This study was performed in strict accordance with the recommendations of the Institute for Laboratory Animal Research’s 2011 Guide for the Care and Use of Laboratory Animals, under the auspices of an animal care and use protocol approved by the Louisiana State University Institutional Animal Care and Use Committee (15-061). All animal experiments were performed according to ARRIVE guidelines.

*Animals*

Naïve, adult male Sprague Dawley rats were used in this experiment. Rats were bred in the LSU School of Veterinary Medicine vivarium and were between 10 and 12 weeks of age when the study was initiated. Rats were pair-housed in standard plastic microisolator cages and had access to food and water ad libitum. The cages were maintained in ventilated racks (8X5) and each cage was assigned a rack location at random to ensure even distribution of rack location. The vivarium room was kept on a 12 hour light/dark cycle (0700-1900); room temperature was maintained at 20 ± 1 °C and humidity ranged from 23% to 42%. Predator exposures were performed using two cats, one male and one female (age 9, Harlan Laboratories, Indianapolis, IN, and age 12, Tulane University, New Orleans, LA, respectively). Cats were housed in an open room maintained at the same light/dark cycle, temperature, and humidity as the rat room. This experiment was initially performed and subsequently replicated in two independent cohorts of animals. Six groups of rats were used in this experiment across two independent cohorts: Control (n=6), PE/PSS (n=5), PE/PSS + DMT 2 mg/kg (n=7), PE/PSS + DMT 4 mg/kg (n=3), PE/PSS + Harmaline (n=6), PE/PSS + Harmaline +
DMT (n=7). The PE/PSS + DMT 4 mg/kg group was less effective in normalizing ROS and was therefore not replicated and was not considered in the final analysis, but ROS data for that group is available in the supplemental material. (Figure C. 16.a, b.) Controls were not exposed to any form of stress and were left double housed in the vivarium until the end of the experiments when they were sacrificed on the same day as experimental animals for cohort 1 and the day after experimental animals in cohort 2 (day 39). This difference was due to time restraints associated with EPR measures and the larger N. No significant between cohort differences emerged in the EPR measures between cohort 1 and 2, verifying that this small difference in relative sac day did not impact the results. (Figure C. 16.a-d.)

**Stress induction**

Animals were pseudo-randomly assigned into either “control,” “PE/PSS,” “PE/PSS + DMT,” “PE/PSS + HL,” and “pharmahuasca” (PE/PSS + HL + DMT) such that animal date of birth was balanced between groups (n=5-7/ group). The following day, all PE/PSS rats began the predator exposure and psychosocial stress regimen, first published and validated by Zoladz, et al., designed to produce a pre-clinical PTSD-like phenotype that closely mimics signs and symptoms of human PTSD patients.54-58 PE/PSS induction were performed as previously described.20

**Drug treatment**

31 days post predator exposure and psychosocial stress procedure, animals were treated with DMT (2 mg/kg body weight IP), HL (1.5 mg/kg body weight IP), or DMT + HL (2 mg/kg and 1.5 mg/kg IP, respectively) every other day for a total of 3 doses. Drug dosages were selected based on existing literature and a preliminary study testing two
doses of DMT, 2 mg/kg and 4 mg/kg IP (Figure C. 1.a,b.) 2 mg/kg DMT was the optimal
dose to normalize free radical production in PE/PSS animals. The dose of HL was
chosen with the primary objective of ensuring sufficient MAO-inhibition. DMT (Cat.#
SML0791, Lot# 083M4613V, ≥97% HPLC, Origin: India) and HL (Cat.# 51330; Lot#
BCBK5380V, ≥95%) were purchased from Sigma Aldrich for the preliminary cohort. For
the second cohort, DMT was purchased from Cayman Chemical (Cat.# 13959; Lot#
04647877, Origin: MI, USA). DMT was solubilized in 100% ethyl alcohol using a table-
top vortex and diluted with sterile saline such that final ETOH concentrations were
below 8%, the acceptable level for use as a vehicle. After stress induction, PE/PSS,
DMT, HL, and pharmahuasca (DMT + HL) animals were interperitoneally injected first
with HL (1.5 mg/kg IP) or vehicle and immediately afterwards with DMT (2 mg/kg IP) or
vehicle, as applicable. PE/PSS, DMT, and HL animals were injected with vehicle on the
same schedule as animals receiving two drug injections. Experimenters were not
blinded to the drugs administered or group assignments during drug treatment.
Immediately after treatment, animals returned to their home cages, observed for 30 min,
and returned to the vivarium. Initially, we planned to compare these data with orally
gavaged Ayahuasca from our collaborator, Dr. Jordi Riba, to study the differences
caused by route of administration and additional components of the traditional
Ayahuasca brew. While no animal exhibited any concerning physiological side effects,
they exhibited behavioral signs of distress and were extremely resistant to taking a
second dose of Ayahuasca. These animals chewed through the plastic gavage tube, a
behavior we have never before witnessed. Based on these effects, we determined that
it was not ethical to continue studying the oral administration of whole plant-derived Ayahuasca in rats.

*Electron Paramagnetic Resonance Spectroscopy:*

ROS were measured in fresh brain tissue (HC and PFC) as previously published by our lab.\textsuperscript{20,106} At the time of sacrifice, an aliquot of chopped and hand homogenized pieces of tissue were immediately plunged into ice cold buffer containing 25 uM of DF, 2.5 uM of DETC, and the spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH) (200 µM), which was contained within a 24 well plate submerged in ice. CMH broadly binds to reactive oxygen species (ROS), which are produced in relative proportion to *in vivo* production at physiological pH and temperature. Therefore, we incubated tissue in this CMH containing buffer at 37 °C for 30 minutes immediately prior to EPR measurement to produce a controlled release of ROS. The 24 well plate containing tissue + buffer were subsequently plunged directly back into ice to stop the reaction and iquot of incubated probe media were then taken in 50 ul disposable glass capillary tubes (Noxygen Science Transfer and Diagnostics) for determination of ROS production. EPR measurements were performed using an EMX ESR eScan BenchTop spectrometer and super high quality factor microwave cavity (Bruker Company, Germany) under the following settings: center field g = 2.002, field sweep 9.000 G, microwave power 20 mW, modulation amplitude 1.90 G, conversion time 10.24 ms, time constant 81.92 ms, receiver gain 3.17 x 10\textsuperscript{3}. Time Scan mode was used for the detection of ROS with the averaging of EPR amplitude every 10 scans over 10 min. These experiments were performed at at 37°C under 20 mm Hg of oxygen partial pressure using the Gas-Controller NOX-E.4-GC (Noxygen Science Transfer and
Diagnostics GmbH). EPR measurements were normalized by total sample protein using a Pierce BCA Protein Assay Kit (catalog number: 23225). Experimenters performed EPR and protein estimation using sample numbers and brain region information (eg. PFC-12, HC-12, etc) only and were therefore blinded to sample group assignments. Group assignments were unblinded upon statistical analysis.

As this experiment was performed in two separate cohorts with each group equally represented within each cohort, as expected, small but significant differences in baseline levels of ROS production in control groups were observed between the two cohorts; therefore, data were normalized between cohorts to control animals such that both groups could be considered together for overall statistical analysis. The overall pattern of ROS induction in PE/PSS and attenuation by drug treatments were comparable between groups in all cases with the exception of several animals exhibiting increased ROS production in the HL group. (Figure C. 1.)

RNA sequencing:
Whole PFC tissue was placed in Trizol and stored at -80 °C until RNA extraction. Total RNA was extracted using a Qiagen Lipid Tissue Mini Kit Cat. No. 74804, and two aliquots were prepared, one for RNA sequencing, and one for real time RT-PCR confirmation of those data. RNA quality and quantity were measured by Nanodrop Spectrophotometer ND-1000 UV/Vis and by nucleotide fragment analysis on an Advanced Analytical Fragment Analyzer. RNA quality number in all samples was ≥ 6.8. 3-4 samples per group were chosen based on optimal RNA quality and shipped to Novogene corporation at UC Davis for RNA sequencing on the Illumina platform. cDNA library construction was performed with 1 ug of RNA using NEBNext® Ultra 2 RNA
Library Prep Kit for Illumina® (cat NEB #E7775, New England Biolabs, Ipswich, MA, USA) according to the manufacturer’s protocol. Novogene enriched the mRNA using oligo (DT) beads and followed this with two rounds of purification and fragmented randomly by adding fragmentation buffer. The first strand of cDNA was synthesized using random hexamers primer. Next, dNTPs, RNase H, DNA polymerase 1, and a custom second strand synthesis buffer (Illumina) were added to produce the second strand (ds-cDNA). Subsequently, terminal repair, poly-adenylation and sequencing adaptor ligation were performed, followed by size selection and PCR enrichment. This resulted in 250-350 bp insert libraries, which were quantified using a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Walkham, MA, USA) and quantitative PCR. An Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) was used to determine size distribution. Sequencing was performed on an Illumina NovaSeq 6000 Platform (Illumina, San Diego, CA, USA) using a paired-end 150 run (2x150 bases) Q30≥80% at 20M raw reads/sample. Alignment was performed using hisat2, v2.1.

Real-Time PCR analysis:
Semi-quantitative real-time RT-PCR (n=4/group) was used to confirm the mRNA levels of a small subset of significantly differentially expressed genes elucidated by RNA sequencing in the PFC (Igf2, Igfbp2, Slc6a13, Clip3). Very little PFC tissue sample remained after aliquoting tissue samples for EPR and RNAseq, but we chose to confirm this small set of genes based on their contribution to the PE/PSS phenotype. All 4 genes were run on at least 3 replicates across 2 PCR runs, totaling 6 runs/ gene/ sample. Total RNA extraction was performed as described above. cDNA synthesis was performed using a Bio-Rad iScript cDNA synthesis kit (Catalog # 1708891) as
previously described. Gene expression was calculated by ΔΔCT and was normalized to GAPDH mRNA levels. These data are presented as fold change of the gene of interest relative to control animals. (Figure 4.6.c–j.) Table of primers (Figure C. 17.) 2/4 measured genes showed the similar differential expression between PCR and RNAseq. (Figure 4.6.c–j.) 2/4 measured genes showed higher variability in PCR than RNAseq as expected from the relative (PCR) vs absolute (RNAseq) quantification of transcripts by each technique (Figure 4.6.c–j.), but showed the same pattern of expression between groups, overall confirming the RNAseq data.

**Cell Culture Methods:**

Human embryonic kidney (HEK) 293 cells stably expressing the human 5-HT2A receptor (HEK-h2A; $B_{\text{max}} = 1600$ fmol/mg protein) were a kind gift from Dr. David Nichols (University of North Carolina, Chapel Hill). These cells were routinely cultured and maintained in DMEM (Gibco, Cat.# 11054-020) supplemented with 10% (v/v) fetal bovine serum (Gibco, Cat.# 16000-044; Lot# 2216190RP), 2 mM GlutaMAX™, 100 units/mL penicillin, 100 µg/mL streptomycin, and 100 µg/mL Zeocin™ within an incubator controlled at 37 °C, 95% humidity and 5% CO2.

**Calcium Flux Assay:**

$G_{\alpha q}$-mediated calcium flux downstream of 5-HT2A receptor activation was determined using the HEK-h2A cell line. Cells were seeded in DMEM (Gibco, Cat.# 11054-020) supplemented with 10% (v/v) fetal bovine serum (Gibco, Cat.# 16000-044; Lot# 2216190RP), 2 mM GlutaMAX™, 100 units/mL penicillin, 100 µg/mL streptomycin, and 100 µg/mL Zeocin™ onto 96-well Poly-D-Lysine plates with clear bottoms (12,000 cells/well) and cultured at 37 °C, 95% humidity and 5% CO2. 48 hours following plating,
media was aspirated and replaced with DMEM supplemented with 1% dialyzed fetal bovine serum, 2mM GlutaMAX™, and 100 µg/mL Zeocin for an additional 12 hour incubation at 37 °C, 95% humidity and 5% CO2. On the day of the experiment, cells were washed once with HBSS supplemented with 20 mM HEPES, loaded with 75 µL of 3 µM Fluo-2 AM HA (Ion Indicators, LLC) diluted in HBSS–HEPES buffer, incubated for 1 hour at 37 °C, 95% humidity and 5% CO2, washed again with HBSS–HEPES, and maintained in 50 µL HBSS–HEPES at 25 °C. The plates of dye-loaded cells were placed into a FlexStation 3 microplate reader (Molecular Devices, LLC) to monitor fluorescence (excitation, 485 nm; emission, 525 nm; cutoff, 515 nm). Plates were read for 20 s (bottom read, 2 s interval, 12 reads/well, PMT: medium) to establish baseline fluorescence and then challenged with DMT, HL, or 5-HT diluted in a range of 10 pM to 100 µM or buffer and read for an additional 80 s. Each concentration point was tested in triplicate. After obtaining a calcium mobilization trace for each sample, the peak fluorescence in each well minus mean baseline fluorescence (ΔF) was divided by mean baseline fluorescence (F) to give ΔF/F. Data were normalized to the maximum peak fold-over-basal fluorescence produced by 5-HT (100%) and baseline fluorescence (0%) and analyzed using nonlinear regression curve-fitting routines in GraphPad Prism 9.0 (GraphPad Software, Inc.). HL freebase was dissolved in DMSO and the final concentration of DMSO in assay plate was ≤ 1%.

*Competition Binding Assay:*

HEK-h2A cells were subcultured and grown in DMEM (Gibco, Cat.# 11054-020) supplemented with 10% (v/v) fetal bovine serum (Gibco, Cat.# 16000-044; Lot# 2216190RP), 2 mM GlutaMAX™, 100 units/mL penicillin, 100 µg/mL streptomycin, and
100 µg/mL Zeocin™ until 90% confluent. The cells were then serum-starved in DMEM (Gibco, Cat.# 11054-020) supplemented with 2 mM GlutaMAX™, 100 units/mL penicillin, 100 µg/mL streptomycin, and 100 µg/mL Zeocin™ for 12 hours. Membranes were prepared by scraping and homogenizing on ice and centrifuged at 2,000 x g for 10 minutes at 4 °C. Membranes were resuspended in cold binding buffer (50 mM Tris-HCl, 10 mM MgCl₂, 0.1 mM EDTA, pH 7.4), homogenized, and centrifuged again. After resuspension in cold binding buffer, 1 mL aliquots were distributed to pre-chilled 1.5 mL microcentrifuge tubes and centrifuged at 13,000 rpm for 20 minutes at 4 °C. Supernatant was aspirated immediately and pellets stored at -80°C until needed. On the following day, one pellet was resuspended in 1 mL cold binding buffer and protein concentration quantified using a Quick Start™ Bradford Protein Assay Kit 2 (Bio-Rad, Cat.# 500-0202). On day of assay, the following components were diluted in cold binding buffer in a 96 well 2 mL deep well polypropylene plate and incubated at room temperature on a platform rocker for 1 hour: 25 µg membrane preparation, 1 nM [³H]ketanserin hydrochloride (PerkinElmer, Part# NET791025UC), and test compound diluted in binding buffer (DMT: 20 nM to 30 µM; HL: 200 nM to 300 µM; 5-HT: 100 pM to 10 µM). Mianserin (100 µM) was used to define nonspecific binding. Each concentration point was tested in triplicate. Samples were filtered onto a 0.25% polyethylenimine-coated UniFilter-96 GF/C microplate (PerkinElmer, Part# 6055690) using a PerkinElmer FilterMate™ cell harvester and counted in MicroScint™-20 cocktail (PerkinElmer, Part# 6013621) on a PerkinElmer Microbeta² System at 57% efficiency. Ki values were generated using the GraphPad Prism 9.0 “one site – fit Ki” function (GraphPad...
Software, Inc.). HL freebase was dissolved in DMSO and the final concentration of DMSO in assay plate was ≤ 3%.

Statistical analysis:

Data are presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism 9.0. Between group differences in variance were calculated using the nonparametric Kruskal-Wallis test for EPR. Multiple comparisons were performed using the two stage linear set-up procedure of Benjamini, Krieger, and Yekutieli. A nonparametric test was chosen for simplicity due to the nature of the data and because the assumption of homoscedasticity for ANOVA was not met. A one way ANOVA + Tukey’s post hoc test was used for the PCR validation of the RNAseq data. P values less than 0.05 were considered statistically significant. To test whether the differentially expressed genes in the PE/PSS vs control are significantly enriched in the human PFC DEGs (Girgenti et al) as opposed to the rat non DEGs (background=12,964) (Figure C. 18.), we performed a one-sided Fisher’s exact test where the null hypothesis is that there is no significant association between the rat and the human DEGs. Overlaps were obtained between protein coding orthologous genes. Spearman’s R was calculated to test the correlation between the log2 fold changes of the overlapping genes between the human and rat studies. We used Spearman’s R because the data were not normally distributed.

RNA-seq analysis:

HTSeq gene counts obtained from Novogene were used to perform tests in DESeq2 v1.29.6. Prior to running tests for differential gene expression, we corrected unwanted variation (noise stemming from technical and biological factors unmodelled in
the experiment) using RUVseq \textsuperscript{110} to increase power and biological insight specific to the experimental design. \textsuperscript{111} Briefly, after normalizing the counts (upper quantile) using the RUVr function we estimated the variables that capture the unwanted variation by factor analysis on deviance residuals after a running a regression of the counts on the covariates of interest. (Figure C. 19.) Those variables were included in the design formula in DESeq2. For the tests run in DESeq2 we used un-normalized HTSeq counts after filtering genes with less than 10 reads in at least 3 samples. Statistical significance was set at FDR<0.1 (Benjamini-Hochberg (BH) adjusted p value). \textsuperscript{112} This FDR was chosen based on a previous work in which LC-MS based proteomics and IPA analysis were used to analyze the effects of 5-methoxy-DMT on human cerebral organoids. \textsuperscript{65}

For clustering analysis (PCA and unsupervised hierarchical clustering heatmaps), we used the variance stabilized RUVseq corrected counts. Variance stabilization was performed with the functions in the vsn package. \textsuperscript{113,114} Hierarchical clustering was performed and plotted using the functions in the ComplexHeatmap package \textsuperscript{115} Briefly, we calculated the Euclidean distances and used Ward’s linkage to obtain the clusters. Volcano plots were plotted with Enhanced Volcano in R. \textsuperscript{116} Gene Set Enrichment Analysis (GSEA) was performed for the genes that passed the FDR .1 threshold in pairwise comparisons with gene ranks calculated from shrunken log fold2 changes \textsuperscript{117} corrected by the corresponding FDR, in WebGestalt \textsuperscript{118} using the Gene Ontology (GO) and KEGG pathway functional databases. Ingenuity Pathway Analysis (IPA) pathway analysis used the same data sets, but instead plugged in gene ensemble IDs, FDR, and fold change. Rnorvegicus genes were mapped to human orthologues for pathway analysis in IPA.
4.5. Notes


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Chapter 5: Summary and Conclusions

5.1. Summary and Conclusions

The research presented in the previous three chapters supports the idea that aberrantly expressed allostatic load (AL) and metabolic factors in the dHPC 1) are associated with avoidance of stress-paired stimuli and 2) are caused by trauma + chronic stress. This exhibits both an association of AL factors with the development of a trauma associated phenotype and a causal relationship between trauma and these factors. The major limitation of this work is the lack of behavioral testing in Experiments 2 and 3, but other studies from the Francis and Gilpin labs, and from other groups have previously provided this connection, as outlined in the introduction.

We observed that ROS production was lower in Av and correlated strongly with dHPC pattern separation. Av also exhibited lower maximal and spare respiratory capacity and lower insulin signaling transcripts that inversely correlated with avoidance behavior and exhibited elevated mitochondrial and metabolic transcripts that were correlated to avoidance. Rats that exhibited the worst HPC pattern separation tended to exhibit the highest levels of ROS (3/4 rats). Previous data from the Gilpin lab exhibits lower stress induced CORT in Av. In experiment 2, we observed increased ROS production, CHOL and TG and lower overall lipid species not including those targets, indicating that HPC AL factors often follow similar trajectories as they do in the periphery. PCR data confirmed some of these changes. However, we observed surprisingly lower MDA in PE/PSS animals, indicating lower levels of oxidized polyunsaturated fats, a marker of oxidative stress. Lower MDA indicates adaptation to stress-induced elevated ROS and its sequela and this is bolstered by the observed
increases in DHA in the HPC. Finally, we show that DMT and pharmahuasca normalize ROS production and stress induced transcriptional changes in the PFC that overlap with human PTSD, exhibiting the capacity of 5HT2A agonists to reset stress-induced aberrations in metabolic and allostatic load related factors.

The purpose of the stress response is to: 1) upregulate energetic substrate availability to the brain during stress and 2) provide long term protective effects to help resolve the stress response and prepare the organism for future stressors (adaptation). According to Martin Picard and Bruce McEwen, the mitochondria are the central orchestrator of these events across the entire organism. The stress response at the CNS level (allostasis) requires memory and the HPC is required for explicit memory formation. The HPC is highly susceptible to the deleterious effects of stress and becomes dysfunctional after extreme or chronic stress and this effect is mediated by allostatic load factors, many of which are mitochondrially regulated. PTSD is associated with allostatic load and HPC memory processing deficits, which may directly prevent recovery from the disorder. The study of the collective contribution of these factors to PTSD and other psychiatric disorders is still in its infancy, but the work herein offers a significant contribution to our understanding of this field.

Avoiders exhibit a distinct functional and metabolic hippocampal phenotype after predator odor stress: connections and implications

Based on the available information, the most elegant explanation of these data is that previously measured lower stress-induced CORT and increased vHPC Src-1 in Av represent chronic differences in HPA axis and glucocorticoid signaling in these animals. This is speculative however because CORT measures were acute and Src-1 was measured only 48 hours post exposure, while the measures in this study were all
performed at 16 days post stress. Reduced GCC signaling, as observed in human PTSD, may lead to reduced insulin and IGF1/2R signaling, reduced energetic substrate transporter expression and activity and subsequently reduced energetic substrate availability to the mitochondria, which leads to reduced maximal and spare respiratory capacity. Reduced spare capacity may be related to altered HPC function through altered HPC mean firing rates. Increases in mitochondrial, ribosomal, and metabolic gene expression may be compensatory. CORT also reduces antioxidant gene expression and therefore the observed increase in antioxidant expression may derive from reduced CORT expression in Av. Lower ROS production could derive from either increased antioxidant gene expression and/or lower spare capacity or other GCC associated factors.

We observed bidirectional changes between Av and NAv in numerous measures. Avoiders only differed from unstressed controls in seahorse extracellular flux analysis (SEFA) measures of maximal and spare respiratory capacity with NAv being highly variable and exhibiting a mean between control and Av. NAv never differed from control in any measure. In all cases, when changes are described as bi-directional, I refer to divergent expression about the unstressed control group in which the Av and NAv significantly differed. Av and NAv exhibited bidirectional changes relative to unstressed controls in 1) object pattern separation (OPS) effect size (Figure 2.2.), 2) electron paramagnetic resonance spectroscopy (EPR) measure of ROS production (Figure 2.2., Figure C. 3, 3.) DEG analysis of RNA Sequencing data displayed on heatplot (Figure 2.4), 4) WGCNA analysis of RNA Sequencing data displayed as box and whisker plots for the indianred4 module and the magenta module. (Figure 2.5.), and 5.) Antioxidant
gene expression. (Figure C. 3.a, b, c.) These bidirectional changes exhibit the clear phenotypic differences in metabolic factors between Av and NAv and the SEFA measures further demonstrate mitochondrial differences in Av. The main purpose of the stress response is adaptation to metabolic demands and the prevention and repair of metabolically induced damage associated with stress. Based on this idea and the clear exhibition of bidirectional changes relative to control between Av and NAv across behavioral, functional, and molecular levels of biological organization, I propose that Av and NAv as groups exhibit at least two distinct adaptive responses to predator urine stress. The Av phenotype appears very specific, while the NAv phenotype is more diverse and may contain two subgroups. For this reason, I focused on Av in my summary schematic in Chapter 2. (Figure 2.6.) The existence of two distinct stress response phenotypes may represent the beginning of separation into the different allostatic load phenotypes mentioned in Chapter 1. If true, this could have massive impact on our understanding of stress physiology and the basis of chronic disease.

Confirming the roles of these interdependent factors in HPC function and stress-induced deficits in pattern separation, which is dysregulated in numerous psychiatric diseases, would allow for the identification of optimal targets for intervention. Further, such a study could confirm the differences in PS performance between Av and NAv and its relationship to ROS, and confirm the existence of two NAv subgroups (NAVPS+, NAVPS-). One could then analyze the differences in each parameter between NAVPS+ and NAVPS- and determine the role of spare capacity and insulin signaling in this distinction. If NAVPS- is confirmed as a distinct phenotype, it would represent a third phenotype that emerges in response to predator odor stress. In light of the potentially adaptive changes
observed in Av such as reduced ROS production and the preservation of moderate
OPS performance after stress, the NAvPS- group may in fact be the group that
experiences the most deleterious effects of stress on the HPC. Whitaker 2015 exhibited
a substantial range of CORT expression in NAv, from ~400 – ~800 ng/ml, while Av
ranged from ~200 – 600 ng/ml.7 It would be interesting to determine where NAvPS- fall
amongst the NAv, and the relationship between CORT both immediately after trauma
and at the time of sacrifice, and ROS production. If NAvPS- do indeed represent a distinct
behavioral phenotype and generally exhibit the highest levels of ROS, as we observed,
if our hypothesis is true, they should also exhibit the highest level of CORT. If so, the
NAvPS- may represent animals whose CORT expression overwhelmed endogenous
buffering systems and subsequently crossed into AOL and toxic stress. It would be
assumed that this toxic stress is responsible for their clear deficits in pattern separation.
If true, this would raise the question as to if they lack CPA because they do not have the
capacity to remember the distinction between the two environments, rather than altered
central amygdala (CeA) activation, as would be expected in NAvPS+.11 Further studies
paring object pattern separation and measures of CeA activation in these animals would
be required to determine if this is the case. This information would provide a substantial
update to our understanding of this stress model and adaptive stress response
phenotypes in general.

*PE/PSS is associated with increased factors of allostatic load in the hippocampus and exhibits reduced MDA despite increased ROS, indicating an adaptive response to stress: connections and implications*

In Experiment 2, we observed that major contributing factors to allostatic load (AL) in
the periphery, elevated ROS production, cholesterol (CHOL), cholesterol esters (ChE),
Triglycerides (TG), and reduced phosphatidylcholine (PC), phosphatidyl serine (PS), and malondialdehyde (MDA) in the HPC of PE/PSS rats. We also observed increased docosahexaenoic acid (DHA) concentrations in PE/PSS, which is a potent antioxidant and neuroprotective polyunsaturated fatty acid that may help explain reduced MDA concentrations in that group despite increased ROS production.

Raman represents a major breakthrough in label free imaging that is particularly useful for in situ imaging of lipid species, but is currently being adapted for protein measures and in vivo real time imaging.12 If that is accomplished, Raman could revolutionize biomedicine by allowing for the label free measurement of diverse biomolecules in real time. These data exhibit a surprising and important characteristic of the stress response, the shifting of homeostatic set points to prevent toxic stress. In this study, we observed a several fold increase in ROS production in PE/PSS animals and many of the sequela of oxidative stress including increased CHOL and TG levels, but we also observed reduced phospholipids, which exhibited a positive correlation to MDA concentrations, and increased DHA, which is protective against oxidative stress. Together, these results exhibit the capacity of biological systems to adapt to even extreme and chronic stress. However, results from David Diamond’s lab do indicate that PE/PSS animals exhibit deficits in HPC-dependent cognitive tasks, like 24 hour novel object recognition (NOR), indicating that while adaptation has occurred, preventing further oxidative stress, the deleterious effects of stress on the HPC have likely already accrued.13-18

An interesting connection between experiment one and experiment two is the possible link between corticosterone and ROS production. The Gilpin model exhibits
attenuated stress-induced CORT in Av compared to NAv and GCC signaling abnormalities in the amygdala, hypothalamus, and HPC. Alternatively, Dr. Joseph Francis’ lab exhibited increased CORT in PE/PSS rats relative to control rats at sacrifice after the entire 30 day stress regiment. The Gilpin model is associated with reduced CORT production after stress in Av and reduced ROS production in that group, and the Francis model exhibits increased CORT at sacrifice (~Day 31-35 depending on treatments and behavioral measures) and increased ROS levels. This connection is particularly interesting in light of the aforementioned known relationship between CORT production and ROS/OXS.

DMT and pharmahuasca reduce ROS production, inflammatory gene expression, exhibits psychoplastic transcriptional effects in PFC of the PE/PSS rat model, and rescue 9 and 14 overlapping differentially expressed genes between the PE/PSS model and human PTSD: connections and implications

The results observed in this experiment provide supporting evidence for the present literature suggesting that DMT and pharmahuasca exhibit neuroprotective and psychoplastogenic effects. We observed that these compounds reduce ROS production, mitochondrial gene expression and ectopic insulin-like growth factor gene expression, while increasing insulin/IGF receptor gene expression and the expression of many downstream effectors including the mTOR and CREB IPA pathways, all of which are major regulators of metabolic function and protein synthesis. Like the other two studies, these effects have substantial implications for the design of therapeutic interventions that normalize brain energy metabolism and allostatic load after stress and trauma. Collectively, these data support other studies that suggest that DMT and pharmahuasca treatment normalize two major factors associated with allostatic load, ROS production and inflammatory gene transcription. These compounds may also
normalize ectopic growth factor expression and upregulate genes and pathways that are permissive for neuroplasticity. Future investigations should extend these observations to the HPC paired with object pattern separation in order to confirm these salutary effects at the behavioral level. In Experiment 2, we observed increased cholesterol, triglycerides, and other alterations to lipid species in the HPC, which also contribute to AL in the periphery. Future investigations should also consider these factors and Seahorse extracellular flux analysis in evaluations of DMT, pharmahuasca, and other 5HT2A agonist psychedelics in animal models of stress.

**5.2. Explanatory Framework**

PTSD is associated with broad metabolic dysregulation, which is evident in its comorbidities. One meta-analysis reported that the prevalence of metabolic syndrome (MetS) in PTSD was 38.7%, nearly double (relative risk, 1.82; 95% confidence interval 1.72-1.92) that of the general population. Other MetS factors were also reported in this study: hyperglycemia, 36.1%; hypertriglyceridemia, 45.9%; lower high-density lipoprotein cholesterol, 46.4%; abdominal obesity, 49.3%; and hypertension, 76.9%.¹⁹ The accumulating evidence suggests that the mitochondria are a possible central mediator of these interrelated effects.²⁰

Allostatic load factors are identical to most factors of MetS, both are highly associated with PTSD, and both sets of factors may be driven by alterations to the mitochondria. I propose here, in line with the work of Picard, and McEwen, that these alterations are driven by differences in HPA axis function, which are also linked to the observed alterations to insulin signaling and systemic energetic homeostasis in PTSD, animal models, and in this work.¹⁴,²¹-²⁵ Mitochondria regulate GCC signaling through
the HPA axis and GCC regulate systemic mitochondria, exhibiting the bidirectionality of
every link in the causal chain across each of these systems and evidence suggests
more potential causal links than I could possibly describe in detail here.

Bringing it all together: a Bio-Contextual Affective Model of Traumatic Stress
A physiological model

HPA axis activation is (present and previous) experience-dependent. Both explicit
episodic memories and present affective state mediate the interpretive cognitive
framework that activates the stress response through the HPA axis. Glucocorticoids
(GCC), like cortisol in humans and corticosterone (CORT) in rats are generated in the
mitochondria of the adrenal glands in the last step of the HPA axis. GCC signal directly
to glucocorticoid receptors (GCR) on mitochondria in every cell of the body, modulating
gene expression and function and therefore systemic respiration and metabolism. GCC
also regulate insulin signaling in every cell of the body, which regulates systemic
energetic substrate availability and controls the HPA axis in addition to the canonical
negative feedback mechanism. Chronic or developmental stress leads to dysregulation
of the HPA axis and therefore the chronic dysregulation of systemic metabolism and in
tandem with life stress, often leads to allostatic overload (AOL) and toxic stress, which
may predispose to psychiatric disease, like PTSD. Neurons use over 80% of their
reserve respiratory capacity when firing and are therefore highly metabolically sensitive.
Spare respiratory capacity regulates HPC mean firing rate, a critical setpoint for HPC
function and is directly dependent upon insulin signaling. The hippocampus (HPC) is
particularly sensitive to GCC and other stress factors and therefore their neurons
represent one canary in the coal mine that presents stress effects earlier than other
brain regions. Chronic stress and PTSD are associated with HPC pattern separation
and contextual memory processing deficits, metabolic syndrome, and high allostatic load. Stress and GCC have been shown to induce HPC insulin resistance, mitochondrial deficits, and changes to HPC mean firing rate (MFR), which result in functional deficits. Deficits in HPC pattern separation and contextual memory processing lead to disruptions in CNS allostatic regulation and affective and behavioral dysregulation in addition to dysregulated metabolism and dysregulated control of the HPA axis, beginning the loop again.

A cognitive model

PTSD is cognitively associated with a traumatic event that leads to a meaning violation that disrupts models of self and world, which explains 48% of the variability in CAPS-5 symptoms. Associative and contextual memory processing deficits may be underpinned by pattern separation deficits in the DG-CA3 circuit and may contribute to avoidance and reexperiencing symptoms in PTSD and the persistent inability to integrate a traumatic memory with ones model of self and world. This produces substantial cognitive dissonance and uncertainty, which in addition to (genetic, chronic stress or developmental stress-induced) memory processing deficits originating in the medial temporal lobe, dysregulate CNS allostatic processing and affect. Dysregulated CNS allostatic processing dysregulates behavior, the primary contributor to metabolic output, and alters metabolism through the HPA axis, regulation of the autonomic nervous system and direct efferent visceromotor connections. Visceromotor control regions (anterior cingulate) may also contribute to altered GI function and nutrient absorption, regulating access to dietary nutrients.

Connecting the model systems
These two models intersect at the mitochondria as both a systemic and local regulator of metabolism and brain function under stress. The top down and bottom up streams converge at the HPA axis, but immediately once the HPA axis is activated, the stress response is under mitochondrial control, which integrates both top down and bottom up information as systemic metabolic regulation under stress.

5.3. Resetting Trauma-Induced Energetic Dysregulation in Post-Traumatic Stress Disorder (PTSD)

Wolkowitz et al. produced a table exemplifying possible novel strategies for treating metabolic alterations in PTSD.\textsuperscript{20,27} They highlight that peroxisome proliferator-activated receptor (PPAR) agonists may be particularly useful for improving insulin resistance as may be insulin sensitizers like metformin. They also mention that mitochondrial antioxidants, like COQ10 or antioxidant system activators, like quercetin and resveratrol (SIRT1), oleanolic acid derivatives (NRF2), or ATP production enhancers, like creatine, carnitine, and lipoic acid may also be useful.\textsuperscript{20}

Interestingly, DMT and pharmahuasca reduced ROS production, the goal of many of these recommended treatments, and normalized stress-induced ectopic insulin-like growth factor expression also observed in human PTSD. Furthermore, it reduced inflammatory gene transcripts overlapping with human PTSD and reduced transcription of pathways associated with oxidative stress. Pharmahuasca exhibited the most substantial effects in all of these domains.

PTSD is associated with the inability to cognitively integrate traumatic memories with models of the self and world, a process that may be undermined by reduced pattern separation, cognitive flexibility, and contextual memory processing.
Pharmahuasca treatment may improve pattern separation and contextual memory processing, and possibly cognitive flexibility through the aforementioned normalization of aberrant allostatic load factors and metabolism. However, 5-HT$_{2A}$ agonist psychedelics, like DMT also induce critical period reopening and neuroplasticity, which may contribute to enhanced memory processing and ability for traumatic memory integration.

The work included in this manuscript support an emerging new model of PTSD as a memory processing disorder in which stress arises from the inability of dysregulated memory processing circuits to integrate a traumatic event, which dysregulates allostatic processing at the CNS and mitochondrial levels. A chronic inability to integrate a traumatic event leads to post-traumatic stress disorder (PTSD) and, when persistent, leads to chronic metabolic dysregulation. Genetics, epigenetics, environmental context, socioeconomic status, and pre-existing traumatic stressors or chronic stress are all predispositional factors for the development of PTSD, likely through convergent effects on allostatic processing and resulting increases in AL.

Evidence is accumulating that numerous allostatic load and metabolic factors increase the risk of developing PTSD, which, if true, exhibits the bidirectional relationships between trauma, stress, and allostatic load. Stress dysregulates metabolism, increasing allostatic load. Trauma dysregulates neural models of self and world, which dysregulate metabolism, increasing allostatic load. The reverse may also be true, dysregulated metabolism and allostatic load dysregulate contextual memory processing systems, leading to an increased propensity to develop PTSD upon exposure to a traumatic event. Intervening at any step in this cycle or in external contributing factors may yield
improvements, but holistic approaches may be necessary to facilitate recovery in some PTSD patients.

In translating this work into the clinic, factors associated with metabolic dysfunction and allostatic load can be measured in PTSD patients, who can then be phenotyped into the AL categories presented in chapter 1.1. Specific treatment of these phenotypes in combination with psychotherapeutic interventions, optimally paired with psychoplastogens or compounds that reopen critical periods, will allow for addressing the systemic disorder, preventing metabolic or allostatic load factors from interfering with memory integration, and for the facilitation of memory integration itself and the resolution of PTSD.

5.4 Notes


APPENDIX A. SUPPLEMENTAL INFORMATION FOR CHAPTER 2

Figure A. 1. WGNA analysis. a) WGCNA analysis identified 23 modules. b) Module-trait relationship analysis revealed that two of these (Indianred4, and Magenta) were significantly correlated with time spent in the odor compartment in the CPA test.
Figure A. 2. Cell type specific enrichment of genes in the significant modules using the hypergeometric test. a) The indianred4 module was significantly enriched for neurons, myelinating, and newly-formed oligodendrocytes, and oligodendrocyte progenitor cells (OPC). b) No significant enrichment of any cell type was seen in the magenta module.
Figure A. 3. a) Gpx4 gene expression, b) Sirt2 gene expression, c) Txn1 gene expression d) Electron paramagnetic resonance (EPR) reactive oxygen species (ROS) data for all three groups.
APPENDIX B. SUPPLEMENTAL INFORMATION FOR CHAPTER

Figure B. 1 a) Representative Raman heatmap of cardiolipin (CL) in control. b) Representative Raman heatmap of CL in PE/PSS. c) Quantification of Raman heatmaps for control vs PE/PSS. d) LC-MS data for CL. e) Representative Raman heatmap of phosphatidylinositol (PI) in control. f) Representative Raman heatmap of phosphatidylinositol (PI) in PE/PSS. g) Quantification of Raman heatmaps for control vs. PE/PSS. h) LC-MS data for PI. i) Whole HPC tracing used for Raman quantification of the dorsal hippocampus. j) Tracing of HPC subregions (CA1, CA3, DG) used for subregion specific analysis of cholesterol concentrations. k) Quantification of total measured phospholipids between control and PE/PSS. l) qRT-PCR data for LXRB between control and PE/PSS. m) qRT-PCR data for LDLR between control and PE/PSS. n) qRT-PCR data for HMGCR between control and PE/PSS. o) qRT-PCR data for ABCA1 between control and PE/PSS.
Table B.1. Table of Primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5’ → 3’)</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDLR</td>
<td>ATTCCAAACTCCACTCCATCTC</td>
<td>GCTGCTTCTCATCCCTCCAAA</td>
</tr>
<tr>
<td>HMGCR</td>
<td>AAGAGTCGCTGTGTTCATCTC</td>
<td>CCTGCTTGTACTCTGCTCTCAAC</td>
</tr>
<tr>
<td>LXRβ</td>
<td>GATCTGGGATGTCATGAGTAG</td>
<td>GAAGCGTCCATCTGCAAGGTA</td>
</tr>
<tr>
<td>Lrp1</td>
<td>TGTGAAGTGCTCCTGCTATG</td>
<td>GCCTTTGGAAGAGATGATGA</td>
</tr>
<tr>
<td>Cyp46(a1)</td>
<td>ACTCCTGCTCACCCTCTAAT</td>
<td>CAGTGTGGTCAGTGTTTAGAG</td>
</tr>
<tr>
<td>ABCA1</td>
<td>TTGGATTCCGGCTGTAGTATTT</td>
<td>GGACACTGAGGTGGAAGATTG</td>
</tr>
<tr>
<td>Srebf1</td>
<td>CGACTACATCCGCTTCTACG</td>
<td>AAGCTGACACCAGGTCTTTTC</td>
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<tr>
<td>Srebf2</td>
<td>AGGTCTAGGGATGGGTGAAA</td>
<td>GTGGAAGGACAGGACAATTA</td>
</tr>
<tr>
<td>APOE</td>
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<td>CCATCAGTACCCTCAGGCTTCT</td>
</tr>
<tr>
<td>Cyp27(a1)</td>
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<td>CACACCAGTCACCTCCCTTGT</td>
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<tr>
<td>GAPDH</td>
<td>AGACAGCGGCATCTTCTTGT</td>
<td>CTTGCGTGAGGTAGTGT</td>
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</table>
APPENDIX C. SUPPLEMENTAL INFORMATION FOR CHAPTER 4

Caption on following page

\(^4\) Caption on following page
Figure C.1. Heat plot for pairwise comparisons between control, predator exposure/psychosocial stress (PE/PSS), and PE/PSS + treatment groups. The heatmap illustrates hierarchical clustering of the scaled counts (z-scores) for a set of biologically relevant differentially expressed genes (FDR<0.1).

Figure C.2.a. Volcano plot and b. Heat plot: PE/PSS vs DMT (all genes = 3869). Blue points: Log2FC <1 Red points: Log2FC>1
Figure C.3.a. Volcano plot and b. Heat plot: PE/PSS vs Harmaline (all genes = 5278).
Blue points: Log2FC < 1 Red points: Log2FC > 1
Figure C.4.a. Volcano plot and b. Heat plot: PE/PSS vs pharmahuasca (all genes = 2976). Blue points: Log2FC < 1; Red points: Log2FC > 1.
Figure C.5. (a-i) Selected differentially expressed genes (DEG) observed by RNAseq between control and predator exposure + psychosocial stress (PE/PSS) animals and the effects of PE/PSS + DMT, PE/PSS + harmaline, and PE/PSS + pharmahuasca on their expression. Significance notations are plotted from the bioinformatics analysis used to produce differentially expressed genes (DEG).
Figure C.6. Gene Set Enrichment Analysis (GSEA) for Gene Ontology (GO) cellular component and GO Biological Process analysis of predator exposure + psychosocial stress (PE/PSS) vs PE/PSS + DMT, PE/PSS + Harmaline, and PE/PSS + pharmahuasca. a) GO cellular component for DMT b) GO biological process for DMT c) GO cellular component for Harmaline d) GO biological process for Harmaline e) GO cellular component for pharmahuasca f) GO biological process for pharmahuasca
Figure C.7. KEGG pathway analysis of predator exposure + psychosocial stress (PE/PSS) vs PE/PSS + DMT, PE/PSS + Harmaline, and PE/PSS + pharmahuasca a) DMT b) Harmaline c) pharmahuasca
Figure C.8. Effects of predator exposure + psychosocial stress (PE/PSS) + DMT vs PE/PSS + Vehicle on the IPA NRF2-mediated Oxidative Stress Response pathway. Red indicates upregulated transcripts; green indicates downregulated transcripts and
red + green indicates uncertainty in the direction of modulation. White indicates parts of the pathway that do not significantly differ between groups.

Figure C.9. Schematic of transcriptional changes in the IPA G\textsubscript{q} signaling pathway between PE/PSS + DMT vs PE/PSS animals. Red indicates upregulated transcripts and green indicates downregulated transcripts and grey indicates uncertainty in the direction of modulation. White indicates parts of the pathway that do not significantly differ between groups.
Figure C.10. Effects of predator exposure + psychosocial stress (PE/PSS) + Harmaline vs PE/PSS animals on the IPA $\alpha$ signaling pathway. Red indicates upregulated transcripts; green indicates downregulated transcripts and red + green indicates uncertainty in the direction of modulation. White indicates parts of the pathway that do not significantly differ between groups.
Figure C.11. Effects of predator exposure + psychosocial stress (PE/PSS) + pharmahuasca vs PE/PSS animals on IPA Neuroinflammation pathway. Red indicates upregulated transcripts; green indicates downregulated transcripts and red + green indicates uncertainty in the direction of modulation. White indicates parts of the pathway that do not significantly differ between groups.

Figure C.12. Effects of predator exposure + psychosocial stress (PE/PSS) + pharmahuasca vs PE/PSS + Vehicle on IPA pathway for CREB signaling. Red indicates upregulated transcripts; green indicates downregulated transcripts and red + green indicates uncertainty in the direction of modulation. White indicates parts of the pathway that do not significantly differ between groups.
Figure C.13. Schematic of transcriptional changes in the IPA Synaptogenesis pathway between predator exposure + psychosocial stress (PE/PSS) + pharmahuasca vs PE/PSS + Vehicle treated animals. Red indicates upregulated transcripts; green indicates downregulated transcripts and red + green indicates uncertainty in the direction of modulation. White indicates parts of the pathway that do not significantly differ between groups
Figure C.14. Effects of predator exposure + psychosocial stress (PE/PSS) + pharmahuasca vs PE/PSS animals on the IPA Long Term Potentiation Pathway. Red indicates upregulated transcripts; green indicates downregulated transcripts and red + green indicates uncertainty in the direction of modulation. White indicates parts of the pathway that do not significantly differ between groups.
Figure C.15. Effects of predator exposure + psychosocial stress (PE/PSS) + pharmahuasca vs PE/PSS animals on IPA Opioid signaling pathway. Red indicates upregulated transcripts; green indicates downregulated transcripts and red + green
indicates uncertainty in the direction of modulation. White indicates parts of the pathway that do not significantly differ between groups.

Figure C.16. Individual cohort data. Preliminary cohort data (a,b). Replicate cohort data (c,d). Statistics were not performed on the first cohort data alone as n sizes were too small (n=2-4/group). Effects of DMT 2 mg/kg, Harmaline 1.5 mg/kg, DMT 2 mg/kg + Harmaline 1.5 mg/kg, and DMT 4mg/kg + Harmaline 1.5 mg/kg on total ROS production in the prefrontal cortex and hippocampus of predator exposed rats + daily psychosocial
stress (PE/PSS) a) Total ROS in the prefrontal cortex: control vs PE/PSS and PE/PSS + treatment. b) Total ROS production in the hippocampus: control vs PE/PSS and PE/PSS + treatment. c) Effects of DMT 2 mg/kg, Harmaline 1.5 mg/kg, and DMT 2 mg/kg + Harmaline 1.5 mg/kg on total ROS production in the prefrontal cortex and d) hippocampus of Sprague Dawley rats with and without PE/PSS

Figure C.17. PCR primer table

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<tr>
<th>Gene</th>
<th>Forward 5' Sequence</th>
<th>Reverse 3' Sequence</th>
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<td>GAPDH F</td>
<td>5' CCC TGT TGC TGT AGC CAT ATT 3'</td>
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<td>GAPDH R</td>
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<tr>
<td>IGF2 F</td>
<td>5' TAC CAG CGA GAG CCA GTA A-3'</td>
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<td>IGF2 R</td>
<td>5' GCC AAG CGA TAG AGA CAG ATA AA 3'</td>
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<td>IGFBP2 F</td>
<td>5' GTG ATG GGT GTG GAG GAT TT 3'</td>
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<tr>
<td>IGFBP2 R</td>
<td>5' AGG AAC GGA GGT ACA GGT TA -3</td>
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<tr>
<td>Clip3 F</td>
<td>5' GCA TCT CGT ATC CAG AGG ATT G -3'</td>
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<td>Clip3 R</td>
<td>5' CTG TTG TGA AGG TGC GTT TG 3'</td>
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</tr>
<tr>
<td>Slc6a13 F</td>
<td>5' GGT TTC CCT ATC TCT GCT ACA AG 3'</td>
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<tr>
<td>Slc6a13 R</td>
<td>5' CTC CAG GAA GAA GAC AGG AAT G 3'</td>
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</tr>
<tr>
<td>Data analyzed</td>
<td>DEGs in Rats</td>
<td>Non DEGs in Rats</td>
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<tr>
<td>-------------------------------------------</td>
<td>--------------</td>
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<tr>
<td>Genes overlapping with Girgenti et al.</td>
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<td>Odds ratio</td>
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<td>Reciprocal of odds ratio</td>
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<tr>
<td>P value &lt;0.0001</td>
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Figure C.18. Contingency table and inputs to the one tailed Fishers exact test.
Figure C.19. (a-b): Hierarchical clustering before and after applying RUVseq
Figure C.20. Hypothetical schema of the interaction of N,N-DMT with the 5HT$_{2a}$R and the Sigma1 receptor to modulate ROS production, reduce inflammation, and to induce neuroplasticity. Created with BioRender.com
LIST OF REFERENCES:


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Yin, Y., Hua, H., Li, M., Liu, S., Kong, Q., Shao, T., . . . Jiang, Y. (2016). mTORC2 promotes type I insulin-like growth factor receptor and insulin receptor activation through the tyrosine kinase activity of mTOR. *Cell Res, 26*(1), 46-65. doi:10.1038/cr.2015.133


CURRICULUM VITAE

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Education

B.A. Department of Philosophy, Louisiana State University, Baton Rouge, LA.
Focus: Philosophy of Mind

B.S. Department of Psychology, Louisiana State University, Baton Rouge, LA.
Focus: Physiological Psychology

Ph.D. Candidate: Department of Comparative Biomedical Sciences, LSU School of Veterinary Medicine, Baton Rouge, LA.

Post-Doctoral Fellowship: Translating Psychedelic Research Program (TrPR), Weill Institute for Neurosciences, University of California San Francisco

Dissertation Committee

Mentor: Dr. Joseph Francis
Co-mentor 2017-2019: Dr. Charles Lee
Co-mentor 2019-2022: Dr. Nick Gilpin
Dr. Charles Nichols
Dr. Steven Barker
Deans representative: Dr. Meghan Papesh (Year 1-4)
Deans representative: Dr. Graca Vicente (Year 5)

Positions and employment

05/2007- 08/2007 Medical charts assistant: Surgical Specialty Center, Baton Rouge, LA
High school summer job

01/2006- 08/2016 General Manager: Major Kelley’s Tree Service, Baton Rouge, LA
Family business for which I worked from high school until I began my PhD

2014-2015 Research Assistant: Nutritional Neuroscience and Aging Lab- Pennington Biomedical Research Center, Baton Rouge, LA

2015-2016 Research Assistant: Comparative Biomedical Sciences, Louisiana State University

2016- Present Graduate Assistant: Comparative Biomedical Sciences, Louisiana State University

2017-2018 Social Chair: Graduate Student Organization LSU SVM

2018- 2021 Treasurer: Graduate Student Organization LSU SVM

November 2018- December 2019 MAPS (Multidisciplinary Association for Psychedelic Studies) Night Attendant 1529 River Oaks Rd W New Orleans, LA 70123
December 2019- Current **MAPS Night Attendant** Phase 2/3 MDMA-assisted psychotherapy research with MAPS/MPBC 320 N. Carrollton, Suite 202, New Orleans, LA 70119

2019- 2022 **Graduate Representative to CBS Faculty**, Comparative Biomedical Sciences, LSU

2020- Current **Gratis trainee** - Gilpin lab, Department of Physiology, LSU Health Sciences Center New Orleans

2022- Current **Post-Doctoral Fellow** - Translational Psychedelic Research Program, Weill Institute for Neurosciences, University of California San Francisco

**Professional Memberships and Honors/Awards**

- 2010-2014 Psi Chi Psychology Honor Society
- 2017- current Society for Neuroscience
- 2016 - current American Association for the Advancement of Science (AAAS)
- 2020 - 2021 1st place presentation CBS research retreat
- 2020 - 2021 Heidi Lott Scholarship Award Winner
- 2021 – 2022 3rd place presentation CBS research retreat

**Community involvement**

**LSU School of Veterinary Medicine Open House 2017**
I operated a booth where I taught visiting children from the community to extract DNA from strawberries in order to spread the wonders of science to Baton Rouge.

**LSU School of Veterinary Medicine Open House 2018**
I operated a booth where I taught visiting children from the community to make simple electrophysiological recordings from the leg of a cricket to spread the wonders of science to Baton Rouge.

**Scientific/ academic presentations**

* When multiple years are listed, the most recent presentation is listed here

- **Comparative Biomedical Sciences Research Retreat**, LSU arboretum- 2017, 2018, 2019, 2020, 2021, 2022*- Conditioned place avoidance of a bobcat urine associated spatial context is associated with a distinct behavioral and hippocampal phenotype - 15 minute talk

- **Phi zeta, research emphasis day**, LSU- 2017, 2018, 2019, 2020*- Avoidance of a trauma related context in a predator urine exposure model of PTSD is associated with reduced synaptic associated transcripts in the dorsal hippocampus D. Parker Kelley, D. Singh, L. Souza, N. Gilpin, J. Francis - Poster presentation

- **Lunch and Science, Comparative Biomedical Sciences**, LSU- 2018- Investigations in a murine model of PTSD D. Parker Kelley, Katy Venable, Brad Wilson, Mike Metchnikoff, Phillip Ebenezer, Steven Barker, Joseph Francis - 1 hour talk

- **Society for Neuroscience, San Diego 2018**- An investigation into the effects of hippocampal IL-1β and tryptophan catabolites (TRYCATS) in a predator exposure model of PTSD D. Parker Kelley, Philip Ebenezer, Katy Venable, Charles Lee, Joseph Francis - Poster presentation

- **Society for Neuroscience, Chicago 2019, nanosymposium**- trauma and stress: adaptive mechanisms – Transcriptomic alterations in the dorsal hippocampus of a predator urine exposure

New Orleans Psychedelic Society (with Dr. Jon Cogburn) 2019: “Anatman on demand: Implications of the mass availability of selfless experiences for the psychology and philosophy of mind” - 1 hour talk and subsequent group discussion

Dissertation proposal seminar, Comparative Biomedical Sciences, LSU 2020- Transcriptomic changes underpinning contextual memory processing deficits in the dorsal hippocampus of a predator urine exposure model of PTSD: Role of IGF2 (2021) D. Parker Kelley, D. Singh, L. Souza, N. Gilpin, J. Francis - 1 hour talk

Post-Doctoral Fellowship Interview, Translational Psychedelic Research Program, UCSF- Molecular homeostasis in stress and trauma- 1 hour talk

Dissertation Defense, Comparative Biomedical Sciences, LSU 2022- Evaluation of Hippocampal Allostatic Load-Associated Factors in Animal Models of Post-Traumatic Stress Disorder: Relevance to Human PTSD- 1 hour talk

Publications

D. Parker Kelley, Katy Venable, Aspasia Destouni, Gerald Billac, Philip Ebenezer, Krisztian Stadler, Charles Nichols, Steven Barker, Joseph Francis (2021) Pharmahuasca and DMT rescue ROS production and differentially expressed genes after predator and psychosocial stress: relevance to human PTSD. ACS Chemical Neuroscience


Under Review

Michael F. Salvatore, Ella A. Kasanga, D. Parker Kelley, Katy E. Venable, Mark A. Cantu, Jennifer Terrebonne, Donald K. Ingram (2021) Calorie restriction intervention at advanced middle age stalls aging-related motor decline with increased expression of D1 receptor in substantia nigra and increased GFR-α1 and GLT-1 in striatum: Aging Cell

D. Parker Kelley†, Ardalan Chaichi†, Alexander Duplooy, Dhirendra Singh, Manas Gartia, Joseph Francis Increased ROS production is associated with increased cholesterol, altered lipid expression in the hippocampus after predator exposure and psychosocial stress: RAMAN imaging + Lipidomics. Acta Neuropathologica (February 2022) † indicates shared 1st authorship

Scientific work in preparation (completed studies) (in order of predicted publication)

D. Parker Kelley, Lucas A Souza, Shea Cruise, Alexander Duplooy, Dhirendra Singh, Nick Gilpin, Joseph Francis Conditioned place avoidance (CPA) is associated with a distinct Hippocampal phenotype: Role of Pattern Separation, IGF1/2, ROS, and Mitochondria. eLife (March 2022)


Philosophical/ Ethics work in progress

Boots on the (actual) ground; Huxleyan perception, thalamocortical gating, and Dennett’s anosognosic theory of perception D. Parker Kelley†, Jon Cogburn†, indicates shared 1st authorship

Do psychedelic experiences change beliefs and values?: ethical implications for the use of psychedelics in mental health treatment. Michelle Matvey, D. Parker Kelley, Josh Woolley

Clinical trials

https://clinicaltrials.gov/ct2/show/NCT04398784

Teaching experience

LSU department of Philosophy and Religious Studies: Philosophy of Mind: Theories of Consciousness (2020) – Guest Instructor with Dr. Jon Cogburn- My role was to present a focused neuroscience lecture at the beginning of most classes (~20 min) that provided neuroscientific, physiological, and psychological context for the reading for that day.

LSU department of Philosophy and Religious Studies: Kant and Mind (2021)- Guest Instructor with Dr. Jon Cogburn
- My role was to provide scientific context to Kant’s claims regarding the self and fundamental mental faculties, particularly spatial perception.

Mentoring experience

1. Jordan Malatesta, DVM student in the summer scholars program at LSU SVM where I was his direct mentor and was supervised by Dr. Joseph Francis. I taught Jordan the basics of molecular biology including western blotting and PCR.

2. Michael Mechikoff, At the time, Mike was completing his BS in Biology at the Airforce Academy and received summer training in our lab on the PTSD model in collaboration with Lt. Colonel Brad Wilson, PhD: I trained Mike in the elevated plus maze, fear conditioning and western blotting. Mike is now a M.S. student at Purdue.

3. Alexander Duplooy, Xander holds a B.S. in Biology (2021): Xander is an RA in our lab and I am currently Xander’s direct supervisor. I taught Xander PCR and we are currently working on endocannabinoid signaling in both the predator exposure and predator scent stress models of PTSD.

4. Henry Crull, Henry holds a B.S. in Biology (2019): Henry is an RA in our lab working on statistical analysis of behavior and physiological endpoints in our clinical trial using antioxidant supplementation to treat depression and anxiety in a rural population. I trained Henry in the
interpretation of human psychometric data and am overseeing Henry’s role in statistical analysis for the depression/anxiety study.

Skills and techniques
All of the following are assumed to be at full proficiency unless otherwise specified

Clinical:
Administration and analysis of clinical interviews:
- **SIGH-D** - Structured interview for the Hamilton Depression Scale
- **IDS-C** - Inventory of Depressive Symptomology- Clinician administered

Administration and analysis of clinical surveys:
- **MDI** - Major Depression Inventory
- **LSEQ** - Leeds Sleep Evaluation Questionnaire
- **GAD-7** - Generalized Anxiety Disorder-7 Questionnaire
- **VFFQ** - Venable Food Frequency Questionnaire – food frequency questionnaire that Katy Venable, a member of our team developed for the purpose of group randomization in our antioxidant treatment for depression and anxiety study

Physiological:
- Development of remote multisite sample collection, storage, and management protocols for the collection of: blood, plasma, serum, samples for molecular (ELISA, Luminex, RNAseq) and electron paramagnetic resonance (EPR) analysis of free radical production and biomarkers of depression

Statistics, bioinformatics and coding experience:
- **SPSS** (basic understanding from undergrad general statistics class)
- **SAS** (basic understanding from intermediate statistics in the psychology department)
- **Prism 9** complete proficiency
- **IPA pathway analysis** (currently developing an understanding through the analysis of RNA sequencing data)
- **Gene Ontology (GO), KEGG, Panther, Wikipathway pathway analysis** (proficient to calculate ranks from DSEQ2/ RUVseq data and generate pathway analysis)
- **G Power**- Proficient at power analysis for clinical trials and preclinical work and the calculation of effect size

Preclinical and molecular

Animal models and behavioral analysis
- Predator exposure (PE) and psychosocial stress (PSS) model of PTSD
- Fear conditioning of predator exposure (FC)
- Predator urine (bobcat) exposure model of PTSD in contextual place avoidance (CPA) paradigm
- Lipopolysaccharide (LPS) model of inflammatory depression
- Sucroese preference depression test
- Object pattern separation (OPS)
- Context object discrimination (COD)
- Novel object recognition (NOR)
- Elevated Plus Maze (EPM)
- Longitudinal activity analysis with activity chambers and Versamax analyzer
- Stone T maze measure of spatial memory

Surgeries and tissue extraction in rats
- Fresh or frozen brain tissue slicing and punching
  - vmPFC
  - dmPFC
  - dHC
  - dHC CA3-DG circuit
  - vHC
  - Amygdala
  - Substantia Nigra
  - Striatum
- Perfusion with PBS and paraformaldehyde in preparation for IHC
- Stereotaxic surgeries for the implantation of cannula in to the brain of rats

Molecular biology
- Basic cell culture experience in N2A Murine neuronal cell line and RAW macrophage cell line
- Protein estimation
- Western blotting
- Total RNA, miRNA, mRNA extraction and quantitative/ qualitative analysis by Nanodrop spectrophotometer
- Polymerase Chain Reaction (PCR) and CNDA synthesis
  - qRT-PCR
  - PCR + Electrophoresis for genotyping, primer verification, etc
- Enzyme linked immunosorbert assay (ELISA)
- Lumine/plex assays
- HPLC/ LC-MS analysis of neurotransmitters and Tryptophan/ Kynurenine catabolites (experience, but not expertise)
- Electron Paramagnetic Resonance Spectroscopy (EPR) for the quantification of free radicals in tissue
- Seahorse extracellular flux analysis
- Immunohistochemistry (IHC)
- Confocal microscopy
- Nanozoomer microscopy
• Standard light microscopy