DIFFERENTIAL PROGRESSION OF COGNITIVE AND MOOD-RELATED PHENOTYPES IN A MOUSE MODEL OF CHRONIC DEPRESSION

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Abstract

Depressive disorders remain highly prevalent and lack effective treatments. Mounting evidence suggests that disrupted cognitive processes, such as uninhibited negative thought patterns ("rumination"), play a significant role in the development of Major Depressive Disorder (MDD), either alongside or preceding serotonin-related mood imbalances. However, the underlying mechanisms of prodromal cognitive symptoms necessitate further investigation. This study explored the differential progression of cognitive and mood-related phenotypes in BALB/c mice subjected to the unpredictable chronic mild stress (UCMS) model. Following two UCMS durations—short-term (2–3 weeks) and long-term (5–6 weeks)—we observed the emergence of distinct symptom sets. Differences in neurobiological processes and substrates were also seen, including alterations to regional metabolic activity. Additionally, sex differences in behavioural measures were identified, in line with previous research pointing to sex-specific vulnerabilities to chronic stress. Altogether, our findings demonstrate the emergence of cognitive deficits associated with dysregulated inhibitory mechanisms in the early stages of depressive-like symptom onset, and may inform the development of preventive treatment strategies.

Keywords: depressive symptom chronology, early depression mouse model, SK3 channels

Declaration of Co-Authorship

I hereby declare that this thesis incorporates material from joint research supervised by Dr. Francis Bambico. Derek Wan-Yan-Chan aided in behavioural testing and contributed RNAscope data and interpretations. S. M. Nageeb Hassan aided in surgical procedures, biosensor recordings, and dissections. Haley Winsor and Areej Khan aided in UCMS procedures, behavioural testing, and coding. Brandon Hannam and Nada Abdelhalim also aided UCMS procedures and behavioural testing. Yellow Martin aided in ELISA preparation and procedures. Dr. Francis Bambico and Ashutosh Patel contributed electrophysiology recording data. In all cases, primary contributions were performed by myself.

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1-EBIO	1-ethyl-2-benzimidazolinone
5-HT	5-hydroxytryptamine
5-HT1A	5-hydroxytryptamine 1A
5CSRTT	5-choice serial reaction time task
8-OH-DPAT	8-Hydroxy-2-(di-n-propylamino)tetralin
ACTH	Adrenocorticotropic hormone
ADTs	Antidepressant treatments
ANOVA	Analysis of variance
AP	Anterior-to-posterior
APA	American Psychiatric Association
ASST	Attentional set shifting task
BDNF	Brain-derived neurotrophic factor
BK	Large-conductance calcium-activated potassium channel
BLA	Basolateral complex of the amygdala
BORIS	Behavioral Observation Research Interactive Software
CBT	Cognitive-behavioural therapy
CeA	Central nucleus of the amygdala
CRH	Corticotropin releasing hormone
CTR	Control
D1R	Dopamine receptor
DA	Dopamine
DEX	Dexamethasone
dlPFC	Dorsolateral prefrontal cortex
DMN	Default mode network
dmPFC	Dorsomedial prefrontal cortex

DRN	Dorsal raphe nucleus
DSM-5	*Diagnostic and Statistical Manual of Mental Disorders (5th ed.)*
DST	Dexamethasone suppression test
ELISA	Enzyme-linked immunosorbent assay
ES	Escapable stress
FC	Functional connectivity
fMRI	Functional magnetic resonance imaging
FosB	FBJ murine osteosarcoma viral oncogene homolog B
GABA	Gamma-aminobutyric acid
GR	Glucocorticoid receptor
HC	Hippocampus
HPA	Hypothalamic-pituitary-adrenal
IP	Intraperitoneally
IS	Inescapable stress
LED	Light-emitting diode
LT-UCMS	Long-term unpredictable chronic mild stress
MDD	Major Depressive Disorder
MDE	Major depressive episode
ML	Medial-to-lateral
mPFC	Medial prefrontal cortex
nA	Nanoamperes
NAc	Nucleus accumbens
NFκB	Nuclear factor-kappa B
NMDA	N-methyl-D-aspartate
NSFT	Novelty-suppressed feeding test
NT	Neurotensin

OXTR	Oxytocin receptor
PET	Positron emission tomography
PFC	Prefrontal cortex
PND	Post-natal day
PrL	Prelimbic
PV	Parvalbumin
PVN	Hypothalamic Paraventricular Nucleus
RGB	Red, green, and blue
SAT	Spontaneous alternation test
SEM	Standard error of the mean
sgPFC	Subgenual prefrontal cortex
SIT	Social interaction test
SK1	Small conductance calcium-activated potassium channel (subtype 1)
SK2	Small conductance calcium-activated potassium channel (subtype 2)
SK3	Small conductance calcium-activated potassium channel (subtype 3)
SKC	Small conductance calcium-activated potassium channel
SSRIs	Selective serotonin reuptake inhibitors
SST	Somatostatin
ST-UCMS	Short-term unpredictable chronic mild stress
UCMS	Unpredictable chronic mild stress
vmPFC	Ventromedial prefrontal cortex
WHO	World Health Organization

Differential Progression of Cognitive and Mood-Related Phenotypes in a Mouse Model of Chronic Depression

Mental health conditions continue to necessitate urgent advancements in understanding, treatment, and prevention. Their societal burden is tremendous: mental health conditions are the leading cause of years lived with disability (World Health Organization [WHO], 2022a) and account for over US$1 trillion in lost productivity annually (WHO, 2022b). Among the many diagnostic categories, depressive disorders are considered most prevalent. They are marked by heterogeneous sets of symptoms, including persistent low moods and/or loss of pleasure in daily activities, while being accompanied by a mix of other cognitive, behavioural, or physical manifestations (Malhi & Mann, 2018; WHO, 2019). Over time, these disorders can severely limit one's ability to carry out normal daily functioning.

While treatments for depressive disorders have existed for some time—biochemical etiologies of affective conditions have been proposed since the 1960s (Coppen, 1967)—global incidence has not seemed to decrease proportionally. Indeed, Ormel et al. (2022) coined the discrepancy a "treatment-prevalence paradox." Despite ongoing refinements to antidepressant treatments (ADTs) and increasing access over the past several decades, meta-analytic findings suggest unchanging and even significant increases in prevalence (Ormel et al., 2022), including pronounced increases among younger individuals and vulnerable populations (Weinberger et al., 2018). Various explanations for this paradox have been proposed: prominent among them include overestimates in the short- and long-term efficacy of conventional ADTs due in part to low generalizability in clinical trial samples (Ormel et al., 2019, 2022); limited understanding of the enduring psychopharmacological effects of ADTs in relation to relapse (Harmer et al., 2017), especially in adolescents and young adults (Murphy et al., 2021); changes in attitude towards mental health conditions (Ormel et al., 2022); excessive focus on a categorical classifications of disease (Fried, 2015, 2022; Fried & Nesse, 2015; Sibille & French, 2013); and, perhaps the most significant, the attenuation of the "serotonin hypothesis," which has long been influential in our

understanding of the pathophysiology of depression and thus the development of conventional ADTs (Moncrieff et al., 2022; Shilyansky et al., 2016).

It has been said that two individuals who qualify for the same diagnosis may not share a single symptom in common (Fried & Nesse, 2015). Operationalizing depressive disorders has been an enduring challenge across research domains, hindering the development of efficacious therapies. Numerous factors, such as the nature of environmental stressors, duration and severity symptoms, age of onset, sex, heritability, and interindividual differences, have been considered in establishing diagnostic subtypes (Gotlib et al., 2020; Lye et al., 2020; Rush et al., 2003; Snyder, 2013; WHO, 2019; Zimmerman et al., 2008). However, given the myriad of interactions that can occur amongst these factors, it is no surprise that common symptom profiles have not yet materialized (Fried & Nesse, 2015), or that the search for robust biomarkers has yielded equivocal findings (Sibille & French, 2013). The delicate argument of whether mental health conditions and their vulnerability factors are better comprehended psychologically or biologically seems to carry some weight in this definitional challenge (Kendler, 2016; Koster et al., 2017). The notion that depressive disorders can even be considered "latent entities" with labels has also been discussed (Fried, 2015; Kendler, 2016), and has led some to call for the adoption of more nuanced and individualized approaches to screening and treatment, e.g., machine learning-powered network frameworks that organize around central symptoms (Borsboom & Cramer, 2013; Fried, 2015, 2022; Harald & Gordon, 2012; Taubitz et al., 2022).

Nevertheless, extensive clinical and preclinical research has informed the development of current depressive disorder subtypes in an effort to facilitate the identification of therapies. Common clinical diagnoses include Major Depressive Disorder, Adjustment Disorder, Seasonal Affective Disorder, and Late-Life Depression (American Psychiatric Association [APA], 2013; WHO, 2019). Broadly, these tend to fall under two prototypes: acute non-recurrent or chronic-recurrent (Malhi & Mann, 2018; Schramm et al., 2020). Major Depressive Disorder

(MDD) is considered to be most clinically prevalent, and is of particular concern given its high rate of relapse (Bromet et al., 2011; Malhi & Mann, 2018; Monroe & Harkness, 2022). Each episode tends to increase the probability of further episodes, and life course recurrence is considered to range anywhere from 30–90% (Gotlib et al., 2020; Kessler & Bromet, 2013; Monroe & Harkness, 2022; Sabille & French, 2013). The study herein will be centred around this classification.

The next section will review the common features and current treatments of MDD, the role of chronic stress and rumination in depressive symptom onset, and the selection of rodent models that best approximate these conditions. A discussion on the novel mechanisms being explored by our lab will then be provided, followed by an overview of the present phenotyping study, which aims to extend knowledge on the mechanisms underlying the onset of putative cognitive prodromal symptoms in mice.

1.1 Major Depressive Disorder

The APA (2013) classifies MDD as discrete, often recurrent depressive episodes, at least 2 weeks in duration, with "clear-cut changes in affect, cognition, and neuro-vegetative functions." It is considered unipolar, absent of the manic symptoms that also manifest in bipolar disorder (APA, 2013). If episodes recur for a prolonged duration, the condition can be characterized as chronic-recurrent depression; further, if symptoms are consistently presented for at least 2 years, the condition evolves to persistent depressive disorder or dysthymia (Malhi & Mann, 2018).

1.1.1 Symptomatology, Chronicity, and Translatability

Depressed individuals present a vast array of symptoms spanning several categories. Based on the DSM-5 (APA, 2013), there are nine categories considered in a clinical diagnosis: depressed mood (e.g., feelings of sadness or irritability), loss of interest or pleasure (anhedonia), significant changes in appetite or weight, sleep disturbances, psychomotor changes (e.g., in

observable speed and movement patterns), low energy (e.g., reduced efficiency in completing routine tasks), feelings of worthlessness or excessive guilt, impaired cognitive abilities (e.g., in concentrating or making decisions), and thoughts of death or suicide. As well, a diagnosis requires that significant impairment in important areas of functioning (e.g, social relationships, occupation) is present, and that symptoms are not directly attributed to the effects of substances (e.g., drug abuse, medication side effects) or separate medical conditions (APA, 2013). While a minimum of five symptoms are required for a clinical diagnosis (including the requirement of depressed mood or anhedonia), presentation of an insufficient number or severity (i.e., a subthreshold syndrome) may still be informative and predictive (Malhi & Mann, 2018).

Prodromes refer to the initial symptoms and signs that precede the onset of a disease. They can offer valuable insights into early indicators and warning signs of MDD, facilitating early detection and intervention (Benasi et al., 2021; Monroe & Harkness, 2022). The study of prodromes also contributes to a deeper understanding of disease progression and underlying mechanisms, enabling the development of preventive strategies (Gotlib et al., 2020). At this time, however, early prognostic indicators of MDD, especially those that predict more chronic cases, are not nearly robust and require further research (Monroe & Harkness, 2022). Recent reviews have only found modest correlations in prodromal symptoms and cite methodological challenges given the DSM-5 does not distinguish between primary and secondary MDD, as is commonly done in general medicine (Benasi et al., 2021). As discussed, MDD often exhibits a lifelong and recurrent trajectory. This is typically characterized by episodes of escalating severity, diminishing therapeutic response, and shorter periods of remission (Sibille & French, 2013). In cases where prevention strategies fail and initial therapies are not adequately targeted, the risk of progression to a chronic illness becomes substantial. Thus, the importance of extending knowledge in this area of research is paramount.

Rodent models have been extensively used to study specific symptoms associated with depression given their translatability and scalability. They continue to be the gold-standard

organisms used in a wide range of experimentation in biomedical research (Gururajan et al., 2019). Observable behaviours, such as reduced interest in pleasurable activities (e.g., sucrose consumption), changes in grooming and locomotion, and altered social interactions, can be measured through highly validated assays (Belzung & Lemoine, 2011; Planchez et al., 2019). Additionally, physiological changes associated with depression, especially when correlated with changes in behaviour, play a key role in the discovery of new biomarkers and treatments. These can include genetic, epigenetic, biochemical, neurophysiological, neurotrophic, and network factors (Bambico & Belzung, 2013). It is important to note, though, that aspects of depression, particularly those related to the subjective experience of emotions and thoughts, cannot be directly modeled in animals. Since we cannot know precisely what they are thinking, there are obvious limitations in capturing the full range of depressive symptoms observed in humans (Gururajan et al., 2019). However, by focusing on observable behaviours and homologous physiological changes, investigators can still gain valuable insights that inform further replication across species.

1.1.2 The State of SSRIs

It is no surprise that the precise pathophysiology of depression remains elusive. One of the pervasive theories (the “serotonin hypothesis”) involves a persistent deficiency in the synaptic content of the monoamine transmitter 5-hydroxytryptamine (5-HT), which is produced in neurons originating from the raphe nuclei (Berger et al., 2009; Moncrieff et al., 2022). A class of pharmaceuticals that target this deficiency, selective-serotonin reuptake inhibitors (SSRIs), has been found to improve mood at the neuropsychological level through reducing negative affective bias—the inclination to concentrate on, interpret, and recall negative information (Harmer et al., 2017). However, the delayed onset of their clinical effects (4–8 weeks), chronic administration requirements (>6 months), lack of responsiveness in many patients (<50% achieve remission), and the need to account for the reversal of various depression symptoms after administration, combine to profoundly challenge a solely neurotransmitter-based

explanation (French et al., 2014; Harmer et al., 2017; Leuchter et al., 2009; Moncrieff et al., 2022; Trivedi et al., 2006).

Mounting clinical and preclinical evidence strongly supports the idea that disrupted cognitive processes are not just epiphenomena following the emergence of 5-HT imbalances, but play a significant role in the pathogenesis of depression and require further attention (Abela & Hankin, 2011; Austin et al., 2001; Hammar & Årdal, 2009; Kalueff & Murphy, 2007; Richardson et al., 2022). In the next section, the concept of chronic stress will be discussed in relation to its role in sustained negatively-valenced thought processes and the progression of cognitive deficits in the context of depression onset.

1.1.3 Chronic Stress and Known Biomarkers of MDD

Stress is said to arise when environmental demands—particularly those that are *uncontrollable* and *unpredictable*—exceed one's inherent regulatory capacity (Koolhaas et al., 2011). In other words, only when physiologically demanding circumstances cannot be anticipated or quickly recovered from do they become aversive. Maier et al. (2006) demonstrated the fundamental concept of stress controllability by measuring activation patterns in the dorsal raphe nucleus (DRN) and ventromedial prefrontal cortex (vmPFC), regions associated with stress detection and evaluation, respectively. By correlating behavioural and extracellular measures in response to two conditions, inescapable stress (IS) and escapable stress (ES), they provided strong evidence that perceived control (ES) effectively downregulated the overall stress response in subsequent trials compared with IS. Crucially, while the DRN still responded to the stressor, the vmPFC inhibited the stress response when a sense of controllability was present. Feeling able to exert control over stressors (e.g., through learned coping mechanisms) is a protective factor against the development of stress-induced disorders, while the inverse represents a vulnerability factor (Amat et al., 2005; Maier et al., 2006). Moreover, when stress is perceived as irresolvable over an extended duration, it can result in adverse impacts on cognitive and emotional processing, as well as detrimental changes in brain

tissue. These combined effects have been linked to the subsequent development of neuropsychiatric disorders (Popoli et al., 2012).

Among biomarkers in a subset of MDD patients are abnormally heightened metabolic activity in key limbic regions (as measured by glucose metabolism), including the ventral regions of the PFC and the amygdala, and decreased metabolic activity in the dorsolateral PFC (dlPFC) (Drevets et al., 1997; Kennedy et al., 2001; Price & Drevets, 2010). Clinical studies have also consistently linked depression and other stress-related disorders with volume alterations, neuronal atrophy, and disrupted connectivity within the PFC (Drevets et al., 2008; Price & Drevets, 2010). Further, levels of neurotrophic factors, synaptic proteins, and spine density have all been shown to decrease in the PFC as detected in MDD animal models (Liu et al., 2012).

Anatomically, the rodent PFC shares similar efferent and afferent patterns of connectivity with primates, including homologous functions across the medial PFC (mPFC; e.g., prelimbic, infralimbic, and anterior cingulate) (Öngür and Price, 2000). Of note, the mPFC plays an important role in the integration of cognitive and affective behaviour, and the regulation of autonomic and neuroendocrine functions—all critical for translating stressful experiences into adaptive behaviour (McKlveen et al., 2015). The mPFC responds to stress and modulates the response to stress through regulation of the hypothalamic paraventricular nucleus (PVN) which, in turn, controls sympatho-adrenal and hypothalamic–pituitary–adrenal (HPA) activity (McKlveen et al., 2015).

Proper functioning of the negative feedback loop of the HPA axis is crucial to normal stress response and recovery. HPA hyperactivity/basal hypercortisolemia is considered to be a prominent feature in about 50% of MDD patients (Arana et al., 1985; Holsboer, 1983), with clinical manifestations characterized by a resistance to negative feedback on cortisol secretion in response to the dexamethasone (synthetic glucocorticoid) suppression test (DST) (Stokes et al., 1984), and by increased adrenocorticotropic hormone (ACTH) and cortisol in response to the combined dexamethasone suppression/corticotropin releasing hormone (CRH) stimulation

(DEX/CRH) test (Heim et al., 2008). The latter (DEX/CRH test) is a highly sensitive biomarker (about 90% in age-matched MDD patients) used to evaluate HPA homeostasis that is unconfounded by acute stress (Oshima et al., 2001). Key limbic regions, particularly the PFC and hippocampus (HC) that have a high density of glucocorticoid receptors (GRs), are involved in the negative regulation of glucocorticoid secretion (Feldman & Conforti, 1985; Feldman & Weidenfeld, 1999; Mizoguchi et al., 2003).

1.1.4 Rumination and Excitatory–Inhibitory Imbalances

Stress controllability and HPA axis dysregulation are considered to be intertwined with a progressive disinhibition of negative thought patterns in MDD. The longstanding Response Styles Theory (Nolen-Hoeksema, 1991) holds that persistent patterns of negative thinking tend to emerge first before the more advanced mood-related symptoms involved in full depressive episodes. A common construct of negative thinking is rumination. It refers to the tendency to excessively focus or dwell on negative thoughts, emotions, and problems, which can lead to a prolonged sense of distress and an impaired ability to problem-solve (Nolen-Hoeksema et al., 2008). Ruminating tends to occur when individuals feel more certain of their circumstances and view past events as uncontrollable, which can evoke feelings of helplessness and potentially bias subsequent information processing through reinforcement (Diener et al., 2009; Harmer et al., 2017; Nolen-Hoeksema et al., 2008). Studies have shown that individuals scoring high in rumination are unable to inhibit emotional information, place heightened focus on irrelevant negative stimuli, and spiral between disparate negative thoughts much more easily (Joormann, 2006; Nolen-Hoeksema et al., 2008). Rumination has also been consistently shown to be a vulnerability factor in experiencing the onset of major depressive episodes (MDEs), having a previous history of MDEs, and having significantly longer durations of future MDEs (Abela & Hankin, 2011; Kinderman et al., 2013; Michalak et al., 2011; Michl et al., 2013; Philippi et al., 2018; Spasojević & Alloy, 2001; Spinhoven et al., 2018).

The propensity to engage in ruminative behaviours may be associated with altered recruitment of mechanisms that amplify self-referential thought and negative affect. Early neuroimaging studies cited in Nolen-Hoeksema et al. (2008) indicated that the mPFC, a central locus in emotional processing and self-regulatory behaviour, is highly active during ruminative processes, along with the amygdala (Ray et al., 2005; Siegle et al., 2002). More recent studies have expanded these findings, highlighting functional connectivity (FC) involving the default mode network (DMN). A key node of the DMN is the ventral subregion of the mPFC (vmPFC), which is considered central in self-referential thought processes and emotional regulation (Cooney et al., 2010; Lemogne et al., 2009, Philippi et al., 2018; Qin & Northoff, 2011; Zhou et al., 2020). More specifically, the vmPFC has garnered attention for its role in relaying inhibitory signals from the dorsolateral PFC (dlPFC) to the amygdala—the area of the brain well known for recognition of the emotional aspects of information and the generation of negative emotions (Ghashghaei et al., 2007; Johnstone et al., 2007; Siegle & Thayer, 2003). The disruption of cortical inhibitory control has been posited as a mechanism facilitating sustained amygdala activity through positive feedback loops, given the known synergistic ("top-down" and "bottom-up") relationship between the vmPFC and amygdala (Ghashghaei et al., 2007; Johnstone et al., 2007; Siegle & Thayer, 2003). In sum, this delicate bidirectional balance is believed to be disturbed in MDD, with the amygdala exerting aberrant influence over core PFC processes (Johnstone et al., 2007).

While the concept of rumination cannot be directly modelled in animals given its inherent language-driven introspective processes (e.g., "inner speech"; Alderson-Day & Fernyhough, 2015), observing similar patterns in FC paralleled with the onset of depressive-like symptoms may yield additional insights into the pathophysiology of depression. Intriguingly, emerging preclinical research has pointed to potential homologous processes in rodent models of chronic stress. In a study of chronic restraint stress on rats, Henckens et al. (2015) reported heightened DMN-like FC (along with somatosensory and visual networks) in rats, indicative of

stress-related psychopathology. Further, Grandjean et al. (2016) reported heightened activation of prefrontal and cingulate cortices following a murine model of chronic psychosocial stress, resembling DMN FC. Taken together with the human neuroimaging studies discussed, dysfunctional inhibitory processing involving several PFC regions appear to be core to stress-induced rumination (like) behaviour and represent a putative mechanism for the pathogenesis of depression.

In addition to metabolic, HPA, and FC biomarkers, there have been many reports of chronic stress-induced alterations in excitatory-inhibitory balance involving prefrontal GABAergic and glutamatergic neurotransmission. A variety of higher-order functions, including working memory, decision-making, and emotional regulation are dependent on maintaining a delicate equilibrium between excitation and inhibition within the PFC (Fuster & Bressler, 2015). Indeed, reductions in GABA have been observed in depressed individuals, and deficient GABAergic signaling has been proposed as a mechanism underlying emotional dysregulation (Ghosal et al., 2017; Page et al., 2019; Shepard & Coutellier, 2018). In the PFC, local GABAergic interneurons—predominantly somatostatin (SST^+) and parvalbumin (PV^+)—are known to exert regulatory control over glutamatergic output neurons (Ghosal et al., 2017). Since these excitatory projections target various subcortical and brainstem regions involved in mood and emotion (e.g., central nucleus of the amygdala [CeA], dorsal raphe nucleus [DRN]), reduced upstream inhibition—or *disinhibition*—can result in a cascade of deleterious effects (Ghosal et al., 2017). Furthermore, enduring alterations in the synaptic integrity and function of principal excitatory neurons has been attributed to stress-induced release of glucocorticoids, and is characterized by neuronal atrophy, loss of synaptic connections, and even excitotoxic damage mediated by increased extracellular glutamate (Ghosal et al., 2017; Harmer et al., 2017; Papoli, 2012). In sum, these findings highlight the intricate interplay between stress, GABA and glutamate signalling, cognitive function, and emotional processing.

Additional support for the role of cognitive-affective deficits associated with PFC disinhibition in MDD relates to the reversal effects of cognitive therapy in humans. Cognitive-based interventions, such as cognitive-behavioural therapy (CBT), have demonstrated the ability to enhance PFC activity and effectively restructure maladaptive thinking (Pradhan et al., 2015). They have also been shown to be more effective in preventing relapse compared to conventional ADTs (Pradhan et al., 2015). Additionally, CBT specifically targeting rumination symptoms in residual depression has been shown to yield generalized improvements in depression and comorbidity measures, and hypothesized to reduce hyperconnectivity between the DMN and the sgPFC (Hamilton et al., 2015; Watkins et al., 2007).

1.2 Modelling MDD Onset in Mice

Models of chronic depression range from inducing mild to intense stressors that last anywhere from hours to weeks. Common examples include chronic social defeat, learned helplessness, corticosterone manipulation, and unpredictable chronic mild stress (Gururajan et al., 2019; Planchez et al., 2019). Among these, the unpredictable chronic mild stress (UCMS) model is regarded for its face, construct, and predictive validity (and overall reliability) in capturing key aspects of depression (Belzung & Lemoine, 2011; Nollet, 2021; Willner, 2017a, 2017b). Briefly, it involves exposing mice to a series of mild, unpredictable stressors over an extended period. A key aspect is stress (un)controllability, and the model can be likened to the persistent and frequently unmanageable stressors experienced by humans, such as workplace burnout, socioeconomic challenges, among other psychosocial stressors (Kessler & Bromet, 2013; Lam et al., 2022; Maslach & Leiter, 2016).

UCMS has been shown to induce depressive-like behaviours in mice, including anhedonia, social withdrawal, and cognitive impairments (Banasr et al., 2007; Willner, 2017b). Underlying these behaviours, this model has been found to disrupt neurobiological systems known to be involved in stress response, including the HPA axis, leading to dysregulation of stress hormones (Nollet, 2021; Willner, 2017b). In contrast to models using brief acute stressors,

the development of depressive-like symptoms over several weeks is considered to make the model more translationally relevant (Frisbee et al., 2015). Allostatic load is another potential translational aspect—the cumulative burden of chronic stress over time is considered to lead to a cascade of deleterious physiological effects (Guidi et al., 2020). Furthermore, UCMS has been adapted to model the progression of various symptoms over shorter and longer periods (e.g., Bambico et al., 2009; Farooq et al., 2018; Woodward et al., 2023; Zurawek et al., 2019), making it an attractive paradigm to investigate differential phenotype progression.

1.3 Potential Pathophysiological Roles of SK3 and NF-κB

The molecular mechanisms mediating chronic stress-induced dysfunction in the PFC are not fully understood. An avenue of active investigation relates to the impaired expression of inhibitory elements (Faber, 2009; Faber & Sah, 2003; Gargus, 2006). Small-conductance, calcium-activated potassium channels (SKC/KCa2) stand out as one of the most heterogeneous and ubiquitous inhibitory ion channels. Their role in producing medium afterhyperpolarization of neurons shortly after the action potential peak is well-established (Faber, 2009; Kshatri et al., 2018). Three subtypes of SKCs have been identified (SK1/KCa2.1 to SK3/KCa2.3), and are known to be driven by the genes Kcnn1–3. All three share a similar molecular architecture as Shaker-like voltage-gated potassium channels. Each subunit has six membrane-spanning hydrophobic alpha-helical domains, and three associated subunits (protein phosphatase-2A, casein kinase-2, and calmodulin elements) are involved in channel conductance through allosteric modulation; the calmodulin subunit is thought to account for the sensitivity to calcium transients within intracellular microdomains (Faber, 2009; Faber & Sah 2010; Kshatri et al., 2018). SKCs thus have the unique ability to couple intracellular calcium concentration with low threshold changes in potassium conductance and membrane potential. Calcium influx via postsynaptic muscarinic or NMDA receptors can therefore also modulate SKC activity (Faber, 2009; Giessel & Sabatini, 2010; Ngo-Anh et al., 2005). Accordingly, this regulates postsynaptic potentials, burst-firing, interspike interval distribution, and spike frequency adaptation.

Through these mechanisms, SKCs play a role in mediating activity-dependent and long-term potentiation-like plasticity that affect limbic behavioural function (e.g., emotion regulation and motivation), as well as stress adaptation (Faber, 2009; Faber & Sah, 2003, 2007; Kshatri et al., 2018; Nasheed et al., 2022).

Recent preclinical studies have shown SKC subtype-3 (SK3) overexpression or hyperactivity in the DRN and PFC (Sargin et al., 2016; Qu et al., 2020; Bambico et al., 2020). Following the UCMS model of depression, drug-mediated blockade of SKC conductance, instigated by muscarinic receptor inhibition, effectively led to depolarization-induced plasticity detected in the prelimbic (PrL) subregion of the mPFC (Bambico et al., 2020). This effect was associated with a rapid antidepressant-like response. Pharmacological inhibition or genetic deactivation of SK3 has also shown antidepressant-like activity (Nashed et al., 2022). By contrast, stress-induced glucocorticoid release is known to modulate calcium mobilization and rapidly enhance the transcription and expression of SKC via glucocorticoid type II receptors (Kye et al., 2007; Levitan et al., 1991; Shipston et al. 1996; Tian et al., 1998). In addition, evidence for epigenetic regulation under pathological conditions has also been recently found (Cadet et al., 2017).

A potential driver of SK3 upregulation is signalling pathways involved in inflammation. Kye et al. (2007) demonstrated that the SK2 promoter was transcriptionally enhanced by both glucocorticoids and nuclear factor-kappa B (NF-κB), which is considered to regulate the expression of proinflammatory cytokines, chemokines, and adhesion molecules. When activated in response to psychosocial stressors, NF-κB has been found to promote the transcription of these proinflammatory mediators, contributing to the initiation and amplification of inflammatory responses (Bierhaus et al., 2003; Kye et al., 2007). Intriguingly, genetic deactivation of the large-conductance calcium-activated potassium (BK) channels resulted in the dampened response of the HPA axis to restraint stress (Brunton et al., 2007), whereas activation of SK2 in the hypothalamic PVN by 1-EBIO upregulated SK2 expression and diminished

stress-induced visceral sensitivity (Ji et al., 2021). Thus, SKCs and associated elements may serve as therapeutic molecular targets for rapid and effective relief of stress-induced conditions such as depression.

1.4 Study Overview

Given the waning of the "serotonin hypothesis," the investigation of additional depressive disorder etiologies—particularly those related to cognitive symptoms—is paramount (Moncrieff et al., 2022; Shilyansky et al., 2016). The present phenotyping study aimed to extend knowledge into behaviours and mechanisms underlying prodromal rumination-like cognitive symptoms in mice. The UCMS model and relevant behavioural assays were employed, followed by biosensor recordings of glucose activity in the mPFC and DRN in response to a DEX (GR agonist) challenge. Along with assessing the differential metabolic activation of the mPFC and DRN, it was determined whether the metabolic status is dependent on the UCMS stage: 2–3 weeks for early symptoms (short-term; ST-UCMS) vs. 5–6 weeks for later symptoms (long-term; LT-UCMS). Additionally, given its potential role in SKC upregulation, NF-κB concentrations were analyzed through ELISA. Finally, RNAscope carried out by Wan-Yan-Chan (2023) were referenced to provide insight into differential alterations of cortical SK3 expression in GABAergic interneurons following ST-UCMS, along with *in vivo* electrophysiology pilot data (unpublished) measuring basal firing rates of GABAergic SST^+ interneurons to corroborate regional metabolic statuses.

Our hypotheses were as follows: (a) ST-UCMS stressed mice will show significant deficits in cognitive-affective behavioural measures (social interaction test, splash test, spontaneous alternation test), but not yet in behavioural measures of anxiety or anhedonia (novelty-suppressed feeding test, sucrose test); (b) ST-UCMS stressed mice will show a significant increase in metabolic activity in the mPFC, but not yet in the DRN, in response to GR agonist challenge (DEX); (c) LT-UCMS stressed mice will show significant behavioural measures of anxiety and anhedonia (previously listed tests), along with deficits across measures of

cognitive-affective behavioural measures (previously listed tests); and finally, (d) LT-UCMS stressed mice will show a significant decrease in metabolic activity in both the mPFC and DRN in response to DEX.

Additionally, sex differences related to emotional dysregulation as a result of chronic stress have been previously documented: women have been shown to be twice as likely to experience emotional dysregulation after stress, leading to substantially higher psychopathology (Armstrong et al., 2018; Kessler et al., 2005). Behavioural and biomarker sex differences in rodents, including alterations to prefrontal GABAergic interneurons and brain-derived neurotrophic factor (BDNF), have also been documented in female mice (Monteggia et al., 2007; Page & Coutellier, 2019; Woodward et al., 2023). Thus, it was predicted that sex effects related to emotional loading may emerge in ST-UCMS stressed female mice across the aforementioned cognitive-affective behavioural measures.

2.0 Methods

2.1 Animals

Given their reliable responsiveness to stress-based paradigms (Ibarguen-Vargas et al., 2008; Malki et al., 2015; Yalcin et al., 2008), and akin to how personality in humans can predict susceptibility to stress (Lye et al., 2020), the inbred BALB/c mouse strain was chosen for this study (Charles River Laboratories, PND 42–76; equal numbers female/male). The sample sizes were as follows: n = 36 for ST-UCMS (18 stress, 18 controls) and n = 16 for LT-UCMS (8 stress, 8 controls). Upon arrival, mice were randomly split into separate standard laboratory rooms—UCMS and control—and habituated for a period of one week. Control mice were group-housed by sex to eliminate the stress of single housing (2–3 per cage; ear tagged for identification), while the experimental mice were singly housed per the UCMS protocol (Nollet, 2021). Both UCMS and control rooms maintained a 12-h reverse light-dark cycle, except for when UCMS mice were subjected to light disturbance stressors. Both rooms maintained a constant temperature (20–23°C) and all mice were given *ad libitum* access to food and water.

All conditions and procedures were conducted in accordance with the Canadian Council on Animal Care Guidelines and approved by Memorial University of Newfoundland's Animal Care Committee.

2.2 UCMS Model of Depression

The UCMS model was selected given its widely reported translatability, validity, and consistency in eliciting depressive-like behaviours. The model involves exposing mice to a series of mild stressors with unpredictable timing. Due to its very nature, the selection, sequencing, frequency, and duration of stressors in UCMS varies significantly (e.g., Belzung & Lemoine, 2011; Nollet, 2021; Willner, 2017a, 2017b). The following commonly described stressors were included in this study: cage tilt (45° or -45°), aversive smell exposure (granularized fox urine), light cycle disruption (overnight illumination), damp bedding (25–30°C tap water), bedding removal, shallow bath (25–30°C tap water), confined space/restraint (100 ml shakers with glass windows and air holes), stroboscope (continuously rotating RGB LEDs), high frequency sound exposure (ultrasonic pest repellers), and oscillation in restraint (orbital mixer at 100 rpm). Three stressors occurred daily, scheduled pseudorandomly throughout the morning, afternoon, and evening, Monday through Friday. (Sucrose testing occurred Saturday through Sunday; see Sucrose Test.) Stressors lasted 30–180 minutes, with the exception of those scheduled overnight. See Table 1 for a representative weekly schedule. To address potential confounding factors between stress and control groups, control animals were exposed to handling and 60-min exposure to small novel objects, scheduled pseudorandomly, Monday through Friday, 2–3 times per week. Finally, previous studies have shown some depressive-like symptoms begin to emerge following 2 weeks of UCMS, while more advanced profiles including anhedonia-like symptoms can take 5 weeks or more (Bambico et al., 2009; Farooq et al., 2018). Thus, two UCMS lengths were defined: 2–3 weeks (short-term stress; ST-UCMS group) and 5–6 weeks (long-term stress; LT-UCMS group).

2.3 Behavioural Tests

2.3.1 Sucrose Test

Reduced consumption of palatable solutions, e.g., containing sucrose, serves as a well-established behavioural indicator of a deficit in hedonic experiences, or a state resembling depressive behaviour (Pothion et al., 2004; Moreau et al., 1992; Willner et al., 1987). Testing of sucrose consumption was performed to ascertain depressive-like symptom progression following LT-UCMS exposure, and to verify no anhedonia-like behaviour was emerging following ST-UCMS exposure. Both UCMS and control groups were tested for sucrose solution consumption compared to water following each week of the stress paradigm. Testing occurred between Saturday 15:00 and Sunday 10:00, and no UCMS stressors were scheduled on these days. Within this window, all mice were singly housed and given 50 mL of 4% sucrose solution and 50 mL tap water in separate cups. The cups were partially covered to prevent buildup of debris and secured in a plastic holder at the front of the cage. Both cups were weighed (in grams to two decimal places) before and after the testing period to determine differential consumption. While sucrose preference to water following UCMS is considered a reliable measure of anhedonia for rats and some mice breeds (e.g., C57BL/6), it has been shown to be an insensitive measure for several mice strains, including BALB/c (Surget & Belzung, 2008). Accordingly, per Strekalova and Steinbusch (2010), sucrose consumption was determined by comparing absolute intake between groups.

2.3.2 Novelty-Suppressed Feeding Test

Novelty-suppressed feeding (hyponeophagia) is a longstanding measure of anxiety commonly used in stress-based models of depression. It assesses latency to eating in a novel environment following a period of food deprivation (Deacon, 2011; Samuels & Hen, 2011). The test was performed to assess anxiogenic-like symptoms following long-term stress exposure, and to assess any emergence of this behaviour following short-term stress exposure. Approximately

50% of individuals diagnosed with depression also meet the criteria for a co-occurring anxiety disorder, and more than 90% experience symptoms of anxiety (Kennedy, 2022; Regier et al., 1998). Thus, this assay is highly predictive of depression-related dysfunction. In our experiment, food was removed from homecage hoppers 14–18 hours prior to testing. Mice were brought to a novel, brightly lit testing room and individually placed at one end of a plexiglass arena (60 x 25 x 25 cm) with white walls and black floor. A highly palatable but novel substance (a single Froot Loop®) was placed at the centre of the opposite end, approximately 10 cm from the end wall. Once the divider was lifted from the starting chamber, the timer was started, and mice were able to roam the arena for up to 5 minutes. The latency to consumption, defined as eating continuously for 2–3 seconds (Deacon, 2011), was recorded by the experimenter who was positioned several metres from the chamber.

2.3.3 Social Interaction Test

The social interaction test (SIT) has been in use for nearly 50 years as a measure of anxiety, and is considered highly naturalistic given the behavioural endpoints are related to social investigation of conspecifics (File & Seth, 2003). Generally, rodents have a natural tendency to spend more time with conspecifics (sociability) and show greater interest in exploring new, unfamiliar conspecifics over those already known (social novelty) (Kaidanovich-Beilin et al., 2011). Since short-term stress exposure was not expected to result in significant changes in anxiety (Shepard et al., 2016), the test was selected more as a measure of cognitive functioning; complex decision making is involved in social affiliation/motivation, social memory, and novelty seeking (Bicks et al., 2015; Xing et al., 2021). With this in mind, following File and Seth (2003), a familiar environment (i.e., a testing room and arenas the mice were already habituated to) and dim lighting were employed to reduce an anxiogenic response. Two successive trials were performed, each 5 minutes in duration. In the first, two wire mesh cages (10 × 6.5 × 5 cm) were placed at opposite ends of the aforementioned arena—one empty and one with a conspecific. (Cages of this type allow for visual and olfactory interactions, but

prevent direct contact.) The mice were first placed in the centre zone, video recording was started, divider doors were removed. After 5 minutes of free exploration, the dividers were replaced, mice were moved back to the centre zone, and a novel conspecific was brought to the previously empty cage. Following removal of the dividers, the mice were then given 5 more minutes to explore freely. EthoVision XT (Nodulus) was then used to analyze behavioural differences, with 3 cm interaction zones defined around each cage. The endpoints analyzed, based on Moy et al. (2004), were sociability (an index of the time spent engaging with a conspecific vs. empty cage) and social novelty (an index of the time spent engaging with the novel vs. familiar conspecific).

2.3.4 Splash Test

Apathy relates to a lack of emotion, interest, or concern; experimentally, it is the observable reduction of voluntary, goal-directed behaviours (Levy & Dubois, 2006). The splash test is considered to quantify grooming motivation, and is said to be a reliable measure of apathetic behaviour in the context of depression models (Isingrini et al., 2010; Levy & Dubois, 2006; Willner, 2005). Given the underlying mechanisms of apathy are considered to have a cognitive component (i.e., elaborating plans for ongoing or forthcoming behaviours) (Levy & Dubois, 2006), the test was chosen as a measure of potential cognitive dysfunction following short-term stress exposure. The procedure consisted of squirting a 10% sucrose solution on the mouse's dorsal coat (two sprays at close proximity) in a familiar testing arena. Immediately following the application of the sucrose solution, a timer was started and mice were video recorded from above for a period of 5 minutes. BORIS (behavioural Observation Research Interactive Software; Friard & Gamba, 2016) was used to quantify grooming duration. Per Kalueff and Tuohimaa (2004), the following grooming behaviours were captured: nose/face grooming (strokes along the snout), head washing (semicircular movements over the top of the head and behind the ears), and body grooming (licking of the body fur). To prevent experimenter bias throughout analysis, coders were blinded to the treatment group.

2.3.5 Spontaneous Alternation Test

The spontaneous alternation test (SAT) is another well-validated assay of cognitive behaviour in rodents. It relies on the innate inclination of rodents to favour exploring new arms of a maze rather than familiar ones (d'Isa et al., 2021). Critical to this task is spatial working memory, a cognitive buffer that stores spatial information and allows for its manipulation to guide decision-making (d'Isa et al., 2021; Malenka et al., 2009). Given spatial working memory relies on intact prefrontal cortical functions (Kraeuter et al., 2019), and that the task is generally not considered emotionally loaded (Petchkovsky & Kirkby, 1970), SAT serves as a useful evaluation of non-affective cognitive behaviour following short- and long-term chronic stress exposure. The procedure involved placing mice in the centre of a white-walled, Y-shaped maze on a black floor (three open arms 35 × 5 cm with a 15 cm central radius, with 120° angles between arms). Mice were videotaped from above for a period of 7 minutes. EthoVision XT (Nodulus) was then used to quantify an alternation index, defined as Total Alternations ÷ Max Alternations. To successfully alternate, mice needed to recall which arms had previously been visited; fewer reentries resulted in a higher score.

2.4 mPFC and DRN Glucose Quantification

Mice underwent a surgical procedure to insert guide cannulas at the regions of interest (Figure 1), into which glucose-sensitive biosensor probes with integrated Ag/AgCl (Pinnacle Technology Inc.) were inserted to quantify changes in metabolic activity throughout a dexamethasone (DEX) challenge. The following sections outline the surgical, recording, and data processing procedures.

2.4.1 Surgical Procedure

Mice were first habituated to the surgery room 30 minutes prior to surgery. Mice were first anesthetized with isoflurane in an induction chamber, then transferred to the stereotaxic frame (David Kopf Instruments), where a maintenance flow of anesthetic was kept throughout

the procedure. Meloxicam (4 mg/kg) was administered subcutaneously for analgesia and Opthagel was applied for corneal protection. The skull was then exposed, cleaned of connective tissue, and dried. Two holes were drilled relative to bregma: one to access the (right) mPFC (anterior-posterior [AP]: 1.9 mm, medial-lateral [ML]: -0.3 mm) and the other to access the DRN (AP: -4.3 mm, ML: 0 mm). Cannula guides were stereotaxically lowered to the appropriate depths (dorsal-ventral: -2.5 mm, from the surface of the brain, for both mPFC and DRN). Then, dental ceramic compound was applied, first surrounding the guides, then over the rest of the exposed skullcap. Once the compound solidified, isoflurane was gradually reduced and mice were injected intraperitoneally (IP) with a maintenance dose of urethane anesthetic (0.75 mg/kg).

2.4.2 DEX Challenge

Immediately following the surgical procedure, mice were rested on a warming pad, the biosensor probes were inserted, and simultaneous activity recording from the two regions was initiated through Sirenia® Acquisition software (Pinnacle Technology Inc.). A 30 minute baseline was established, then the DEX challenge was started. Mice were injected IP every 30 minutes with DEX (Sigma; saline vehicle) following a dose response curve (0.1 mg/kg → 1.6 mg/kg), for a total of five injections. 30 minutes after the final injection, recordings were stopped and mice were euthanized through cervical dislocation. Brains were extracted and immediately flash-frozen on dry ice, then stored at -80°C.

2.4.3 Data Capture and Processing

Throughout a 180-minute recording period, second-by-second changes in metabolic activity were simultaneously measured in the mPFC and DRN. Electrodes transduced signals initiated from the biosensors' enzyme-mediated processing of glucose concentration to a preamplifier (Pinnacle Technology Inc.), which boosted the signals into nanoamperes (nA). Digital readouts were recorded through Sirenia® Acquisition software (Pinnacle Technology

Inc.). To analyze these data, time bins were formed to obtain average activity over each one-minute interval (60 rows = 1 bin). The final 10 minutes of each animal's 30-minute baseline period were averaged to form baseline measurements by region. Each subsequent bin was then calculated for its percent change from baseline: ([raw data] – [baseline]) / [baseline] × 100. Average changes from baseline were then calculated.

2.5 ELISA for NF-κB

As discussed, SKCs have been shown to be transcriptionally enhanced through the pro-inflammatory transcription factor NF-κB (Kye et al., 2007). The brain samples collected were thus analyzed for NF-κB concentrations through ELISA (BioMatik, EKN47464-96T). Tissue homogenates from the two target regions were prepared following the manufacturer's protocol then immediately stored at -20°C. On the day of the procedure, kit components and samples were brought to room temperature (18-25°C), the standard was prepared, and incubation and concentration readings were performed following the manufacturer's protocol. A BioTek Epoch 2 Microplate Spectrophotometer with Gen5™ software was used for incubation and data capture. The standard curve and analysis/modelling was performed using GainData (Arigo Biolaboratories). A Five Parameter Logistic (5PL) Regression model was used (R^2 = 0.988).

2.6 Statistical Analysis

Jamovi (The Jamovi Project, 2022) was used for all statistical analyses. Data throughout are displayed as the mean ± standard error of the mean (SEM). Statistical analyses were performed using t-tests as appropriate. ST-UCMS and LT-UCMS experiments were conducted independently, each with its own PND-paired control group; consequently, analysis for each group was performed and presented separately. Sex differences were also analyzed independently by subgroup (e.g., 'ST-UCMS females' vs. 'ST control females'). If the assumptions of variance homogeneity and normality of data distribution were not satisfied,

nonparametric tests were employed as alternatives. Differences were considered statistically significant at $p < .05$. The following significance indicators are used in graphs: $*p < .05$; $**p < .01$; $***p < .001$. Cohen's *d* was used to report effect size, aside from nonparametric tests (e.g., Mann–Whitney *U*), for which rank-biserial correlation (r_{rb}) was used.

3.0 Results

3.1 Anhedonia-Like Symptoms in LT-UCMS, Not ST-UCMS

As discussed, several weeks of UCMS have been shown to reliably induce anhedonia-like symptoms in mice. Reduced sucrose consumption is considered indicative of the reduced motivational and reward-seeking behaviours seen in MDD. In line with our hypotheses, no significant difference in absolute sucrose consumption were seen between ST stress and ST control animals following 2 weeks of UCMS, $t(10.2) = -.150$, $p = .558$, $d = -.075$, while significantly less sucrose was consumed by LT stressed mice vs. LT controls following 6 weeks, $t(10.9) = -1.987$, $p = .036$, $d = -.994$ (Figure 2).

3.2 Anxiety-Like Symptoms in LT-UCMS, Not ST-UCMS

Several weeks of UCMS have also been shown to reliably induce anxiety-like symptoms in mice as measured by the novelty-suppressed feeding test. Also in line with our hypotheses, Mann–Whitney *U* tests indicated no significant differences in latency to feeding between ST-UCMS and ST controls, $U = 145.0$, $p = .395$, $r_{rb} = .052$, while LT-UCMS resulted in a significant increase in latency to feeding for stressed mice vs. LT controls, $U = 13.0$, $p = .025$, $r_{rb} = .594$ (Figure 3).

3.3 Sex Differences in Social Interaction in ST-UCMS

Alternations in social interactions, including social motivation and social memory, are well documented across the development of depressive disorders, and are closely related to disruption to cognitive, affective, and reward-seeking processes (Bicks et al., 2015). Sociability, a measure of social motivation, is defined as an index of how much time the mouse spent

investigating the novel mouse compared with the empty cage (the first phase of the test). In our experiment, this was shown to be insignificant for both ST-UCMS vs. ST controls, $t(34) = .038$, $p = .515$, $d = .013$, and LT-UCMS vs. LT controls, $t(14) = 1.32$, $p = .896$, $d = .660$, indicating there were no confounding effects of social aversion on the second phase (Figure 4).

Preference for social novelty (the second phase of the test) saw no significant differences for ST-UCMS vs. ST controls overall, $t(32) = -1.227$, $p = .114$, $d = -.421$; however, when analyzing by sex (independent comparisons by subgroup), it was found that ST-UCMS stressed females spent significantly less time investigating the novel conspecific compared with ST female controls, $t(14) = -2.363$, $p = .017$, $d = -1.191$ (Figure 4). No significant differences for LT-UCMS vs. LT controls were observed, $t(14) = .109$, $p = .543$, $d = .055$, potentially indicative of stress adaptation (Figure 4).

3.4 Reduction in Grooming Behaviours in ST-UCMS

In the splash test, time spent grooming (duration) and the delay to first grooming behaviour (latency) are indicative of reduced motivation. While no significant differences for ST-UCMS were found overall for duration, $t(27.3) = -1.550$, $p = .067$, $d = -.516$, or latency, $t(29.4) = -1.040$, $p = .154$, $d = -.346$, when analyzing by sex (independent comparisons by subgroup), two differences were identified: ST-UCMS males spent significantly less time grooming compared with ST male controls, $t(10.8) = -2.015$, $p = .035$, $d = -.950$, and ST-UCMS females took significantly longer to initiate grooming compared with ST female controls, $t(11.5) = 1.796$, $p = .049$, $d = .847$ (Figure 5). No significant differences were seen for LT-UCMS vs. LT controls, in duration, $t(14) = -1.199$, $p = .125$, $d = -.600$, or latency, $t(14) = .630$, $p = .731$, $d = .315$. This is potentially indicative of a waning effect of the UCMS procedure related to the cognitive control and adaptation aspects of stress controllability (Koolhaas et al., 2011).

3.5 Spatial Working Memory Deficits in LT-UCMS, Not ST-UCMS

In the spontaneous alternation Y-maze task, an evaluation of spatial working memory, no significant differences were seen for ST-UCMS, $t(31) = .716$, $p = .760$, $d = .250$, while LT-UCMS led to a significant decrease in successful alternations for stressed mice vs. LT controls, $t(14) = -2.02$, $p = .032$, $d = 1.01$ (Figure 6). The differences in performance are potentially explained by the previously discussed supposition that early cognitive deficits in depression are considered to have more emotional loading (affective and motivational components), compared with broad cognitive deficits also encompassing basic functions such as working memory.

3.6 Differential Alternations in mPFC and DRN Activity for ST- and LT-UCMS

Simultaneous glucose biosensors were used to record neurochemical concentration changes throughout a DEX (glucocorticoid receptor [GR] agonist) challenge. Differential alterations in regional activity were detected in both regions of interest, in both UCMS groups. These are presented below by brain region.

3.6.1 Significantly Higher mPFC Activity Following ST-UCMS, Insignificant Difference in mPFC Activity Following LT-UCMS

A Mann–Whitney *U* test indicated that ST-UCMS stressed mice showed a significantly higher percent activity change from baseline in the mPFC across the recording duration compared with ST controls, $U = 6.00$, $p = .037$, $r_{rb} = .657$ (Figure 7). On the other hand, a Mann–Whitney *U* test indicated that LT-UCMS did not lead to a significantly lower percent activity change from baseline in the mPFC of stressed mice across the recording duration vs. LT controls, $U = 8.00$, $p = .210$, $r_{rb} = .360$ (Figure 7).

3.6.2 No Detectable Changes in DRN Activity Following ST-UCMS, Significantly Lower DRN Activity Following LT-UCMS

No detectable differences in percent activity change were seen in ST-UCMS vs. ST controls in the DRN across the recording duration, $U = 14.00$, $p = .639$, $r_{rb} = .200$ (Figure 8). On the other hand, LT-UCMS showed a significantly lower percent activity change from baseline in the DRN across the recording duration compared to LT controls, $U = 1.00$, $p = .008$, $r_{rb} = .920$ (Figure 8).

3.7 Trending NF-κB Concentration Increase in mPFC for ST-UCMS (ELISA)

A Mann–Whitney *U* test indicated that there was an insignificant, but potentially trending, higher level of concentration of NF-κB in the mPFC for the ST-UCMS vs. ST controls, $U = 1.00$, $p = .095$, $r_{rb} = .800$ (Figure 9). No significant differences in concentrations for the same groups were observed in the DRN, $U = 9.00$, $p = .635$, $r_{rb} = .100$ (Figure 9).

4.0 Discussion

This study investigated the emergence of depressive-like phenotypes in BALB/c mice following two UCMS durations: short-term (2–3 weeks; ST-UCMS) and long-term (5–6 weeks; LT-UCMS). Given the putative role of prodromal cognitive symptoms related to negative thought patterns in MDD, we sought to determine if early cognitive deficits, accompanied by disruptions to prefrontal inhibitory processes, would emerge in mice. A range of behavioural assays, simultaneous biosensor recordings of metabolic activity, and proinflammatory transcription factor concentration measurements—along with contributed RNAscope and electrophysiology data—combine to evolve our understanding of the neurobiological mechanisms underlying the onset of depressive-like symptoms following distinct temporal trajectories of chronic unpredictable stress.

Our results indicate the appearance of a cognitive-affective phenotype following short-term chronic stress exposure, and both a cognitive- and mood/anhedonic-related

phenotype following longer-term exposure. Additionally, sex differences in some behavioural tests were observed and seem to parallel sex-specific vulnerability factors seen by others studying chronic stress models. Alongside behavioural changes, we observed potential pathophysiological changes in regional metabolic activity and lower firing rates of GABAergic interneurons (SST) and serotonergic neurons (5-HT). These differences in inhibitory capacity are potentially explained by higher SK3 expression observed in mPFC GABAergic interneurons, and trending increases in proinflammatory transcription factor NF-κB. The sections that follow integrate our findings, review our experimental limitations, and provide directions for future research.

4.1 Chronicity and Sex Differences in Depressive-Like Behaviours

Revisiting our hypotheses related to the behavioural measures, we expected ST-UCMS would result in significant differences in behavioural measures of cognitive-affective dysfunction, but not yet in measures of anxiety or anhedonia. Partially consistent with our hypotheses, we saw significant results in two tests with cognitive components: the social novelty measure of the social interaction test, as well as the latency and duration measures of the splash test. And consistent with our hypotheses, we saw no significant differences for this group in sucrose consumption or the novelty-suppressed feeding test—tests related to anhedonia and anxiety.

The social interaction test (SIT), which serves as an indicator of sociability (social motivation) and social memory (recognizing others previously encountered), holds significant relevance in the testing of cognitive processing; impairments in these measures have long been implicated as hallmarks across psychiatric disorders (Bicks et al., 2015; Kaidanovich-Beilin et al., 2011). Rodents exhibit an inherent inclination to spend more time with conspecifics and display heightened interest in exploring unfamiliar peers compared to familiar ones, reflecting their sociability and response to social novelty (Kaidanovich-Beilin et al., 2011). Complex decision-making processes underlie social motivation, social memory, and novelty seeking.

Collectively, successful social cognition entails the intricate integration of various behavioural aspects, encompassing salience, reward-seeking, motivation, self and other awareness, and adaptive adjustment within groups (Bicks et al., 2015; Xing et al., 2021). The ability to discern between familiar and novel conspecifics, for instance, plays a pivotal role in establishing preferential social interactions crucial for survival (Bicks et al., 2015). Notably, the mPFC assumes a vital regulatory role in social cognition, with evidence suggesting homologous regions between the rodent and human mPFC, including the ventromedial prefrontal cortex (vmPFC) and dorsomedial prefrontal cortex (dmPFC) (Bicks et al., 2015).

In our experiment, sociability, which was defined as an index of how much time the mouse spent investigating the novel conspecific compared with the empty cage (phase one), did not differ between control and stressed groups (both ST-UCMS and LT-UCMS), indicating no confounding effects of social aversion for social novelty (phase two). This result is unsurprising given differences in this facet have typically been associated with more intense paradigms, such as the chronic social defeat model of depression (e.g., Okamura, 2022). Additionally, related to our hypothesis of disrupted cognitive circuits following chronic stress, it has been documented that both inhibition and transection of the mPFC have not been shown to reduce social investigation, which indicates it is not a primary modulator of this behaviour (Gonzalez et al., 2000; Xing et al., 2021).

In the second phase of the test, no significant differences for ST-UCMS were observed overall; however, after further analysis it was found that ST-UCMS females spent significantly less time investigating the novel conspecific compared with female ST controls. This result was intriguing as it seems to parallel recent evidence of sex-specific trajectories of anxiety- and depressive-like symptoms onset following UCMS. Female mice have been shown to experience chronic stress-induced emotional dysfunction sooner than their male counterparts (e.g., 4 weeks vs. 8), including in measures of anxiety, social behaviour, and working memory (Page & Coutellier, 2019; Woodward 2023). While sex effects are often attributed to the phase of the

female estrus cycle, this variability has also been questioned (Prendergast et al., 2014; Ramos-Ortolaza et al., 2017). Woodward et al. (2023) have speculated that the differences in cognitive processing of emotionally laden behaviours following stress are in part due to the neuroadaptations underlying differential increases in FosB activation in mPFC PV^{+} neurons. Further, Barko et al. (2019) have shown that chronic stress increased GABA-related and glutamate-related gene expression in the mPFC of female (but not male) mice. Female prefrontal neurons projecting to the amygdala have also displayed dendritic changes following periods of stress (Shansky et al., 2010). While not yet fully understood, any potential sex differences influencing this result are also said to vary along a spectrum, with some individuals expressing full resilience while others expressing high vulnerability (Woodward et al., 2023).

Related to the mechanisms underlying social memory encoding, recent discoveries employing optogenetics have implicated certain subpopulations of mPFC neurons, described as thin-tufted dopamine receptor (D1R)-expressing and oxytocin receptor (OXTRs)-expressing glutamatergic pyramidal neurons, as being involved in the production of engram cells (Tan et al., 2019; Xing et al., 2021). Excitatory-inhibitory imbalance in neighbouring circuits following chronic stress exposure could be affecting these subpopulations. Considering that both studies exclusively involved male mice, replication with females is necessary to strengthen the confidence in this interpretation.

Contrary to our initial hypothesis where we indicated LT-UCMS would also result in significant changes in cognitive measures, we saw no significant differences between stressed and LT control mice in SIT, overall or by sex. Looking at the literature, this is perhaps unsurprising as Nazir et al. (2022) also saw no effect in UCMS with BALB/c under similar conditions, and we speculate that this could again be indicative of stress adaptation following several weeks of the UCMS procedure (Koolhaas et al., 2011).

The splash test, considered a quantitative test of goal-directed behaviour (grooming), is considered to be one of the most consistent assays in demonstrating behavioural deficits

associated with disorders involving the PFC (Levy & Dubois, 2006). The associated depressive symptom, apathy, involves disruption to normal cognitive, emotional, or social functioning; in humans, these can include showing less persistence in maintaining activities or conversations, taking longer to make decisions, showing less interest in personal health or image, and diminished emotions or social interactions, among other changes (Steffens et al., 2022). While cognitive elements were considered to be involved in the splash test, a reduction in grooming behaviour compared to ST controls for ST-UCMS stressed mice, but not LT-UCMS stressed mice vs. LT controls, was unexpected given apathy is generally associated with more advanced depression symptomatology and relates more to executive function and dopamine transmission than affective processing (Floresco & Magyar, 2006; Steffens et al., 2022). The dlPFC has been found to be recruited during motivationally-related tasks (Levy & Dubois, 2006), and is not generally associated with the previously discussed stress controllability processes of the vmPFC. Recent discussion, however, has brought to light that studies on apathy have largely measured older adults with more advanced disease states (and low sample sizes) (Steffens et al., 2022). It is important to consider the modulating aspects of more medial circuits given their role of integrating information related both to the external (sensory) and internal (limbic) spheres (Bonelli & Cummings, 2007). Indeed, as part of the frontostriatal circuit, the dorsomedial and ventromedial PFC (dmPFC and vmPFC) have also been shown to be functionally active both in motivational states and within the DMN (Andrews-Hanna et al., 2010; Bonelli & Cummings, 2007; Xu et al., 2016). As inferred by Levy and Dubois (2006), marked sensitivity to emotionally negative situations may induce negative bias, interfering with attentional resources and executive functioning, and potentially resulting from contrasting alternations in metabolic activity in neighbouring circuitry. Thus, given the suspected role of recent chronic stress in increasing levels of negative bias resulting in heightened DMN activity in the early stages of depressive symptoms, this hyperactivity may be “distracting” mice from their normal grooming behaviours and potentially relate to the anticipation of stressors.

For LT-UCMS, the lack of significant difference in the splash test may indicate that these anticipatory behaviours had subsided due to chronic stress adaptation (Koolhaas et al., 2011). Further, in rodents, contextual variables related to the environment and their predispositions are considered to play an important role in motivated behaviours (Berridge, 2004). As discussed, BALB/c mice are known to be sensitive to the effects of stress, so there may also be an effect of strain differences at play.

Turning to the spontaneous alternation Y-maze task, contrary to our initial hypothesis, we saw no significant differences between ST-UCMS stressed mice and ST controls. We had anticipated reduced alternations in stressed mice compared to controls given the test was considered reliant on cognitive functioning. We did however observe a significant difference between LT-UCMS stressed mice and LT controls, which aligned with our hypothesis. Our results parallel a previous study demonstrating that inescapable stress led to impaired retrieval of previously visited maze arms (Bats et al., 2001). In this case, the authors thought the reduction in spatial memory could be resulting from the rapid activation of GRs within the hippocampus (HC), a brain area known for its involvement in this particular cognitive function. Impaired spinogenesis, synaptogenesis, and neurogenesis in the HC (i.e., an overall decrease in PFC–HC activity) is also expected following longer-term chronic stress (Bambico & Belzung, 2013; Grimm et al., 2015; Willner, 2017). Hock and Bunsey (1998) reviewed the literature on spatial memory in rats and noted the essential role of the dorsal hippocampus, rather than ventral, in spatial memory. This fits with our understanding of how neurogenesis through the ventral HC (anterior HC in humans) may play a role in mediating antidepressant effects related to affect and stress (Mahar et al., 2014). Perhaps the lack of spatial working memory deficits seen in ST-UCMS stressed mice is related to evidence that the mPFC is thought to control internal risk assessment (a highly emotional process) through connections with the ventral HC (McNaughton & Corr, 2018); it is in the ventral region that we would expect dysfunction following shorter-term chronic stress. McNaughton and Corr (2018) also touched on the

potential overlap between risk assessment and rumination, summarizing it as less of a proximal threat and more of a slower reprocessing of information internally, involving the scanning of memories. This may also relate to Roise and Sahakian's (2013) contrast of "hot" (emotion-laden) and "cold" (emotion-*independent*) cognition in humans. Cold cognitive impairments are considered to be reliably present in an MDD diagnosis, and are distinct from the more affective-laden cognitive deficits hypothesized in more prodromal symptomatology.

Finally, revisiting the results of our sucrose consumption and novelty-suppressed feeding test (NSFT), there were no significant differences seen for ST-UCMS stressed mice vs. ST controls, while LT-UCMS stressed mice showed significant reductions in both measures vs. LT controls. Several weeks of UCMS have been shown to reliably induce anhedonia-like symptoms in mice; moveover, the sucrose test is considered confirmation that UCMS is effective (Pothion et al., 2004; Willner et al., 1987); reduced sucrose consumption is considered indicative of reduced motivational and reward-seeking behaviours, as seen in MDD. In line with our hypotheses related to the progression of anhedonia-like symptoms, no significant differences in absolute sucrose consumption were found following 2 weeks of chronic stress (ST-UCMS), while significantly less sucrose was consumed following 6 weeks. These effects have been repeatedly demonstrated to be associated with dysfunction of the HPA axis, as well as decreased release of dopamine (DA) in the nucleus accumbens (NAc) in response to reward (along with increasing DA release in the NAc in response to aversive stimulation (Di Chiara et al., 1999; Willner, 2017).

Several weeks of UCMS have also been shown to reliably induce anxiety-like symptoms in mice through the NSFT. This assay is based on the natural conflict between the drive to explore a novel environment and the motivation to consume food (Deacon, 2011). We interpret that the lack of significant differences in the ST-UCMS group may be attributable to an activated adaptive stress response system that aids in coping with the immediate challenges, especially in younger organisms (Amat et al., 2006; McEwen & Morrison, 2013). The initial response to stress is considered to involve the activation of the HPA axis, leading to the release of stress

hormones such as corticosterone. This acute stress response can enhance cognitive and emotional functioning, which might explain the absence of anxiety-like behaviours in the NSFT during the early stages of stress exposure (McEwen & Morrison, 2013). In contrast, LT-UCMS is considered to lead to maladaptive changes in the brain and dysregulation of stress response systems. Prolonged activation of the HPA axis can result in an exaggerated stress response, altered neurotransmitter signaling, and structural changes in brain regions involved in emotional regulation, such as the prefrontal cortex, amygdala, and hippocampus. These alterations contribute to the development of anxiety-like behaviours (McEwen, 2008).

4.2 Elucidating ST-UCMS and LT-UCMS Behavioural Deficits

To explore the potential pathophysiological phenomena underlying the ST- and LT-UCMS behavioural profiles observed, we captured and analyzed regional metabolic activity following evoked stress response (dexamethasone [DEX]), firing rates of mPFC inhibitory interneurons (SST) and DRN serotonergic neurons, concentration levels of a proinflammatory transcription factor (NF-κB), and co-expression of ionic-level adaptations (SK3) with GABAergic interneurons (PV^+). First discussed are the simultaneous glucose biosensor recordings in the mPFC and DRN, where differential alterations in regional activity were detected in both groups.

4.2.1 ST-UCMS mPFC Hyperactivation

Consistent with our hypothesis, evoked stress response through IP injection of DEX on ST-UCMS led to a significant increase in metabolic activity in the mPFC as measured by glucose-sensitive biosensors (Figure 7). DEX is a glucocorticoid receptor (GR) agonist and is thus expected to mimic glucocorticoid increase. Glutamatergic pyramidal neurons are considered to be representing a large portion of this metabolic activity; they are known to be ubiquitous in the mPFC and for having a high density of GRs (Brown et al., 2005; Wellman, 2001). Given ST-UCMS was observed to show hyperactivation in the mPFC compared to the non-stressed ST controls, a potential sensitization effect (i.e., a tonic increase of corticosterone)

has occurred. Short-term stress has been shown to amplify stress responses of these neurons, while, in a biphasic manner, dampening their response following longer-term stress (Yuen et al., 2012). It has also been shown to alter dendritic morphology of layer II–III pyramidal neurons in the mPFC (Brown et al., 2005). While an increase in mPFC activity during stress has also been associated with stress resilience (Amat et al., 2006; Sinha et al., 2016), behavioural data for this sample suggest some deficits are present, and a lack of resilience may relate to the stress sensitivity of BALB/c mice (Ibarguen-Vargas et al., 2008; Malki et al., 2015; Yalcin et al., 2008). A potential mechanistic explanation for this lies in the reduction of inhibitory activity on pyramidal neurons through GABAergic interneurons. Given chronic stress, even in the short term, can instigate a cascade of proinflammatory effects, it is hypothesized that an upregulation of SK3 channels is resulting in a *disinhibitory* effect on mPFC pyramidal activity through impaired GABAergic interneuron firing. This explanation is supported by recent data (also using BALB/c mice) showing that ST-UCMS results in an increase in SK3 co-expression with GABAergic (SST^+ and PV^+) interneurons (Figure 10; Wan-Yan-Chan, 2023). Furthermore, *in vivo* electrophysiology pilot data have shown decreased basal firing rates of GABAergic SST interneurons (Figure 11; unpublished).

Findings from the LT-UCMS group, however, were not consistent with our hypothesis. We had suspected that longer-term chronic stress would result in hypoactivation of mPFC activity, in line with metabolic studies on both humans and animals (Kennedy et al., 2001; Price & Drevets, 2010; Yuen et al., 2012). Although not significant, the mean difference was lower for LT-UCMS, and the lack of a trend or significance in the opposite direction is potentially indicative of a gradual reversal from the hyperactivation seen in ST-UCMS stressed mice. Further, it is worth noting that some chronic stress investigators have used 8 weeks or more for their longer-term models (e.g., Frisbee et al., 2015). Given our paradigm was 5–6 weeks, potentially extending the duration in future experiments would bring the hypoactivation towards significance, as would be expected for vulnerable phenotypes (Vialou et al., 2014).

Additionally, a higher variance in LT control animals was observed in the LT-UCMS recordings. This is potentially explained by the fact that controls in this group were exposed to much more handling and environmental manipulation between novelty exposure sessions and sucrose testing over the course of the additional 2–4 weeks. Frequent environmental change and handling are known to be stressful (e.g., Gouveia & Hurst, 2017). Given the potential for individual differences as well (Zurawek et al., 2019), it is possible that some in the control group presented heightened sensitivity to the DEX challenge. Likewise, it is also worth noting that GR desensitization within HPA-axis has shown mixed findings in human studies, with only around 50% of patients showing dysfunction (Arana et al., 1985; Holsboer, 1983). In any case, further replication to increase LT-UCMS sample sizes would potentially address this variability.

4.2.2 LT-UCMS DRN Hypoactivation

Also consistent with our hypotheses, LT-UCMS led to a significant decrease in metabolic activity in the DRN in response to DEX vs. LT controls, while ST-UCMS did not show a significant difference vs. ST controls (Figure 8). To reiterate, since DEX is a glucocorticoid receptor (GR) agonist, it is expected to mimic glucocorticoid increase. Given LT-UCMS was observed to show hypoactivation, a potential desensitization effect has occurred. Previous studies investigating the effects of chronic stress-induced anhedonia-like behaviours on rodents have also observed similar alterations. *In vivo* electrophysiology on 5-HT1A autoreceptors in the DRN revealed a desensitization effect in response to 8-OH-DPAT as well as a decrease in the number of spontaneously active 5-HT neurons (Bambico et al., 2009), while *in vitro* brainstem slices were shown to have a reduced response to the partial 5-HT1A agonist ipsapirone (Froger et al., 2004). Notably, this latter desensitization finding was shown to be dependent on glucocorticoid activity. Additionally, decreases in the density of 5-HT1A autoreceptors and reductions in 5-HT1A receptor binding have been previously observed (Arango et al., 2001; Drevets et al., 2007). Together, these findings suggest that chronic stress is associated with overall attenuation of 5-HT neuronal activity. Given DRN-originating axons are known to

extensively innervate virtually all 5-HT receptor-expressing corticolimbic structures involved in mood regulation and the stress response (Mahar et al., 2014), the observed hypoactivation and corroboration by previous studies provide a plausible explanation for the anhedonic-like behaviour seen in LT-UCMS. Regarding ST-UCMS, although insignificant, the lower mean difference appears to be in the direction of LT-UCMS and may be indicative of an early desensitization effect. Further, consistent with our findings, *in vivo* electrophysiology pilot data show significantly decreased firing rate of 5-HT neurons following LT-UCMS, as well as no difference following ST-UCMS (Figure 12; unpublished). And to note, higher variance was also seen in DRN activity for the LT control group. Our interpretations of this are detailed in the previous section.

4.2.3 Trending NF-κB Concentration Increases in mPFC Following ST-UCMS

Finally, through ELISA we observed a trending increase in nuclear factor-kappa B (NF-κB) concentration in the mPFC for ST-UCMS stressed mice vs. ST controls, and no significant difference in the DRN. NF-κB, a transcription factor in the expression of proinflammatory molecules, is known to be activated in response to psychosocial stressors and contribute to the initiation and amplification of inflammatory responses (Bierhaus et al., 2003; Kye et al., 2007). This exploratory finding suggests a potential association between NF-κB and the upregulation of SK3 channels, which has been hypothesized to be an underlying mechanism involved in the onset of early (mPFC-mediated) cognitive symptoms in depression. These results align with previous research showing that SK2 expression is enhanced by NF-κB, indicating a potential involvement of inflammatory pathways in the regulation of SK channels (Kye et al., 2007). They also align with recent data from Wan-Yan-Chan (2023), who saw no significant increase in SK3 expression in the DRN following ST-UCMS. Given that SK channels and their associated elements have been implicated as therapeutic targets for stress-induced conditions such as depression (Ji et al., 2021), further investigation of the NF-κB-mediated inflammatory cascade may shed light on novel treatment strategies. It is worth noting that the present

experiment had a small sample size (CTR: $n = 4$; ST-UCMS: $n = 5$), emphasizing the need for cautious interpretation and the necessity of replication.

4.3 Mechanisms Underlying the Deleterious Effects of Rumination-Like Behaviour

To our knowledge, no studies have combined the techniques herein to model prodromal cognitive symptoms related to negative thought patterns in MDD-like onset in mice. While extant literature has implicated dysfunctional upstream inhibition at mPFC GABAergic PV^+ and SST^+ interneurons resulting in overactive pyramidal output neurons, and even excitotoxicity (Ghosal et al., 2017; Harmer et al., 2017), studies investigating cellular mechanisms underlying these alterations following a paradigm like ST-UCMS are lacking. Taken together, our data suggest that short-term chronic stress may lead to rumination-like symptoms through alterations in inhibitory mechanisms, and bring further support to a more "cognitive theory" of depression. A preliminary mechanistic diagram proposing the temporal progression of disinhibited cognitive dysfunction brought on by repetitive negative thought processes (potentially due in part to SKC upregulation), and their eventual progression to more advanced mood symptoms, is illustrated in Figure 12. Further elucidations of these potential mechanisms may enable improved prevention strategies, including more treatment specificity in the early stages of MDD onset.

4.4 Limitations

While the social interaction test and splash test do contain elements of mPFC-mediated cognitive processing, they are limited in their direct testing of cognitive symptoms like decision making and attention. Follow-up studies incorporating additional assays that more specifically validate dysfunction related to cognitive judgements following ST-UCMS would add further support for the hypothesis that cognitive symptoms are the first to emerge. Examples include the attentional set shifting task (ASST), a test of cognitive flexibility (Heisler et al., 2015), and

the 5-choice serial reaction time task (5CSRTT), an operant-based test of attention and impulse control (Asinof & Paine, 2014).

Further, while behavioural assays following 2–3 and 5–6 weeks of UCMS offer some insight into the time-dependent trajectory of depressive-like symptom development, classical behavioural tests have a limited ability to provide more granular symptom progression tracking given they are known to be stressful with several environmental changes, repeated handling, and some potentially aversive manipulations (e.g., Gouveia & Hurst, 2017). Moreover, control mice in both groups underwent all behavioural tests prior to testing for glucose activation in the mPFC and DRN. Given that some control mice may have been more susceptible to stress, some potentially exhibited more of a response to DEX throughout the biosensor recordings. To reduce the chance of this confounding the activational recordings, assuming a large enough sample size, the controls could be randomly split into two groups: behavioural controls and biosensor controls. Another related consideration has to do with chronic unpredictable stress models broadly. Krishnan and Nestler (2011) among others have questioned its construct validity given the stressors are physical (e.g., restraint, strobe lights, circadian disruptions) rather than *social*, and are unlikely to be encountered by rodents in the wild. They proposed that models of psychosocial stress, e.g. chronic social defeat, may carry higher strength in modelling affective symptoms given they rely on innate social behaviour.

Like many other lines of research, another consideration in the design of experiments would be to test in the context of the estrus cycle. Previous work has highlighted alterations in both baseline and stress-induced anxiety-like behaviours, as well as changes in GABAergic neurons, depending on the phase of the female estrus cycle (e.g., Ramos-Ortolaza et al., 2017). While Prendergast et al. (2014) point out that the effects of the estrus cycle have been overblown and do not typically present a significant variability on experimental outcome compared to average variability in males, the context of environmental manipulations such as UCMS have

indeed been shown to disrupt the normal estrus cycle, leading to hormonal fluctuations that can impact various physiological and behavioural measures (Gruene, 2015; McCarthy, 2010).

Finally, some results reported and cited herein represent preliminary and exploratory data sets. Replication to increase power would add confidence and potentially reveal further phenotypic elements related to chronic stress, the emergence of prodromal depressive-like symptoms, vulnerability factors, pathophysiological correlates, and sex-specific trajectories.

4.5 Future Directions

There are a number of ways this research could be corroborated, and even extended to support clinical applications. First, there is a need for further replication of ST-UCMS behavioural assays and metabolic activation patterns to validate previous findings and enhance the robustness of the experimental results. Replicating NF-κB concentration changes following ST-UCMS and extending this analysis to LT-UCMS would also provide a more comprehensive understanding of the neuroinflammatory response to chronic stress. Utilizing fiber photometry to causally link neuronal activity through intracellular Ca2+ level alterations while the animal is behaving could elucidate the causal relationship between neuronal activation patterns and behaviour. Additionally, the investigation of SK3, PV^{+}, and SST^{+} IN as potential pathophysiological correlates and therapeutic targets, including testing known SK3 blockers, offers promising avenues for the development of novel interventions for stress-related disorders. Finally, positron emission tomography (PET) scans have been used for some time in investigating functional changes in depressed patients (e.g., Drevets et al., 1997; Kennedy et al., 2001). Exploring the clinical translatability of simultaneous mPFC/DRN glucose biosensors to PET scans represents a novel approach to the identification of early biomarkers of depression, with significant implications for diagnosis and treatment.

In the literature review for this study, another research avenue related to novel targets for negative affect encoding stood out. Emerging research suggests that neurotensin (NT), a neuropeptide involved in regulating emotions and modulating the brain's reward and stress

systems (primarily in the basolateral complex of the amygdala [BLA]), may play a role in the encoding and processing of positive and negative emotional experiences (Li et al., 2022). Given the widely accepted role of negative bias in depressive disorders and other mental health conditions, being able to target the very source of valence is intriguing. Although the potential targeting of NT is still in its early stages, combined with advent of circuit-based approaches to drug discovery and delivery, it may hold promise in alleviating the rigid negative thought patterns current ADTs often cannot adequately address (Harmer et al., 2017; Li et al., 2022; Ressler & Mayberg, 2007; Spellman & Liston, 2020; Stone & Hernandez, 2023).

5.0 Conclusion

In summary, we have demonstrated distinct depressive-like phenotypes for BALB/c mice following shorter and longer durations of UCMS. Our results combine to evolve our understanding of the behavioural manifestations and neurobiological mechanisms underlying the onset of early depressive-like symptoms following chronic stress, including cognitive function and emotional processing, SKC upregulation, and GABA and glutamate signalling. The research theory and design herein may be informative in the development of a preclinical model of early cognitive symptoms, which would enable investigation of related mechanisms and interventions. With upwards of 75% of mental health conditions considered to start before the age of 24, and accumulating evidence that many spur from unaddressed abnormalities in negatively biased (“hot”) cognition (Owens et al., 2012; Roiser & Sahakian, 2013), continued research into more targeted and robust prevention and treatment related to rumination-like symptoms appears to be paramount in addressing the prevalence predicament we are facing.

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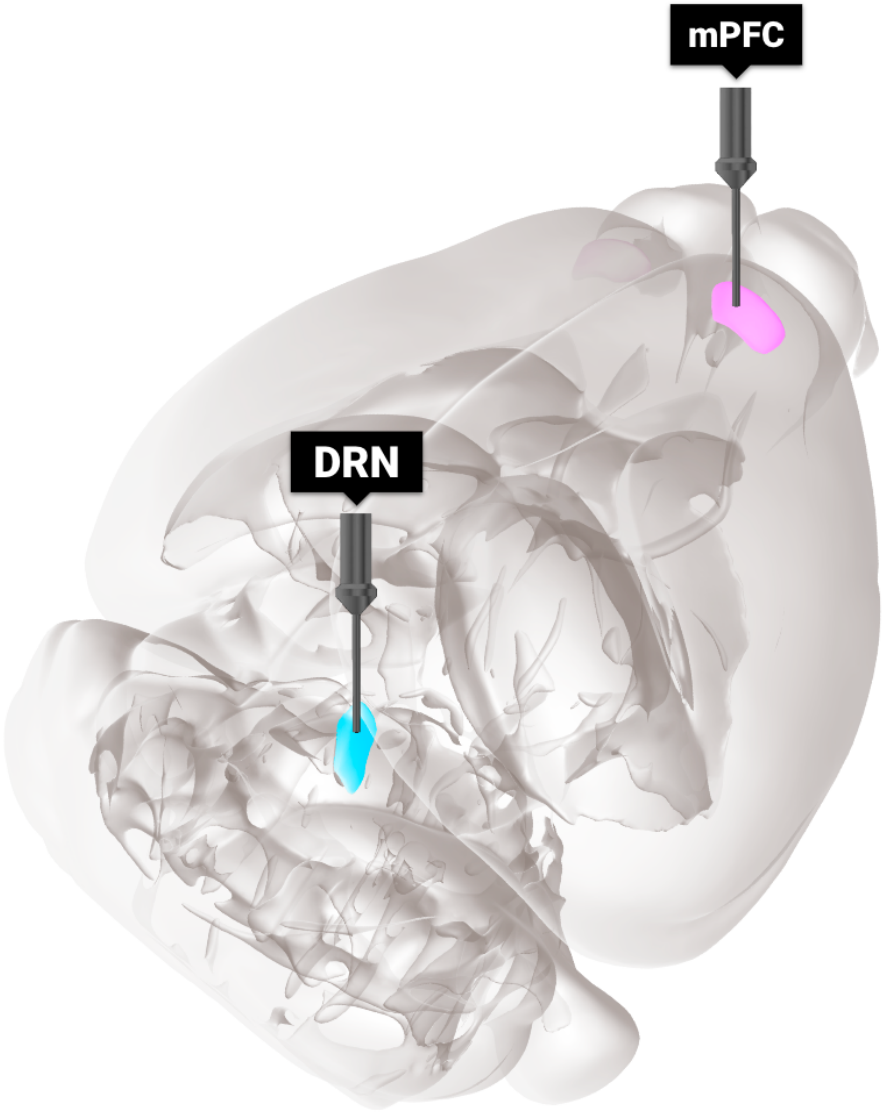
Appendix A

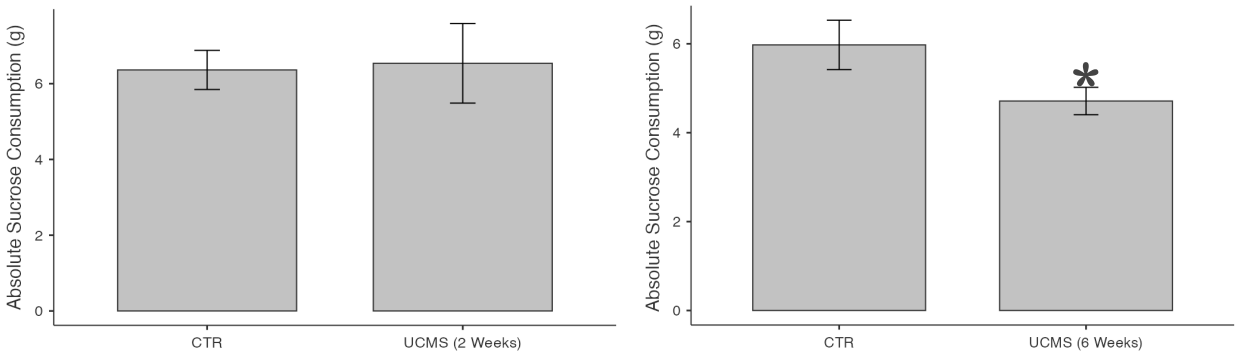
Figure 1: Graphical Representation Showing Regions of Interest in the Mouse Brain



mPFC: Medial prefrontal cortex; DRN: Dorsal raphe nucleus

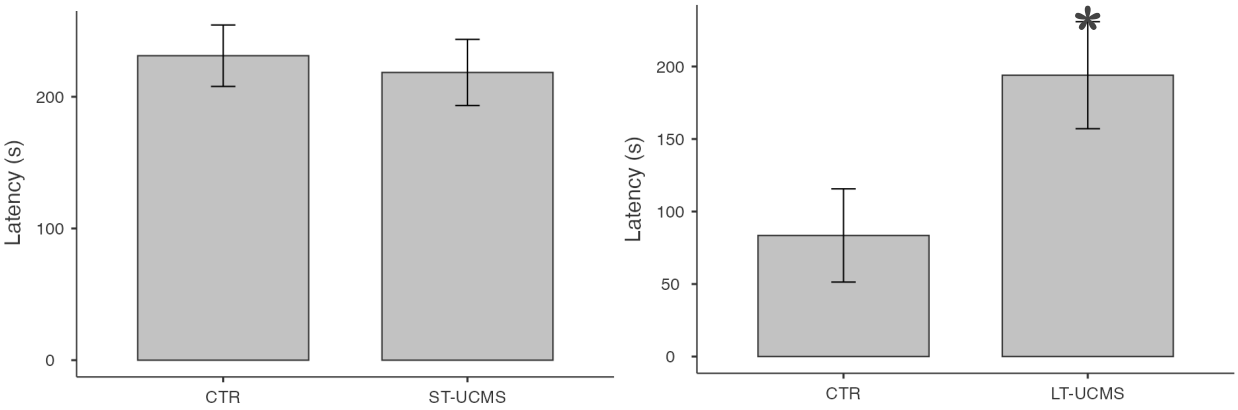
Brain model image credit: Allen Brain Explorer® beta. Allen Institute for Brain Science. https://connectivity.brain-map.org

Figure 2: Differences in Sucrose Consumption at 2 vs. 6 Weeks of UCMS



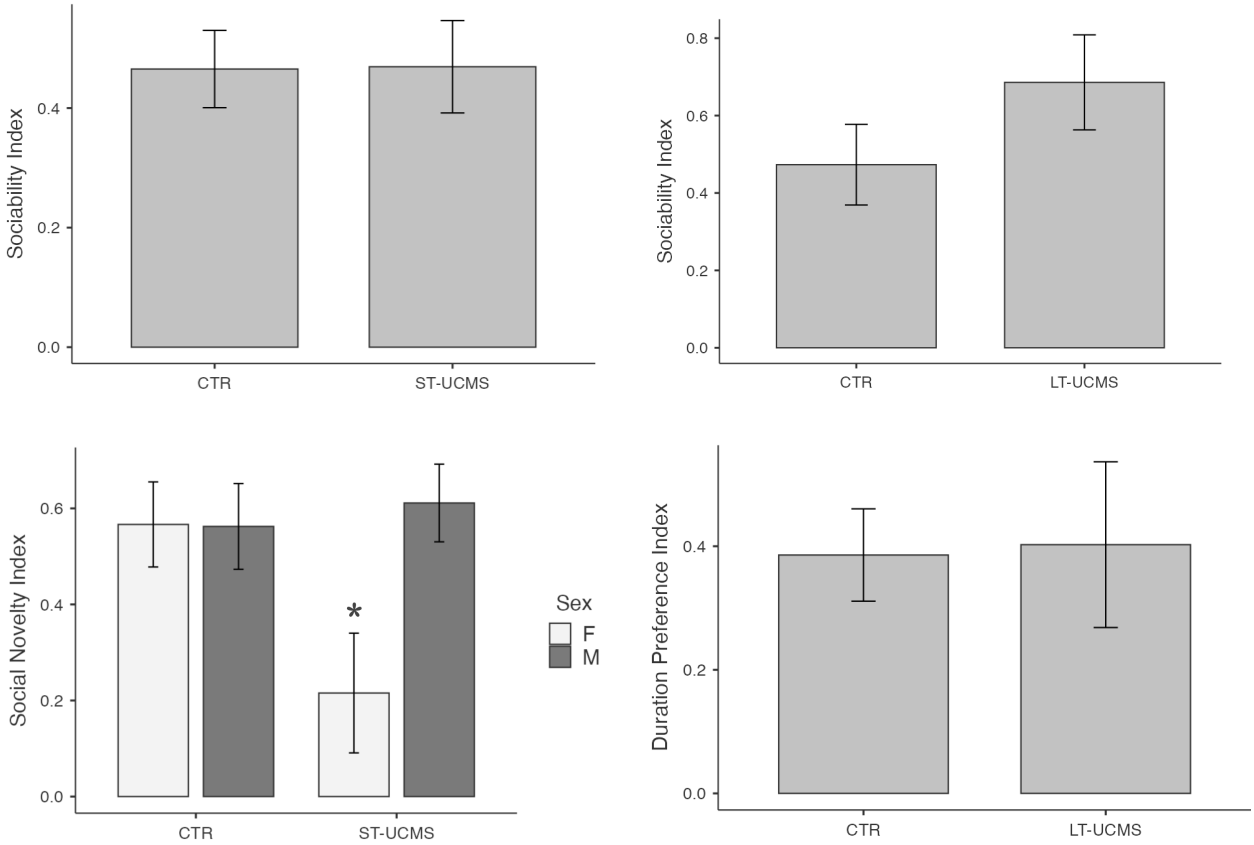
*Graphs show means ± standard error of the mean (SEM). After 2 weeks of UCMS, stressed mice (n = 8) did not show a significant difference in sucrose consumption vs. controls (n = 8) (**left**), while after 6 weeks of UCMS, stressed mice showed significantly lower sucrose consumption (**right**), indicative of anhedonia-like behaviour.*

Figure 3: Differences in Latency to Feeding in the Novelty-Suppressed Feeding Test



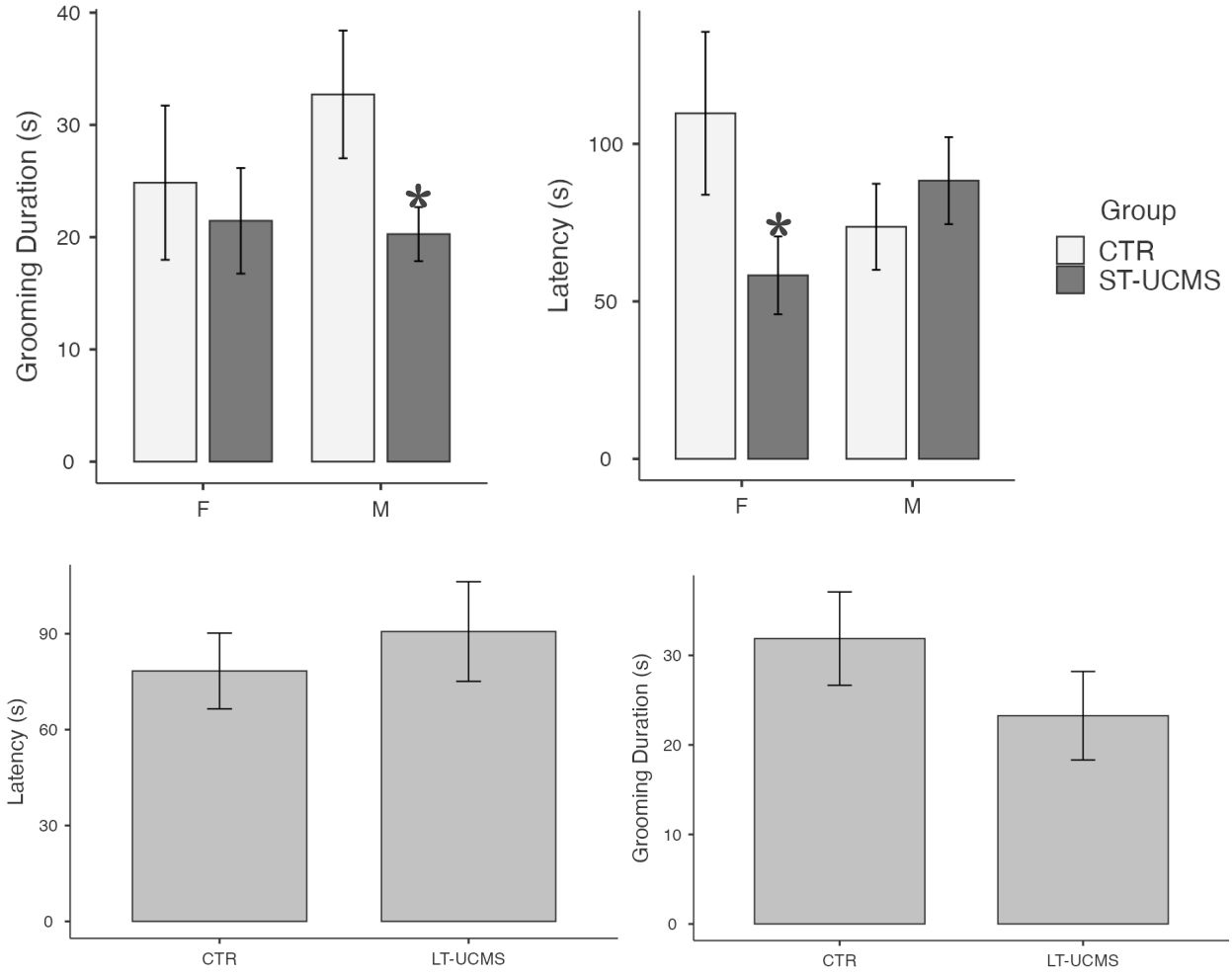
*Graphs show means ± SEM. No significant differences were seen for ST-UCMS stressed mice (n = 17) vs. ST controls (n = 18) (**left**), while LT-UCMS stressed mice (n = 8) showed significantly longer latency to feeding compared to LT controls (n = 8) (**right**), indicative of anxiety-like behaviour.*

Figure 4: Differences in Social Novelty Preference in the Social Interaction Test



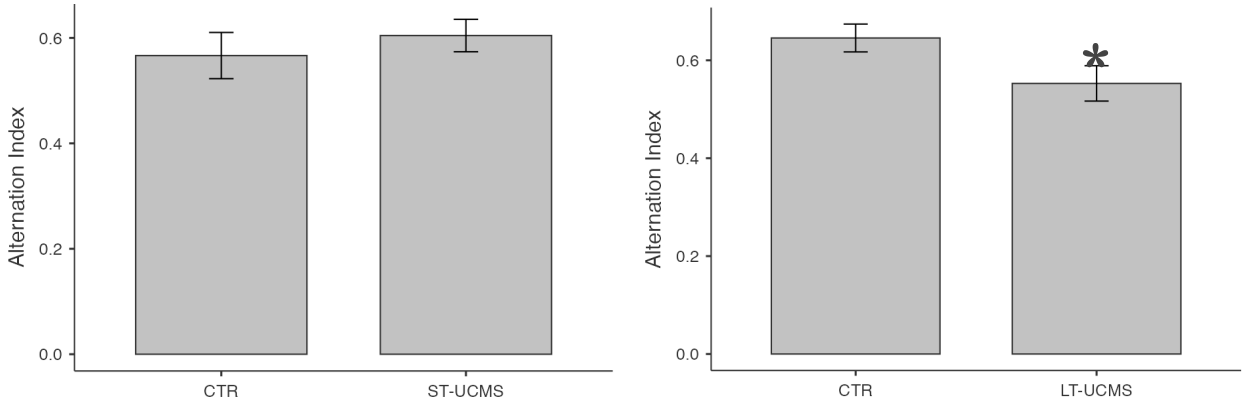
*Graphs show means ± SEM. No significant differences were seen in sociability (i.e., novel conspecific vs. empty cage), for either UCMS group (ST: **top-left**; LT: **top-right**), combined or by sex, indicating there were no confounding effects of social aversion for all analyses. While no significant differences for ST-UCMS were found overall, a sex difference was identified (**bottom-left**): ST-UCMS stressed females (n = 7) spent significantly less time investigating the novel conspecific compared with female ST controls (n = 9). No significant differences were seen for LT-UCMS stressed mice (n = 8) vs. LT controls (n = 8), combined or by sex (**bottom-right**).*

Figure 5: Differences in Grooming Behaviours in the Splash Test



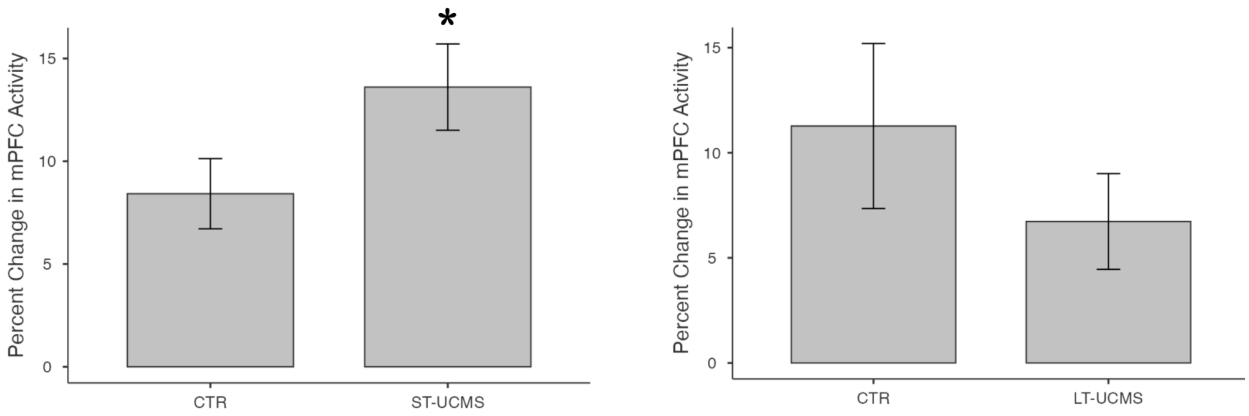
*Graphs show means ± SEM. While no significant differences for ST-UCMS were found overall, two significant differences were seen when analyzing by sex: ST-UCMS males (n = 9) spent significantly less time grooming compared with ST male controls (n = 9) (**top-left**), while ST-UCMS females (n = 9) took significantly longer to initiate grooming compared with ST female controls (n = 9) (**top-right**). No significant differences were seen for LT-UCMS (n = 8) vs. LT controls (n = 8), in latency (**bottom-left**) or duration (**bottom-right**).*

Figure 6: Alternation Index in the Spontaneous Alternation Y-Maze Test



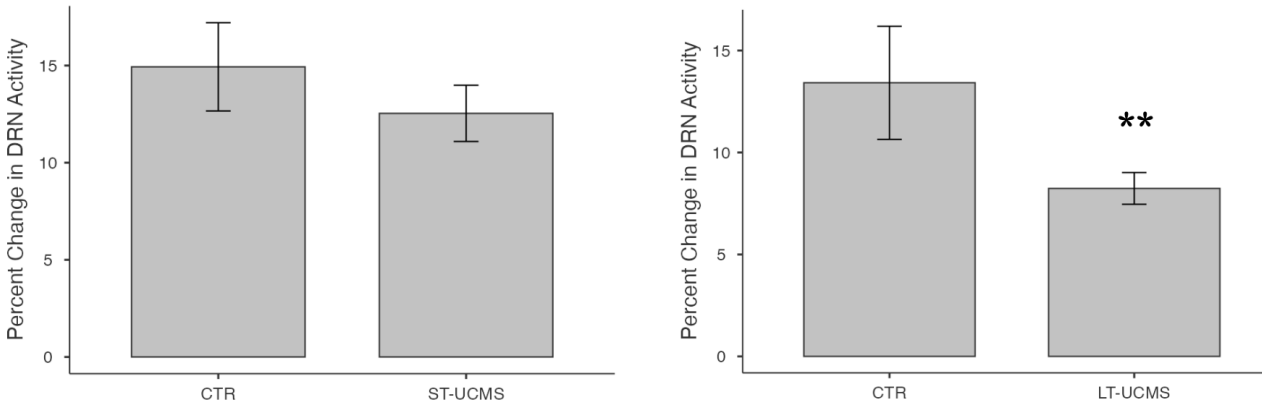
*Graphs show means ± SEM. No significant differences were seen for ST-UCMS (n = 17) vs. ST controls (n = 16) (**left**). LT-UCMS stressed mice (n = 8) showed significantly lower performance in the alternation task vs. LT controls (n = 8) (**right**), indicative of deficits in spatial working memory.*

Figure 7: Percent Change in mPFC Metabolic Activity (Biosensor Recordings)



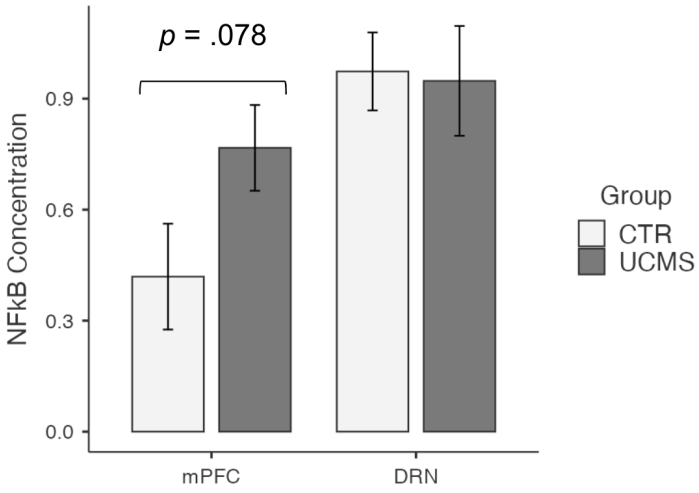
Graphs show means ± SEM. ***Left:*** *ST-UCMS (CTR: n = 5 [2 females, 3 males]; ST-UCMS: n = 7 [4 females, 3 males]).* ***Right:*** *LT-UCMS (CTR: n = 5 [3 females, 2 males]; LT-UCMS: n = 5 [2 females, 3 males]). Following DEX administration, significantly higher metabolic activity was observed in ST-UCMS stressed mice. A higher variance in control animals was observed in LT-UCMS. This is potentially explained by the fact that controls in this group were exposed to much more handling and environmental manipulation between novelty exposure sessions and sucrose testing over the course of the additional 2–4 weeks. As previously discussed, studies have also shown that the BALB/c strain is generally more sensitive to stress; given the potential for individual differences it is possible that some presented heightened sensitivity to the DEX challenge. See Discussion for more details.*

Figure 8: Percent Change in DRN Metabolic Activity (Biosensor Recordings)



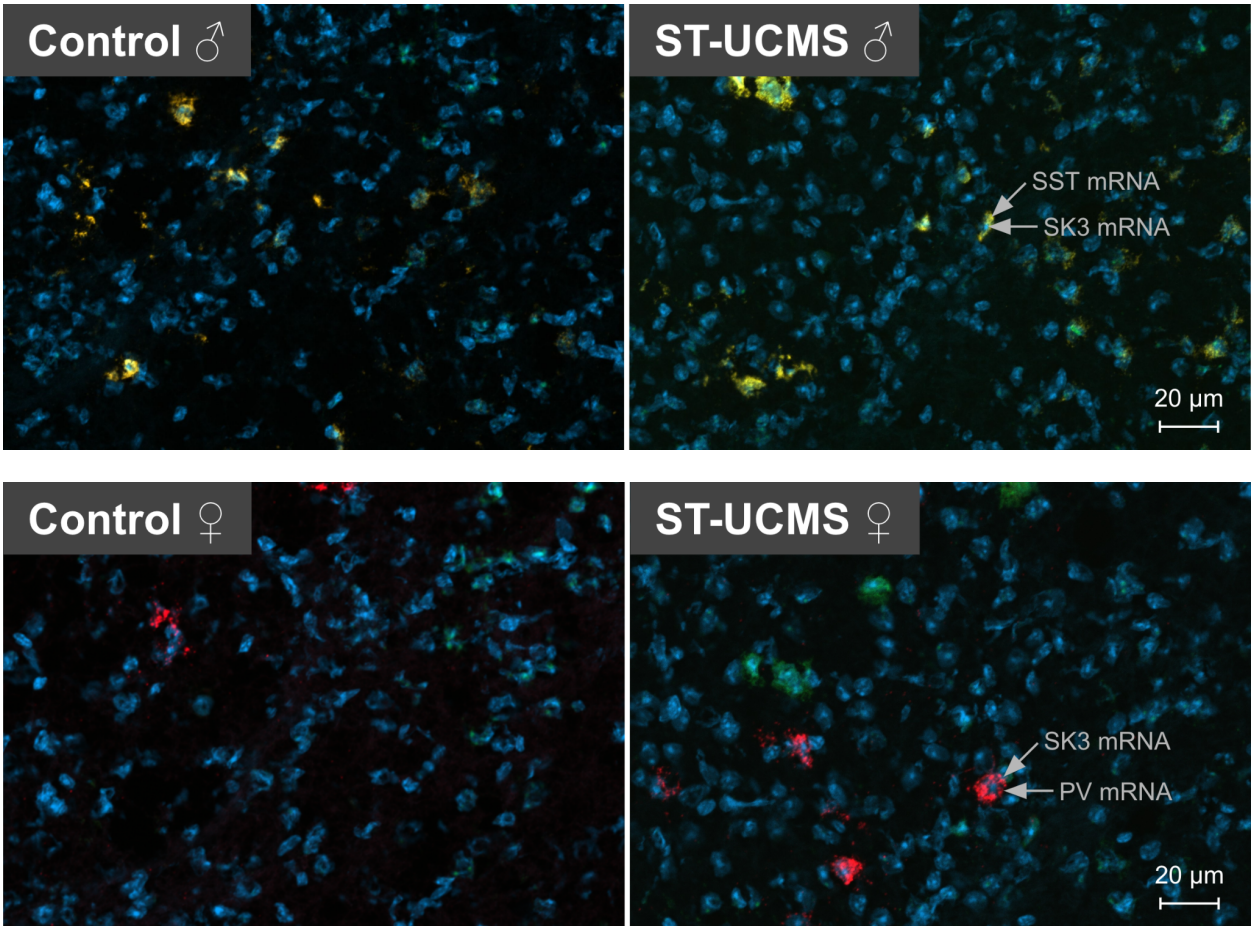
Graphs show means ± SEM. ***Left:*** *ST-UCMS (CTR: n = 5 [2 females, 3 males]; ST-UCMS: n = 7 [4 females, 3 males]).* ***Right:*** *LT-UCMS (CTR: n = 5 [3 females, 2 males]; LT-UCMS: n = 5 [2 females, 3 males]). Following DEX administration, significantly lower metabolic activity was observed in LT-UCMS stressed mice. No significant difference was seen for ST-UCMS.*

Figure 9: NF-κB Concentration Differences Following ST-UCMS (ELISA)



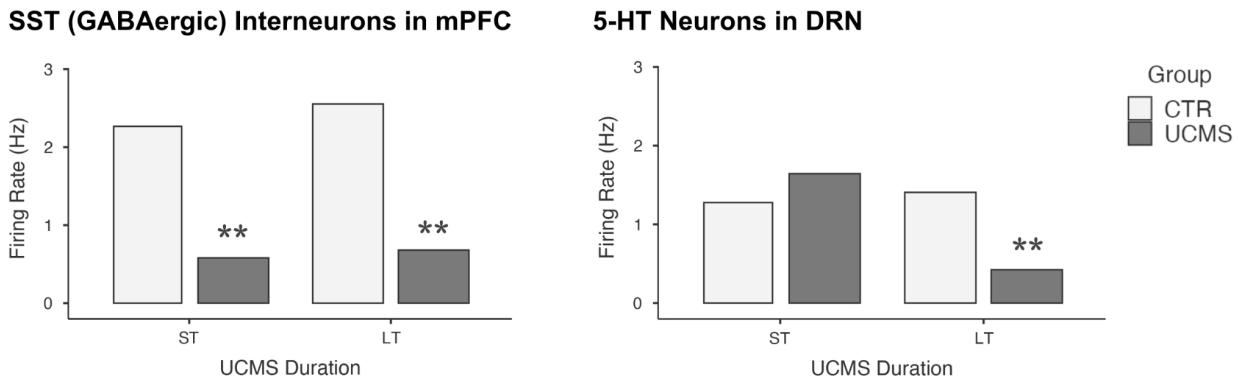
Graphs show means ± SEM. ST-UCMS: n = 6 (3 females, 3 males); controls: n = 4 (2 females, 2 males). ST-UCMS stressed mice show a trending result towards a higher concentration of NF-κB in the mPFC vs. controls. No significant difference was seen in the DRN.

Figure 10: Differences in Cortical SK3 Expression Following ST-UCMS (RNAscope)



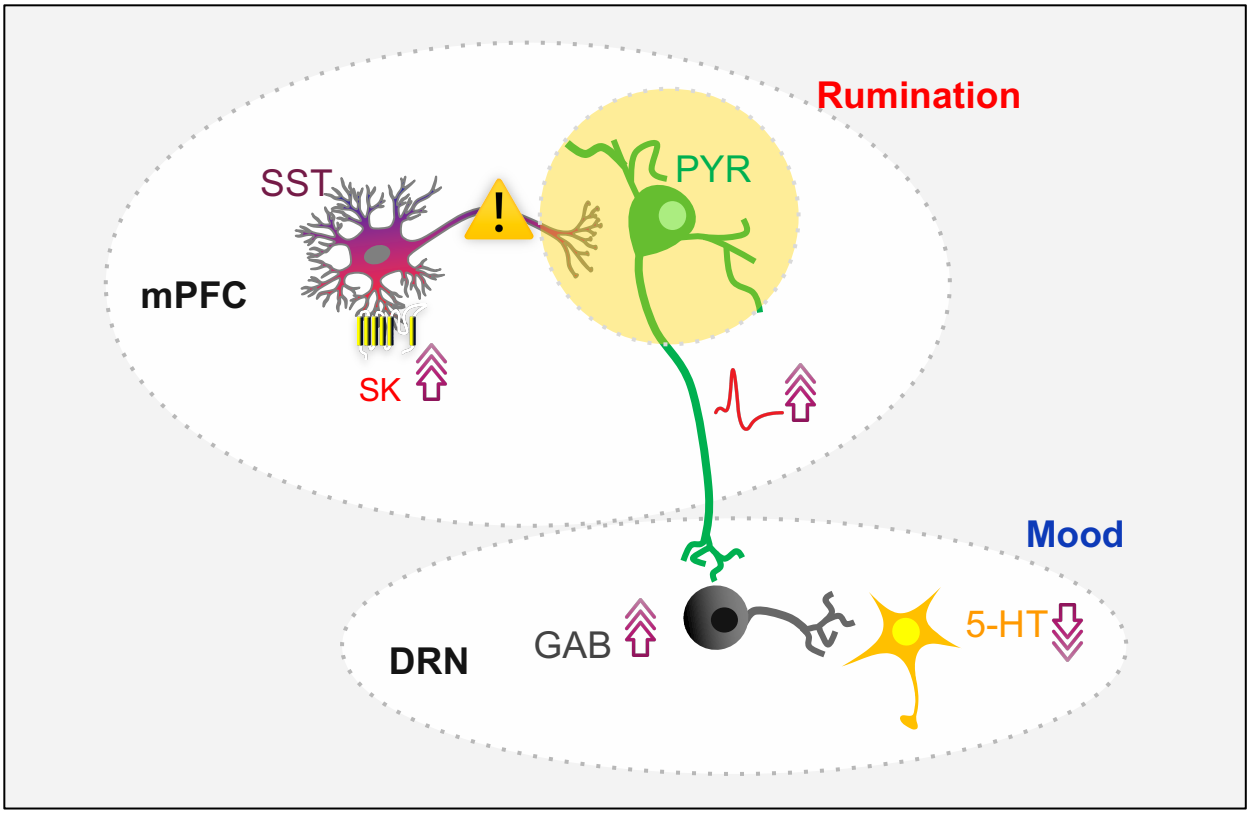
*Note: Qualitative inspections by Wan-Yan-Chan (2023) have revealed increases in cortical SK3 co-expression with SST⁺ (**top set**) and PV⁺ (**bottom set**) interneurons following ST-UCMS. Green (TSA Vivid dye 520) indicates SK3 mRNA (Mm-Kcnn3); yellow (TSA Vivid dye 570) indicates SST mRNA (Mm-Sst-C2); red (TSA Vivid dye 650) indicates PV mRNA (Mm-Pvalb-C3); blue (DAPI) indicates cell nuclei (reagent kit V2). All probes, reagents, and dyes were obtained from Advanced Cell Diagnostics, Inc. (Newark, USA).*

Figure 11: Differences in Basal Firing Rate (Electrophysiology)



Note: In vivo*, extracellular electrophysiology recordings were performed prior to this project in a pilot study (unpublished) exploring differences in PFC SST interneuron (**left**) and DRN 5-HT neuron activity (**right**) following ST-UCMS and LT-UCMS. Decreased basal activity of PFC SST interneurons was seen following both ST- and LT-UCMS. Decreased basal activity of DRN 5-HT neurons was only seen following LT-UCMS.*

Figure 12: Illustration of Proposed Temporal Circuit Dynamics



Note: See 4.3 Mechanisms Underlying the Deleterious Effects of Rumination-Like Behaviour.

Appendix B

Table 1: Weekly UCMS Stressor Schedule Example

	Monday	Tuesday	Wednesday	Thursday	Friday
Morning	45° cage tilt (2 hr)	Oscillation (30 min)	Aversive smell (2 hr)	Water in cage (2 hr)	High frequency sound (30 min)
Afternoon	High frequency sound (30 min)	-45° cage tilt (3 hr)	Restraint (30 min)	No bedding (2 hr)	Oscillation (45 min)
Evening	Stroboscope (overnight)	Damp bedding (overnight)	Overnight illumination	45° cage tilt (1 hr)	Damp bedding (2 hr)

The timing, duration, and sequencing of stressors were pseudorandom to introduce an element of unpredictability. Sucrose testing occurred between Saturday 15:00 and Sunday 10:00. No stressors occurred on these days.