EXPOSURE TO STRESS DURING ADOLESCENCE ALTERS SAFETY LEARNING AND EMOTIONAL BEHAVIOURS THAT PERSIST INTO ADULTHOOD

A Thesis Submitted to the Committee on Graduate Studies
in Partial Fulfillment of the Requirements for the Degree of Master of Science
in the Faculty of Arts and Science

TRENT UNIVERSITY

Peterborough, Ontario, Canada

Psychology MSc. Graduate Program

January 2026

ABSTRACT

Exposure to Stress During Adolescence Alters Safety Learning and Emotional Behaviours that Persist into Adulthood

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Stress during adolescence has profound effects on psychological, behavioral, and neurobiological outcomes in adulthood. This study investigates the impact of adolescent stress on safety learning, anxiety- and depressive-like behaviors, and associated neurocircuitry using a rat model. Adolescent male Long Evans rats underwent an unpredictable intermittent stress regimen, followed by behavioral testing and immunohistological analyses in adulthood. It was confirmed that stress impaired safety learning and increased fear generalization. Behavioral assays revealed heightened anxiety- and depressive-like phenotypes in stressed rats, evidenced by reduced open-arm exploration in the elevated plus maze and increased immobility in the forced swim test, although limited changes in sucrose preference were noted during habituation. Immunohistological findings showed reductions in hippocampal neurogenesis (DCX+ cells) and disruptions in GABAergic interneuron plasticity (PV+/PNN+ populations) within the medial prefrontal cortex. These alterations suggest that adolescent stress leads to long-term changes in brain regions associated with emotional regulation and stress resilience.

Keywords: Adolescent Stress, Safety Learning, Anxiety-Like Behaviour, Depressive-Like Behaviour, Neuroplasticity, Medial Prefrontal Cortex, Hippocampus

Acknowledgments

I would like to express my heartfelt gratitude to my supervisor, Dr. Neil M. Fournier, for his invaluable guidance, support, and mentorship throughout my master's program and thesis project. I am also deeply thankful to my lab mates for their friendship and encouragement during my time at Trent. Your support—both in the lab and beyond—meant so much. In particular, I would like to thank Gillian and Trixia, whose dedication and hard work were essential to the completion of this project.

I am profoundly grateful to my family and my partner for their unwavering love and encouragement throughout this journey. To my mom—thank you for being my rock, for your constant belief in me, and for your strength during a time when we truly leaned on each other. Your resilience and love have been my greatest source of inspiration.

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Chapter 1: Introduction

1.0 Introduction

Stress is a complex biological and psychological phenomenon that can affect an organism's homeostasis, affecting physiological and psychological functioning (Herman et al., 2016). It is broadly defined as a series of events that begin with the perception of a stimulus or stressor by the brain, triggering a cascade of neurobiological responses that ultimately evoke adaptive reactions such as the well-documented "fight" or "flight" responses (Dhabhar & McEwen, 1997). While acute stress can be beneficial for survival by enhancing alertness and preparedness, chronic stress can have severe detrimental effects on health, predisposing individuals to various psychiatric and physiological conditions (Dhabhar, 2014).

Exposure to stress, particularly in early life and during critical developmental periods such as adolescence, can be a significant risk factor for the emergence of mental health disorders in adulthood, including anxiety and depression (Kessler et al., 2010; Lemoult et al., 2020; Ma et al., 2021). Adolescence is a particularly vulnerable stage of neurodevelopment, marked by ongoing transitions and remodelling in brain regions responsible for emotional regulation and cognitive processes (Spear, 2000). Stress during this period can lead to long-lasting changes in neural circuits, protein expression, and synaptic plasticity, particularly in brain regions associated with stress regulation, such as the hypothalamic-pituitary-adrenal (HPA) axis, hippocampus, amygdala, and medial prefrontal cortex (mPFC). These neurobiological alterations may increase susceptibility to psychopathologies such as post-traumatic stress disorder (PTSD), acute stress disorders, depression, and anxiety (Tsoory et al., 2006).

Research has also highlighted the significant rise in stress-related mental health issues, particularly among younger populations. During the COVID-19 pandemic, global stress levels

increased by 13%, while rates of mental health disorders, such as anxiety and depression, rose by 9%, creating a correlational connection between stress and mental health (Dragioti et al., 2022). Notably, younger individuals exhibited a higher prevalence of stress-related disorders compared to older adults, suggesting that they may be particularly vulnerable to its adverse effects (McGinty et al., 2020; Varma et al., 2021). The cumulative impact of stress, particularly when experienced during sensitive developmental windows, has led to increasing interest in identifying the underlying neurobiological and anatomical mechanisms that mediate these effects, as changes in neural circuits and protein expression involved in moderating stressors undergo adverse changes. Understanding how stress exposure influences the adolescent brain and contributes to increased vulnerability to psychopathologies is crucial for developing targeted interventions and prevention strategies.

Such disruptions can have long-lasting consequences on neural systems that underlie emotional regulation, cognitive flexibility, and stress reactivity. Therefore, investigating the mechanisms by which stress alters developmental trajectories is essential for understanding how stress-related psychopathologies emerge and persist.

This research aims to examine how stress during mid-adolescence impacts behavioural outcomes and neurobiological markers associated with emotional regulation and safety learning in adulthood. Specifically, this study aims to determine whether adolescent stress alters performance in safety learning paradigms, increases anxiety- and depressive-like behaviours, and produces measurable changes in hippocampal neurogenesis (DCX+ cells) and plasticity-related markers (PV+, PNN+) in the medial prefrontal cortex. Through a rat model of unpredictable intermittent stress, the goal is to understand how stress during a critical developmental window may contribute to long-term vulnerability to psychopathology.

1.1 Adolescence and Neurodevelopment

Adolescence is a socially constructed developmental period that occurs between childhood and adulthood, characterized by profound physiological, neurological, and behavioural changes (Arellano, 2024). A defining feature of this transition is puberty, marked by significant hormonal fluctuations that influence both brain and behaviour. During adolescence, critical maturation of brain regions involved in higher-order cognitive functions occurs alongside ongoing neurodevelopmental processes that shape emotional regulation, decision-making, and learning. In humans, adolescence is typically defined as spanning approximately 12 to 18 years of age (Arellano, 2024). As such, adolescence represents a pivotal developmental bridge between childhood and adulthood, during which substantial biological and psychological reorganization takes place.

Neurodevelopment is a dynamic process that begins prenatally and continues throughout early adulthood. During early childhood, the brain undergoes rapid growth, characterized by synaptogenesis, increased cortical volume, and heightened plasticity. Processes like synaptic pruning and myelination help refine neural circuits to enhance the efficiency of neural signalling. Synaptic pruning eliminates unutilized or weak neural connections, strengthening important pathways**.** Myelination insulates axons to increase the speed of signal transmission between neurons. Adolescence, typically defined as ages 12–18 in humans, marks a critical phase in these processes for functional and structural reorganization (Spear, 2000). This period is sometimes referred to as the "second wave" of brain maturation, following the rapid growth of childhood (Dow-Edwards et al., 2019; Gogtay et al., 2004; Mills et al., 2016).

In contrast to the relatively thick cortex during early childhood, the thinning of the cortex during adolescence marks a shift toward greater neural precision and specialization. Simultaneously, white matter volume increases due to continued myelination, especially in long-range tracts such as the frontostriatal and prefrontal-limbic pathways. This myelination supports faster signal transmission and better integration across distant brain regions (Aubert-Broche et al., 2013; Giedd et al., 1999). The myelination of these pathways is particularly critical as it improves the communication between the prefrontal cortex and limbic regions, which are essential for emotional regulation and decision-making. These processes enable more advanced regulatory functions but also create a critical period of vulnerability. Increased stress during mid-adolescence can significantly disrupt these processes, potentially affecting the maturation of circuits involved in emotion regulation and fear processing and thus increasing the risk of psychiatric outcomes in adulthood.

Brain regions responsible for different aspects of behaviour and cognition mature at different rates during adolescence. Notably, the limbic system, including the amygdala, hippocampus, and nucleus accumbens, matures relatively early and is responsible for emotional regulation, reward processing, and risk evaluation. In contrast, the medial prefrontal cortex (mPFC), which governs higher-order executive functions such as working memory, impulse control, and decision-making, undergoes the most prolonged development, continuing to mature well into early adulthood (Huttenlocher, 1979; Petanjek et al., 2011). The delayed maturation of the mPFC contributes to an imbalance between emotional responses and cognitive control often seen during adolescence, where emotional impulses are stronger than the ability to regulate them. The mPFC includes functionally specialized subregions such as the cingulate cortex area 1 (Cg1), prelimbic (PrL), and infralimbic (IL) cortices, which are critical for regulating behaviour

and emotion. For instance, the PrL regulates conditioned fear responses, while the IL is involved in fear extinction and emotional inhibition, key processes in adaptive emotional regulation (Giustino & Maren, 2015).

Although these subregions of the rodent brain do not map one-to-one onto the human brain compared to a rodent brain, they are broadly homologous to regions such as the anterior cingulate cortex (Cg1), dorsomedial prefrontal cortex (PrL), and ventromedial prefrontal cortex (IL) in humans—areas that also regulate emotion, valuation, and the modulation of limbic activity. This developmental asynchrony between the limbic system and prefrontal cortex is critical in understanding the behavioural patterns observed in adolescence, such as increased risk-taking, heightened emotional reactivity, and greater susceptibility to peer influence (Grüne et al., 2017; Somerville et al., 2018; Spear, 2000). This heightened sensitivity to emotional stimuli and the inability to regulate these responses appropriately is thought to contribute to adolescent vulnerability to stress-related mental health issues.

Adolescence is a sensitive period during which stress exposure can actively shape the trajectory of brain development. Unlike in adulthood, where stress effects tend to be more transient, stress during adolescence can recalibrate neurobiological systems and alter the maturation of neural circuits in lasting ways (McEwen & Morrison, 2013). This recalibration is not limited to immediate effects on the brain but can lead to long-lasting shifts in how the brain responds to future stressors. For example, chronic stress in adolescence has been shown to alter glucocorticoid receptor expression in key stress-regulating areas like the hippocampus and amygdala, which can compromise the brain's ability to regulate stress later in life (McEwen & Morrison, 2013). Stress exposure during this period can also lead to changes in synaptic plasticity, dendritic spine density, and neuroinflammatory pathways, which in turn may

contribute to emotional dysregulation and vulnerability to psychopathology (Eiland & Romeo, 2013).

This experience-dependent plasticity can interfere with normative processes such as pruning and myelination, reshaping the brain's developmental path at a critical time for network consolidation. Although not all stress-induced changes are maladaptive, research suggests that early-life stress can increase the sensitivity of brain circuits to future stress, contributing to emotional dysregulation later in life. Such changes are often associated with reduced capacity for fear extinction and deficits in safety learning processes, critical for distinguishing between safe and unsafe environments (Eiland & Romeo, 2013). Deficits in safety learning—particularly when stress occurs during adolescence—may contribute to the onset of neuropsychiatric disorders such as anxiety, depression, and post-traumatic stress disorder (PTSD).

These changes are not always maladaptive, but their long-term effects depend on the stressors' timing, intensity, and context (Bangasser & Wiersielis, 2018). This is particularly evident in adolescence, where stress exposure can exacerbate vulnerabilities to psychopathology. Early-life stress can also influence stress responsivity and emotional regulation strategies into adulthood. Moreover, sex differences in stress response, driven by hormonal changes during puberty and differences in corticolimbic connectivity, may help explain gender disparities in mood and anxiety disorders during both adolescence and adulthood (Bangasser & Wiersielis, 2018).

In conclusion, adolescence represents a critical neurobiological period where genetic and environmental factors shape the brain. While processes like synaptic pruning, myelination, and plasticity facilitate cognitive and emotional maturation, they also make the adolescent brain particularly vulnerable to the adverse effects of stress. Understanding how stress exposure during

this critical developmental window impacts safety learning, emotional regulation, and long-term vulnerability to psychiatric disorders is essential for developing interventions aimed at promoting resilience and mitigating the impact of stress on adolescent brain development. These developmental processes underscore the importance of early interventions to address environmental stressors and promote healthy brain development.

1.2 Stress and Neuropathology

Stress has been extensively studied as a significant risk factor for the development of psychopathological conditions such as depression and anxiety. Numerous studies have highlighted the strong association between high stress levels and mental health disorders, emphasizing the vulnerability of particular demographic groups, specifically adolescents and young adults. A global cross-sectional survey conducted during the COVID-19 pandemic revealed that 77% of individuals reported at least moderate stress levels, with 35% experiencing mild or higher levels of depression and 59% meeting the criteria for clinical anxiety (Varma et al., 2021). This study found that younger individuals were disproportionately affected, a trend that aligns with findings among young Canadians, who report higher rates of stress, depression, and anxiety compared to their older counterparts (Nwachukwu et al., 2020). Further supporting this pattern, a meta-analysis by Ma et al. (2021) concluded that adolescents aged 13–18 years, experience significantly higher rates of stress, depression, and anxiety compared to younger children. These findings consistently identify adolescents as a particularly vulnerable group to the mental health consequences of stress.

One potential explanation for the heightened vulnerability of adolescents to stress-related psychopathologies is the stress sensitization model, which suggests that individuals who experience early life stress or childhood adversities develop an increased sensitivity to stress

over time. This heightened sensitivity renders them more susceptible to developing depressive symptoms following exposure to subsequent stressors. The model is rooted in disruptions in neurobiological stress regulation mechanisms and maladaptive cognitive changes that arise due to early adverse experiences (Hammen et al., 2005). Research has provided empirical support for this model, demonstrating that ongoing stress during adolescence can act as a bridge between earlier negative experiences in childhood and the development of depression later on. For instance, Hazel et al. (2008) found that adolescents who endured ongoing stress at age 15 were significantly more likely to develop depression by late adolescence. Further, McLaughlin et al. (2010) reported that individuals with a history of multiple childhood adversities were at a greater risk of developing major depression, PTSD, and anxiety disorders when faced with recent life stressors compared to those without such early adversities.

The long-term effects of childhood stress extend beyond adolescence, influencing mental health well into adulthood. Clark et al. (2010) demonstrated that the adverse effects of childhood adversity persist from early adulthood to mid-life, with each additional childhood adversity increasing the likelihood of developing psychopathologies in mid-life. These enduring consequences are partly explained by the neurobiological changes induced by stress exposure in childhood. Stress in children manifests through heightened emotional reactivity, increased internalization, fear generalization, and impulsivity (Heleniak et al., 2016). These behavioural responses are closely linked to alterations in the limbic and cortical structures, particularly the amygdala and medial prefrontal cortex (mPFC), which play crucial roles in emotion regulation and cognitive processing (Sheridan & McLaughlin, 2014).

A second hypothesis suggests that experiences of adversity lead to an acceleration of neural development, particularly in brain regions crucial for emotional processing and regulation,

such as the amygdala and mPFC (Callaghan & Tottenham, 2016). Studies have found that the connectivity between the amygdala and mPFC shows accelerated growth and maturation in children raised without supportive or consistent caregiving, often resembling a more "adult-like" pattern following deprivation (Gee, Gabard-Durnam, et al., 2013; Gee, Humphreys, et al., 2013). Regarding the amygdala's structural development, maternal deprivation early in life has been linked to atypically large amygdala volumes in children aged 5–16 (Tottenham et al., 2010). Moreover, high rates of childhood adversity are associated with larger left amygdala volumes in early adolescence (Whittle et al., 2013). Although the functional significance of this accelerated amygdala maturation is still debated, studies suggest that children exhibiting a more mature pattern of amygdala-mPFC connectivity tend to show less anxiety than those with a more immature pattern, indicating that early maturation of the amygdala-mPFC circuit may support emotional regulation, at least in the short term (Gee, Humphreys, et al., 2013). However, some studies have reported smaller amygdala volumes following early life stress, particularly in adolescents (Hanson et al., 2012) and preschoolers (Luby et al., 2013). These mixed results may reflect the complex effects of repeated exposure to stress, which could lead to opposing structural adaptations within the amygdala.

The body's physiological response to stress is primarily mediated by the hypothalamic-pituitary-adrenal (HPA) axis. Upon encountering stress, the hypothalamus releases corticotropin-releasing hormone (CRH) from the paraventricular nucleus (PVN), which subsequently stimulates the anterior pituitary gland to release adrenocorticotropic hormone (ACTH). ACTH then prompts the adrenal cortex to release cortisol, a glucocorticoid hormone, which activates the sympathetic autonomic nervous system and enhance the body's fight-or-flight response (Herman et al., 2016; Helfrich-Forsster, 2017; Herrero et al., 2015). The hippocampus and amygdala, key

limbic system components, regulate cortisol levels through glucocorticoid receptors, providing feedback to the HPA axis (Labar & Cabeza, 2006). While the hippocampus typically inhibits excessive stress responses, the amygdala reduces inhibitory control, amplifying emotional reactivity. The mPFC plays a regulatory role by modulating brain activity in response to stress, although it may become dysregulated under chronic stress conditions (Herman et al., 2012).

Early life stress also induces structural changes in the mPFC, a brain region critical for regulating cognitive and emotional responses to stress and threat (McKlveen et al., 2015). Neuroimaging studies have consistently shown that adults with PTSD (Li et al., 2014) or major depression exhibit reduced gray matter volume in the mPFC, accompanied by morphological abnormalities (Grieve et al., 2013; Singh et al., 2013). The mPFC follows a prolonged developmental trajectory, maturing later than other cortical brain regions (Brenhouse et al., 2011). This extended maturation period makes the mPFC particularly vulnerable to stress. Structural reductions in the mPFC have been found in children exposed to neglect, abuse, or poverty (Hanson et al., 2010; Hanson et al., 2012), which may contribute to functional impairments.

These findings highlight stress's complex and enduring impacts on neurobiological and psychological development. To understand potential adaptive processes in the face of adversity, it is important to consider mechanisms such as safety learning, which may serve to buffer or regulate stress-related responses.

1.3 Safety Learning

Safety learning involves associating specific cues with the absence of threat, leading to adaptive responses that inhibit fear and promote survival (Laing et al., 2022). Although the neurobiological mechanisms underlying safety learning are not yet fully understood, emerging

evidence suggests that it plays a critical role in psychological well-being, particularly fear regulation and inhibition. A deeper understanding of safety learning could inform therapeutic strategies for psychological disorders such as anxiety and depression. By enhancing safety learning, it may be possible to improve symptom management and overall mental health.

Studies using human and animal models have examined the neural circuits involved in safety learning. The acquisition of safety cues typically involves the formation of conditioned associations between neutral stimuli and the absence of threat, resulting in suppressed fear responses. This process has been linked to activation in the amygdala, medial prefrontal cortex (mPFC), and other regions associated with emotion and memory, such as the hippocampus (Grunfeld, 2022). The amygdala detects threats and learns to associate safety cues with the absence of danger. In conjunction with the mPFC, which helps regulate fear responses, and the hippocampus, which supports context-based discrimination between threatening and non-threatening stimuli, these regions form a network critical for effective safety learning. Disruptions in any part of this circuitry can impair safety learning and contribute to persistent anxiety.

The ability to learn and utilize safety cues changes across development. Research indicates that children show a reduced ability to distinguish between threat and safety cues, while adolescents become more adept at this distinction as they approach adulthood. This developmental progression reflects the maturation of brain regions involved in emotional regulation, particularly the amygdala and mPFC (Grasser & Jovanovic, 2021). Understanding this trajectory is important, as delays or disruptions in this process may increase vulnerability to psychological disorders such as anxiety.

The effects of stress on safety learning have also been studied. Both chronic and acute stress have been found to interfere with the ability to process and respond to safety cues. For example, acute stress impairs the expression of learned safety, potentially increasing the risk of developing anxiety disorders later in life (Wang et al., 2024). Similarly, pre-adolescent stress has been shown to have long-term consequences on anxiety-like behaviours and safety learning, with effects that can persist into adulthood (Meyer et al., 2021). Stress-related changes in brain regions such as the mPFC, amygdala, and thalamus have been linked to altered threat discrimination and increased fear generalization (Grunfeld, 2022). These disruptions may make it more difficult to distinguish between safe and threatening cues, contributing to heightened anxiety and stress-related symptoms. Overall, as the neurobiological understanding of safety learning continues to evolve, it offers critical insights into how safety cues are processed and how dysfunction in these processes can contribute to the development and persistence of anxiety and other psychological disorders.

1.4 Comparative Neurodevelopment (Humans vs Rodents)

Adolescence is a critical neurodevelopmental period characterized by dynamic brain structure, connectivity, and behaviour changes. Understanding these developmental processes in humans can be supported by studies in rodents, given key homologous stages and timelines. The adolescent period in humans typically spans 12–18 years of age, corresponding to approximately postnatal days (P)33-46 in rats (Marty et al., 2003; Bell, 2018). Earlier stages of human development—such as infancy (1 month–2 years) and childhood (2–11 years)—roughly map onto (P)7–35 in rats, while the peripubertal period (11–14 years in humans) aligns with (P)35–55 in rats, a time when major hormonal and neurodevelopmental shifts occur (Bell, 2018; Beckman

& Feuston, 2003; Picut & Ziejewski, 2018). These cross-species alignments validate the use of rats to investigate adolescent-specific brain mechanisms.

During adolescence, both humans and rodents experience a temporal mismatch in brain maturation; limbic structures such as the nucleus accumbens mature earlier than the medial prefrontal cortex (mPFC), a region involved in executive control and emotion regulation (Andersen et al., 2000; Teicher et al., 1995). This mismatch contributes to heightened emotionality and risk-taking behaviours—a hallmark of adolescence—and may partially explain increased vulnerability to stress-related psychopathology during this period.

Rats, like humans, also display a surge in social interaction and play behaviour during adolescence, which is regulated by the mPFC and associated limbic structures (Panksepp, 1981; Pellis & Pellis, 1998). This behavioural parallel reinforces the translational value of rodent models for investigating how stress during this sensitive window could alter typical socioemotional development. While social play is not the direct focus of the present thesis, it provides a behavioural context in which adolescent brain plasticity is evident and susceptible to perturbation.

Adolescent brain development is a period of heightened plasticity and remodelling, particularly in the mPFC, and exposure to stress during this stage may induce long-lasting alterations in neurobiological and behavioural outcomes. Indeed, stressors during this window have been shown to impair fear extinction, alter connectivity in stress-responsive networks, and predispose individuals to anxiety and depressive disorders in both rodent and human studies (Giustino & Maren, 2015). By aligning the developmental windows of rats and humans, this thesis aims to investigate how stress during early childhood and adolescence, (P)31–43 in rats, affects neural plasticity and safety learning mechanisms that, if disrupted, may contribute to the

onset of psychiatric illness later in life. The use of rats in this context is methodologically feasible and biologically meaningful, given the conserved features of adolescent brain and behavioural development across species.

1.5 Stress in Rodent Models

These processes are synonymous with rat models used to emulate the effect of stress, making rats an appropriate subject group. Stress studies using rodents have primarily focused on early weaning of the pup from its mother. These studies have found increased activation in the amygdala, precocious myelination of axons in the basolateral amygdala, hyperconnectivity between the amygdala and the hippocampus and mPFC, reduced spine density, and long-term potentiation impairment (Moriceau et al., 2009; Ono et al., 2008; Johnson et al., 2018; Radley et al., 2005). Further studies have also found alterations in the mesocorticolimbic pathway, decreased hippocampal volume (found in subfields CA1, CA3, and the dentate gyrus), dendritic loss in the mPFC, and decreased plasticity between the mPFC and amygdala, specifically in juvenile subjects persisting into adulthood (Fragale et al., 2017; Isgor et al., 2004; McCormick et al., 2007). These neurobiological changes produced effects of increased avoidance behaviours, anxiety-like behaviours, and impairments in memory (Isgor et al., 2004; McCormick et al., 2011).

Regarding rodent studies, experiencing stress in adolescence has also been shown to influence depression and anxiety-like behaviours. Pohl and colleagues (2007) showed that male and female adolescent rats exhibited amplified anxiety-related behaviours, such as increased shock probe burying and increased escape responses in the elevated plus maze, after exposure to intermittent physical stressors in comparison to controls. Another study identified that a difference in stress impact on depression and anxiety-like behaviours exists between juvenile

stress and adolescent stress in rats. The rats in the juvenile stress group (PD28) are in their early adolescence which begins at 21 to 34 days of age. The adolescent stress group (PD34) are in the mid-adolescence or pre-pubertal period that starts from 34 to 46 days (Tirelli et al., 2003). Specifically, exposure to short-term stress in juvenility has a greater augmenting effect than in adolescence, as indicated by reduced exploration, poor avoidance learning, and learned helplessness-like behaviours (Tsoory & Richter-Levin, 2006). Later work using the same short-term stressors and a predator scent stressor both resulted in increased anxiety and depression-like behaviours that lasted into adulthood (Tsoory et al., 2007).

Expanding on these findings, Tirelli (2003) investigated differences in stress responses between juvenile rats (21-34 days of age) and adolescent rats (34-46 days of age). The study found that exposure to short-term stress had a more significant impact on the juvenile group, as indicated by decreased exploration, poor avoidance learning, and increased learned helplessness behaviours in behavioural tasks, similar to the Tirelli et al. (2003) study. However, both groups exhibited increased rates of anxiety and depressive-like symptoms that persisted into adulthood (Tirelli et al., 2003). These findings highlight the potential long-term consequences of early-life stress, particularly in the mesocorticolimbic pathway, and make the adolescent group an area of further interest for subsequent research.

A subsequent follow-up study by Wilkin and colleagues (2012) presented that stress during early adolescence or juvenile period increased the duration of burying behaviour in the shock-probe test, increased immobility in forced swim test, and decreased open-arm exploration in the elevated plus maze compared to controls. This supports previous findings and emphasizes that the effects of stress on psychopathology in adulthood may depend on the timing of stress exposure in adolescence. Interestingly, the same study found that mid-adolescent stress leads rats

to display greater risk-taking behaviour as implied by increased open-arm activity. This contrasts with the stress sensitization model, nonetheless, it may reflect the increased risky behaviours observed in human adolescents. These general observations of increased anxiety and depression-like behaviours were also observed by Yohn and Blendy (2017) who used a chronic unpredictable stress paradigm starting in early adolescence. More specifically, rats in this study were exposed to three stressors each day for 12 days, whereas the rats in the study of Wilkin et al., (2012) experienced three stressors a day randomly for 6 days within 12 days.

1.6 Molecular and Cellular Mechanisms of Stress

Stress exposure during critical developmental periods induces molecular and cellular changes that can shape long-term brain function. These changes manifest in alterations to neuroplasticity, neuronal maturation, and synaptic organization, influencing cognitive and emotional processes. Parvalbumin (PV) interneurons, perineuronal nets (PNNs), and doublecortin (DCX) are examples of those affected by stress. The dysregulation of these cellular mechanisms may thereby link stress to psychiatric disorders, including anxiety and depression. Understanding how stress disrupts these mechanisms is essential for identifying therapeutic interventions to mitigate the long-term consequences of early-life stress.

1.6.1 Structural Plasticity: Parvalbumin and Perineuronal Nets

Structural plasticity refers to the brain's ability to reorganize its architecture in response to environmental stimuli, learning, or injury, and is a key component of neuroplasticity (Gage, 2004). Unlike synaptic plasticity, which involves changes in the strength of connections between neurons, structural plasticity encompasses modifications to the physical structure of neurons, including the growth of dendritic branches, the formation of new synapses, and the rearrangement of existing neural circuits (Gage, 2004). These changes are essential for long-term

learning, memory consolidation, and adaptive behaviours, and are particularly prominent during critical developmental periods. However, stress exposure during these sensitive periods can interfere with the brain's ability to undergo structural plasticity, leading to lasting alterations in neuronal networks. Stress can impact the development of key cellular structures, such as parvalbumin (PV) interneurons and perineuronal nets (PNNs), which are crucial for maintaining the stability and function of neural circuits (Catale et al., 2022). Disruptions in these structures can impair cognitive and emotional processes, including safety learning and fear regulation, making the brain more vulnerable to neuropsychiatric disorders such as anxiety and depression (Catale et al., 2022). Understanding how stress affects structural plasticity through alterations in PV and PNNs is crucial for identifying potential therapeutic targets to mitigate the long-term consequences of early-life stress and promote healthier brain development.

Parvalbumin (PV) interneurons play a significant role in the relationship between stress and neurobiology, particularly in neurodevelopmental and neuropsychiatric disorders (Woodward & Coutellier, 2021). PV interneurons, a GABAergic interneuron in the cortex, are sensitive to stress due to their extended development from the neonatal stage through adolescence. In a study by Page (2019), increased prefrontal PV expression in female mice under stress correlated with anxiety-like behaviours, suggesting a link between stress-induced changes in prefrontal PV cells and emotional behaviours. Additionally, chronic stress can affect PV interneurons in the limbic system, particularly in the hippocampus, altering the GABAergic network and potentially contributing to mood disorders (Zaletel et al., 2016; Rossetti et al., 2018). The role of PV interneurons in safety learning is also crucial, as these cells help regulate the inhibition of fear and facilitate the expression of safety memories. Stress-induced disruptions in PV interneurons can impair the brain's ability to inhibit fear in response to safety cues, leading

to difficulties in learning and maintaining these cues (Ruzicka et al., 2022). Therefore, the interplay between stress and PV interneurons can lead to neurobiological changes that may contribute to stress-related neuropsychiatric disorders later in life.

Perineuronal nets (PNNs) are crucial in regulating synaptic plasticity and emotional regulation by stabilizing synapses through stressful experiences. PNNs are structures found within the extracellular matrix surrounding cell bodies. It has been found that stress can alter the structure and intensity of PNNs. Catale and colleagues (2022) found that adult stress tends to increase PNN density, while early-life stress disrupts the maturation of PNNs in the anterior cingulate cortex (ACC) and impairs synaptic plasticity. Additionally, behavioural deficits, including anxiety, have been connected to alterations in PNNs as they are essential not only for synaptic integrity but also for closing developmental periods (Li et al., 2023). Early-life stress can profoundly impact PNN development, leading to disrupted inhibitory signalling and increased vulnerability to affective disorders into adulthood (Catale et al., 2022). PNNs also play a significant role in consolidating safety memories by regulating the plasticity of synapses involved in forming and expressing safety cues. Disruptions in PNN structure, particularly under stress, may impair the brain's ability to stabilize safety memories, making it more difficult to differentiate between safe and threatening cues (Ruzicka et al., 2022). Understanding how stress responses impact labeled cell PNN counts and intensity, can offer insights into psychiatric disorders and their therapeutic targets (Laham & Gould, 2022; Morphett et al., 2024).

The interplay between PV and PNNs is vital to understanding neurodevelopment and the advancement of psychopathologies. PV interneurons are influenced by the PNNs surrounding them, particularly in the mPFC (Wingert & Sorg, 2021). In an autism spectrum disorder (ASD) model, changes in PV expression and the density of PNNs disrupt GABAergic inhibitory

neurons, leading to imbalances in brain activity and have been linked to the development of ASD (Ueno et al., 2017). In a study by Li (2023), a valproic acid (VPA) model of ASD treatment showed through an immunofluorescent marker, PV intensity decreased, PNN intensity increased, indicating an abnormal relationship between the two elements, possibly contributing to the disorder's pathophysiology. Therefore, the vulnerability of PV interneurons and PNNs in the mPFC may play a role in cognitive challenges (Ueno et al., 2017). Disruptions in both PV interneurons and PNNs may contribute to deficits in safety learning, preventing the effective inhibition of fear and altering the brain's ability to encode and express safety memories appropriately (Ruzicka et al., 2022).

In the study by Ueno et al. (2017), the effects of chronic stress during adolescence on perineuronal nets (PNNs) and prefrontal cortex development were examined using male Sprague-Dawley rats exposed to stress for 7, 15, or 35 consecutive days beginning at postnatal day 28. The researchers found that chronic stress delayed the maturation of PNNs in both the medial prefrontal cortex (mPFC) and orbitofrontal cortex (OFC), regions critical for cognitive flexibility and executive function. This disruption in PNN development was associated with impairments in behaviours reliant on these brain regions, suggesting that adolescence represents a sensitive period during which stress can produce long-lasting structural and functional changes (Ueno et al., 2017). Given that PNNs are known to regulate synaptic stability and critical period plasticity, these findings have important implications; stress-induced delays in PNN maturation may hinder the refinement of inhibitory circuits and contribute to enduring deficits in prefrontal-dependent behaviours (Ueno et al., 2017). This work supports the notion that adolescent stress increases vulnerability to psychiatric disorders by altering the trajectory of neural development in the prefrontal cortex.

Moreover, another study by Ueno et al. (2018) explored the effects of juvenile stress on PV neurons and PNNs, shedding light on how early-life stress can influence neurodevelopment. In this study, juvenile stress resulted in neurodevelopmental disorder-like behaviours, including anxiety-like behaviours in the stressed mice. Alternatively, the fluorescence intensity of WFA-positive PNNs decreased in the brains of juvenile-stressed mice, which may signify increased brain vulnerability. This suggests that juvenile stress impacts the brain's vulnerability to neuropsychiatric disorders, as it alters both the behaviour and the neurobiological processes involving PV neurons and PNNs; however the patterns of these changes should be further explored.

1.6.2 Hippocampal Neurogenesis: Doublecortin (DCX)

Neurogenesis is the process by which new neurons are generated, and it plays a crucial role in maintaining brain plasticity throughout life, particularly in areas like the hippocampus, which is involved in learning, memory, and emotional regulation (Shors et al., 2013). During neurogenesis, precursor cells proliferate and differentiate into new neurons that integrate into existing neural circuits (Shors et al., 2013). This process is essential for cognitive functions such as memory formation and the regulation of emotional responses. However, neurogenesis is highly sensitive to environmental factors, including stress, which can profoundly affect the formation and maturation of new neurons. Stress, especially during critical developmental periods like adolescence, can disrupt the neurogenic process, impairing the maturation of neurons and affecting the structure of brain regions such as the hippocampus. Doublecortin (DCX), a protein expressed in immature neurons, is a key marker of neurogenesis. Chronic stress has decreased the proliferation of DCX-positive neurons and impaired their maturation, particularly in the hippocampal dentate gyrus, a region critical for learning and memory (Harbi et

al., 2021). Stress-induced alterations in DCX expression can have long-lasting effects on neurogenesis, influencing cognitive functions like memory and the brain's ability to distinguish between safety and threat cues. Disruptions in neurogenesis, particularly in the context of stress, may impair the formation of new associations and the brain's processing of fear, contributing to the development of psychiatric disorders such as anxiety and depression. Understanding how stress affects neurogenesis, primarily through alterations in DCX expression, provides critical insights into the neurobiological mechanisms underlying stress-related cognitive and emotional dysregulation.

Doublecortin (DCX) is a microtubule-associated protein expressed in immature neurons and plays a pivotal role in neuronal development, particularly in the hippocampus (Dioli et al., 2019). Research has shown that chronic stress significantly impacts the density and dendritic complexity of DCX-positive neurons, especially in the hippocampal dentate gyrus, where alterations in DCX expression can vary depending on the stage of neuronal maturation and the specific sublayer involved (Dioli et al., 2019). The hippocampus, a critical region for learning and memory, undergoes neurogenesis throughout life, with significant changes during adolescence, a period particularly vulnerable to stress (Borsini et al., 2023).

In adolescent rodents, stress has been shown to reduce hippocampal cell proliferation and neuroplasticity, which may contribute to behavioural changes, including depressive-like behaviours (Borsini et al., 2023). Chronic stress suppresses the maturation of newly generated neurons, leading to altered dendritic complexity and a decline in synaptic plasticity in the dentate gyrus (Chen et al., 2015). These disruptions may affect cognitive functions, such as memory, and contribute to psychiatric symptoms in later life. These changes in neurogenesis and synaptic plasticity are highly relevant to safety learning, as the hippocampus plays a crucial role in

encoding the brain's adaptation to new memories, including the discrimination between safety and threat cues. Impairments in hippocampal neurogenesis may hinder the formation of new associations for effective safety learning.

The impact of stress on DCX expression has been investigated in multiple studies, indicating that stress reduces DCX+ cells in the hippocampus. It was found that acute stress during adolescence can impair spatial learning and memory, associated with reduced neurogenesis and disruptions in neuronal maturation (Kutsuna et al., 2012). These impairments may extend to difficulties in learning safety cues, as distinguishing between safe and threatening environments depends on a flexible and adaptive neural network. When stress interferes with the maturation of DCX-positive neurons, it may reduce the brain's capacity to process and retain safety signals, potentially impairing fear inhibition and emotional regulation.

Moreover, stress-related changes in neurogenesis can persist into adulthood, with some studies showing long-term impairments in cognitive functions and depressive-like behaviours (Borsini et al., 2023). Such disruptions may undermine the capacity to learn safety cues throughout life, leading to chronic difficulties in distinguishing between safety and threat, a hallmark of anxiety disorders.

Notably, chronic social stress, such as social defeat stress, has been shown to decrease the survival of adult-born neurons in the hippocampus, impairing synaptic maturation. While these neurons may survive the stress, their dendritic complexity and the development of synaptic properties are altered, specifically in terms of N-methyl-D-aspartate receptor subunit composition (Chen et al., 2015). This alteration in synaptic maturation is thought to hinder the typical functional integration of newly generated neurons, affecting cognitive and emotional behaviours. When applied to safety learning, this disruption may compromise the brain's ability

to effectively encode and utilize safety signals, leading to heightened fear responses and a reduced ability to regulate emotions in the presence of safety cues.

The evidence underscores that stress can significantly impact DCX expression and neurogenesis, disrupting neuronal maturation and cognitive functions. These changes may contribute to the development of psychiatric disorders, particularly in vulnerable periods like adolescence, when the brain is undergoing critical developmental processes. Given the role of DCX in neurogenesis and synaptic plasticity, these disruptions are likely to affect the formation and expression of safety memories, contributing to difficulties in safety learning and the regulation of fear responses.

1.7 Conclusion

This introduction has explored the impact of adolescent stress on behavioural outcomes and neurobiology while emphasizing the role of specific brain regions. Adolescence is a period of vulnerability in which stress can induce long-term psychopathologies to develop later in life. These psychological outcomes are due to neural circuits impacting emotional regulation, cognitive functioning, and stress resilience.

Empirical findings suggest a connection between stress and the impairment of safety learning due to increased fear generalization, thereby contributing to anxiety and depressive-like symptoms. The complex interplay between stress exposure, neuroplasticity, and behavioural changes underscores the importance of early identification and intervention to mitigate long-term consequences.

Rodent models provide valuable insights into the mechanisms by which stress affects the brain. Findings in rodent models parallel those observed in human studies, reinforcing the translational value of preclinical research to understand stress-related psychopathologies. There

is an increased need for continued research in this area. By investigating these topics further, targeted interventions aimed at enhancing stress resilience can help reduce the risk of anxiety and depressive symptoms in individuals exposed to early life stress.

1.7.1 Research Significance and Rationale

The present study seeks to bridge the gap in understanding how adolescent stress influences neurobiological markers associated with safety learning and affective behaviours in adulthood. While fear conditioning and extinction are well studied, there is a lack of information on the long-lasting effects of early life stress on safety learning. Additionally, the combined analysis of distinct cellular markers to provide possible explanations to behavioural outcomes is a cornerstone of the present study. This study uses an intermittent physical stress paradigm to examine how stress alters DCX expression in the hippocampus and PV/PNN expression in the mPFC (Wilken et al., 2014). This study will comprehensively analyze how adolescent stress impacts adult neurobehavioral outcomes by assessing behavioural outcomes, such as safety conditions, anxiety-like behaviours (explored through the elevated plus maze and open field task), and depressive-like behaviours (explored through sucrose consumption and forced swim task). By understanding these effects, potential therapeutic interventions for stress-related psychopathologies may be improved.

1.7.2 Hypotheses and Study Objectives

The objectives of this study aimed to (1) determine the impact of prior adolescent stress (mid-adolescent period; PD 31-43) on later adult (PD 70) safety learning and emotional behaviour; (2) determine the impact of prior adolescent stress on adult hippocampal neurogenesis; and (3) determine the impact of prior adolescent stress on markers of plasticity.

It is hypothesized that adolescent stress will reduce DCX+ cell counts in the hippocampus, reflecting impaired neurogenesis. Additionally, stressed rats will exhibit decreased PV expression and increased PNN expression in the mPFC. The stressed rats will demonstrate higher anxiety and depressive-like symptoms in behavioural tasks and show impairment in safety learning.

By addressing these objectives, the study aims to clarify the relationship between adolescent stress, neurobiological changes, and long-term psychological outcomes, contributing to a deeper understanding of stress-induced vulnerabilities in adulthood.

Chapter 2: Adolescent Stress Induces Long-Lasting Deficits in Safety Learning, Emotional Regulation, and Reward Sensitivity

2.0 Introduction

Adolescence is a critical developmental period in which extensive neurobiological adaptations set the stage for cognitive and emotional processes into adulthood. Exposure to stress during this vulnerable period has been shown to alter brain function and structure thereby shaping cognitive and emotional processes, increasing the risk of neuropsychiatric disorders. Behaviourally, these effects often manifest as heightened anxiety- and depressive-like responses, impaired emotional regulation, and alterations in learning processes (Pohl et al., 2007; Wilkin et al., 2012). For example, stressed rodents consistently show reduced exploration in behavioural tests such as the elevated plus maze and increased immobility in the forced swim test (Yohn & Blendy, 2017). It is important to note that these stress-induced changes not only affect basic neurobiological functions but also influence specific behavioral processes, such as emotional regulation, decision-making, and learning which can be examined through behavioural testing.

Research indicates that stress makes neural circuits and brain regions, specifically the hippocampus and mPFC, involved in emotional regulation, susceptible to dysregulation (Kim et al., 2015; McEwen et al., 2016). While previous research has examined the impact of chronic stress in adulthood, fewer studies focus on the long-lasting effects of early-life stress, specifically on safety learning, into adulthood. Exposure to stress during this sensitive window can recalibrate stress-responsive neural circuits, alter synaptic plasticity, and produce lasting changes in behaviour and cognition (Spear, 2000; McEwen & Morrison, 2013).

Since safety learning - the ability to associate specific cues with the absence of threat – is crucial for adaptive stress responses, investigating its neurobiological underpinnings in stressed rodent models is essential. Safety learning depends on the coordinated activity of the mPFC,

hippocampus, and amygdala (Grunfeld, 2022). Disruptions in these circuits can impair the ability to discriminate between safe and threatening contexts, leading to maladaptive generalization of fear (Meyer et al., 2021). Chronic or unpredictable stress has been shown to interfere with the acquisition and expression of safety cues, often resulting in persistent anxiety-like states and reduced behavioural flexibility (Wang et al., 2024). The present behavioural chapter explores these relationships, focusing on whether exposure to unpredictable intermittent stress during early-life produces measurable changes in behavioural tasks.

2.1 Methodology

2.1.1 Subjects

Male Long Evans rats were obtained from Charles River Laboratories (St. Constant, QC, Canada) and sourced from six distinct litters. The rats arrived at our facility at 22 days of age and were randomly distributed to mitigate potential litter effects at the time of group assignment. The rats were paired housed in microisolated cages under a 12:12 h light/dark cycle (lights on at 0700 h) with food and water freely available unless otherwise specified. All procedures were conducted in accordance with the Canadian Council on Animal Care and approved by Trent University's Animal Care Committee.

2.1.2 Intermittent unpredictable stress exposure

Prior to beginning stress, rats randomly assigned to either stress (n=12) or non-stress (n=12) conditions and housed individually for the duration of the experiment. Rats in the stress condition were exposed to one of three stressors—water immersion, elevated platform and footshock stress (procedures adapted from Wilkins et al., 2012). The stressors were applied randomly across postnatal days (PND) 31 to 43, with only one stressor used per session and no

stressor administered more than twice over the 12-day period. Stress sessions were conducted at random times throughout the day in testing rooms separate from the animals' housing area. Rats in the non-stress condition received brief handling on the stress days to control for handling effects.

Water immersion stress

Rats were individually transported in their home cage to a testing room containing the water immersion apparatus. The water immersion tank was a plastic container (40 cm x 40 cm x 60 cm) that was filled with water (24°C ± 2°C) to a height of 6 cm (for the first exposure) or 8 cm (for the second exposure). During the exposure, rats were individually placed into black PVC restraint tubes (7 cm x 20 cm) that were secured into the immersion tank by a Plexiglas stabilizing plate (35 cm x 50 cm). This enabled the rat to sit or stand in the water without movement. A ventilated Plexiglas lid (40 cm x 40 cm) covered the restrainer tubes to prevent escape. Rats were placed into the water immersion tank for 45 minutes and monitored during this time. Following the exposure, the rat was removed and dried off with a towel before being returned to their home cage. After each session, the immersion tank was emptied and cleaned with Oxivir Five 16 Concentrate (Johnson Diversy, Canada) disinfectant and refilled with fresh water.

Elevated platform stress

Rats were individually transported in their home cage into a brightly lit room and immediately placed on a platform (either 10 cm x 10 cm or 15 cm x 15 cm) that was mounted atop of a Plexiglas tower (10 cm x 10 cm x 100 cm). The platform was positioned in the circular container (150 cm diameter) lined with towels. Each rat remained on the elevated platform for 30 minutes. If the rat jumped or fell from the platform it was promptly returned to the platform by the experimenter and the session resumed. At the end of the exposure, the rat was returned to its home

cage and colony room. The platform was cleaned and disinfected between animals with Oxivir Five 16 Concentrate (Johnson Diversy, Canada).

Foot shock stress

Rats were transported in their home cage to separate room for foot shock stress. Rats were placed in a Plexiglas chamber (25.4 x 25.4 x 36.5 cm, Ugo Basile) housed in a sound-attenuating cabinet. The chamber had a standard grid floor consisting of 21 stainless steel rods (4 mm diameter, 1 cm distance) connected to an adjustable shock generator (Ugo Basile, Varese, Italy) to deliver a scrambled foot shock. A ventilation fan produced a constant background noise of 55 dB within the sound-attenuating cabinet. The chamber was illuminated by a 2.5 W white LED light. At the end of the 5-minute exposure, the rat was returned to their home cage and transported back to their colony room. The boxes were cleaned with Oxivir Five 16 Concentrate after each rat.

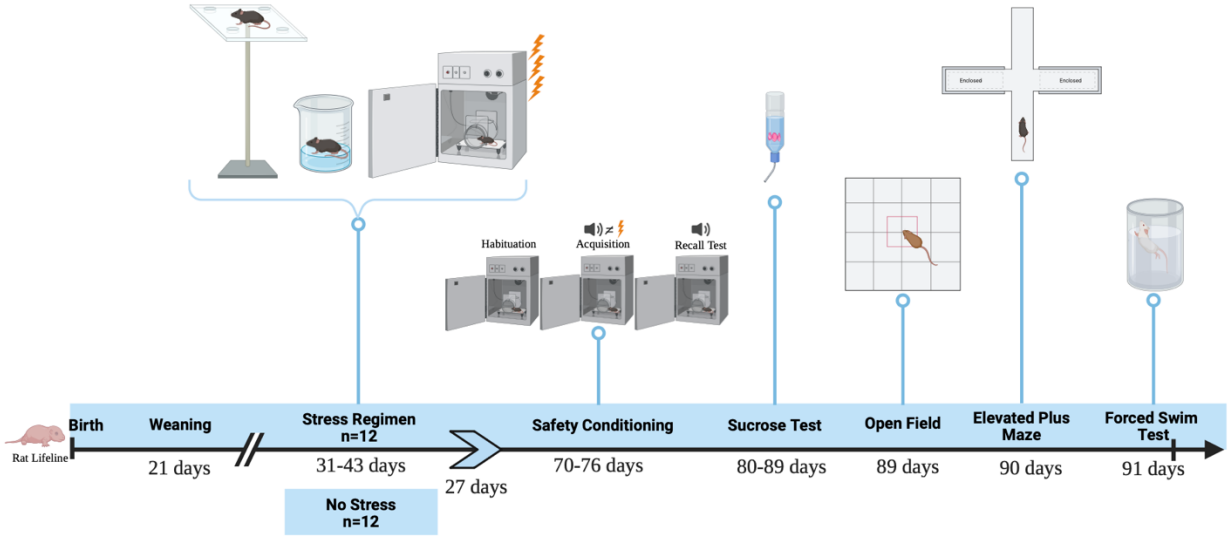

Figure 1: Experimental timeline. Rats have experienced a test regimen for 6 of the 12 days (p31-43). After 27 days of rest, safety conditioning began (p70-76). The rats were subjected to the sucrose consumption test (p80-89), open field (p89), elevated plus maze (p90), and forced swim test (p91).

2.1.3 Behavioural Testing

After the 12-day stress exposure period, all rats were left undisturbed in the colony for 27 days, aside from routine weighing and regular cage cleaning. Behavioral testing (described below) began on PND 70 and was completed on PND 91 (Figure 1).

Safety Conditioning

Safety conditioning occurred in the same chambers as described above but modified to include checkerboard walls and a 2% (v/v) lemon-scented odor. Auditory stimuli (20s, 80 dB) were delivered through a loudspeaker mounted on the ceiling of the cabinet. All sessions were video recorded using an overhead webcam connected to a laptop computer. Chambers were cleaned with 70% ethyl alcohol following each session and the odor was replaced.

Safety conditioning was carried out over seven consecutive days. On Days 1-3, rats were habituated to the modified chambers for 22 minutes per day, during which no shocks or were presented. On Days 4-6, rats underwent safety conditioning consisting of five explicitly unpaired presentations of unconditioned stimulus (US, foot shock, 1s, 0.7 mA) and CS (tones, 20s, 80 dB) presentations. The time between the US and CS was explicitly offset by 120s (range: 100-140s) and occurred in a pseudo-randomized order across the conditioning days.

On Day 7, a retention test was conducted. After transportation from the colony, the rats were immediately inside the conditioning chamber. Following a 90s baseline period, three test tones (20s, 80 dB) were delivered, each separated by an interval of 90s. Defensive freezing, which was defined as the absence of observable movement except those necessary for respiration, was measured using an automated freeze detection system (AnyMaze). The percentage of freezing displayed during each test tone was used as a measure of conditioned safety. The test compared

freezing durations 20 seconds prior to and after the onset of the tone. Following the test, the rats were returned to their home cages and the chambers were cleaned with 70% ethanol.

Sucrose Consumption

Sucrose consumption, a well validated measure of anhedonia, began four days after the completion of safety conditioning. Starting on PND 80, waterspouts were removed from the home cages and replaced with plastic drinking bottles to habituate the animals to the new drinking system over two days. Water consumption was monitored at 1 hr, 24 hr and 48 hr time points. On PNDs 82-84, the water bottles were replaced with new bottles containing 1.8% sucrose solution. Sucrose intake was monitored at 1 hr, 24 hr and 48 hrs intervals. After the 48-hr acclimation session to the sucrose solution, the rats' home cages and lids were changed to eliminate potential food residue, and the animals underwent a 24-hr period of food and water deprivation. On PND 85, both chow and a 1.8% sucrose solution were reintroduced. Sucrose intake was recorded at 1 hr and 24 hrs intervals. On PND 86, the sucrose bottles were replaced with standard water bottles for a 24 hour re-acclimatation period. This was followed by a second 24 hr deprivation period with additional cage changes to prevent access to residue food particles. On PND 88, the rats were returned to standard chow and water consumption was measured at 1 hr and 24 hr intervals. Sucrose consumption was calculation as a ratio of sucrose intake to water intake at the 1 hr and 24 hr time points to control for individual differences in fluid consumption

Open Field

On PND 89, the rats were transported to a testing room with the stainless-steel open field arena (60cm x 60cm x 60cm) with a small amount of corn bedding covering its floor. At the

beginning of the session, the rat was placed at one of the four corners of the arena with its nose facing the wall. The rats were allowed to explore the arena for 10 minutes. The session was recorded with a camera situated above the open field and analyzed using AnyMaze software (Stoelting, Wood Dale, IL). The open field was digitally divided into a center zone, which was defined as a 576 cm^2 square in the middle of the open field, and a peripheral zone, which was considered the space between the walls of the enclosure and the middle zone border (i.e. the 3024 cm^2 area surrounding the middle zone). The total distance travelled, the mean movement velocity, time spent immobile, and the time spent and distance travelled in the center zone was recorded. After testing each animal, the arena was cleaned with Oxivir Five 16 concentrate (Diversey Inc. Canada).

Elevated Plus Maze

On PND 90, the rats were transported to testing room containing an elevated plus maze (EPM). The EPM was elevated 50 cm and consisted of two enclosed arms (50 cm x 10 cm x 40 cm) and two open arms (50 cm x 10 cm). The EPM was placed in the center of a homogeneously illuminated room. Each rat was placed in the intersection between the arms facing the open arm opposite to the investigator. Each session was video recorded for 5 min, and the rat's position was determined by automatic video tracking (AnyMaze, Stoelting, Co.). The percentage of open arm entries, time in open arms, time in closed arms (s), and time in the center square (s) were recorded. After testing each animal, the elevated plus maze was cleaned with Oxivir Five 16 concentrate (Diversey Inc. Canada).

Forced Swim Test

On PND 91**,** rats were transported in their home cages to the testing room for a single session of the forced swim test, a widely used assay of behavioral despair. Each rat was placed for 10 minutes into a Plexiglas cylinder (20 cm diameter, 50 cm height) filled with water (22–24 °C) to a depth of 30 cm. Following the session, the rat was removed, gently dried with a towel, and placed under a heat lamp for 30 minutes. The water was changed, and the cylinder cleaned with Oxivir Five 16 concentrate (Diversey Inc. Canada) between animals.

Forced swim sessions were video recorded and later scored offline for the total duration of immobility. Immobility was defined as the absence of active swimming, with the rat either floating passively or making only minimal movements necessary to keep its head above water, without leaning against the cylinder wall.

2.2 Results

2.2.1 Adolescent stress impairs adult safety learning

To address whether exposure to intermittent stress during adolescence disrupted the later ability to learn safety signals, adult rats underwent safety conditioning beginning on PND 70, which corresponded to 26 days after their final stress exposure. We first acclimated rats to the conditioning chambers for three days before safety conditioning. Examination of the freezing behaviour over this period revealed a significant Day x Group interaction [$F(2,44)=32.89$, $p<.001$, partial $\eta^2=.599$] and main effect for Group [$F(1,22)=12.70$, $p<.002$, partial $\eta^2 =.366$]. The stress group displayed initially higher levels of freezing than the controls on the first two days of acclimation ($p < .037$), but this difference was no longer significant by the third day ($p=.645$) (**Figure 2A**). However, the stressed-exposed rats showed a significant reduction in freezing

particularly between Days 1 and 2 ($p < .003$) indicating gradual but eventual habituation to the chambers. These findings align with past reports of impaired habituation following early-life stress and support the interpretation that adolescent stress enhances initial reactivity to novel environments.

Conditioning

After acclimation to the conditioning chambers, all animals underwent of safety conditioning during which five tones and fives foot shocks were delivered in an explicitly unpaired manner. To examine the development of safety learning, a 2 (Group: Stress vs. Non-Stressed) x 3 (Day: Day 1 through. Day 3) x 5 (Tone: Tones 1 through 5) repeated measures ANOVA was used to examine tone-evoked freezing during conditioning. The three-way interaction between Group, Day, and Tone was not significant [$F(8,176) = 1.03$, $p=.414$, partial $\eta^2=.045$]. However, multiple two-way interactions were found. Significant Group × Tone [$F(4,88) = 3.23$, $p=.016$, partial $\eta^2=.128$] and Day × Tone [$F(8,176) = 2.02$, $p = .047$, partial $\eta^2 = .084$] interactions were observed. There were also main effects of Day [$F(2,44)=38.87$, $p < .001$, partial $\eta^2=.639$] and Tone [$F(4,88)= 2.46$, $p= .051$, partial $\eta^2 =.100$]. No other main or interaction effects reached statistical significance (All $p > .10$). Both groups exhibited reduced freezing to the tones from Day 1 to Day 2 ($p < .001$) and from Day 2 to Day 3 ($p < .001$) which is consistent with the development of safety learning across training (**Figure 2B**). Further analysis revealed that stressed animals froze significantly more than non-stressed controls during presentation of the first tone on the second ($p = .015$) and third days ($p = .028$) of conditioning. However, both groups eventually converged demonstrating similar freezing to later tones presented during each training session. Polynomial contrast analyses confirmed this observation and found a significant linear trend in freezing across tones in stressed

animals on Day 3 of conditioning [$F(1,22)=6.89$, $p=.015$, partial $\eta^2=.239$] indicative of a steeper decline in freezing levels across the tone blocks, whereas non-stressed controls showed a flatter response profile across the tone blocks.

Recall Test

To assess whether animals had learned that the tone presented during conditioning served as a "safety signal", we performed a recall test twenty-four hours after the final day of conditioning. During the recall test, freezing behavior was measured during the 20-second period immediately preceding each tone (reflecting context-driven fear) and during the 20-second tone presentation itself. If the tone had acquired the properties of a "safety signal", then a reduction in freezing from the pre-tone to the tone period would serve as evidence of safety learning.

A 2 (Group: stress vs. non-stressed) x 3 (Block: 1-3) x 2 (Tone: pre-tone vs. tone) repeated measures ANOVA was conducted to examine safety memory recall. There were significant main effects of Tone [$F(1,22) = 19.73$, $p <.001$, partial $\eta^2 = .473$], Group [$F(1,22)=30.55$, $p<.001$, partial $\eta^2 =.581$], and Block [$F(2,44)=6.60$, $p=.003$, partial $\eta^2=.232$]. However, there was no significant interaction between Group and any of these variables. As shown in **Figure 2C**, the stressed group exhibited higher overall freezing during the pre-tone and tone periods than non-stressed controls (Stressed, $84.09 \pm 5.86\%$ vs. Non-stressed, $47.36\% \pm 4.11\%$, $p<.001$). When collapsing across the blocks, the non-stressed group displayed significantly higher freezing during the pre-tone periods than during tone presentations (paired t-test, $t(11)=3.36$, $p=.006$, **Figure 2D**). In contrast, the stressed group did not show a significant difference between pre-tone and tone freezing levels ($p=.186$) suggesting impaired safety recall. To further examine this effect, we computed an overall safety suppression score for each rat by subtracting the average freezing during the tones from the

average freezing during the pre-tone periods. The safety suppression score was significantly greater for the non-stressed controls compared to the stress group (Mann-Whitney U=35.50, p=.035, **Figure 2E**) indicating the tones elicited a stronger suppression of freezing in the control animals.

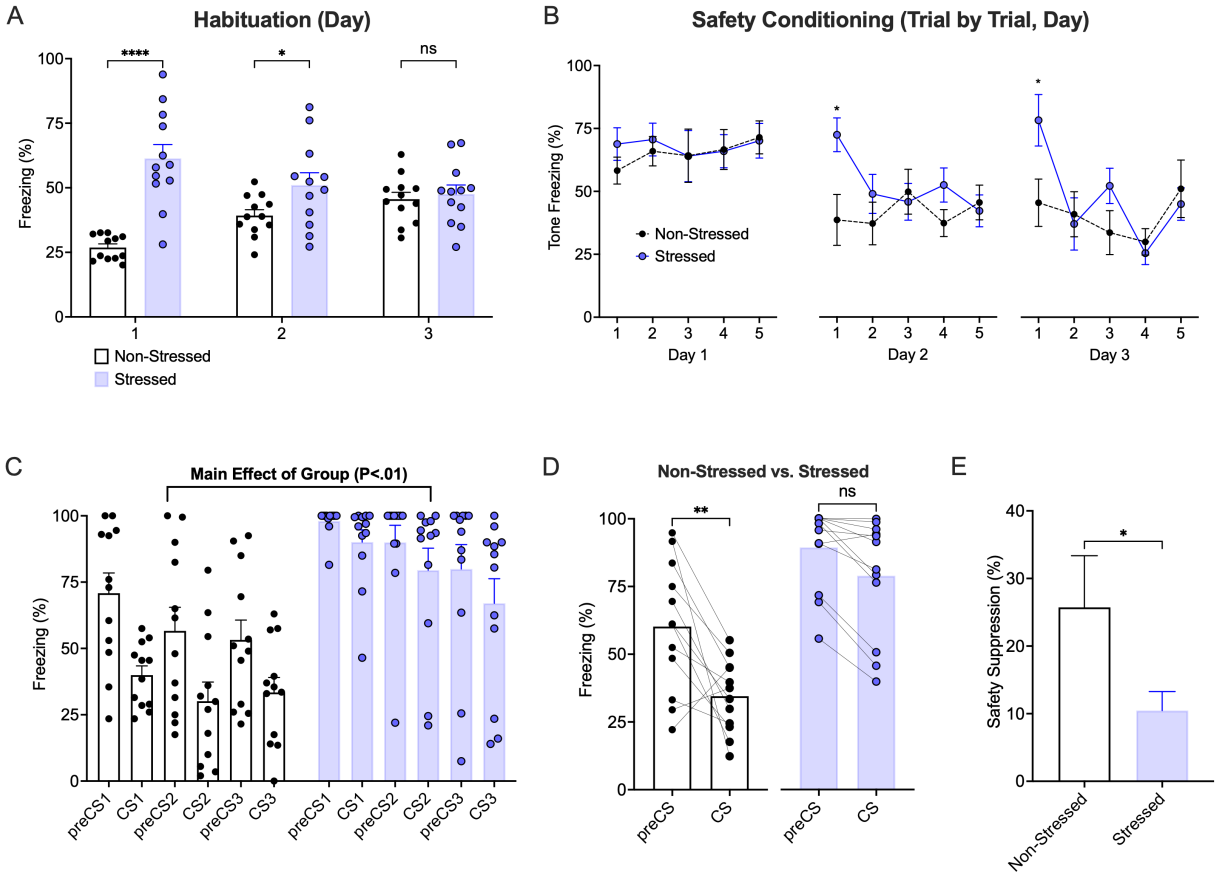

Figure 2: Effects of adolescent stress on safety learning and recall. (A) acclimation, (B) safety conditioning, (C) recall, (D) tone vs. pre-tone, (E) suppression recall

2.2.2 Adolescent stress decreases sucrose consumption

To assess the impact of prior adolescent stress on the consumption of a palatable sucrose solution during adulthood, we measured each rats' sucrose preference by calculating their sucrose-to-water consumption ratios. We compared these ratios after 1 hr, 24 hrs, and 48 hrs access to sucrose and water solutions during the habituation phase (when food was still available). A 2

(Group: Stressed vs. Control) × 3 (Interval: 0–1 hr, 1–24 hr, 24–48 hr) repeated measures ANOVA examining the sucrose consumption ratios revealed a significant main effect of Time [$F(2,42)=25.44$, $p<.001$, partial $\eta^2 =.548$] indicating an overall increase in sucrose consumption during the first 24 hrs after presentation. While there was no significant main effect for Group [$F(1,21)=1.99$, $p=.173$, partial $\eta^2 =.087$], the Time x Group interaction approached significance [$F(2,42)=2.06$, $p=.086$, partial $\eta^2 =.110$). Follow-up comparisons showed that adult rats exposed to adolescent stress had significantly lower sucrose consumption ratios compared to non-stressed controls particularly during final 24 to 48 hr interval of habituation [$F(1,21)=6.12$, $p<.022$, partial $\eta^2 = .226$, **Figure 3A**]. To determine if the groups differed in the development of sucrose preference over time, we computed change scores in the sucrose-to-water ratios for the total (1-48 hr) and early (1-24 hr) intervals of the habituation period. A comparison of these scores revealed a significant group difference ($p = .039$, **Figure 3B**) indicating that non-stressed control rats showed an increase in preference for the sucrose solution over the habituation period, whereas stressed rats showed a relative decrease in sucrose preference. In contrast, no significant group difference was observed when comparing the change in sucrose preference between the mid (1–24 hr) and late (24–48 hr) intervals ($p = .51$). This suggested that the differences in sucrose consumptions between these groups developed gradually over habituation period, rather than occurs abruptly during the final 24 hr period of exposure

Because an acute period of fluid and food deprivation can enhance sensitivity to reward, we also examined sucrose and water consumption following an overnight period of deprivation. Sucrose-to-water intake ratios were computed after 1 hr and 24 hrs following the reintroduction of food. A 2 (Group: Stress vs. Control) × 2 (Time: 1 hr, 24 hrs) ANOVA revealed no significant main effects or interaction effects on the sucrose preference ratios [All $Fs < 0.388$, All $ps > 0.540$,

Figure 3C], indicating that adolescent stress did not affect sucrose consumption when driven by physiological needs, i.e. such as after an acute period of deprivation.

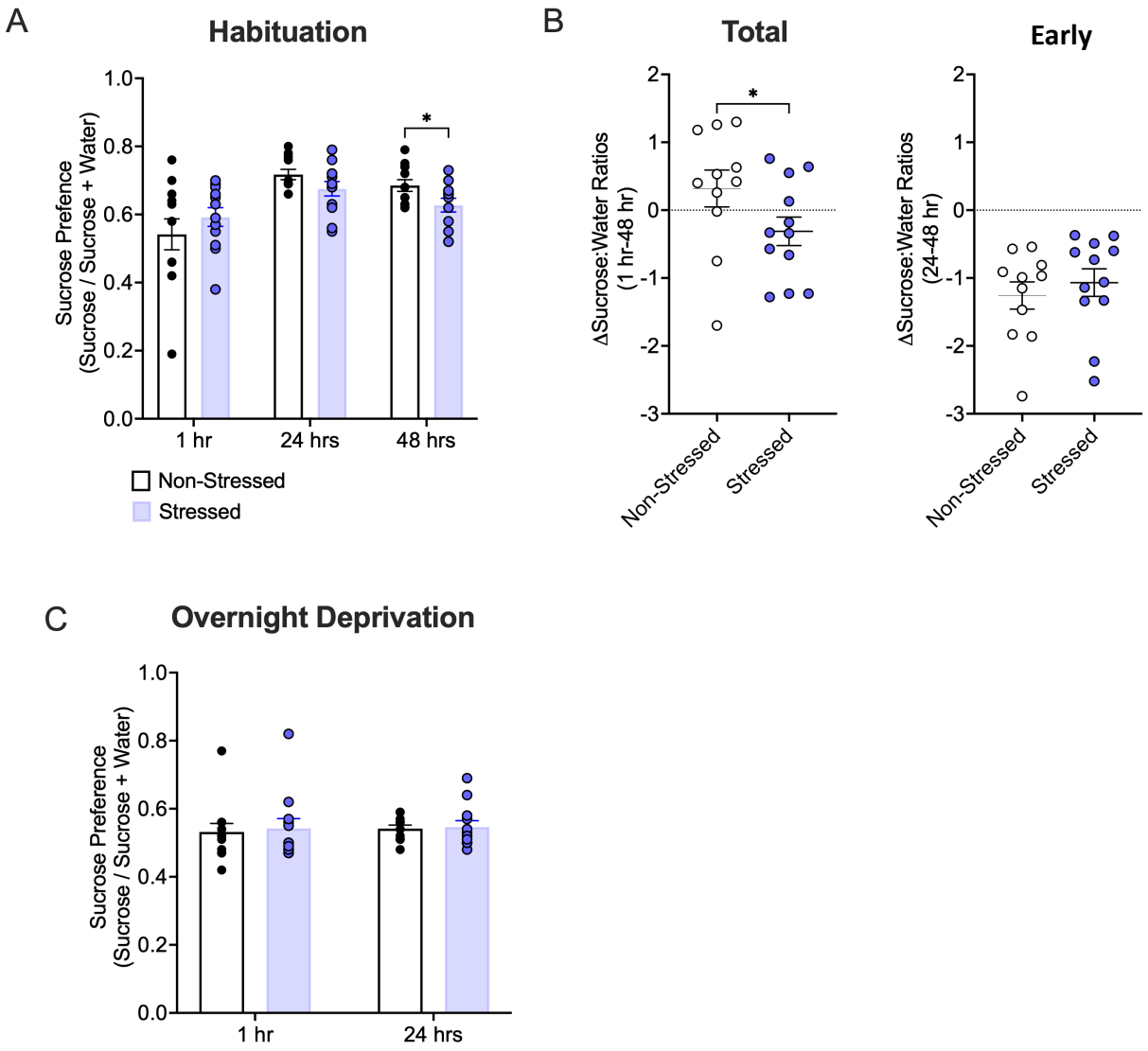

Figure 3: Effects of adolescent stress on sucrose preference in adulthood. (A) habituation period preference, (B) sucrose preference during 1-48hr, (C) sucrose preference after food deprivation

2.2.3 Adolescent stress causes mild anxiogenic responses in adulthood

To determine if adolescent stress exposure impacted exploratory behavior and anxiety-like responses during exposure to a novel environment, rats were tested in the open field. Total distance

traveled and mean locomotor speed were used as primary indicators of general locomotion, while time spent in the center vs. periphery zones, distance traveled in each zone, and zone-specific average movement speed was analyzed as indices of exploratory behavior and thigmotaxis.

As shown in **Figure 4**, adult rats that had been previously exposed to adolescent stress showed reduced exploratory activity. Specifically, the stressed rats traveled significantly less overall distance [Student t-test, $t(22) = 2.29$, $p = .032$, **Figure 4A**] and reduced movement speed during exploration [Student t-test, $t(22) = 2.25$, $p = .035$, **Figure 4B**] than the non-stressed controls. However, in comparison to non-stressed control rats, zone-specific analyses indicated that the stress group traveled less distance [$t(22) = 2.61$, $p = .016$] and spent less time in the peripheral zone [$t(22) = 2.18$, $p = .040$]. There were no differences found in either the average movement speed or the number of entries into the peripheral zone (All *ps* > .465). Interestingly, although the stressed group spent significantly more time in the center zone of the open field arena than the non-stressed group ($p = .040$, **Figure 4E),** there were no group differences in either the distance traveled or in the entries made into this zone (All *ps* > .648). However, stressed rats displayed significantly lower movements speed in the center zone (Stressed, **4**.68 ± 0.63 cm/s vs. Non-stressed, 7.56 ± 1.11 cm/s, p = .034), indicating that the occupancy of the central zone was not associated with a corresponding increase in exploratory activity within in this zone.

To further examine the nature of anxiogenic changes in these animals, we tested rats in the elevated plus maze. The elevated plus-maze (EPM) test is one of the most frequently used behavioral assays to evaluate anxiety-related behavior in rodents. Compared to non-stressed controls, rats previously exposed to adolescent stress spent significantly less time in the open arms [$t(22) = 2.15$, $p = .042$, **Figure 4K**] suggestive of an anxiogenic profile in the elevated plus maze (**Figure 4F**). While the number of open arm entries was also reduced in the stress group, this group

difference did not reach statistical significance [Stress, 2.33 ± 0.58 vs. Non-stressed, 3.58 ± 0.45**,** p=.105, **Figure 4H**]. There were also no group difference in the time or entries made into the closed arms [All ps > .446, **Figure 4G,J**]. Lastly, the total frequency of arm entries was not statistically significant [Stress**,** 16.33 ± 2.08 vs. Non-stressed**,** 19.50 ± 1.38**,** p=.219] suggesting similar levels of locomotor across between the groups.

To assess approach–avoidance conflict behavior, we computed the ratio of time spent in the center hub relative to time spent in the open arms of the elevated plus maze. This ratio is considered an index of risk assessment reflecting hesitation or decision-making at the transition point between exploration and threat avoidance. A higher proportion of rats from the stress group failed to explore the open arms (25%) compared to non-stressed controls (8.3%). However, among the rats that did explore both zones, a significant group difference in center-to-open time ratios was observed (p = .024, **Figure 4L**), with the stressed group displaying higher ratios than the non-stressed controls. These findings indicate that adult rats previously exposed to adolescent stress engaged in more frequent or prolonged risk assessment during exploration of the elevated plus maze.

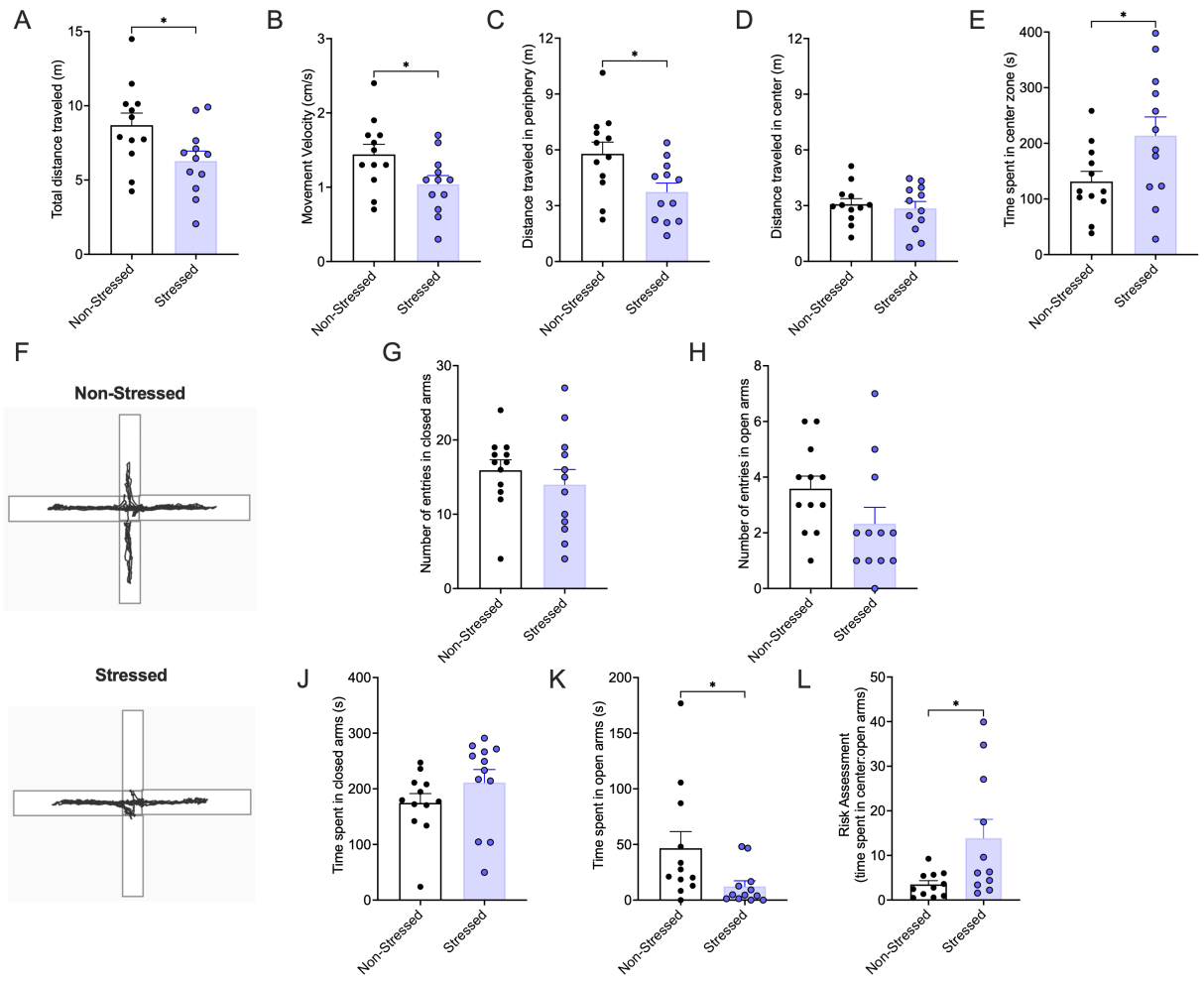

Figure 4: Adolescent stress reduces exploratory activity and increases anxiety-like behaviour. Stressed rats show reduced locomotion and speed in the open field, altered zone use, and less time in open arms of the elevated plus maze, with higher risk-assessment

2.2.4 Adolescent stress increased behavioral despair

To examine the effect of adolescent stress on the development of behavioural despair, we examined adult rats during a 10-minute single forced swim session. As shown in **Figure 5A**, adults rats exposed to adolescent stress spent more time immobile than non-stressed controls [$t(22)=2.58$, $p<.0172$]. We also partitioned the total 10-minute session into successive 2-minute epochs to determine when during 10-minute forced swim test groups differed. A repeated measures ANOVA revealed a significant interaction between Time and Group [$F(1,22)=4.65$, $p<.042$, partial η^2 =

.174] and significant main effects of Time [F(4,88)=88.72, p<.001, partial η^2 = .801] and Group [F(1,22)=6.65, p<.017, partial η^2 = .232]. Post hoc comparisons showed that immobility differences between the stressed and non-stressed groups was most pronounced during the first two minutes (epoch 1, p<.001) and last four minutes (epochs 4 and 5, p<.034) of the forced swim test (**Figure 5B**).

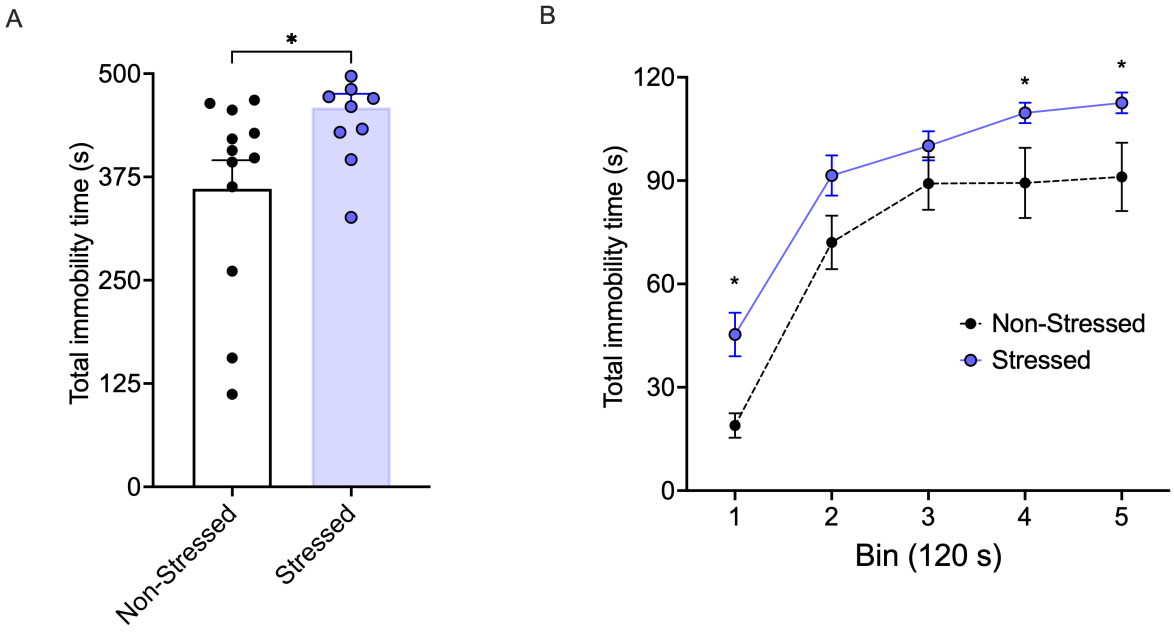

Figure 5: Adolescent stress demonstrates despair in forced swim task. (A) immobility over the 10 minute period, (B) immobility broken down into 2 minute epochs

2.3 Discussion

Overall, early-life exposure to unpredictable intermittent stress resulted in long-lasting behavioural alterations into adulthood. Through behavioural testing, it was evident that the stressed group experienced impaired safety learning and recall, anhedonia, anxiety-like behaviours, and despair behaviours. In the safety learning task, the stressed group showed heightened freezing during the acclimation phase, suggesting an increased reactivity to novel environments. During the actual safety conditioning, these rats froze earlier to tone presentations on days 2 and 3,

indicating a delayed discrimination between threat and safety cues. In the recall task, the stressed rats also had increased freezing behaviours, whereas the controls did not, showing an impaired safety memory recall. This all suggests that early-life stress disrupts the ability to process safety signals and remember them. Therefore, the deficit seen here is with memory, as trials demonstrate that although they learned what the tone meant, the memory (**Figure 2B;** shown on days 2 and 3 in initial tone presentations) took longer to process and remember for the stressed group. Once the safety cue was remembered, we can see their freezing behaviour become more similar to the non-stressed group.

In the sucrose consumption task, the stressed group consumed less sucrose during the habituation period, showing a potential reduction in sensitivity to natural rewards. Like humans, we would expect the rats to want a sugary treat, but this was not evident in the stressed group. There were no group differences after food and water deprivation overnight, implying that the physiological need of hunger overrides the stress-related deficits in the hedonic motivation.

In the open field task, the stressed rats displayed lower locomotion and slower speed when exploring the apparatus. There was also slower and less activity in the centre zones, indicating reduced exploration and possibly anxiety-like behaviours, as rodents will naturally stay towards the periphery to avoid threat of predators. Similarly, in the elevated plus maze, the stressed rats spent less time in the open arms, supporting an anxiogenic phenotype. They also engaged in more risk-assessment behaviours, showing altered decision making in potentially threatening context. Lastly, in the forced swim task, the stressed rats spent more time immobile, especially at the beginning and end of the session compared to their control counterparts. This suggests a greater behavioural despair with reduced coping or escape directed behaviour in stressful situations as

from the beginning to end, they showed less effort to try to escape the apparatus, giving up earlier than the controls.

These behavioural results align with prior work in the field, showing that adolescent stress disrupts common behavioural outcomes in these tasks. The safety learning impairments are comparable to humans in the way that early-life adversity can lead to overgeneralization of fear and difficulty distinguishing between safe and threatening cues. The anhedonia and anxiety-like behaviours are also consistent with rodent models of depression following early-life stress, as seen in Pohl et al, (2007). Therefore, adolescence is a sensitive neurodevelopmental period where stress can cause long-lasting maladaptive behavioural responses. These findings may help explain the links between early-life adversity and its connection to adult-onset psychological issues like anxiety and depression or impaired emotional learning.

However, there are some future directions that may expand the research generalizability. Firstly, only male rodents were tested, there are expected sex differences in behavioural outcomes based on literature, therefore future studies should include sex differences. These behavioural changes were also only observed in adulthood, but the phases between were not assessed. Therefore, we question how quickly these behavioural changes would occur if the rodents were placed in these tasks earlier or possibly later. The neurobiological underpinnings will be examined through histological assessments in the next chapter.

Chapter 3: Adolescent Stress Reduces Hippocampal Neurogenesis and Alters mPFC Inhibitory Circuitry

3.0 Introduction

The hippocampus plays an important role in stress responses and is linked to memory processing, consolidation, and safety learning. During adolescence, neural plasticity can be impacted by external environmental influences like chronic stress, and therefore affect the development of neurons in this brain region. Doublecortin-positive (DCX+) cells mark immature neurons and can provide insight into the neurogenic activity of the hippocampus. DCX counts are a reliable measure of ongoing neurogenesis, essential for maintaining cognitive flexibility, leaning, and emotional regulation. Stress has been linked to a reduction in DCX+ cells, resulting in long-term impacts on behaviour (Levone et al., 2014).

The mPFC plays an important role in information, cognitive, and emotional processing, as well as motivation. Parvalbumin-positive (PV+) and perineuronal nets (PNNs) in the mPFC contribute to synaptic plasticity and emotional regulation. Dysregulation in these structures has been identified through early-life stress studies, which can lead to impaired inhibition and increased anxiety (Ruzicka et al., 2022). Stress during specifically vulnerable developmental periods can further disrupt PV and PNN maturation in the mPFC, particularly in the cingulate cortex (CG1). These disruptions may weaken inhibitory control, altering fear responses, increasing anxiety-like behaviours, and present deficits in safety learning.

Based on the previous behavioural analysis, it is important to understand the neurobiological underpinnings of those behavioural differences. Therefore, a histological analysis of DCX in the hippocampus, and PV/PNN in the mPFC was conducted to provide critical insights into the cellular mechanisms though which adolescent stress may alter neurogenesis and influence cognitive and behavioural outcomes in adulthood.

3.1 Methodology

3.1.1 Perfusion and Tissue Preparation

Rats were deeply anesthetized 90 min after forced swim testing with sodium pentobarbital (340 mg/ml, Euthanosl, Merck Animal Health Canada) and then underwent transcardiac perfusion with ice cold 0.1 M phosphate buffered saline (PBS; pH = 7.4) followed by ice cold 4% (w/v) formaldehyde fixative (pH = 7.4) that was freshly prepared from depolymerized paraformaldehyde. The brains were extracted and post-fixed in the same fixative overnight at 4°C. After fixation, the brains were sectioned on vibrating microtome in the coronal plane at a thickness of 40 µm. All sections were stored at 4°C in PBS containing 0.01% (w/v) sodium azide for future histological analysis (Leica VT1000 S, Lecia Biosystems Inc).

3.1.2 Doublecortin (DCX) Immunohistochemistry

Following perfusion and sectioning, free-floating coronal sections (40 µm) were processed to visualize immature neurons by following the DCX protocol described by Horsey et al. (2020). The Doublecortin stain started with 5 washes in a 0.1 M tris-buffered saline (TBS) for 10 minutes each. The tissue was then put into a blocking solution containing 5% normal goat serum (NGS), 0.5% TBS with Triton X-100 (TBSx), and 1% bovine serum albumin (BSA) for one hour. Then the sections were incubated with rabbit anti-doublecortin (1:1000) overnight at 4 °C with consistent agitation. Following this, they were then rinsed in TBS 3 times for 10 minutes each. The sections were then placed in 1% H_2O_2/TBS for 30 minutes. Sections were then submerged for 1 hour in biotinylated goat anti-rabbit IgG secondary antibody (1:200 in TBSx). Three more TBS washes for 10 minutes were completed followed by the avidin-biotin complex (ABC; 1:500) for 1 hour. After that, the tissue was placed in a 0.175 M acetate buffer (pH=6.8) twice for 5 minutes each. Then, using a 0.025% diaminobenzidine (DAB) with 0.002% hydrogen peroxide and

4.1672% nickel ammonium sulfate dissolved in TBS was used for approximately 8 minutes. The sections were then rinsed in a 0.1 M acetate buffer twice for 5 minutes each. Lastly, sections were rinsed in TBS 3 times for 10 minutes each. All steps were performed with consistent agitation. After the stain was completed, sections were mounted onto slides and cover slipped for imaging (Horsey et al., 2020).

3.1.3 Doublecortin Stereology and Quantification

Once slides were prepared for imaging, DCX cells were quantified using the optical fractionator probe in Stereo Investigator (MBF Bioscience) on a motorized microscope with a 100x oil immersion objective. The region of interest was first outlined at a low magnification (4x), then oil was applied for the high magnification objective. Focus was adjusted manually using the fine knob and joystick to define both the top and bottom thickness of the tissue with guard zones at around 2.5 µm on both ends. The section sampling fraction (ssf) was 1/6 and the area of the counting frame was 8019 μm^2. The height of the dissector (h) was set to 15 µm based on guidelines set forth by Horsey et al. (2020).

Systematic random sampling was applied with six tissue sections per animal being analyzed. The counting frames were predefined within the granule cell layer and subgranular zone. The cells were then counted according to the stereological rules based on included and excluded boundary zones. After completing each section, the stage was manually moved to the next section and the process repeated. Counts from both hemispheres were combined to yield total estimates per animal. Data per animal was averages across the sampled sections and statistical analyses were all completed through JASP software.

3.1.4 Parvalbumin and Perineuronal Nets (PV & PNN) Immunohistochemistry

Brain sections were processed for immunofluorescence staining of parvalbumin (PV) and perineuronal nets (PNN), following a parvalbumin antibody and biotinylated Wisteria Floribunda Agglutinin (WFA). The sections were first washed in phosphate buffer saline (PBS) 6 times for 5 minutes each. Then they were placed in PBS containing 0.3% Triton-X-100. The sections were then placed in a 5% normal goat serum (NGS), 1% bovine serum albumin (BSA), and 0.3% Triton-X-100 in PBS block to reduce nonspecific binding. The tissue was then incubated overnight at 4°C in a primary solution containing mouse anti-parvalbumin IgG (1:1000) and biotin WFA (1:200) diluted in the blocking solution. All subsequent steps were conducted in a dark room to minimize photobleaching. Sections were then washed in PBS 6 times for 5 minutes each. The sections were then placed in a secondary solution with Alexa Flour 488-conjugated goat anti-mouse serum (1:500) and streptavidin-Cy3 (1:500) in PBS containing 0.3% Triton X-100 in a dark room. After an additional 3 PBS washes for 5 minutes each, the tissue was placed in a Hoechst 33342 (1:2000) solution. Following that, an additional 2 PBS washes for 5 minutes each was done, and the sections were then mounted onto Superfrost Plus slides, air dried and cover slipped using an anti-fade medium (ProLong Gold). Slides were then sealed with nail polish and stored at 4 °C in the dark until imaging.

3.1.5 Parvalbumin and Perineuronal Nets Quantification

Fluorescent images were acquired using a confocal microscope with appropriate filter settings for the stain conducted. The regions of interest (ROIs) were manually traced using the polygon tool in ImageJ (Fiji) based off of the Paxinos and Watson Rat Brain Atlas. The regions of interest were manually defined with a focus on the CG1, PrL, and IL regions in the mPFC. PV and PNN cells were counted using the Cell Counter plugin. Colours were assigned to distinguish

between the different cells, in this case green for PV and red for PNN. Each cell was manually clicked and recorded. Binary masks were generated from threshold channels to assess colocalization between PV and PNN cells. Multiple fields (6) per ROI were analyzed for each animal. Images were converted to 8-bit grayscale, channels were split, and background subtraction was applied to enhance clarity. All imaging and analysis settings were kept consistent across samples. All statistical analyses were conducted using JASP software.

3.2 Results

3.2.1 DCX Effect of adolescent stress on adult hippocampal neurogenesis.

We examined the effects of adolescent stress on levels of adult hippocampal neurogenesis by quantifying the number of doublecortin-positive (DCX+ cells), a widely used marker to assess numbers of neuroblasts or immature neurons in rodents, non-human primates, and humans. Using unbiased stereological procedures, we found that rats exposed to adolescent stress showed a 20.4% reduction in the number of DCX+ cells compared to non-stressed controls [$t(22)=2.61$, $p=.016$, **Figure 6**]. Further examination suggested that DCX+ cells were uniformly decreased across the dorsal and ventral hippocampus. These findings indicate that adolescent stress induced a persistent but moderate decrease in levels of adult hippocampal neurogenesis.

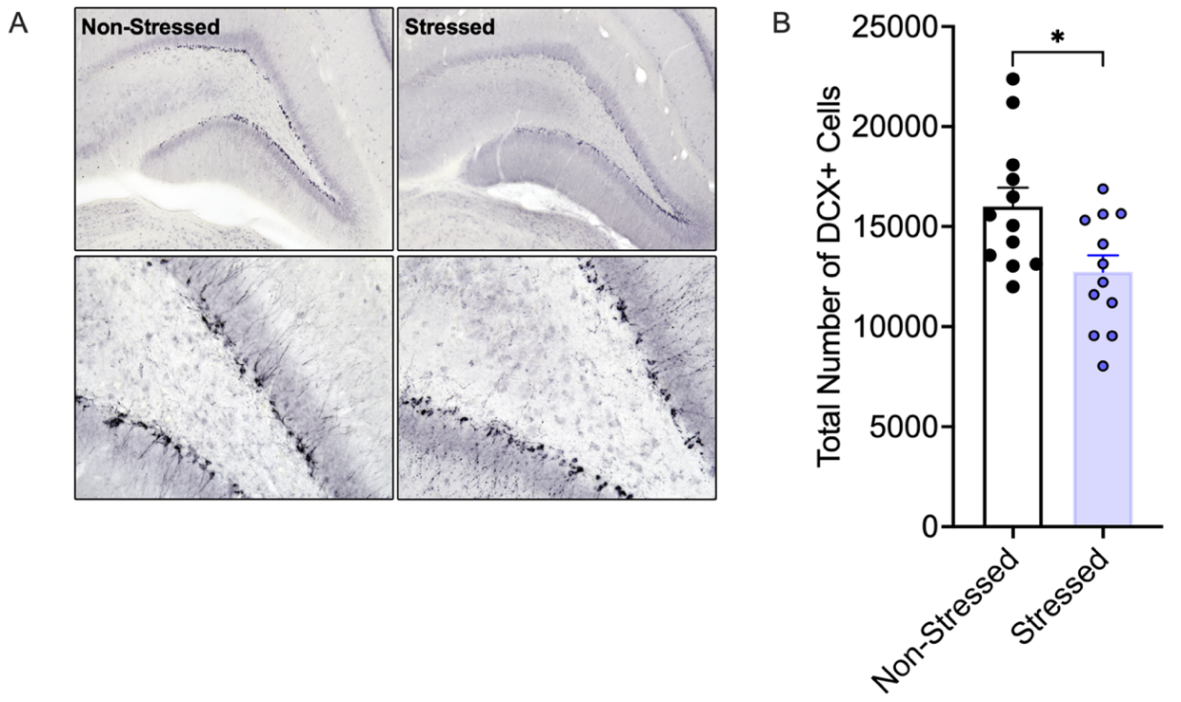

Figure 6: DCX+ cells within the hippocampus in the no stress vs stress group

3.2.2 Parvalbumin and Perineuronal Nets effect on GABA Interneuron Plasticity. Counts of PV and PNN immunofluorescence in the mPFC were conducted within the cingulate cortex (CG1), prelimbic cortex (PrL), and infralimbic cortex (IL). Significant group differences were observed in the CG1 region; CG1: PV+/PNN+ [t(13)=3.63, p =.003], CG1: PV+/PNN- [t(13)=-2.77, p=.016], and CG1: PV−/PNN+ [t(13)=-2.21, p =.045] conditions **(Figure 7).** These results indicate robust contrasts between the control and stressed groups, suggesting significant alterations in PV+/PNN+, PV+/PNN−, and PV-/PNN+ populations, reflecting changes in the stability and plasticity of GABAergic interneurons. The decrease in PV−/PNN+ and PV+/PNN- cells further highlights potential disruptions in the protective role of PNNs around interneurons, which may contribute to impaired synaptic regulation and functional connectivity in the mPFC.

Interestingly, no significant differences were found in the prelimbic cortex (PrL) and infralimbic cortex (IL) regions (all $p > .05$), suggesting that the observed stress-induced effects on PV and PNN expression may be localized to specific subregions of the mPFC, such as the cingulate cortex (CG1). This regional specificity highlights the need for further investigation into how different areas of the mPFC contribute to stress-related neural and behavioural outcomes.

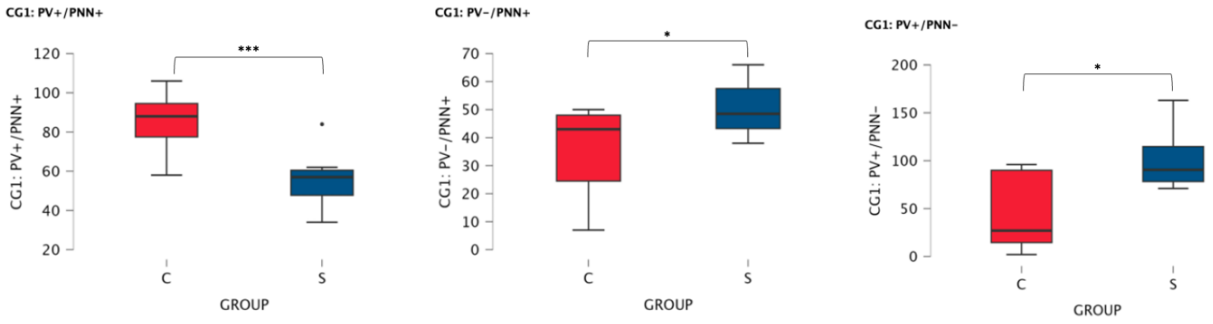

Figure 7: All CG1 region counts for (A) PV+,PNN+, (B) PV-,PNN+, (C) PV+, PNN-, demonstrating significant differences between groups. p<.05, p<.01**, p<.001****

3.3 Discussion

Overall, in the DCX analysis, there was an evident decrease in DCX+ cells during adulthood for the stressed rat group, suggesting a reduction in hippocampal neurogenesis. This reduction was consistent across both the dorsal and ventral hippocampus, indicating a more widespread effect than an impact on a specific sub-region. This result may explain the behavioural outcomes of reduced memory and safety learning processes in the stressed rodents, as well as reduced emotional regulation and cognitive flexibility. This further aligns with the literature in the field. Levone and collogues (2014) found that chronic stress exposure would supress neurogenesis and lead to long-term behavioural changes. Therefore, the present results present support for the view that adolescent stress induced neurobiological changes that persist into adulthood, possibly contributing to the development of maladaptive behavioural phenotypes.

Significant alterations in the mPFC in relation to PV/PNN counts were also found, but more specifically in the CG1 subregion. The prelimbic (PrL) and infralimbic (IL) regions were also analyzed, revealing no significant findings. This decrease in PV+/PNN- and PV-/PNN+ cells may therefore indicate a disruption in inhibitory behaviours and emotional regulation. This may lead to a reduced inhibitory control in the mPFC and thereby behavioural deficits, as observed in the behavioural data (Ruzicka et al., 2022).

The overarching result of the immunohistological analysis aligns with previous literature. Both the hippocampus and the mPFC, specifically CG1, have been altered due to adolescent stress. The reduced neurogenesis may result in impaired memory and adaptability, explaining the deficits in safety learning, while the alterations in PV and PNN cells may explain the dysregulated emotional processing. These neurobiological changes further align with the observed behavioural changes, reinforcing the concept that early-life stress leaves lasting effects on the brain. This further supports that adolescence is a sensitive period where stress can impact structural and functional plasticity and regulation.

Although these results support the hypotheses, it is important to consider what future research should focus on. The sample size was relatively small, reducing the ability to generalize findings. Also, only male rats were used for this study, where sex differences may be evident, it is important to explore females as well for stress dependent sex effects.

Chapter 4: Discussion

The objective of this study was to determine if adolescent stress impacts adult safety learning and thereby the onset of anxiety and depressive-like symptoms in adulthood. This was investigated using an unpredictable intermittent physical stress regimen during the adolescent period (P31-43) and later testing adult rats for safety learning and anxiety-like (open field, elevated plus maze) and depressive-like behaviours (sucrose consumption, forced swim test).

4.0 Adolescent stress impact on safety learning

Aligning with the hypotheses, adolescent stress impaired both the acquisition and recall of safety learning in adulthood. During acclimation, the stressed group displayed significantly higher freezing on the first two days compared to controls, suggesting heightened reactivity to a novel environment (Christianson et al., 2012; Park et al., 2018), although this difference was no longer present by Day 3 due to gradual habituation. These findings align with past reports of impaired habituation following early-life stress and support the interpretation that adolescent stress enhances initial novelty reactivity (Chaby et al., 2015).

During safety conditioning, both groups exhibited reduced freezing to tones across days, indicating safety learning**.** However, stressed animals froze significantly more than controls to the first tone on Days 2 and 3, suggesting delayed discrimination between threat and safety cues early in each training session. This also indicated a problem with memory, as the safety cue took multiple trials for the stressed group to remember what it meant**.** By later tones, both groups converged in freezing levels, indicating that the stressed group was capable of learning but required more exposures to reduce fear and remind them what the tone meant**.** Polynomial contrasts further revealed a steeper decline in freezing across tones on Day 3 in stressed rats**,** consistent with this slower acquisition pattern. This partially contrasts with prior findings that

adolescent stress does not impact adult associative learning (Chaby et al., 2015) and may indicate that there are additional neural connections beyond the traditionally proposed overlapping fear and safety circuitry (Christianson et al., 2008; Shelton & Davidson, 2004).

In the recall test, controls displayed clear safety recall**,** with reduced freezing during the tone compared to the pre-tone periods, indicating that the tone had acquired safety signal properties. In contrast, the stressed group maintained similarly high freezing in both periods**,** showing no significant safety suppression effect**.** Safety suppression scores confirmed significantly impaired recall in the stressed group compared to controls. This pattern is consistent with prior research demonstrating that adolescent stress heightens fear responses (Zhang & Rosenkranz, 2013; Lukkes et al., 2009) and aligns with evidence that stress does not typically alter freezing to the shock itself but increases overall fearful behaviour (Barbayannis et al., 2017).

These findings indicate that adolescent stress not only increases initial reactivity to novel environments but also slows the acquisition of safety learning and disrupts the ability to recall safety signals**.** While both groups could eventually learn the safety association during training, the stressed rats' inability to retrieve this information the following day suggests a deficit in safety memory consolidation or retrieval**.** Given the complex interplay between stress and safety learning—which share yet oppose aspects of neural circuitry—future research should investigate the timing, type, and duration of adolescent stress exposure to determine the conditions under which safety learning is most profoundly affected (Arruda-Carvalho et al., 2017).

4.1 Impact of adolescent stress on adult depressive-like behaviour

The findings of this study provide mixed evidence for the effects of adolescent stress on depressive-like behavior in adulthood. The sucrose consumption test, a measure of anhedonia,

revealed some moderate differences between the adolescent stress and control groups, indicating possible effects of adolescent stress on sucrose preference. This result aligns with studies such as Wang et al. (2019), where chronic social stress during adolescence reduced sucrose preference in adulthood but the strength of this finding differs. These discrepancies may stem from differences in stress protocols, species, or strain used in research.

The Long Evans rats used in this study may partly explain the lack of anhedonia, as this strain is not genetically predisposed to affective disorders and has shown resilience to stress-induced changes in sucrose consumption (Murray et al., 2013). This suggests that strain-specific differences in stress susceptibility could influence the outcomes of stress-related behavioral assays. Additionally, the intermittent stress regimen employed here may have been less impactful than chronic stress paradigms in altering sucrose preference. These findings underscore the variability in results across studies, with stress exposure in rodents sometimes increasing, decreasing, or having no effect on sucrose consumption (e.g., Calvez & Timofeeva, 2016; Matthews et al., 1995; Ozkartal et al., 2018).

In contrast, the forced swim test yielded significant differences between groups, with the adolescent stress group exhibiting higher levels of immobility compared to controls. This finding aligns with prior research demonstrating increased immobility following adolescent stress, as observed by Wilkin et al. (2012) and Iniguez et al. (2014). Immobility in the forced swim test is often interpreted as a passive coping strategy and is considered indicative of increased vulnerability to depressive-like states (Commons et al., 2017). Although the test has faced criticism for its ecological validity—measuring coping responses rather than strictly depressive behaviour—it remains a widely used and robust measure of behavioural changes following stress.

Interestingly, while the hypothesis that adolescent stress would increase depressive-like behaviour was partially supported, the results highlight a dissociation between two facets of depressive-like behaviour: anhedonia and passive coping. While sucrose consumption did not reflect anhedonia, the increased immobility in the forced swim test suggests that adolescent stress significantly impacted coping behaviours. This divergence may indicate that different stress paradigms or timing of stress exposure target distinct neural circuits or behavioural outcomes.

Overall, the findings emphasize the importance of considering factors such as strain differences, stress regimen, and exposure timing when interpreting adolescent stress's effects. Future research could explore whether alternative stress paradigms, including chronic or early developmental stress, yield more consistent effects on anhedonia and depressive-like behaviour. Such studies would provide a more comprehensive understanding of the long-term consequences of adolescent stress.

4.2 Impact of adolescent stress on adult anxiety-like behaviour

The findings of this study highlight the complex relationship between adolescent stress and anxiety-like behaviours in adulthood. In the open field test, the adolescent stress group did not differ from the distance travelled in the center zone **(Figure 7).** This result was unexpected, as it was hypothesized that stressed rats would avoid the center—a behaviour typically associated with anxiety. However, closer analysis revealed that the stress group exhibited significantly higher levels of immobility while in the center, suggesting a lack of exploration rather than reduced anxiety. Increased immobility has been identified as a marker of anxiety-like behavior in rodents (Brockhurst et al., 2015; Rebolledo et al., 2017), though this interpretation is complicated by the seemingly paradoxical increase in time spent in the center zone.

One explanation for this discrepancy could be the starting position of the rats, which faced the center at the beginning of the test. This might have contributed to their prolonged presence in the center as they became immobile shortly after entering. Reduced locomotion in the stress group, characterized by slower speeds and shorter distances traveled, further supports the notion of decreased exploratory drive (Sestakova et al., 2013; Geerse et al., 2006). Prior research has linked reduced locomotion in the open field to increased corticosterone levels (Umrykhin & Grigorchuk, 2017) and stress exposure during adolescence (Wang et al., 2018; Caruso et al., 2018). Although reduced locomotion often reflects anxiety-like behavior, the conflicting evidence from center-zone immobility precludes definitive conclusions about anxiety levels in the open field test.

Importantly, the decrease in locomotion observed in the open field raises concerns about its potential impact on freezing behavior during safety conditioning. However, freezing levels during the final session of habituation **(Figure 3)** did not differ significantly between groups, suggesting that reduced locomotion likely did not influence freezing behavior during safety acquisition.

The elevated plus maze provided clearer evidence of anxiety-like behavior in the adolescent stress group. These rats spent significantly less time in the open arms compared to the control group, indicating a decreased drive to explore these areas and increased anxiety-like behavior (Pellow et al., 1985; Walf & Frye, 2007). This finding aligns with previous studies showing that intermittent stress during adolescence reduces exploration of the open arms in the elevated plus maze (Wilkin et al., 2012). Similar results have been observed in other stress paradigms, including maternal separation (Jin et al., 2018) and social defeat (Watt et al., 2009).

Overall, while the open field test yielded ambiguous results due to conflicting measures of anxiety-like behavior, the elevated plus maze findings strongly support the hypothesis that adolescent stress induces anxiety-like phenotypes in adulthood. These results emphasize the importance of using multiple behavioral assays to capture the nuanced effects of stress on anxiety and highlight the need for further research to disentangle the relationship between locomotion, exploration, and anxiety.

4.3 Immunohistology

Immunohistology analysis in this study revealed a significant reduction in DCX+ cells within the dentate subgranular zone and granule cell layer of the hippocampus in the stressed rats. This finding suggests impaired adult hippocampal neurogenesis, a process critical for memory formation, emotional regulation, and stress resilience. Reduced neurogenesis in the hippocampus is consistent with existing literature on stress and mood disorders, as the hippocampus plays a central role in regulating the stress response and managing emotional experiences (Boldrini et al., 2013). Impaired neurogenesis, particularly in response to chronic stress, could contribute to the depressive- and anxiety-like behaviors observed in the current study. The hippocampus is known to be highly sensitive to stress, with prolonged stress exposure leading to cellular atrophy and functional impairments (Kim et al., 2015). Given its pivotal role in regulating emotional responses, reductions in hippocampal neurogenesis might not only exacerbate the vulnerability to stress-related mood disorders but may also impair cognitive processes such as memory and spatial navigation, further compounding the effects of stress.

These findings support the hypothesis that disrupted neurogenesis may serve as a key biological mechanism linking stress exposure to mood disorders. In particular, it is possible that reduced hippocampal neurogenesis contributes to the heightened depressive-like behaviors

observed in the forced swim test, as well as the anxiety-like behaviors seen in the elevated plus maze. The hippocampus is implicated in both the regulation of anxiety responses and the integration of fear learning and reductions in neurogenesis in this region could impair the adaptive processing of emotional stimuli.

In addition to changes in hippocampal neurogenesis, the current study also examined the effects of adolescent stress on GABAergic interneurons in the medial prefrontal cortex (mPFC), a region critical for higher-order cognitive functions such as decision-making, emotional regulation, and the top-down regulation of fear responses. Parvalbumin (PV) and perineuronal net (PNN) counts in the mPFC revealed significant differences in the cingulate cortex (CG1), with increased cell counts in both PV-/PNN+ and PV+/PNN− cells in stressed rats. These findings suggest that stress has a localized impact on GABAergic interneurons, leading to disruptions in synaptic stability and plasticity in the CG1 region of the mPFC which is essential for modulating fear and safety learning (Li et al., 2023).

Interestingly, these stress-induced changes in the mPFC were not observed uniformly across all subregions. While significant reductions were observed in the cingulate cortex (CG1), no such changes were found in the prelimbic (PrL) or infralimbic (IL) regions. This regional specificity suggests that stress-induced alterations in GABAergic interneuron function may not be equally distributed throughout the mPFC, potentially contributing to localized functional impairments. The heightened effects in the CG1 region may be particularly relevant to the behavioral outcomes observed in the study, as the cingulate cortex is involved in the regulation of emotional responses, including anxiety-like behaviors and passive coping strategies (Utashiro, 2024). These findings align with the increased immobility and reduced exploration seen in the

open field test and the elevated plus maze, suggesting that the specific changes in the mPFC may contribute to the development of anxiety-like behaviors in stressed rats.

The decreased stability and plasticity of GABAergic interneurons, particularly those in the CG1 region, could impair the PFC's ability to regulate emotional processing and behavioral responses to stress. Given the mPFC's role in controlling fear and safety learning, alterations in this region may lead to dysregulated fear responses and impaired adaptive coping strategies. Stress-induced changes in the mPFC's GABAergic circuitry may also exacerbate the vulnerability to mood disorders, further supporting the idea that neuroplastic changes in key brain regions underpin the observed behavioral outcomes (Yang et al., 2021).

4.4 Future Directions

The present study has several aspects that should be addressed in future research to improve our understanding of the relationship between adolescent stress, safety learning, and associated neurocircuitry.

4.4.1 Timing of Stress Exposure. The age range for stress exposure (P31–P43) may have missed critical developmental windows of vulnerability. Previous research has shown that early adolescence (P22–33) and mid-adolescence (P35–46) have distinct effects on adult anxiety- and depressive-like behaviors in response to stress (Wilkin et al., 2012). Moreover, the developmental trajectory of medial prefrontal cortex (mPFC) connections to the basolateral amygdala (BLA) suggests that significant neuroplastic changes occur between P10 and P30, with glutamatergic synapses strengthening and plateauing by P30 (Arruda-Carvalho et al., 2017). Stress exposure starting at P31 may therefore occur after critical periods for disrupting "top-down" processing to the extent that safety learning is impaired. Future studies should investigate the impact of stress

during earlier developmental stages, such as early adolescence (P22–33) or pre-adolescence (P14–25), to identify critical windows of vulnerability and their effects on safety learning and neurocircuitry.

4.4.2 Sex Differences. This study exclusively examined male rats, neglecting potential sex differences in stress responses and affective outcomes. Depression and anxiety disorders are more prevalent in females, and sex differences in stress responses emerge during adolescence (Seney & Sibille, 2014; Dalla et al., 2010; Berslau et al., 2017). Therefore, females would possibly produce differing stress effects compared to males. Rodent studies should further include both sexes to ensure findings are applicable to the general population.

4.5 Conclusion

This study demonstrated that adolescent stress leads to depressive- and anxiety-like behaviors in adulthood, as shown by increased immobility in the forced swim test and reduced exploration in the elevated plus maze. These behaviors reflect passive coping strategies and are associated with alterations in amygdala and mPFC function. Other changes, including reduced hippocampal neurogenesis and disrupted GABAergic interneuron plasticity in the mPFC, provide a potential neurobiological basis for these outcomes. This study also found impairments in safety learning, characterized by slower acquisition and impaired recall. Future research should investigate earlier periods of stress vulnerability, explore sex differences in stress responses, and further examine how structural and functional changes in safety learning neurocircuitry contribute to long-term behavioral outcomes. Understanding these mechanisms could guide therapeutic strategies to mitigate the lasting effects of adolescent stress and enhance resilience.

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