Does psychosocial stress experienced at different points across the rat lifespan cause sex-specific changes in spatial learning and memory and plasticity-related proteins?

by

Saeideh Davari Dowlatabadi

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Examining Committee Membership

The following served on the Examining Committee for this thesis. The decision of the Examining Committee is by majority vote.

External Examiner	Dr. Gerlinde Alexandra Metz Professor
Co-supervisor	Dr. John G. Mielke Associate Professor
Co-supervisor	Dr. Reza Ramezan Continuing Lecturer
Internal Member	Dr. Peter Hall Professor
	Dr. Diane Williams Continuing Lecturer
Internal-external Member	Dr. Michael Beazely Associate Professor

Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Overview

Considering that susceptibility to a range of diseases appears strongly influenced by both sex and exposure to social stress, there is a need to evaluate how adverse experiences across the lifespan (alone and in combination) may affect brain development and function, and whether these interactions are modified by sex. Therefore, we examined two models of social stress, Chronic Early-Life Social Isolation (CELSI; an early-life psychosocial stressor) and Bystander Stress (ByS; an adult psychosocial stressor), individually and together, to determine how they affected the hippocampus (a structure important for learning and memory) in both male and female rats.

Objectives

Various models have been proposed to help explain how early-life and adulthood stressors interact to affect disease vulnerability. In particular, the match/mismatch hypothesis suggests that early-life experiences can most clearly affect disease risk when they are incongruent with experiences during adulthood. However, since we had insufficient background information about the individual effects of the selected psychosocial stress models (especially the ByS model), we began by exploring some of the critical behavioural and biochemical traits of CELSI and ByS models in both male and female rats. After this, we examined the potential impact of post-weaning chronic social isolation on hippocampus-dependent behavioural and biochemical responses to bystander stress in adulthood. Potential changes in spatial learning and memory performance were evaluated along with changes in the expression of several glutamate receptor subunits (including GluA1, GluA2, GluN1, GluN2A,

and GluN2B) and PSD-95 in the septal and temporal poles of the hippocampus. The highlighted proteins were selected given that they are upstream elements of hippocampal synaptic plasticity and play an important role in learning and memory. Importantly, all assessments of the behavioural and biochemical data were done using sex as a variable.

Methods

For the first study, upon weaning, male and female siblings from 15 Sprague-Dawley rat litters were stratified by sex and then randomly assigned to either group housing (3 animals/cage), or social isolation (1 animal/cage) for 7 weeks (that is, CELSI). Spatial learning and memory were then tested over 5 days using the Morris water maze (MWM). Next, the animals were euthanized, and a variety of stress-sensitive biometrics (body weight, adrenal gland weight, liver weight, retroperitoneal fat pad weight, serum corticosterone levels, liver lipid content, and non-fasting blood glucose) were collected. Lastly, to determine whether CELSI affected neural cell density, the expression of key neuronal and glial proteins (PSD-95 and GFAP, respectively) was assessed in isolated hippocampal tissue using Western blotting.

In the second study, bystander stress was applied to adult male and female rats twice per day for 5 consecutive days. To remove the potential influence of behavioural testing on the expression of plasticity-related proteins in the hippocampus, the study was completed in two parts: 10 groups were examined using the MWM and 6 groups were used for tissue harvesting after the stress paradigm. Following the hippocampal dissection, septal and temporal poles were isolated. Then, after preparing enrichments of synaptic terminals, Western blotting was used to measure the expression of key AMPA and NMDA receptor subunits (GluN1, GluN2A, GluN2B, GluA1, and GluA2), as well as PSD-95.

In the final study, to investigate the effect of early-life stress on response to adulthood stress (by considering match/mismatch theory), after undergoing CELSI (or group housing), animals were placed in standard housing conditions for 6 months and then underwent ByS. Once the ByS paradigm was completed, MWM data were collected. After the collection of behavioural data, hippocampal tissue was harvested, synaptoneurosomes were prepared, and Western blotting used to examine the expression of AMPA and NMDA receptor subunits, as well as PSD-95, in the septal and temporal poles.

Results

Unexpectedly, in study one, socially isolated male and female rats displayed stronger spatial learning and memory ability than group housed rats. As well, socially isolated male rats exhibited increased expression of PSD-95 (a key neuronal cell marker). However, there was no clear effect of housing condition on stress-sensitive biometrics, or the hippocampal expression of GFAP.

In study two, no significant differences were observed between the spatial learning and memory performance of control and bystander stress male and female rats. However, the expression of GluA2, GluN2A, and GluN2B was dramatically decreased in the septal pole of the hippocampus in male rats exposed to bystander stress, when compared to male bystander control rats. In contrast, levels of GluA2 were clearly increased in the temporal pole of the hippocampus from female ByS rats compared to female ByC rats. Lastly, expression of PSD-95 was found to be greater in the temporal pole of male ByS rats relative to female ByS rats.

The results of study three indicate that male rats performed better on the spatial learning task than female rats within the matched control situation (group housing during the post-

weaning period followed by the ByC group during adulthood). Surprisingly, the learning performance of male animals that experienced the mismatch 1 condition was also superior to that of female animals (that is, the animals underwent social isolation followed by ByC). In alignment with our hypothesis, male animals exposed to a stressful situation both during development and in adulthood (that is, with matched stress exposures: SI-ByS) displayed better spatial learning on day four than animals that were raised in group housing, but experienced bystander stress in adulthood (a mismatched situation: GH-ByS). In terms of protein expression, a significant increase in GluN1 expression was observed in the hippocampal septal pole of male rats exposed to matched stressful situations (SI-ByS) as compared to matched control male rats (GH-ByC).

Conclusion

Our findings reveal that there are sex-based, long-standing effects of early-life adversity on later stress exposure with regards to hippocampal-dependent behaviour. In particular, our results indicate that spatial learning performance was best on the match condition on adversity (SI-ByS), but only in male rats. The finding supports the match/mismatch theory in that the outcome from dealing with an adult psychosocial stressor similar to the environment encountered during early life (match) differs from that observed when dealing with incongruent psychosocial situations encountered across the lifespan (mismatch) and, notably, that male and female rats are uniquely affected.

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Table of Contents

List of Figures

List of Tables

Chapter 1
Introduction

1.1 Overview

The development of the brain, and consequently behaviour, is a lifelong process influenced by a complicated interaction of genetic and environmental factors (Kolb et al., 2014). Interestingly, genome-wide association scans have not been successful in identifying significant genes associated with psychiatric illnesses, which suggests that environmental factors may play a lead role in shaping pathological brain development and function (Abdolmaleky, 2014). Therefore, positive, or negative experiences could add up to shape brain development in a way that may have lifelong effects on physical and mental health.

Mental health has been incorporated into sustainable development goals in recent years, illustrating the increasing recognition of its important role in achieving global development goals. Social support from friends and family during stressful times can increase the likelihood of effectively managing stressors or recovering from mental health disorders (Escalera et al., 2019; Inagaki, 2018; Lee et al., 2018). Conversely, negative social interactions, which may include threatening or unpleasant social situations, can lead to psychosocial or social relationship stress for individuals. Every individual experiences stress from time to time, as it is the nature of the daily life and a consequence of both good and bad events. Even though stress is unpleasant, it does not constitute a medical condition. However, stress can have important effects on the actions of many systems, especially regions of the nervous system (such as the hippocampus) that may increase the risk for developing neuropsychiatric disorders, such as depression and dementia. More seriously, stress and subsequent changes are

associated with higher rates of morbidity and mortality (Kopp & Réthelyi, 2004), including a higher risk for mental illness and suicide (Paykel, 1976). Therefore, stress has been identified as a "worldwide epidemic" by the World Health Organization as a result of the magnitude of its harmful effects (World Health Organization, 2007).

A rich literature has demonstrated that social stress, which is the result of one's relationships and other features within their social environment, is a main risk factor for several psychiatric disturbances, including bipolar disorder and depression (Lee et al., 2010; Lex et al., 2017). Moreover, an extensive body of literature shows that Early Life Stress (ELS), including maternal separation or social isolation, affects brain development (Alquicer et al., 2008; Fone & Porkess, 2008). Recent research conducted on humans and animals suggests that exposure to stress during early life could elevate the possibility of developing neurological, psychiatric, and behavioural disorders, such as depression, anxiety, and schizophrenia later in life (Grippo et al., 2007; Heim et al., 2004; Reinwald et al., 2018; Thorsell et al., 2006). Notably, the prevalence of these disorders is highly sex-dependent (Kendler et al., 1995; Piccinelli & Wilkinson, 2000), but little is known about how the mechanisms that connect social stress to brain development and function are impacted by sex-related differences.

There can be little doubt that a thorough understanding of the causes and effects of stress across the life course, as well as the relationship between stress and mental health, is vital to public health. Also, identifying the neuronal circuits associated with stress, as well as their interactions with mediator molecules, is crucial for not only understanding the physiological stress response, but also its psychological implications. Put simply, having a better understanding of stress will enable us to deal with its consequences more effectively.

Considering that susceptibility to mental disease appears to be strongly influenced by both exposure to social stress and sex, there is a need to evaluate how adverse experiences across the lifespan may interact to impact brain development and function, and whether these interactions are modified by sex.

1.2 Study Rationale

As mentioned, social stress precipitates a wide spectrum of mental and physical changes with major public health significance. However, we have an incomplete understanding about how social stress affects brain development and higher functions of the nervous system, such as spatial learning and memory. Moreover, the role that sex may play in moderating the interaction between stress and the brain is also an area that has received limited attention. To better understand how social stress can affect brain development, two models of social stress (chronic early-life social isolation, or CELSI, a developmental stressor, and bystander stress, or ByS, an adult stressor), alone and in combination, will be investigated. In this regard, my research seeks to fill knowledge gaps by answering the following primary research questions, *"Does experiencing psychosocial stress in early life (CELSI) affect the behavioural and biochemical responses to psychosocial stress encountered in adulthood (ByS)?"*. If so, *"Do male and female animals exhibit different responses?"*.

1.3 Objectives

While a large body of evidence has shown that physical stress can greatly influence brain development and function, there has been little research conducted concerning the effect that social stress may have on the brain. Through careful examination of the effect of CELSI and ByS on different aspects of the brain (proteins to behaviour), we planned to collect evidence

as to whether or not these forms of stress are just as impactful as the more frequently studied forms of physical stress. Therefore, my thesis work developed along three phases, as follows:

Phase 1: The first phase of this project examined the effects of chronic early-life social isolation on male and female rats. Following seven weeks of exposure to CELSI, we used different measures (behavioural and biochemical) to accomplish the following objectives (Figure 1.1).

Objective 1: To determine whether CELSI affects the expression of key neuronal and glial structural proteins (PSD-95 and GFAP, respectively) in the hippocampus.

Objective 2: To investigate the impact of CELSI on a number of stress-sensitive biometrics, such as serum CORT level and the weight of adrenal glands, retroperitoneal fat pads, and the liver.

Objective 3: To uncover whether CELSI influences behaviour as it relates to hippocampal-dependent learning and memory by testing spatial learning and memory using the Morris water maze.

Objective 4: To explore possible sex-specific effects of CELSI on protein expression, biometrics, and behaviour by using both male and female animals.

Phase 2: In the second phase, we applied "bystander stress" (ByS) to adult male and female rats for five consecutive days, and measured its effects on the brain at both the behavioural (cohort 1) and protein (cohort 2) levels (Figure 1.2).

Objective 1: To determine whether bystander stress affects the expression and cellular distribution of a series of plasticity-related proteins (e.g., GluN1, GluN2A, GluN2B, GluA1, GluA2, and PSD-95) in septal and temporal poles of the hippocampus.

Objective 2: To investigate the influence of ByS on hippocampal-dependent behaviour by testing spatial learning and memory using the Morris water maze.

Objective 3: To explore possible sex-specific effects of ByS on plasticity-related protein expression and behaviour by using both male and female animals.

Phase 3: In the final phase, we examined whether experiencing CELSI changes the behavioural and biochemical responses to ByS in male and female rats to see if exposure to early-life stress influences the effect of social stress in adulthood (Figure 1.3).

Objective 1: To determine whether experiencing both early life (CELSI) and adulthood stress (ByS) affects the expression and cellular distribution of a series of plasticity-related proteins (e.g., GluN1, GluN2A, GluN2B, GluA1, GluA2, and PSD-95) in septal and temporal poles of the hippocampus.

Objective 2: To investigate the influence of experiencing both early life (CELSI) and adulthood stress (ByS) on hippocampal-dependent behaviour by testing spatial learning and memory using the Morris water maze.

Objective 3: To explore possible sex-specific effects of experiencing both early life (CELSI) and adulthood stress (ByS) on plasticity-related protein expression and behaviour by using both male and female animals.

Notably, the closure of the University's animal facilities and labs to address COVID-19 caused significant disruption to our work (we were required to pause the work from March 2020 until September 2020). Therefore, we had to unexpectedly change the study design; instead of proceeding directly from CELSI into ByS, a gap of approximately 6 months was inserted between the post-weaning stressor (CELSI) and the adulthood stressor (ByS).

1.4 Experimental Hypotheses

The core experimental expectations were as follows:

1. Elevated serum CORT levels would be observed in rats exposed to CELSI in comparison to control rats; also, stressed rats were expected to have heavier adrenal glands, retroperitoneal fat pads, and liver (since these changes require time to develop, they were only measured during phase one).

2. Impaired spatial learning and memory would be observed in rats subjected to CELSI, ByS, or their combination compared to rats in the respective control groups due to stress-induced changes in hippocampal synaptic plasticity.

3. The expression of plasticity-related proteins (particularly, the glutamate receptor subunits GluN1, GluN2A, GluN2B, GluA1, and GluA2), as well as PSD-95, would be altered in stressed rats when compared to the respective control rats.

4. Sex-dependent responses would be observed with regards to how the animals react to CELSI, ByS, or their combination associated with spatial learning and memory task performance, stress sensitive biometrics, and the expression of plasticity-related proteins in the whole hippocampus (phase 1), and in septal and temporal poles of the hippocampus (phases 2 and 3).

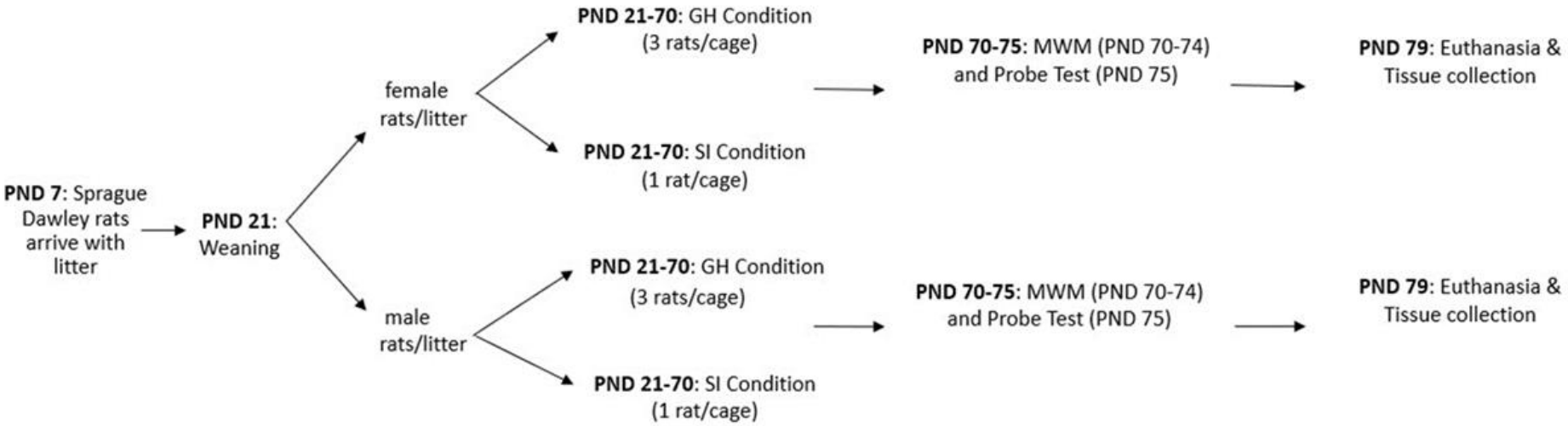


Figure 1.1. Chronic early-life social stress (CELSI) paradigm for 15 rat litters. **PND**: Post-natal Day, **GH**: Group Housed, **SI**: Socially Isolated, **MWM**: Morris Water Maze.

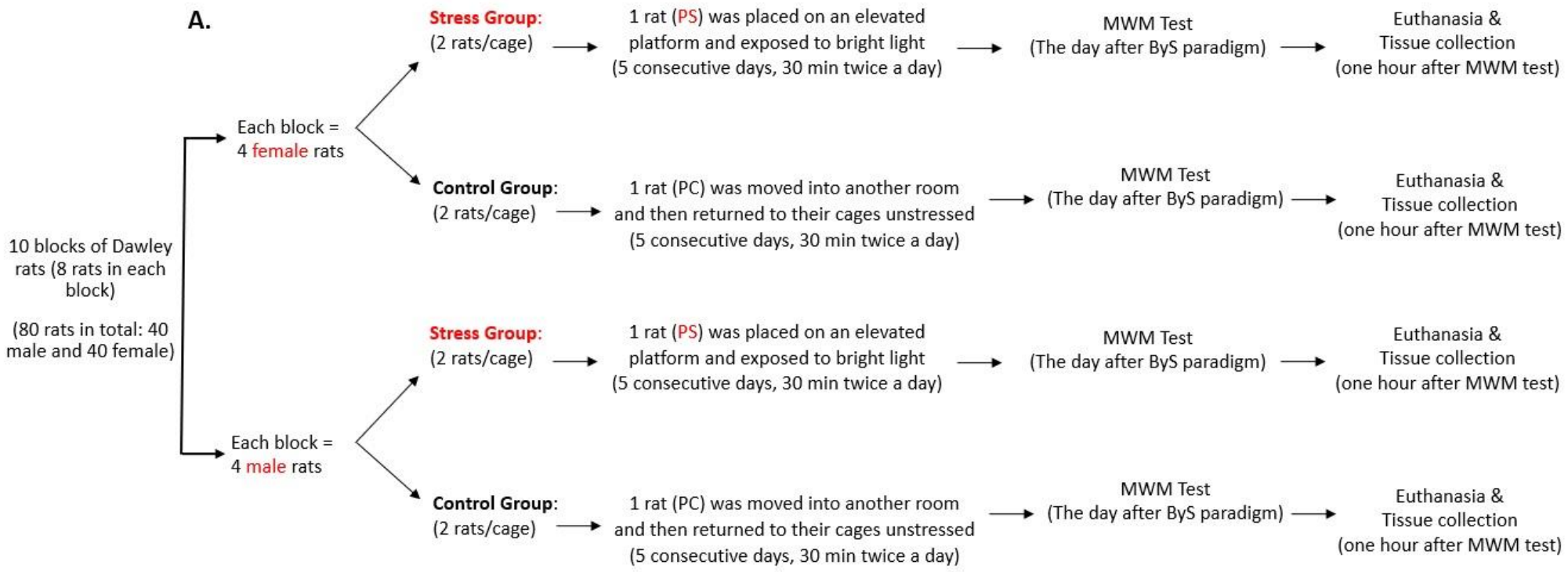


Figure 1.2. A) Cohort 1, Bystander stress paradigm. **PS:** Platform Stress, **PC:** Platform Control, **ByS:** Bystander Stress, **MWM:** Morris Water Maze, **Block:** experimental unit: one set of animals with each condition represented. **Tissue collection:** brain (septal and temporal hippocampus).

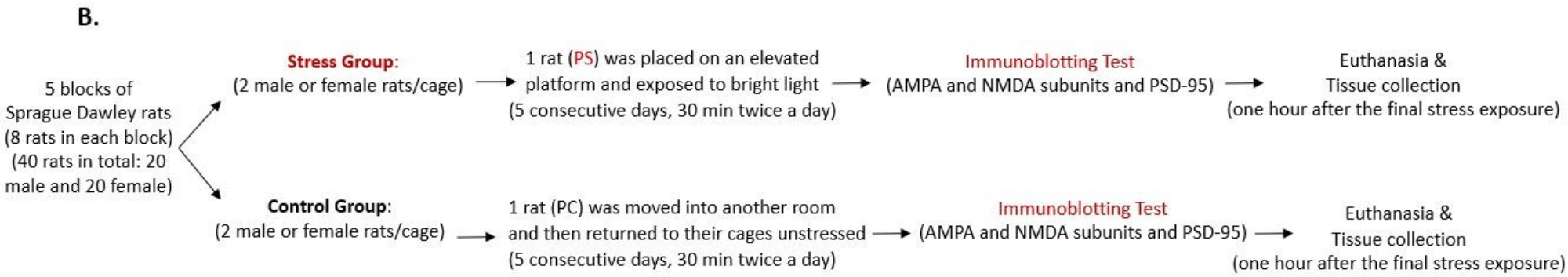


Figure 1.2. B) Cohort 2, Bystander stress paradigm. **PS:** Platform Stress, **PC:** Platform Control, **ByS:** Bystander Stress, **MWM:** Morris Water Maze, **Block:** experimental unit: one set of animals with each condition represented. **Tissue collection:** brain (septal and temporal hippocampus). Brain tissue samples were collected one hour after the stress paradigm instead of having the animals complete the MWM testing.

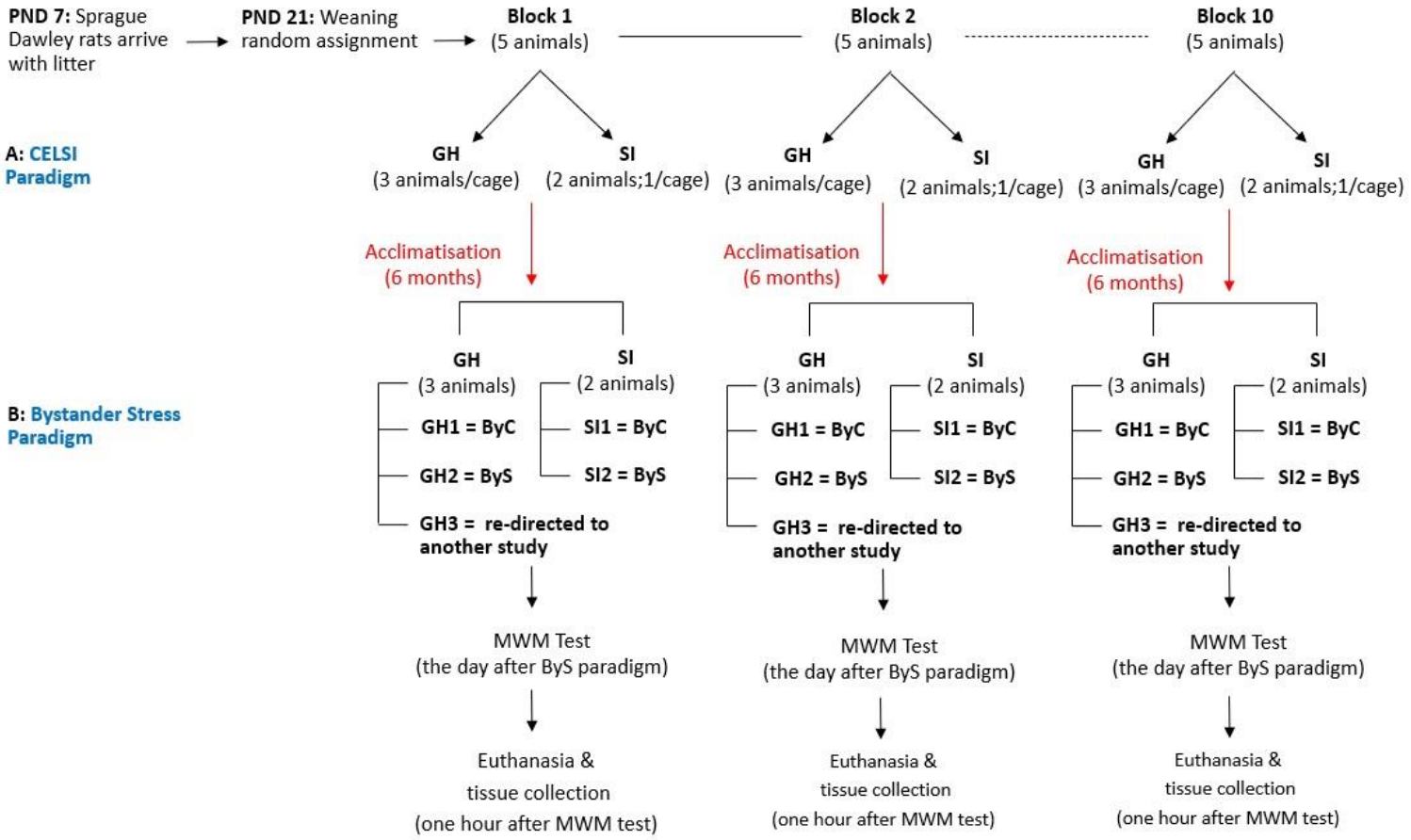


Figure 1.3. The combination of CELSI and ByS. **PND**: Post-natal Day, **GH**: Group Housed, **SI**: Socially Isolated, **ByS**: Bystander Stress, **MWM**: Morris Water Maze.

Chapter 2
LITERATURE REVIEW

2.1 Stress: Sources and Types

The maintenance of life depends on maintaining a constant internal milieu (homeostasis) despite changes in the environment (Bernard, 1957; Cannon, 1929). As a result, stress may be defined as the effects of anything that poses a significant threat to homeostasis (Selye, 1956). Following from the noted definition, "stressors" are perceived or actual threats to an organism, and the organism's reaction is the "stress response." Despite the fact that stress responses evolved as adaptive processes and that transient stress responses are essential for survival, prolonged stress responses can adversely affect the body's functioning, as observed in chronic stress (McEwen, 2000). As a result, to fully comprehend the concept of stress and its manifestations, it is necessary to understand the fundamental aspects of its dynamic process. There are several factors that are crucial to consider when evaluating stress, including the source of stress (stressor), its duration and severity, the response elicited, and the mediators involved; as well, we need to consider how the response may alter the physiological functioning of the body.

According to Chamberlain (1979), stress sources can be classified into six categories: Task, Role, Physical, Primary Social, Secondary Social, and Individual or Internal Stress (Figure 2.1): 1. Task-based stress is caused by difficulty in accomplishing the task itself, such as difficulties related to the technical, mechanical, or intellectual aspects of the performance.

2. Role-based stress resulting from the expectations of others regarding the individual activities, their efforts to influence performance, and any uncertainties that may arise due to those expectations. 3. Physical environment can be changed by conditions such as extreme temperatures, excessive noise, or crowding. 4. Primary social relationship stress refers to interpersonal stress resulting from close and/or important relationships, and can be caused by either a work colleague or a family member or friend who has direct influence over the individual. 5. Secondary social relationship stress is a type of interpersonal stress that arises from people who may influence the subject's life, but only indirectly (not as close/important as primary individuals), such as neighbors, distant employees, and politicians. 6. Individual stress is caused by a person's internal feelings about themselves. Thus, individuals may experience this type of stress internally because of feelings about their capabilities and inadequacies, their self-confidence, their concerns, or psychological problems that stem from personal illness, or their general nervousness (Chamberlain, 1979).

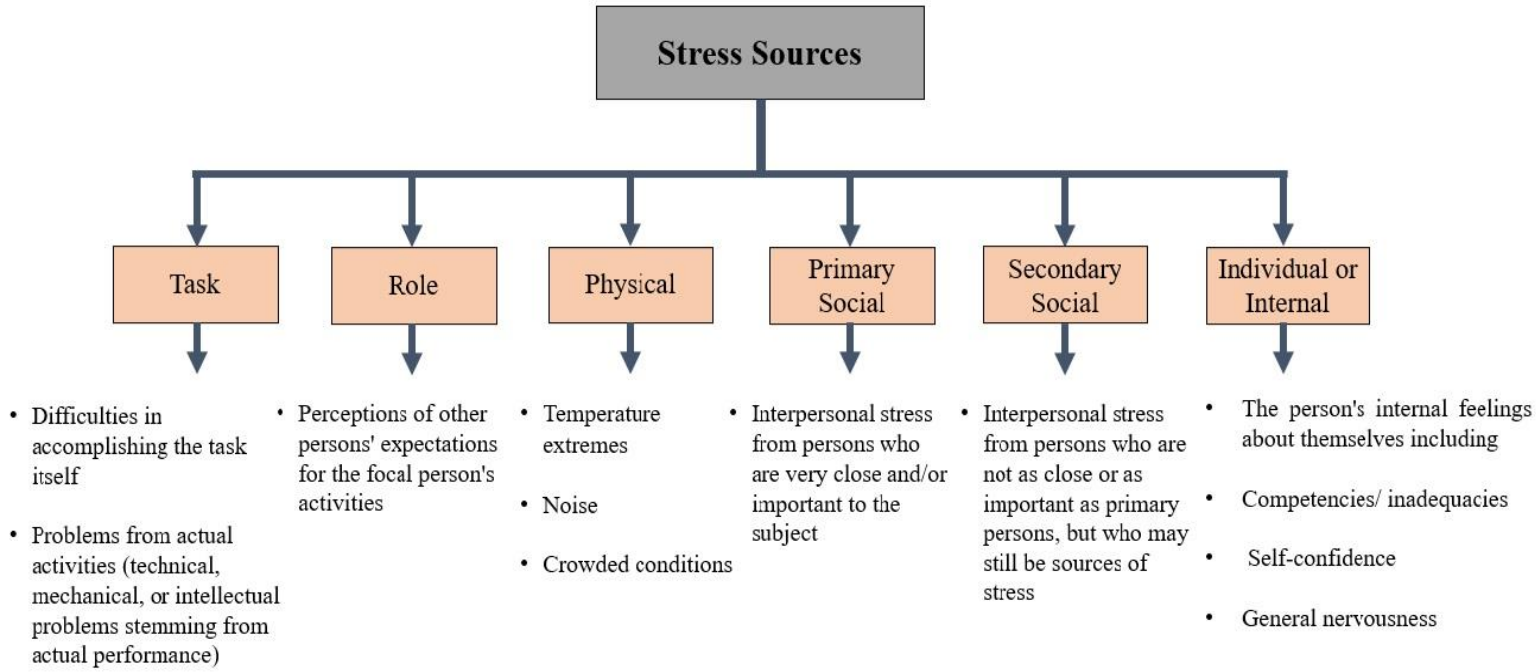


Figure 2.1. Chamberlain's classifications of sources of stress: Task, Role, Physical, Primary Social, Secondary Social, and Individual or Internal Stress.

2.1.1 Acute, Episodic Acute, and Chronic Stress (positive to toxic stress responses)

The American Psychological Association defines three types of stress - acute stress, episodic acute stress, and chronic stress - since periodicity and duration are important factors that influence the stress response. Acute and chronic stress can be considered as poles of a spectrum and there are different types of stress with varying durations and intensities between these two poles, including episodic acute and traumatic stress.

The most common form of stress is acute stress, which is triggered by a temporary stimulus that lasts between minutes and hours; as a result, the body's stress system is activated for a short period of time. Acute stress is typically associated with the fight-or-flight response to manage dangerous situations, but it may also result from experiencing something new or exciting, such as an unpredictable event or situation. Notably, acute stress can be associated with eustress (positive stress), a beneficial response to stress that promotes health, motivation, and performance, as well as psychological well-being, which represents the exhilarating feeling of taking risks, overcoming challenges, and receiving positive results. This type of stress, as an essential component of healthy development, is characterized by a healthy self-esteem, strong impulse control, and the ability to make decisions (McEwen, 2016). Therefore, this positive type of stress enhances resilience by strengthening the body's adaptation mechanisms and serving as a biological warning system (Salleh, 2008). Although acute stress can have long-term biological or behavioural effects if it is severe enough, the human stress response system is generally capable of coping with such situations.

Episodic acute stress refers to the high-stress situations where acute stresses occur frequently and/or periodically, for example, working under tight deadlines on a regular basis. We are unable to return to a relaxed and calm state when we are experiencing this type of stress, which often leaves us feeling as if we are moving from one crisis to another. Episodic acute stress that persists over a prolonged period of time may be harmful, resulting in physiological symptoms (Bakker et al., 2011). Negative experiences, such as those that may lead to episodic acute stress, can be considered tolerable stress if the individual has the resources and support needed to cope with the problem (McEwen, 2016; Shonkoff et al., 2009). Further, the tolerable stress may activate the body's alert system in advance of more severe, longer-lasting challenges, preparing the individual for upcoming challenges.

Chronic stress, triggered by persistent stimuli or very frequent activation of the body's stress system, several hours per day for several weeks or months (Dhabhar & McEwen, 1997), is associated with the development and progression of a wide range of negative health outcomes in both humans and animals including negative impact on brain function and behaviour (De Kloet et al., 2005; Lupien et al., 2009; Shonkoff et al., 2009). In other words, chronic exposure to stress may compromise biological resilience, impairing homeostatic function and predisposing individuals to adverse physical and mental conditions (Goldstein & McEwen, 2002). Importantly, early adverse life experiences may interfere with the brain's ability to tolerate such stress, and may affect the availability of the internal and external resources necessary to cope with negative experiences (such as social support, impulse control, and self-esteem).

Consequently, chronic stress (especially that occurring early in the lifespan) including physical or emotional abuse, economic hardship without any support, neglect or violence, substance abuse or mental illness, can trigger a toxic stress response/distress (prolonged, or permanent negative physiological reaction to stress), which may result in adverse behavioural, physiological, and psychological consequences (Franke, 2014; McEwen, 2016; Salleh, 2008). It should be noted that stress responses can vary depending on factors such as the duration and severity of the stressor, genetics, development, coping mechanisms, and historical events (adverse life events, trauma, abuse) (Figure 2.2).

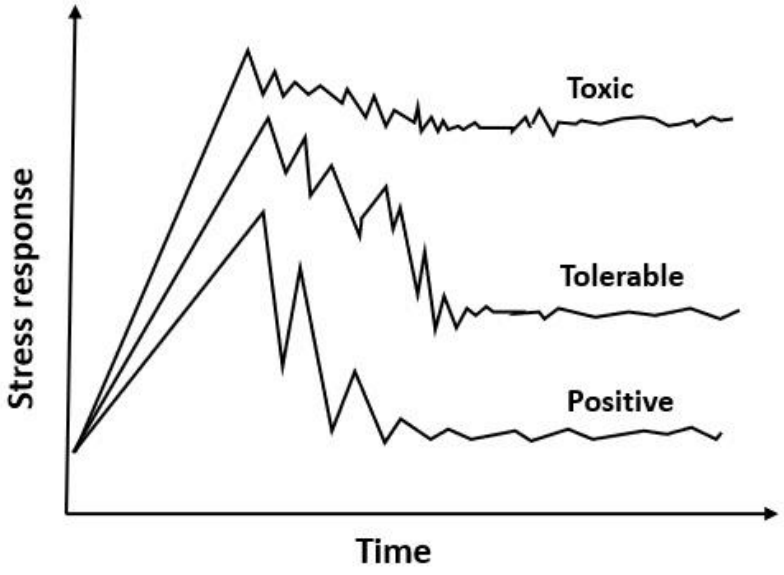


Figure 2.2. The relative effect and time course of types of stress response (acute or positive, tolerable, and chronic or toxic).

2.1.2 Environmental, Psychological, and Biological Stress

The concept of stress can also be described from an environmental, psychological, and biological perspective. According to the environmental approach, stress is defined as a change arising from an assessment of one's experience or environment. The frequency and severity of significant events requiring adaptation over a particular time period can be used to measure such changes (Clark et al., 2007). The biological aspect of the stress is characterized by the activation of a number of physiological response systems, such as the sympathetic-adrenomedullary system (SAM), the hypothalamic-pituitary-adrenocortical axis (HPA), and the immune system (Clark et al., 2007; Salleh, 2008). According to these perspectives, stress occurs when environmental demands disrupt a person's perception of their ability to adapt, and this change can set the stage for the development of disease.

2.1.3 Physiological and Psychosocial Stress

It has been demonstrated that physiological stress and psychosocial stress can be distinguished by the conditions that cause the stress. The term physiological stress refers to a condition where the individual's internal environment is disturbed (for example, by starvation, or the experience of unpleasant sensations) and this disruption triggers mechanisms intended to restore homeostasis, but that can result in tissue damage, pain, dehydration, malnutrition, and oxidative stress (Colaianna et al., 2013; Kogler et al., 2015). Therefore, physiological stress activates the body's stress response when it is confronted with a situation that requires a "fight-or-flight" response involving motoric-sensory processing as well as self-referential working memory (Kogler et al., 2015). For example, your heart races before giving a

presentation as a result of cellular changes caused by your body trying to prepare for what you are about to do.

While physiological stress stimulates the fight-or-flight response, psychosocial stress often arises from the need to regulate emotions and goal-oriented behaviour (Kogler et al., 2015). “Psychosocial stress refers to socially derived, conditioned, and situated psychological processes that stimulate any or all of the many manifestations of dysphoric affect falling under the rubric of subjective distress” (Kaplan, 1983). In other words, psychosocial stress is the consequence of an imbalance between the intensity of adverse experiences in daily life and our ability to cope with them, and refers to stress that occurs in the course of social interaction with others (interpersonal, family, and societal) including major life events such as the death of a loved one, divorce, moving, job loss, and major illness or injury (Pryce & Fuchs, 2017; Serido et al., 2004; Slavich, 2016). Psychosocial stress may also be caused by social evaluation due to the unpredictable nature of certain interactions or outcomes. Therefore, those who are exposed to socially risky situations, such as being evaluated by the community or excluded from it, may experience psychosocial stress (Dickerson & Kemeny, 2004; Kogler et al., 2015; Pruessner et al., 2010). Consequently, psychosocial stress may result in dysfunctional intrapersonal emotional and behavioural states that may contribute to the destruction of interpersonal networks and social relationships (Laelia et al., 2006), creating a vicious cycle.

2.1.4 Social Relationship Stress

No man is an island,
Entire of itself,
Every man is a piece of the continent,
A part of the main…
John Donne (1572-1631)

Despite having been written 400 years ago, this poem has a profound meaning for us today, especially during the COVID pandemic, when there was social isolation and lockdowns, along with a need for mutual responsibility and respect (Seffusatti, 2022). No person is an island by themselves, but is interconnected with other individuals, sectors, and institutions; thus, disruption in one area of a person's life could have detrimental consequences in another area (Pearlin, 1989). As a result, being associated with a person who is directly affected by stress can be regarded as a source of social relationship stress. Depending on the significance and closeness of the person experiencing direct stressors, social relationship stress can be classified as primary or secondary.

2.1.4.1 Primary Social Relationship Stress

Stress associated with primary social relationships is stress arising from relationships with individuals who are very close to and/or important to the subject. Stress resulting from social interactions may take the form of interpersonal conflicts, competition, disagreements with family members, friends, colleagues, or even enemies who can have a direct impact on a person’s life (Chamberlain, 1979). As an example, sharing a space or role with a person experiencing a primary stressor may result in stress (Pearlin, 1989).

2.1.4.2 Secondary Social Relationship Stress

This type of social stress is interpersonal stress from persons who are not as close or as important as primary persons, but who may still be sources of stress. Distant employees, distant relatives (note that distance may only be psychological), neighbors, politicians, or any other people who do not matter to you in a direct way may be examples of persons who may influence the subject's life but only in an indirect way (Chamberlain, 1979).

2.2 Animal Stress Models

There are two major types of stress models in animals: physical stress and psychological stress (Liu et al. 2018). Typically, in physical stress models, animals are exposed to primary environmental or physical stressors directly. For example, chronic unpredictable mild stress (CUMS) is considered as a physical stress model. Over the course of several weeks, at unpredictable times, animals are exposed to a series of minor stressors including wet bedding, cage tilt, light-dark reversal, water/food deprivation, immobilization, crowding, loud noise, forced swimming, tail pinch, and cold swim (Liu et al., 2018; Shao et al., 2013).

In contrast, psychosocial stressors are applied to animals in psychosocial stress models in a manner that may be primary or secondary (direct or indirect). Social defeat (SD), for instance, is a model that is based on the introduction of an "intruder" into the cage of a resident (an aggressive male animal). A serious defeat is achieved when the intruder surrenders or takes a supine position for approximately five seconds, with a maximum interaction time of five minutes. The intruder is then placed in a mesh cage within the resident's cage, where it can see, hear, and smell the resident, but not engage in direct physical. This procedure takes approximately one hour to be completed (Liu et al., 2018).

To apply psychosocial stress in different studies of this thesis, chronic early life social isolation (CELSI) and bystander stress models were used as primary and secondary social relationship stressors, respectively, to investigate their effects on the expression of important hippocampal plasticity-related proteins and spatial learning and memory in male and female rats (further information will be provided in the following chapters).

2.3 Social Stress and the Hypothalamic-Pituitary-Adrenal (HPA) Axis

Both the central and peripheral nervous systems are involved in the stress response. In particular, the stress response involves the HPA axis (Figure 2.3), the efferent sympathetic-adrenomedullary system (SAM), and parts of the parasympathetic branch of the autonomic nervous system (ANS) (Chrousos, 2002; Chrousos & Gold, 1992; Habib et al., 2001). Stressors trigger the secretion of two peptide hormones from the paraventricular nucleus (PVN) in the hypothalamus; arginine vasopressin (AVP) and corticotropin-releasing hormone (CRH). AVP activates vasoconstriction and reabsorption of water by the kidneys, which increase arterial blood pressure. CRH stimulates the synthesis and secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland (Maniam et al., 2014). ACTH is transported to the adrenal cortex via the bloodstream, where it stimulates the production of cortisol, a glucocorticoid hormone (GC). Cortisol then circulates throughout the body, affecting various cells. The primary role of cortisol in the stress response is to redirect cellular functions from long-term metabolic processes to immediate survival systems, which includes suppressing bodily functions not required for the stress response. The brain utilizes cortisol to maintain balance by reallocating energy and glucose to vital organs (Kolb & Whishaw, 2001). Notably,

cortisol is the major glucocorticoid hormone in humans, while corticosterone is the major GC hormone in rodents (Cottrell & Seckl, 2009; Welberg & Seckl, 2001).

Two main subtypes of receptors, with a similar protein structure, mediate glucocorticoid actions throughout the body: the mineralocorticoid receptor (MR), which is activated by low circulating levels of glucocorticoids, and the glucocorticoid receptor (GR), which is activated by relatively high concentration of glucocorticoids. Upon activation, the receptors translocate to the nucleus where they prompt changes in gene expression, which has long-lasting effects on the structure and function of cells (Kloet et al., 2009).

Glucocorticoids also have a significant role in controlling HPA axis activity via rapid binding to glucocorticoid receptors in the hypothalamus and pituitary gland. As cortisol/corticosterone rises to a certain point, further production of CRH and ACTH is suppressed and the HPA stress response is basically stopped. In addition, stress responses are also regulated by negative feedback mechanisms involving the hippocampus and prefrontal cortex (PFC), which are also mediated by GR and MR receptors (Nicolaides et al., 2015). Also, both the hippocampus and prefrontal cortex send glutamatergic projections to GABAergic PVN projecting neurons in regions such as the bed nucleus of the stria terminalis, suggesting these two structures may work either independently, or in tandem to inhibit PVN activation. Nevertheless, if the HPA axis is chronically activated and cortisol levels remain elevated for a long time, a desired state of homeostasis cannot be achieved. As a result, inhibition of growth hormone, muscle wasting and fatigue, gastrointestinal issues, a decreased inflammatory response, deficits in emotional regulation, impaired executive function, diminished self-

regulatory behaviour, and suppression of the immune system can occur (Cohen et al., 2007; Herman et al., 2003).

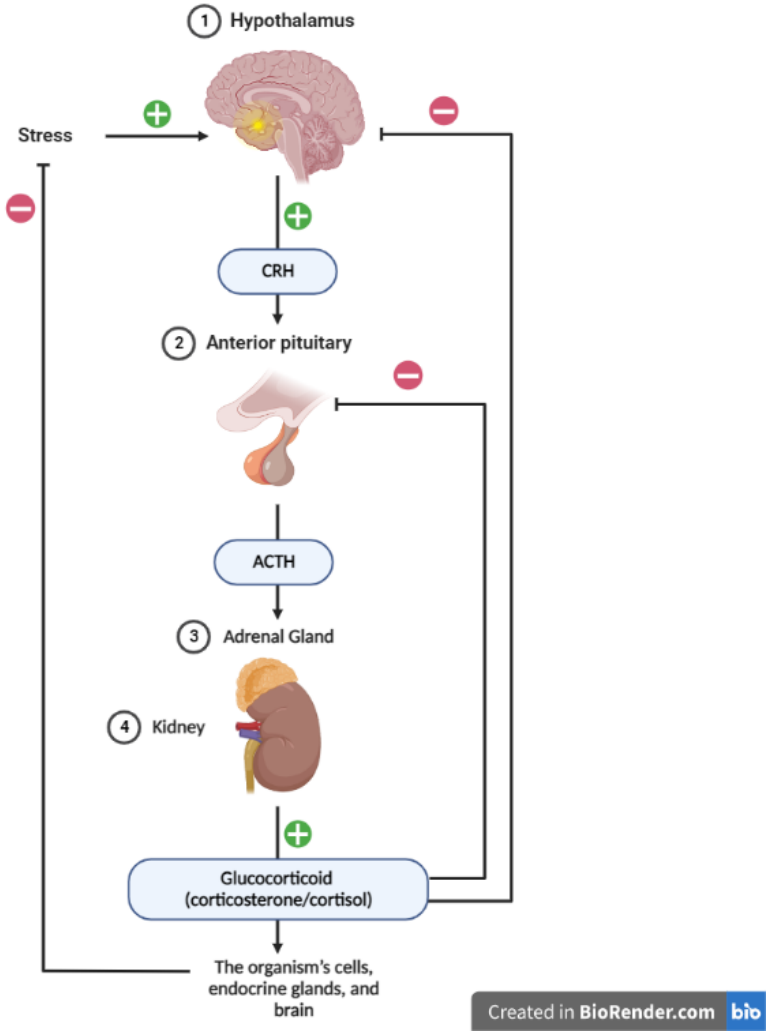


Figure 2.3. The Hypothalamic-pituitary-adrenal (HPA) axis. CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropin hormone.

2.4 The Hippocampus

Due to the enrichment of MRs and GRs in the hippocampus, plasticity, neuronal function, and the integrity of this structure are particularly sensitive to stress (Kloet et al., 2009; McEwen, 2000b). The hippocampus (HP) belongs to the limbic system and is located within the medial temporal lobe of each hemisphere. Across mammalian species, the HP is involved in learning and memory formation and the consolidation of information. The hippocampal formation comprises several components, such as the dentate gyrus (DG), subiculum, and the hippocampus proper, also known as Cornu Ammonis (CA). The CA region can be further divided into sub-fields, including CA1 and CA3. Given that the morphology and function of the hippocampus is conserved across mammalian species, it is widely used for comparative neurological studies (Anand & Dhikav, 2017).

The hippocampus contains unilateral excitatory synapses that link the DG, CA3, and CA1 subfields, forming what is known as the trisynaptic circuit. The entorhinal cortex sends information to the hippocampus via the perforant pathway, which then passes through the dentate gyrus and into the CA3 region of the hippocampus. From here, the CA3 pyramidal cells divide axons into two pathways. The first pathway involves commissural fibers that connect with the hippocampus on the opposite side of the brain. The second pathway forms the final connections of the trisynaptic circuit, which includes the Schaffer collaterals and the apical and basal dendrites of the pyramidal cells in the CA1 subfield. These cells then send information back to the entorhinal cortex. At the same time, the distal apical dendrites (stratum lacunosum moleculare) in the CA1 region also receive input directly from the entorhinal cortex through the temporoammonic pathway (Figure 2.4) (Kajiwara et al., 2008).

The prevailing consensus regarding trisynaptic loop projections is that they are purely excitatory and require regional GABAergic inhibitory interneurons to modulate their activation (Cobb et al., 1995; Freund & Gulyas, 1997). This pathway is strongly linked to hippocampal dependent learning processes, and disrupting any combination of the dentate gyrus, CA3, and CA1 nodes can result in negative effects on episodic, spatial, and declarative memory across different species (Squire, 1992; van Strien et al., 2009).

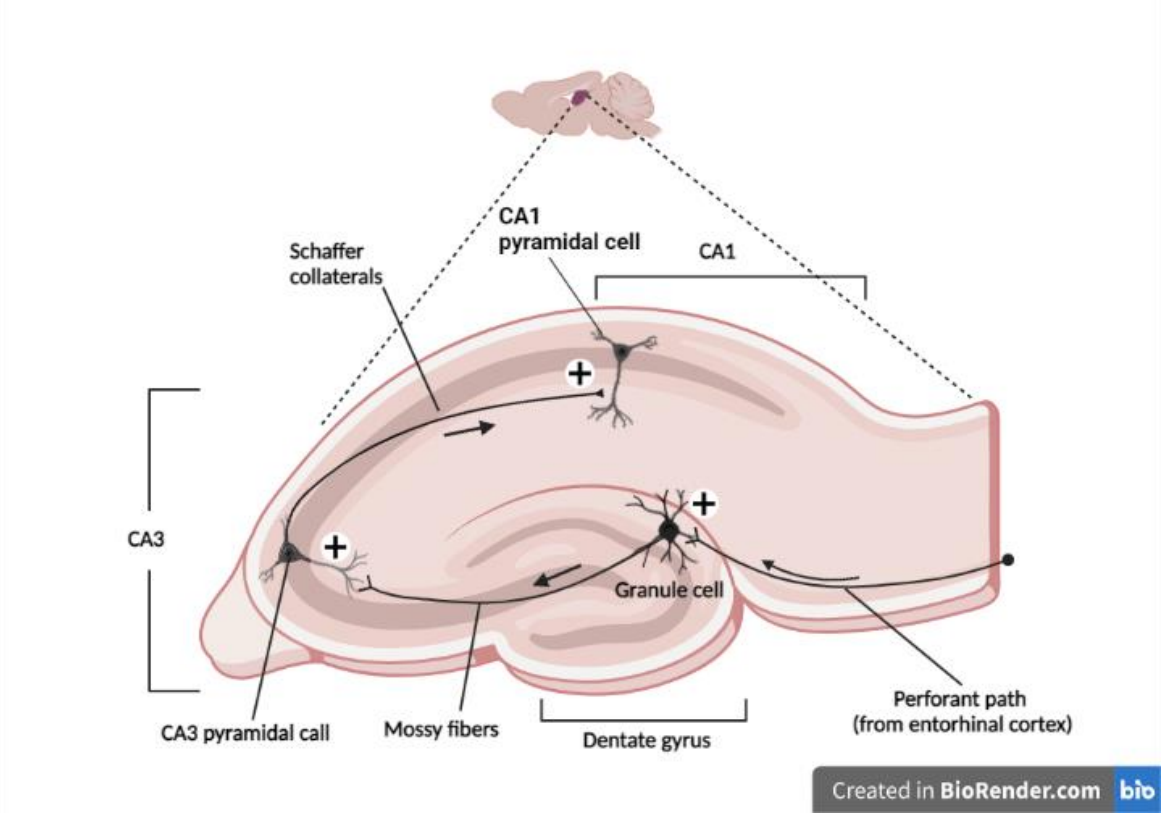


Figure 2.4. The cellular structure of the hippocampal formation. Excitatory axons from the perforant pathway connect to the granule cells of the DG, which project to the pyramidal cells of the CA3 sub-field. In turn, excitatory connections from the CA3 form the Schaffer collaterals that synapse on the apical and basal dendrites of pyramidal cells in the CA1 sub-field. The major output of the hippocampus is formed by the CA1 pyramidal cells, which project to the entorhinal cortex (Amaral & Witter, 1989). Created with BioRender.com.

2.4.1 Heterogeneity of the Hippocampus

Human and animal studies propose that the septal (dorsal) and temporal (ventral) hippocampal regions may make different contributions to emotional responses, spatial learning, anxiety, and even decision making (Ito & Lee, 2016; Moser et al., 1993; Nadel, 1968; Schumacher et al., 2018). Septal/temporal and dorsal/ventral terms are usually used interchangeably to describe poles of the hippocampus. The functional distinctions may be partially due to the difference in anatomical projections from hippocampal sub-regions (septal and temporal) to varying cortical and subcortical structures (Swanson & Cowan, 1977).

The septal pole is predominantly involved in cognitive functions, whereas the temporal pole plays a more significant role in processing emotions and regulating stress responses (Fanselow & Dong, 2010; Herman et al., 1995). Furthermore, it has been shown that a lesion of the temporal pole leads to decreased fear and anxiety (Trivedi et al., 2004), while a lesion of the septal pole impairs learning, memory, and spatial navigation performance (Zhang et al., 2004). It has been demonstrated that the ventral subiculum can limit the increase in corticosterone following restraint stress without affecting basal levels of glucocorticoids, suggesting that it projects to the paraventricular nucleus (PVN) in the hypothalamus, which regulates the hypothalamic-pituitary-adrenal axis (HPA) (Herman et al., 1995; Herman & Mueller, 2006).

Additional support for functional differences across the longitudinal axis is provided by experiments that illustrate differential susceptibility to anoxic injury (Ashton et al., 1989; Rami et al., 1997). One explanation for hippocampal heterogeneity and functional segregation along the hippocampal longitudinal axis that has emerged is biochemical data

demonstrating layer-specific septotemporal gradients of key glutamatergic receptors (AMPAR and NMDAR; please refer to section 2.6) across the hippocampus (Martens et al., 1998). Taken together, these data highlight the importance of considering differences across the hippocampal axis when assessing hippocampal data.

2.5 Synaptic Plasticity

Repeated activation of one neuron by another inducing long-lasting cellular changes that increase, or decrease connectivity between the two neurons is the defining feature of synaptic plasticity, which is thought to be a critical component of the neural mechanisms underlying learning, memory, and development in neural circuits (Hebb, 1949). In other words, synaptic plasticity is the ability of neurons to modify their synaptic activities as a result of specific activation patterns. Bliss and Lømo, (1973) found that high-frequency electrical stimulation applied to the perforant pathway of the hippocampus increases the magnitude of field excitatory post-synaptic potentials (fEPSP) measured in the dentate gyrus (Bliss & Lømo, 1973). The pattern of synaptic stimulation causes a long-lasting enhancement of synaptic transmission that has come to be called long-term potentiation. Today, long-term potentiation (LTP) and long-term depression (LTD) are considered as the two main forms of synaptic plasticity in the CNS that are caused by a long-lasting increase, or decrease in synaptic strength, respectively. Both processes are thought to be involved in learning and memory and various physiological and pathological processes (Abbott & Nelson, 2000; Massey & Bashir, 2007).

2.6 Glutamate Receptor Mediated Learning and Memory

Basal excitatory synaptic transmission is predominantly governed by ionotropic glutamate receptors (Huganir & Nicoll, 2013). These receptors are also associated with many

forms of synaptic plasticity such as LTP and LTD, learning, and memory formation mechanisms. Based on their pharmacology and structural similarities, the ionotropic glutamate receptors are divided into three groups: AMPA, NMDA, and Kainate receptors (Hollmann et al., 1989). In various parts of the brain, particularly in the hippocampus, AMPA and NMDA receptors play an essential role in modulating synaptic plasticity and mediating learning and memory functions (Li & Tsien, 2009; Shepherd, 2012). Most glutamatergic synapses throughout the brain have these receptors colocalized within the postsynaptic density, thereby mediating the postsynaptic currents that occur at excitatory synapses (Pinheiro & Mulle, 2006). Kainate receptors have been found to be functionally unconventional among the ionotropic glutamate receptors. In contrast to AMPA and NMDA receptors, they are distributed throughout the brain rather than being primarily located in excitatory postsynaptic signaling complexes. In addition to modulating synaptic transmission and neuronal excitability, kainate receptors are associated with metabotropic signaling pathways (Contractor et al., 2011; Huettner, 2003). Considering these differences, we focused our study on a few key subunits of the AMPA and NMDA receptors.

2.6.1 AMPARs (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors)

AMPARs are transmembrane ionotropic glutamate receptors, which are responsible for mediating most of the excitatory synaptic transmission in the brain. These receptors form homo- or hetero-tetrameric complexes consisting of various combinations of subunits (GluA1-4) (Dingledine et al., 1999), although most AMPARs in the hippocampus are formed from GluA1/GluA2 or GluA2/GluA3 subunits (Hayashi et al., 2000; Wenthold et al., 1996). GluA1/A2 heteromeric receptors are dominant in various brain regions, such as the nucleus

accumbens, dorsal striatum, prefrontal cortex, and adult hippocampus (Reimers et al., 2011; Wenthold et al., 1996), whereas GluA3 and GluA4 are predominantly expressed in the cortex and cerebellum, respectively (Italia et al., 2021; Schwenk et al., 2014). In addition, GluA4 is primarily expressed in neurons in the early stages of development and participates in plasticity events that occur during synapse maturation (Zhu et al., 2000). In general, as a result of proteomic and genetic analyses, it has been demonstrated that GluA1 and GluA2 form the majority of AMPAR subunits in hippocampal neurons (Italia et al., 2021; Lu et al., 2009; Schwenk et al., 2014). Due to these differences, we concentrated our study on the GluA1 and GluA2 subunits of the AMPA receptors.

Moreover, data support two different pathways governing the trafficking (that is, the movement of receptors from storage vesicles to the membrane and back) of AMPARs, and suggest various functions for each AMPAR subunit type within a synapse (Lee et al., 2004; Shi et al., 2001). GluA1-containing AMPA receptors are often involved in LTP induction and synaptic plasticity. Under basal conditions, GluA2-GluA3 heteromeric receptors replace GluA1-containing receptors in a constitutive pathway, which sees the receptors cycling between the postsynaptic membrane and intracellular compartments (Hayashi et al., 2000; McCormack et al., 2006; Zhu et al., 2000).

2.6.2 NMDARs (N-methyl-D-aspartate receptors)

The NMDARs are a type of ionotropic glutamate receptor activated by the binding of glutamate and glycine, and play a critical role in signal transduction and neural plasticity underlying learning and memory formation, as well as brain development (Paoletti et al., 2013; Sheng et al., 1994). The NMDARs are heterotetramers that may be formed by combinations

of three different subunits: GluN1 (found in all NMDARs), GluN2 (A-D) and GluN3 (A and B). A heterotetramer of NMDA receptors is formed by two obligatory GluN1 subunits and two regionally localized GluN2 subunits. It has been found that the GluN3A and GluN3B subunits have an inhibitory effect on receptor function and, in the absence of GluN2, they assemble with GluN1 to form NMDA receptors (Pachernegg et al., 2012). As well, NMDAR subunit expression can be considerably influenced by both the stage of development and brain region (Paoletti et al., 2013). For example, animal and human studies have indicated the expression of GluN2B is most prevalent in prenatal neurons, while the GluN2A expression becomes more prevalent around the time of birth (Law et al., 2003; Ritter et al., 2001).

The activities of the NMDAR regulate the structure and strength of dendrites and synapses since these receptors are attached to a complex network of signalling proteins that regulate synaptic transmission and cytoskeletal proteins (Husi et al., 2000; Husi & Grant, 2001). Therefore, alterations in the proteins involved directly with NMDA receptors, such as PSD-95 (which is attached to GluN2 subunits), may affect the size and density of dendritic spines (Prange & Murphy, 2001; Vickers et al., 2006). Studies have indicated that since PSD-95 has an essential role in concentrating NMDA receptors at synapses (Kim et al., 1996; Migaud et al., 1998), some behavioural disorders triggered by social stress, including CELSI, may involve abnormal expression of PSD-95 (Zhao et al., 2009).

2.7 Stress and Neural Changes

Animal studies reveal that increases in corticosterone/cortisol (CORT) as a result of exposure to chronic stressors from the prenatal period through adulthood is associated with structural changes, such as atrophy of the dendrites in the hippocampus, reduction of neurogenesis, disruption of synaptic plasticity, and alterations to volume of the amygdala (Holmes & Wellman, 2009; Huizink et al., 2004; Liston et al., 2006; McEwen, 1999). Experimental studies on subordinate rats and tree shrews have illustrated that apical dendrites of CA3 pyramidal neurons are remodelled as a consequence of psychosocial stress, similar to what has been seen after applying restraint stress in rats (Joëls et al., 2004; McEwen, 2004; Park et al., 2001; Sapolsky, 1999b). As a result of increased serum glucocorticoid levels, the length and the number of branches in apical dendrites are decreased in the hippocampus, especially in the CA3 region, causing hippocampal-dependent cognitive impairment (Sapolsky, 1999a). Moreover, since stress increases circulating adrenal steroid levels in the hippocampus, stressful situations may influence the creation of dentate gyrus granule neurons in adult animals (Bartanusz et al., 1995; Krugers et al., 1993). Altogether, stress may cause structural, cellular and molecular changes that result in changes to hippocampal-dependent functions, such as cognitive and memory impairments (Bianchi et al., 2006; Sandi, 2004).

2.8 Stress and Spatial Learning and Memory

The processing of spatial information depends on the maintenance of hippocampal circuits, and social stress may weaken the performance on spatial learning and memory tasks as a result of dendritic atrophy in the CA3 (caused by increased adrenal steroids and excitatory amino acids), reduced numbers of neurons and spine density, changes to the hippocampal

cytoskeleton (caused by changing in levels of α-tubulin isoforms associated with microtubule dynamics), and disrupted synaptic connections (Bianchi et al., 2006; Kolb et al., 2014; McEwen, 1999). Overall, these structural changes may make the hippocampus vulnerable to damage and eventually could lead to cell death (Kolb et al., 2014; Mychasiuk et al., 2011). Therefore, the behavioural impairments seen in stressed animals may be considered (at least in part) a consequence of dendritic degeneration and synaptic loss.

Human studies have indicated that early life social stress caused by poverty-related adversity reduces the volume of gray matter in the frontal and temporal cortex as well as the hippocampus. Additionally, this exposure can lead to changes in the physiological response to stress, resulting in the potential for harmful effects of stress-related hormones on the developing brain, and resulting in negative cognitive, emotional, and behavioral consequences (Coley et al., 2015; Deater-Deckard et al., 2010). Moreover, research has shown the quality of parenting that children receive in early childhood and the number of stressful life events they experience can have significant effects on the volume of the hippocampus (Luby et al., 2013).

It is believed that ionotropic glutamate receptors (specifically, AMPARs and NMDARs) are essential for the development of hippocampal synaptic plasticity, as well as cognitive functions dependent on the hippocampus, such as spatial learning and memory. Furthermore, since glutamate receptors have been shown to be affected by stress, it is likely that hippocampal functions may be disrupted by stress (Derkach et al., 2007; Kim & Diamond, 2002). Therefore, changes in signal transduction pathways, play a significant role in the effects of stress on the brain (Duman, 2002; Duman et al., 2000). As a result of these factors, along with the abnormal expression of AMPARs and NMDARs found in a variety of neurologic

conditions (Blanke & VanDongen, 2009; Henley et al., 2011), we sought to determine whether psychosocial stressors (CELSI and ByS) affect the expression of some key plasticity-related proteins in glutamate receptors (specific details relating to stress and its effects on NMDA and AMPA receptors, as well as spatial learning and memory performance will be discussed within later sections).

2.9 Stress and Biometrics

Serum levels of CORT are considered an appropriate biomarker of stress since the HPA axis is involved in both acute and chronic stress (Nater et al., 2013). However, whether isolation rearing affects basal plasma CORT levels remains unclear. In particular, psychosocial stress models, including social isolation, have been reported to elevate, reduce, or leave basal plasma CORT levels unaffected (Heidbreder et al., 2000; Schrijver et al., 2002). In addition, stress and its subsequent metabolic dysfunction may affect several other biometrics such as organ weights (Nater et al., 2013; Robb et al., 2017; Sellers et al., 2007).

Since the adrenal gland is an essential part of both the HPA axis and the sympatho-adrenomedullary system, increased adrenal function and weight in male rats is not surprising, and is thought to occur as a result of the hyperplasia and hypertrophy associated with increased maximal corticosterone responses to ACTH (Ulrich-Lai et al., 2006). With regards to social isolation, there are various findings; some research states that isolation rearing increases the weight of the adrenal glands in male, or female rats (Hatch et al., 1965; Syme, 1973). However, (Fone & Porkess, 2008) demonstrate that although social isolation increases the level of ACTH, there is no effect on adrenal gland weight.

Results have indicated that stress impairs hepatic blood flow and triggers natural killer cell activity in the liver, leading to liver damage and cell death (Chida et al., 2006). Natural killer cells are an important component of the innate immune system and play a crucial role in response to viral infections and tumors. As well, body and fat pad weight are considered stress markers that allow for a better understanding of the metabolic consequences of stress. For example, Bartolomucci et al. (2009) found that social isolation in adult male mice decreased body and perigonadal fat pad weight. Furthermore, their results showed a decrease in perigonadal, perirenal and retroperitoneal fat pad weight in dominant mice compared to the control group (Bartolomucci et al., 2009).

2.10 Social Stress and Sex

The prevalence of psychiatric disorders influenced by stress varies significantly by sex (Kendler et al., 1995; Piccinelli & Wilkinson, 2000). Animal studies suggest that female rodents exhibit higher basal levels of corticosterone compared to males (Malisch et al., 2009a). Furthermore, females display more pronounced and enduring adrenocorticotropic hormone and corticosterone responses to stress when compared to males (Larkin et al., 2010; Young et al., 2001). In addition, Leedy et al. (2013) found that social stress in female rats causes behavioural changes (reduced activity in open field testing, and alterations in social interest) and reduction of the basal dendrite lengths of CA3 hippocampal neurons (Leedy et al., 2013).

On the other hand, human studies showed that cortisol reactivity in men is about twofold higher than women to psychosocial stressors such as public speaking and mental arithmetic tasks (Kirschbaum et al., 1992), while stress reactivity in women seems to be higher in social rejection conditions (Stroud et al., 2002).

Even though men show higher HPA axis reactivity, women are found to be more likely to develop stress-related disorders (Juster et al., 2019; Kendler et al., 2002). Furthermore, it seems males suffer more from disorders with developmental origins, whereas females suffer more from disorders with adult onset (Joel & McCarthy, 2017). In addition, biomedical research is increasingly recognizing that there is a need to investigate areas in which sex may play an important role. Overall, measuring biological sex differences will provide insights into sex-dependent variation in stress physiology and the development of sex-specific diseases.

Chapter 3

CHRONIC EARLY LIFE SOCIAL ISOLATION ENHANCES SPATIAL MEMORY IN MALE AND FEMALE RATS

3.1 ABSTRACT

Objectives

Social adversity during childhood and adolescence can alter brain development in ways that may increase the likelihood of many prominent mental illnesses. To determine the underlying mechanisms, several animal models have been developed, such as Chronic Early-Life Social Isolation (CELSI), which involves rats isolated for several weeks after weaning. Although such a paradigm does cause many consistent changes in adult behaviour, one area where uncertainty exists concerns its effect upon hippocampal-dependent learning and memory.

Methods

To help sort out how CELSI affects spatial learning and memory, male and female siblings from 15 Sprague-Dawley rat litters were stratified by sex and then randomly assigned to either group-housing (3 animals/cage), or social isolation (1 animal/cage) for 7 weeks. Spatial learning and memory were then tested over 5 days using the Morris water maze. Next, the animals were euthanised, and stress-sensitive biometrics, including serum corticosterone levels, were collected. Lastly, to determine whether CELSI affected neural cell density, the expression of key neuronal and glial proteins (such as PSD-95 and GFAP, respectively) was assessed in isolated hippocampal tissue using immunoblotting.

Results

Notably, both male and female rats that had experienced post-weaning social isolation displayed stronger spatial learning and memory abilities than their group-housed counterparts. As well, socially isolated male rats exhibited a clear increase in expression of PSD-95. However, housing condition did not seem to affect either stress-sensitive biometrics, or hippocampal GFAP expression. Our results support the possibility that CELSI may enhance some aspects of hippocampal-dependent behaviour in a fashion similar among male and female rats.

3.2 INTRODUCTION

A broad array of evidence has revealed that early-life adversity can alter mammalian development in a dramatic fashion (Hanson & Gluckman, 2014; Nelson et al., 2020). For example, social stressors encountered before adulthood may alter brain development in a way that increases the likelihood of many prominent mental illnesses, such as depression and schizophrenia (Heim et al., 2004; Morgan & Fisher, 2007). Not surprisingly, adult rodents that have experienced psychosocial stress early in the lifespan often display features that reflect certain aspects of neuropsychiatric illness (Bolton et al., 2018; Fone & Porkess, 2008; Wang et al., 2020).

Although several rodent models of early-life adversity have been developed, one of the commonly studied involves keeping animals socially isolated from the time of weaning (around 21 days of age) to some point in early adulthood (Fone & Porkess, 2008; Lapiz et al., 2003; Walker et al., 2019). As with other similar models, the exposure to chronic, early-life social isolation (CELSI), which is also described as post-weaning social isolation, or isolation rearing, clearly affects adult behaviour. Perhaps the most consistently observed change caused by CELSI is novelty-induced hyperactivity (Fone & Porkess, 2008), however, other notable effects have also been found, such as impaired pre-pulse inhibition (Geyer et al., 1993), altered responses to psychomotor stimulants (Noschang et al., 2021), and enhanced anxiety-related behaviour in the elevated plus maze (Weiss et al., 2004).

Despite being able to exert a clear influence upon many facets of adult behaviour, the effect that CELSI can have upon learning and memory appears somewhat variable. For example, post-weaning social isolation has been found to cause a robust deficit in passive

avoidance learning (Gardner et al., 1975), a selective impairment after a shift in behavioural response rules within the radial arm maze (Schrijver & Würbel, 2001), a lack of difference in time spent exploring new and familiar items in a novel object recognition task (Bianchi et al., 2006), and a clear reduction in time spent freezing following observational fear conditioning (Yusufishaq & Rosenkranz, 2013). However, such a consistent pattern of undesirable change has not been observed when examining the spatial learning and memory ability of animals that experienced social isolation before adulthood.

Given its widespread acceptance as an index of cognitive function, several approaches to measuring spatial learning and memory have been developed, however, the Morris water maze has become one of the most common (Vorhees & Williams, 2006). Indeed, several groups have applied the method in trying to understand how exposing rats to CELSI affects their spatial learning ability in adulthood, and the results have been inconsistent. In particular, several reports found the acquisition of a spatial memory to be unaffected by post-weaning social isolation (Han et al., 2011; Hellemens et al., 2004; Lapiz et al., 2001; Quan et al., 2010; Schrijver et al., 2004), whereas two reports found the stressor impaired learning ability (Lu et al., 2003; Cevik et al., 2018) and another two observed an improvement in performance (Pisu et al., 2011; Wongwitdecha & Marsden, 1996). Similarly, of those reports that also examined spatial memory using a probe test, four failed to observe an influence of early-life housing condition (Cevik et al., 2018; Han et al., 2011; Hellemens et al., 2004; Schrijver et al., 2004), one observed an impairment (Quan et al., 2010), and two reported an improvement (Lapiz et al., 2001; Pisu et al., 2011).

Since a lack of clarity existed regarding how post-weaning social isolation affects the development of brain regions important for the acquisition and recall of spatial memories, the primary objective of our study was to examine Morris water maze performance in animals that had been either group-housed, or socially isolated for a 7-week period after weaning (this was the average length of isolation used in the previous reports that we were able to locate). As a companion to our behavioural analysis, we also assessed cellular structure within both groups of animals by measuring the relative expression of post-synaptic density 95 protein, which is abundantly expressed within glutamatergic synapses (Kennedy, 1997); and glial fibrillary acidic protein, which is an intermediate filament protein found in astrocytes (Middeldorp & Hol, 2011).

Unexpectedly, each one of the previous reports that examined CELSI and adult performance in the Morris water maze used only male rats. The omission of female rats from earlier reports is quite striking when considering the wide range of evidence revealing differences in the organisation and regulation of the stress response between male and female rodents (Bale & Epperson, 2015; Heck & Handa, 2019). Carrying on from this point, previous research has also drawn attention to sexual dimorphism among rats with regards to spatial learning and memory (Safari et al., 2021; Williams & Meck, 1991), at least some of which may be attributable to sex-dependent effects on how the animals react to stress associated with task performance (Beiko et al., 2004; Perrot-Sinal et al., 1996). As a result, a secondary objective of our study was to determine whether the effect of CELSI upon adult water maze performance could be moderated by sex.

3.3 MATERIALS AND METHODS

3.3.1 Animals and Social Isolation Protocol

Each week a Sprague-Dawley rat litter containing a dam and 5 male and 5 female pups arrived at our facility on post-natal (PND) 7 from Envigo (the exact number of litters varied according to the experimental measure). The animals were housed in polypropylene cages containing wood chip bedding and PVC tubes for enrichment. They were maintained in a temperature-controlled room (21◦C) with a 12:12-hour reverse light:dark cycle (lights on at 10:00 p.m.). The animals were given ad libitum access to standard rodent chow (Teklad 22/5, Envigo) and water. Upon weaning (PND 21-28; decision based upon pup size and eating behaviour), male and female siblings were stratified by sex and then randomly assigned to either the group housed (GH: 3 animals/cage), or the social isolation (SI: 1 animal/cage) condition for 7 weeks (figure 3.1). For both conditions, cage cleaning was limited to once a week to keep interaction to a minimum. After seven weeks, 2-3 GH rats and 1-2 SI rats from each litter, depending on other experiments being done in parallel, underwent behavioural testing. All animals were handled in accordance with procedures approved by the University of Waterloo Animal Care Committee.

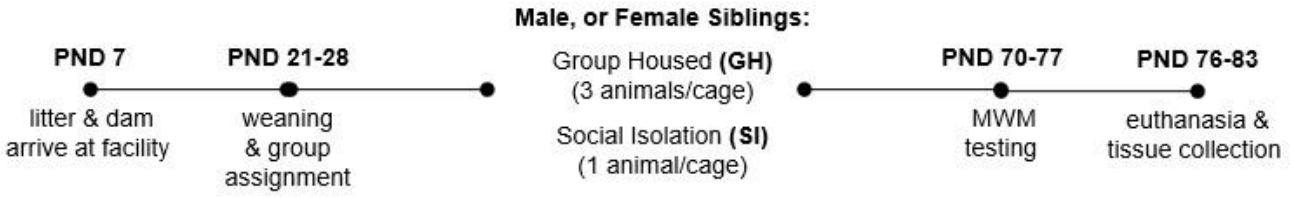


Figure 3.1. Chronic early-life social stress (CELSI) paradigm. Based upon observations of pup size and eating behaviour, animals were weaned between PND 21-28 and placed in sex-specific cages containing either 3 animals (Group-Housed; GH), or 1 animal (Social Isolation; SI). The housing conditions were maintained for 7 weeks (until PND 70-77), when the animals underwent Morris water maze (MWM) testing, followed by euthanasia and tissue collection.

3.3.2 Morris Water Maze Testing

Beginning the day after 7 weeks of social isolation, spatial learning and memory performance were assessed in the Morris water maze (MWM). Testing was done between 13:00 and 15:00 under low light conditions using a circular black plastic tank (175 cm in diameter, 70 cm in depth) containing no proximal cues and filled with water (50 cm in depth, 22°C ± 1°C). The pool was divided into four equal quadrants: northeast (NE), southeast (SE), southwest (SW), and northwest (NW). A circular platform (17.5 cm in diameter) was submerged 1.5-2 cm below the water surface and located in one quadrant in a fixed position throughout the experiment. Captured videos were analysed for behavioural variables (escape latency and distance travelled to locate the platform) with video tracking software (EthoVision XT8). All experiments were performed in a dimly lit room at the same time each afternoon, and the animals were placed into the room for thirty minutes prior to testing.

Over four consecutive days, the animals underwent four learning trials each day. Each trial was started by releasing an animal into the water maze, facing the perimeter, at one of the four starting points (north, south, east, or west). Each rat was allowed up to 60 s to find the hidden platform and was allowed to stay on the platform for 30 s. Those animals that failed to complete the task within 60 s were guided to the platform and were allowed to stay on the platform for 30 s. Following the completion of a trial, animals were dried and returned to their home cages and an inter-trial interval of approximately 10 minutes permitted.

On the day following the end of the learning phase, the memory phase (probe test) was done: the platform was removed, and each animal was placed in the drop location farthest from

where the platform had been and allowed to swim for 30 s. The time spent in the quadrant where the platform was formerly present was then measured (figure 3.2).

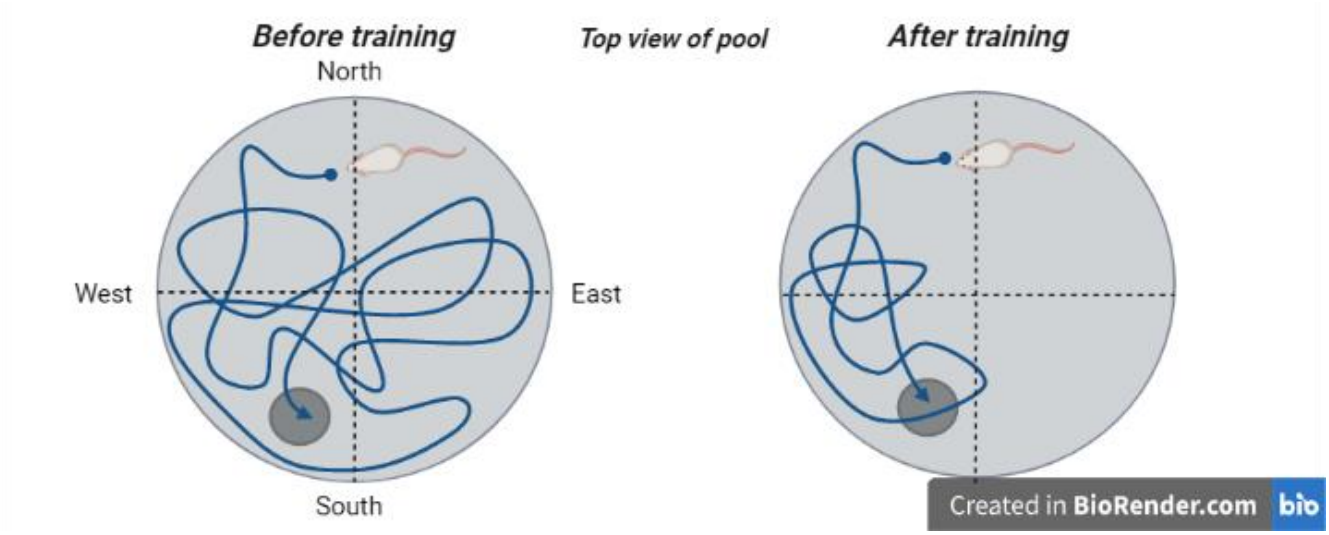


Figure 3.2. Morris water maze is a behavioural test designed to evaluate spatial learning and memory. A metal pool filled with water is used as the testing area. A hidden platform is submerged just below the surface of the water. By locating the platform, the animal is able to escape the water, most often with the aid of visual cues.

3.3.3 Biometrics and Extraction of the Hippocampus

Between 22-24 h following the probe test for each set of animals, the body weight of one rat of each sex from each housing condition was measured. Afterwards, anaesthesia was induced by placement in a chamber with >60% CO_2 and then animals were promptly euthanised by decapitation. Brains were rapidly removed (~60 s) and submerged in ice cold artificial cerebrospinal fluid that contained (in mM) 124.0 NaCl (Sigma-Aldrich; all subsequent reagents from Sigma-Aldrich, unless otherwise noted), 26.0 $NaHCO_3$, 10.0 glucose, 10.0 HEPES, 2.0 $CaCl_2$, 3.0 KCl, 1.0 $MgSO_4$, and 1.2 NaH_2PO_4 and was equilibrated with carbogen (95% O_2/5% CO_2) and held at pH 7.37–7.43. Next, the two hippocampi were dissected, snap-frozen (using liquid N_2), and stored at -80˚C. Concurrent with the brain dissection, trunk blood was collected along with a set of organs (adrenal glands, retroperitoneal fat pads, and liver) that were weighed. In all cases, raw values for organ weights were standardised to an animal's body weight.

A small volume of the trunk blood was used immediately after collection to measure non-fasting blood glucose levels using a standard glucose meter. The remaining blood was left to coagulate at room temperature for 20 min, and then centrifuged at 1000 × g for 10 min at 4°C. Following centrifugation, the supernatants (representing the serum portion of the blood) was collected and stored at -80°C. To measure serum corticosterone (CORT) levels, samples were run in triplicate using a commercially available ELISA kit (Cayman Chemicals) according to the manufacturer`s instructions.

3.3.4 Liver Lipid Analysis

For a subset of animals, the lipid content of a 1 g piece of the liver (typically from the median lobe) was determined using the Folch method (Folch, Lees, & Stanley, 1957). Briefly, the liver sample was manually homogenised in 5 mL of Type 1 (deionised) water, and then 10 mL of methanol was added to the mixture, which was then shaken briefly. Next, 5 mL of chloroform was added to the tube, which was then shaken briefly before being placed on ice for 1 h. After the incubation, a further 5 mL of chloroform was added and was followed by 5 mL of 1.0 M potassium chloride (the tube was shaken briefly after each addition). The tube was then placed on ice for 20 min, followed by centrifuged at 3000 rpm for 20 min, which caused the mixture to separate into 3 layers. The top layer was aspirated and the middle layer gently moved aside to permit the bottom layer to be decanted into a pre-weighed beaker. The beaker was placed in a fumehood overnight and then into an oven (at 60°C) for 5-10 min (to remove residual moisture) before being weighed.

3.3.5 SDS-PAGE and Immunoblotting

Using a Potter-Elvehjem homogeniser, tissue samples were manually homogenised at 4°C in lysis buffer [100 mM NaCl, 25 mM EDTA, 10 mM Tris, 1% (v/v) Triton X-100, 1% (v/v) NP-40] supplemented with a protease inhibitor cocktail (containing AEBSF, aprotinin, bestatin hydrochloride, E-64, leupeptin hemisulfate salt, and pepstatin A). Each homogenate was centrifuged at 1000 x g for 10 min at 4°C, and the resulting supernatant (representing the post-nuclear fraction) was collected. Protein concentrations were determined using the DC Protein Assay kit (BioRad) according to the manufacturer's instructions.

Equal amounts of protein (10 µg; determined to be within the linear range for each of the antibodies used in the study) were denatured in sample buffer [0.0625 M Tris, 2% (v/v) glycerol, 5% (w/v) sodium dodecyl sulfate (SDS), 5% (v/v) β-mercaptoethanol, and 0.001% (w/v) bromophenol blue, pH 6.8] at 95ºC for 5 min. Samples were loaded in duplicate and separated electrophoretically using a 10% SDS-polyacrylamide gel at 200 V for 1 h, and were then electroblotted onto polyvinylidene fluoride membranes via wet transfer (35 V at 4ºC for 16 h). Following transfer, blots were incubated with Ponceau S solution, washed with Type 1 water, imaged using a gel documentation system (Synoptics), and then either dehydrated and stored, or used directly for immunoblotting.

Each membrane was cut horizontally at the 75 kDa mark to permit the simultaneous detection of both target epitopes. Membranes were blocked for 1 h at room temperature in 5% (w/v) skim milk powder prepared in tris-buffered saline with Tween-20 [TBS-T; 20 mM Tris, 140 mM NaCl, 0.1% Tween-20 (v/v), and pH 7.6], and then incubated with primary antibodies overnight at 4°C. Each primary antibody solution was prepared at 1:1000 in blocking buffer: anti-PSD-95 (mouse; Millipore, cat. MABN68) and anti-GFAP (mouse; Millipore, cat. MAB3402). Following primary antibody incubation, membranes were washed 3 x 10 min with TBS-T and were then incubated for 1 h at room temperature with a secondary antibody solution (goat anti-mouse immunoglobulin G-horseradish peroxidase; Santa Cruz Biotechnology, cat. SC-2005) prepared at 1:5000 in blocking buffer. Following secondary antibody incubation, membranes were again washed 3 x 10 min with TBS-T, and then treated with enhanced chemiluminescence solution (Millipore).

Signals from antibody complexes were captured using a gel documentation system, and the optical density of each target band from within the linear range of exposures was measured using ImageJ and then standardised to its respective whole-lane Ponceau S optical density (also measured using ImageJ) to account for loading variation. After calculating the average of the standardised duplicate samples on a blot, a ratio was constructed between the sex-matched SI and GH values (that is, percent of sex-matched GH sibling) to account for inter-blot variability.

3.3.6 Statistical Methods

Typically, in the learning phase of the Morris water maze, animals that do not complete the task within an allotted amount of time are guided to the platform (Vorhees & Williams, 2014); as a result, the true escape latency and path length required by these animals remains unknown. Although putting in place a performance limit during task acquisition reflects an attempt to reduce variation, the trade-off resulting from such an intervention is that the truncated measurements are not as informative as uncensored data. When a trial is limited to 60 s (as was the case in the current study), an animal that accomplishes the task in 60 s is considered to have performed no differently than an animal that might have required much longer time to complete the task. As a result, the sample mean of the time to accomplish the task underestimates the true mean. Indeed, the underestimation of the true performance that results from censoring will lead to a systematic bias in analytical tools that make use of means and standard deviations, such as the ANOVA-type models often used with water maze data (Jahn-Eimermacher et al., 2011). Beside the issue imposed by the noted systematic bias, the underlying assumptions of mean-based ANOVA models are usually violated when applied to censored data.

While one remedy to address the problems resulting from censored data would be to use a statistical method that uses median values (given that a median would not be as affected by censoring as a mean), a more common statistical approach with such data involves the application of survival analysis. Rather than focusing on group means, survival analysis relates the probability of an event (in our case, finding the hidden platform) to variables of interest (here, animal sex and housing condition; henceforth referred to as covariates). Put simply, a survival model will connect learning-related measures (such as escape latency) to the covariates of interest while accounting for censoring.

One popular choice among survival models is the Cox Proportional Hazard (CPH) model (Cox, 1972), which is, essentially, a regression model that assumes changing a covariate has a multiplicative effect on the hazard rate (for our work, the hazard rate is taken to mean the rate at which animals in a particular group located the platform at different points in time). As noted by its name, the proportionality of hazards (which reflects stability in the ratio of hazards between groups over time) is a crucial assumption in the CPH model and when this assumption is violated, procedures such as stratification are among the potential solutions to the problem.

To investigate the effects of sex and housing condition on swim speed, probe test performance, body weight, organ weights, serum corticosterone level, liver lipid content, and fasting blood glucose, we used ANOVA models in which an animal's litter was regarded as a blocking variable. To confirm the appropriateness of the chosen ANOVA models, exploratory data analysis was completed using residual diagnostic plots as well as assessments of normality (using both visual examination of QQ-plot and the Shapiro-Wilk test) and homogeneity of

variance (using both a residual vs. fitted values plot and the Fligner-Killeen test). Whenever a statistically significant p value (< .05) was observed at the omnibus level (that is, when at least one of the groups was found to be different from the others), Tukey's HSD was used as the post-hoc test to examine differences among the groups.

To assess the influence of sex and housing condition upon neural protein expression, we used a one-sample t-test to assess statistical significance ($p < .05$) with 100% as our theoretical comparator. Given that we constructed a ratio of each SI value to its sex-matched GH counterpart, we expected that the average of these ratios would be very close to 100% if SI had no effect on protein expression. Based on our exploratory data analysis (using the approaches outlined above), we felt that our data satisfied the primary assumptions required for the use of a parametric test.

To assess the practical significance of relevant group differences across our dependent measures (with the exception of spatial learning ability), we applied Cohen's d to standardize the difference between group means based on their pooled standard deviation. As a standard convention, Cohen's d was interpreted as a small ($d = 0.2$), moderate ($d = 0.5$), or large ($d = 0.8$) effect size.

3.4 RESULTS

3.4.1 Performance During Learning Trials

The average swim speed of each animal (GH – male/female, N = 35; SI – male/female, N = 20) during the probe test was measured to determine whether any observed differences in task acquisition might be attributable to variability in either motivation to complete the task and/or sensorimotor abilities (e.g., swimming ability). Our analysis revealed that swim speed in our sample did not differ according to either sex, or housing condition (figure 3.3A).

Prior to analysing the data gathered during the learning and probe trials, we determined which of the performance measures we collected (escape latency and path length) would form the basis for our analysis. To do this, we measured the correlation between our two dependent measures, and found that they were highly correlated (Pearson's r = 0.95; figure 3.3B), which matches the degree of association observed previously (Vorhees & Williams, 2014). Given that escape latency is the more easily collected of the two measures (in that tracking software is not required) and would therefore be likely to have broader use among investigators, we decided to build our analyses around this performance feature.

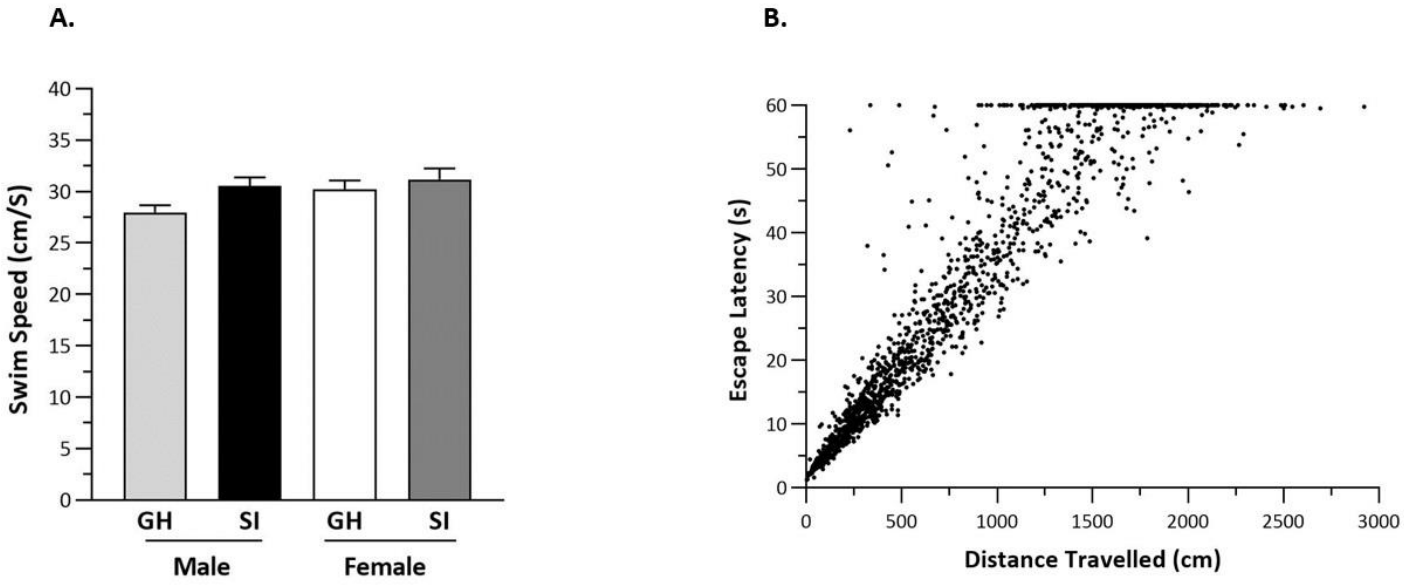


Figure 3.3. General water maze performance characteristics. (A) The graph presents the average swim speed recorded for each group during the probe test. Each bar presents swim speed ± SEM. (B) The graph illustrates the correlation (Pearson's r) between both recorded measurements of maze performance (distance travelled and escape latency). The darker regions represent overlapping data points. The two measures were found to be highly correlated (r = 0.95). **SEM**, standard error of the mean

As noted earlier (section 2.6), using means and standard deviations is not well suited to data sets wherein measurements have been censored. However, to allow the reader to compare our results to the majority of historical data, we have illustrated them using both the standard format (with means and SEMs; figure 3.4A) and medians and inter-quartile ranges (given their reduced sensitivity to censored data; figure 3.4B). Based on performance during the fourth day of learning trials, male SI rats had an average escape latency that was 28% lower than that of the male GH rats, and female SI rats had an average escape latency that was 32% lower than that of the female GH rats. Furthermore, the mean escape latency of male SI rats was 2.5% lower than female SI rats and was 9% lower in male GH rats than female GH rats.

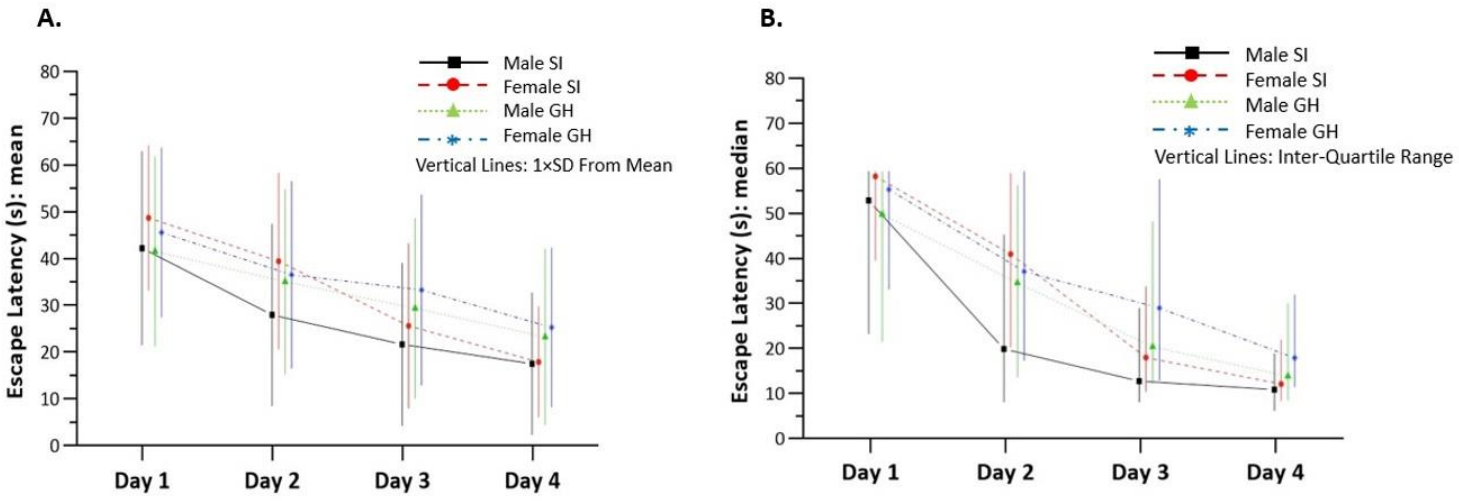


Figure 3.4. Standard presentation for water maze performance over the learning phase. The graphs present either the mean and SD (A), or the median and IQR (B) of escape latency recorded for each group on each training day. Considering performance during the fourth day, male SI rats had an average escape latency that was 28% lower than that of male GH rats, and female SI rats had an average escape latency that was 32% lower than that of female GH rats. **GH**, group-housed; **IQR**, inter-quartile range; **SD**, standard deviation; **SI**, social isolation

To address the weakness of ANOVA-based models in the face of censored data, we employed survival analysis to examine the learning trial data. As mentioned in section 2.6, our survival analysis model (CPH) rests on the assumption of proportional hazards; since this assumption did not hold true for sex on either day 1, or day 4 we used an alternative approach. At first, we wanted to use stratification to resolve the violation of the proportionality assumption, however, since we found an interaction between sex and housing condition, we were unable to do so. As a result, we used marginal models to allow both different baseline hazards and different effects of housing condition on hazard rates for male vs. female rats (that is, we constructed separate models for male and female animals). Separate models were fitted to male and female data for days 1 and 4 and were then compared (figures 3.5A and 3.5B). Given that the performance was highly similar on the first day of the learning phase and clearly different on the last day, we focused on these two days.

On day 1, the hazard ratio for the GH and SI male rats was 1, with a 95% confidence interval of (0.71, 1.4), which means that both groups had the same probability of finding the platform at any point in time. The hazard ratio for the GH and SI female rats on day 1 was 0.9, with a 95% confidence interval of (0.64, 1.3), which indicates that they too had the same probability of finding the platform at any point in time.

On day 4, the hazard ratio for GH and SI male rats was 1.5, with a 95% confidence interval of (1.1, 1.9), which reveals that the SI group was 1.5 times as likely to find the platform at any time compared to the GH group. The hazard ratio for the GH and SI female rats was 1.7, with a 95% confidence interval of (1.3, 2.3), which shows that was 1.7 time as likely for animals in the SI group to find the platform at any time relative to those in the GH group.

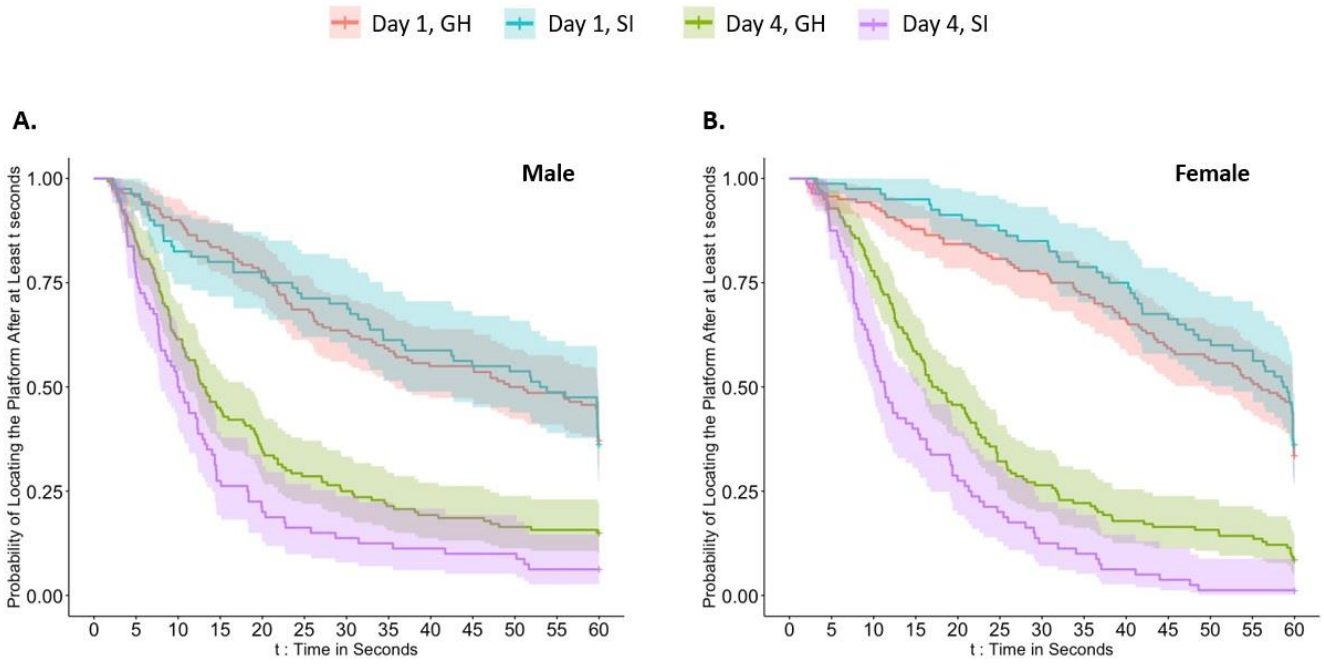


Figure 3.5. Probability of locating the escape platform displayed by male (A) and female (B) rats for trials on days 1 and 4 of the learning phase. A significant difference between the performance of animals on day 1 and day 4 in all groups ($p < .0001$) indicates that each group improved over the course of the learning trials. However, while there was not a clear difference between SI and GH groups on day 1, by day 4 the hazard ratio between the groups grew to about 1.5 for both male and female animals. **GH**, group-housed; **SI**, social isolation

3.4.2 Performance During the Probe Test

For the probe test, all animals were allowed to search the maze for 30 s on the day following the final learning trial (figure 3.6). Given that its underlying assumptions were met (homogeneity of variance, normality, and uncorrelated residuals), we proceeded to use an ANOVA model to analyse the results. Since there was no interaction between our two main factors (sex and housing condition) [$F(1, 92) = .03$, $p = .86$], we first examined the main effect of housing condition [$F(1, 93) = 8.2$, $p = .005$] and found those animals that underwent social isolation spent approximately 25% more time in the target quadrant. Indeed, post-hoc comparisons clearly revealed that the time spent in the target quadrant was greater in both male ($p = .026$; $d = 0.53$) and female ($p = .026$; $d = 0.60$) rats that experienced social isolation. In contrast, sex did not seem to have either a statistical, or practical effect upon performance of animals during the probe test (GH-male vs. GH-female: $p = .99$, $d = 0.009$; SI-male vs. SI-female: $p = .99$, $d = 0.067$).

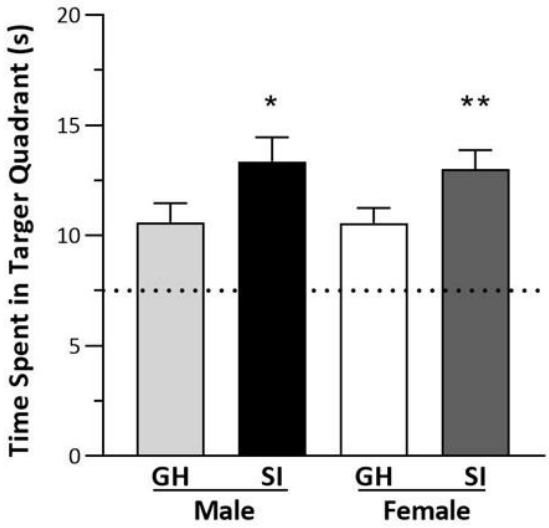


Figure 3.6. Time spent in the target quadrant during the probe test. The amount of time spent in the target quadrant was significantly different across housing condition in both male (*p = .026, d = 0.53) and female (**p = .026, d = 0.60) rats that experienced post-weaning social isolation. The data are displayed as mean ± SEM. **GH**, group-housed; **SEM**, standard error of the mean; **SI**, social isolation

3.4.3 Body weight, Organ Weights, and Metabolic Parameters

Since our data were judged to have met the necessary underlying assumptions, we used an ANOVA model to analyse our results, and did not observe a significant interaction between housing condition and sex for any of the biometric variables. As a result, we went on to analyse only the main effect of housing condition within our data, since sex based biometric differences on their own were expected. Regardless of the biometric examined, a statistically significant difference between our two housing conditions was not apparent following any of our planned pairwise comparisons (table 1).

Biometric	**Group Housed: male** mean ± SEM, N	**Social Isolation: male** mean ± SEM, N	***p* value** GH-M vs SI-M	**Effect Size (d)**	**Group Housed: female** mean ± SEM, N	**Social Isolation: female** mean ± SEM, N	***p* value** GH-F vs SI-F	**Effect Size (d)**
body weight (g)	334 ± 2.9 25	329 ± 2.8 28	.55	0.17	214 ± 3.0 25	210 ± 2.7 30	.55	0.03
adrenal gland weight (% BW)	0.0152 ± 0.000553 25	0.0151 ± 0.000521 28	.99	0.24	0.0292 ± 0.000553 25	0.0291 ± 0.000521 30	.99	0.14
liver weight (% BW)	3.54 ± 0.04 25	3.60 ± 0.04 28	.71	0.15	3.20 ± 0.04 25	3.24 ± 0.04 30	.71	0.16
RP fat pad weight (% BW)	0.38 ± 0.01 22	0.40 ± 0.01 27	.50	0.34	0.31 ± 0.01 22	0.33 ± 0.01 27	.50	0.12
Serum CORT (pg/mL)	14.0 ± 3.4 10	12.3 ± 3.4 10	.97	0.14	14.0 ± 3.4 10	12.4 ± 3.4 10	.97	0.12
Liver Lipid Content (mg/g)	37.0 ± 0.9 10	35.0 ± 0.9 10	.23	0.82	44.0 ± 0.9 10	42 ± 0.9 10	.23	0.41
Non-fasting Blood Glucose (mmol/L)	7.23 ± 0.40 10	7.24 ± 0.40 10	1.00	0.02	7.60 ± 0.40 10	7.60 ± 0.40 10	1.00	0.05

Table 3.1. General physiological characteristics of male and female animals in each treatment group. BW, body weight; **CORT**, corticosterone; **d**, Cohen's d effect size measure; **GH-F**, group-housed female; **GH-M,** group-housed male; **N**, sample size; **RP**, retroperitoneal; **SEM**, standard error of the mean; **SI-F**, socially isolated female; **SI-M**, socially isolated male

3.4.4 Neural Protein Expression

Male rats that experienced CELSI displayed a level of PSD-95 protein expression that was about 12% greater than what was measured in group-housed animals ($t = 8.74$, $p = .0003$, $d = 3.55$; $N = 6$; figure 3.7). In contrast, housing condition during the weeks after weaning did not appear to affect PSD-95 expression in female animals ($t = 0.14$, $p = .89$, $d = 0.05$; $N = 8$). With regards to GFAP, an extended period of post-weaning social isolation did not seem to alter its expression in either male ($t = 0.97$, $p = .36$, $d = 0.34$; $N = 8$), or female ($t = 0.55$, $p = .60$, $d = 0.22$; $N = 6$) animals.

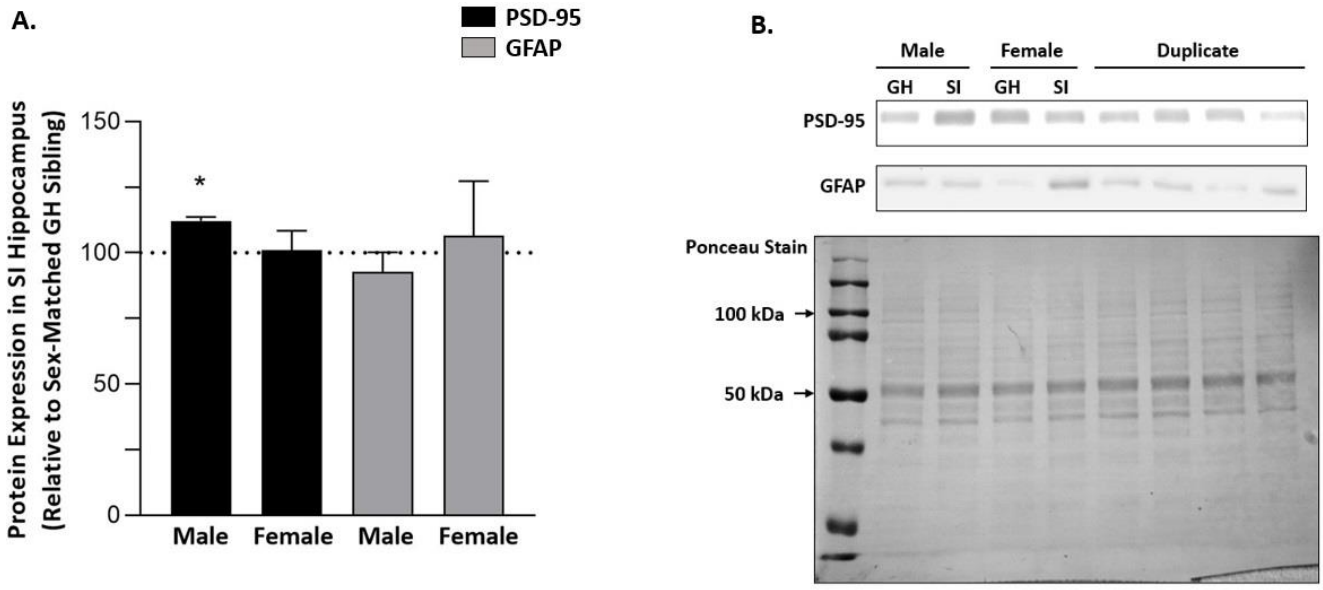


Figure 3.7. Immunoblotting-based measurement of important structural proteins in neurones (PSD-95) and glia (GFAP) across treatment groups. A) The graph presents the average protein expression of each SI group member standardised to its respective sex-matched GH sibling (N = 6). Hippocampal PSD-95 expression was about 12% greater in the socially isolated male rats relative to their group-housed counterparts (*p = .0003). B) Representative immunoblots for each antibody (upper images) and Ponceau S staining for the same blot (lower image). **GFAP**, glial fibrillary acidic protein; **GH**, group-housed; **PSD-95**, post-synaptic density protein 95; **SI**, social isolation

3.5 DISCUSSION

3.5.1 CELSI-mediated Changes to Spatial Learning and Memory

The primary goal of our study was to contribute to the ongoing discussion around whether post-weaning social isolation affects the development of brain regions important for the acquisition and recall of spatial memories. Although male and female rats from both housing conditions were able to successfully navigate the water maze, the spatial learning ability of the CELSI animals was clearly stronger. Indeed, our socially isolated animals displayed an escape latency on the final training day that was approximately 30% quicker than their group-housed counterparts, which is a magnitude of difference that overlaps quite well with the enhanced performance observed in two earlier studies that used male rats (Pisu et al., 2011; Wongwitdecha & Marsden, 1996). However, our results stand in contrast to most of the earlier work on this topic, which suggested that social isolation during the post-weaning period does not alter place learning (Table 2).

After examining the ability of our animals to learn the water maze task, we assessed the persistence of their hippocampal-dependent spatial memory 24 h after the last learning trial by measuring time spent in the target quadrant throughout a probe trial. Again, whereas previous studies did not tend to observe an effect of CELSI in the probe test (Table 2), we found that socially isolated animals (regardless of sex) spent about 25% more time in the target quadrant than control animals. As well, our observation of improved spatial memory in the CELSI animals generally agrees with the sort of difference observed in both of the earlier reports that found early-life social isolation caused animals to spend more time than control animals in the region around the escape platform (Lapiz et al., 2001; Pisu et al., 2011).

Although there may be many reasons for results to vary across studies (not the least of which is simple sampling variation), two of the usual suspects tend to be differences in the animal model and differences in how the dependent measure is collected. When reflecting upon our results, we considered both of the noted points, and were not able to find a strong argument to be made in either case. When comparing our findings against earlier work done in the same species (Table 3.2), no clear pattern around the animal model emerged; for example, in both the Sprague-Dawley and Lister Hooded strains, CELSI was observed to either have no effect on place learning, or to cause an improvement in ability. As well, a lack of effect of CELSI upon learning trial performance was observed in studies where the isolation period stretched from 2 to 12 weeks, with improvements and impairments being seen in studies where the isolation period varied within this range. In a similar fashion, most studies used the same basic approach when examining task acquisition (3-4 trials per day for 4-5 days), suggesting that performance differences were not likely attributable to animals having more/fewer opportunities to learn the task.

Since there are no apparent technical reasons to explain why our results disagreed with many earlier reports, we went on to consider what biological mechanisms might be responsible for the improved performance we observed among those rats that experienced post-weaning social isolation. After considering various possibilities, we were left with one that seemed particularly compelling: *developmental conditioning*. Built using several decades worth of epidemiological and experimental evidence, developmental conditioning is a framework that suggests animals make use of environmental information received prior to adulthood to calibrate their developmental trajectory to improve their chances of survival (Gluckman and

Hanson, 2014). Indeed, the basic elements of developmental conditioning have recently been used to develop the match/mismatch hypothesis, which suggests that mild to moderate adversity experienced during development can allow an individual to weather similar conditions later in life better than those individuals wherein there is a mismatch between early-life and adult conditions (Schmidt, 2011). With developmental conditioning in mind, the social isolation stress experienced early in the lifespan by some of the animals in our study may be viewed as having shaped their cognitive development in a way that enabled them to face a subsequent adulthood stressor (the water maze task) more successfully than their control counterparts.

Of course, the distal explanation for our observation offered by the match/mismatch hypothesis requires a more proximal mechanism, which may be found in the activity of glucocorticoids. Although often viewed solely as a key player in the unfolding of the classical stress response, glucocorticoids have become recognised as important mediators of memory formation (Joels et al., 2012; Schwabe et al., 2012); in particular, within rats, intraperitoneal injection of these hormones after training improved water maze performance (Sandi et al., 1997), while intracerebroventricular administration of glucocorticoid receptor antagonists after training led to impaired memory consolidation (Oitzl & de Kloet, 1992). With the above points in mind, we believe that isolation rearing led the affected animals to become hyper-responsive to the stress they experienced while completing the water maze, and that this enhanced response took the form of increased glucocorticoid release, which facilitated learning and memory acquisition. Indeed, a substantial amount of work has revealed that early-life adversity can alter glucocorticoid regulation via epigenetic changes that adjust activity of the HPA axis

(Anacker et al., 2014). As well, an earlier report by Pisu et al. (2011) observed that training in the Morris water maze caused a substantial increase in plasma corticosterone levels among rats that experienced 4-5 weeks of post-weaning social isolation (whereas their group-housed counterparts displayed a decrease in corticosterone levels).

A wide variety of reports have established that chronic stress experienced *during adulthood* is not only able to affect several areas of rodent cognition, but also that female animals tend to show greater resilience (Luine et al., 2017). Unfortunately, only a limited amount of research has been done to examine sexual dimorphism after post-weaning social isolation stress, and these data have not allowed a similarly clear pattern to emerge; for example, previous work with this form of social adversity has revealed reduced social preference among male animals (Kinley et al., 2021), but slower emergence into an unfamiliar space by female animals (Einon and Morgan, 1977). Although previous evidence did not clearly suggest the sort of change that we should have seen after CELSI, there was an expectation that we were likely to have observed some sort of sex-based difference in spatial learning and memory. Although the reason we did not find sexual dimorphism is not immediately apparent, its absence does support the idea that *when* during the lifespan a stressor is experienced will influence whether (and how) male and female animals will be uniquely affected. That is, whereas the experience of extended stress during adulthood is likely to result in a greater effect upon male spatial learning and memory ability (Conrad, 2010), such a pattern may not emerge when the stressor is present during the peri-adolescent period.

Author, Year	Sex, Strain	Light Cycle	Start of Isolation	Length of Isolation	Sample Size	Acquisition Trials	Probe Test	Outcome: Acquisition	Outcome: Probe Test
Cevik et al., 2018	male, Wistar	reverse	PND 29	8 weeks	8	4 trials/day for 4 days	yes; 24 h after last acquisition trial	SI animals displayed escape latency and distance travelled ~40% greater than GH on day 1	SI animals entered target quadrant ~30% more than GH (difference not statistically significant)
Han et al., 2011	male, Sprague -Dawley	standard	PND 21	2 weeks	9	4 trials/day for 4 days	yes; 24 h after last acquisition trial	no main effect of housing condition	no main effect of housing condition
Hellemens et al., 2004	male, Long-Evans	reverse	PND 21	12 weeks	12	12 trials/day for 2 days	yes; 24 h after last acquisition trial	no main effect of housing condition	no main effect of housing condition
Lapiz et al., 2001	male, Lister Hooded	standard	PND 21	4 weeks	6	4 trials/day for 4 days	yes; 24 h after last acquisition trial	no main effect of housing condition	SI animals entered target annulus ~40% more than GH
Lu et al., 2001	male, Sprague -Dawley	standard	PND 22	4 and 8 weeks	8 - 9	3 trials/day for 7 days	no	statistically significant differences between groups on days 4-7; on day 7, escape latency of the SI group was ~40% greater than GH regardless of isolation period	---

Table 3.2. Previous studies examining the effect of post-weaning social isolation upon hippocampal-dependent spatial learning and memory performance measured using the Morris water maze. **GH**, group-housed; **PND**, post-natal day; **SI**, social isolation

3.5.2 Neural Protein Expression Following CELSI

Over several decades, the view gradually emerged that prolonged exposure to either stressors, or stress-hormones (particularly, adrenal steroids) can promote changes in neuronal structure (over the short-term), or neuronal survival (over the long-term) (Sapolsky, 1996). Importantly, such changes have been seen with post-weaning social isolation. For example, Fabricius et al., (2010) observed that CELSI was able to reduce hippocampal volume in male animals by about 15%, while Silva-Gomez et al. (2003) found that this type of adversity was associated with reductions of greater than 50% in spine density from the hippocampal CA1 region of male rats. Given that the sort of structural changes observed with CELSI should be clearly related with altered protein expression, we expected to find obvious group difference in both PSD-95 and GFAP.

Although we did find that PSD-95 expression was different across our groups, we were surprised to find that its levels were *greater* in socially isolated male rats than their group housed counterparts. Along with the previously noted decline in hippocampal spine density (Silva-Gomez et al., 2003), CELSI has also been associated with reduced synaptophysin expression (of ~10%) in the dentate gyrus of male rats (Varty et al., 1999), and a reduction of about 25% in PSD-95 expression in the PFC of female rats (Hermes et al., 2011); taken together, the cited literature suggests that we should have found a reduction in PSD-95. Interestingly, however, our observation does agree with a report that found isolation rearing caused about a 10% increase in PSD-95 mRNA within the male hippocampus. Although the notion has been advanced that developmental isolation may cause either an increase, or decrease in synaptic density (and the expression of synaptic markers, such as PSD-95) based

upon when during the lifespan the isolation occurs (Li et al., 2021), both the Hermes et al. report (that showed a decrease in PSD-95 protein expression) and the Zhao et al. report (that showed a 10% increase in PSD-95 mRNA levels) used an isolation procedure almost identical to ours (Sprague-Dawley rats isolated at weaning for approximately 55 days). As a result, the true nature of how CELSI affects PSD-95 expression (either at the level of message, or product), and the reason for its sex-based variation remain uncertain.

Given the clear CELSI-related changes in brain volume observed by Fabricius et al. and the relative abundance of astrocytes within the mammalian brain, we expected to see levels of GFAP that were different across our groups. However, the direction in which the change would occur was unclear to us at the outset of the study. That is, previous work has shown that administration of the stress hormone corticosterone to male rats for 12 days caused about a 15% decrease in GFAP expression in the hippocampus (O'Callaghan et al., 1989), while having male rats undergo a weeklong period of activity-related stress caused a rise in GFAP levels in the same region (Lambert et al., 2000). As well, isolation of male animals early in the peri-adolescent period PND 21-34) has been associated with an approximate doubling of GFAP levels within the prefrontal cortex (although the animals were re-socialised prior to protein measurement; Sun et al., 2017). Given that elevated GFAP expression is considered reflective of a reactive gliosis meant to assist the brain in tolerating injury (Eddleston & Mucke, 1993), the lack of notable differences among our groups would suggest that CELSI was not causing the level of stress that would typically be needed to motivate an astrocytic response.

3.5.3 The Effects of CELSI on Body weight, Organ Weights, and Metabolic Parameters

Along with behavioural and neural changes, pre-weaning social isolation has also been observed to influence structural development outside of the nervous system. Perhaps, the most basic feature that has been studied is body weight, and, like other measures, the pattern of observation has not been consistent. That is, although most of the reviewed studies (with male animals) reported that post-weaning separation for at least three weeks did not affect body weight (Cevik et al., 2018; Cowley & Widdowson, 1965; Hellemens et al., 2004; Ko & Liu, 2015; Moore, 1968; Morinan & Leonard, 1980; Ryu et al., 2009; Sanchez et al., 1998; Weiss et al., 2004), one study did find an increase of about 10% (Weintraub et al., 2010) whereas two others showed decreases of about 10% (Cruz et al., 2015; Hatch et al., 1965). Of the studies that used female animals, two reported no change in body weight (Weintraub et al., 2010; Weiss et al., 2004), two others found an increase of 10-20% (Morgan & Einon, 1975; Syme, 1973), and another observed a decrease of ~20% (Hatch et al., 1965). Taken together, the historical data would suggest that CELSI is not likely to have an appreciable effect upon the body weight of either male, or female rats, which agrees with our observations.

Beyond body weight, we examined whether post-weaning social isolation might have affected the weight of several organs (relative to body weight), given that such changes are often regarded as signs of stress, toxicity, or metabolic dysfunction (Sellers et al., 2007). Although some degree of liver hypertrophy was expected, given the organ's contributions to the altered macronutrient metabolism that coincides with the stress response, we did not observe any changes in liver weight. Similarly, we did not find that isolation rearing affected levels of visceral fat mass, despite increases in adipose tissue often being seen with social

isolation, albeit in mice (Schipper et al., 2018). Notably, relative liver and fat pad weight do not appear to have been previously examined following post-weaning social isolation in rats.

The final organ that we examined was the adrenal gland, which is (arguably) among the most stress-sensitive tissues. Interestingly, social isolation was not found to affect relative adrenal gland weight in our study, which agrees with the general pattern observed for male animals (Gamallo et al., 1986; Gentsch et al., 1981; Hatch et al., 1963; Hatch et al., 1965; Holson et al., 1991; Moore, 1968; Morinan & Leonard, 1980; Sanchez et al., 1998; Weiss et al., 2004). Although several earlier reports also failed to observe a change in female animals (Holson et al., 1991; Moore, 1968; Weiss et al., 2004), there are an equal number that did measure an increase of 15-20% (Hatch et al., 1963; Hatch et al., 1965; Syme, 1973) (all of the studies with female animals used a period of isolation lasting 12-16 weeks), which suggests that adrenal hyperplasia may be one outcome that could reasonably be expected following isolation rearing in female rats.

Along with organ weights, we also examined terminal plasma CORT levels, given that a change in the amount of the hormone would intuitively be expected following an extended period of early-life social isolation. Indeed, several earlier reports using either male (Gamallo et al., 1986; Serra et al., 2018), or female (Hatch et al., 1965) rats showed increases in basal CORT levels of 25-40% after periods of post-weaning social isolation lasting from 4 – 13 weeks. Somewhat unexpectedly, we did not find that CORT levels varied between our treatment groups, however, this appears to agree with many earlier reports that used either male (Cevik et al., 2018; Gentsch et al., 1981; Hatch et al., 1965; Holson et al., 1991; Morinan & Leonard, 1980; Weiss et al., 2004), or female (Holson et al., 1991; Weiss et al., 2004) rats.

Potentially, the lack of a change in plasma CORT may reflect a protective developmental adaptation to the experience of social isolation during the period of the lifespan covering the post-weaning to early-adulthood stages. Interestingly, we located earlier reports that observed CELSI-related *decreases* of at least 50% in blood CORT levels in either male (Pisu et al., 2016; Sanchez et al., 1998), or female (Pisu et al., 2016) rats. Taken together, the data that we and others have collected would suggest that CELSI may not affect CORT levels in either male, or female rats, but that future work should not necessarily assume this to be the case.

3.5.4 General Conclusions

Using a statistical method more suitable for analysing the sort of censored data typically seen during the acquisition phase of the standard Morris water maze, our results demonstrate that post-weaning social isolation can improve the spatial learning and memory ability of both male and female rats. Given the lack of a notable difference in either stress-sensitive biometrics, or a consistent change in hippocampal PSD-95 protein expression across male and female rats, a clear reason for the apparently beneficial effect of CELSI remains unclear. However, in an effort to uncover the responsible mechanism, future work should consider how CELSI may have affected plasticity-related proteins within the hippocampus and whether isolated animals display a stress response to water maze testing that is different from their group-housed counterparts.

Chapter 4

DOES BYSTANDER STRESS AFFECT SPATIAL LEARNING AND MEMORY AS WELL AS HIPPOCAMPAL PLASTICITY-RELATED PROTEINS IN ADULT MALE AND FEMALE RATS?

4.1.1 ABSTRACT

Objective

The purpose of this study was to determine whether indirect psychosocial stress induced by the Bystander Stress (ByS) model could affect brain areas important for spatial learning and memory in adult rats.

Methods

Rat siblings from each of the 16 litters were randomly assigned to same-sex pairs (4 pairs from each litter; two pairs of male and two pairs of female rats). Twice daily for 5 consecutive days, one rat from each stress set was placed on an elevated platform for 30 min (Platform Stress, PS). The cage-mates of PS animals were considered the ByS rats (i.e., those receiving indirect stress). Each set also had Platform Control (PC) animals that were simply moved to another room twice daily for 30 min. The cage-mates of each PC animal were considered the Bystander Control animals (ByC). In cohort 1, spatial learning and memory were assessed in 10 litters over 5 days using the Morris water maze. In cohort 2, the septal and temporal poles of the hippocampus were harvested from 6 litters one hour after the last stress exposure; subsequently, synaptic proteins were enriched from the dissected samples using the synaptoneurosome preparation technique. Following this, Western blotting was used to examine differences in synaptic proteins of interest.

Results

Our findings suggest that ByS did not significantly affect spatial learning and memory in either male, or female rats. Biochemically, ByS was characterized by a decrease in glutamate receptor subunit expression in male rats compared to the ByC group, especially in the septal pole of the HP. In particular, ByS significantly reduced the expression of GluA2 (61%), GluN2A (20%), and GluN2B (24%). However, in female rats, GluA2 significantly increased (58%) in the temporal pole of ByS rats compared to ByC rats. As well, there was a significant difference in GluA2 levels between the hippocampal septal and temporal poles of female ByS rats (GluA2 levels were 76% greater in the temporal pole). In addition, PSD-95 levels in the temporal pole were 99% higher in male ByS rats compared to female ByS rats.

In summary, bystander stress appeared to affect the synaptic expression of certain plasticity-related proteins in both a sex and region-dependent manner. While no statistical significance was observed between male and female rats for any particular measure, except for PSD-95 in the temporal pole, male rats displayed more overall changes compared to female rats, which suggests that the effects of ByS on plasticity-related protein expression in the hippocampus were influenced by the sex of an animal.

4.2 INTRODUCTION

Stress models in rats involve physical, psychological stress (Liu et al., 2018), or both at the same time. For physical stressors, animals are directly exposed to some form of stress, such as restraint stress, or forced swimming. On the other hand, psychosocial stressors can be either direct (such as the maternal deprivation and social defeat stress models), or indirect (such as bystander stress). As compared to non-social, or physiological paradigms, psychosocial stress paradigms have been increasingly recognized as being more relevant to the understanding of human conditions (Slattery & Cryan, 2014). That is, animals exposed to social stressors have exhibited behavioural, physiological, and cellular changes that are relevant to humans exposed to psychological distress (Pryce & Fuchs, 2017). For example, dendritic reorganization in response to chronic social stress from a dominant animal has been well documented in certain animal models (McKittrick et al., 2000); as well, it has been demonstrated that reduced dendritic branching and spine densities in CA3 were associated with higher levels of anxiety and longitudinal depression scores (Soetanto et al., 2010). These observations, as well as the fact that stress has been associated with a number of mental illnesses including MDD, generalized anxiety disorder, and PTSD, have resulted in numerous studies using animal models of psychosocial stress to investigate the effects of stress on humans (Fuchs et al., 2001).

Indirect stress paradigms work based on the premise that subjects experience stress when forced to observe other animals in distress. As a result, witness animals communicate their empathy with the distressed animals by changing their vocalizations and pain behaviours, such as writhing and licking (Langford et al., 2006; Mychasiuk, Gibb, et al., 2011). Building

on this earlier work, "bystander stress" is an indirect psychosocial stress model where an animal is exposed to stress caused by living in the same cage as another animal that has experienced direct (primary) stress (Mychasiuk, Gibb, et al., 2011; Mychasiuk, Schmold, et al., 2011).

Initially developed by Mychasiuk et al., the researchers utilized the bystander stress paradigm to assess the effects of the stressor on pre-natal brain development. From gestational days 12 to 16, pregnant rats were housed with another female rat that had been exposed to platform stress (a direct form of psychosocial stress wherein an animal is placed on an elevated platform twice per day over several days). It was found that the global DNA methylation, gene expression, and dendritic organization of offspring were significantly altered (Mychasiuk, Gibb, et al., 2011; Mychasiuk, Schmold, et al., 2011) suggesting that the offspring were stressed in different ways, resulting in variable effects on brain development. In two studies, Mychasiuk only examined the effects of pre-natal bystander stress; due to the lack of information regarding how bystander stress influences the adult brain, particularly, the function of brain regions associated with spatial learning, we investigated Morris water maze performance in male and female animals exposed either to platform (direct), or bystander (indirect) stress as adults.

Human and animal studies have identified structural and biochemical changes in the brain associated with psychosocial stress and stress-induced disorders such as anxiety and depression (Heim et al., 2004; Reinwald et al., 2018). Interestingly, one area that has been found to be affected by stress is the hippocampus, which has been the subject of considerable scientific interest in recent years, as it plays a significant role in cognition and emotion,

particularly in consolidating information and for learning and retaining spatial information. Experimental studies on different species of animals have shown that stress exposure leads to a decrease in apical dendrite length and branching, as well as a decrease in dendritic spine density in the hippocampal formation. In particular, the CA3 subfield of the hippocampus appears to be most affected, but changes have also been reported in the CA1 subfield and the dentate gyrus of the hippocampus (Magariños et al., 1996; Vyas et al., 2002). Moreover, stress can have a detrimental effect on neuronal proteins by altering their structure and function (McEwen & Sapolsky, 1995).

One group of proteins that may be affected by stress is the ionotropic glutamate receptors, which allow the passage of ions through the cell membrane and are classified into three groups depending on their structural similarity: AMPA, NMDA, and Kainate (KA) receptors. These receptors are responsible for the majority of excitatory neurotransmission in the brain, and they have been implicated as playing a role in the effects of stress on the hippocampus (Goodwani et al., 2017; Popoli et al., 2012).

AMPA receptors are responsible for fast synaptic transmission in the central nervous system. These receptors are composed of four subunits GluA1-4 that are derived from different genes and that combine to form tetramers. The modulation of the AMPA receptors is believed to underlie the plasticity of excitatory synaptic transmission in the brain, as AMPA receptors play a major role in fast excitatory synaptic transmission (Benke et al., 1998; Song & Huganir, 2002). Most synaptic AMPARs in the hippocampus contain the GluA1 subunit, the only subunit that has the capability of forming calcium permeable homotetramers (Whitehead et al., 2017). Furthermore, GluA2 is another important subunit associated with channel resistance to

calcium ions. Consequently, AMPA receptors without GluA2 pass sodium and calcium ions, resulting in increased conductance (Shepherd, 2012).

NMDA receptors are heteromeric complexes that interact with a variety of intracellular proteins through three subunits (GluN1, GluN2, and GluN3) and become activated when glutamate and glycine are bound to them (Furukawa et al., 2005). NMDA receptors are heterotetramers composed of two GluN1 subunits, which are obligatory subunits, and two GluN2A-D subunits, which are modulatory subunits (Salussolia et al., 2011). To activate the NMDA receptor, glutamate must bind to the GluN2 subunits, glycine must bind to the GluN1 subunits, and membrane depolarization is required to remove the Mg^{2+} blocking the channel pore (Hood et al., 1989; Panatier et al., 2006). The GluN2B subunit is believed to be responsible for the receptor's basic structure and function, as it forms the glutamate binding site and controls the Mg^{2+} block. Furthermore, the GluN2B subunit has been found to modulate a variety of cognitive functions, including learning and memory (Kristiansen et al., 2007). As humans and animals reach sexual maturity, GluN2B levels decrease relative to GluN2A levels, causing the juvenile brain to be more plastic than the adult brain due to the higher levels of GluN2B (Priestley et al., 1995).

The postsynaptic density protein (PSD-95) plays an important role in anchoring synaptic proteins and is almost exclusively found in the postsynaptic density of excitatory neurons. PSD-95 forms a multimeric scaffold at postsynaptic sites that promotes the clustering of receptors, ion channels, and signaling proteins (Hunt et al., 1996). The GluN2A and GluN2B subunits of the NMDAR are connected to PSD-95 via a postsynaptic protein called Neto1 and are involved in modulating synaptic plasticity (Li & Tsien, 2009; Ng et al., 2009).

AMPA and NMDA receptors are essential upstream players regulating synaptic plasticity in various parts of the brain, especially the hippocampus, and thereby help to mediate learning and memory functions (Li & Tsien, 2009; Shepherd, 2012). These receptors are colocalized in the postsynaptic density of most glutamatergic synapses, where they help to influence postsynaptic excitatory currents (Pinheiro & Mulle, 2006). While kainate receptors are distributed throughout the brain, they are not as widespread in excitatory postsynaptic signaling complexes as the AMPA and NMDA receptors. Based on these differences, our study focused on key subunits of the AMPA and NMDA receptors.

As mentioned before, exposure to stressors can alter the structure and function of neuronal proteins, resulting in reductions in the complexity and density of dendritic spines (Ma et al., 2021; McEwen & Sapolsky, 1995). Notably, this kind of change has been observed with only a single social stress experience. For example, Krugers et al., (1993) observed that 24 hours after a single hour of social defeat stress in rats, the glutamate neurotransmitter's impact on hippocampal CA3 neurons was altered due to a significant increase in the NMDA/AMPA binding ratio in CA3 stratum oriens and CA3 stratum radiatum areas. As well, the expression of key plasticity-related proteins, such as glutamate receptor subunits, at synaptic terminals determines several changes downstream in the process of signal transduction (Yuen et al., 2009). Stress has been demonstrated to reduce the expression of the GluA1 subunit of AMPA receptors, as well as PSD-95, in the CA1 region of the hippocampus and impairs AMPAR mediated synaptic excitation (Huganir & Nicoll, 2013; Kallarackal et al., 2013; Ma et al., 2021). Contrary to this, high levels of corticosterone have been reported to increase synaptic insertion of AMPA receptors containing GluA2 in hippocampal primary cultures and

hippocampal slices, enhancing AMPA receptor mediated synaptic transmission (Krugers et al., 2010). Moreover, chronic stress has been shown to increase glutamate activity via post-synaptic mechanisms, through the regulation of NMDA receptors. For example, stress leads to an increase in the levels of the obligatory GluN1 subunit, as well as the GluN2A and GluN2B subunits, especially in the ventral hippocampus (Calabrese et al., 2012; Elhussiny et al., 2021).

To our knowledge, no literature has examined the effects that an indirect psychosocial model of stress might have on the synaptic expression of excitatory receptor subunits. For the noted reasons, as a complement to our behavioural analysis, we investigated the relative expression of AMPA and NMDA receptor subunits (GluA1, GluA2, GluN1, GluN2A, and GluN2B,) as well as PSD-95, in septal and temporal poles of the hippocampus in ByS and ByC groups. In this regard, the objectives of the study were as follows:

Objective 1: To investigate the influence of ByS on hippocampal-dependent behaviour by testing spatial learning and memory using the Morris water maze.

Objective 2: To determine whether bystander stress affects the expression of a series of plasticity-related proteins (GluN1, GluN2A, GluN2B, GluA1, GluA2, and PSD-95) in septal and temporal poles of the hippocampus.

Objective 3: To explore possible sex-specific effects of ByS on plasticity-related protein expression and behaviour by using both male and female animals.

4.3 MATERIALS AND METHODS

4.3.1 Animals and Stress Procedures

All animals were maintained on a 12:12-hour reverse light:dark cycle in a temperature-controlled room (22°C) and given access to food and water *ad libitum*. For the present study, 128 Sprague-Dawley rats from sixteen litters were used. Each litter of rats was stratified by sex (4 male and 4 female rats per litter); subgroups were then formed by randomly dividing the rats into stress and control groups. For the stress groups, rats housed in pairs were randomly assigned to either the platform stress (PS; N = 10), or bystander stress (ByS; N = 10) condition. In each control group, animals were randomly assigned either to the platform control (PC; N = 10), or to the bystander control (ByC; N = 10) condition.

The ByS model was used to induce indirect social stress in adult animals. For five days, PS rats were placed on an elevated Plexiform platform (92.5 cm tall, 18.5 x 18.5 cm) and exposed to bright light for 30 minutes twice daily (9:00 to 9:30 and 15:00 to 15:30); the cage-mate of each PS rat was considered a ByS rat. The platform stress procedure has been shown to alter rat vocalizations and behaviour, and to evoke a strong stress reaction in the affected animal (Mychasiuk, Gibb, et al., 2011; Mychasiuk, Schmold, et al., 2011); therefore, to avoid any unintended effects of vocalizations from the stressed rats, the stress and control groups were kept in separate rooms. Furthermore, to control for the influence of removing an animal from a cage, one rat from each "control group" was moved to another room twice daily for 30 minutes at the same times and then returned to their home cage (Platform Control, PC). The cage-mate of each PC rat was considered a ByC rat. Each procedure ended with the PS and PC rats being returned to their respective cages with the ByS and ByC rats (figure 4.1). Through

social interaction with the PS rats, the ByS rats represent our secondary psychosocial stress model and are assumed to have experienced stress indirectly.

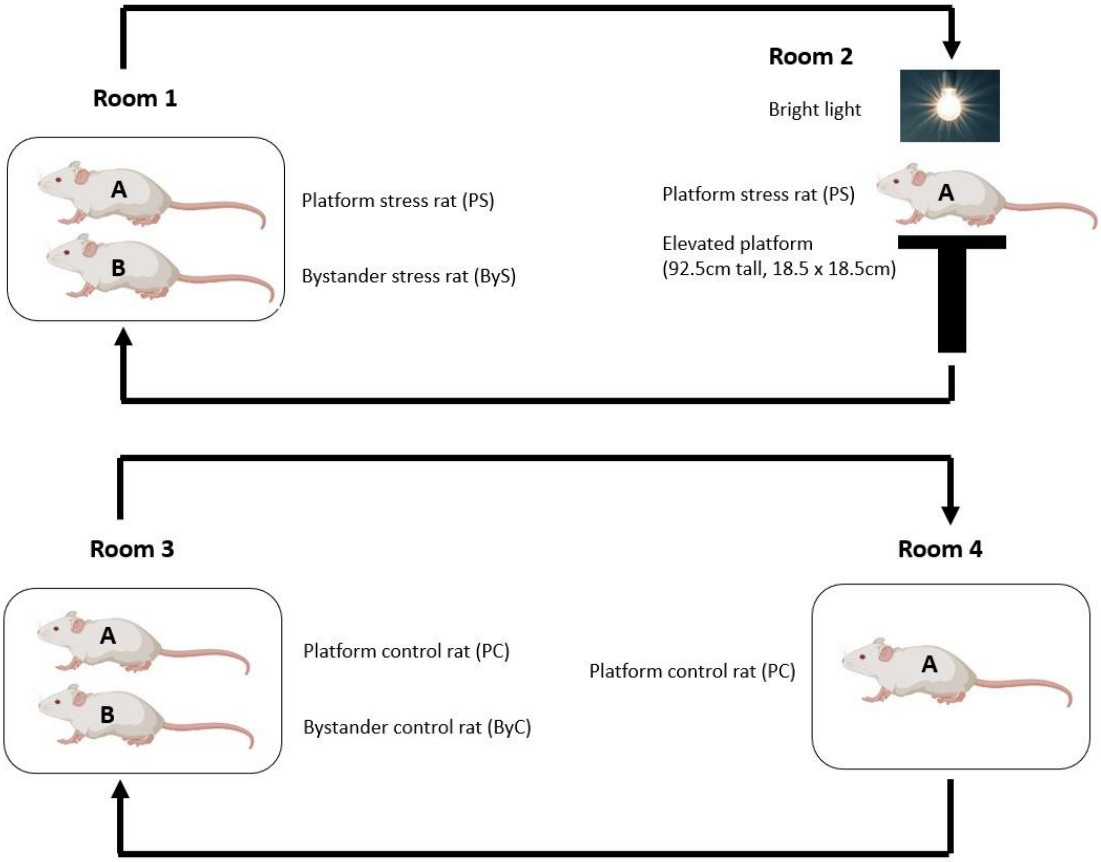


Figure 4.1. Schematic illustration of the Platform stress and Bystander stress protocols. A PC rat was taken out of its home cage and put in a separate room each time a PS rat underwent the stress protocol. On completion of PS, the PS rat was returned to its home cage (which housed the ByS rat) and the PC rat was placed back in its home cage (which housed the ByC rat).

4.3.2 Morris Water Maze (MWM)

Beginning the day after the final application of platform stress, spatial learning and memory performance were assessed using the Morris water maze (MWM) (figure 4.2). Animals were placed in the testing room thirty minutes prior to behavioural trials, which occurred between 13:00 and 15:00 under low light conditions using a circular black plastic tank (175 cm in diameter, 70 cm in depth) containing no proximal cues and filled with water (50 cm in depth, 22°C ± 1°C). The pool was divided into four equal quadrants: northeast (NE), southeast (SE), southwest (SW), and northwest (NW). A circular platform (17.5 cm in diameter) was submerged 1.5-2 cm below the water surface and located in one quadrant in a fixed position throughout the experiment. Captured videos were analysed for behavioural variables (escape latency and distance travelled to locate the platform) with video tracking software Noldus Ethovision XT v8.5 video tracking system (Noldus Information Technology, Leesburg, VA, USA).

Over four consecutive days, the animals underwent four learning trials each day. Each trial was started by releasing an animal into the water maze, facing the perimeter, at one of the four starting points (north, south, east, or west), which were selected in a pseudorandom order. Each rat was allowed up to 60 s to find the hidden platform and was then allowed to stay on the platform for 30 s. Those animals that failed to complete the task within 60 s were guided to the platform and were allowed to stay on the platform for 30 s. Following the completion of a trial, animals were dried and returned to their home cages and an inter-trial interval of approximately 10 minutes permitted.

On the day following the end of the learning phase, the memory phase (probe test) was done: the platform was removed, and each animal was placed in the drop location farthest from where the platform had been and allowed to swim for 30 s. The time spent in the quadrant where the platform was formerly present was then measured.

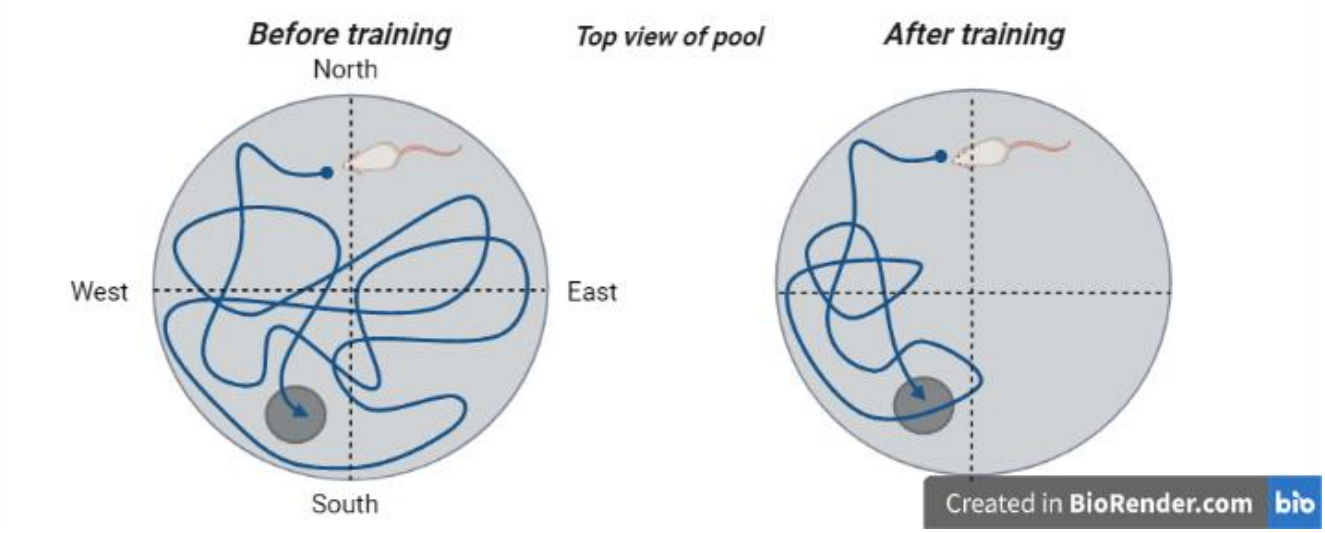


Figure 4.2. The Morris water maze is a behavioural test designed to evaluate spatial learning and memory. A pool filled with water is used as the testing area. A hidden platform is submerged just below the surface of the water. Using visual cues, the animal can locate the platform to escape the water. Over time, the ability of the animal to locate the escape platform is expected to improve and the measurement of this improvement (as shown by either escape latency, or distance travelled) is taken as an index of learning and memory.

4.3.3 Extraction of the Hippocampus

The brain isolation procedure was performed one hour following the last stress procedure on the fifth day. The animals were anaesthetized with >60% CO_2 and then immediately decapitated. Brain tissue was rapidly removed and immediately placed in chilled (4^oC) oxygen-rich (95% O_2: 5% CO_2) ACSF composed of: 124.0 mM NaCl, 3.0 mM KCl, 1.2 mM NaH_2PO_4/H_2O, 1.0 mM $MgSO_4/7H_2O$, 2.0 mM $CaCl_2/2H_2O$, 26 mM $NaHCO_3$, 10.0 mM D-Glucose, 10.0 mM HEPES buffer with a pH of 7.37-7.43 and osmolarity of 300-320 mOsm. Dissection of the two hippocampi was then performed, and three-millimetre segments of both hippocampal septal and temporal poles were removed, immediately snap-frozen (using liquid N_2), and stored at $-80^{\circ}C$.

4.3.4 Preparation of Synaptoneurosomes

The synaptoneurosome preparation (SNP) technique was used to enrich synaptic terminals containing the proteins of interest. Each piece of frozen HP tissue was placed directly into a 2 mL Potter-Elvehjem homogeniser, and manually homogenized over ice using 30 strokes with a total of 750 μL of chilled, 1X modified Krebs buffer (composed of 11.85 mM NaCl, 0.47 mM KCl, 0.118 mM $MgCl_2 \cdot 6H_2O$, 0.25 mM $CaCl_2 \cdot 2H_2O$, 0.118 mM KH_2PO_4) with 24.90 mM $NaHCO_3$ and 10.00 mM glucose added (pH adjusted to 7.40 with HCl) and supplemented with the P8340 protease inhibitor cocktail (AEBSF, Aprotinin, Bestatin hydrochloride, E-64, Leupeptin hemisulfate salt, Pepstatin A) and sodium orthovanadate (Na_3VO_4) (stored at -20°C). To allow gravity sedimentation of nuclear material after homogenization, samples were decanted into an Eppendorf tube and placed on ice for around

10 minutes. For quality control purposes, a small portion (100 µL) of the supernatant was aliquoted as whole homogenate (WH).

The sample was filtered through three pre-wet (using 1X Krebs buffer) Millipore nylon net filters (100 µm pore size) held in a 25-mm stainless-steel Millipore syringe filter holder. The collected filtrate was filtered again through one pre-wet Millipore nitrocellulose filter (5 µm pore size) held in a 13-mm stainless steel Millipore syringe filter holder. The final filtrate was spun at 1000 x g for 15 minutes at 4°C. The supernatant, representing the cytosolic fraction, was removed, and the pellet, representing the synaptoneurosome fraction, was suspended in 200 µL of 1X modified Krebs buffer with protease inhibitor and sodium orthovanadate added. The protein concentration of each re-suspended synaptoneurosome fraction was determined with a BioRad DC protein assay kit according to the manufacturer's instructions. The rest of each sample was aliquoted in 50 µL fractions and stored at -80°C.

4.3.5 Quality Control Blotting

To ensure the effectiveness of the synaptoneurosome preparation in enriching synaptic terminals, quality control immunoblotting was performed using PSD-95 and GFAP antibodies. At the post-synaptic density, PSD-95 protein plays a key role in synaptic plasticity, synapse formation, and the reconstruction of synaptic connections. As such, it is present in greater quantities in SNPs than in whole homogenates. Glial Fibrillary Acid Protein (GFAP), however, is an intermediate filament protein located in the cytoskeleton of astrocytes and should not be present at synaptic terminals; therefore, WHs should exhibit greater levels of this protein. By providing a positive and negative control, respectively, PSD-95 and GFAP helped demonstrate

the effectiveness of the SNP protocol at enriching the synaptic region. After normalization to Ponceau, SNPs showed a higher optical density of PSD-95 than WH, and a much lower optical density of GFAP than WH, indicating an enrichment of synaptic terminals. Only after quality control tests to confirm an enrichment of synaptic terminals (figure 4.3), were SNPs used for immunoblotting.

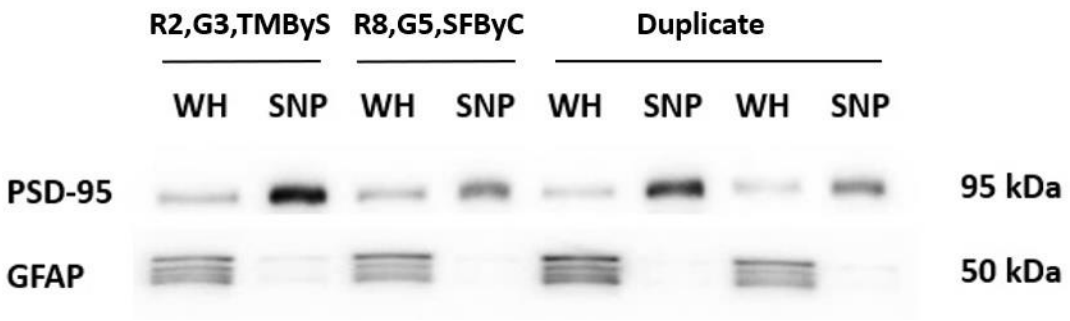


Figure 4.3. Quality control assessment of synaptoneurosome samples. In order to ensure the effectiveness of the SNP protocol, a quality control step was conducted on randomly selected samples, rat 2 from group 3 (temporal pole of male ByS rat; R2,G3,TMByS) and rat 8 from group 5 (septal pole of female ByC rat; R8,G5,SFByC). The representative blots demonstrate the expression of PSD-95 (95 kDa) and GFAP (50 kDa) in both whole homogenates (WH) and synaptoneurosome preparations (SNP). Relative differences in the expression of GFAP and PSD-95 between the SNP and WH were taken to confirm synaptic enrichment.

4.3.6 Standard Curve Optimization Blotting

The optimal protein loading concentration for each sample was determined by performing an optimization blot using PSD-95 and GFAP antibodies. A 10% SDS gel was loaded sequentially with 5 µg, 10 µg, 15 µg, and 20 µg of sepal and temporal hippocampus SNPs from one of our samples. In order to obtain a stable, clear, and unsaturated signal, the optimal loading concentration was determined to be 10 µg.

4.3.7 SDS-PAGE and Western Blotting

The effects of ByS on expression of PSD-95 and the synaptic expression of GluN1, GluN2A, GluN2B, GluA1, and GluA2 (important subunits of NMDA and AMPA receptors) in the septal and temporal hippocampus in male and female stressed and control adult rats was measured using Western blotting. Samples were thawed on ice and then denatured in sample buffer (0.0625 M Tris, 2% [v/v] glycerol, 5% [w/v] sodium dodecyl sulfate [SDS], 5% [v/v] β-mercaptoethanol, and 0.001% [w/v] bromophenol blue, pH 6.8) at 95ºC for 5 min, except for those probed for GluN1, which required that samples were loaded without being heated (they were simply left at room temperature for 5 minutes).

Considering that the stress and control groups included both males and females, eight gels were run for each group: four gels for male rats and four gels for female rats. Protein samples (e.g., male ByS-septal HP; male ByS-temporal HP; male ByC-septal HP; male ByC-temporal HP) and their duplicates were loaded into the 10% SDS-polyacrylamide gels and electrophoresed at 200 V for around 1 hour. Upon completion, the gels were removed from the apparatus and soaked for 15 minutes in chilled transfer buffer. The proteins were then transferred onto pre-treated PVDF membranes using a sandwich assembly in chilled transfer

buffer [composed of 25 mM Tris, 192 mM glycine, 20% (v/v) methanol] at 4ºC, 90 V for 120 min. Blots were then incubated with Ponceau S solution for around 10 min, washed with deionized water, air dried, and then imaged in order to verify that the protein samples transferred correctly.

Membranes were blocked using either 5% (w/v) bovine serum albumin (BSA), or 5% (w/v) non- fat milk in Tris-buffered saline with Tween-20 (TBST) for 1 h at room temperature, and then incubated with relevant antibodies overnight at 4ºC. All primary antibodies were diluted to an optimized concentration in blocking buffer specific to the application. On the following day, membranes were incubated in either rabbit, or mouse IgG secondary antibody (Santa-Cruz 1:5000, or 1:2000) for 1 h. After decanting the secondary antibody solution, TBS-T was used to wash the membranes for 3 to 5 minutes. Following this, the blots were covered for 5 minutes with either an enhanced chemiluminescence (ECL) solution, Immobilon Classico, or an ECL plus solution, Immobilon Crescendo Western HRP Substrate (Millipore). A gel documentation system, SNAPGENE, was then used to obtain densities of the protein bands by exposing the blots at intervals ranging from 30 seconds to 5 minutes.

Afterwards, membranes were stripped using 1X Re-Blot Plus stripping solution for 25 minutes at room temperature and then TBS-T was used to wash membranes (three times for five minutes). Once the blocking buffer had been reapplied to the membranes for 20 minutes, the membranes were incubated overnight at 4°C with the next antibody. Following application of the secondary antibody, the signals were detected using the SNAPGENE software.

Depending on the type of blot being performed, membranes were incubated with two of the following antibodies: anti-total GluA1 rabbit monoclonal (*Abcam Cat #183797, 1:500 5% NFDM/TBST*) and anti-total GluN2A rabbit monoclonal (*Cell Signaling Cat #4205S, 1:1000*); anti-total GluA2 rabbit monoclonal (*Cell Signaling Cat #13607S, 1:1000*) and anti-total GluN2B rabbit monoclonal (*Cell Signaling Cat #4207S, 1:1000*). Since samples needed to be treated differently to obtain GluN1 signals (that is, without heating the samples prior to gel loading), one membrane from each group was incubated with anti-total GluN1 rabbit monoclonal (*Millipore Cat #05432, 1:500*). The last membrane of each group was used for PSD-95 using anti-PSD-95 (mouse; *Millipore, cat. MABN68*), since the secondary antibody was different from the other antibodies (mouse anti-mouse). Primary antibodies were prepared in either 5% NFDM/TBST, or 5% BSA/TBST (as appropriate) at 4°C overnight. Mouse anti-rabbit IgG-HRP, or Mouse anti-mouse IgG-HRP conjugated secondary antibody (*Santa-Cruz 1:5000 or 1:2000*) was applied the next day, and the signals detected using the SNAPGENE software. Finally, the NIH ImageJ software was used to conduct a densitometric analysis of all saved images.

4.3.8 Western Blot Image Analysis

The NIH Image J software was used to extract all data, which was then compiled in Microsoft Excel spreadsheets, and the data were analysed in GraphPad Prism version 9. To determine if bystander stress affected the expression of our proteins of interest, we measured the optical density of bands in each blot and their respective Ponceau stains (to control for loading variation). For each blot, each duplicate band was normalised to its corresponding Ponceau staining, and then the duplicate values were averaged and expressed as a percentage

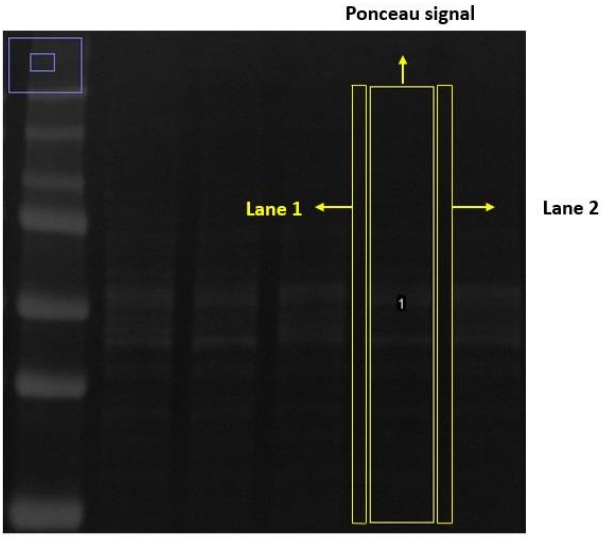
of the value measured for the corresponding control group; for example, the levels of the bystander stress protein were expressed as a percentage of the bystander control (figure 4.4). Then, to analyse the Western blot data, a two-way ANOVA was conducted where sex and brain region (septal and temporal HP) were considered as factors that might influence the effect of bystander stress on the expression of AMPA and NMDA subunits (GluN1, GluN2A, GluN2B, GluA1, and GluA2), as well as PSD-95.

Analyzing WB images (ponceau image) using "Image J" software:

- ✓ Make sure images are in TIF format (click on "File" on the toolbar, then "open image" and then choose your image file)
- ✓ Adjust the type of images for 8-bit (Image _Type _8-bit)
- ✓ Click on "Edit", click on "invert", the background should be black and the bands should be white
- ✓ Fit the rectangular shape around the lane (ponceau signal/lane1/lane2)
- ✓ Analyze_ Set measurements_ unchecked everything except for integrated density
- ✓ Analyze_ Measure (gives us the OD number)
- ✓ Then in Excel do true signal calculation based on the below formula:

OD = protein/ponceau signal + background signal (BG)

True signal = OD – BG signal (lane 1 + lane 2)



Analyzing WB images (protein signals), using "Image J" software:

Follow the protocol in the previous slide regarding analyzing the ponceau image.

OD = protein/ponceau signal + background signal (BG)

True signal = OD – BG signal

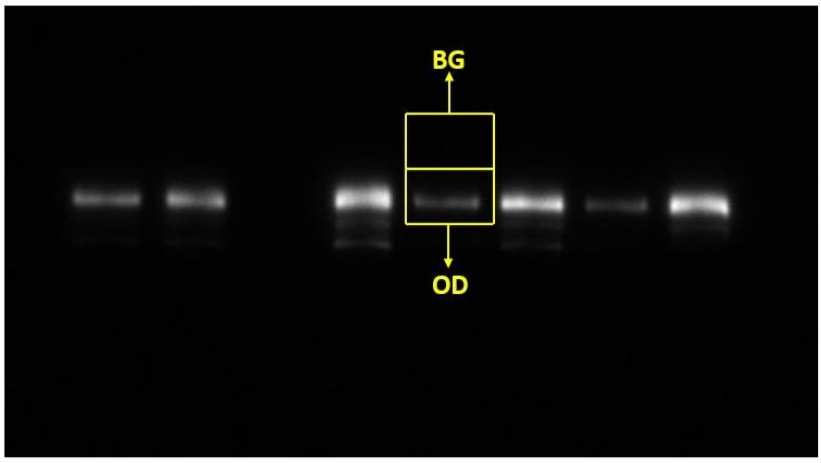


Figure 4.4. Procedure used to analyse Western blot images with Image J software.

4.3.9 Statistical Methods

To investigate the effects of bystander stress and sex on spatial learning, we used time-to-event statistical framework (survival analysis) where the Cox proportional hazard model was employed. Here, hazard rate refers to the rate at which the animals in a particular group locate the platform over time. Additionally, ANOVA models were used to examine the impact of stress and sex on probe test performance, with the litter as a blocking variable. Using residual diagnostic plots and assessments of normality and homogeneity of variance, both exploratory and inferential residuals analysis were performed to confirm the appropriateness of the chosen ANOVA models. If a statistically significant p-value (< .05) was observed at the omnibus level, Tukey's HSD was used as the post-hoc test to examine differences between the groups (see section 2.6 of chapter 3 for a more extensive discussion of this part).

To assess the influence of stress and sex upon neural protein expression, we used a one-sample t-test to assess statistical significance ($p < .05$) with 100% as our theoretical comparator. Given that we constructed a ratio of each BySham value to its sex-matched, ByControl counterpart, we expected that the average of these ratios would be very close to 100% if BySham had no effect on protein expression. Additionally, we screened the immunoblotting data for outliers (see section 3.3.6 for more information).. Based on our diagnostic plots and tests (using the approaches outlined above), we felt that our data satisfied the primary assumptions required for the use of a parametric test.

To assess the practical significance of relevant group differences across our dependent measures (with the exception of spatial learning ability), we used Cohen's d, which standardises the difference between group means according to their pooled standard deviation.

When interpreting Cohen's d, we applied the standard conventions for a small (d = 0.2), moderate (d = 0.5), and large (d = 0.8) effect size.

4.4 RESULTS

The learning trial data were analysed using survival analysis to overcome the inappropriateness of ANOVA-based models when censored data are present. As discussed in chapter 3 (CELSI study), we employed techniques from survival analysis. In particular, the Cox proportional hazard (CPH) model was used to analyse the learning phase data. Moreover, the average swim speed of each animal (ByS – male/female, N = 10/sex; ByC – male/female, N = 10/sex; PS – male/female, N = 10/sex; PC – male/female N = 10/sex) during the probe test was measured to determine whether any observed differences in task acquisition might be attributable to variability in either motivation to complete the task and/or sensorimotor abilities (e.g., swimming ability). We did not find any evidence that swim speed differs according to the group assignment [$F(3, 72) = 1.07$, $p = .36$)] (figure 4.5).

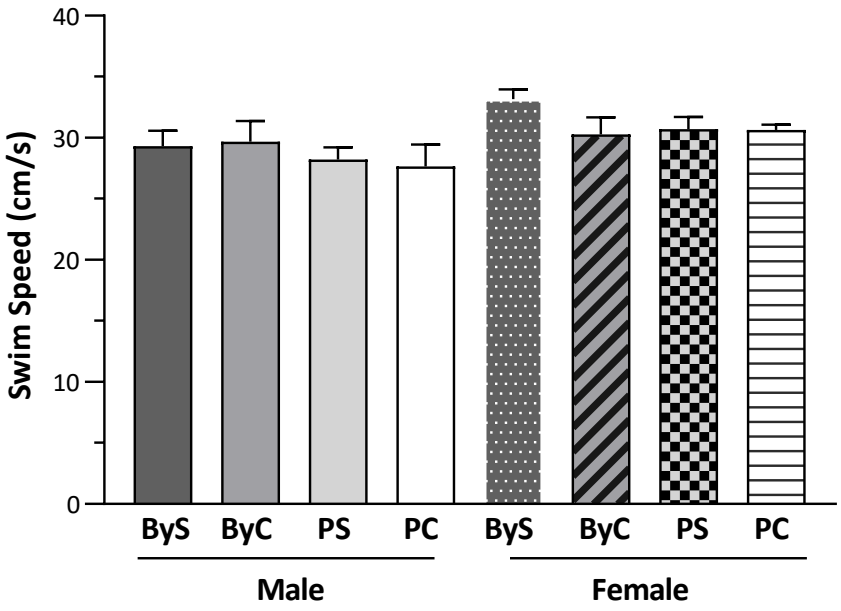


Figure 4.5. Swim speed. The graph presents the average swim speed as a general water maze performance characteristic recorded for each group during the probe test. The data are displayed as mean ± SEM. ByC, bystander control; ByS, bystander stress; PC, platform control; PS, platform stress; SEM, standard error of the mean

4.4.1 The Effect of Bystander Stress on Spatial Learning and Memory in Adult Rats

On day 1, the hazard ratio for the ByS and ByC male rats was 1.3, with a 95% confidence interval of (0.67, 2.4), which means that both groups had the same probability of finding the platform at any point in time. The hazard ratio for the ByS and ByC female rats on day 1 was 0.9, with a 95% confidence interval of (0.49, 1.7), which indicates that they too had the same probability of finding the platform at any point in time (table 1, figure 4.6a).

On day 4, the hazard ratio for ByS and ByC male rats was 0.98, with a 95% confidence interval of (0.63, 1.5), suggesting that both groups were equally likely to locate the platform at any given time. The hazard ratio for the ByS and ByC female rats was 1.4, with a 95% confidence interval of (0.87, 2.2), indicating the same probability of finding the platform at any point in time. The results of our study suggest that ByS did not affect the spatial learning and memory ability of male and female rats over the 4 consecutive days of learning trials (table 4.1, figure 4.6a).

In terms of the comparison between PS and PC on day 1, the hazard ratio for the PS and PC male rats was 0.92, with a 95% confidence interval of (0.53, 1.6), indicating that both groups were equally likely to find the platform at any given time. The hazard ratio for the PS and PC female rats on day 1 was 1.4, with a 95% confidence interval of (0.81, 2.6), suggesting that they also had the same likelihood of finding the platform (table 4.1, figure 4.6b).

On day 4, the hazard ratio for PS and PC male rats was 0.94, with a 95% confidence interval of (0.59, 1.5), suggesting that both groups had an equal chance of finding the platform at any given time. The hazard ratio for the PS and PC female rats was 1.3, with a 95% confidence interval of (0.83, 2.1), indicating that the platform would be found at the same

probability regardless of time. Overall, PS had no significant influence on the spatial learning and memory of male and female rats over a period of 4 consecutive days of learning trials (table 4.1, figure 4.6b).

With regards to the effect of PS on learning performance among male and female rats, weak evidence for a difference in spatial learning performance was observed ($p = .086$) on day 4. The hazard ratio for the male and female PS rats was 0.67, with a 95% confidence interval of (0.42, 1.1), which confirms that animals in the PS male group were 0.67 time as likely to find the platform at any time relative to PS females (table 4.1, figure 4.6d).

	Hazard ratios (day 1)	95% CI of mean	Hazard ratios (day 4)	95% CI of mean
Male: ByS vs. ByC*	1.3	(0.67 , 2.4)	0.98	(0.63 , 1.5)
Male: PS vs. PC*	0.92	(0.53 , 1.6)	0.94	(0.59 , 1.5)
Female: ByS vs. ByC*	0.92	(0.49 , 1.7)	1.4	(0.87 , 2.2)
Female: PS vs. PC*	1.4	(0.81 , 2.6)	1.3	(0.83 , 2.1)
ByS: Male vs. Female*	1.2	(0.66 , 2.3)	0.9	(0.57 , 1.4)
ByC: Male vs. Female*	0.89	(0.47 , 1.7)	1.3	(0.83 , 2.0)
PS: Male vs. Female*	0.83	(0.47 , 1.5)	0.67	(0.42 , 1.1)
PC: Male vs. Female*	1.3	(0.74 , 2.3)	1.0	(0.64 , 1.6)

Table 4.1. The hazard ratios for key learning trials among ByS vs ByC and PS vs PC animals in the Morris water maze. The table shows the potential of each group to find the platform on days 1 and 4. There is no evidence to support a difference among the different groups (all hazard ratios are, statistically speaking, equal to 1). In each case, * shows the reference group. ByC, bystander control; ByS, bystander stress; PC, platform control; PS, platform stress

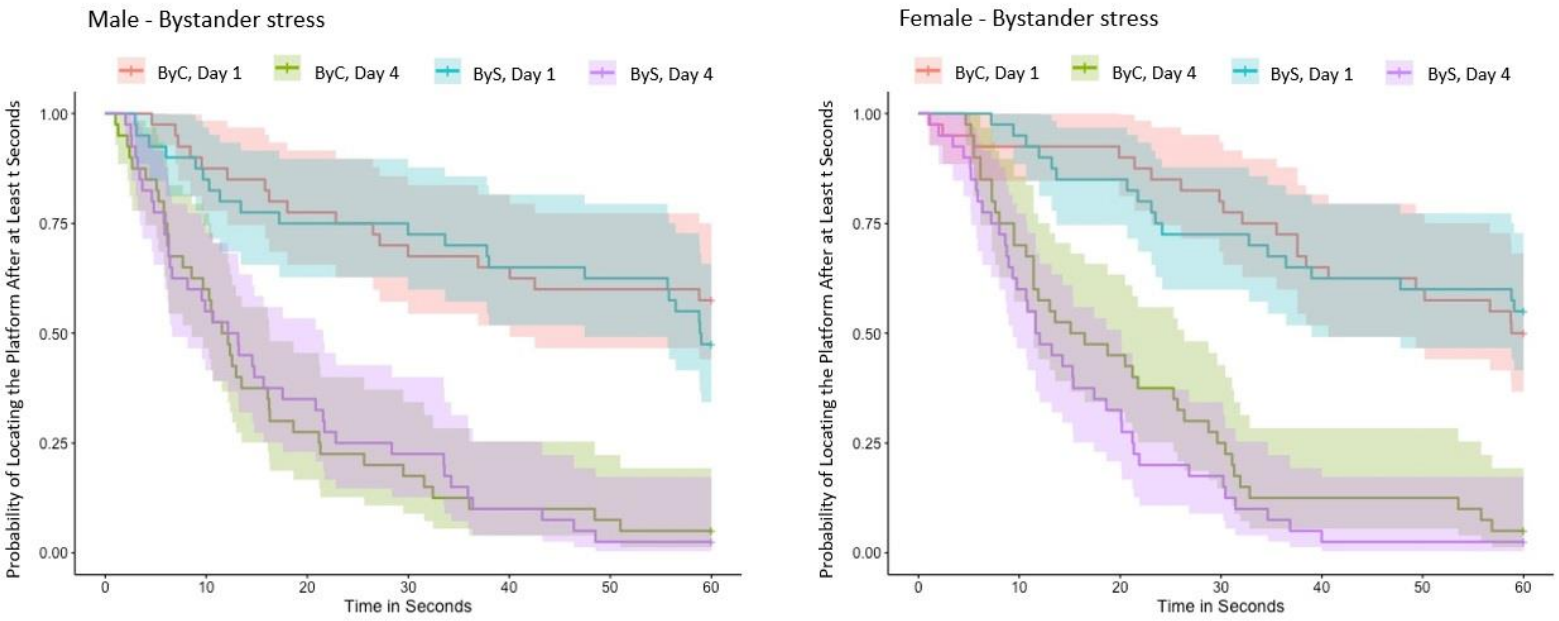


Figure 4.6a. Probability of locating the escape platform displayed by male and female bystander stress/control rats for trials on days 1 and 4 of the learning phase. The solid lines represent the Kaplan-Meier estimates of the survival functions, and the shaded areas show the corresponding 95% confidence interval for each survival function. A significant difference between the performance of ByS and ByC animals on day 1 and day 4 in male and female groups ($p < .0001$) indicates that each group improved over the course of the learning trials. ByC, bystander control; ByS, bystander stress

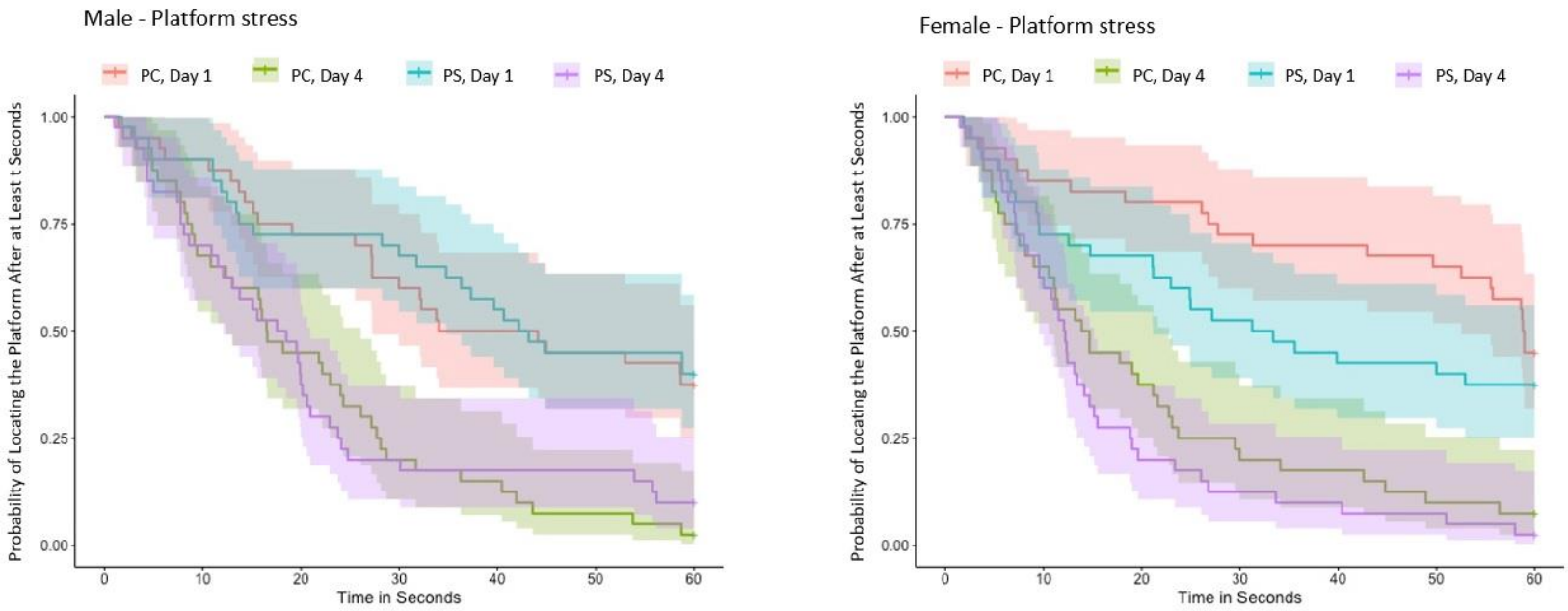


Figure 4.6b. Probability of locating the escape platform displayed by male and female platform stress/control rats for trials on days 1 and 4 of the learning phase. The solid lines represent the Kaplan-Meier estimates of the survival functions, and the shaded areas show the corresponding 95% confidence interval for each survival function. A significant difference between the performance of PS and PC animals on day 1 and day 4 in male and female groups ($p < .0001$) indicates that each group improved over the course of the learning trials. PC, platform control; PS, platform stress

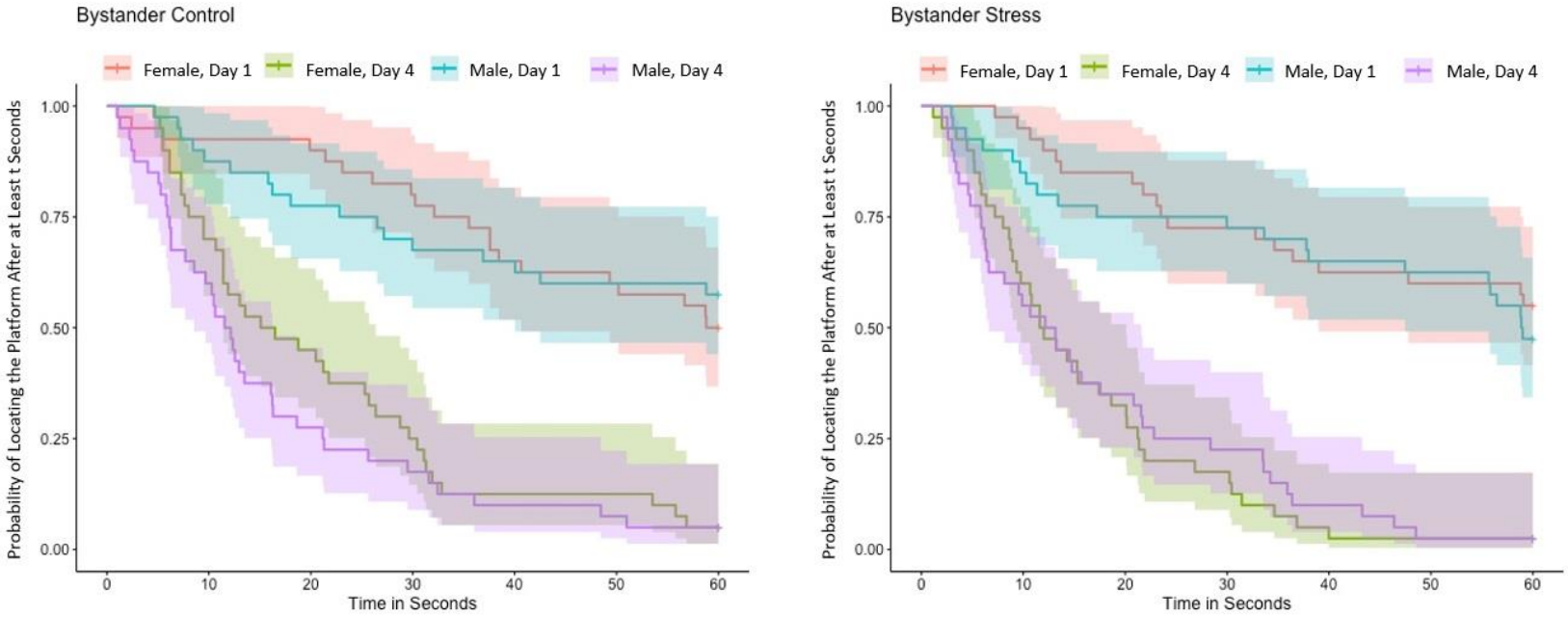


Figure 4.6c. Probability of locating the escape platform displayed by bystander stress/control rats for trials on days 1 and 4 of the learning phase. The solid lines represent the Kaplan-Meier estimates of the survival functions, and the shaded areas show the corresponding 95% confidence interval for each survival function. A significant difference between the performance of male and female animals on day 1 and day 4 in ByC and ByS groups ($p < .0001$) indicates that each group improved over the course of the learning trials. ByC, bystander control; ByS, bystander stress

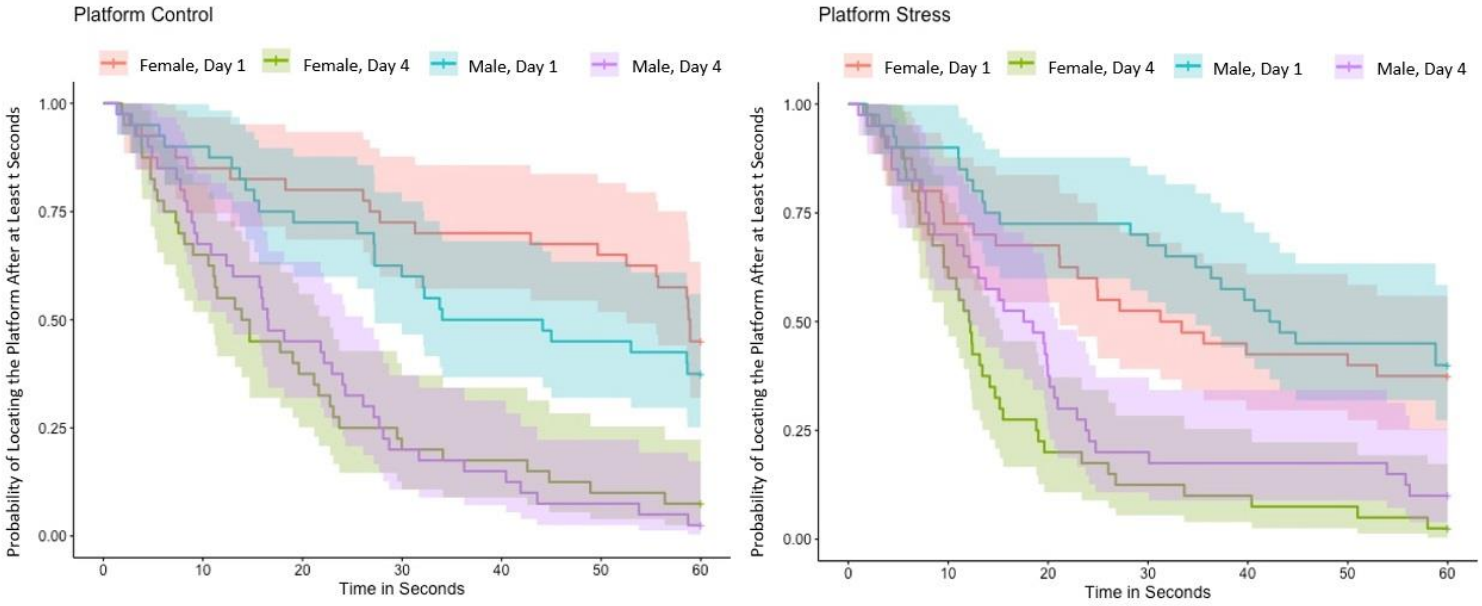


Figure 4.6d. Probability of locating the escape platform displayed by platform stress/control rats for trials on days 1 and 4 of the learning phase. The solid lines represent the Kaplan-Meier estimates of the survival functions, and the shaded areas show the corresponding 95% confidence interval for each survival function. A significant difference between the performance of male and female animals on day 1 and day 4 in PC and PS groups ($p < .0001$) indicates that each group improved over the course of the learning trials. Also, weak evidence on difference in spatial learning performance between male and female PS rats was observed ($p = .086$) on day 4. PC, platform control; PS, platform stress

4.4.2 Performance During the Probe Test

During the probe test, all animals were allowed to search the maze for 30 s on the day following the completion of the 4-day learning period. Given that its underlying assumptions were met (homogeneity of variance, normality, and uncorrelated residuals), we proceeded to use an ANOVA model to analyse the results. Since there was no interaction between our two main factors (sex and stress) [F (3, 72) = 1.7, p = .17], we examined the main effects of stress [F (3, 72) = 1.8, p = .14) and sex (F (1, 72) = .006, p = .9], and found that neither one had a statistically significant effect upon performance of animals during the probe test (figure 4.7).

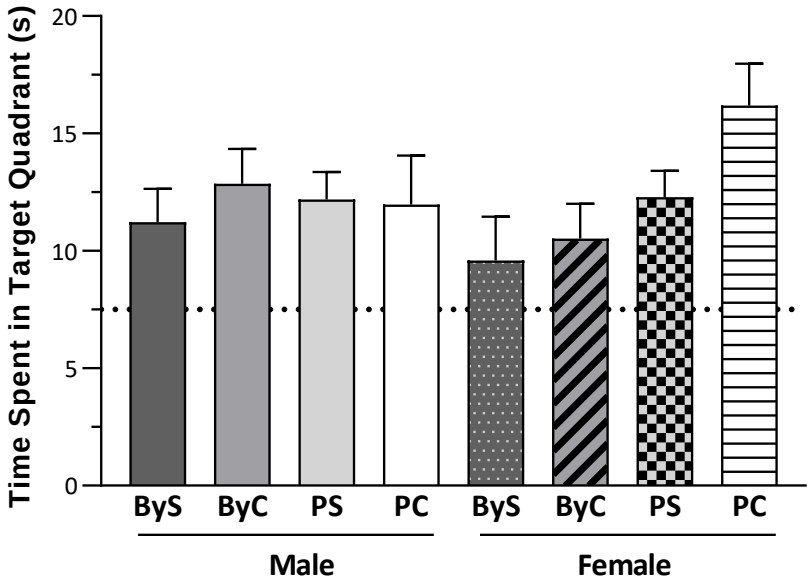


Figure 4.7. Time spent in target quadrant during the probe test. The amount of time spent in the target quadrant was not significantly different across groups in either male, or female rats. The data are displayed as mean ± SEM. ByC, bystander control; ByS, bystander stress; PC, platform control; PS, platform stress; SEM, standard error of the mean

4.4.3 Neural Protein Expression

Immunoblotting was conducted for the septal and temporal poles of ByS and ByC for both male and female rats. The data for six plasticity related proteins were analysed using standard parametric models (one-sample t-test and two-way ANOVA) given that the necessary underlying assumptions were met (homogeneity of variance, normality, and uncorrelated residuals). The data are presented as $\overline{X} \pm$ SEM. A one-sample t-test was used to compare each ByS group with its control ByC group (for example, male ByS septal and male ByC septal) to assess statistical significance ($p < .05$) with 100% as our theoretical comparator (shown as a dotted line in the figures). Moreover, to compare the expression of proteins within ByS groups (such as male ByS septal versus female ByS septal) by considering the effects of sex and region, a two-way ANOVA was used.

4.4.3.1 Bystander Stress Did Not Significantly Alter Total GluA1 Expression Regardless of Region, or Sex.

While the total GluA1 expression was decreased slightly in the septal pole of the male (% ByC: 81 ± 9%) and female rats (% ByC: 91 ± 12%) and increased slightly in the temporal pole of male (% ByC: 111 ± 46%) and female (% ByC: 146 ± 45%) rats, statistically significant changes were not observed (figure 4.8, table 4.2). As well, there was no significant effect of sex on changes caused by ByS [$F (1, 17) = 0.51$, $p = .5$] on total GluA1 (table 4.3).

4.4.3.2 Bystander Stress Substantially Altered Total GluA2 Expression in a Sex- and Pole-Specific Manner.

Total GluA2 expression levels decreased significantly in the hippocampal septal pole in male ByS rats [% ByC: 38.64 ± 2.15%, $t (2) = 28.57$, $p < .001$], but not in female ByS rats

(figure 4.9, table 4.4). Moreover, in female rats, ByS caused a notable increase in GluA2 in the temporal pole [% ByC: 157.7 ± 10.44%, t (2) = 5.52, p < .001], but such a change was not found in the male rats (% ByC: 98.3 ± 29.5%). In other words, bystander stress affected the septal pole of the HP in male rats, and the temporal pole of the HP in female rats, but in opposite directions (decrease for males and increase for females). As a result, our ANOVA-based analysis showed pole-specific [F (1, 10) = 16.61, p = .002] and sex-specific [F (1, 10) = 9.518, p = .01] differences due to ByS (figure 4.9, table 4.4). In fact, sex and pole accounted for 27.99% and 48.84% of the total variance, respectively. In particular, within female ByS rats, total GluA2 expression was higher in the temporal compared to septal pole of the HP (p = .028, table 4.5).

4.4.3.3 Bystander Stress Does Not Alter Total GluN1 Expression Regardless of Region, or Sex.

We did not observe a statistically significant change in the expression of total GluN1 in either the septal (% ByC: 89.86 ± 13.07%) or temporal (% ByC: 93.66 ± 11.51%) poles of the HP in male rats after bystander stress (figure 4.10, table 4.6). Similarly, total GluN1 expression in both septal (% ByC: 91.53 ± 5.58%) and temporal (% ByC: 92.47 ± 8.28%) poles in female rats did not change appreciably relative to their respective controls. Additionally, there was no statistically significant influence of either sex [F (1, 14) = 0, p = .98], or pole [F (1, 14) = 0.06, p = .8] on changes caused by ByS on total GluN1 expression (table 4.7).

4.4.3.4 Bystander Stress Decreases Total GluN2A Expression in the Hippocampal Septal Pole of Male Rats.

In male rats, ByS caused a statistically significant decrease in total GluN2A expression of the septal pole of the HP [% ByC: 80.42 ± 5.85%, t (3) = 3.34, p = .04] compared to the same pole of the ByC rats (figure 4.11, table 4.8). Conversely, in the temporal pole of the female rats, total GluN2A increased after ByS (% ByC: 130.9 ± 35.77%), albeit not to a degree that was statistically significant. No evidence was found for a notable change in the total expression of GluN2A following ByS in the temporal pole of either the male (% ByC: 105.7 ± 27.72%), or female (% ByC: 105 ± 17.04%) rats. Moreover, the effect of ByS did not seem to be significantly affected by sex [F (1, 13) = 1.1, p = .3], or region [F (1, 13) = 1.16, p = .3] (table 4.9).

4.4.3.5 Bystander Stress Decreased Total GluN2B Expression in the Septal Pole of the Hippocampus in Male Rats.

We found evidence that bystander stress reduced the expression of GluN2B at the septal pole of the HP in male rats [% ByC: 76.06 ± 4.2%, t (4) = 5.68, p = .004] (figure 4.12, table 4.10). Male ByS rats also showed a decrease in total GluN2B expression in the temporal pole, but this change was not statistically significant (% ByC: 79.02 ± 11%). We did not find any evidence of change in the total expression of GluN2B in either the septal (% ByC: 101.4 ± 19.5%), or temporal pole (% ByC: 105.7 ± 19.08%) of the female rats following ByS. Furthermore, the effect of ByS was not significantly influenced by sex [F (1, 15) = 3.24, p = .09], or pole [F (1, 15) = 0.06, p = .8] (table 4.11).

4.4.3.6 Bystander Stress Alters Total PSD-95 Expression in the Hippocampal Temporal Pole of Male and Female Rats in Opposite Directions.

There was a significant influence of sex on the changes caused by ByS on total PSD-95 expression [$F(1, 16) = 8.6$, $p = .009$], with sex accounting for 29.72% of the total variance (table 4.12). In contrast, pole had no significant influence on ByS effects on PSD-95 expression [$F(1, 16) = 0.49$, $p = .49$]. In the temporal pole of male rats, ByS increased total PSD-95 expression, albeit not to a significant degree (% ByC: 155.6 ± 26.43%) (figure 4.13, table 4.13). In contrast, ByS decreased PSD-95 expression in female rats, but again the change was not statistically significant (% ByC: 56.14 ± 21.15%). However, the difference of PSD-95 expression in the temporal pole between male and female ByS animals was significant ($p = .01$). Furthermore, PSD-95 expression in the septal pole of male (% ByC: 101.3 ± 10.97%) and female (% ByC: 82.08 ± 19.07%) ByS rats were not changed to an appreciable degree.

To give a broader view of the results, effect sizes (in this case, Cohen's d) were computed to determine the practical significance of the findings. The effect sizes for bystander stress gave insights different from the p-values, for the changes in AMPAR and NMDAR subunit proteins, as well as PSD-95 were large in most cases, even though differences based on p value were often not statistically significant; key exceptions included the difference of GluA2 expression between septal and temporal poles ($p = .028$, $d = 3.1$) in female rats and the difference of PSD-95 expression in the hippocampal temporal pole between male and female ByS rats ($p = .01$, $d = 1.85$).

Notably, the effect size results show that the influence of bystander stress on total GluA1 expression in the hippocampal temporal pole of male and female rats ($d = 1.32$) and in

septal and temporal poles of female rats (d = 0.75), which were not statistically significant, crossed the threshold for practical significance. Also, the effect of bystander stress on total GluA2 expression in septal and temporal poles of male rats (d = 1.6) and between the hippocampal septal (d = 4.5) and temporal (d = 1.5) poles between male and female rats crossed the threshold for practical significance despite not being statistically significant.

Regarding the effect sizes of NMDAR subunit proteins, the effect of bystander stress on total GluN2A expression between septal and temporal poles of male (d = 0.63) and female (d = 0.9) rats and in the hippocampal septal (d = 0.87) poles between male and female rats, which were not statistically insignificant, crossed the threshold for practical significance. Also, total GluN2B expression between the hippocampal septal (d = 0.8) and temporal (d = 0.83) poles between male and female rats, which were not statistically insignificant, crossed the threshold for practical significance. Finally, the effect of bystander stress on total PSD-95 expression between septal and temporal poles of male (d = 1.2) rats, which was not statistically insignificant, crossed the threshold for practical significance. It is noteworthy that not finding statistical significance for a practically significant difference can be related to lower power due to smaller sample sizes.

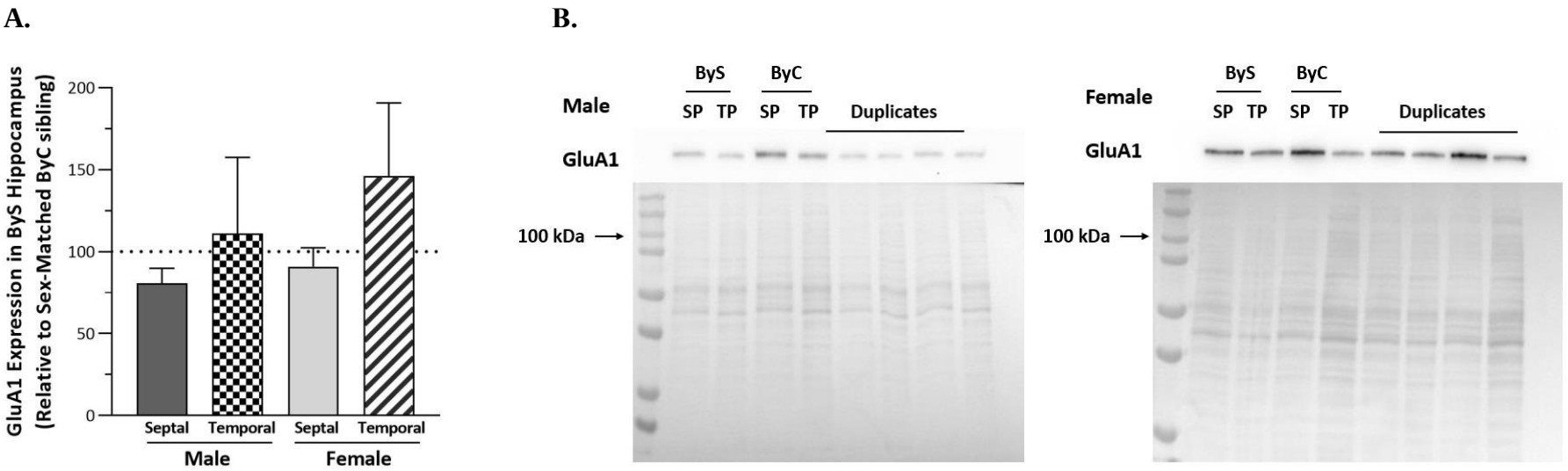


Figure 4.8. Total GluA1 expression in the hippocampal septal and temporal poles of the bystander stressed male and female rats. (A) The summary graph presents the effect of ByS on total GluA1 expression in the hippocampal septal and temporal poles of male and female rats. Each septal, or temporal ByS sample was taken as a percentage of their respective control group (septal, or temporal ByC). (B) Representative immunoblot images of total GluA1 expression in the hippocampal septal and temporal poles of male and female ByS rats, and their respective control groups. Male ByS septal (N = 5), Male ByS temporal (N = 5), Female ByS septal (N = 6), Female ByS temporal (N = 5). All data are expressed as mean ± SEM.

ByS, bystander stress; ByC, bystander control; SP, septal pole of the hippocampus; TP, temporal pole of the hippocampus

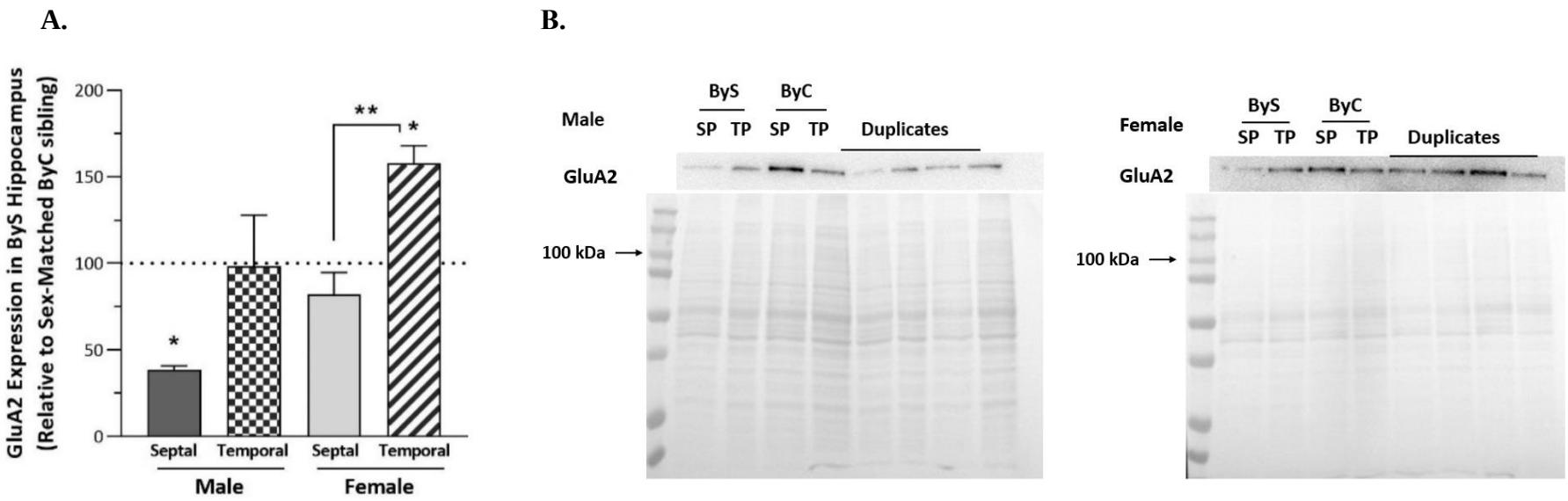


Figure 4.9. Total GluA2 expression in the hippocampal septal and temporal poles of the bystander stressed male and female rats. (A) The summary graph presents the effect of ByS on total GluA2 expression in the hippocampal septal and temporal poles of male and female rats. Each septal, or temporal ByS sample was taken as a percentage of their respective control group (septal, or temporal ByC). (B) Representative immunoblot images of total GluA2 expression in the hippocampal septal and temporal poles of male and female ByS rats, and their respective control groups. Male ByS septal (N = 3), Male ByS temporal (N = 3), Female ByS septal (N = 5), Female ByS temporal (N = 3). All data are expressed as mean ± SEM. *statistically significant p-value < .05

ByS, bystander stress; ByC, bystander control; SP, septal pole of the hippocampus; TP, temporal pole of the hippocampus

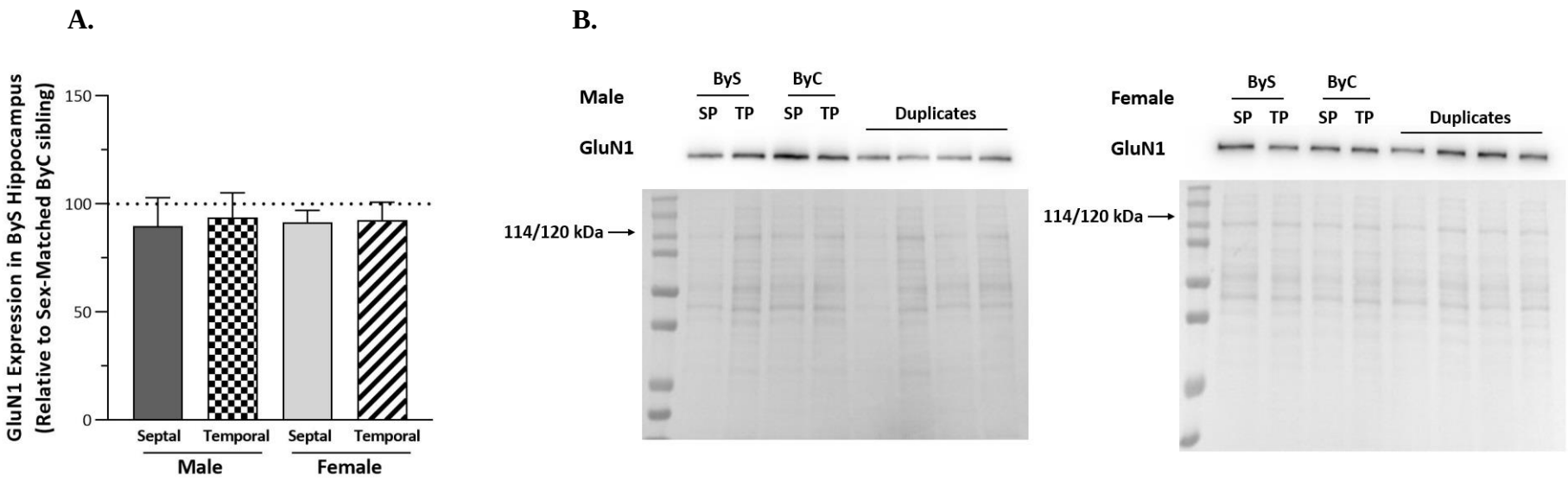


Figure 4.10. Total GluN1 expression in the hippocampal septal and temporal poles of the bystander stressed male and female rats. (A) The summary graph presents the effect of ByS on total GluN1 expression in the hippocampal septal and temporal poles of male and female rats. Each septal, or temporal ByS sample was taken as a percentage of their respective control group (septal, or temporal ByC). (B) Representative immunoblot images of total GluN1 expression in the hippocampal septal and temporal poles of male and female ByS rats, and their respective control groups. Male ByS septal (N = 4), Male ByS temporal (N = 4), Female ByS septal (N = 5), Female ByS temporal (N = 5). All data are expressed as mean ± SEM.

ByS, bystander stress; ByC, bystander control; SP, septal pole of the hippocampus; TP, temporal pole of the hippocampus

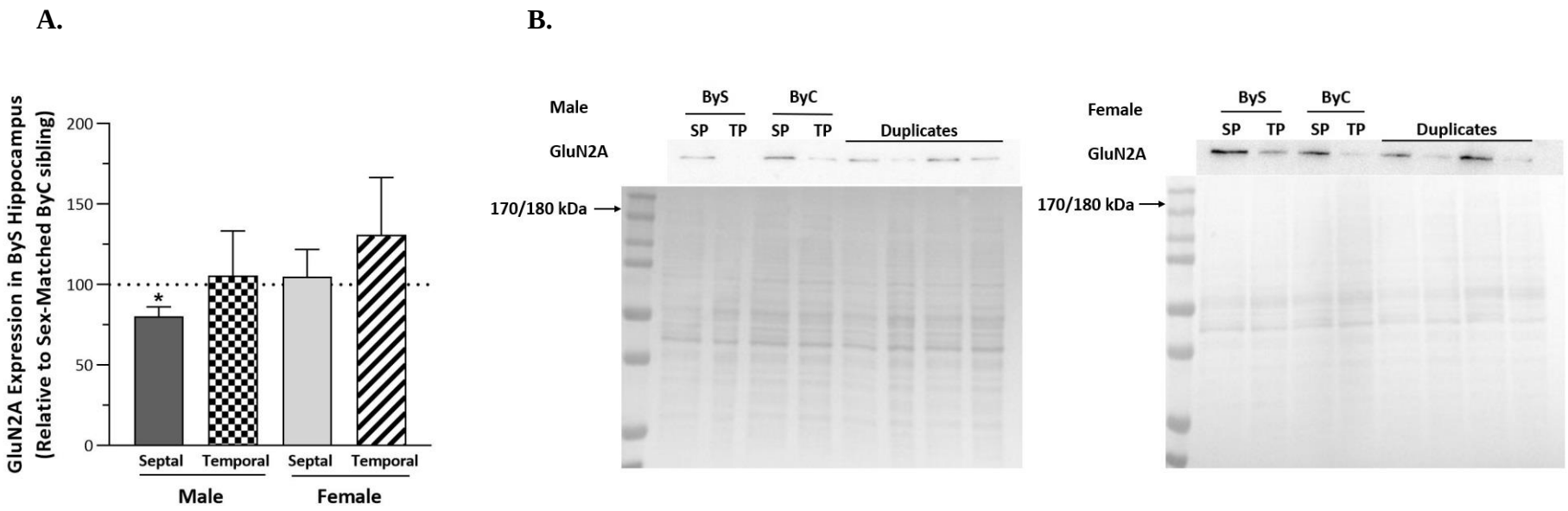


Figure 4.11. Total GluN2A expression in the hippocampal septal and temporal poles of the bystander stressed male and female rats. (A) The summary graph presents the effect of ByS on total GluN2A expression in the hippocampal septal and temporal poles of male and female rats. Each septal, or temporal ByS sample was taken as a percentage of their respective control group (septal, or temporal ByC). (B) Representative immunoblot images of total GluN2A expression in the hippocampal septal and temporal poles of male and female ByS rats, and their respective control groups. Male ByS septal (N = 4), Male ByS temporal (N = 4), Female ByS septal (N = 5), Female ByS temporal (N = 4). All data are expressed as mean ± SEM. *statistically significant p-value < .05

ByS, bystander stress; ByC, bystander control; SP, septal pole of the hippocampus; TP, temporal pole of the hippocampus

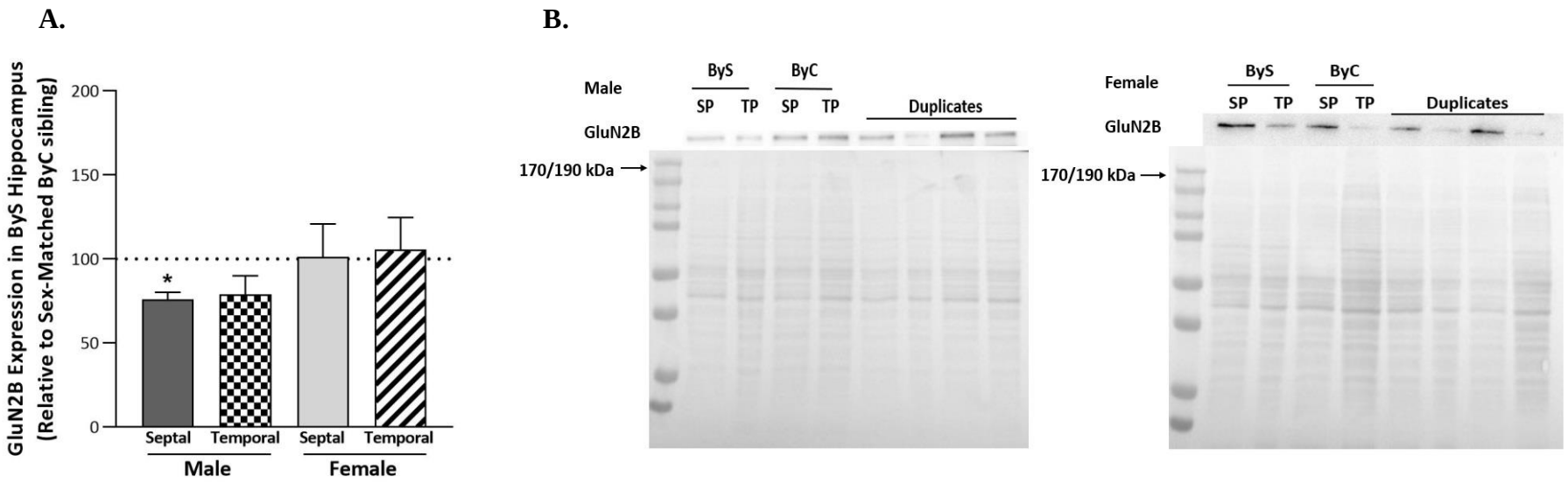


Figure 4.12. Total GluN2B expression in the hippocampal septal and temporal poles of the bystander stressed male and female rats. (A) The summary graph presents the effect of ByS on total GluN2B expression in the hippocampal septal and temporal poles of male and female rats. Each septal, or temporal ByS sample was taken as a percentage of their respective control group (septal, or temporal ByC). (B) Representative immunoblot images of total GluN2B expression in the hippocampal septal and temporal poles of male and female ByS rats, and their respective control groups. Male ByS septal (N = 5), Male ByS temporal (N = 5), Female ByS septal (N = 5), Female ByS temporal (N = 4). All data are expressed as mean ± SEM. *statistically significant p-value < .05

ByS, bystander stress; ByC, bystander control; SP, septal pole of the hippocampus; TP, temporal pole of the hippocampus

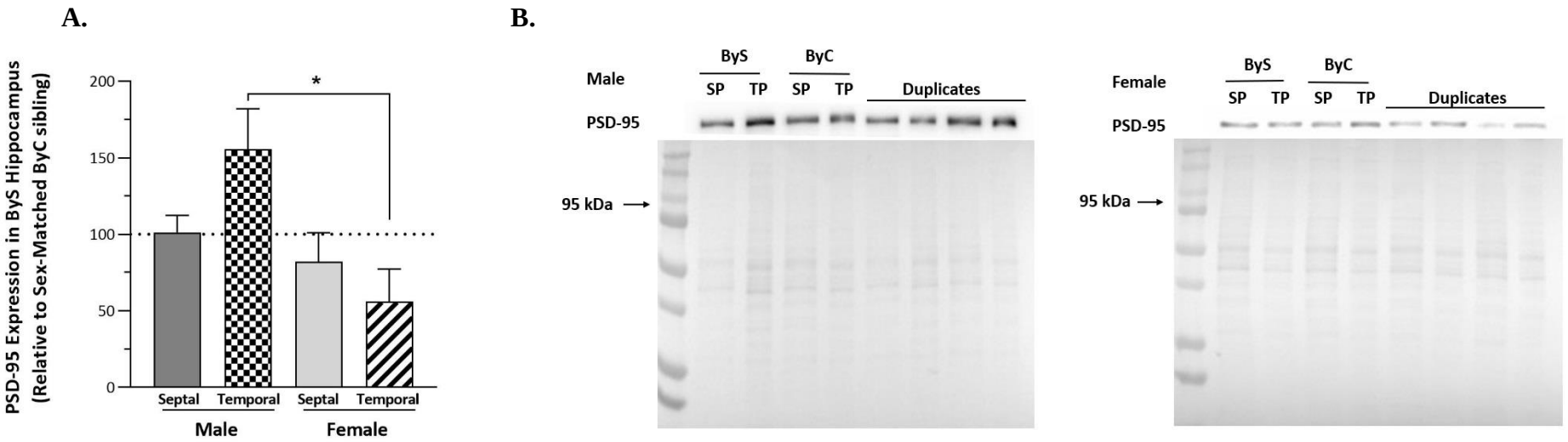


Figure 4.13. Total PSD-95 expression in the hippocampal septal and temporal poles of the bystander stressed male and female rats. (A) The summary graph presents the effect of ByS on total PSD-95 expression in the hippocampal septal and temporal poles of male and female rats. Each septal, or temporal ByS sample was taken as a percentage of their respective control group (septal, or temporal ByC). (B) Representative immunoblot images of total PSD-95 expression in the hippocampal septal and temporal poles of male and female ByS rats, and their respective control groups. Male ByS septal (N = 5), Male ByS temporal (N = 5), Female ByS septal (N = 5), Female ByS temporal (N = 5). All data are expressed as mean ± SEM. *statistically significant p-value < .05

ByS, bystander stress; ByC, bystander control; SP, septal pole of the hippocampus; TP, temporal pole of the hippocampus

Bystander stress (ByS)	Region	Mean (%ByC)	Percent change	N	T-statistic	p value
Males	Septal	80.68	-19.32	5	2.1	.1
	Temporal	111.1	11.10	5	0.23	.82
Females	Septal	90.83	-9.16	6	0.78	.46
	Temporal	146.2	46.15	5	1.03	.35

Table 4.2. Summary of the analyses for total GluA1.

GluA1 expression in the septal and temporal poles of the ByS rats relative to sex matched ByC siblings (%ByC) were compared to the expected value (theoretical mean) in both male and female rats. Theoretical mean used for each comparison = 100.

Source of Variation	% of total variation	p value			
Interaction	0.79	.69			
Poles	9.44	.19			
Sex	2.62	.48			
ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Interaction	808.4	1	808.4	F (1, 17) = 0.15	p = .69
Poles	9589	1	9589	F (1, 17) = 1.83	p = .19
Sex	2665	1	2665	F (1, 17) = 0.51	p = .48
Residual	88759	17	5221		

Table 4.3. Two-way ANOVA table for total GluA1.

Male and female rats were compared for significant differences in GluA1 expression in the septal and temporal poles of the ByS rats relative to their sex matched ByC siblings (%ByC).

Bystander stress (ByS)	Region	Mean (%ByC)	Percent change	N	T-statistic	p value
Males	Septal	38.64	-61.36	3	28.57	.001*
	Temporal	98.33	-1.67	3	0.05	.96
Females	Septal	81.86	-18.14	5	1.41	.23
	Temporal	157.7	57.72	3	5.52	.03*

Table 4.4. Summary of the analyses for total GluA2.

GluA2 expression in the septal and temporal poles of the ByS rats relative to sex matched ByC siblings (%ByC) were compared to the expected value (theoretical mean) in both male and female rats. Theoretical mean used for each comparison = 100. ***** denotes p value < .05

Source of Variation	% of total variation	p value			
Interaction	0.69	.63			
Poles	48.84	.002			
Sex	27.99	.01			
ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Interaction	218.0	1	218.0	F (1, 10) = 0.23	p = .63
Poles	15313	1	15313	F (1, 10) = 16.61	p = .002*
Sex	8775	1	8775	F (1, 10) = 9.51	p = .01*
Residual	9219	10	921.9		

Table 4.5. Two-way ANOVA table for total GluA2.

Male and female rats were compared for significant differences in GluA2 expression in the septal and temporal poles of the ByS rats relative to their sex matched ByC siblings (%ByC). ***** denotes p value < .05

Bystander stress (ByS)	Region	Mean (%ByC)	Percent change	N	T-statistic	p value
Males	Septal	89.86	-10.14	4	0.77	.49
	Temporal	93.66	-6.34	4	0.55	.62
Females	Septal	91.53	-8.47	5	1.51	.2
	Temporal	92.47	-7.52	5	0.9	.41

Table 4.6. Summary of the analyses for total GluN1.

GluN1 expression in the septal and temporal poles of the ByS rats relative to sex matched ByC siblings (%ByC) were compared to the expected value (theoretical mean) in both male and female rats. Theoretical mean used for each comparison = 100.

Source of Variation	% of total variation	p value			
Interaction	0.15	.88			
Poles	0.44	.8			
Sex	0.004	.98			
ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Interaction	9.03	1	9.03	F (1, 14) = 0.02	p = .88
Poles	24.96	1	2496	F (1, 14) = 0.06	p = .8
Sex	0.25	1	0.25	F (1, 14) = 0.0006	p = .98
Residual	5638	14	402.7		

Table 4.7. Two-way ANOVA table for total GluN1.

Male and female rats were compared for significant differences in GluN1 expression in the septal and temporal poles of the ByS rats relative to their sex matched ByC siblings (%ByC).

Bystander stress (ByS)	Region	Mean (%ByC)	Percent change	N	T-statistic	p value
Males	Septal	80.42	-19.58	4	3.34	.04*
	Temporal	105.7	5.7	4	0.2	.85
Females	Septal	105	4.9	5	0.29	.78
	Temporal	130.9	30.87	4	0.86	.45

Table 4.8. Summary of the analyses for total GluN2A.

GluN2A expression in the septal and temporal poles of the ByS rats relative to sex matched ByC siblings (%ByC) were compared to the expected value (theoretical mean) in both male and female rats. Theoretical mean used for each comparison = 100. ***** denotes p value < .05

Source of Variation	% of total variation	p value			
Interaction	0.001	.98			
Poles	7.68	.3			
Sex	7.24	.31			
ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Interaction	0.41	1	0.41	F (1, 13) = 0.1548	p = .98
Poles	2759	1	2759	F (1, 13) = 1.836	p = .3
Sex	2600	1	2600	F (1, 13) = 0.5104	p = .31
Residual	30793	13	2369		

Table 4.9. Two-way ANOVA table for total GluN2A.

Male and female rats were compared for significant differences in GluN2A expression in the septal and temporal poles of the ByS rats relative to their sex matched ByC siblings (%ByC).

Bystander stress (ByS)	Region	Mean (%ByC)	Percent change	N	T-statistic	p value
Males	Septal	76.06	-23.94	5	5.68	.004*
	Temporal	79.02	-20.98	5	1.9	.12
Females	Septal	101.4	1.43	5	0.07	.94
	Temporal	105.7	5.71	4	0.29	.78

Table 4.10. Summary of the analyses for total GluN2B.

GluN2B expression in the septal and temporal poles of the ByS rats relative to sex matched ByC siblings (%ByC) were compared to the expected value (theoretical mean) in both male and female rats. Theoretical mean used for each comparison = 100. ***** denotes p value < .05

Source of Variation	% of total variation	p value			
Interaction	0.01	.96			
Poles	0.34	.8			
Sex	17.75	.09			
ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Interaction	2.03	1	2.03	F (1, 15) = 0.002	p = .96
Poles	61.60	1	61.60	F (1, 15) = 0.06	p = .8
Sex	3189	1	3189	F (1, 15) = 3.24	p = .09
Residual	14751	15	983.4		

Table 4.11. Two-way ANOVA table for total GluN2B.

Male and female rats were compared for significant differences in GluN2B expression in the septal and temporal poles of the ByS rats relative to their sex matched ByC siblings (%ByC).

Bystander stress (ByS)	Region	Mean (%ByC)	Percent change	N	T-statistic	p value
Males	Septal	101.3	1.32	5	0.12	.9
	Temporal	155.6	55.60	5	2.1	.1
Females	Septal	82.08	-17.92	5	0.93	.4
	Temporal	56.11	-43.86	5	2.07	.1

Table 4.12. Summary of the analyses for total PSD-95.

PSD-95 expression in the septal and temporal poles of the ByS rats relative to sex matched ByC siblings (%ByC) were compared to the expected value (theoretical mean) in both male and female rats. Theoretical mean used for each comparison = 100.

Source of Variation	% of total variation	p value			
Interaction	13.57	.06			
Poles	1.69	.49			
Sex	29.72	.009			
ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Interaction	8043	1	8043	F (1, 16) = 3.94	p = 0.06
Poles	1004	1	1004	F (1, 16) = 0.49	p = 0.49
Sex	17614	1	17614	F (1, 16) = 8.64	p = 0.009*
Residual	32597	16	2037		

Table 4.13. Two-way ANOVA table for total PSD-95.

Male and female rats were compared for significant differences in PSD-95 expression in the septal and temporal poles of the ByS rats relative to their sex matched ByC siblings (%ByC).

***** denotes p value < .05

	Bystander Stress			
	Male		Female	
	Septal	Temporal	Septal	Temporal
Total GluA1	↓ (19%)	↑ (11%)	↓ (9%)	↑ (46%)
Total GluA2	↓ **(61%)***	↓ (1.7%)	↓ (18%)	↑ **(58%)***
Total GluN1	↓ (10%)	↓ (6%)	↓ (8.5%)	↓ (7.5%)
Total GluN2A	↓ **(20%)***	↑ (6%)	↑ (5%)	↑ (31%)
Total GluN2B	↓ **(24%)***	↓ (21%)	↑ (1.4%)	↑ (6%)
Total PSD-95	↑ (1.3%)	↑ (56%)	↓ (18%)	↓ (44%)

Table 4.14. Summary of all findings.

↑,↓, signifies an increase and decrease respectively in the hippocampal septal and temporal poles in ByS rats compared to their control group (ByC)

***** denotes p value < .05 in the ByS compared to the ByC group

↑,↓, denotes p value < .05 between/within septal and temporal poles of the ByS rats

4.5 DISCUSSION

4.5.1 The Effect of Bystander Stress on Spatial Learning and Memory

In this exploratory study, we sought to determine whether bystander stress (an indirect psychosocial stressor) experienced during adulthood would affect one of the core brain regions responsible for the acquisition and retrieval of spatial memories. As platform stress was a part of the bystander stress paradigm, we also investigated the effects of platform stress on spatial learning and memory. Male and female rats from both the bystander and platform stress groups navigated the water maze successfully and their learning performance improved over four days, as expected. However, the spatial learning abilities of the ByS, or PS animals were not significantly different from those of the ByC, or PC groups, regardless of their sex. As the next step in investigating hippocampal-dependent ability, spatial memory was evaluated 24 hours after the last learning trial by measuring the amount of time each animal spent in the target quadrant during a probe trial. Again, we found that male and female ByS and PS animals performed similarly to their control counterparts (ByC and PC, respectively).

Several factors need to be considered to interpret our findings, including the intensity of stress induced by the paradigms used in our study and the possibility of stress induction in the control groups. A variety of human and animal social stress models have been investigated to determine whether they affect spatial learning and memory, and some types of stress have been found to either improve (Pisu et al., 2011; Schwabe et al., 2007), or impair (Cevik et al., 2018; Quan et al., 2010) learning and memory performance. Learning and memory performance has also been found to be unaffected in some models of stress (Hellemans et al.,

2004). However, no research has examined the effects of the bystander stress model (or a similar model of secondary stress) on spatial learning and memory.

Indeed, the two previous studies that have used bystander stress applied the paradigm as a model for gestational stress, not as a secondary psychosocial stress model for adult rats (Mychasiuk, Gibb, et al., 2011; Mychasiuk, Schmold, et al., 2011). According to the earlier behavioural results, bystander stress offspring spent less time facing upwards on the platform during the negative geotaxis task, and locomotor behaviour in female offspring was reduced (Mychasiuk, Schmold, et al., 2011). Considering the absence of information available regarding the effects of secondary psychosocial stress, specifically the bystander stress model, on spatial learning and memory in rats, comparing our results with earlier results is challenging. However, some human studies have evaluated behavioural and biochemical effects (but not spatial learning and memory) of secondary (indirect) psychosocial stress under conditions that can be considered close to the bystander stress model. For example, anxiety and depression caused by secondary traumatic stress, or vicarious trauma (Ariapooran et al., 2022; Dunkley & Whelan, 2006). Vicarious trauma (VT), secondary traumatic stress (STS), compassion fatigue, traumatic countertransference, emotional contagion, countertransference, posttraumatic stress disorder, and shared trauma (Branson, 2019) are terms that have been developed to describe situations similar to those of bystander stress. Although these terms are used in different circumstances with various intensities, they all describe negative physiological, neurological, mental, and cognitive changes that may occur to people who engage in an empathetic relationship with a traumatized individual (Branson, 2019).

As platform stress is a primary psychosocial stressor (a stress induced directly by a particular event, in this case height, light, and fear of predation), we expected that there would be a greater effect on behaviour than that seen with our indirect/secondary stressor (ByS), however, there were no clear behavioural differences between them in our study. The lack of a difference may have resulted because our platform control rats were not the most suitable comparison group for our PS animals, since they may have experienced some level of stress as a result of being taken to a separate room during the bystander paradigm. Also, given that the type of bystander stress paradigm in our study (twice a day for five days) can be classified as mild to moderate for adult rats, it may not have been sufficient to affect hippocampal-dependent learning and memory between ByS and ByC rats, regardless of their sex.

4.5.2 AMPAR and NMDAR Expression Following Bystander Stress

Ionotropic glutamate receptors (specifically, AMPARs and NMDARs) play an essential role in hippocampal synaptic plasticity and, consequently, in hippocampus-dependent cognitive functions, including spatial learning and memory. Moreover, as glutamate receptors have been shown to be affected by stress, hippocampal functions may be disrupted by stress, mediated in part by glutamate receptor activation (Derkach et al., 2007; Kim & Diamond, 2002; Kim & Yoon, 1998; Krugers et al., 1993). There is also evidence that the reduction of glutamate receptor function is associated with a decline in cognitive function, whereas overstimulation is associated with the development of certain neurodegenerative diseases (Blanke & VanDongen, 2009; Henley et al., 2011). In other words, modifications in neuronal plasticity, such as alterations in signal transduction pathways, are major ways by which stress can influence the brain (Duman, 2002; Duman et al., 2000). Taking into account these factors,

as well as the fact that AMPARs and NMDARs are abnormally expressed in a number of neurologic and pathological conditions (Blanke & VanDongen, 2009; Henley et al., 2011), one of our objectives in this study was to examine whether secondary psychosocial stress affects the expression of certain plasticity-related proteins from the family of glutamate receptors, and, if so, whether these changes differ across hippocampal region and by sex.

With regards to the effect of bystander stress on AMPAR subunits, we investigated the expression of total GluA1 and GluA2 in the hippocampal septal and temporal poles in male and female rats (table 4.14). Overall, male and female rats that experienced bystander stress did not exhibit any statistically significant differences in GluA1 expression in the hippocampal septal, or temporal pole compared to their respective control groups. In contrast, studies have shown that chronic unpredictable stress for six to seven weeks (Kallarackal et al., 2013) and twice a day for four weeks (Ma et al., 2021) decreases the expression of GluA1 subunits of AMPA receptors in the CA1 region of the hippocampus and inhibits AMPAR-mediated synaptic excitation. The reason for the difference between our observations and those of earlier reports may be that the stress intensity in our study was insufficient to alter the expression of GluA1. Additionally, stress duration, animal age, and possibly other factors may also contribute to differences in these findings.

Total GluA2 expression levels were significantly decreased in the hippocampal septal pole in male ByS rats (61%). Moreover, in female rats, ByS caused a significant increase in GluA2 in the temporal pole (58%). As well, strong evidence for a difference between how the temporal and septal poles of female rats were affected by ByS in terms of the expression of total GluA2 was observed. Notably, ByS caused pole- and sex-specific changes in GluA2

expression. For male rats, our results are consistent with the findings of Bonini et al. (2016), who observed that stress induced a rapid decrease in the expression of GluA2 at postsynaptic spines in prefrontal and frontal cortex due to acute foot shock stress. It should be noted, however, that the type of stress (foot shock) and the location of the brain examined (PFC) were different from those in our study. Moreover, our findings for female animals align with an earlier study by Groc et al. (2008) that showed high levels of corticosterone increased the membrane expression and synaptic insertion of GluA2-containing AMPARs in the hippocampus (Groc et al., 2008), enhancing synaptic transmission through AMPARs (Krugers et al., 2010). Despite the fact that bystander stress was associated with significant GluA2 alteration based on poles and sex, spatial learning and memory were unaffected by these alterations. It is possible that the changes in GluA2 expression were not large enough to cause any behavioural changes. Furthermore, our results are consistent with the results indicating that glucocorticoids affect GluA2 subunits more than GluA1 subunits (Groc et al., 2008; Martin et al., 2009), suggesting corticosteroid hormones may persistently be responsible for regulating synaptic transmission through GluA2-A3 containing AMPARs rather than through GluA1-A2 containing AMPARs (Conboy & Sandi 2010). An additional important consideration is that the mentioned studies used immunohistochemistry and live cell imaging techniques for whole hippocampus, whereas hippocampal septal and temporal synaptoneurosome were used in our study.

For the purpose of examining whether bystander stress alters NMDAR subunit expression, the expression of GluN1, GluN2A, and GluN2B subunits was evaluated in the hippocampal septal and temporal poles of male and female rats (table 4.14). GluN1 expression

was not significantly altered by bystander stress regardless of sex. However, bystander stress did significantly decrease total expression of both GluN2A (20%) and GluN2B (24%) in the septal pole of the hippocampus in male rats. Furthermore, bystander stress significantly altered total PSD-95 expression in the hippocampal temporal pole of male and female rats, but in opposite directions (56% increase in males and 44% decrease in females).

The effects of stress on AMPAR and NMDAR subunits have already been demonstrated in previous research, and results have varied according to the stress protocol, species, strain, and brain area examined. For example, Calabrese et al., (2012) reported that six weeks of chronic mild stress (a variable series of mild and unpredictable stressors, such as food or water deprivation, crowding, isolation, soiled cages, immobilization, and light on overnight) did not alter AMPAR subunit expression in the dorsal (septal pole), or ventral (temporal pole) hippocampal areas in synaptosomal fractions of adult male rats. However, our results indicated pole-specific changes regarding GluA2 expression in septal and temporal poles of both ByS male and female rats (reduction in the septal pole of male and increase in the temporal pole of female rats). Regarding the expression of NMDAR subunits, our findings also did not align with their finding that chronic mild stress increases GluN2A (around 40%) and GluN2B (around 25%) expression in the ventral hippocampus of male rats (we found a 20% and 24% reduction in the expression of GluN2A and GluN2B, respectively). In addition, consistent with our results, the level of GluN1 did not change (Calabrese et al., 2012).

In another study by Pacheco et al., (2017) a two-fold increase in GluN1 expression was found in the dorsal hippocampus of adult male rats following chronic restraint stress (2.5 hours for fourteen consecutive days), however, GluN2A and GluN2B expression did not change.

Further, Fumagalli et al. (2009) examined prenatal stress (from embryonic day 14 until delivery, 45 min, three times a day) for its effects on the glutamatergic synapse's response to acute swimming stress (5 minutes) at adulthood as well as potential sex differences. They conducted protein analyses on rat hippocampal crude synaptosomal fractions. In male rats exposed to prenatal stress, total levels of GluA1 increased significantly (by 11% in male rats exposed to only prenatal stress, and by 15% in male rats exposed to both prenatal stress and acute swim stress in adulthood). Moreover, female animals that had been subjected to prenatal stress and sham treatments showed an increase in GluA1 levels, 29% and 24% respectively. Both male and female rats did not show any changes in the total levels of GluA2, GluN1, GluN2A, and GluN2B subunits in the hippocampus following prenatal restraint stress, or after the acute swim stress in adulthood.

Our findings do not appear to be in agreement with those of previous studies, and the differences may be attributable to the ways in which our stress protocol was distinct from the ones used in these earlier reports. Our study utilized indirect/secondary psychosocial stress, which fundamentally differs from acute, or chronic physical stress (particularly, in terms of intensity and duration). As well, for our research, synaptoneurosome preparations of the hippocampal septal and temporal (presynaptic compartments attached to a postsynaptic element) for both males and females were used, however, many of the previous studies used crude synaptosomal fractions (which consist of the presynaptic compartment/spine) (Calabrese et al., 2012; Fumagalli et al. 2009; Pacheco et al., 2017).

We hypothesized that ByS would alter the expression of the AMPAR and NMDAR subunit proteins based on sex and pole. In the data, other than total GluN1 expression, there

were statistically significant differences in protein expression found across the septal and temporal poles (between ByS and ByC, between/within male and female rats). In general, our assumptions appear to be supported by the results. In addition, our data suggest that the septal pole of the hippocampus in males and the temporal pole of the hippocampus in females are more sensitive to secondary psychosocial stress exposure, which predominantly altered the expression of AMPAR and NMDAR subunits that may influence the function of these areas as these proteins are essential for the plasticity of hippocampal synapses. As mentioned before, AMPAR and NMDAR subunits are responsible for the plasticity of excitatory synaptic transmission in the brain, and their function has been implicated in the effects of stress on the hippocampus (Goodwani et al., 2017; Popoli et al., 2012). In addition, increasing the expression of these proteins is associated with depression (Karolewicz et al., 2009). Conversely, inactivating these proteins leads to anxiolytic and antidepressant effects in rodents (Boyce-Rustay & Holmes, 2006). These biochemical changes, however, did not result in behavioural changes at the level of spatial memory, or learning in our study. Furthermore, our findings suggest that total GluN2A and GluN2B may be more sensitive to psychosocial stress in male rats (particularly in the septal pole) compared to female rats. In terms of GluA2 and PSD-95, this sensitivity was observed in both male and female rats.

One of the potential mechanisms behind the protein-related changes observed in this study is epigenetic change. Stressors can affect specific epigenetic marks such as DNA methylation and histone modifications that subsequently alter gene expression levels and protein production (McGowan et al., 2009). The bystander stress mechanism is not yet fully understood, but with regards to the other types of stress, such as maternal care adversity, can

lead to changes in the expression of hippocampal glucocorticoid receptors via epigenetic process (Franklin et al., 2012). The adult offspring of high compared to low maternal care mothers show epigenetic changes in promoters, exons, and gene ends associated with higher transcriptional activity across many genes (McGowan et al., 2011).

Taken together, the data in this study suggest that following secondary psychosocial stress, there tends to be altered expression of AMPAR and NMDAR subunits, as well as PSD-95 at synaptic sites. Bystander stress as a secondary psychosocial stressor appeared to selectively affect certain plasticity-related proteins across the hippocampal poles. Furthermore, male rats displayed more changes compared to female rats, which indicated sex-dependent effects of ByS on plasticity-related proteins expression in the hippocampus. However, since the intensity of bystander stress may be mild for adult rats, the changes in plasticity-related proteins may not be sufficient to affect spatial learning and memory. Therefore, in the snapshot taken one hour following the last MWM trial, we observed a change in the expression of the AMPAR and NMDAR subunits resulting synaptic transmission changes in the poles of the hippocampus; however, in this stress model and with this level of intensity, biochemical changes did not lead to behavioural changes.

Chapter 5

DOES EXPERIENCING CHRONIC EARLY LIFE SOCIAL ISOLATION AFFECT THE BEHAVIOURAL AND BIOCHEMICAL RESPONSE TO BYSTANDER STRESS LATER IN LIFE? EVALUATING THE MATCH/MISMATCH HYPOTHESIS

5.1 ABSTRACT

Objective

In light of the match/mismatch hypothesis, we sought to determine whether experiencing chronic early life social isolation (CELSI) changes behavioural and biochemical responses to bystander stress (ByS) in adulthood to see if exposure to early life stress could influence the effect of social stress in adulthood.

Methods

Male and female siblings from 10 Sprague-Dawley rat litters were stratified by sex and then randomly assigned to either group-housing (3 animals/cage), or social isolation (1 animal/cage) for 7 weeks. A six-month gap was then applied, followed by the ByS paradigm (twice daily for five consecutive days), which provided animal groups with stress and control matches (SI-ByS and GH-ByC) and mismatches (SI-ByC and GH-ByS). Spatial learning and memory were then tested over 5 days using the Morris water maze. Next, the animals were euthanised, and brain tissue was harvested. After this, synaptoneurosomes were prepared and Western blotting completed to assess the effect of the match-mismatch paradigm on the expression of AMPAR and NMDAR subunits (GluA1, GluA2, GluN1, GluN2A, and

GluN2B), as well as PSD-95, in the hippocampal septal and temporal poles of male and female rats from five of the litters.

Results

From the behavioural perspective, male rats in the stress matched situation (SI-ByS) showed better spatial learning performance compared to those in mismatch 2 (GH-ByS). In other words, experiencing psychosocial stress during both development and adulthood improved spatial learning in male rats. Furthermore, there was evidence of sexual dimorphism in spatial learning among rats exposed to the mismatch 1 situation (SI-ByC); male rats performed significantly better than female rats. This result suggests that social isolation during the developmental period conditions spatial learning performance in adulthood in a sex-dependent manner. In terms of immunoblotting, SI-ByS was generally characterized by an increase in the obligatory NMDA receptor subunit (GluN1) expression (24.8%) in the septal pole of the male HP, when compared to the GH-ByS group. In addition, a significant increase in total PSD-95 expression (46%) was observed in the hippocampal temporal pole of female SI-ByS rats, in comparison to the septal pole. Taken together, our biochemical results and behavioural observations were clearly correlated and support the match/mismatch hypothesis with a sex-specific approach, showing that male rats exposed to the stress matched condition had better spatial learning performance than those exposed to the mismatch 2 (GH-ByS) condition where only adulthood stress was epxerienced. Furthermore, these findings suggest that early social isolation may have lifelong effects, specifically in male rats.

5.2 INTRODUCTION

In both human and animal studies, stressful experiences during early life are associated with an increased risk of later-life neurological, psychiatric, and behavioural disorders, such as depression, anxiety, and schizophrenia (Grippo et al., 2007; Heim et al., 2004; Reinwald et al., 2018; Thorsell et al., 2006). Possibly, individuals may be more vulnerable to stress depending on their developmental history. In other words, similar adult stressors may have very different effects across individuals due to variability in their early life experiences (Caspi et al., 2003; Gluckman et al., 2007).

Early-life experiences have been proposed to influence brain development and lead to long-term effects in two main ways: 1) the developmental constraints hypothesis (also known as cumulative stress theory, or the "double-hit" hypothesis) and 2) the predictive adaptive response hypothesis (PAR), or match/mismatch hypothesis (Gluckman et al. 2005a, 2005b). According to the first possibility (cumulative stress theory/"double-hit" hypothesis) exposure to early life adversity is costly and such experiences (the first hit) are more likely to disrupt neural system evolution and increase vulnerability to later life stressors (the second hit). Such a pattern is associated with an increased risk of developing mental health disorders with each additional stressor (Choy et al., 2008; Walker et al., 2009), for example, schizophrenia (Choy & van den Buuse, 2008), or anxiety (Imanaka et al., 2006, Diehl et al., 2007). In other words, the more stress a person is exposed to, the more detrimental the outcome may be. Alternatively, the match/mismatch hypothesis suggests that early life experiences "condition" the brain in a way that may alter how later-life stressors become biologically embedded (Belsky & Pluess, 2009; Frankenhuis & Del Giudice, 2012; Ricon et al., 2012; Schmidt, 2011). Hence, stressful

childhood may prepare the individual for a stressful adulthood through "adaptive programming" where there is a match between events experienced over time; however, in the event that a mismatch occurs across childhood and adulthood events, then the outcome might be negative and behavioural problems are likely to arise (Nederhof & Schmidt, 2012). While there is some evidence that early life experiences are associated with adaptive, or maladaptive stress responses (Power et al., 2013; Santarelli et al., 2014a; Zalosnik et al., 2014), the exact relationship remains unclear, especially in relation to learning and memory functions.

As noted in an earlier section, psychiatric disorders caused by social stress are highly sex-dependent (Kendler et al., 1995; Piccinelli & Wilkinson, 2000), yet little is known about whether early-life adversity impacts the effects of social stress later in life uniquely in men and women (or male and female animals). Animal studies have shown that male and female rodents respond to stress differently, with females having higher baseline corticosterone levels (Malisch et al., 2009a) and greater and more persistent adrenocorticotropic hormone and corticosterone responses (Larkin et al., 2010; Young et al., 2001). Further, social stress has been reported to result in behavioural changes specific to female rats (reductions in activity in open field tests, as well as changes in social interest) and reduced basal dendrite lengths in CA3 hippocampal neurons (Leedy et al., 2013).

Stress-induced behavioural changes may be influenced by the neural expression of plasticity-related genes, as a result, some individuals are able to adapt to environmental changes effectively, while others are unable to do so (McEwen & Gianaros, 2011). For example, BDNF is downregulated by chronic stress, which may contribute to cellular remodeling and atrophy of vulnerable neurons and distal dendrites (Charney et al. 2004), as

well as impairment of synaptic plasticity (McEwen & Gianaros, 2011). Also, maternal nursing adversity (such as decreased licking/grooming) is known to be associated with the development of proper behavioural responses during adulthood and may influence the NR3C1 gene in the hippocampus, which has been implicated in the regulation of glucocorticoid receptors and the HPA axis by modulating the availability of the stress hormone (Weaver et al. 2004). Studies have also demonstrated that synaptic plasticity-related proteins, including AMPA and NMDA receptor subunits, are affected by stress (Bartanusz et al., 1995; Calabrese et al., 2012; Kurgers et al., 2010), especially in regions of the nervous system (such as the hippocampus) that may increase risk for developing neuropsychiatric disorders, such as depression and dementia (Sestito et al., 2011).

Unlike wild animals, which experience a complex life environment that includes a mixture of stressful situations during their developmental and adulthood periods, experimental animals live in standard conditions in research facilities and, according to research protocols and factors evaluated in research studies, their environmental factors (light, noise, bedding, housing, etc.) can be altered over the course of their life (prenatal, postnatal, childhood, adulthood, and so on) to induce stress. Indeed, the majority of stress-related research uses a mismatch model in which animals are stressed only in childhood, or only in adulthood. However, there are no studies in the literature comparing the effects of match/mismatch situations on AMPA and NMDA plasticity-related proteins (GluA1, GluA2, GluN1, GluN2A, and GluN2B), as well as PSD-95.

To test the match/mismatch hypothesis in rats, we examined whether the interaction between social isolation early in life and bystander stress in adulthood would affect

hippocampal-dependent spatial learning and memory and the expression of NMDA and AMPA receptor subunits in the hippocampus of male and female rats. In other words, we examined whether experiencing CELSI changes the behavioural and biochemical responses to ByS in male and female rats. We hypothesized that animals which experienced either matched stressed, or unstressed environments, would exhibit better performance in MWM compared to animals with mismatched early and adult life environments. In this regard, the objectives of the study were as follows:

Objective 1: To investigate the influence of experiencing both early life (CELSI) and adulthood stress (ByS) on hippocampal-dependent behaviour by testing spatial learning and memory using the Morris water maze.

Objective 2: To determine whether experiencing both early life (CELSI) and adulthood stress (ByS) affects the expression of a series of plasticity-related proteins (GluA1, GluA2, GluN1, GluN2A, GluN2B, and PSD-95) in the septal and temporal poles of the hippocampus.

Objective 3: To explore possible sex-specific effects of experiencing both early life (CELSI) and adulthood stress (ByS) on plasticity-related protein expression and behaviour by using both male and female animals.

5.3 MATERIALS AND METHODS

5.3.1 Animals and Stress Protocols

Each week a Sprague-Dawley rat litter containing a dam and 5 male and 5 female pups arrived at our facility on post-natal (PND) 7 from Envigo (in total, 10 litters were used). The animals were housed in polypropylene cages containing wood chip bedding and PVC tubes for enrichment. They were maintained in a temperature-controlled room (21◦C) with a 12:12-hour reverse light:dark cycle (lights on at 10:00 p.m.). The animals were given ad libitum access to standard rodent chow (Teklad 22/5, Envigo) and water. Upon weaning (PND 21-28; a decision based upon both pup size and eating behaviour), male and female siblings were stratified by sex and then randomly assigned to either the group housed (GH: 3 animals/cage), or the social isolation (SI: 1 animal/cage) condition for 7 weeks (figure 5.1). For both conditions, cage cleaning was limited to once a week to keep interactions to a minimum.

Following CELSI, the socially isolated animals from each litter were placed in sex-specific pairs and, along with their matched GH animals, were maintained under standard housing conditions for 6 months. After the 6-month hiatus, two of the animals from each GH set were randomly assigned to either the ByC, or ByS condition; similarly, the SI animals from each litter were randomly assigned to either the ByC, or ByS condition. After assignment to either the ByC, or ByS condition, each animal from the CELSI phase of the study was then paired with an age and sex-matched companion animal that filled the role of either the platform stress, or platform control cagemate, as needed. For example, a GH male animal that had been assigned to the ByC condition would have been paired with an age-matched naïve animal that

was assigned to the PC condition. One week after the new pairs were established, the ByS paradigm was conducted (please see the Methods section of chapter 4 for more information).

As a result, four stress-related situations were created for each sex, two matches and two mismatches (figure 5.2):

1) Male and female rats that experienced both models of stress, 7 weeks of chronic early-life social isolation and 5 days of bystander stress (ByS) during adulthood, were regarded as stress matched (SI-ByS).

2) Male and female rats that experienced the unstressed/control situation during both development (group-housed: GH) and adulthood (bystander control: ByC), were regarded as control matched (GH-ByC).

3) Male and female rats that experienced CELSI during development (post-weaning social isolation: SI) and the bystander control condition during adulthood were regarded as mismatch 1 (SI-ByC).

4) Male and female rats that experienced the control situation during development (group-housed: GH) and bystander stress (ByS) during adulthood were regarded as mismatch 2 (GH-ByS).

5.3.2 Behavioural and Biochemical Analyses

The day after the ByS paradigm was completed, Morris water maze data (four days of learning and one day for the memory test) were collected, and brain tissue was then harvested (applying methods discussed in chapter 4). Subsequently, synaptoneurosomes were prepared and Western blotting was used to assess the effect of each match-mismatch paradigm on the expression of AMPAR and NMDAR subunits (GluA1, GluA2, GluN1, GluN2A, and

GluN2B), as well as PSD-95, in the hippocampal septal and temporal poles of male and female rats randomly selected from five of the ten litters (once again, the related methods are described in detail in chapter 4).

Although several combinations of early-life and adult experiences were possible with our experimental design, for this project we chose to focus our biochemical examination upon the four groups of most immediate interest: male-mismatch 2, male-stress match, female-mismatch 2, and female-stress match. That is, when collecting Western blotting results, we did not evaluate the other possible combinations since their experiences were similar to that of either group-housed, or socially isolated rats in the CELSI project (chapter 3). For instance, in the mismatch 1 situation (SI in the developmental period followed with ByC in adulthood), rats were only exposed to a stressor early in life. Similarly, rats in the control match situation did not experience any stressors during their developmental period, or during their adulthood. The statistical methods used for analysing behavioural and immunoblotting data were described in chapter 3.

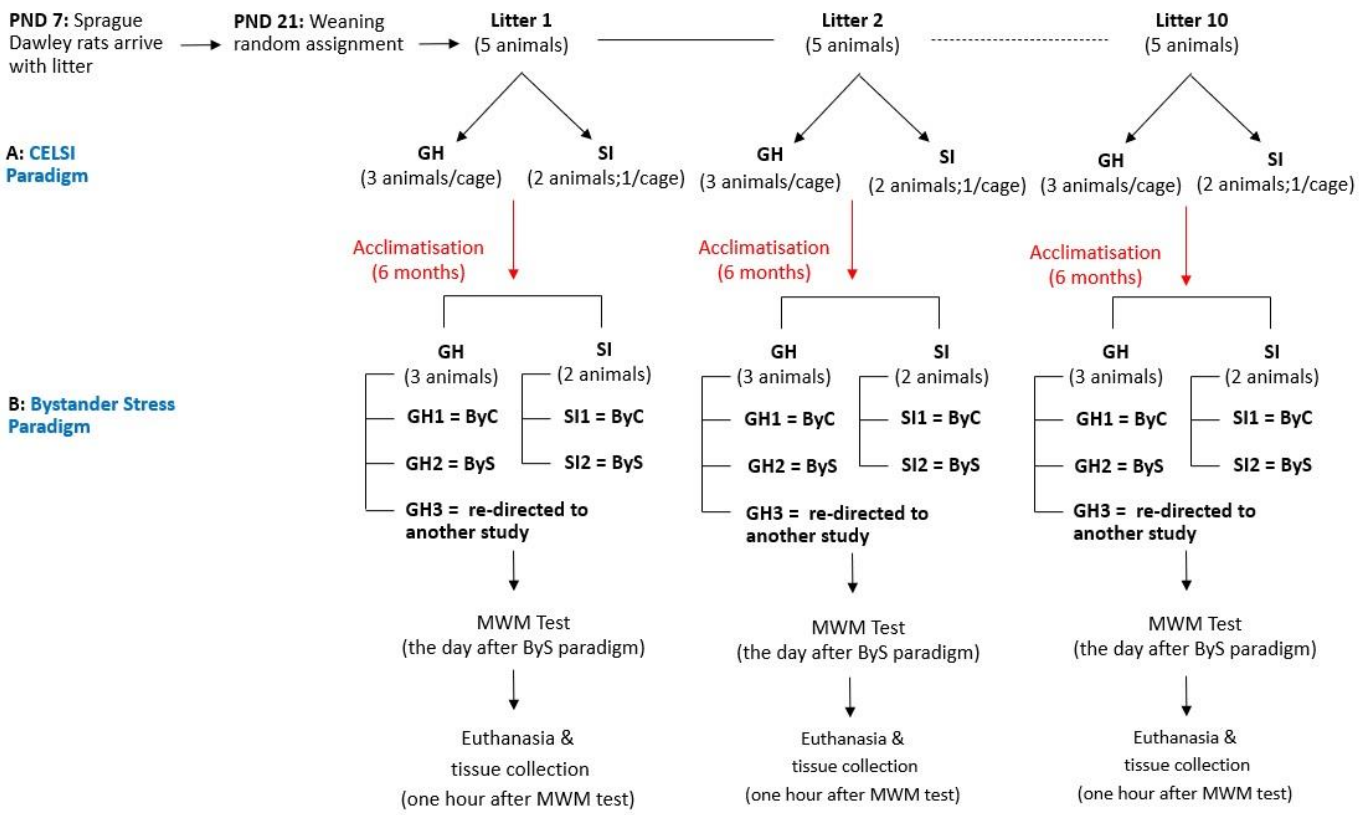


Figure 5.1. A combination of CELSI and ByS was used to develop the match/mismatch paradigms. In each litter, there are five males and five females, but for simplicity's sake, only five males are shown. **PND**: Post-natal Day, **GH**: Group Housed, **SI**: Socially Isolated, **ByC**: Bystander Control, **ByS**: Bystander Stress, **MWM**: Morris Water Maze

Early Life	Adulthood	Life Stage Comparison
Group House (GH)	Bystander Control (ByC)	Control Match
Chronic Early Life Social Isolation (CELSI)	Bystander Stress (ByS)	Stress Match
Chronic Early Life Social Isolation (CELSI)	Bystander Control (ByC)	Mismatch 1
Group House (GH)	Bystander Stress (ByS)	Mismatch 2

Figure 5.2. Four different stress-related situations were created for each sex (two matches and two mismatches). GH: Group Housed, **SI**: Socially Isolated, **ByC**: Bystander Control, **ByS**: Bystander Stress

5.4 RESULTS

To overcome the shortcomings of ANOVA-based models when censored data are present, the learning trial data were analysed using survival analysis. Our analysis of the learning phase data was conducted using the Cox proportional hazards model as discussed in chapter 3 (CELSI study). Additionally, to determine whether the observed differences in learning and memory phases are due to variation in motivation to complete the task or sensorimotor abilities including swimming ability, we measured the average speed at which each animal swam during the probe test (GH-ByS – male/female, N = 10/sex; GH-ByC – male/female, N = 10/sex; SI-ByS – male/female, N = 10/sex; SI-ByC – male/female N = 10/sex). Our analysis (two-way ANOVA, stress and sex as factors) revealed that swim speed in our sample did not differ according to whether animals experienced the matched, or mismatched stress conditions [$F(3, 72) = 0.36$, $p = .78$)] (figure 5.3).

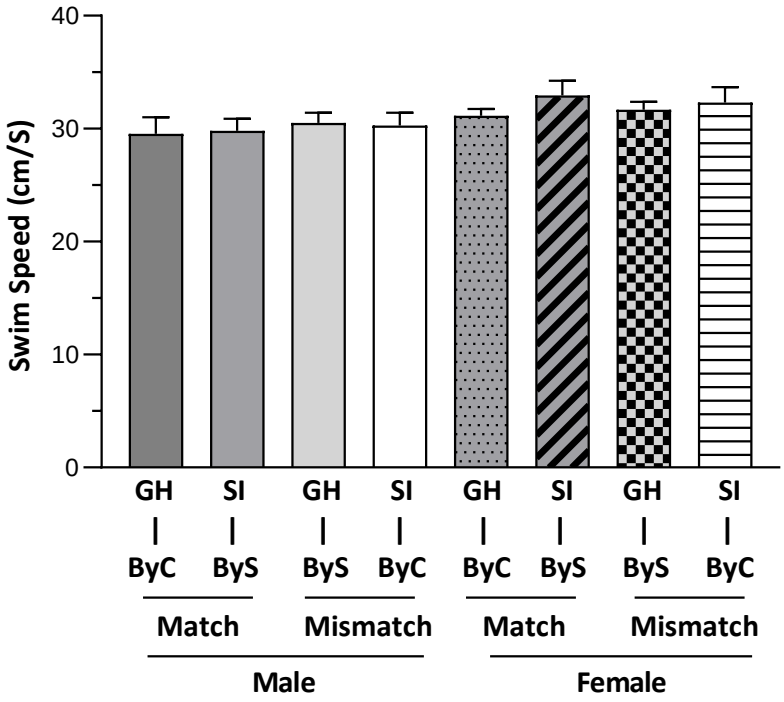


Figure 5.3. Swim speed. The graph presents the average swim speed as a general water maze performance characteristic recorded for each group during the probe test. Each bar presents mean + SEM. **GH-ByC**, group housed-bystander control; **GH-ByS,** group housed-bystander stress; **SI-ByC**, social isolation-bystander control; **SI-ByS**, social isolation-bystander stress; **SEM**, standard error of the mean

Evaluating the probability of locating the escape platform in male and female rats of all groups showed a significant difference between the spatial learning performance on day 1 and day 4 ($p < .0001$) indicating that each group improved over the course of the learning trials (figure 5.4 A-D).

5.4.1 On Day One, Statistically Significant Differences in Spatial Learning were Observed Between Male and Female Rats for The Control Match Condition

On day 1, the hazard ratio for male and female GH-ByC rats (matched on the control conditions) was 1.9, with a 95% confidence interval of (1, 3.6), which indicates that the male GH-ByC group was 1.9 times as likely to find the platform at any time compared to the female GH-ByC group; this difference was statistically significant [(male vs. female)-GH-ByC, $p = .033$] (table 5.2, figure 5.5A&C).

5.4.2 On Day Four, Statistically Significant Differences in Spatial Learning were Observed Between Male and Female Rats for Mismatch 2

On day 4, the hazard ratio for male and female SI-ByC rats (mismatch 1) was 1.9, with a 95% confidence interval of (1.2, 3), indicating that the male SI-ByC group was 1.9 times as likely to find the platform at any time compared to the female SI-ByC group; this difference was statistically significant [(male vs. female)-SI-ByC, $p = .0064$] (table 5.2, figure 5.5B).

5.4.3 On Day Four, Male Stress Matched Rats, Performed Better Than Male Mismatch 2 Ones in Learning Phase of MWM

On day 4, the hazard ratio for male SI-ByS and GH-ByS rats was 1.6, with a 95% confidence interval of (1, 2.4), indicating that the SI-ByS group was 1.6 times as likely to find the platform at any time compared to the GH-ByS group [p = .049] (table 5.1, figure 5.4D).

The analysis of the learning phase data from the other groups did not reveal any hazard ratios statistically different from 1, suggesting that the other groups had the same probability of finding the platform at any point in time (table 5.1 and 5.2, figure 5.5A-D).

Taken together, the data revealed a difference in learning performance between male and female rats in the control match (GH-ByC) condition on day one, and in the mismatch 1 condition (SI-ByC) on day 4. In other words, male rats performed better in the learning phase regardless of their experiences during the developmental period, however, the difference was more distinct when the animals experienced SI. This result suggests that social isolation during development is related to spatial learning performance in adult rats in a sex-dependent manner. Additionally, the stress match (SI-ByS) male rats performed better than the male mismatch 2 (GH-ByS) rats, indicating that experiencing stress during the developmental period may have conditioned them to better tolerate adulthood stress. Although the difference of spatial learning performance in MWM between male GH-ByS and male SI-ByS seem to have relatively weak statistical support (p = .049), observing such a difference after six months (which is approximately equivalent to eighteen years of human life) is valuable and worth noticing.

	Hazard ratios (day 1)	95% CI of mean	Hazard ratios (day 4)	95% CI of mean
M-SI: ByS vs. ByC Male (stress match vs. mismatch 1)	1.1	(0.65 ,1.9)	1.1	(0.69 ,1.7)
F-SI: ByS vs. ByC Female (stress match vs. mismatch 1)	0.93	(0.52 ,1.7)	1.3	(0.84 , 2.1)
M-ByS: GH vs. SI Male (stress match vs. mismatch 2)	1.2	(0.67,2.00)	1.6*	(1, 2.4)
F-ByS: GH vs. SI Female (stress match vs. mismatch 2)	1.1	(0.62 ,2.1)	0.94	(0.6 , 1.5)

Table 5.1. The hazard ratios for male and female rats in the Morris water maze across stress conditions. The table shows comparisons between different groups in terms of the likelihood of finding the platform at any given time. While there was no evidence to support a difference between most situations (that is, the hazard ratios were, statistically speaking, equal to 1), there was evidence to support a difference in learning performance between male GH-ByS and SI-ByS rats on day four. * shows the statistically significant hazard ratio and the underline shows the reference group. **SI-ByC**, social isolation-bystander control; **SI-ByS**, social isolation-bystander stress; **GH-ByS,** group housed-bystander stress; **SEM**, standard error of the mean

	Hazard ratios (day 1)	95% CI of mean	Hazard ratios (day 4)	95% CI of mean
GH-ByC: F vs. M (control match)	1.9*	(1 , 3.6)	1.6	(0.99 , 2.4)
SI-ByS: F vs. M (stress match)	1.4	(0.8,2.5)	1.5	(0.93,2.3)
SI-ByC: F vs. M (mismatch 1)	1.2	(0.67,2.1)	1.9*	(1.2,3.0)
GH-ByS: F vs. M (mismatch 2)	1.3	(0.75,2.4)	0.92	(0.59 , 1.4)

Table 5.2. The hazard ratios for male and female rats in the Morris water maze based on sex (male vs. female). The table shows comparisons between different groups in terms of the likelihood of finding the platform at any given time between male and female rats. Data analysis revealed that control match (GH-ByC) group on day one, as well as the mismatch 1 (SI-ByC) group on day four, displayed evidence of differences in spatial learning performance between male and female rats. * shows statistically significant hazard ratios and the underline shows the reference group. **GH-ByC**, group housed-bystander control; **GH-ByS,** group housed-bystander stress; **SI-ByC**, social isolation-bystander control; **SI-ByS**, social isolation-bystander stress; **SEM**, standard error of the mean

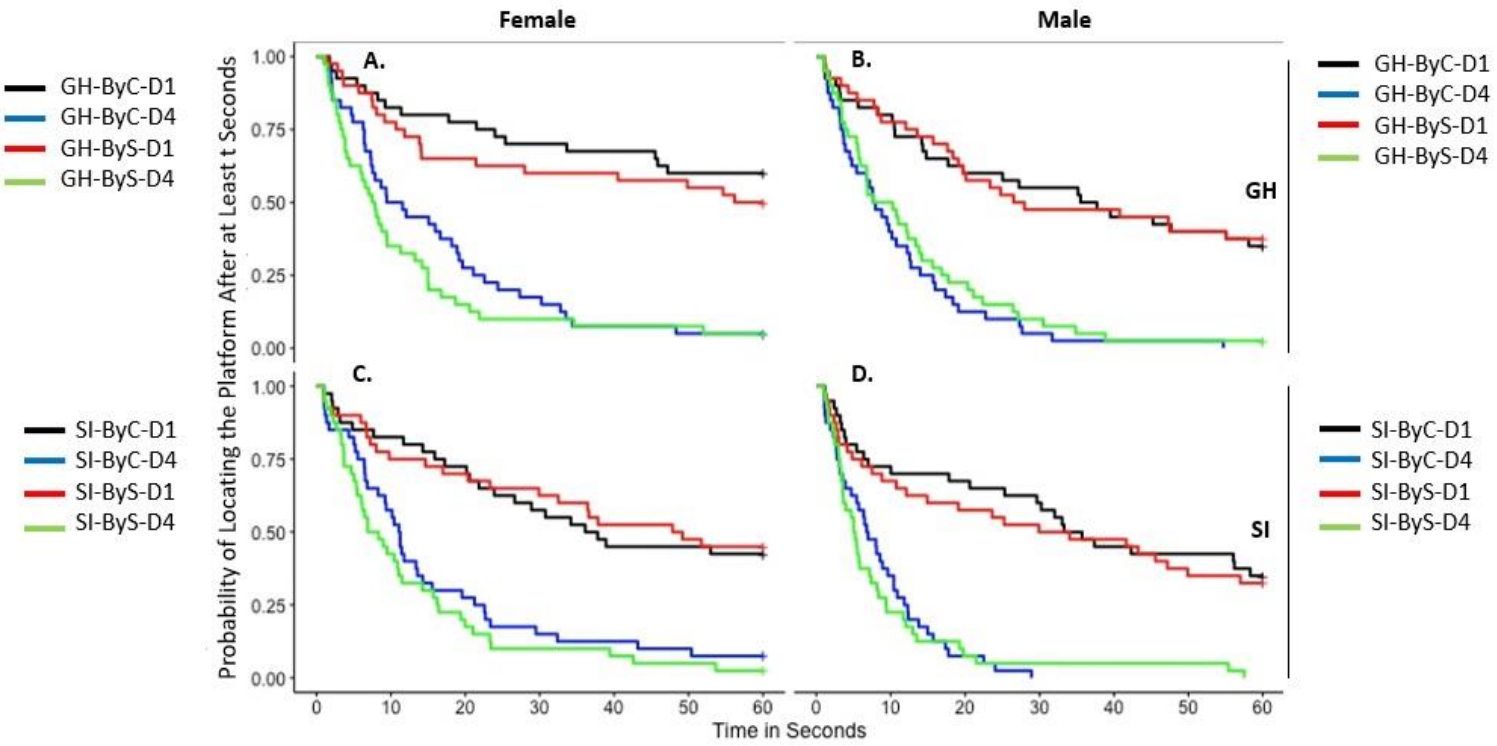


Figure 5.4 A-D. Probability of locating the escape platform displayed by male and female rats in matched and mismatched situations for trials on days 1 and 4 of the learning phase. A significant difference was observed between the performance of male and female animals on day 1 and day 4 across all groups ($p < .0001$), indicating that each group improved their performance over the course of the learning trials. **GH-ByC**, group housed-bystander control; **GH-ByS,** group housed-bystander stress; **SI-ByC**, social isolation-bystander control; **SI-ByS**, social isolation-bystander stress

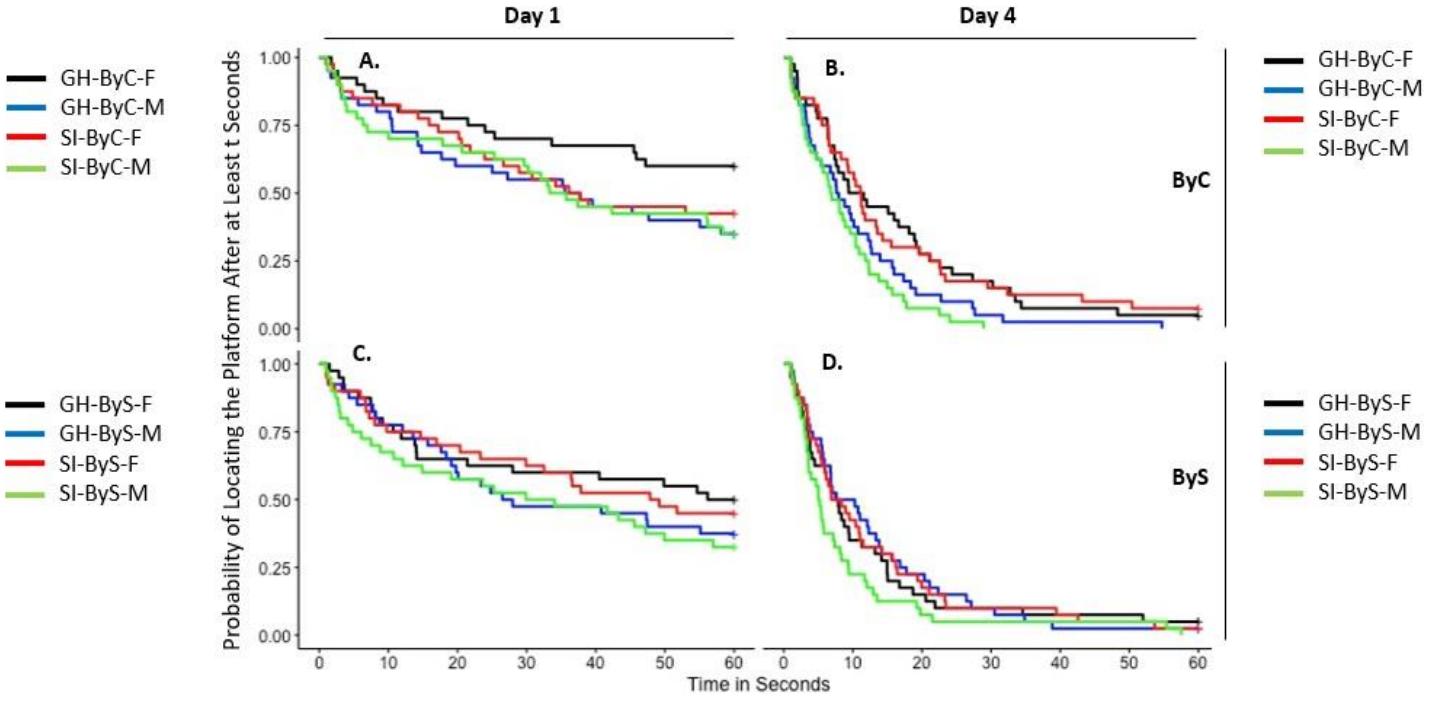


Figure 5.5 A-D. Probability of locating the escape platform by male and female rats on days 1 and 4. GH-ByC, group housed-bystander control; **GH-ByS,** group housed-bystander stress; **SI-ByC**, social isolation-bystander control; **SI-ByS**, social isolation-bystander stress

5.4.4 Performance During the Probe Test

Performance during the probe test was measured by the time spent in the target quadrant. Since the underlying assumptions of the model were met, ANOVA was used to analyse the data. Notably, there was no interaction between the two main factors, sex and stress [$F(3, 72) = 0.05$, $p = .98$]. Moreover, the main effect of stress was not statistically significant [$F(3, 72) = 0.55$, $p = .64$], in contrast to the significant effect of sex [$F(1, 72) = 7.56$, $p = .007$]. In general, different types of matched and mismatched stress situations did not appear to affect the performance of animals during the probe test (figure 5.6).

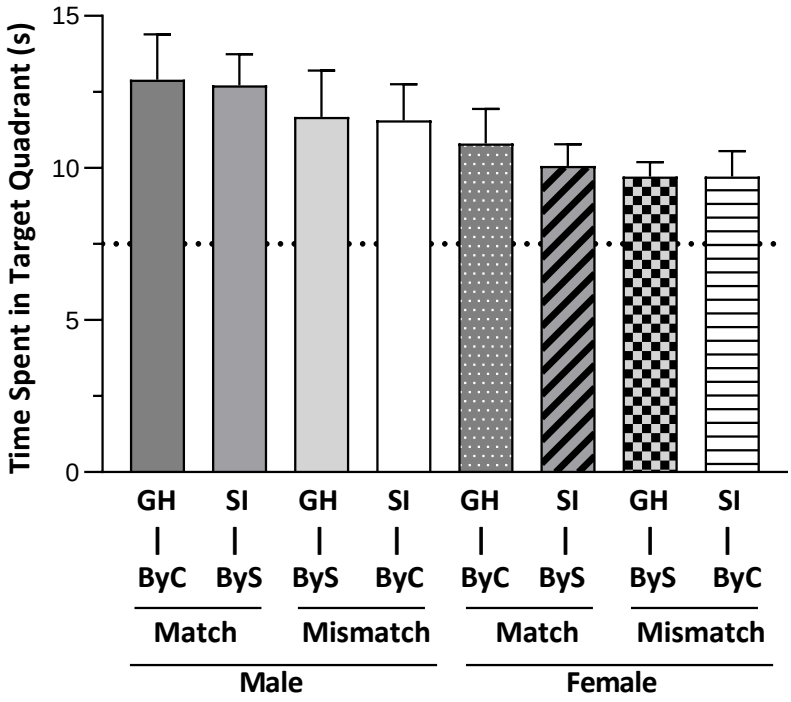


Figure 5.6. Time spent in target quadrant during the probe test. The amount of time spent in the target quadrant was not significantly different across male and female rats that experienced matched, or mismatched stress conditions over the lifespan. The data are displayed as mean ± SEM and the dashed horizontal line presents the performance that would be expected were animals to perform at the level of chance. **GH-ByC**, group housed-bystander control; **GH-ByS,** group housed-bystander stress; **SI-ByC**, social isolation-bystander control; **SI-ByS**, social isolation-bystander stress; **SEM**, standard error of the mean

5.4.5 Neural Protein Expression

In both males and females, immunoblotting was conducted on the septal and temporal poles of rats exposed to ByS in adulthood, after either matched (SI), or mismatched (GH) conditions earlier in the lifespan. Six plasticity-related proteins were analysed using the appropriate parametric models (one-sample t-test and two-way ANOVA) and, in all cases, the underlying assumptions were met (homogeneity of variance, normality, and uncorrelated residuals).

5.4.5.1 SI-ByS Did not Significantly Alter Total GluA1 Expression Regardless of Sex.

Among the male stress matched rats (SI-ByS), total GluA1 expression did not change in either the hippocampal septal (% GH-ByS: 104.4 ± 2.96%, $t(4) = 1.48$, $p = .21$), or temporal (% GH-ByS: 112.2 ± 14.68%, $t(4) = 0.83$, $p = .45$) poles. Furthermore, there was no statistically significant difference in GluA1 in the hippocampal septal (% GH-ByS: 110.6 ± 8.83%, $t(4) = 1.19$, $p = .29$) and temporal (% GH-ByS: 102.7 ± 19.72%, $t(4) = 0.13$, $p = .89$) poles of female SI-ByS rats (figure 5.7, table 5.3 & 5.4).

5.4.5.2 SI-ByS Did not Significantly Alter Total GluA2 Expression Regardless of Sex.

In the hippocampal septal (% GH-ByS: 114.4 ± 7.76%, $t(4) = 1.85$, $p = .13$) and temporal (% GH-ByS: 117.1 ± 17.74%, $t(4) = 0.96$, $p = .38$) poles of male SI-ByS rats, and septal (% GH-ByS: 105.3 ± 11.29%, $t(4) = 0.46$, $p = .66$) and temporal (% GH-ByS: 129.8 ± 20.15%, $t(4) = 1.48$, $p = .21$) poles of female SI-ByS rats, GluA2 expression did not change in a statistically significant manner (figure 5.8, table 5.5 & 5.6).

5.4.5.3 SI-ByS Altered Total GluN1 Expression in the Hippocampal Septal Pole in Male Rats.

Total GluN1 expression levels were significantly increased in the hippocampal septal pole in male SI-ByS rats [% GH-ByS: 124.8 ± 6.13%, t (3) = 4.05, p = .02] compared to their GH-ByS counterparts. In contrast, GluN1 expression levels remained unchanged following SI-ByS in the hippocampal temporal pole in male rats [% GH-ByS: 94.39 ± 4.96%, t (4) = 1.13, p = .32]. As well, no appreciable differences were found in either the septal [% GH-ByS: 99.13 ± 11.10%, t (4) = 0.07, p = .94], or temporal pole [% GH-ByS: 107.5 ± 9.27%, t (4) = 0.81, p = .46] of female rats. Moreover, there was an interaction between poles and sex [F (1, 15) = 5.19, p = .037], (figure 5.9, table 5.7 & 5.8).

5.4.5.4 SI-ByS Did not Significantly Alter Total GluN2A Expression Regardless of Sex.

The expression of total GluN2A remained unchanged in the septal [% GH-ByS: 120.1 ± 12.13%, t (4) = 1.65, p = .17] and temporal poles [% GH-ByS: 109.2 ± 17.63%, t (4) = 0.52, p = .62] of the HP in SI-ByS male rats compared to their GH-ByS counterparts. Similarly, total GluN2A expression did not change in either the septal [% GH-ByS: 108.6 ± 11.74%, t (4) = 0.73, p = .5], or temporal [% GH-ByS: 123.1 ± 17.55%, t (4) = 1.31, p = .25] poles in female SI-ByS rats compared to their respective controls (GH-ByS) (figure 5.10, table 5.9 & 5.10).

5.4.5.5 SI-ByS Did not Significantly Alter Total GluN2B Expression Regardless of Sex.

In male SI-ByS rats, total GluN2B expression in the septal [% GH-ByS: 122.2 ± 11.16%, t (4) = 1.99, p = .11] and temporal [% GH-ByS: 106.7 ± 21.01%, t (4) = 0.32, p = .76]

poles of the HP was not appreciably different from the same pole of the GH-ByS rats (figure 5.11, table 5.11 & 5.12). Likewise, GluN2B levels did not demonstrably change either in septal [% GH-ByS: 118.5 ± 11.92%, t (4) = 1.55, p = .19], or temporal [% GH-ByS: 127.3 ± 15.75%, t (4) = 1.73, p = .15] poles of the female SI-ByS rats.

5.4.5.6 SI-ByS Significantly Altered Total PSD-95 Expression in the Hippocampal Temporal Pole of Female Rats.

In the septal [% GH-ByS: 97.38 ± 7.18%, t (4) = 0.36, p = .73] and temporal [% GH-ByS: 98.58 ± 17.80%, t (4) = 0.07, p = .94] poles of the male SI-ByS rats and the septal [% GH-ByS: 97.57 ± 3.28%, t (3) = 0.74, p = .51] pole of the female SI-ByS rats, PSD-95 expression appeared unchanged (figure 5.12, table 5.13 & 5.14). However, in the temporal pole of female rats, SI-ByS caused an increase in total PSD-95 expression, which was significant compared to the septal pole [difference between means ± SEM: 46.10 ± 15.05, t (6) = 3.06, p = .02]. In addition, sex [F (1, 14) = 3.16, p = .09] and pole [F (1, 14) = 3.44, p = .08] did not significantly affect the changes caused by SI-ByS on total PSD-95 expression.

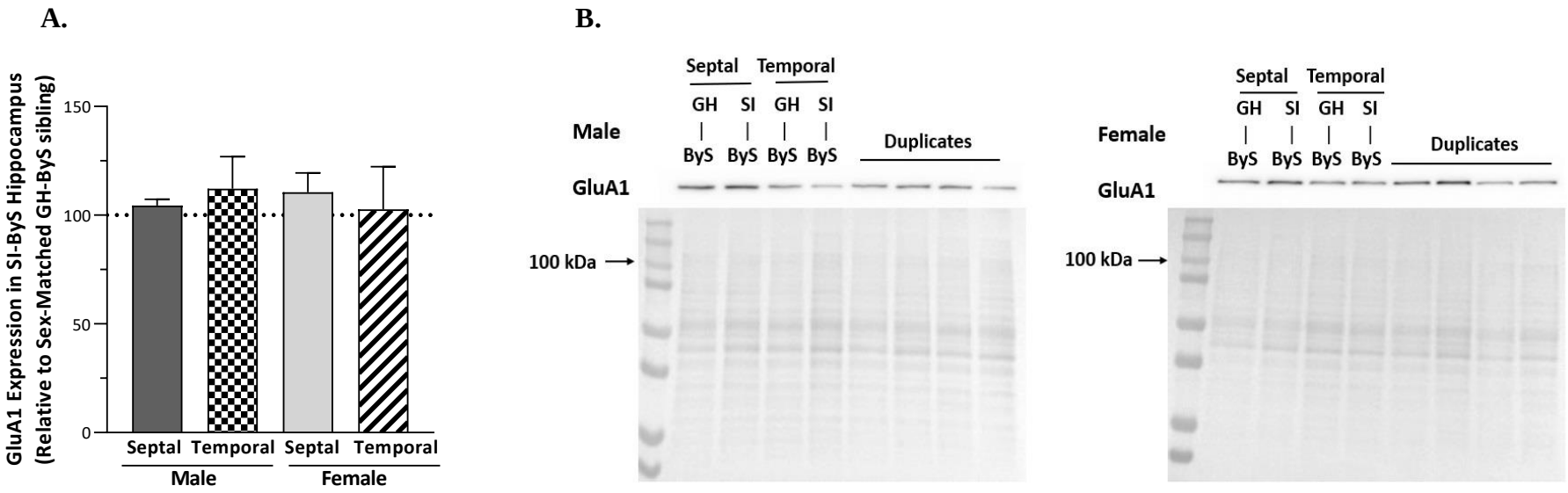


Figure 5.7. Total GluA1 expression in the hippocampal septal and temporal poles of male and female SI-ByS rats. (A) The summary graph presents the effect of SI-ByS on total GluA1 expression in the hippocampal septal and temporal poles of male and female rats. Each septal, or temporal SI-ByS sample was taken as a percentage of their respective control group (that is, septal, or temporal GH-ByS). (B) Representative immunoblot images of total GluA1 expression in the hippocampal septal and temporal male and female SI-ByS rats, and their respective control groups. Male GH-ByS septal (N = 5), Male SI-ByS temporal (N = 5), Female GH-ByS septal (N = 5), Female SI-ByS temporal (N = 5). All data are expressed as mean ± SEM. **GH-ByC**, group housed-bystander control; **GH-ByS,** group housed-bystander stress; **SI-ByC**, social isolation-bystander control; **SI-ByS**, social isolation-bystander stress

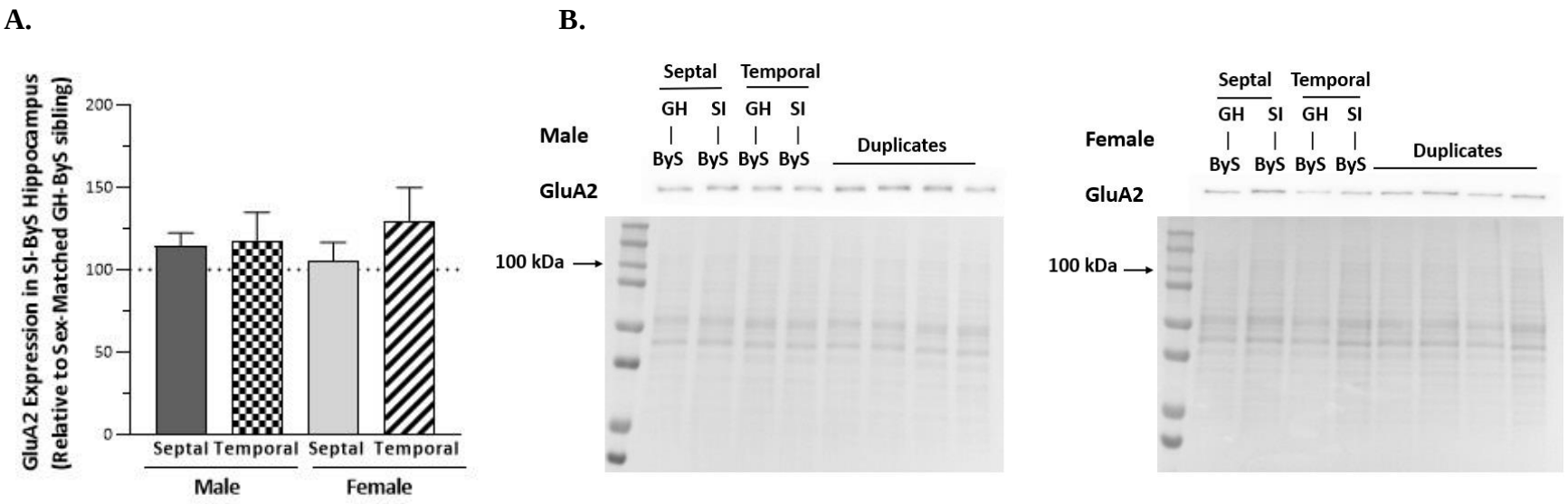


Figure 5.8. Total GluA2 expression in the hippocampal septal and temporal poles of male and female SI-ByS rats. (A) The summary graph presents the effect of SI-ByS on total GluA2 expression in the hippocampal septal and temporal poles of male and female rats. Each septal, or temporal SI-ByS sample was taken as a percentage of their respective control group (that is, septal, or temporal GH-ByS). (B) Representative immunoblot images of total GluA2 expression in the hippocampal septal and temporal male and female SI-ByS rats, and their respective control groups. Male GH-ByS septal (N = 5), Male SI-ByS temporal (N = 5), Female GH-ByS septal (N = 5), Female SI-ByS temporal (N = 5). All data are expressed as mean ± SEM. **GH-ByC**, group housed-bystander control; **GH-ByS,** group housed-bystander stress; **SI-ByC**, social isolation-bystander control; **SI-ByS**, social isolation-bystander stress

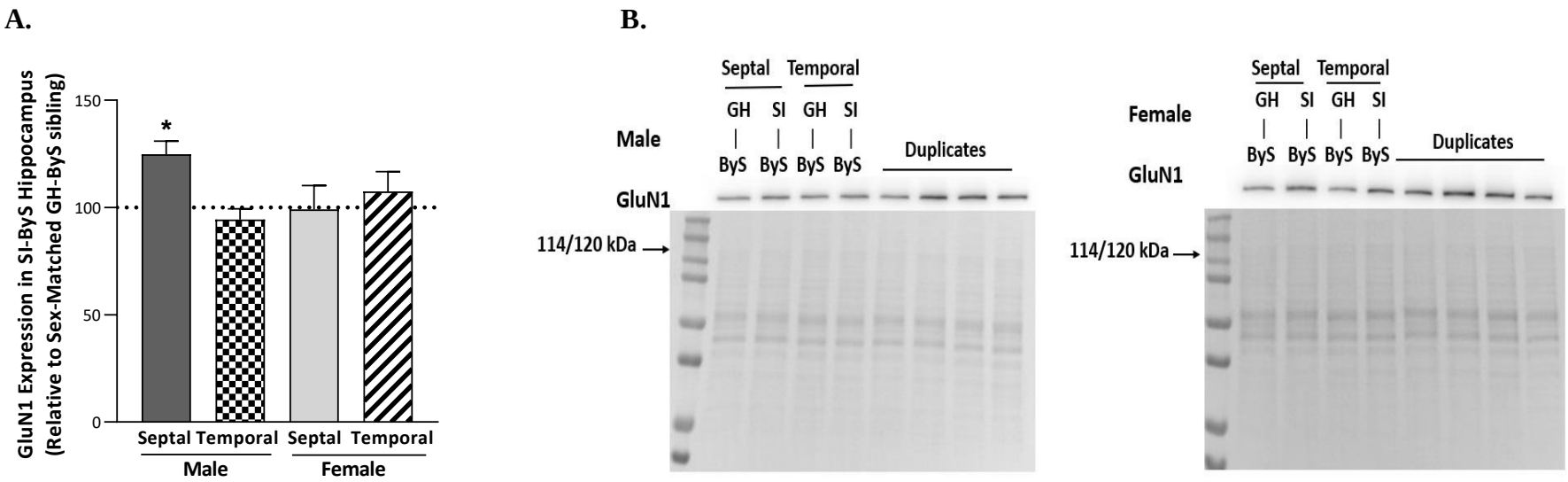


Figure 5.9. Total GluN1 expression in the hippocampal septal and temporal poles of male and female SI-ByS rats. (A) The summary graph presents the effect of SI-ByS on total GluN1 expression in the hippocampal septal and temporal poles of male and female rats. Each septal, or temporal SI-ByS sample was taken as a percentage of their respective control group (that is, septal, or temporal GH-ByS). (B) Representative immunoblot images of total GluN1 expression in the hippocampal septal and temporal male and female SI-ByS rats, and their respective control groups. Male GH-ByS septal (N = 4), Male SI-ByS temporal (N = 5), Female GH-ByS septal (N = 5), Female SI-ByS temporal (N = 5). All data are expressed as mean ± SEM. *statistically significant p-value of < .05. **GH-ByC**, group housed-bystander control; **GH-ByS,** group housed-bystander stress; **SI-ByC**, social isolation-bystander control; **SI-ByS**, social isolation-bystander stress

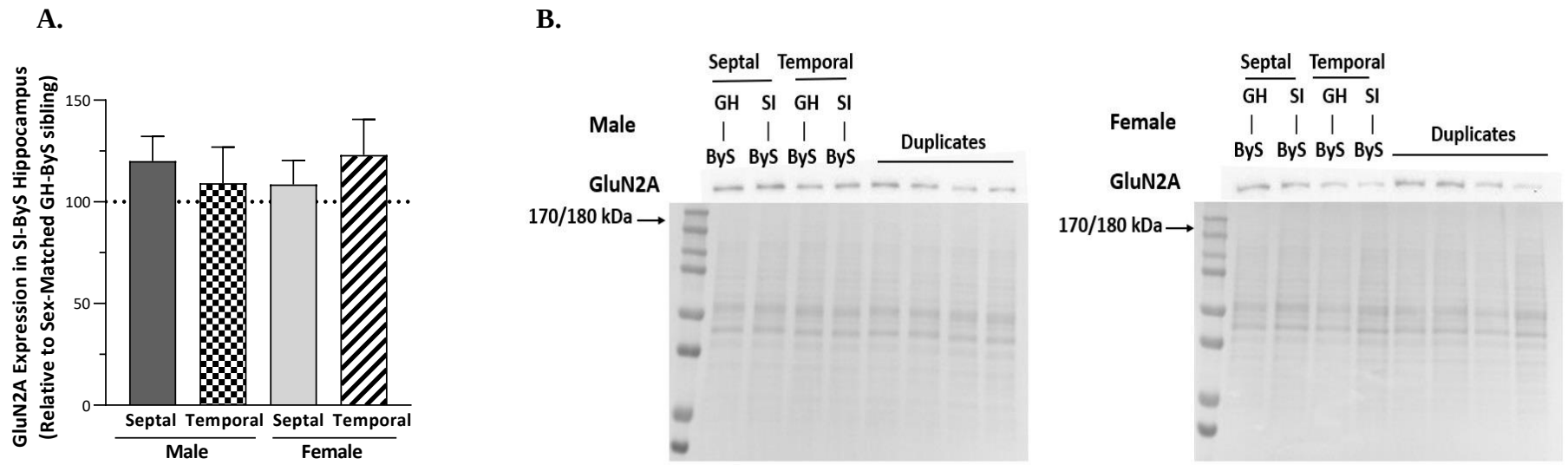


Figure 5.10. Total GluN2A expression in the hippocampal septal and temporal poles of male and female SI-ByS rats. (A) The summary graph presents the effect of SI-ByS on total GluN2A expression in the hippocampal septal and temporal poles of male and female rats. Each septal, or temporal SI-ByS sample was taken as a percentage of their respective control group (that is, septal, or temporal GH-ByS). (B) Representative immunoblot images of total GluN2A expression in the hippocampal septal and temporal male and female SI-ByS rats, and their respective control groups. Male GH-ByS septal (N = 5), Male SI-ByS temporal (N = 5), Female GH-ByS septal (N = 5), Female SI-ByS temporal (N = 5). All data are expressed as mean ± SEM. **GH-ByC**, group housed-bystander control; **GH-ByS,** group housed-bystander stress; **SI-ByC**, social isolation-bystander control; **SI-ByS**, social isolation-bystander stress

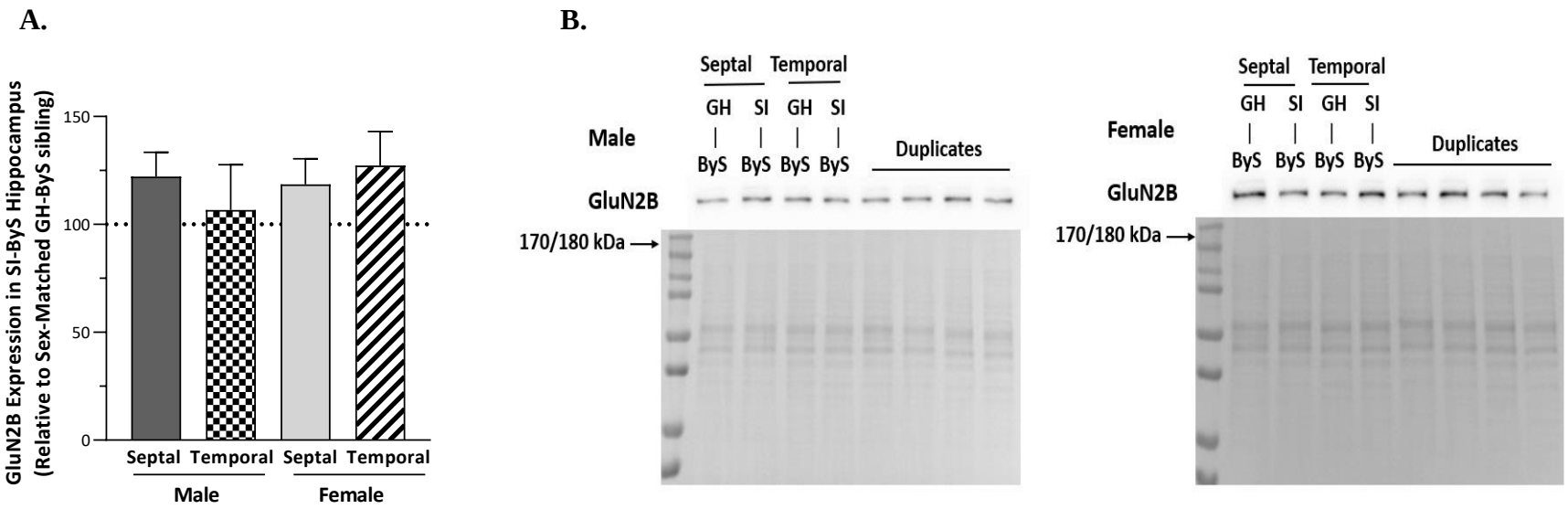


Figure 5.11. Total GluN2B expression in the hippocampal septal and temporal poles of male and female SI-ByS rats. (A) The summary graph presents the effect of SI-ByS on total GluN2B expression in the hippocampal septal and temporal poles of male and female rats. Each septal, or temporal SI-ByS sample was taken as a percentage of their respective control group (that is, septal, or temporal GH-ByS). (B) Representative immunoblot images of total GluN2B expression in the hippocampal septal and temporal male and female SI-ByS rats, and their respective control groups. Male GH-ByS septal (N = 5), Male SI-ByS temporal (N = 5), Female GH-ByS septal (N = 5), Female SI-ByS temporal (N = 5). All data are expressed as mean ± SEM. **GH-ByC**, group housed-bystander control; **GH-ByS,** group housed-bystander stress; **SI-ByC**, social isolation-bystander control; **SI-ByS**, social isolation-bystander stress

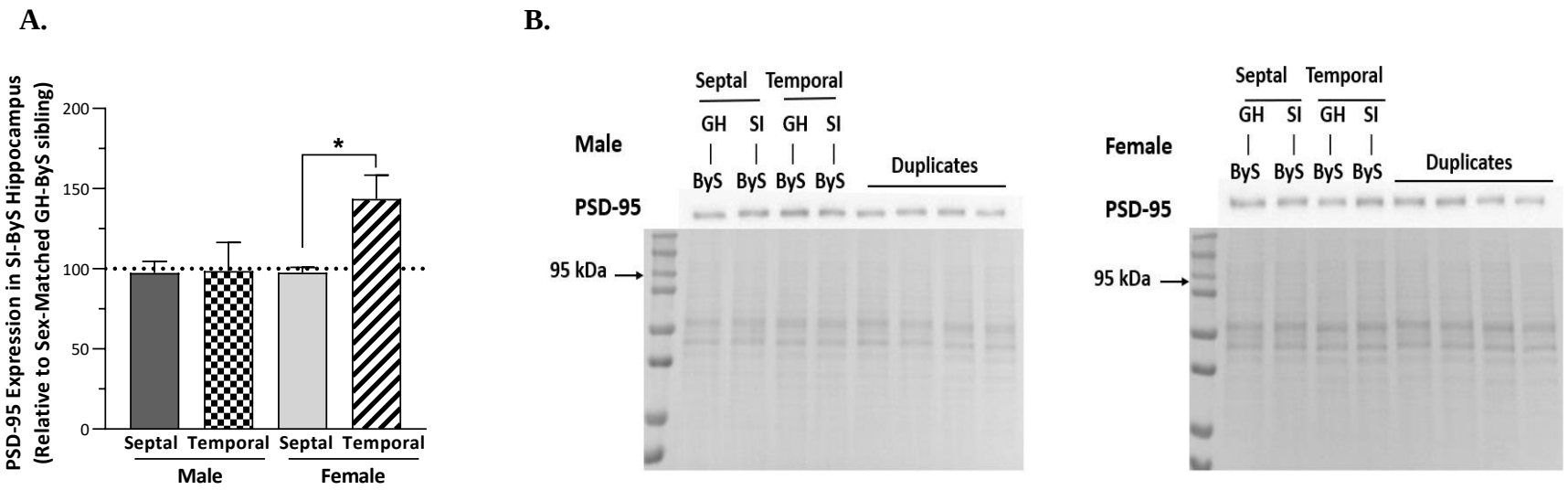


Figure 5.12. Total PSD-95 expression in the hippocampal septal and temporal poles of male and female SI-ByS rats. (A) The summary graph presents the effect of SI-ByS on total PSD-95 expression in the hippocampal septal and temporal poles of male and female rats. Each septal, or temporal SI-ByS sample was taken as a percentage of their respective control group (that is, septal, or temporal GH-ByS). (B) Representative immunoblot images of total PSD-95 expression in the hippocampal septal and temporal male and female SI-ByS rats, and their respective control groups. Male GH-ByS septal (N = 5), Male SI-ByS temporal (N = 5), Female GH-ByS septal (N = 4), Female SI-ByS temporal (N = 4). All data are expressed as mean ± SEM *statistically significant p-value of <0.05. **GH-ByC**, group housed-bystander control; **GH-ByS,** group housed-bystander stress; **SI-ByC**, social isolation-bystander control; **SI-ByS**, social isolation-bystander stress; **SEM**, standard error of the mean

SI-ByS	Region	Mean (%GH-ByS)	Percent change	N	T-statistic	p value
Males	Septal	104.4	4.39	5	1.48	.21
	Temporal	112.2	12.23	5	0.83	.45
Females	Septal	110.6	10.58	5	1.19	.29
	Temporal	102.7	2.65	5	0.13	.89

Table 5.3. Summary of the one-sample t-test analyses for total GluA1.

The septal and temporal poles of the SI-ByS rats were compared to the septal and temporal poles of the GH-ByS rats, respectively, in both male and female rats. Theoretical mean used for each comparison = 100.

Source of Variation	% of total variation	p value
Interaction	2.19	.55
Poles	6.76	.99
Sex	0.1	.89

ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Interaction	310.5	1	310.5	F (1, 16) = 0.35	p = .55
Poles	0.009	1	0.009	F (1, 16) = 1.108	p = .99
Sex	14.37	1	14.37	F (1, 16) = 0.016	p = .89
Residual	13826	16	864.1		

Table 5.4. Summary of the two-way ANOVA test for total GluA1.

Male and female rats were compared for significant differences in total GluA1 levels caused by SI-ByS in the hippocampal septal and temporal poles.

SI-ByS	Region	Mean (%GH-ByS)	Percent change	N	T-statistic	p value
Males	Septal	114.4	14.42	5	1.85	.13
	Temporal	117.1	17.12	5	0.96	.38
Females	Septal	105.3	5.3	5	0.46	.66
	Temporal	129.8	29.84	5	1.48	.21

Table 5.5. Summary of the one-sample t-test analyses for total GluA2.

The septal and temporal poles of the SI-ByS rats were compared to the septal and temporal poles of the GH-ByS rats, respectively, in both male and female rats. Theoretical mean used for each comparison = 100.

Source of Variation	% of total variation	p value			
Interaction	3.02	.47			
Poles	4.7	.37			
Sex	0.08	.90			
ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Interaction	596.5	1	296.5	F (1, 16) = 0.52	p = .47
Poles	927	1	927	F (1, 16) = 0.81	p = .37
Sex	16.16	1	16.16	F (1, 16) = 0.01	p = .9
Residual	18174	16	1136		

Table 5.6. Summary of the two-way ANOVA test for total GluA2.

Male and female rats were compared for significant differences in total GluA2 levels caused by SI-ByS in the hippocampal septal and temporal poles.

SI-ByS	Region	Mean (%GH-ByS)	Percent change	N	T-statistic	p value
Males	Septal	124.8	24.85	4	4.05	.02*
	Temporal	94.39	-5.61	5	1.13	.32
Females	Septal	99.13	-0.87	5	0.07	.94
	Temporal	107.5	7.52	5	0.81	.46

Table 5.7. Summary of the one-sample t-test analyses for total GluN1.

The septal and temporal poles of the SI-ByS rats were compared to the septal and temporal poles of the GH-ByS rats, respectively, in both male and female rats. Theoretical mean used for each comparison = 100.

Source of Variation	% of total variation	p value			
Interaction	23.79	.03			
Poles	7.66	.21			
Sex	2.49	.47			
ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Interaction	1776	1	1776	F (1, 15) = 5.19	p = .03
Poles	572.6	1	572.6	F (1, 15) = 1.67	p = .21
Sex	186.1	1	186.1	F (1, 15) = 0.54	p = .47
Residual	5130	15	342		

Table 5.8. Summary of the two-way ANOVA test for total GluN1.

Male and female rats were compared for significant differences in total GluN1 levels caused by SI-ByS in the hippocampal septal and temporal poles.

SI-ByS	Region	Mean (%GH-ByS)	Percent change	N	T-statistic	p value
Males	Septal	120.1	20.05	5	1.65	.17
	Temporal	109.2	9.24	5	0.52	.62
Females	Septal	108.6	8.57	5	0.73	.5
	Temporal	123.1	23.06	5	1.31	.25

Table 5.9. Summary of the one-sample t-test analyses for total GluN2A.

The septal and temporal poles of the SI-ByS rats were compared to the septal and temporal poles of the GH-ByS rats, respectively, in both male and female rats. Theoretical mean used for each comparison = 100.

Source of Variation	% of total variation	p value			
Interaction	4.23	.41			
Poles	0.08	.9			
Sex	0.03	.93			
ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Interaction	799.8	1	799.8	F (1, 16) = 0.7	p = .41
Poles	16.87	1	16.87	F (1, 16) = 0.01	p = .9
Sex	6.88	1	6.88	F (1, 16) = 0.006	p = .93
Residual	18078	16	1130		

Table 5.10. Summary of the two-way ANOVA test for total GluN2A.

Male and female rats were compared for significant differences in total GluN2A levels caused by SI-ByS in the hippocampal septal and temporal poles.

SI-ByS	Region	Mean (%GH-ByS)	Percent change	N	T-statistic	p value
Males	Septal	122.2	22.23	5	1.99	.11
	Temporal	106.7	6.73	5	0.32	.76
Females	Septal	118.5	18.53	5	1.55	.19
	Temporal	127.3	27.34	5	1.73	.15

Table 5.11. Summary of the one-sample t-test analyses for total GluN2B.

The septal and temporal poles of the SI-ByS rats were compared to the septal and temporal poles of the GH-ByS rats, respectively, in both male and female rats. Theoretical mean used for each comparison = 100.

Source of Variation	% of total variation	p value			
Interaction	3.64	.44			
Poles	0.27	.83			
Sex	1.76	.59			
ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Interaction	738.4	1	738.4	F (1, 16) = 0.61	p = .44
Poles	55.71	1	55.71	F (1, 16) = 0.04	p = .83
Sex	357.2	1	357.2	F (1, 16) = 0.29	p = .59
Residual	19118	16	1195		

Table 5.12. Summary of the two-way ANOVA test for total GluN2B.

Male and female rats were compared for significant differences in total GluN2B levels caused by SI-ByS in the hippocampal septal and temporal poles.

SI-ByS	Region	Mean (%GH-ByS)	Percent change	N	T-statistic	p value
Males	Septal	97.38	-2.62	5	0.36	.73
	Temporal	98.58	-1.42	5	0.07	.94
Females	Septal	97.57	-2.43	4	0.74	.51
	Temporal	143.7	43.66	4	2.97	.05

Table 5.13. Summary of the one-sample t-test analyses for total PSD-95.

The septal and temporal poles of the SI-ByS rats were compared to the septal and temporal poles of the GH-ByS rats, respectively, in both male and female rats. Theoretical mean used for each comparison = 100.

Source of Variation	% of total variation	p value			
Interaction	13.48	.09			
Poles	14.95	.08			
Sex	13.7	.09			
ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Interaction	2240	1	2240	F (1, 14) = 3.1	p = .09
Poles	2485	1	2485	F (1, 14) = 3.44	p = .08
Sex	2277	1	2277	F (1, 14) = 3.16	p = .09
Residual	10088	14	720.6		

Table 5.14. Summary of the two-way ANOVA test for total PSD-95.

Male and female rats were compared for significant differences in total PSD-95 levels caused by SI-ByS in the hippocampal septal and temporal poles.

5.5 DISCUSSION

5.5.1 The Effect of Matched and Mismatched Stress Conditions Across the Lifespan on Spatial Learning and Memory

By exposing rats to chronic social isolation, or group housing during a substantial portion of post-natal development, followed by exposure to either bystander stress, or bystander control conditions in adulthood, we examined whether matched/mismatched stress situations could affect the ability of the hippocampus to acquire and recall spatial memories. Over the course of the four days of the learning phase, rats from all possible matched and mismatched situations could successfully navigate the water maze. However, on day one, the spatial learning ability of the male animals was significantly higher in the group where control conditions were matched across the lifespan (GH-ByC), and these animals found the hidden platform 1.9 times faster than their female counterparts. We did not expect this result since we did not observe a difference between male and female control rats in MWM spatial learning performance in our previous studies when either group-housed, or bystander control was used. Similarly, on day four, male rats from the early-life stress mismatch group (mismatch 1, SI-ByC) found the platform 1.9 times faster than female rats from the same condition. Considering that the animals in this study would have been several months older than the animals in the earlier studies, this difference could be attributed to their age.

Furthermore, male SI-ByS (stress matched) rats performed better than male GH-ByS (mismatch 2) rats. Considering that the results of the previous chapter showed that ByS did not affect water maze performance, this result indicates that the SI-ByS combination enhanced

spatial learning performance in male rats. Based on our previous results (CELSI study, chapter 3) and the match/mismatch hypothesis, we expected this result. In other words, in a similar manner as CELSI findings demonstrated the improvement in performance resulting from SI, this study showed the improvement in learning performance as a result of SI x ByS for male rats. Therefore, SI either alone, or with the adulthood stressor (ByS) enhanced spatial learning performance in male rats. This result indicates that even six months, which is equivalent to approximately eighteen years on the human life scale (Sengupta, 2013), after being exposed to social isolation, there is a sex-specific effect in different stages of spatial learning.

The persistence of hippocampal-dependent spatial memory in our animals was assessed 24 hours after the last learning trial by measuring the time spent in the target quadrant during a probe trial. No significant differences regarding time spent in the target quadrant were observed between any of the groups, although we expected to see a difference at least between rats from control match and mismatch 1, since their conditions were similar to those of the CELSI study rats (control match was similar to the GH condition and mismatch 1 was similar to the SI situation). Therefore, this result was not consistent with our CELSI study outcomes, where socially isolated male and female rats showed better spatial memory compared to GH rats. It may be the result of the six-month interval between the developmental stressor (CELSI) and the adulthood stressor (ByS) that all rat groups performed approximately the same in the spatial memory phase. Probably during the interval, biochemical changes reversed, which resulted in differences in behaviour.

5.5.2 AMPAR and NMDAR Subunits Expression Following Bystander Stress

A significant objective of this study was to examine whether the match/mismatch stress paradigm altered the expression of certain plasticity-related proteins belonging to the glutamate receptor family, as well as whether any observed changes differed by sex in the septal and temporal poles of the hippocampus. Regarding the effects of the match/mismatch stress paradigm on AMPAR subunits, we examined the expression of total GluA1 and GluA2 in the hippocampal septal and temporal poles of male and female rats (table 5.15). No statistically significant difference in total GluA1 and GluA2 expression was observed in the hippocampal septal and temporal poles of the male and female SI-ByS rats compared to GH-ByS rats. Notably, GluA2 expression level was 29.8% greater in the temporal pole of female SI-ByS rats compared to their GH-ByS counterparts, which, while not statistically significant, was above our "threshold of interest" (20%).

In order to determine whether bystander stress alters the expression of NMDAR subunits, the GluN1, GluN2A, and GluN2B subunits were evaluated in the hippocampal septal and temporal poles of male and female stress matched (SI-ByS) rats compared to control matched (GH-ByC) rats (table 5.15). Total GluN1 expression was significantly increased by SI-ByS in the hippocampal septal pole in male rats (24.8%). For GluN2A and GluN2B, an increase was observed in septal male SI-ByS (20 and 22.2%) and temporal SI-ByS female (23 and 27.3%) compared to control matched rats. Our threshold of interest was exceeded by these changes, although they were not statistically significant. Moreover, the temporal pole of female stress matched rats showed an increase (43.6%) in PSD-95 expression compared to female control matched rats. Again, even though these changes were not statistically significant, they

exceeded our threshold of interest. In addition, there was a significant difference in PSD-95 expression between the septal and temporal poles in female SI-ByS rats. In general, the stress matched situation resulted in statistically significant increases in the total expression of GluN1 and PSD-95, which are synaptic plasticity-related proteins associated with the function of NMDA receptors. The AMPA receptor subunits of both male and female rats were not significantly altered by exposure to the stress matched condition.

5.5.3 What Is the Reason(s) for the Difference in Spatial Learning Performance Between Male and Female Rats?

The difference between male and female rats can be explained by the fact that male and female rodents respond to stress differently. In particular, female rats exposed to social stress display behavioural changes (such as increased sedentary behaviour in open field tests) and their basal dendrite lengths in the CA3 hippocampal neurons are reduced (Leedy et al., 2013). Chronic stress triggers dendritic retraction in the CA3 region, disrupting the hypothalamic-pituitary-adrenal axis and dysregulation of the release of glucocorticoids. Combining hippocampal CA3 dendritic retraction with increased glucocorticoid release leads to impairments in spatial memory (Conrad, 2006). Furthermore, according to our CELSI study results (please see chapter 3), social isolation experienced early in the lifespan improved spatial memory and increased synaptic plasticity-related proteins (such as PSD-95) in male rats and it is possible that a similar mechanism may have occurred here. It is noteworthy that in the CELSI study, experiencing early life social isolation significantly influenced spatial memory performance (that is, probe test performance) in both male and female rats, whereas in study three, it appears that early life social isolation influenced spatial learning rather than memory.

Moreover, in the CELSI study, since the assumption of proportional hazards was not valid for sex on either day 1, or day 4, and an interaction between sex and housing conditions was observed, separate statistical models for males and females were conducted. As a result, the direct comparison of males and females was therefore not conducted in the CELSI study. More specifically, the results of the third study showed significant differences in spatial learning (rather than in spatial memory, as seen in the CELSI study) between male and female mismatched 1. Male rats exposed to social isolation during their developmental period and bystander control during adulthood (mismatch 1, SI-ByC) performed better in the late stages of spatial learning (day four) as compared to their female counterparts.

As mentioned above, these results can be explained by a sex-specific stress response which results in a variety of biochemical and anatomical changes in the brain. In our study, experiencing social isolation in early life improved the late stage of spatial learning in male mismatch 1 rats (SI-ByC) compared to females. These findings suggest that various stress models can affect learning at different stages of development, likely due to changes in the expression level of synaptic plasticity-related proteins at specific hippocampal poles.

5.5.4 What Explains the Lack of Differences in Spatial Memory Performance Between GH Rats and SI Rats in this Study Compared to the CELSI Study (chapter 3)?

The control match and mismatch 1 rats were exposed to approximately the same conditions as the GH or SI rats in study one (CELSI study, chapter 3), so we expected similar results. In spite of this, no statistically significant differences were observed within males or females regardless of developmental stress situations (either GH or SI). The results of this study are not in accordance with those of study one in which SI males and females showed

better spatial memory in comparison to their same sex counterparts. However, it should be noted that after seven weeks of social isolation in study three, same sex SI rats were paired for a six-month period before water maze testing. As a result, it is possible that the social isolation induced biochemical changes that could have explained the behavioural changes seen in the CELSI study were washed out during the 6-month interval.

5.5.5 Comparison of the Results with Other Studies

The match/mismatch hypothesis has been studied in a limited number of reports using different species, different types of early-life and adulthood stressors, as well as different levels of intensity and duration, and these data have not revealed a clear pattern. According to the match/mismatch hypothesis, early life experiences should have the potential to alter how adulthood stressors affect the brain's response to stress, thereby affecting cognitive functions including learning and memory. In this regard, a stressful childhood can help prepare an individual for a stressful adulthood by utilizing "adaptive programming", which matches events experienced over a long period of time; however, if there is a mismatch between childhood and adulthood events, it may result in negative outcomes, including behavioural problems.

In support of the match/mismatch hypothesis, Zalosnik et al. (2014) found that rats exposed to mismatch situations (non-maternally separated and stressed animals) performed poorly on hippocampal-dependent memory retrieval tasks (contextual fear conditioning test) compared to rats in the matched situation (maternally separated and stressed animals). During their study, male Wistar rats were subjected to daily maternal separation for 4.5 hours between postnatal days 1 and 21, and then, from postnatal day 50, they were exposed to a chronic

unpredictable stress paradigm for 24 days. Similarly, Santarelli et al., (2014) investigated the role of early life experience and adult environment in the development of anxiety-like and depression-like behaviours in female Balb/c mice. Based on their findings, it was evident that animals with mismatched environmental conditions behaved differently than animals with matched environmental conditions regarding anxious, social, and depressive phenotypes. In addition to using limited nesting and bedding materials to induce stress during childhood (PND 2-14), mice were also socially isolated during adulthood (PND 50-97) in their study (Santarelli et al., 2014a). Our findings are in part consistent with the Zalosnik, et al. (2014) study, since male rats in our stress matched situation exhibited superior spatial learning abilities in MWM. However, since we found no significant difference in spatial memory in male and female rats exposed to either match, or mismatch conditions, our memory results are also somewhat different from those of Zalosnik, et al. (2014), who concluded that memory retrieval was weaker in non-maternally separated and stressed animals (mismatched situation) than in control animals. Notably, they used different types of stress to create match/mismatch situations, as well as a different behavioural test.

Based on the results of the study, it appears that the different stress conditions associated with the combination of early life and adulthood environments had some effects on hippocampal dependent spatial learning. In particular, male stress matched rats (SI-ByS) performed better than male mismatch 2 (GH-ByS) rats in the MWM task suggesting that experiencing social stress during the developmental period had strong sex-specific effects on stress responses in adulthood, even after 6 months living in the group housing condition. Moreover, the matched stress situation (SI-ByS) altered the expression of some plasticity

related proteins in the hippocampus (GluN1 expression in the septal pole of male SI-ByS rats clearly increased compared to the septal pole of male GH-ByS rats, and there was a significant increase in the total expression of PSD-95 in the temporal pole of the female SI-ByS rats in comparison to the septal pole). Our biochemical results and behavioural observations were clearly correlated, with higher levels of GluN1 expression observed in the septal pole of male stress matched rats that did better in spatial learning than male mismatched 2 rats. The septal pole has been found to be strongly correlated with cognitive function (Fanselow & Dong, 2010; Herman et al., 1995). Moreover, damage to the septal pole adversely affects learning, memory, and spatial navigation (Zhang et al., 2004). The increased GluN1 expression found in the hippocampal septal pole of male stress-matched rats may have helped strengthen NMDA receptor function that, in turn, helped strengthen spatial learning.

Taken together, these findings provide support for the match/mismatch hypothesis in a sex-specific manner, showing that male rats exposed to stress condition across the lifespan have better spatial learning performance than those exposed to only adulthood stress (the mismatch 2 condition). Furthermore, these findings suggest that early social isolation may have lifelong effects, specifically in male rats.

	Social Isolation-Bystander Stress (SI-ByS)			
	Male		Female	
	Septal	Temporal	Septal	Temporal
Total GluA1	↑ (4.4%)	↑ (12.2%)	↑ (10.5%)	↑ (2.6%)
Total GluA2	↑ (14.4%)	↑ (17.1%)	↑ (5.3%)	↑ (29.8%)
Total GluN1	↑ **(24.8%)***	↓ (-5.6%)	↓ (-0.8%)	↑ (7.5%)
Total GluN2A	↑ (20%)	↑ (9.2%)	↑ (8.5%)	↑ (23%)
Total GluN2B	↑ (22.2%)	↑ (6.7%)	↑ (18.5%)	↑ (27.3%)
Total PSD-95	↓ (-2.6%)	↓ (-1.4%)	↓ (-2.4%)	↑ (43.6%)

Table 5.15. Summary of all findings involving plasticity-related proteins.

↑,↓ signifies an increase and decrease respectively in the hippocampal septal and temporal poles in SI-ByS rats compared to their control group (GH-ByS)

***** denotes p value < .05 in the SI-ByS compared to the GH-ByS group

↑,↓, denotes p value < .05 between/within septal and temporal poles of the SI-ByS rats

Chapter 6
SUMMARY

6.1 Summary of Main Findings

(Grippo et al., 2007; Heim et al., 2004; Reinwald et al., 2018; Thorsell et al., 2006). Notably, the prevalence of these disorders is highly sex-dependent (Kendler et al., 1995; Piccinelli & Wilkinson, 2000), but little is known about how the mechanisms that connect social stress to brain development and function are impacted by sex-related differences.

To better understand how social stress can affect brain development, two models of social stress (chronic early-life social isolation, or CELSI, a developmental stressor, and bystander stress, or ByS, an adult stressor), alone and in combination, were investigated in Chapters 3, 4, and 5 using behavioural and biochemical measurements. In this regard, the dissertation sought to fill knowledge gaps by answering the following primary research questions: *"Does experiencing psychosocial stress in early life (CELSI) affect the behavioural and biochemical responses to psychosocial stress encountered in adulthood (ByS)?"* and *"Do male and female animals exhibit different responses?"*.

We began in chapter 3 by investigating the influence that chronic early-life social isolation (CELSI) may have on spatial learning and memory and the expression of key neuronal and glial structural proteins (PSD-95 and GFAP, respectively) in the hippocampus, as well as the impact of CELSI on a number of stress-sensitive biometrics, such as the level of serum CORT and the weight of the adrenal glands, retroperitoneal fat pads, and liver. Notably, both male and female rats that had experienced post-weaning social isolation displayed stronger spatial learning and memory abilities than their group-housed counterparts. As well,

socially isolated male rats exhibited a clear increase in expression of PSD-95. However, housing condition did not seem to affect either stress-sensitive biometrics, or hippocampal GFAP expression. Our results support the possibility that CELSI may enhance some aspects of hippocampal-dependent behaviour in a fashion similar among male and female rats.

In the second study (chapter 4), we applied "bystander stress" (ByS) to adult male and female rats for five consecutive days and measured its effects on spatial learning and memory (cohort 1) and the synaptic expression of a series of plasticity-related proteins (i.e., GluN1, GluN2A, GluN2B, GluA1, GluA2, and PSD-95) in septal and temporal poles of the hippocampus (cohort 2). Neither male nor female rats were significantly affected by ByS in terms of spatial learning and memory. Our findings suggest that bystander stress appeared to affect the synaptic expression of certain plasticity-related proteins in both a sex and region-dependent manner (GluA2, GluN2A, GluN2B). While no statistical significance was observed between male and female rats for any particular measure, except for PSD-95 in the temporal pole, male rats displayed more overall changes compared to female rats, which suggests sex-dependent effects of ByS on plasticity-related protein expression in the hippocampus.

In study three (chapter 5), we examined whether experiencing CELSI changes the behavioural and biochemical responses to ByS in male and female rats to see if exposure to early-life stress influences the effect of social stress in adulthood by measuring hippocampal-dependent behaviour (through testing spatial learning and memory using the Morris water maze) and evaluating the synaptic expression of a series of plasticity-related proteins (GluN1, GluN2A, GluN2B, GluA1, GluA2, and PSD-95) in septal and temporal poles of the hippocampus. From the behavioural perspective, there was evidence of sexual dimorphism in

spatial learning among rats exposed to the control match (GH-ByC) situation on the first spatial learning training day; as well, on the final training day male rats from the stress match (SI-ByS) and mismatch 1 (SI-ByC) situations performed significantly better than their female counterparts. As a result, social isolation during the post-weaning developmental period may be related to spatial learning performance in adulthood in a sex-dependent manner. Further, male stress match rats (SI-ByS) demonstrated better spatial learning performance than male mismatch 2 rats (GH-ByS). In addition, the SI-ByS effect was generally characterized by a greater expression of the NMDA receptor obligatory subunit (GluN1) in the septal pole of the HP compared to GH-ByS. Additionally, a significant increase in total PSD-95 expression (46%) was observed in the hippocampal temporal pole of female SI-ByS rats compared with the septal pole. Overall, our results and behavioural observations are clearly correlated and support the match/mismatch hypothesis using a sex-specific perspective. As a result of these findings, it is possible that early social isolation may have lifelong effects, particularly in male rats.

6.1.1 Study One Main Findings (CELSI)

- Both female and male rats that had experienced post-weaning social isolation exhibited superior spatial memory abilities when compared with their counterparts that were housed in groups.
- PSD-95 expression increased about 12% in socially isolated male rats.
- CELSI enhanced hippocampal-dependent behaviour in a fashion similar among male and female rats.

6.1.2 Study Two Main Findings (ByS)

- ByS did not significantly affect spatial learning and memory in either male, or female rats.
- Bystander stress appeared to affect the synaptic expression of certain plasticity-related proteins in both a sex and region-dependent manner.
- ByS significantly reduced the expression of GluA2 (61%), GluN2A (20%), and GluN2B (24%) in the septal pole of male rats.
- In female rats, GluA2 expression was significantly increased (58%) in the temporal pole of ByS rats compared to sex-matched ByC rats. GluA2 expression increased 76% greater in the temporal pole compared to septal pole due to ByS in female rats.
- The clearest sex-based change was a 99% difference in PSD-95 levels observed at the temporal pole between male and female ByS rats.

6.1.3 Study Three Main Findings (CELSI + ByS)

- Male rats in the stress matched situation (SI-ByS) showed better spatial learning performance than those in mismatch 2 (GH-ByS), supporting the match/mismatch hypothesis in a sex-specific manner.
- There was evidence of sexual dimorphism in spatial learning among rats exposed to the mismatch 1 situation (SI-ByC) with male rats performing significantly better than female rats.
- The stress matched situation (SI-ByS) effect was generally characterized by an increase in expression of the obligatory NMDA receptor subunit (GluN1; 24.8%) in male rats in the septal pole of the HP compared to the GH-ByS group.

- A significant increase in total PSD-95 expression (46%) was observed in the hippocampal temporal pole of female SI-ByS rats in comparison to the septal pole.
- Early social isolation may have lifelong effects, specifically in male rats.

6.2 Statistical Approach

Due to the increasing importance of data-based decisions in modern society, it is imperative that researchers pay closer attention to their data analytics and statistical methodologies. A proper statistical approach assists researchers in learning from data and avoiding common errors that can lead to inaccurate conclusions. As an example from the current dissertation, the underlying assumptions of mean-based ANOVA models are violated when applied to censored data, such as those collected during the learning phase of the MWM. To address the weakness of ANOVA models in the face of censored data, unlike the vast majority of previous studies, we employed survival analysis (the Cox Proportional Hazard model) to examine the learning trial data (for more information, please see Chapter 3). Appropriateness of the statistical methods employed are crucial in the validity of the conclusions drawn from the study.

To assess the relationship between plasticity-related proteins and behavioral outcomes, linear models were employed. However, no significant evidence supporting a link between cognitive function and the measurements of subunits was found. From a statistical standpoint, this lack of significance could be attributed to insufficient data or a genuine absence of a connection. Nevertheless, it is important to note that at the biological level, there may not necessarily be a direct association between the expression of receptor subunits and the behavioral changes induced by stress.

6.3 Limitations

Psychosocial stress model interactions are challenging, and the current experimental design had several limitations. For instance, depending upon the nature of the social interaction, group housing experience may be considered either positively (social enrichment), or negatively (as a source of social stress). Moreover, keeping siblings together is not the same as housing non-related adult rats due to the possibility that different interactions, such as personal attachments, might exist between siblings and not between non-siblings, which may have an impact on the level of stress. Therefore, our study three results should be interpreted by considering that the platform stress and platform control animals for each group were non-related adults. It is possible that the amount of bystander stress would have been different if the platform stress rats were siblings, and this could have impacted the results, including spatial learning and memory. The following are some other limitations of my dissertation study:

- All of the animals used in my work were shipped to the University's animal facility. In order to facilitate the stress recovery process and minimize the effects of shipping, all animals underwent a period of stabilization and acclimation prior to use in our experiments, however, the possibility exists that stress associated with the shipping process may have affected our results. Although, shipment to shipment variability was considered in my analytical model, since we did not have any animals that were not shipped, I could not directly investigate the effect that shipping-related stress may have had on our outcome measures.

- Continuing on from the last point, the rats used in studies one and three were shipped early in their lives (in the first post-natal week), a period when animals are highly sensitive to environmental changes. Early life stress, such as maternal separation, has been demonstrated to negatively impact brain development (Fone & Porkess, 2008). There is evidence that early experiences that lead to stress may be associated with an increased risk of neurological, psychiatric, and behavioral disorders, such as depression, anxiety, and schizophrenia, later in life (Reinwald et al., 2018; Thorsell et al., 2006). Notably, shipping stress has been shown to have deleterious effects and to alter drug treatment responses (Wiley & Evans, 2009), as well as increases the vulnerability of offspring to neurotoxicity due to valproate exposure in pregnant women (Ogawa et al., 2007). However, there is limited available data regarding the duration of physiological stress indicators before, during, and after transportation. Consequently, it is crucial to determine whether the experience of shipping stress at different developmental and adolescent stages may impact study outcomes and interpretations.
- Whole hippocampal homogenates were used in study one, while synaptoneurosomes of the hippocampal septal and temporal poles were used in studies two and three. Due to differences in both the tissue that was used and in the method of homogenization, directly comparing results from study one with those of studies two and three is not possible. In addition, the proteins that were examined in study one was different from studies two and three (with the exception of PSD-95).

- We experienced significant disruptions as a result of the closure of the University's animal facilities and labs to address COVID-19. In particular, we had to change the design of the final study unexpectedly; instead of proceeding directly from CELSI to ByS, a gap of approximately 6 months was inserted between the post-weaning stressor (CELSI) and the adulthood stressor (ByS).
- As part of study one, the evaluation of CORT and other stress sensitive biometrics was conducted, but these were not collected in studies two and three due to a lack of human resources and COVID (the laboratory's capacity was limited after re-opening, and we were not able to collect blood and organs simultaneously with the removal of the hippocampus).
- Studies two and three had a small-modest sample size for immunoblotting, which may have affected the statistical power of the test in some cases (although this may be acceptable given that these were exploratory studies).
- Female rats were not assessed for estrous phase, which may have influenced the expression of proteins and behaviour.

6.4 Future Considerations

Stress can have important effects on the actions of many systems, especially regions of the nervous system that may increase the risk for developing neuropsychiatric disorders, such as depression and dementia. Even more seriously, stress and subsequent changes are associated with higher rates of morbidity and mortality (Kopp & Réthelyi, 2004), including a higher risk for mental illness and suicide (Paykel, 1976). As a result of the magnitude of its harmful effects, stress has been identified as a "worldwide epidemic" by the World Health Organization

(World Health Organization, 2007). The importance of understanding the causes and effects of stress across the life course, as well as the relationship between stress and mental health, cannot be overstated. It is also crucial to identify the neuronal circuits that are associated with stress, as well as their interactions with mediator molecules, in order to better understand not only the physiological aspect of stress, but also its psychological impacts. Put simply, we will be better able to deal with the consequences of stress more effectively if we have a better understanding of it. In spite of the large number of potential future research opportunities, the following section will focus on a few of the most important ones.

- In future studies, measuring basal and stress-induced CORT levels might provide insight into how CORT levels are related to stressors applied to each sex, providing clues to possible mechanisms that might underlie sexual dimorphism in response to psychosocial stress models.
- In the second study, PS was compared to PC, and ByS to ByC; however, these comparison groups may not be the most accurate because both control groups experienced short periods of social isolation. It is therefore necessary to conduct further research on spatial learning and memory, as well as stress-related effects on protein expression using cage-controls that will not be subjected to any social isolation, or movement. Furthermore, in contrast to studies two and three, previous studies have generally employed whole cell homogenates, or crude synaptosomal fractions, which may not provide a clear picture of synaptic changes. As a result, using the SNP technique in future studies examining the effects of different stress models could be helpful to more precisely measure protein-level changes at the synaptic level.

- It appears that bystander stress may cause both pole-specific and sexually dimorphic changes in the same direction, such as the reduction in the expression of AMPAR (specifically GluA2) and NMDAR (specially GluN2A and GluN2B) subunits in the hippocampal septal pole of male rats. Subsequent studies are needed to confirm whether male rats are more susceptible to stress-induced changes in AMPAR and NMDAR subunit proteins due to bystander stress (and other possible psychosocial stress models), and what might be the broader behavioural consequences of these changes using tests that examine other features of behaviour.
- Evaluating the expression of some of the scaffolding proteins that hold the AMPA and NMDA receptor subunits, as well as their trafficking within the synapse, may provide a more comprehensive picture of the changes in synaptic plasticity associated with stress-induced behaviour.
- Different types of stress models used in match/mismatch situations can elicit a specific stress response and may affect behaviour and biochemistry differently. In other words, stress-coping responses are adaptive when they are compatible with current stress conditions, whereas they are maladaptive when they are not compatible with current stress conditions. For instance, the acquisition of an active response to stress during early life may culminate in the development of appropriate coping responses during adulthood. In this regard, the exposure to developmental social isolation may alter synaptic plasticity in a manner that causes an appropriate stress response in adulthood when exposed to social isolation. Therefore, it would be beneficial to conduct future studies evaluating stress models with the same and different developmental and

adulthood stressors, in order to understand the possible effects of exposure to similar and unrelated stressors.

6.5 Conclusions

Animals with mismatched environmental conditions generally differed from those with matched environmental conditions, particularly in terms of behavioural characteristics. Our results further support the match/mismatch hypothesis and demonstrate how aversive conditions during the post-weaning developmental period can shape an individual to be optimally adapted to similarly adverse conditions later in life.

This dissertation attempted to evaluate the different effects on male and female animals in response to developmental and adulthood stressors and their combination. Consequently, we considered sex as a variable in each of our studies, which is not the usual practice. This dissertation highlighted the importance of studying both sexes and understanding sex differences (and similarities) in response to psychosocial stress and provided a context for the debate surrounding whether the response to psychosocial stress may be an adaptive process. Based on our findings, sex has a general influence on how stressors affect behavioural performance.

References

Abbott, L. F., & Nelson, S. B. (2000). Synaptic plasticity: taming the beast. *Nature Neuroscience*, *3*(11s), 1178.

Abdolmaleky, H. M. (2014). Horizons of psychiatric genetics and epigenetics: Where are we and where are we heading? In *Iranian Journal of Psychiatry and Behavioural Sciences*.

Alquicer, G., Morales-Medina, J. C., Quirion, R., & Flores, G. (2008). Postweaning social isolation enhances morphological changes in the neonatal ventral hippocampal lesion rat model of psychosis. *Journal of Chemical Neuroanatomy*, *35*(2), 179–187. https://doi.org/https://doi.org/10.1016/j.jchemneu.2007.10.001

Amaral, D. G., & Witter, M. P. (1989). The three-dimensional organization of the hippocampal formation: a review of anatomical data. *Neuroscience*, *31*(3), 571–591.

Anand, K. S., & Dhikav, V. (2017). Hippocampus in health and disease : An overview. *Annals of Indian Academy of Neurology*. https://doi.org/10.4103/0972-2327.104323

Ashton, D., Van Reempts, J., Haseldonckx, M., & Willems, R. (1989). Dorsal-ventral gradient in vulnerability of CA1 hippocampus to ischemia: a combined histological and electrophysiological study. *Brain Research*, *487*(2), 368–372.

Bakker, J., Pechenizkiy, M., & Sidorova, N. (2011). What's your current stress level? Detection of stress patterns from GSR sensor data. *2011 IEEE 11th International Conference on Data Mining Workshops*, 573–580.

Bale, T. L., & Epperson, C. N. (2015). Sex differences and stress across the lifespan. *Nature Neuroscience*, *18*(10), 1413–1420.

Bartanusz, V., Aubry, J.-M., Pagliusi, S., Jezova, D., Baffi, J., & Kiss, J. Z. (1995). Stress-induced changes in messenger RNA levels of N-methyl-D-aspartate and AMPA receptor subunits in selected regions of the rat hippocampus and hypothalamus. *Neuroscience*, *66*(2), 247–252.

Beiko, J., Lander, R., Hampson, E., Boon, F., & Cain, D. P. (2004). Contribution of sex differences in the acute stress response to sex differences in water maze performance in the rat. *Behavioural Brain Research*, *151*(1–2), 239–253.

Bellinger, L., Lilley, C., & Langley-Evans, S. C. (2004). Prenatal exposure to a maternal low-protein diet programmes a preference for high-fat foods in the young adult rat. *British journal of Nutrition*, *92*(3), 513-520.

Belsky, J., & Pluess, M. (2009). Beyond diathesis stress: differential susceptibility to environmental influences. *Psychological Bulletin*, *135*(6), 885.

Bernard, C. (1957). *An introduction to the study of experimental medicine* (Vol. 400). Courier Corporation.

Bianchi, M., Fone, K. F. C. C., Azmi, N., Heidbreder, C. A., Hagan, J. J., & Marsden, C. A.

(2006). Isolation rearing induces recognition memory deficits accompanied by cytoskeletal alterations in rat hippocampus. *European Journal of Neuroscience*, *24*(10), 2894–2902. https://doi.org/10.1111/j.1460-9568.2006.05170.x

Blanke, M. L., & VanDongen, A. M. J. (2009). Activation Mechanisms of the NMDA Receptor. In A. M. Van Dongen (Ed.), *Biology of the NMDA Receptor*. CRC Press/Taylor & Francis.

Bliss, T. V. P., & Lømo, T. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *The Journal of Physiology*, *232*(2), 331–356.

Boldrini, M., Underwood, M. D., Hen, R., Rosoklija, G. B., Dwork, A. J., John Mann, J., & Arango, V. (2009). Antidepressants increase neural progenitor cells in the human hippocampus. *Neuropsychopharmacology*, *34*(11), 2376–2389.

Bolton, J. L., Ruiz, C. M., Rismanchi, N., Sanchez, G. A., Castillo, E., Huang, J., Cross, C., Baram, T. Z., & Mahler, S. V. (2018). Early-life adversity facilitates acquisition of cocaine self-administration and induces persistent anhedonia. *Neurobiology of Stress*, *8*, 57–67.

Branson, D. C. (2019). Vicarious trauma, themes in research, and terminology: A review of literature. *Traumatology*, *25*(1), 2.

Buwalda, B., Stubbendorff, C., Zickert, N., & Koolhaas, J. M. (2013). Adolescent social stress does not necessarily lead to a compromised adaptive capacity during adulthood: A study on the consequences of social stress in rats. *Neuroscience*, *249*, 258–270. https://doi.org/10.1016/j.neuroscience.2012.12.050

Calabrese, F., Guidotti, G., Molteni, R., Racagni, G., Mancini, M., & Riva, M. A. (2012). Stress-induced changes of hippocampal NMDA receptors: modulation by duloxetine treatment. *PloS One*, *7*(5). https://doi.org/10.1371/JOURNAL.PONE.0037916

Cannon, W. B. (1929). Organization for physiological homeostasis. *Physiological Reviews*, *9*(3), 399–431.

Caspi, A., Sugden, K., Moffitt, T. E., Taylor, A., Craig, I. W., Harrington, H., McClay, J., Mill, J., Martin, J., & Braithwaite, A. (2003). Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. *Science*, *301*(5631), 386–389.

Cevik, O. S., Sahin, L., & Tamer, L. (2018). Long term treadmill exercise performed to chronic social isolated rats regulate anxiety behaviour without improving learning. *Life Sciences*, *200*(January), 126–133. https://doi.org/10.1016/j.lfs.2018.03.029

Chamberland, D. C. (1979). Sources of stress pshchological structure and coping response. Indiana University, Kelley School of Business.

Charney, D. S., & Manji, H. K. (2004). Life stress, genes, and depression: multiple pathways lead to increased risk and new opportunities for intervention. *Science's STKE*, *2004*(225), re5-re5.

Chida, Y., Sudo, N., & Kubo, C. (2006). Does stress exacerbate liver diseases? *Journal of Gastroenterology and Hepatology*, *21*(1), 202–208.

Choy, K. H. C., de Visser, Y., Nichols, N. R., & van den Buuse, M. (2008). Combined neonatal stress and young-adult glucocorticoid stimulation in rats reduce BDNF expression in hippocampus: effects on learning and memory. Hippocampus, 18(7), 655-667.

Choy, K. H. C., & van den Buuse, M. (2008). Attenuated disruption of prepulse inhibition by dopaminergic stimulation after maternal deprivation and adolescent corticosterone treatment in rats. *European Neuropsychopharmacology*, *18*(1), 1-13.

Clark, M. S., Bond, M. J., Hecker, J. R., Clark, M. S., Bond, M. J., Environmental, J. R. H., Clark, M. S., Bond, M. J., & Hecker, J. R. (2007). Environmental stress , psychological stress and allostatic load. *Psychology, Health & Medicine*, *12*(1), 18–30. https://doi.org/10.1080/13548500500429338

Cobb, S. R., Buhl, E. H., Halasy, K., Paulsen, O., & Somogyi, P. (1995). Synchronization of neuronal activity in hippocampus by individual GABAergic interneurons. *Nature*, *378*(6552), 75–78. https://doi.org/10.1038/378075a0

Cohen, S., Janicki-Deverts, D., & Miller, G. E. (2007). Psychological stress and disease. In *Journal of the American Medical Association*. https://doi.org/10.1001/jama.298.14.1685

Colaianna, M., Schiavone, S., Zotti, M., Tucci, P., Morgese, M. G., Bäckdahl, L., Holmdahl, R., Krause, K.-H., Cuomo, V., & Trabace, L. (2013). Neuroendocrine profile in a rat model of psychosocial stress: relation to oxidative stress. *Antioxidants & Redox Signaling*, *18*(12), 1385–1399.

Coley, R. L., Lynch, A. D., & Kull, M. (2015). Early exposure to environmental chaos and children's physical and mental health. *Early Childhood Research Quarterly*, *32*, 94–104.

Conrad, C. D. (2006). What is the functional significance of chronic stress-induced CA3 dendritic retraction within the hippocampus?. *Behavioral and cognitive neuroscience reviews*, *5*(1), 41-60.

Cottrell, E., & Seckl, J. (2009). Prenatal stress, glucocorticoids and the programming of adult disease. In *Frontiers in Behavioural Neuroscience* (Vol. 3, p. 19). https://www.frontiersin.org/article/10.3389/neuro.08.019.2009

Cox, D. R. (1972). Regression Models and Life-Tables. *Journal of the Royal Statistical Society, Series B*, *34*(2), 187–220.

De Kloet, E. R., Joëls, M., & Holsboer, F. (2005). Stress and the brain: from adaptation to disease. *Nature Reviews Neuroscience*, *6*(6), 463–475.

Deater-Deckard, K., Sewell, M. D., Petrill, S. A., & Thompson, L. A. (2010). Maternal working memory and reactive negativity in parenting. *Psychological Science*, *21*(1), 75–79.

Derkach, V. A., Oh, M. C., Guire, E. S., & Soderling, T. R. (2007). Regulatory mechanisms of AMPA receptors in synaptic plasticity. *Nature Reviews Neuroscience*, *8*(2), 101–113.

Dhabhar, F. S., & McEwen, B. S. (1997). Acute stress enhances while chronic stress suppresses cell-mediated immunity in vivo: A potential role for leukocyte trafficking. *Brain, Behaviour, and Immunity*, *11*(4), 286–306. https://doi.org/10.1006/brbi.1997.0508

Dickerson, S. S., & Kemeny, M. E. (2004). Acute stressors and cortisol responses: a theoretical integration and synthesis of laboratory research. *Psychological Bulletin*, *130*(3), 355.

Diehl, L. A., Silveira, P. P., Leite, M. C., Crema, L. M., Portella, A. K., Billodre, M. N., ... & Dalmaz, C. (2007). Long lasting sex-specific effects upon behavior and S100b levels after maternal separation and exposure to a model of post-traumatic stress disorder in rats. *Brain research*, *1144*, 107-116.

Dingledine, R., Borges, K., Bowie, D., & Traynelis, S. F. (1999). The glutamate receptor ion channels. *Pharmacological Reviews*, *51*(1), 7–62.

Duman, R. S. (2002). Pathophysiology of depression: The concept of synaptic plasticity. *European Psychiatry*, *17*(SUPPL. 3), 306–310. https://doi.org/10.1016/S0924-9338(02)00654-5

Duman, Ronald S., Malberg, J., Nakagawa, S., & D'Sa, C. (2000). Neuronal plasticity and survival in mood disorders. *Biological Psychiatry*, *48*(8), 732–739. https://doi.org/10.1016/S0006-3223(00)00935-5

Escalera, C., Santoyo-Olsson, J., Stewart, A. L., Ortiz, C., & Nápoles, A. M. (2019). Social Support as a Moderator in the Relationship between Intrusive Thoughts and Anxiety among Spanish-speaking Latinas with Breast Cancer. *Psycho-Oncology*.

Fone, K. C. F., & Porkess, M. V. (2008). Behavioural and neurochemical effects of post-weaning social isolation in rodents—Relevance to developmental neuropsychiatric disorders. *Neuroscience & Biobehavioural Reviews*, *32*(6), 1087–1102. https://doi.org/https://doi.org/10.1016/j.neubiorev.2008.03.003

Franke, H. A. (2014). Toxic stress: effects, prevention and treatment. *Children*, *1*(3), 390–402.

Frankenhuis, W. E., & Del Giudice, M. (2012). When do adaptive developmental mechanisms yield maladaptive outcomes? *Developmental Psychology*, *48*(3), 628.

Franklin, T. B., Saab, B. J., & Mansuy, I. M. (2012). Neural mechanisms of stress resilience and vulnerability. Neuron, 75(5), 747-761.

Freund, T. F., & Gulyas, A. I. (1997). Inhibitory control of GABAergic interneurons in the hippocampus. *Canadian Journal of Physiology and Pharmacology*, *75*(5), 479–487.

Fuchs, E., Flügge, G., Ohl, F., Lucassen, P., Vollmann-Honsdorf, G. K., & Michaelis, T.

(2001). Psychosocial stress, glucocorticoids, and structural alterations in the tree shrew hippocampus. *Physiology and Behaviour*, *73*(3), 285–291. https://doi.org/10.1016/S0031-9384(01)00497-8

Gardner, E. B., Boitano, J. J., Mancino, N. S., D'Amico, D. P., & Gardner, E. L. (1975). Environmental enrichment and deprivation: effects on learning, memory and exploration. *Physiology & Behaviour*, *14*(3), 321–327.

Geyer, M. A., Wilkinson, L. S., Humby, T., & Robbins, T. W. (1993). Isolation rearing of rats produces a deficit in prepulse inhibition of acoustic startle similar to that in schizophrenia. *Biological Psychiatry*, *34*(6), 361–372.

Gluckman, P. D., Hanson, M. A., & Spencer, H. G. (2005). Predictive adaptive responses and human evolution. *Trends in ecology & evolution*, *20*(10), 527-533.

Gluckman, P. D., Hanson, M. A., Spencer, H. G., & Bateson, P. (2005). Environmental influences during development and their later consequences for health and disease: implications for the interpretation of empirical studies. Proceedings of the Royal Society B: Biological Sciences, 272(1564), 671-677.

Gluckman, P. D., Hanson, M. A., & Beedle, A. S. (2007). Early life events and their consequences for later disease: a life history and evolutionary perspective. *American journal of human biology*, *19*(1), 1-19.

Goldstein, D. S., & McEwen, B. (2002). Allostasis , Homeostats , and the Nature of Stress. *International Journal on the Biology of Stress*, *5*(1), 55–58. https://doi.org/10.1080/10253890290012345

Goodwani, S., Saternos, H., Alasmari, F., & Sari, Y. (2017). Metabotropic and ionotropic glutamate receptors as potential targets for the treatment of alcohol use disorder. In *Neuroscience and Biobehavioural Reviews* (Vol. 77, pp. 14–31). https://doi.org/10.1016/j.neubiorev.2017.02.024

Grippo, A. J., Gerena, D., Huang, J., Kumar, N., Shah, M., Ughreja, R., & Carter, C. S. (2007). Social isolation induces behavioural and neuroendocrine disturbances relevant to depression in female and male prairie voles. *Psychoneuroendocrinology*, *32*(8–10), 966–980.

Hanson, M. A. and, & Gluckman, P. D. (2014). Early developmental conditioning of later health and disease: physiology or pathophysiology? *Physiological Reviews*.

Hatch, A. M., Wiberg, G. S., Zawidzka, Z., Cann, M., Airth, J. M., & Grice, H. C. (1965). Isolation syndrome in the rat. *Toxicology and Applied Pharmacology*, *7*(5), 737–745.

Hayashi, Y., Shi, S.-H., Esteban, J. A., Piccini, A., Poncer, J.-C., & Malinow, R. (2000). Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction. *Science*, *287*(5461), 2262–2267.

Hebb, D. O. (1949). The organization of behaviour; a neuropsycholocigal theory. *A Wiley Book in Clinical Psychology.*, 62–78.

Heck, A. L., & Handa, R. J. (2019). Sex differences in the hypothalamic–pituitary–adrenal axis' response to stress: an important role for gonadal hormones. *Neuropsychopharmacology*, *44*(1), 45–58.

Heidbreder, C. A., Weiss, I. C., Domeney, A. M., Pryce, C., Homberg, J., Hedou, G., Feldon, J., Moran, M. C., & Nelson, P. (2000). Behavioural, neurochemical and endocrinological characterization of the early social isolation syndrome. *Neuroscience*, *100*(4), 749–768.

Heim, C., Plotsky, P. M., & Nemeroff, C. B. (2004). Importance of studying the contributions of early adverse experience to neurobiological findings in depression. *Neuropsychopharmacology*, *29*(4), 641–648.

Hellemans, K. G. C., Benge, L. C., & Olmstead, M. C. (2004). Adolescent enrichment partially reverses the social isolation syndrome. *Developmental Brain Research*, *150*(2), 103–115. https://doi.org/10.1016/j.devbrainres.2004.03.003

Henley, J. M., Barker, E. A., & Glebov, O. O. (2011). Routes, destinations and delays: Recent advances in AMPA receptor trafficking. In *Trends in Neurosciences* (Vol. 34, Issue 5, pp. 258–268). https://doi.org/10.1016/j.tins.2011.02.004

Herman, J. P., Figueiredo, H., Mueller, N. K., Ulrich-Lai, Y., Ostrander, M. M., Choi, D. C., & Cullinan, W. E. (2003). Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo–pituitary–adrenocortical responsiveness. *Frontiers in Neuroendocrinology*, *24*(3), 151–180.

Hollmann, M., O'Shea-Greenfield, A., Rogers, S. W., & Heinemann, S. (1989). Cloning by functional expression of a member of the glutamate receptor family. *Nature*, *342*(6250), 643.

Holmes, A., & Wellman, C. L. (2009). Stress-induced prefrontal reorganization and executive dysfunction in rodents. *Neuroscience & Biobehavioural Reviews*, *33*(6), 773–783.

Huizink, A. C., Mulder, E. J. H., & Buitelaar, J. K. (2004). Prenatal stress and risk for psychopathology: specific effects or induction of general susceptibility? *Psychological Bulletin*, *130*(1), 115.

Husi, H., & Grant, S. G. N. (2001). Isolation of 2000-kDa complexes of N-methyl-D-aspartate receptor and postsynaptic density 95 from mouse brain. *Journal of Neurochemistry*, *77*(1), 281–291.

Husi, H., Ward, M. A., Choudhary, J. S., Blackstock, W. P., & Grant, S. G. N. (2000). Proteomic analysis of NMDA receptor–adhesion protein signaling complexes. *Nature Neuroscience*, *3*(7), 661.

Imanaka, A., Morinobu, S., Toki, S., & Yamawaki, S. (2006). Importance of early environment in the development of post-traumatic stress disorder-like behaviors. *Behavioural brain research*, *173*(1), 129-137.

Inagaki, T. K. (2018). Neural mechanisms of the link between giving social support and health. *Annals of the New York Academy of Sciences*, *1428*(1), 33–50.

Ito, R., & Lee, A. C. H. (2016). The role of the hippocampus in approach-avoidance conflict decision-making: Evidence from rodent and human studies. *Behavioural Brain Research*, *313*, 345–357. https://doi.org/https://doi.org/10.1016/j.bbr.2016.07.039

Jahn-Eimermacher, A., Lasarzik, I., & Raber, J. (2011). Statistical analysis of latency outcomes in behavioural experiments. *Behavioural Brain Research*, *221*(1), 271–275. https://doi.org/10.1016/j.bbr.2011.03.007

Joel, D., & McCarthy, M. M. (2017). Incorporating sex as a biological variable in neuropsychiatric research: where are we now and where should we be? *Neuropsychopharmacology*, *42*(2), 379.

Joëls, M., Karst, H., Alfarez, D., Heine, V. M., Qin, Y., Riel, E. van, Verkuyl, M., Lucassen, P. J., & Krugers, H. J. (2004). Effects of chronic stress on structure and cell function in rat hippocampus and hypothalamus. *Stress*, *7*(4), 221–231.

Juster, R.-P., de Torre, M. B., Kerr, P., Kheloui, S., Rossi, M., & Bourdon, O. (2019). Sex Differences and Gender Diversity in Stress Responses and Allostatic Load Among Workers and LGBT People. *Current Psychiatry Reports*, *21*(11), 110.

Kajiwara, R., Wouterlood, F. G., Sah, A., Boekel, A. J., Baks-te Bulte, L. T. G., & Witter, M. P. (2008). Convergence of entorhinal and CA3 inputs onto pyramidal neurons and interneurons in hippocampal area CA1—an anatomical study in the rat. *Hippocampus*, *18*(3), 266–280.

Kaplan, H. B. (Ed.). (1983). *Psychosocial Stress: Trends in Theory and Research*. Academic Press.

Kendler, K. S., Gardner, C. O., & Prescott, C. A. (2002). Toward a comprehensive developmental model for major depression in women. *American Journal of Psychiatry*, *159*(7), 1133–1145.

Kendler, K. S., Walters, E. E., Neale, M. C., Kessler, R. C., Heath, A. C., & Eaves, L. J. (1995). The Structure of the Genetic and Environmental Risk Factors for Six Major Psychiatric Disorders in Women: Phobia, Generalized Anxiety Disorder, Panic Disorder, Bulimia, Major Depression, and Alcoholism. *JAMA Psychiatry*, *52*(5), 374–383. https://doi.org/10.1001/archpsyc.1995.03950170048007

Kennedy, M. B. (1997). The postsynaptic density at glutamatergic synapses. *Trends in Neurosciences*, *20*(6), 264–268. https://doi.org/10.1016/S0166-2236(96)01033-8

Kessler, R. C. (2003). Epidemiology of women and depression. *Journal of Affective Disorders*, *74*(1), 5–13.

Kim, E., Cho, K.-O., Rothschild, A., & Sheng, M. (1996). Heteromultimerization and NMDA receptor-clustering activity of Chapsyn-110, a member of the PSD-95 family of proteins. *Neuron*, *17*(1), 103–113.

Kim, J. J., & Diamond, D. M. (2002). The stressed hippocampus, synaptic plasticity and lost memories. *Nature Reviews Neuroscience*, *3*(6), 453–462. https://doi.org/10.1038/nrn849

Kim, J. J., & Yoon, K. S. (1998). Stress: metaplastic effects in the hippocampus. *Trends in Neurosciences*, *21*(12), 505–509.

Kirschbaum, C., Wüst, S., & Hellhammer, D. (1992). Consistent sex differences in cortisol responses to psychological stress. *Psychosomatic Medicine*, *54*(6), 648–657.

Kloet, E. R. D. E., Vreugdenhil, E., Oitzl, M. S., Pharmacology, M., & Amsterdam, L. (2009). Brain Corticosteroid Receptor Balance in Health and Disease. *Endocrine Reviews*, *19*(3), 269–301. https://academic.oup.com/edrv/article-abstract/19/3/269/2530808

Kogler, L., Mueller, V. I., Chang, A., Eickhoff, S. B., Peter, T., Gur, R. C., & Derntl, B. (2015). Psychosocial versus physiological stress - meta-analyses on deactivations and activations of the neural correlates of stress reactions. *Neuroimage*, *119*, 235–251. https://doi.org/10.1016/j.neuroimage.2015.06.059.Psychosocial

Kolb, B., Harker, A., Mychasiuk, R., de Melo, S. R., & Gibb, R. (2017). Stress and prefrontal cortical plasticity in the developing brain. In *Cognitive Development* (Vol. 42, pp. 15–26). https://doi.org/10.1016/j.cogdev.2017.01.001

Kolb, B., Mychasiuk, R., & Gibb, R. (2014). Brain development, experience, and behaviour. In *Pediatric Blood and Cancer*. https://doi.org/10.1002/pbc.24908

Kolb, B., & Whishaw, I. Q. (2001). An introduction to brain and behaviour. In *An introduction to brain and behaviour*. (pp. xx, 601–xx, 601). Worth Publishers.

Koolhaas, J. M., Bartolomucci, A., Buwalda, B., de Boer, S. F., Flügge, G., Korte, S. M., Meerlo, P., Murison, R., Olivier, B., & Palanza, P. (2011). Stress revisited: a critical evaluation of the stress concept. *Neuroscience & Biobehavioural Reviews*, *35*(5), 1291–1301.

Kopp, M. S., & Réthelyi, J. (2004). Where psychology meets physiology: chronic stress and premature mortality—the Central-Eastern European health paradox. *Brain Research Bulletin*, *62*(5), 351–367.

Krugers, H. J., Koolhaas, J. M., Bohus, B., & Korf, J. (1993). A single social stress-experience alters glutamate receptor-binding in rat hippocampal CA3 area. *Neuroscience Letters*, *154*(1–2), 73–77.

Kurgers, H. J., Hoogenraad, C. C., & Groc, L. (2010). Stress hormones and AMPA receptor trafficking in synaptic plasticity and memory. *Nature Reviews Neuroscience*, *11*(October), 675–681.

Laelia, G., Apicella, L., Brakarsh, J., Dube, L., Jemison, J., Kluckow, M., Smith, T., & Snider, L. (2006). *Orphans and vulnerable youth in Bulawayo, Zimbabwe: An exploratory study of psychosocial well-being and psychosocial support programs*.

Langford, D. J., Crager, S. E., Shehzad, Z., Smith, S. B., Sotocinal, S. G., Levenstadt, J. S., Chanda, M. L., Levitin, D. J., & Mogil, J. S. (2006). Social modulation of pain as evidence for empathy in mice. *Science*, *312*(5782), 1967–1970. https://doi.org/10.1126/SCIENCE.1128322/SUPPL_FILE/LANGFORD.SOM.PDF

Lapiz, M. D.S., Fulford, A., Muchimapura, S., Mason, R., Parker, T., & Marsden, C. A. (2003). Influence of postweaning social isolation in the rat on brain development, contioned behaviour, and neurotransmission. *Neuroscience and Behavioural Physiology*, *33*(1), 13–29. https://doi.org/10.1023/A:1021171129766

Lapiz, Maria Danet S., Mateo, Y., Durkin, S., Parker, T., & Marsden, C. A. (2001). Effects of central noradrenaline depletion by the selective neurotoxin DSP-4 on the behaviour of the isolated rat in the elevated plus maze and water maze. *Psychopharmacology*, *155*(3), 251–259. https://doi.org/10.1007/s002130100702

Larkin, J. W., Binks, S. L., Li, Y., & Selvage, D. (2010). The Role of Oestradiol in Sexually Dimorphic Hypothalamic-Pituitary-Adrena Axis Responses to Intracerebroventricular Ethanol Administration in the Rat. *Journal of Neuroendocrinology*, *22*(1), 24–32.

Law, A. J., Weickert, C. S., Webster, M. J., Herman, M. M., Kleinman, J. E., & Harrison, P. J. (2003). Expression of NMDA receptor NR1, NR2A and NR2B subunit mRNAs during development of the human hippocampal formation. *European Journal of Neuroscience*, *18*(5), 1197–1205.

Lea, A. J., Altmann, J., Alberts, S. C., & Tung, J. (2015). Developmental constraints in a wild primate. *The American Naturalist*, *185*(6), 809–821.

Lee, Park, H. E., An, S. R., Park, J. Y., Yoon, H.-K., Ko, Y.-H., & Han, K.-M. (2018). Social support moderates association between posttraumatic growth and trauma-related psychopathologies among victims of the Sewol Ferry Disaster. *Psychiatry Research*, *272*(August 2018), 507–514. https://doi.org/10.1016/j.psychres.2018.12.168

Lee, S., Jeong, J., Kwak, Y., & Park, S. K. (2010). Depression research: where are we now? *Molecular Brain*, *3*(1), 8. https://doi.org/10.1186/1756-6606-3-8

Lee, Simonetta, A., & Sheng, M. (2004). Subunit rules governing the sorting of internalized AMPA receptors in hippocampal neurons. *Neuron*, *43*(2), 221–236.

Leedy, G. M., Barrows, L. F., & Clark, S. (2013). Effects of social housing on hippocampal dendrites and behaviour in ovariectomized rats. *Brain Research Bulletin*, *92*, 69–75. https://doi.org/10.1016/j.brainresbull.2012.11.006

Lex, C., Bäzner, E., & Meyer, T. D. (2017). Does stress play a significant role in bipolar disorder? A meta-analysis. *Journal of Affective Disorders*, *208*, 298–308. https://doi.org/https://doi.org/10.1016/j.jad.2016.08.057

Liston, C., Miller, M. M., Goldwater, D. S., Radley, J. J., Rocher, A. B., Hof, P. R., Morrison, J. H., & McEwen, B. S. (2006). Stress-induced alterations in prefrontal cortical dendritic morphology predict selective impairments in perceptual attentional

set-shifting. *Journal of Neuroscience*, *26*(30), 7870–7874.

Lovelock, D. F., & Deak, T. (2020). Acute stress imposed during adolescence has minimal effects on hypothalamic-pituitary-adrenal (HPA) axis sensitivity in adulthood in female Sprague Dawley rats. Physiology & behavior, 213, 112707.

Luby, J., Belden, A., Botteron, K., Marrus, N., Harms, M. P., Babb, C., Nishino, T., & Barch, D. (2013). The effects of poverty on childhood brain development: the mediating effect of caregiving and stressful life events. *JAMA Pediatrics*, *167*(12), 1135–1142.

Lucassen, P. J., Pruessner, J., Sousa, N., Almeida, O. F. X., Van Dam, A. M., Rajkowska, G., Swaab, D. F., & Czéh, B. (2014). Neuropathology of stress. *Acta Neuropathologica*, *127*(1), 109–135.

Luine, V., Gomez, J., Beck, K., & Bowman, R. (2017). Sex differences in chronic stress effects on cognition in rodents. *Pharmacology Biochemistry and Behaviour*, *152*, 13–19.

Lupien, S. J., McEwen, B. S., Gunnar, M. R., & Heim, C. (2009). Effects of stress throughout the lifespan on the brain, behaviour and cognition. *Nature Reviews Neuroscience*, *10*(6), 434.

Magarin˜os, A. M., & McEwen, B. S. (1995). Stress-induced atrophy of apical dendrites of hippocampal CA3c neurons: Involvement of glucocorticoid secretion and excitatory amino acid receptors. *Neuroscience*, *69*(1), 89–98. https://doi.org/10.1016/0306-4522(95)00259-L

Malisch, J. L., Breuner, C. W., Kolb, E. M., Wada, H., Hannon, R. M., Chappell, M. A., Middleton, K. M., & Garland, T. (2009a). Behavioural despair and home-cage activity in mice with chronically elevated baseline corticosterone concentrations. *Behaviour Genetics*, *39*(2), 192–201.

Maniam, J., Antoniadis, C., & Morris, M. J. (2014). Early-Life Stress, HPA Axis Adaptation, and Mechanisms Contributing to Later Health Outcomes . In *Frontiers in Endocrinology* (Vol. 5, p. 73). https://www.frontiersin.org/article/10.3389/fendo.2014.00073

Martens, U., Capito, B., & Wree, A. (1998). Septotemporal distribution of [3 H] MK-801,[3 H] AMPA and [3 H] kainate binding sites in the rat hippocampus. *Anatomy and Embryology*, *198*(3), 195–204.

Massey, P. V, & Bashir, Z. I. (2007). Long-term depression: multiple forms and implications for brain function. *Trends in Neurosciences*, *30*(4), 176–184. https://doi.org/https://doi.org/10.1016/j.tins.2007.02.005

McCormack, S. G., Stornetta, R. L., & Zhu, J. J. (2006). Synaptic AMPA receptor exchange maintains bidirectional plasticity. *Neuron*, *50*(1), 75–88.

McEwen, B. S. (1999). Stress and hippocampal plasticity. *Annual Review of Neuroscience*, *22*(1), 105–122.

McEwen, B. S. (2000a). Allostasis and Allostatic Load: Implications for Neuropsychopharmacology. *Neuropsychopharmacology*, *22*(2), 108–124.

McEwen, B. S. (2000b). The neurobiology of stress: from serendipity to clinical relevance. *Brain Research*, *886*(1–2), 172–189.

McEwen, B. S. (2003). Mood disorders and allostatic load. *Biological Psychiatry*, *54*(3), 200–207.

McEwen, B. S. (2004). Protection and damage from acute and chronic stress: allostasis and allostatic overload and relevance to the pathophysiology of psychiatric disorders. *Annals of the New York Academy of Sciences*, *1032*(1), 1–7.

McEwen, B. S. (2016). In pursuit of resilience : stress, epigenetics, and brain plasticity. *Annals of the New York Academy of Sciences.*, *1373*, 56–64. https://doi.org/10.1111/nyas.13020

McEwen, B. S., & Gianaros, P. J. (2011). Stress-and allostasis-induced brain plasticity. *Annual Review of Medicine*, *62*, 431.

McEwen, B. S., & Sapolsky, R. M. (1995). Stress and cognitive function. *Current Opinion in Neurobiology*, *5*(2), 205–216. https://doi.org/10.1016/0959-4388(95)80028-X

McGowan, P. O., Sasaki, A., D'alessio, A. C., Dymov, S., Labonté, B., Szyf, M., ... & Meaney, M. J. (2009). Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. Nature neuroscience, 12(3), 342-348.

McGowan, P. O., Suderman, M., Sasaki, A., Huang, T. C., Hallett, M., Meaney, M. J., & Szyf, M. (2011). Broad epigenetic signature of maternal care in the brain of adult rats. PloS one, 6(2), e14739.

McKittrick, C. R., Magariños, A. M., Blanchard, D. C., Blanchard, R. J., McEwen, B. S., & Sakai, R. R. (2000). Chronic social stress reduces dendritic arbors in CA3 of hippocampus and decreases binding to serotonin transporter sites. *Synapse*, *36*(2), 85-94.

McLaughlin, K. A., Conron, K. J., Koenen, K. C., & Gilman, S. E. (2010). Childhood adversity, adult stressful life events, and risk of past-year psychiatric disorder: a test of the stress sensitization hypothesis in a population-based sample of adults. *Psychological Medicine*, *40*(10), 1647–1658.

McManus, E., Talmi, D., Haroon, H., & Muhlert, N. (2020). The Effects of Social Stress on Memory: If, How and When. *BioRxiv*.

Middeldorp, J., & Hol, E. M. (2011). GFAP in health and disease. *Progress in Neurobiology*, *93*(3), 421–443. https://doi.org/10.1016/j.pneurobio.2011.01.005

Migaud, M., Charlesworth, P., Dempster, M., Webster, L. C., Watabe, A. M., Makhinson, M., He, Y., Ramsay, M. F., Morris, R. G. M., & Morrison, J. H. (1998). Enhanced long-term potentiation and impaired learning in mice with mutant postsynaptic density-95

protein. *Nature*, *396*(6710), 433.

Morgan, C., & Fisher, H. (2007). Environment and schizophrenia: environmental factors in schizophrenia: childhood trauma—a critical review. *Schizophrenia Bulletin*, *33*(1), 3–10.

Moser, E., Moser, M. B., & Andersen, P. (1993). Spatial learning impairment parallels the magnitude of dorsal hippocampal lesions, but is hardly present following ventral lesions. *The Journal of Neuroscience*, *13*(9), 3916 LP – 3925. https://doi.org/10.1523/JNEUROSCI.13-09-03916.1993

Mychasiuk, R., Gibb, R., & Kolb, B. (2011). Prenatal bystander stress induces neuroanatomical changes in the prefrontal cortex and hippocampus of developing rat offspring. *Brain Research*, *1412*, 55–62. https://doi.org/10.1016/j.brainres.2011.07.023

Mychasiuk, R., Schmold, N., Ilnytskyy, S., Kovalchuk, O., Kolb, B., & Gibb, R. (2011). Prenatal bystander stress alters brain, behaviour, and the epigenome of developing rat offspring. *Developmental Neuroscience*, *33*(2), 159–169. https://doi.org/10.1159/000330034

Nadel, L. (1968). Dorsal and ventral hippocampal lesions and behaviour. *Physiology & Behaviour*, *3*(6), 891–900. https://doi.org/https://doi.org/10.1016/0031-9384(68)90174-1

Nater, U. M., Skoluda, N., & Strahler, J. (2013). Biomarkers of stress in behavioural medicine. *Current Opinion in Psychiatry*, *26*(5), 440–445.

Nederhof, E., & Schmidt, M. V. (2012). Mismatch or cumulative stress: toward an integrated hypothesis of programming effects. *Physiology & Behaviour*, *106*(5), 691–700.

Nelson, C. A., Bhutta, Z. A., Harris, N. B., Danese, A., & Samara, M. (2020). Adversity in childhood is linked to mental and physical health throughout life. *Bmj*, *371*.

Nicolaides, N. C., Kyratzi, E., Lamprokostopoulou, A., Chrousos, G. P., & Charmandari, E. (2015). Stress, the stress system and the role of glucocorticoids. *Neuroimmunomodulation*, *22*(1–2), 6–19.

Noschang, C., Lampert, C., Krolow, R., & De Almeida, R. M. M. (2021). Social isolation at adolescence: a systematic review on behaviour related to cocaine, amphetamine and nicotine use in rats and mice. *Psychopharmacology*, *238*(4), 927–947.

Ogawa, T., Kuwagata, M., Hori, Y., & Shioda, S. (2007). Valproate-induced developmental neurotoxicity is affected by maternal conditions including shipping stress and environmental change during early pregnancy. Toxicology letters, 174(1-3), 18-24.

Organization, W. H. (2007). *Task shifting: rational redistribution of tasks among health workforce teams: global recommendations and guidelines*.

Paoletti, P., Bellone, C., & Zhou, Q. (2013). NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease. *Nature Reviews Neuroscience*,

14(6), 383.

Park, C. R., Campbell, A. M., & Diamond, D. M. (2001). Chronic psychosocial stress impairs learning and memory and increases sensitivity to yohimbine in adult rats. *Biological Psychiatry*, *50*(12), 994–1004.

Paykel, E. S. (1976). Life stress, depression and attempted suicide. *Journal of Human Stress*, *2*(3), 3–12.

Pearlin, L. I. (1989). The sociological study of stress. *Journal of Health and Social Behaviour*, 241–256.

Perrot-Sinal, T. S., Kostenuik, M. A., Ossenkopp, K.-P., & Kavaliers, M. (1996). Sex differences in performance in the Morris water maze and the effects of initial nonstationary hidden platform training. *Behavioural Neuroscience*, *110*(6), 1309.

Piccinelli, M., & Wilkinson, G. (2000). Gender differences in depression: Critical review. *British Journal of Psychiatry*, *177*(6), 486–492. https://doi.org/DOI: 10.1192/bjp.177.6.486

Popoli, M., Yan, Z., McEwen, B. S., & Sanacora, G. (2012). The stressed synapse: the impact of stress and glucocorticoids on glutamate transmission. *Nature Reviews. Neuroscience*, *13*(1), 22–37. https://doi.org/10.1038/nrn3138

Power, R. A., Lecky-Thompson, L., Fisher, H. L., Cohen-Woods, S., Hosang, G. M., Uher, R., Powell-Smith, G., Keers, R., Tropeano, M., & Korszun, A. (2013). The interaction between child maltreatment, adult stressful life events and the 5-HTTLPR in major depression. *Journal of Psychiatric Research*, *47*(8), 1032–1035.

Prange, O., & Murphy, T. H. (2001). Modular transport of postsynaptic density-95 clusters and association with stable spine precursors during early development of cortical neurons. *Journal of Neuroscience*, *21*(23), 9325–9333.

Pruessner, J. C., Dedovic, K., Pruessner, M., Lord, C., Buss, C., Collins, L., Dagher, A., & Lupien, S. J. (2010). Stress regulation in the central nervous system: evidence from structural and functional neuroimaging studies in human populations-2008 Curt Richter Award Winner. *Psychoneuroendocrinology*, *35*(1), 179–191.

Pryce, C. R., & Fuchs, E. (2017). Chronic psychosocial stressors in adulthood: studies in mice, rats and tree shrews. *Neurobiology of Stress*, *6*, 94–103.

Quan, M. N., Tian, Y. T., Xu, K. H., Zhang, T., & Yang, Z. (2010). Post weaning social isolation influences spatial cognition, prefrontal cortical synaptic plasticity and hippocampal potassium ion channels in Wistar rats. *Neuroscience*, *169*(1), 214–222.

Rami, A., Ausmeir, F., Winckler, J., & Krieglstein, J. (1997). Differential effects of scopolamine on neuronal survival in ischemia and glutamate neurotoxicity: relationships to the excessive vulnerability of the dorsoseptal hippocampus. *Journal of Chemical Neuroanatomy*, *13*(3), 201–208.

Reimers, J. M., Milovanovic, M., & Wolf, M. E. (2011). Quantitative analysis of AMPA receptor subunit composition in addiction-related brain regions. *Brain Res, 1367*, 223–233. https://doi.org/10.1016/j.brainres.2010.10.016

Reinwald, J. R., Becker, R., Mallien, A. S., Falfan-Melgoza, C., Sack, M., Clemm von Hohenberg, C., Braun, U., Cosa Linan, A., Gass, N., Vasilescu, A. N., Tollens, F., Lebhardt, P., Pfeiffer, N., Inta, D., Meyer-Lindenberg, A., Gass, P., Sartorius, A., & Weber-Fahr, W. (2018). Neural Mechanisms of Early-Life Social Stress as a Developmental Risk Factor for Severe Psychiatric Disorders. *Biological Psychiatry*, *84*(2), 116–128. https://doi.org/10.1016/j.biopsych.2017.12.010

Ricon, T., Toth, E., Leshem, M., Braun, K., & Richter-Levin, G. (2012). Unpredictable chronic stress in juvenile or adult rats has opposite effects, respectively, promoting and impairing resilience. *Stress*, *15*(1), 11–20.

Ritter, L. M., Unis, A. S., & Meador-Woodruff, J. H. (2001). Ontogeny of ionotropic glutamate receptor expression in human fetal brain. *Developmental Brain Research*, *127*(2), 123–133.

Robb, J.-L., Messa, I., Lui, E., Yeung, D., Thacker, J., Satvat, E., & Mielke, J. G. (2017). A maternal diet high in saturated fat impairs offspring hippocampal function in a sex-specific manner. *Behavioural Brain Research*, *326*, 187–199.

Safari, S., Ahmadi, N., Mohammadkhani, R., Ghahremani, R., Khajvand-Abedeni, M., Shahidi, S., Komaki, A., Salehi, I., & Karimi, S. A. (2021). Sex differences in spatial learning and memory and hippocampal long-term potentiation at perforant pathway-dentate gyrus (PP-DG) synapses in Wistar rats. *Behavioural and Brain Functions*, *17*(1), 1–11.

Salleh, M. R. (2008). Life Event, Stress and Illness. *Malaysian Journal of Medical Sciences*, *15*(4), 9–18.

Sandi, C. (2004). Stress, cognitive impairment and cell adhesion molecules. *Nature Reviews Neuroscience*, *5*(12), 917–930. https://doi.org/10.1038/nrn1555

Santarelli, S., Lesuis, S. L., Wang, X.-D., Wagner, K. V, Hartmann, J., Labermaier, C., Scharf, S. H., Müller, M. B., Holsboer, F., & Schmidt, M. V. (2014a). Evidence supporting the match/mismatch hypothesis of psychiatric disorders. *European Neuropsychopharmacology*, *24*(6), 907–918.

Santarelli, S., Lesuis, S. L., Wang, X. D., Wagner, K. V., Hartmann, J., Labermaier, C., Scharf, S. H., Müller, M. B., Holsboer, F., & Schmidt, M. V. (2014b). Evidence supporting the match/mismatch hypothesis of psychiatric disorders. *European Neuropsychopharmacology*, *24*(6), 907–918. https://doi.org/10.1016/j.euroneuro.2014.02.002

Sapolsky, R. M. (1999a). *<Sapolsky1999.pdf>*. *34*, 721–732.

Sapolsky, R. M. (1999b). Glucocorticoids, stress, and their adverse neurological effects:

relevance to aging. *Experimental Gerontology*, *34*(6), 721–732.

Schmidt, M. V. (2011). Animal models for depression and the mismatch hypothesis of disease. *Psychoneuroendocrinology*, *36*(3), 330–338.

Schrijver, N. C. A., Bahr, N. I., Weiss, I. C., & Würbel, H. (2002). Dissociable effects of isolation rearing and environmental enrichment on exploration, spatial learning and HPA activity in adult rats. *Pharmacology Biochemistry and Behaviour*, *73*(1), 209–224.

Schrijver, N. C., & Würbel, H. (2001). Early social deprivation disrupts attentional, but not affective, shifts in rats. *Behavioural Neuroscience*, *115*(2), 437.

Schumacher, A., Villaruel, F. R., Ussling, A., Riaz, S., Lee, A. C. H., & Ito, R. (2018). Ventral Hippocampal CA1 and CA3 Differentially Mediate Learned Approach-Avoidance Conflict Processing. *Current Biology*, *28*(8), 1318-1324.e4. https://doi.org/https://doi.org/10.1016/j.cub.2018.03.012

Schwabe, L., Oitzl, M. S., Philippsen, C., Richter, S., Bohringer, A., Wippich, W., & Schachinger, H. (2007). Stress modulates the use of spatial versus stimulus-response learning strategies in humans. *Learning & Memory*, *14*(1–2), 109–116.

Seffusatti, N. (2022). "NO MAN IS AN ISLAND": THE POWER OF COMMUNITY. *GC Magazine*.

Sellers, R. S., Mortan, D., Michael, B., Roome, N., Johnson, J. K., Yano, B. L., Perry, R., & Schafer, K. (2007). Society of Toxicologic Pathology position paper: organ weight recommendations for toxicology studies. *Toxicologic Pathology*, *35*(5), 751–755.

Selye, H. (1956). *The stress of life*. McGraw-Hill Book Co.515p.

Sengupta, P. (2013). The laboratory rat: relating its age with human's. *International journal of preventive medicine*, *4*(6), 624.

Serido, J., Almeida, D. M., & Wethington, E. (2004). Chronic Stressors and Daily Hassles : Unique and Interactive Relationships with Psychological Distress *. *Journal of Health and Social Behaviour*, *45*(1), 17–33.

Sestito, R. S., Trindade, L. B., De Souza, R. G., Kerbauy, L. N., Iyomasa, M. M., & Rosa, M. L. N. M. (2011). Effect of isolation rearing on the expression of AMPA glutamate receptors in the hippocampal formation. *Journal of Psychopharmacology*, *25*(12), 1720–1729. https://doi.org/10.1177/0269881110385595

Sheng, M., Cummings, J., Roldan, L. A., Jan, Y. N., & Jan, L. Y. (1994). Changing subunit composition of heteromeric NMDA receptors during development of rat cortex. *Nature*, *368*(6467), 144.

Shi, S.-H., Hayashi, Y., Esteban, J. A., & Malinow, R. (2001). Subunit-specific rules governing AMPA receptor trafficking to synapses in hippocampal pyramidal neurons. *Cell*, *105*(3), 331–343.

Shonkoff, J. P., Boyce, W. T., Mcewen, B. S., & Mcewen, B. S. (2009). Neuroscience,

Molecular Biology, and the Childhood Roots of Health Disparities: Building a New Framework for Health Promotion and Disease Prevention. *Journal of American Medical Association*, *301*(21), 2252–2259. https://doi.org/10.1001/jama.2009.754

Slattery, D. A., & Cryan, J. F. (2014). The ups and downs of modelling mood disorders in rodents. *ILAR Journal*, *55*(2), 297–309. https://doi.org/10.1093/ilar/ilu026

Slavich, G. M. (2016). Life stress and Health: a review of conceptual issues and recent findings. *Teaching of Psychology*, *43*(4), 346–355. https://doi.org/10.1177/0098628316662768.Life

Soetanto, A., Wilson, R. S., Talbot, K., Un, A., Schneider, J. A., Sobiesk, M., Kelly, J., Leurgans, S., Bennett, D. A., & Arnold, S. E. (2010). Association of anxiety and depression with microtubule-associated protein 2–and synaptopodin-immunolabeled dendrite and spine densities in hippocampal CA3 of older humans. *Archives of General Psychiatry*, *67*(5), 448–457.

Squire, L. R. (1992). Memory and the hippocampus: A synthesis from findings with rats, monkeys, and humans. In *Psychological Review* (Vol. 99, Issue 2, pp. 195–231). American Psychological Association. https://doi.org/10.1037/0033-295X.99.2.195

Stroud, L. R., Salovey, P., & Epel, E. S. (2002). Sex differences in stress responses: social rejection versus achievement stress. *Biological Psychiatry*, *52*(4), 318–327.

Swanson, L. W., & Cowan, W. M. (1977). An autoradiographic study of the organization of the efferet connections of the hippocampal formation in the rat. *Journal of Comparative Neurology*, *172*(1), 49–84.

Syme, L. A. (1973). Social isolation at weaning: Some effects on two measures of activity. *Animal Learning & Behaviour*, *1*(3), 161–163.

Thorsell, A., Slawecki, C. J., El Khoury, A., Mathe, A. A., & Ehlers, C. L. (2006). The effects of social isolation on neuropeptide Y levels, exploratory and anxiety-related behaviours in rats. *Pharmacology Biochemistry and Behaviour*, *83*(1), 28–34.

Ulrich-Lai, Y. M., Figueiredo, H. F., Ostrander, M. M., Choi, D. C., Engeland, W. C., & Herman, J. P. (2006). Chronic stress induces adrenal hyperplasia and hypertrophy in a subregion-specific manner. *American Journal of Physiology-Endocrinology and Metabolism*, *291*(5), E965–E973.

van Strien, N. M., Cappaert, N. L. M., & Witter, M. P. (2009). The anatomy of memory: an interactive overview of the parahippocampal–hippocampal network. *Nature Reviews Neuroscience*, *10*, 272. https://doi.org/10.1038/nrn2614

Vickers, C. A., Stephens, B., Bowen, J., Arbuthnott, G. W., Grant, S. G. N., & Ingham, C. A. (2006). Neurone specific regulation of dendritic spines in vivo by post synaptic density 95 protein (PSD-95). *Brain Research*, *1090*(1), 89–98.

Vorhees, C. V., & Williams, M. T. (2014). Assessing spatial learning and memory in rodents. *ILAR Journal*, *55*(2), 310–332. https://doi.org/10.1093/ilar/ilu013

Vorhees, C. V, & Williams, M. T. (2006). Morris water maze: procedures for assessing spatial and related forms of learning and memory. *Nature Protocols*, *1*(2), 848–858. https://doi.org/10.1038/nprot.2006.116

Walker, D. M., Cunningham, A. M., Gregory, J. K., & Nestler, E. J. (2019). Long-Term Behavioural Effects of Post-weaning Social Isolation in Males and Females. *Frontiers in Behavioural Neuroscience*, *13*, 66. https://doi.org/10.3389/fnbeh.2019.00066

Walker, A. K., Nakamura, T., Byrne, R. J., Naicker, S., Tynan, R. J., Hunter, M., & Hodgson, D. M. (2009). Neonatal lipopolysaccharide and adult stress exposure predisposes rats to anxiety-like behaviour and blunted corticosterone responses: implications for the double-hit hypothesis. Psychoneuroendocrinology, 34(10), 1515-1525.

Wang, D., Levine, J. L. S., Avila-Quintero, V., Bloch, M., & Kaffman, A. (2020). Systematic review and meta-analysis: effects of maternal separation on anxiety-like behaviour in rodents. *Translational Psychiatry*, *10*(1), 1–12.

Weaver, I. C., Cervoni, N., Champagne, F. A., D'Alessio, A. C., Sharma, S., Seckl, J. R., ... & Meaney, M. J. (2004). Epigenetic programming by maternal behaviour. *Nature neuroscience*, *7*(8), 847-854.

Weiss, I. C., Pryce, C. R., Jongen-Rêlo, A. L., Nanz-Bahr, N. I., & Feldon, J. (2004). Effect of social isolation on stress-related behavioural and neuroendocrine state in the rat. *Behavioural Brain Research*, *152*(2), 279–295.

Welberg, L. A. M., & Seckl, J. R. (2001). Prenatal Stress, Glucocorticoids and the Programming of the Brain. *Journal of Neuroendocrinology*, *13*(2), 113–128. https://doi.org/10.1111/j.1365-2826.2001.00601.x

Wenthold, R. J., Petralia, R. S., & Niedzielski, A. S. (1996). Evidence for multiple AMPA receptor complexes in hippocampal CA1/CA2 neurons. *Journal of Neuroscience*, *16*(6), 1982–1989.

Wiley, J. L., & Evans, R. L. (2009). To breed or not to breed? Empirical evaluation of drug effects in adolescent rats. International Journal of Developmental Neuroscience, 27(1), 9-20.

Williams, C. L., & Meck, W. H. (1991). The organizational effects of gonadal steroids on sexually dimorphic spatial ability. *Psychoneuroendocrinology*, *16*(1–3), 155–176.

Wongwitdecha, N., & Marsden, C. A. C. A. (1996). Effects of social isolation rearing on learning in the Morris water maze. *Brain Research*, *715*(1–2), 119–124. https://doi.org/10.1016/0006-8993(95)01578-7

Young, E. A., Altemus, M., Parkison, V., & Shastry, S. (2001). Effects of estrogen antagonists and agonists on the ACTH response to restraint stress in female rats. *Neuropsychopharmacology*, *25*(6), 881–891.

Yuen, E. Y., Liu, W., Karatsoreos, I. N., Feng, J., McEwen, B. S., & Yan, Z. (2009). Acute

stress enhances glutamatergic transmission in prefrontal cortex and facilitates working memory. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(33), 14075–14079. https://doi.org/10.1073/pnas.0906791106

Yusufishaq, S., & Rosenkranz, J. A. (2013). Post-weaning social isolation impairs observational fear conditioning. *Behavioural Brain Research*, *242*, 142–149.

Zalosnik, M. I., Pollano, A., Trujillo, V., Suarez, M. M., & Durando, P. E. (2014). Effect of maternal separation and chronic stress on hippocampal-dependent memory in young adult rats: Evidence for the match-mismatch hypothesis. *Stress*, *17*(5), 445–450.

Zhao, X., Sun, L., Jia, H., Meng, Q., Wu, S., Li, N., & He, S. (2009). Isolation rearing induces social and emotional function abnormalities and alters glutamate and neurodevelopment-related gene expression in rats. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *33*(7), 1173–1177. https://doi.org/10.1016/j.pnpbp.2009.06.016

Zhu, J. J., Esteban, J. A., Hayashi, Y., & Malinow, R. (2000). Postnatal synaptic potentiation: delivery of GluR4-containing AMPA receptors by spontaneous activity. *Nature Neuroscience*, *3*(11), 1098.