The Effects of Chronic Early-life Social Isolation on Hippocampal Cell Number and Hippocampal-Dependent Learning and Memory in Male and Female Rats

by

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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

Before, and for a considerable time after birth, the brain is especially plastic and vulnerable to influences from the environment. Exposure to stressful, or adverse, early-life social experiences, such as social isolation, may impact the brain as it develops and raise susceptibility to developing mental and behavioural disorders. Chronic early-life social isolation (CELSI) is stressful for rats subjected to it, and reproduces a number of behavioural, structural, and neurochemical alterations detected in individuals with schizophrenia. Additionally, the hippocampus, a brain region sensitive to stress, is closely involved in learning and memory and early-life stress has been reported to affect its structure, neuroplasticity, and role in learning and memory. Importantly, few social isolation studies involving rodents have compared both sexes as subjects. Thus, this study explored the impacts of CELSI on brain development and behaviour in adult rats of both sexes. Upon weaning (at postnatal day 21), male and female siblings from 10 Sprague-Dawley rat litters were stratified by sex and randomly assigned to either the group housed (3 animals/cage), or the social isolation (1 animal/cage) condition for 7 weeks. As adults, rats were tested using the Morris Water Maze for 5 days to assess spatial learning and memory. Rats were then sacrificed, and stress-sensitive biometrics, including serum corticosterone levels, were collected. Lastly, the effect of CELSI on neural cell density was investigated by examining the expression of important neuronal and glial proteins (such as PSD-95 and GFAP, respectively) in isolated hippocampal tissue via Western blot analysis. Socially isolated female rats exhibited reduced retroperitoneal fat pads weight and a deficit in acquisition on days 1 and 2 of the acquisition phase. Socially isolated male rats exhibited an enhanced acquisition on day 2 of the acquisition phase and an increased expression of a key neuronal cell marker, PSD-95. As a whole, these data propose a greater

negative effect of CELSI on female rats, and, for specific aspects of hippocampal-dependent behaviour, a sexually dimorphic effect of CELSI.

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List of Abbreviations

AChE	Acetylcholinesterase
ACSF	Artificial cerebral spinal fluid
ACTH	Adrenocorticotropic hormone
ANS	Autonomic nervous system
AVP	Arginine vasopressin
Bax	Bcl-2-associated X
Bcl-2	B-cell lymphoma 2
BDNF	Brain-derived neurotrophic factor
Blk	Blank
BSA	Bovine serum albumin
B_0	Maximum binding
CELSI	Chronic early-life social isolation
CORT	Corticosterone
CRH	Corticotropin-releasing hormone
CNS	Central nervous system
CUS	Chronic unpredictable stress
dtn	Determination
DOHaD	Developmental Origins of Health and Disease
E	East
ECL	Enhanced chemiluminescence
ELS	Early-life stress
ELISA	Enzyme-linked immunosorbent assay
GFAP	Glial fibrillary acidic protein
GR	Glucocorticoid receptor
h	Hour(s)
HPA	Hypothalamic-pituitary-adrenal
IQR	Interquartile range
Isolates	Isolation-reared rats
LC	Locus coeruleus
LTP	Long-term potentiation
MAGUK	Membrane associated guanylate kinase
MAP-2	Microtubule associated protein-2
min	Minute(s)
mPFC	Medial prefrontal cortex
MR	Mineralocorticoid receptor
MWM	Morris Water Maze
N	North
NE	Noradrenergic
N-E	North-East
NMDA	*N*-methyl-D-aspartate
NR2	*N*-methyl-D-aspartate receptor 2
NSB	Non-specific binding
MDD	Major depressive disorder
PBP	Primed burst potentiation

PFC	Prefrontal cortex
PND	Postnatal day
PSNS	Parasympathetic nervous system
POMC	Proopiomelanocortin
PPI	Prepulse inhibition
PSD-95	Post-synaptic density protein 95
PVN	Paraventricular nuclei
s	Second(s)
S	South
SAS	Sympathetic-adrenomedullary system
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNS	Sympathetic nervous system
TA	Total Activity
TBS-T	Tris Buffered Saline with Tween-20
W	West

1.0 Overview and Introduction

Adverse social experiences during early-life can elevate susceptibility to the development of mental and behavioural disorders, including depression, anxiety disorders, and substance abuse (Heim & Nemeroff, 2001). Despite other covariates and genetic predispositions that must be considered, about 80% of young adults who stated that they experienced neglect, or abuse in early-life met the diagnostic criteria for at least one or more psychiatric disorders, such as anxiety disorders, affective disorders, schizoaffective disorders, or behavioural disorders (Lukkes et al., 2009). Serious, or long-term adverse experiences during early-life may force significant long-term costs upon a person as well as health, social, and legal systems (Lukkes et al., 2009). Thus, the impacts of adverse early-life experiences on the fundamental neural mechanisms of mood and anxiety states need to be elucidated (Lukkes et al., 2009).

The perinatal brain is highly plastic and considerably vulnerable to environmental influences (Mychasiuk et al., 2011). Studies conducted on laboratory animals and humans have demonstrated that the perinatal environment may significantly influence the developmental outcome of offspring (Kapoor et al., 2006; Seckl, 2004). Additionally, an extensive body of literature demonstrates that exposure of mammals to stressful, or adverse, early-life experiences, including maternal separation or social isolation from conspecifics, affects brain development (Fone & Porkess, 2008; Weiss et al., 2004). Furthermore, social isolation throughout adolescence is a model of adverse early-life experience for laboratory rats that affects adult behavioural and hormonal responses to distinct tasks (Lukkes et al., 2012; Weiss et al., 2004). The structural and behavioural alterations associated with chronic, early life social isolation (CELSI) are comparable to the differences in behaviour seen in human depression and are regarded as a reasonable and practical model of depression (Carnevali et al., 2012; Dronjak et al., 2007; Fuchs & Flügge,

2006; Hall, 1998; Heim & Nemeroff, 2001; Heinrich & Gullone, 2006; Liu et al., 2005; Serra et al., 2007; Spasojević et al., 2007). Additionally, while the causes of schizophrenia are ambiguous, the chance of developing the neuropsychiatric disorder increases with adverse experiences in early-life and genetic events (Gaskin et al., 2014). Specifically, an animal model of chronic early-life social isolation was created to comprehend the effect of early-life stress (ELS) on adult brain structure and its impact on schizophrenia and psychiatric disorders since it replicates several behavioural, structural, and neurochemical modifications observed in individuals with schizophrenia (Castillo-Gómez et al., 2017).

To introduce my work, I will begin by examining literature on how ELS impacts developmental outcomes. Accordingly, I will delve into stress, the stress response, and the biological embedding of stress and the Developmental Origins of Health and Disease (DOHaD) theory. Next, I will cover how CELSI affects the hippocampus with respect to behaviour, plasticity, structure, and biometrics. Subsequently, I will discuss the behavioural task, the Morris Water Maze (MWM), which was used to conduct my research.

My work addressed the effect of CELSI on brain development and behaviour in adult rats. The first experimental question this thesis proposed was, "Does CELSI affect a number of stress-sensitive biometrics, such as corticosterone (CORT) levels and the weight of adrenal glands, retroperitoneal fat pads, and the liver?" The second experimental question this thesis proposed was, "Does CELSI affect glial or neuronal cell numbers in the hippocampus?" The third experimental question this thesis proposed was, "Does CELSI affect behaviour as it relates to hippocampal-dependent learning and memory?" Each of my experimental questions were examined in both male and female animals.

2.0 Literature Review

2.1 Biological Embedding

Biological embedding is the process whereby environmental factors, including social environments and experiences, impact biological processes and developmental phenotypes pertinent to health in a consistent and enduring way (Hertzman & Boyce, 2010; Sasaki et al., 2013). A growing body of research over the decades suggests that early-life environmental experiences have long-lasting effects on health in adulthood (Champagne et al., 2006; Hertzman & Boyce, 2010; Hertzman, 2013; Meaney, 2001). For example, animal studies have indicated that ELS adversely affects neuroplasticity in brain regions like the hippocampus and has an enduring influence on endocrine systems that control the response to psychosocial stressors (McEwen, 2003; Meaney, 2001). Additionally, human studies reveal that the endocrine response to stress is modified in a lasting way through abuse in early-life (Sasaki et al., 2013). These changes take place alongside alterations in the expression of genes, like brain-derived neurotrophic factor (BDNF), which are implicated in neuroplasticity and affective disorders (Sasaki et al., 2013). A body of literature also suggests gene expression of central and peripheral regulators of the hypothalamic-pituitary-adrenal (HPA) axis, such as the glucocorticoid receptor (GR), are modified by ELS (Sasaki et al., 2013).

Understanding of biological embedding originates from psychoneuroimmunology, psychoneuroendocrinology, and studies regarding brain development of primates during critical periods (Hertzman & Wiens, 1996). The neurological system has been shown to communicate with the immune and endocrine systems to influence the body's ability to fight disease and how critical organs function (Hertzman & Wiens, 1996). Consistent variations in the condition of early environments, regarding stimulation as well as emotional and physical support, may influence how

the central nervous system (CNS) is shaped and its neurochemistry in ways that may negatively impact cognitive, social, and behavioural development (Hertzman, 1999). "The life of the HPA axis in society" provides an initial place to find evidence of biological embedding, since the axis is essential for its part in perceiving and reacting to stressful situations (Hertzman, 1999). Glucocorticoids, such as cortisol, or corticosterone (CORT), are secreted by actions of the HPA axis and have vast metabolic effects on organ systems, which, in the short-term, are adaptive, but in the long-term, may be detrimental to organ systems due to excessive exposure (Hertzman, 1999).

Animal studies offer a model of how the HPA axis factors into overall susceptibility and disease (Hertzman, 1999). A study by Meaney (1999) found that interventions in early-life for rats, such as handling, can perpetually condition the manner in which the HPA axis responds to circumstances throughout life. The conditioning merely occurs in a distinct period during early-life, indicating it relies on proper stimulation in a very defined timeframe of opportunity (i.e., a critical period) during brain development (Meaney, 1999; Sapolsky, 1992). Notably, the effects on the functioning of the HPA axis may be permanent (Hertzman, 1999). Rats subjected to handling in the study had a decreased overall exposure of the brain to CORT throughout life (Meaney, 1999). Prolonged and excessive exposure to CORT places certain hippocampal neurons at risk, but handling caused a decline in the rate of hippocampal neurons lost (Meaney, 1999; Sapolsky, 1992). Cognitive functions are sensitive to small amounts of damage to hippocampal neurons (Meaney, 1999; Sapolsky, 1992). Notably, handled rats did not experience as great a decline in cognition due to aging (Meaney, 1999; Sapolsky, 1992). The finding was exhibited by their performance with age in the MWM, which involves a learning task in which rats must locate a

submerged platform in a pool containing opaque water by using visuospatial cues in the environment (Meaney, 1999; Sapolsky, 1992).

2.2 Developmental Origins of Health and Disease (DOHaD)

The DOHaD theory was put forward to explain the observations connecting early-life events to subsequent adult pathology (Heindel & Vandenberg, 2015; Sullivan et al., 2008). DOHaD theorizes that the physiology of an individual changes in response to the environment to which they were exposed early in the lifespan, and that such responses may endure (Sullivan et al., 2008). Therefore, as offspring are exposed to characteristics of the developmental environment, their course of phenotypic development is modified, usually within a standard physiological range (Hanson & Gluckman, 2014). The modification allows for altered responses to future problems, or for suitable responses to comparable environmental issues in nature in the future (Hanson & Gluckman, 2014). Set points of physiological systems, such as those that maintain homeostasis, may be modified by this adaptation and these responses may be maladaptive if what is anticipated in the intrauterine environment does not match with the environment for which an individual is developed (Sullivan et al., 2008).

The fundamental features of DOHaD that result in subsequent adult pathology include developmental plasticity, the mechanism of epigenetic modification, and the outcome of programming (Thiele & Anderson, 2016). Plasticity refers to the potential for a single genotype to generate several phenotypes (Hanson & Gluckman, 2014). Developmental plasticity is based upon the notion that the relationship between phenotype and genotype is not necessarily predetermined and that phenotypic features may be influenced as a result of processes during development (Hanson & Gluckman, 2014). Therefore, plasticity enables the human body to adjust to various external influences and to be malleable to a certain extent in early childhood (Thiele & Anderson,

2016). The heightened sensitivity of the developmental period is perceived as indicative of the plasticity of organisms as they develop (Heindel & Vandenberg, 2015). Physiological changes that promote fitness exist for many postnatal environments, and therefore, if individuals live outside the scope of the anticipated environment, the potential for disease arises (Gluckman et al., 2006).

Epigenetic mechanisms control tissue-specific gene expression throughout differentiation and are a key factor of the processes of developmental plasticity (Gluckman et al., 2008). Particularly, epigenetic modification is vital to developmental processes during gestation (Thiele & Anderson, 2016). Throughout the lifespan, epigenetic modification is caused by internal and external environmental factors that result in non-coding alterations to genes that leave the DNA sequence unmodified, but that increase or decrease gene expression (Heindel & Vandenberg, 2015; Thiele & Anderson, 2016). Consequently, cells and tissues may become susceptible to disease, or function improperly throughout the lifespan (Heindel & Vandenberg, 2015). Developmental periods, such as gestation and the first several years of life, are distinctly vulnerable to environmental deviations due partially to the sensitivity of epigenetic processes to environmental influences while tissues are differentiating (Heindel & Vandenberg, 2015). The first year of postnatal life is a window of heightened developmental susceptibility (Buss et al., 2012). All tissues have a critical time frame in which the epigenome is very sensitive to environmental influences (Heindel & Vandenberg, 2015).

The term, “programming”, pertains to the association between the environment in early-life and the possibility of disease later in the lifespan as well as to the permanent modifications in organ and tissue structure or functional pathways during fetal and early-life development (Hanson & Gluckman, 2014; Thiele & Anderson, 2016). Particular developmental time periods exist in

which various organs and body systems are more vulnerable to programming, or susceptible to disease (Thiele & Anderson, 2016).

2.3 Stress and the Stress Response

The preservation of homeostasis, a complicated dynamic equilibrium, enables life and is continuously tested by detrimental forces (stressors) that may be intrinsic or extrinsic, actual or apparent (Chrousos & Gold, 1992; Habib et al., 2001). To adapt successfully, a stable state is essential and must be preserved through offsetting or regenerating forces, or adaptive responses, including many physical or mental responses that try to offset the impacts of stressors to restore homeostasis (Chrousos & Gold, 1992). Adaptive responses may be specific to the stressor that gives rise to them or widespread, stereotypic, and nonspecific, which typically happens when the extent of the threat to homeostasis surpasses a particular threshold and is described as the "general adaptation syndrome" (Chrousos & Gold, 1992; Tsigos & Chrousos, 2002). Throughout stress, awareness increases, the brain attends to the apparent threat, cardiac output and respiration speed up, catabolism elevates, and circulation is directed to the stimulated brain, heart, and muscles to deliver the most perfusion and energy (Tsigos & Chrousos, 2002). Thus, stress has a distinct form without a certain specific cause (Selye, 1975).

Stress reactions are not entirely the same, or harmful regardless of the stereotypical impacts of neuroendocrine effectors (Chrousos & Gold, 1992; Szabo et al., 2012). Eustress refers to slight, short-lived, and manageable conditions of contested homeostasis that may be regarded as agreeable or stimulating, and that are advantageous stimuli to emotional and intellectual development (Chrousos & Gold, 1992). In contrast, distress refers to states of psychological, or physical distress that are more serious, lengthened, and unmanageable, and that may lead to disease (Chrousos & Gold, 1992).

In response to stress, the human mind and body trigger an intricate range of physiologic and behavioural CNS and peripheral adaptive responses (Chrousos & Gold, 1992; Habib et al., 2001). Several biological systems, such as the endocrine, cardiovascular, metabolic, and immune systems, are activated as part of the physiological stress response (Dich et al., 2014). The stress response is aided along by the stress system, which includes CNS and peripheral components (Chrousos, 2002; Chrousos & Gold, 1992; Habib et al., 2001). The stress system's principal components are situated in the hypothalamus and the brainstem, and in parvocellular neurons of corticotropin-releasing hormone (CRH), arginine vasopressin (AVP) neurons of the paraventricular nuclei (PVN) of the hypothalamus, CRH neurons of the paragigantocellular and parabranchial nuclei of the medulla and the locus coeruleus (LC), as well as predominantly noradrenergic (NE) cell groups in the medulla and pons (LC-NE system) (Charmandari et al., 2005). The stress system's peripheral components are the peripheral branches of the HPA axis, the efferent sympathetic-adrenomedullary system (SAS), and parts of the parasympathetic branch of the autonomic nervous system (ANS) (Chrousos, 2002; Chrousos & Gold, 1992; Habib et al., 2001). The HPA axis and the efferent SAS constitute the effector limbs, which enable the brain to impact each organ in a situation involving threatening stimuli (Tsigos & Chrousos, 2002). The brain sets off a subdivision of vagal and sacral parasympathetic efferents in a differential manner that brings about gut responses to stress (Tsigos & Chrousos, 2002).

HPA axis response plays a role in controlling several of the body's biological systems in order to enable an organism to adjust to their environment and to foster survival (Murphy et al., 2017). Medial parvocellular neurons of the PVN of the hypothalamus release CRH and AVP (Lupien et al., 2009). The main hypothalamic regulator of the pituitary-adrenal axis is CRH, which activates the production and release of adrenocorticotropic hormone (ACTH) from the anterior

pituitary and results in the creation of glucocorticoids via the adrenal cortex (Abou-Samra et al., 1987; Gillies et al., 1982; Lupien et al., 2009).

The last effectors of the HPA axis are glucocorticoids (Tsigos & Chrousos, 2002). Glucocorticoids are pleiotropic hormones that contribute to the regulation of homeostasis of the entire body and the stress response of organisms by largely exerting their effects through intracellular receptors expressed throughout the body (Calogero et al., 1988; Chrousos, 1995; Lupien, et al., 2009; Tsigos & Chrousos, 2002; Wong et al., 1994). As well, glucocorticoids have a vital part in controlling the basal activity of the HPA axis and in bringing the stress response to an end, through their effects on the hypothalamus and the pituitary gland (de Kloet, 1991). Importantly, cortisol is the major glucocorticoid in humans, while corticosterone (CORT) is the major glucocorticoid in rats and mice (Sullivan et al., 2008; Welberg & Seckl, 2001).

Glucocorticoid receptors (GRs) are concentrated in particular brain structures, such as the hippocampus, the prefrontal cortex (PFC), and the hypothalamus (Krolow et al., 2012). The hippocampus, a key target for glucocorticoids, has the greatest number of GRs and is a particularly sensitive brain region to stress (Bianchi et al., 2005; McEwen, 2008; Sapolsky, 2003). GRs act as ligand-activated transcription factors, which control gene expression, and exist in two receptor subtypes - the lower affinity, GR, and the higher affinity, mineralocorticoid receptor (MR) (Lupien et al., 2009; Welberg & Seckl, 2001). GRs mediate the negative feedback regulation of CRH and ACTH secretion (Chrousos, 2002; Chrousos & Gold, 1992; Habib et al., 2001). The total tissue exposure period of an organism to glucocorticoids is restricted by the negative feedback of glucocorticoids on CRH and ACTH secretion, and reduces the impacts of glucocorticoids on catabolism, lipogenesis, and immunosuppression (Charmandari et al., 2005).

Glucocorticoid secretion enhances cardiovascular tone, restrains immune function, and prepares energy stores to allow an individual to deal with its environment and any stressors (Murphy et al., 2017). Consequently, glucocorticoids may have long-term consequences on the actions of brain regions that control their release (Lupien et al., 2009). The CNS, the immune system, and blood pressure regulation are detrimentally impacted when exposure to high glucocorticoid levels endures for longer than normal (Murphy et al., 2017).

A variety of cognitive, emotional, neurosensory, and peripheral somatic signals from different routes are received and combined by the stress system (Charmandari et al., 2005). The behavioural and physical alterations that arise as the stress system becomes activated are, as a whole, described as the stress syndrome (Charmandari et al., 2005). Typically, such alterations are adaptive, restricted in length, and improve an individual's likelihood of survival (Charmandari et al., 2005). The behavioural adaptations that arise are elevated arousal, attentiveness, and observance, enhanced cognition, concentrated attention, elation, improved inability to feel pain, increased core temperature, as well as inhibition of vegetative functions, such as appetite, feeding, and reproduction (Charmandari et al., 2005). The physical adaptations that arise are associated with the behavioural adaptation to stimulate an adaptive redistribution of energy (Charmandari et al., 2005). Notably, oxygen and nutrients are diverted to the CNS and locations of the body that require them most (Charmandari et al., 2005). Consequently, the alterations are exerted in accordance with elevations in cardiovascular tone, the rate of respiration, and intermediate metabolism to stimulate accessibility of key substrates (Charmandari et al., 2005). Functions that work to detoxify the organism are set in motion to clear any unnecessary metabolic products from metabolic alterations linked to stress (Chrousos, 2002; Chrousos, 2007). Moreover, during that

time, digestive function and growth, reproduction, as well as immunity are hindered (Chrousos, 2002; Chrousos, 2007).

The mesocorticolimbic dopaminergic or reward system, the amygdala-hippocampus complex, and the hypothalamic arcuate nucleus proopiomelanocortin (POMC) neuronal system are three additional main constituents of the CNS that the stress system communicates with and that are initiated throughout the course of stress to impact actions of the stress system (Charmandari et al., 2005). As well, the ANS, which regulates a comprehensive variety of functions, quickly reacts to stressors and includes the sympathetic nervous system (SNS) and the parasympathetic nervous system (PSNS) (Chrousos & Gold, 1992; Tsigos & Chrousos, 1994). Either separately, or together, the SNS and the PSNS control the cardiovascular, respiratory, gastrointestinal, renal, endocrine, and additional systems (Gilbey & Spyer, 1993). Effectors of the sympathetic division mainly present an adaptive advantage in stressful circumstances, while the parasympathetic division, which is strongly connected to the functions of the SNS, brings about the opposite effects of the SNS (Chrousos & Gold, 1992).

The impacts of stress at various stages of life act in relation to one another as suggested by research linking exposure to ELS with greater reactivity to stress and cognitive deficits in adulthood (Lupien et al., 2009). The HPA axis is highly vulnerable to early-life programming during fetal and neonatal development as demonstrated by modification of its set point in several studies on a broad variety of species in early-life (Phillips & Jones, 2006; Sullivan et al., 2008; Welberg & Seckl, 2001). Additionally, animal models regarding alterations in HPA activity and the ANS are associated with changes in behaviour (Phillips & Jones, 2006). Furthermore, a stimulus or stressor that takes place at a critical time during fetal or neonatal development may cause long-term changes in structure and physiology (Sullivan et al., 2008). The effects of

extended exposure to glucocorticoids tend to be largely contingent on the time at which stress occurs (Sullivan et al., 2008). Studies on the HPA axis have revealed that it can be modified or programmed prenatally via limited nutrition, an adverse maternal environment, or synthetic glucocorticoid exposure, and postnatally via neonatal handling, maternal deprivation, or infection (Phillips & Jones, 2006).

2.4 Chronic Early-life Social Isolation (CELSI)

Socially interacting with peers is rewarding for adolescent rats (Douglas et al., 2004). However, social interactions are viewed as one of the main sources of chronic stress for social animals, including humans, and as distinctive influences in the evolution of advanced animal species, like mammals (Blanchard et al., 2002; Eskandari Sedighi et al., 2014; Sapolsky, 2005). Animal models of social stress entail single, sporadic, or chronic exposure of a subject animal to other animals of the same species (Blanchard et al., 2001). Nearly every advanced animal species experiences an aspect of chronic, or sporadic, social stress in their lives (Blanchard et al., 2001). Agonistic, or combative, behaviours, leading to injury, fatigue, and, at times, death, frequently arise due to differences involving resources (Blanchard et al., 2001). Spacing for solitary species is founded on avoidant behaviours observed in chance meetings between conspecifics, while interactions involving agonistic behaviours may be a result when avoidance is lengthy, or unfinished (Blanchard et al., 2001). The evolution of stress mechanisms has been mainly driven by social stress in addition to the stress brought on by predation (Blanchard et al., 2001).

For laboratory rats, early-life social isolation from conspecifics is a stressful experience, particularly during adolescence, as suggested by alterations brought on by isolation rearing in behaviours linked to stress and functioning of the HPA axis (Lapiz et al., 2003; Weiss et al., 2004). The concept of the ‘isolation-induced stress syndrome’ in rats originates from studies completed

in the early 1960s and 1970s that noted isolation-reared rats (isolates) were hyper-emotional, anxiogenic, and more reactive than usual to being handled (Hatch et al., 1965; Holson et al., 1991; Koch & Arnold, 1972; Morgan, 1973; Sahakian et al., 1977; Weiss et al., 2004). Social isolation prevents the modification of an animal's adaptive response due to the absence of social stimuli, which are required to adapt to novel conditions (Ishida et al., 2003; Zlatković & Filipović, 2012). The typically social animals and their unusual HPA axis and behavioural response to environmental stimuli are considered a consequence of extended stress induced by isolation rearing (Serra et al., 2005). CELSI of rats or social species is a long-standing model of early-life adversity and stress as well as social deprivation since it leads to anxiety-like behaviour, unusual social behaviour, and increased emotional sensitivity to social interactions (Ahern et al., 2016; Hermes et al., 2011; Stepanichev et al., 2014).

Most studies on social isolation involve housing the rodent by itself for a part of the juvenile period (Hermes et al., 2011). CELSI involves housing adolescent rats individually from the day of weaning, which varies from postnatal day (PND) 21 to PND 28, for four to eight weeks (Ahern et al., 2016; Lukkes et al., 2009; Stepanichev et al., 2014; Tirelli et al., 2003). Following the four to eight week period, testing begins as rats have reached late-adolescence, or young adulthood (Stepanichev et al., 2014). The four to eight week period generally includes the whole adolescent stage, which is from PND 28 to PND 60 (Ahern et al., 2016).

Chronic social isolation from weaning to late-adolescence, or adulthood, denies rats of social contact throughout a critical developmental period wherein social play is developed (Ahern et al., 2016; Weiss et al., 2004). Social play includes abrupt events of motions, such as pouncing on the back of another rodent, pursuing other rodents, grappling with other rodents, and holding other rodents in place (Hermes et al., 2011). Social play extends for a fixed duration and begins

with a plateau in development from PND 24 to PND 52, while reaching its highest point on PND 32 to PND 40 (Hermes et al., 2011). Early-life social play is adaptive for social species like humans and rodents because it enables the habituation of skills required for appropriate social conduct and behaviours for development in adulthood, in addition to cementing forms of social interactions throughout life (Ahern et al., 2016; Hermes et al., 2011). Thus, isolation during the juvenile period may cause a change in rat behaviour comparable to many of the improper social skills seen in human psychiatric illness (Hermes et al., 2011).

Even though, in the majority of studies, socially isolated rats retain olfactory, visual, and auditory contacts through rearing in the same colony as their socially-reared conspecifics, the absence of physical contact results in a variety of behavioural and physiological responses, which largely impact the emotional reactivity of adult rats (Weiss et al., 2004). Since social deprivation intensifies anxiety, the likelihood that isolates would have propensities with regard to greater anxiety in social circumstances, may be indicated through avoidance, or aggression (Hall, 1998). As such, CELSI in rats causes a substantial range of ongoing behavioural changes, while not producing any ongoing change in body weight from age-matched controls (Fone & Porkess, 2008). The behavioural changes seen constitute the 'isolation syndrome' and are in line with the suggestion that rats brought up in social isolation are incapable of properly processing environmental stimuli (Ahern et al., 2016; Fone & Porkess, 2008). The changes include heightened anxiogenic behaviour, increased timidity, hesitancy towards novelty, inadequate recognition of novel objects, poor attentional set-shifting, a heightened inclination to explore, enhanced locomotor activity, and unusual social behaviour (Ahern et al., 2016; McLean et al., 2008; Quan et al., 2010; Vale & Montgomery, 1997). Findings conflict regarding the reaction of rats to novel objects in a known environment, with some studies reporting isolates as more active and exhibiting

a slower adaptation to exploring relative to socially-reared rats, and others reporting no contrast between isolates and socially-reared rats (Schrijver et al., 2002). Rats reared in isolation even show behaviour comparable to rats treated with amphetamine, such as impulsive hyperactivity, persistent reactions in operant conditions, and impaired behaviours concerning routine (Geyer et al., 1993; Schrijver & Würbel, 2001). The social behavioural changes resulting from CELSI are especially noticeable, such as more activity, including repetitive approach and withdraw behaviour, when introduced to an unknown socially housed rat, in comparison to the potential effects of long-term social isolation as a neonate or an adult on behaviour (Hall, 1998). Also, such CELSI-related changes in social behaviour may endure for prolonged periods, or remain permanently (Tulogdi et al., 2014).

At times, rats have also exhibited a deficit in reversal learning, which involves rats completing two tasks wherein they must first suppress the former strategy and then learn the new strategy (Abdul-Monim et al., 2003). Adequate performance depends on complete cognitive capability including adaptability, concentration, and motivation, so as to develop and apply a formerly learned rule (Abdul-Monim et al., 2003). Deficiencies in cognitive abilities linked to the 'isolation syndrome' are associated with animals that cannot effectively complete a serial reversal learning paradigm (the serial conduction of multiple reversals, which stimulates an automatic propensity to switch, potential rule learning, acquirement of a reversal learning set, and future planning for expected reward contingencies) (Abdul-Monim et al., 2003; Izquierdo & Jentsch, 2011).

Social behaviour varies according to sex, which is an important variable in comprehending how circumstances regarding social upbringing affect later behaviour (Ferdman et al., 2007). Accordingly, the effects of CELSI on stress-related behaviours and HPA axis function may be

contingent on the animal's sex (Wall et al., 2012). Female isolates are more active, more susceptible to stereotypy resulting from amphetamines, and not as afraid or likely to groom as male isolates (Ferdman et al., 2007). A study by Wall et al. (2012) found social interactions were increased in socially isolated male and female rats, while a study conducted by Ferdman et al. (2007) found increased social interaction following social isolation in only male, not female rats. Furthermore, despite the lack of agreement in the literature, one change in social behaviour that may be related to social isolation after birth is greater aggression under certain circumstances (Ahern et al., 2016; Ferdman et al., 2007; Hall, 1998; Hermes et al., 2011; Lukkes, 2009; Wall et al., 2012). For example, in tetradic encounters, isolates aim aggression generally at any rat beginning to socially interact with them, while socially housed rats show aggression solely to inferior rats (Hall, 1998). Also, male rats subjected to CELSI are more aggressive, and the aggression brought on by rearing in isolation was aggravated when testing was conducted in a novel environment (Wongwitdecha & Marsden, 1996; Zhao et al., 2009). Even though the majority of studies regarding CELSI have been conducted on male rats, CELSI of female rats may also lead to unusual patterns of social behaviour, such as greater aggression (Wall et al., 2012). Furthermore, studies investigating socially isolated rodents of both sexes are a minority and have found that isolation made the response in both male and female rats more sensitive to systemic amphetamine (Weiss et al., 2001b), as well as interrupted prepulse inhibition (PPI) (Weiss et al., 2001a) and increased anxiety (Weiss et al., 2004) for just male rats (Pietropaolo et al., 2008). Therefore, the authors of these studies suggested that male rats have a greater susceptibility to the influences of social isolation (Pietropaolo et al., 2008).

2.5 Chronic Early-life Social Isolation and Structural Changes

Brain development of rodents is likely to be affected by post-weaning social deprivation since synaptic density and neuronal neurochemical phenotype only develop completely after the end of weaning (Fone & Porkess, 2008; Lapiz et al., 2003). The cortex of rats reared in isolation was revealed to be the thinnest compared to conditions such as enrichment by group housing with toys, standard with two rats per cage, and switching conditions half way through the 12-week rearing period (isolated-enriched, enriched-isolated, isolated-standard, or enriched-standard) (Hellemans et al., 2004). As well, the expression of microtubule associated protein-2 (MAP-2), a neuronal dendritic marker, was reduced in isolated rats, and indicated abnormal dendritic growth, which may also be responsible for the lack of cognitive adaptability observed in isolates (Bianchi et al., 2006). Also, changes in the hippocampus in volume, dendritic branching, dendritic length, spine density, and synaptic plasticity due to CELSI have been observed (Fone & Porkess, 2008). Golgi-Cox staining in the corpus striatum revealed decreased spine density and dendritic arborization on medium spiny neurons (Comery et al., 1995; Comery, et al., 1996). Likewise, Golgi-Cox staining showed a decreased number of pyramidal neurons in the hippocampus (Silva-Gómez et al., 2003). In rats, such alterations accord with the permanent changes in dendritic morphology in the hippocampus caused by social isolation (Comery et al., 1995; Comery, et al., 1996; Silva-Gómez et al., 2003). Furthermore, alterations in synaptic structure brought on by social isolation rearing have also been found in adult animals (Fone & Porkess, 2008; Hermes et al., 2011). For instance, a presynaptic protein marker, synaptophysin, factors largely into the release of neurotransmitters, and, in rats, was markedly down-regulated in the dentate gyrus following eight weeks of social isolation (Hermes et al., 2011; Varty et al., 1999; Zhang et al., 2012). Hence,

impaired synaptogenesis in the hippocampus brought on by social isolation may explain the abnormal behaviours of adult rats (Hermes et al., 2011).

The hippocampal formation completes maturation from PND 30 to PND 40, while cell proliferation in the dentate gyrus may extend far into adulthood (Kaplan & Bell, 1983; Kaplan & Bell, 1984; Lanier & Issacson, 1977). In particular, most granule cells of the gyrus (85%) are postnatally produced with the most rapid increases in cell number occurring from PND 20 to PND 30 and possibly extending into adulthood (Lapiz et al., 2003). The slow sequence of postnatal development causes the brain to be susceptible to early environmental influences (Lapiz et al., 2003). Adrenal steroids have been observed to decrease the rate of neurogenesis of dentate gyrus granule neurons in the adult rat, and through the *N*-methyl-D-aspartate (NMDA) receptor subtype of glutamate receptors, inhibit excitatory input (Cameron & Gould, 1994; Cameron et al., 1995). Since stress increases circulating adrenal steroid levels and glutamate-mediated excitatory input to the hippocampus, stressful situations may modify the creation of dentate gyrus granule neurons in adult animals (Gould et al., 1997; Krugers et al., 1993; Moghaddam et al., 1994; Bartanusz et al., 1995). Additionally, in male rats, adrenal steroids and excitatory amino acids are involved in a reversible atrophy brought on by stress of dendrites in the CA3 region, and apical dendrites are solely affected, leading to impairments in cognition as well as in spatial learning and short-term memory (McEwen et al., 1995; McEwen et al., 1999). For example, hippocampal structural changes exhibited by socially isolated rats include reduced numbers of newborn neurons as well as length of dendrites and of spine density in pyramidal cell dendrites (Bianchi et al., 2006). Thus, stress experienced by isolates may cause changes, including decreased expression of genes associated with development, in neurogenesis in the hippocampus (Quan et al., 2010).

Isolation rearing seems to change several indicators of synaptic function suggestive of alterations in synaptic plasticity (Fone & Porkess, 2008). Post-synaptic density protein 95 (PSD-95), a neurodevelopment-related post-synaptic marker, is a protein enriched in the post-synaptic densities of dendritic spines and a membrane-associated kinase that may be central to piecing together postsynaptic proteins and that, at the synapse, assists in collecting and securing NMDA receptors, mediating synaptic plasticity, and playing a crucial role in the transduction of signals both in the maturing and developed brain (Chen et al., 1998; El-Husseini et al., 2000; Kim et al., 1996; Lim et al., 2003; Migaud et al., 1998; Sans et al., 2000; Zhang et al., 2012; Zhao et al., 2009). PSD-95 does not have enzymatic activity and functions as an adapter molecule via protein–protein interactions brought about by discrete domains (Migaud et al., 1998). Notably, PSD-95 localizes the NMDA receptor to focal clusters in fibroblasts, which signifies the protein may be necessary for concentrating NMDA receptors at synapses (Kim et al., 1996; Migaud et al., 1998). Thus, some behavioural abnormalities brought on by isolation rearing may involve abnormal expression of PSD-95 (Zhao et al., 2009). For example, in the study by Zhao et al. (2009), substantial up-regulation of PSD-95 in the hippocampus was reported in rats in response to rearing in social isolation. Comparably, in the study by Zhang et al. (2012), mice brought up in social isolation displayed substantially elevated protein expression of PSD-95 in the hippocampus in comparison to mice who were socially-reared, which suggested that social isolation impacted the post-synaptic dendritic complexity of the hippocampus.

In addition, when Lister hooded rats were subjected to isolation for eight weeks, the amount of synapses in the molecular layer of the hippocampal dentate gyrus was found to be decreased alongside insignificant decreases in the hippocampal subfields of CA1 and CA3 (Varty et al., 1999). Bianchi et al. (2006) observed isolation rearing brought on hippocampal microtubule

dynamic instability. The "dynamic instability" was indicated by a reduction in hippocampal tyrosinated α-tubulin (Tyr-Tub) and a comparable rise in detyrosinated α-tubulin (Glu-Tub) alongside a reduction in the ratio of Tyr/Glu-Tub excluding whole alterations in overall α-tubulin capacity (Bianchi et al., 2006). Additionally, Sprague-Dawley rats aged two months and isolated for eight weeks exhibited a specific increase of BDNF in the hippocampus as determined through enzyme-linked immunosorbent assay (ELISA) without an accompanying change in BDNF levels in the PFC or striatum (Scaccianoce et al., 2006). Alterations in the morphology of hippocampal neurons may occur with alterations in neurotrophic factors brought on by isolation rearing (Fone & Porkess, 2008). Notably, such results correspond with the finding that neurogenesis and long-term potentiation (LTP) in the hippocampal CA1 region seem to be significantly decreased in isolates (Lu et al., 2003). Each of these alterations suggest that the organization and innervation of the hippocampus may be adversely affected as a consequence of early social deprivation by potentially influencing synaptic plasticity mechanisms reliant on social play (Robbins, 2016).

Astrocytes and microglia are intermediaries of inflammatory reactions within the brain (Gomes et al., 2015). Particularly, astrocytes account for most of the glial cells in the adult brain of mammals and have significant roles in maintaining the normal functioning of neurons in addition to engaging in processes involved in neurotransmitter uptake, preserving ion homeostasis, and neuronal migration throughout development (Gomes et al., 2015; Kanski et al., 2014). The major intermediate filament of astrocytes, glial fibrillary acidic protein (GFAP), is a type III intermediate filament, expressed in nearly all astrocytes, regulated by cytokines and growth factors, and, in immunohistochemical studies, functions as an astrocytic marker to characterize and evaluate astroglial structure and response, respectively (Gomes et al., 2015; Kanski et al., 2014; Sun et al., 2017). In addition to being crucial to stabilizing and preserving the cytoskeleton

of astrocytes, GFAP is implicated in glial cell plasticity and neuroinflammatory actions involving emotion (Rahati et al., 2016; Sun et al., 2017). Neurons have been reported to account for roughly 75% of newly created cells in the adult rat hippocampal dentate gyrus, while GFAP was positive for 15% of the cells and thus, the cells may be astrocytes (Kim et al., 2007; Rietze et al., 2000; Santarelli et al., 2003).

Evidence indicates modification of GFAP expression results in unusual synaptic functioning and behaviour for rodents (Rahati et al., 2016; Sun et al., 2017). Astrocytes transform from their typical condition of quiescence to reactive when stimulated, indicating astrogliosis (Sun et al., 2017). A distinct feature of reactive gliosis, a process mainly associated with brain injury and aging, is increased GFAP expression, and the area of the brain impacted most by reactive gliosis is the hippocampus (Middeldorp & Hol, 2011). In the healthy CNS, several astrocytes do not express identifiable levels of GFAP, even though GFAP is a reliable indicator for reactive astrocytes (Sofroniew & Vinters, 2010). A range of alterations occur due to the reactive astrogliosis response, such as an increase and decrease of astrocytic function, that is contingent on the type and intensity of the damage, and may either advantageously or harmfully impact adjacent neural tissue (Kim et al., 2018; Sofroniew, 2009). Greater than normal expression of GFAP and astrocyte irregularities have been observed in studies regarding schizophrenia, including one that observed increased GFAP levels in schizophrenic patients relative to controls, and another that observed Gunn rats exhibiting behaviour similar to schizophrenia had considerably elevated hippocampal GFAP levels (Feresten et al., 2013; Limoa et al., 2016; Sun et al., 2017).

In rats and tree shrews, chronic social stress has been linked to a decreased hippocampal volume, a particular reduction in the proliferation of cells in the dentate gyrus and PFC, as well as

diminished hippocampal GFAP immunoreactivity (Fuchs et al., 2004; Czéh et al., 2006; Czéh et al., 2007). Furthermore, reductions in GFAP have frequently been observed in animal models of mood disorders, such as depression, stress, and anxiety (Kim et al., 2018). Gosselin et al. (2009) detected GFAP expression was reduced in the hippocampus, PFC, and basolateral amygdala while using the Wistar-Kyoto rat strain model of depression. Notably, Leventopoulos et al. (2007) observed that ELS in Fischer rats brought about by maternal deprivation resulted in reduced expression of GFAP in adulthood in the dentate gyrus, hippocampus, basolateral amygdala, cingulate cortex, and PFC. Thus, in the long-term, ELS may result in alterations in astroglia density in brain regions, such as the hippocampus, associated with stress responses in rats (Leventopoulos et al., 2007).

2.6 Chronic Early-life Social Isolation and Spatial Learning

2.6.1 The Hippocampus

The hippocampus includes the CA1 to CA3 pyramidal cell fields, the hilus, and dentate gyrus granule cells (Jarrard, 1993), and is one of the most sensitive and malleable regions of the brain, along with being very important in cognitive function (McEwen, 2008). In particular, the hippocampus plays a key role in the brain system in charge of learning and memory (Jarrard, 1993; Xiao et al., 2016). Strong evidence throughout the last decades, such as inadequacies maintaining spatial information following impairments to the hippocampal system (CA fields, subiculum, fimbria-fornix transection), exhibit how essential the hippocampus is to spatial learning and memory (de Bruin et al., 1994; Sharma et al., 2010). Specifically, the hippocampus is crucial to declarative memory, which provides the answer to "what", and consists of comprehending details like places, things, individuals, and the significance of such details (Sharma et al., 2010; Squire, 1992).

2.6.2 The Morris Water Maze

The MWM apparatus was first developed in 1981 by Richard G. M. Morris to study spatial localization, learning, and memory in laboratory rats, and has since become a commonly used apparatus in behavioural neuroscience laboratories (Clark & Martin, 2005; D'Hooge & De Deyn, 2001; Morris, 1984; Sharma, et al., 2010). The apparatus is a sizeable circular pool, which contains opaque water and a small, concealed escape platform (D'Hooge & De Deyn, 2001). The platform is concealed by using materials like tempera paint, or polypropylene pellets, which turn the water opaque, or by matching the colour of the platform to the background with a transparent platform that becomes virtually camouflaged (Vorhees & Williams, 2006). The platform does not provide any proximal cues to direct escape behaviour (Vorhees & Williams, 2006). The task serves as a maze because animals have to look for a concealed platform located just underneath the surface of the water in one position (Vorhees & Williams, 2006). Essentially, rodents can swim randomly, or have unsystematic search routes in the pool to escape the water and with practice, normal animals promptly learn to swim straight to the platform from any position (Vorhees & Williams, 2006).

In the task for the MWM, the concealed platform condition requiring animals to use a spatial strategy typically involving distal cues is hippocampus-dependent, while the observable platform condition is hippocampus-independent (Logue et al., 1997). Rats require the hippocampus to solve different spatial situations that entail applying a place strategy and those with hippocampal damage are impaired in the acquisition of place learning while performing the MWM task (de Bruin et al., 1997). Thus, as evidence suggests, the hippocampus is required for acquisition and retrieval of spatial information in addition to consolidation or storage (D'Hooge & De Deyn, 2001).

2.6.3 Sex and the Morris Water Maze

When devising the MWM and interpreting results, certain features of the experimental rats must be considered (D'Hooge & De Deyn, 2001). For example, body weight, physical development, and age, can affect swimming speed (D'Hooge & De Deyn, 2001). In addition, sex and the strain, or species of the animals may impact performance in the MWM (D'Hooge & De Deyn, 2001). Often, male rats execute the task better, which is not solely due to variations in muscle strength or stamina (D'Hooge & De Deyn, 2001). Variations between strains, or species lead to key discrepancies in performance, which largely impact behavioural findings that must be explicated (D'Hooge & De Deyn, 2001). Sex differences and hemispheric asymmetry studies involving acquisition and two reversal tests in the MWM revealed variations amid male and female rodents in their performance in the MWM, as male rats displayed an upper hand in spatial learning (D'Hooge & De Deyn, 2001; Therrien et al., 1982). Even so, male and female rats tested at the age of six months were revealed to have an equal performance, indicating that the sex differences in spatial learning may represent variations in the rate of development of the hippocampus as opposed to variations in the completely mature hippocampus (Bucci et al., 1995).

Sex hormones may underlie the contrasting cognitive performance amid male and female rodents (D'Hooge & De Deyn, 2001). The foundation for the sexual differences in learning involved in the MWM is proposed to be due to changes in the cholinergic system (Berger-Sweeney et al., 1995; D'Hooge & De Deyn, 2001). Particularly, female mice were reported to be more responsive to the impacts of the cholinergic blocker, scopolamine, in comparison to male mice (Berger-Sweeney et al., 1995). As well, estradiol was proposed to interact with the cholinergic system to improve spatial learning (D'Hooge & De Deyn, 2001). Notably, estradiol, for male rats, enhances MWM acquisition (Packard et al., 1996). However, estradiol's enhancing impacts were

inhibited as a result of scopolamine (Packard et al., 1996). Female rats with intact gonads typically performed less well than ovariectomized females (Daniel et al., 1999). Additionally, female rats performed better throughout the estrus phase as opposed to the proestrus phase (Warren & Juraska, 1997). Conversely, MWM acquisition in ovariectomized females and in male rats improved as a result of intrahippocampal estradiol injection, while estrogen treatment noticeably impeded MWM learning in ovariectomized wild types (Packard et al., 1996; Packard & Teather, 1997; Rissman et al., 1999). The dose may determine if estradiol boosts or impedes spatial learning, and appears to involve multiple estradiol receptors (D'Hooge & De Deyn, 2001). In support of this notion, concealed platform MWM acquisition was only enhanced in ovariectomized mice as a result of moderate estrus-like levels of estrogen and not as a result of high proestrus-like levels (Rissanen et al., 1999). However, in transgenic animals missing estrogen receptor-α, estrogen treatment did not impede MWM learning (Rissman et al., 1999).

2.6.4 Social Isolation and the Morris Water Maze

Findings conflict regarding the effect of social isolation on spatial cognition in the MWM (Quan et al., 2010). Fone and Porkess (2008) reviewed the results from multiple studies on the effect of social isolation on acquisition and retention concerning spatial learning. While most groups demonstrate no change in acquisition in the task when a fixed platform position was used (Lapiz et al., 2001; Quan et al., 2010; Schrijver et al., 2004), other groups have observed moderate learning enhancements (Wongwitdecha & Marsden, 1996), or learning impairments (Hellemans et al., 2004; Lu et al., 2003; Wade & Maier, 1986). Performance in reversal learning in the MWM for one group improved due to isolation rearing in comparison to socially housed controls (Wongwitdecha & Marsden, 1996). While noncognitive factors, such as cue inattention as a result of hyperactivity, and more exploratory behaviour, may generally be associated with deficiencies

in spatial learning tasks in rats reared in isolation, it has been reported that such rats are impaired in spatial acquisition learning in the MWM (Jones et al., 1991; Schrijver et al., 2002; Wade & Maier, 1986). Rats brought up in social isolation upon weaning exhibited better retention and impaired reversal learning in line with the initiation of behavioural rigidity or impaired learning of a novel rule (Fone & Porkess, 2008). Fone and Porkess (2008) proposed that such findings indicate that isolation rearing may mainly impact PFC cortico-striatal pathways as opposed to hippocampal pathways, which are, respectively, associated with reversal learning and spatial learning.

2.7 Chronic Early-life Social Isolation and Apoptosis

Apoptosis, or programmed cell death, is a normal element of brain development intended to remove glia and neurons by denying them of target-derived neurotrophic factors when they do not create the appropriate connections (Zhang et al., 2002). Apoptosis may be impacted by early postnatal life (Najbauer & Leon, 1995), and is implicated in structural alterations seen in human and animal models for mood disorders associated with stress (Chatterjee-Chakraborty & Chatterjee, 2010; Krolow et al., 2012). Several diseases, such as depression, involve abnormal or greater than normal apoptosis (Baek et al., 2011). The rearrangement of members of the B-cell lymphoma 2 (Bcl-2) family amid the cytoplasm and mitochondria is the typical hallmark for the start of the proapoptotic process (Djordjevic et al., 2009). Gene products of the Bcl-2 protein family are involved in controlling cell death mediated by mitochondria and include pro-apoptotic in addition to anti-apoptotic proteins, which result in signals that conclude with cell death (Chatterjee-Chakraborty & Chatterjee, 2010; Krolow et al., 2012; Zlatković & Filipović, 2012). Bcl-2-associated X (Bax) protein, a pro-apoptotic protein, and Bcl-2, an anti-apoptotic protein, are important in deciding whether the neuronal cell dies or survives, since Bcl-2 hinders neuronal death through inactivation of Bax by heterodimerization (Chatterjee-Chakraborty & Chatterjee,

2010). Bax is soluble and mainly located in the cytosol, while Bcl-2 is located in mitochondria (Zlatković & Filipović, 2012). The response of cells to death signals sent from mitochondria is regulated by the ratio of Bcl-2 to Bax in mitochondria (Zlatković & Filipović, 2012).

Apoptosis in the dentate gyrus is impacted by exposure to stress and glucocorticoids (Gould et al., 1997). As such, chronic stress may result in the death of hippocampal neurons as an alternative to neural remodeling (Djordjevic et al., 2009; Sapolsky, 2003). Hippocampal GRs are involved in the control of proapoptotic processes (Djordjevic et al., 2009). In a study by Djordjevic et al. (2009), both Bax and Bcl-2 mRNA levels decreased during chronic isolation stress, which involved singly housing adult rats for 21 days. Djordjevic et al. (2009) reported that chronic stress additionally resulted in the movement of Bcl-2 protein from mitochondrial membrane to the cytoplasmic compartment of the hippocampus, in which the soluble form of Bcl-2 increased. Therefore, Djordjevic et al. (2009) determined that during chronic isolation stress, the start of proapoptotic signaling was activated. As well, during chronic isolation stress, the level of Bcl-2 protein in mitochondrial extracts was reduced and the level of Bax protein in the cytoplasm stayed the same, but was reduced in mitochondria (Djordjevic et al., 2009).

The structure of hippocampal parameters, including cell number, can be impacted by chronic stress (Heine et al., 2004). Evidence demonstrates that rats reared in enriched environments, compared to those reared in isolation, grow to have a cerebral cortex of greater weight, more sizeable cell bodies, a larger amount of dendritic spines, and a greater quantity of synapses (Nilsson et al., 1999). Stimulation of endogenous growth factors is presumed to be a mechanism that brings about such alterations in morphology (Nilsson et al., 1999). Alterations in growth factor expression as a result of maternal deprivation may significantly affect brain development (Zhang et al., 2002). Accordingly, when the supply of growth factors becomes

adequately restricted in size, apoptosis of developing neurons occurs by causing the neurons on their own to set off a sequence of biochemical steps that ultimately leads to their shrinkage and death (Zhang et al., 2002). Moreover, due to high glucocorticoid levels from stress, BDNF mRNA may be down-regulated in the dentate gyrus and the ability of granule cells to remain alive may be influenced by a modification in the levels of BDNF (Huot et al., 2002). Famitafreshi et al. (2015) found isolates had reduced levels of BDNF, which mainly assists cholinergic, dopaminergic, and motor neurons to develop and survive, compared to socialized rats. These reduced levels of BDNF are associated with a decreased volume of the hippocampus (Erickson et al., 2010).

2.8 Chronic Early-life Social Isolation and Biometrics

Stress, toxicity, and metabolic dysfunction may affect the value of organ weights, which are often used to assess toxicities related to drugs (Robb et al., 2017; Sellers et al., 2007). For example, changes in liver weight may indicate the presence of cellular alterations, like hepatocellular hypertrophy caused by enzyme induction, or peroxisome proliferation (Sellers et al., 2007). Differences in the weight of adrenal glands can point to hypertrophy, hyperplasia, or atrophy related to stress, endocrine gland diseases, or impacts of drug-related toxicities (Sellers et al., 2007). In particular, isolation rearing has been reported to not affect adrenal gland weight, but does increase levels of ACTH (Fone & Porkess, 2008). However, in a study conducted by Hatch et al. (1965), the weight of the adrenal glands was reported to be significantly larger for female isolates, while similar alterations happened for male isolates, except they were statistically insignificant. Additionally, in a study conducted by Syme (1973), isolates, after 14 weeks, had significantly higher relative and absolute adrenal gland weights compared to control group housed rats. Furthermore, a study by Ieraci et al. (2016) determined that socially isolated adult mice exhibited a reduced difference in weight amid the left and right adrenal gland. The left adrenal

gland weighed more than the right and this result has been consistently described in literature (Droste et al., 2003; Gerendai & Halász, 1997; Ieraci et al., 2016). Also, a significant variance amid the right adrenal gland and the left for group housed mice and no significant variance for socially isolated mice were found (Ieraci et al., 2016). Moreover, a study that individually analyzed both male and female rats found that in adult male rats, isolation stress experienced in the prepubertal period raised adrenal gland weight, but, in females, no difference was found in adrenal gland weight (Krolow et al., 2013). Another study observed that adult female rats subjected to isolation during adolescence displayed significantly greater adrenal gland weights, while male rats subjected to isolation during adolescence had insignificantly smaller adrenal gland weights (Weintraub et al., 2010).

During the adolescent period, stressful experiences may result in extensive and enduring effects on the HPA axis as it develops (Barha et al., 2010). Findings from rodent studies suggest stressful experiences in adolescence may elevate rodents' reactions to stressors in later life (Barha et al., 2010). Particularly, increased levels of CORT and ACTH were found in males in reaction to stress, rather than at baseline (Isgor et al., 2004), and increased levels of CORT were found in females in reaction to stress (Pohl et al., 2007). In response to a stressor, adolescent male and female rats have also displayed longer than normal release of CORT in contrast to adult rats (Romeo et al., 2004a; Romeo et al., 2004b; Romeo & McEwen, 2006; Viau et al., 2005). Additionally, Gomez et al. (2004) discovered acclimatization to chronic stressors did not occur for adolescent male rats in comparison to adult rats who ultimately exhibited a dampened CORT reaction for chronic stressors. As such, adolescents compared to adults are more vulnerable to the harmful consequences of stress on the activity of the HPA axis (Barha et al., 2010). Findings concerning the effect of isolation rearing on basal plasma CORT levels conflict with one another

(Schrijver et al., 2002). Isolation rearing has been reported to elevate, reduce, or leave basal plasma CORT levels unaffected (Schrijver et al., 2002). Specifically, when rearing occurred in standard cages, social isolation left basal CORT levels the same (Schrijver et al., 2002). However, when rearing occurred in wire floor cages, which makes isolation less favourable, social isolation rearing increased basal CORT levels (Heidbreder et al., 2000).

3.0 Study Rationale

Despite the fact that several studies have examined the influence of CELSI on measures of behaviour in rodents, results have varied for nearly all behavioural features (Fone & Porkess, 2008; Lukkes et al., 2009; Schrijver et al., 2002). In particular, the impact of isolation rearing on spatial learning and memory tasks in the MWM has varied (D'Hooge & De Deyn, 2001; Schrijver et al., 2002; Therrien et al., 1982; Wongwitdecha & Marsden, 1996). As well, studies on isolation rearing and the functioning of the HPA axis have been inconsistent with respect to basal plasma CORT levels (Schrijver et al., 2002). In addition, CELSI has led to several alterations in the morphology, structure, and synaptic functioning of the hippocampus that are indicative of changes in plasticity (Fone & Porkess, 2008; Wall et al., 2012). Furthermore, chronic social isolation stress has been shown to impact hippocampal cell number by causing cell death through decreased mRNA levels of Bax and Bcl-2 and initiating proapoptotic signaling due to the relocation of Bcl-2 protein from the mitochondria to the cytoplasm in the hippocampus (Djordjevic et al., 2009). Moreover, only a small number of studies on social isolation in rodents have clearly carried out a comparison with both sexes as subjects (Abramov et al., 2004; Guo et al., 2004; Weiss et al., 2001a; Weiss et al., 2001b; Weiss et al., 2004). Therefore, the research directly addresses gaps in the literature surrounding inconsistencies in the impacts of CELSI on hippocampus-dependent learning and memory, stress-sensitive biometrics, changes in cell number and structure, as well as sex-specific differences in each aspect of the research.

I intended to accomplish the following research goals:

1. Determine how CELSI impacts a number of stress-sensitive biometrics, such as serum CORT levels and the weight of adrenal glands, retroperitoneal fat pads, and the liver.

2. Determine how CELSI affects glial or neuronal cell numbers in the hippocampus by measuring the expression of one key structural protein within each cell type – GFAP for glial cells and PSD-95 for neuronal cells.
3. Determine how CELSI influences behaviour as it relates to hippocampal-dependent learning and memory by testing spatial learning and memory using the MWM.
4. Determine possible sex-specific effects of CELSI on biometrics, behaviour, and glial or neuronal cell numbers in the hippocampus by using and analyzing both male and female animals.

I hypothesized that, in comparison to rats in the control group, rats subjected to CELSI would have elevated serum CORT levels and heavier weights for the adrenal glands, the retroperitoneal fat pads, and the liver.

Additionally, I hypothesized that glial, or neuronal cell numbers in the hippocampus would be reduced in rats subjected to CELSI. I expected this to occur based on reports in the literature that isolates had negative changes in the hippocampus in volume, dendritic branching, dendritic length, spine density, and synaptic plasticity. Furthermore, I hypothesized that rats in the CELSI group would perform less well during the MWM compared to rats in the control group. I anticipated this outcome because a proportion of studies suggest CELSI-related stress impairs learning and memory in the MWM. However, a noticeable proportion of conflicting findings from studies concerning the impact of isolation rearing on acquisition and retention of spatial learning in the MWM indicate a difference in spatial learning may not be observed, potentially due to variations in the species, or strain of rats, and isolation procedures.

Finally, I hypothesized that female rats, compared to male rats, in the CELSI group would have similar serum CORT levels and weights for the adrenal glands, the retroperitoneal fat pads,

and the liver. Based on the literature, I hypothesized that female rats in the CELSI group would have a greater reduction in hippocampal glial and neuronal cell numbers compared to male rats in the CELSI group. Additionally, I hypothesized that male rats in the CELSI group would perform better than female rats in the CELSI group, since male rats have been reported to have an advantage in MWM acquisition training over female rats in the literature due to sexual differences in MWM learning.

4.0 Materials and Methods

4.1 Animals

Each week for a period of 10 weeks, a female, Sprague-Dawley rat and her litter (at PND 7) arrived at our facility (Figure 1); each litter was regarded as a block within the study. On PND 21, the day of weaning, the pups from each litter (N = 10) were stratified by sex (N = 5 male and N = 5 female pups per litter). Next, each sub-group of five animals was randomly separated into either the group housed condition (control group), which involved placing three animals per cage, or the social isolation condition (experimental group), which involved placing both of the remaining animals into separate cages. All rats were maintained on a 12-hour (h) reverse-light cycle (11:00 a.m. to 11:00 p.m.) in a temperature-regulated room (22ºC ± 1ºC), and provided with free access to standard rodent chow and water. After seven weeks (from PND 70 to PND 75), two rats of each sex from the group housed condition and a single rat of each sex from the social isolation condition underwent MWM testing. All animals were handled in accordance with procedures approved by the University of Waterloo Animal Care Committee (ORE 30042).

4.2 Morris Water Maze

The MWM was the behavioural protocol used to evaluate spatial learning and memory. Spatial representations were determined for the MWM as outlined in Standard Operating Procedure (SOP) Central Animal Facility (CAF) OPNHUS 030. The MWM was comprised of a circular black plastic water tank (180 cm in diameter, 50 cm in height) containing no proximal cues and water (30 cm in depth, 22°C ± 1°C in temperature). The testing room was kept at a temperature of 22ºC ± 1ºC. The circular hidden platform (28 cm in height, 18 cm in diameter) was one-tenth the diameter of the water tank and had a rough plastic platform surface to allow friction that enabled subjects to walk and sit. The platform was positioned in the North-East (N-E) quadrant

of the water tank, located 1 cm to 2 cm below the water's surface, and remained in this position all through the study. Four extra-maze visual cues were used in the MWM, with two distal visual cues secured directly above the East (E) and West (W) starting positions of the water tank and two distal visual cues secured to the room walls closest to the E and W starting positions of the water tank, such that they were all visible to the experimental subjects. To allow for consistent lighting around the room and water tank, five lamps were positioned on the floor, or on tables close to the four starting positions (North (N), South (S), E, and W) of the water tank and room lights remained off. A video camera was fixed to the ceiling overhead the middle of the water tank and used to track each rat's escape latency, path length, and swim speed. Videos captured were analyzed for behavioural variables with the video tracking software, Noldus EthoVision XT v8.5.

The MWM test involved a spatial acquisition (learning) phase and a probe phase (spatial memory retrieval test). The spatial acquisition phase was conducted over four consecutive days from PND 70 to PND 74. The experimenter remained in the room as a monitor during testing. Four swimming trials per block from the four starting positions (N, S, E, and W) were conducted for every rat on each day of the spatial acquisition phase. Every one of the four trials began from a different starting position (N, S, E, or W) and the sequence was selected pseudorandomly (not more than once from the same starting position) and differed daily. Rats were allowed up to 60 seconds (s) to locate the hidden platform once they were released facing the water tank wall from one of four starting positions (N, S, E, and W), and were led by the experimenter to the platform if they did not locate the platform by the end of the trial. Once on the platform, every rat was permitted to remain there for 30 s. In between the four swimming trials per block, rats were dried and placed in individual shoebox cages with a heating pad located below for inter-trial intervals ranging from 60 s to 7.5 minutes (min), since six rats per group were run in sequence and inter-

trial intervals times decreased depending on how well rats in each block learned the MWM over the four days.

On PND 75, 24 h following the final day of acquisition, rats underwent one probe trial to evaluate hippocampal-dependent spatial reference memory, and to analyze the strength of learning by determining if the rat recalled the location of the hidden platform. The hidden platform was taken out from the MWM, every rat was released into the quadrant opposite to the location where the platform was once positioned (S water tank wall), and then allowed to swim without restriction for 30 s. Rats were placed in individual shoebox cages with a heating pad located below in anticipation of, and following, each probe trial. Once behavioural testing was completed each day, rats were dried off and placed back into their respective housing conditions.

4.3 Terminal Biometrics

On PND 78, three days (72 h) for male rats, and, on PND 79, four days (96 h) for female rats following the probe test, the body weight of one rat of each sex from each housing condition was measured. Each rat was then anaesthetized with >60% CO_2 via inhalation in an induction chamber, and promptly sacrificed by decapitation. Trunk blood was immediately collected and the adrenal glands, retroperitoneal fat pads, and liver were removed and weighed. Adrenal glands weight, liver weight, and retroperitoneal fat pads weight were calculated as a percentage of body weight to ensure no bias as a result of the rat's body weight. To measure serum CORT levels in the rats, the collected trunk blood from each rat 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 supernatant, or blood serum, was collected and kept at -80°C up to the point of analysis using a corticosterone ELISA kit (Cayman Chemical).

4.4 Corticosterone ELISA Assay

To assess the response of the HPA axis to CELSI in male and female rats, serum CORT levels were measured through ELISA. First, ELISA buffer was prepared by diluting one vial of ELISA Buffer Concentrate (10X) using 90 mL of T1 water and rinsing the vial to eliminate any salts that potentially precipitated. Wash buffer was also prepared by diluting one 5 mL vial of Wash Buffer Concentrate (400X) to a total volume of 2 L with T1 water and adding 1 mL of Polysorbate 20. To create the bulk standard (50 ng/mL), 100 µL of Corticosterone ELISA Standard was transferred to a test tube and 900 µL of T1 water was added for dilution. Eight test tubes were numbered from 1 to 8. A 900 µL aliquot of ELISA buffer was added to tube 1 and 750 µL aliquot of ELISA buffer was added to tubes 2 through 8. From the bulk standard, 100 µL were transferred to tube 1 and mixed completely. A serial dilution of the standard was performed from tube 1 to tube 8, by taking out a 500 µL aliquot and mixing completely. Following this, 100 determination (dtn), where one dtn is the amount of reagent used per well as defined in Cayman Chemical's Corticosterone ELISA kit, Corticosterone Acetylcholinesterase (AChE) Tracer was reconstituted with 6 mL of ELISA Buffer and 100 dtn Corticosterone ELISA Antiserum was reconstituted with 6 mL of ELISA Buffer.

Using the kit's 96 well microtiter plate, which was already coated with the primary corticosterone antibody, 100 µL and 50 µL of ELISA Buffer were loaded into non-specific binding (NSB) wells and maximum binding (B_0) wells, respectively. From tube 8 to tube 1, 50 µL aliquots of Corticosterone ELISA Standard were loaded in duplicate in order from the lowest standard wells to the highest standard wells. Samples were loaded in triplicate by adding 50 µL aliquots to each well. Aliquots of 50 µL of Corticosterone AChE Tracer were added to all wells other than the Total Activity (TA) and Blank (Blk) wells. Aliquots of 50 µL of Corticosterone ELISA Antiserum were

added to all wells other than the TA, the NSB, and the Blk wells. The plate was then covered by plastic wrap and incubated at 4 °C overnight.

Directly prior to use, a 100 dtn vial Ellman's Reagent was reconstituted using 20 mL of T1 water. Wells were cleared and rinsed on five occasions using Wash Buffer. Next, 200 µL aliquots of Ellman's Reagent was loaded in all wells and 5 µL of the Corticosterone AChE Tracer was loaded in the TA well. The plate was covered with plastic wrap and left to develop for 90-120 min using an orbital shaker. The bottom of the plate was cleaned and the plate cover was gently removed to prevent Ellman's Reagent from splashing on the cover. The plate was then read at a wavelength ranging from 405-420 nm, to include the wavelength, 412 nm, at which the product, 5-thio-2-nitrobenzoic acid, of the non-enzymatic reaction of thiocholine with 5,5'-dithio-*bis*-(2-nitrobenzoic acid) is strongly absorbed.

4.5 Brain Extraction, Homogenate Preparation, and Protein Quantification

After euthanasia, each brain was excised from the rat and promptly put into chilled (4°C) artificial cerebral spinal fluid (ACSF) made 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 to 7.43 and an osmolality of 300 mOsm to 320 mOsm. The right hippocampus, left hippocampus, and the PFC were removed (about 60 s). One of the hippocampi and the PFC were snap frozen in 1.5 mL Eppendorf tubes with liquid nitrogen (N_2) and kept at -80°C for later use.

For analysis, each hippocampal sample was homogenized. All necessary instruments were put on ice for a minimum of 10 min before homogenization. For each sample, 15 mL of lysis buffer was made from 14,850 µL of non-ionizing lysis buffer (100 mM NaCl, 25 mM EDTA, 10 mM Tris, 1% (v/v) Triton X-100, 1% (v/v) NP-40) (stored at 4 °C) and 150 µL of a protease inhibitor

cocktail (AEBSF, Aprotinin, Bestatin hydrochloride, E-64, Leupeptin hemisulfate salt, Pepstatin A) (stored at -20°C) and kept on ice. Every snap frozen hippocampal hemisphere was then placed into a 5 mL Potter-Elvehjem homogenizer on ice using chilled forceps, and then homogenized with 3 mL of the lysis buffer. After homogenization, each homogenate was divided in an approximately equal manner between two 1.5 mL Eppendorf tubes and then centrifuged at 1000 × g for 10 min at 4°C. Following centrifugation, the supernatants were collected, pooled, and then divided into four aliquots that were either used for a subsequent protein assay, or placed at -80°C for later use.

A BioRad DC Protein Assay kit was used to ascertain the protein concentration of all snap frozen hippocampal hemisphere homogenates. A standard curve was prepared with bovine serum albumin (BSA). The BioRad DC Protein Assay kit reagents were used to produce the working reagent, reagent A', which contains 20 µL of reagent S (cat# 500-00115) and 1 mL of reagent A (cat# 500-00113). A new and dry 96 well microtiter plate was loaded in triplicate with 5 µL of each standard and lysate sample per block into the specified wells. Following this, 25 µL of reagent A' was added to all wells and the microtiter plate was lightly shaken for 5 s to 10 s to mix reagents. Subsequently, 200 µL of reagent B (cat# 500-00114) was added to all wells and the microtiter plate was lightly shaken for 5 s to 10 s to further mix reagents. Samples were then incubated for 15 min to 20 min without mixing and absorbances were read using spectrophotometry at 750 nm. To ascertain the protein concentration of all unknown samples per block, the optical density of the protein standard, BSA, was contrasted with that of the unknown sample.

4.6 SDS-PAGE and Immunoblotting

All hippocampal homogenate samples were removed from the -80°C freezer and left to defrost on ice. The apparatus for electrophoresis was then assembled, and the required volume of

the defrosted hippocampal samples and the same quantity of 2× sample buffer were added to a smaller Eppendorf tube. The mixture was then vortexed for 1 s to 2 s, centrifuged at 1000 × g for 30 s, and then put into a heating block at 95℃ for 5 min. Subsequently, the tubes were removed using forceps and vortexed for 1 s to 2 s, and pulsed in the centrifuge.

Protein samples (in order from group housed male, socially isolated male, group housed female, to socially isolated female for each block) and their duplicates were loaded into the 10% gels. The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was run at 200 V for about 1 h. When the run was complete, the gels were removed from the apparatus and left to soak for 15 min in chilled transfer buffer in a Petri dish.

The PVDF membranes were pretreated with methanol, T1 water, and transfer buffer for 20 s to 30 s, 2 min, and 5 min, respectively. After this, "sandwiches" containing the gels and PVDF membranes were assembled, and the protein were transferred from the gels to the membranes over 90 min using a current of 350 mA. To confirm whether the protein samples transferred properly, PVDF membranes were incubated with Ponceau S stain, and then destained using several rinses with T1 water. The PVDF membranes were then left to air dry for approximately 15 min and the Ponceau S images were taken and saved. To preserve the membranes when the transfer ended, the blot was labelled and kept at 4℃ until used for immunoblotting.

Prior to immunoblotting, each membrane was cut horizontally into two sections (based on the molecular weights of the proteins of interest; GFAP and PSD-95), rinsed in methanol for 30 s, and then incubated using gentle agitation with blocking buffer made of 5% skim milk powder (w/v) 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) for 1 h at room temperature. Membranes for blocks 1 and 3 were incubated with the primary antibodies (*rabbit anti-PSD-95 affinity purified polyclonal antibody*

(cat. AB9708, EMD Millipore) and *GFAP (GA5) Mouse mAb #3670 (Cell Signaling Technology)*) and membranes for blocks 4, 5, 6, 8, 9, and 10 were incubated with the primary antibodies *(anti-PSD95, clone K28/43 (cat. MABN68, lot 2145078, EMD Millipore)* and *mouse anti-Glial Fibrillary Acidic Protein (GFAP) monoclonal antibody, clone GA5 (cat. MAB3402, EMD Millipore)*) at concentrations of 1:1000 at 4°C overnight. The primary antibody solutions were decanted and 3 × 10 min washes were done using TBS-T. Following this, suitable enzyme-conjugated secondary antibodies *(goat anti-rabbit immunoglobulin G-horseradish peroxidase [IgG-HRP], Santa Cruz Biotechnology, cat. SC 2004, or goat anti-mouse immunoglobulin G-horseradish peroxidase [IgG-HRP], Santa Cruz Biotechnology, cat. SC 2005)* at concentrations of 1:5000 were diluted in 5% (w/v) skim milk/TBS-T blocking buffer and membranes were incubated at room temperature for 1 h using gentle agitation. The secondary antibody solutions were then decanted and 3 × 10 min washes were done using TBS-T. Next, for 1 min to 5 min, blots were covered with enhanced chemiluminescence (ECL) solution, *Immobilon Crescendo Western HRP Substrate (Millipore)*. Blots were then positioned between plastic wrap, and air bubbles were taken out by smoothing over the covered blot. To obtain densities of the protein bands for PSD-95 and GFAP, blots were exposed at intervals from 30 s to 10 min with the gel documentation system, SYNGENE. The points analyzed originated from the detected linear portion of the curve.

4.7 Statistical Analysis

To analyze the effect of social isolation, each experimental data point was standardized to its sex and block-specific control data point; that is, each social isolation value was taken as a percent of its respective sex and group housed value. Data for escape latency and path length from the acquisition trials were averaged for each block of four trials per day to determine the percent group housed block average, while probe trial and swim speed data were averaged for each block

to determine the percent group housed block average. The Wilcoxon signed-rank test (a non-parametric version of the one-sample *t*-test) was used to determine if there was an effect of housing condition on days 1, 2, 3, and 4 of the acquisition phase, the average time spent in the target quadrant during the probe test, swim speed, body weight, liver weight, adrenal glands weight, retroperitoneal fat pads weight, and serum CORT within male rats and within female rats. The Mann-Whitney U test (a non-parametric version of the two-sample *t*-test) was used to determine if there was a difference in the effect of housing condition on days 1, 2, 3, and 4 of the acquisition phase, the average time spent in the target quadrant during the probe test, swim speed, body weight, liver weight, adrenal glands weight, retroperitoneal fat pads weight, and serum CORT between male and female rats.

Outliers in the dataset were excluded by way of Tukey's method (box and whiskers plot) using GraphPad Prism 7. In Tukey's method, the outlier value is contrasted with the calculated upper and lower limits, referred to as "fences (whiskers)", for exclusion (GraphPad Software Inc., 2017; Tukey, 1977). Computation of the fences involves determining the interquartile range (IQR), which is the difference amid the upper quartile (75th percentile) and the lower quartile (25th percentile), for the dataset (Jung & Adeli, 2009). The lower fence is computed by subtracting 1.5 times the IQR from the 25th percentile and the upper fence is computed by adding 1.5 times the IQR to the 75th percentile (Jung & Adeli, 2009). Values not in the computed interval [Q1 – 1.5(IQR), Q3 + 1.5(IQR)] are outliers and can be removed from the dataset (Jung & Adeli, 2009). Tukey's method is appropriate for use in this study, since it is a non-parametric method that avoids any assumptions concerning the underlying distribution of the dataset (Potvin & Roff, 1993). Also, accurate use of Tukey's method is possible even with more than one outlier (Horowitz et al., 2008).

The practical significance of the results were examined using Cohen's *d*, which is an effect size measure that considers the standardized difference between two means, and 95% confidence intervals (CIs) (Nakagawa & Cuthill, 2007). Cohen's *d* was computed with an online calculator (Lenhard & Lenhard, 2016), while 95% CIs were computed using GraphPad Prism 7. Cohen's conventional values for a small effect size ($d = 0.2$), a moderate effect size ($d = 0.5$), and a large effect size ($d = 0.8$) were used for interpreting the biological importance of the standardized effect sizes (Nakagawa & Cuthill, 2007).

5.0 Results

5.1 Morris Water Maze Behavioural Data

5.1.1 The Effects of CELSI on Spatial Memory Acquisition

Spatial learning was evaluated by escape latency (the time elapsed prior to the rat mounting the platform to escape the water) and by path length (distance travelled by the rat from start to mounting the platform to escape the water) (Vorhees & Williams, 2006). Notably, data from the second group housed male rat from block nine was excluded from analysis, since the rat was identified as a nonperformer unable to learn the location of the hidden platform. A decrease in escape latency was seen in rats of both sexes and housing conditions, as displayed in Figure 2.

Analysis through the Wilcoxon signed-rank test indicated no statistically significant effect of housing condition on escape latency for day 1 ($p = 0.92$), day 3 ($p = 0.16$), and day 4 ($p = 0.63$) of training within male rats, and for day 2 ($p = 0.70$), day 3 ($p = 0.77$), and day 4 ($p = 0.38$) of training within female rats (Tables 1 and 2). Computation of Cohen's *d* also indicated a lack of notable effect by housing condition on escape latency over day 1 ($d = 0.09$), day 3 ($d = 0.48$), and day 4 ($d = 0.15$) within male rats, and for day 2 ($d = 0.41$), day 3 ($d = 0.12$), and day 4 ($d = 0.49$) within female rats.

Wilcoxon signed-rank test analysis revealed a statistically significant difference for the effect of housing condition on escape latency for day 2 ($p = 0.04$) of training within male rats and for day 1 ($p = 0.03$) of training within female rats. Cohen's *d* effect sizes generally agreed with the *p* values for within male rats on day 2 ($d = 0.73$) as well as within female rats on day 1 ($d = 0.81$).

Additionally, a statistically significant difference between male and female escape latency based on housing condition was not observed by analysis following the Mann-Whitney U test: day

1 ($p = 0.08$), day 2 ($p = 0.06$), day 3 ($p = 0.63$), and day 4 ($p = 0.63$). Calculation of Cohen's d revealed large effect sizes for day 1 ($d = 0.88$) and day 2 ($d = 0.98$) of the acquisition phase, and small effect sizes for day 3 ($d = 0.32$) and day 4 ($d = 0.19$) of the acquisition phase.

Reductions in path length during the acquisition phase were observed in rats of both sexes and housing conditions as exhibited in Figure 3. No statistically significant differences for the effect of housing condition on path length were observed for day 1 ($p = 0.77$), day 2 ($p = 0.19$), day 3 ($p = 0.43$) and day 4 ($p = 0.70$) of training within male rats and for day 2 ($p = 0.49$), day 3 ($p = 0.85$) and day 4 ($p = 0.38$) of training within female rats following analysis by Wilcoxon signed-rank test. Calculation of Cohen's d suggested no differences for the effect of housing condition on path length for day 1 ($d = 0.21$), day 2 ($d = 0.47$), day 3 ($d = 0.31$) and day 4 ($d = 0.11$) within male rats, and for day 2 ($d = 0.51$), day 3 ($d = 0.02$) and day 4 ($d = 0.22$) within female rats. Additionally, Wilcoxon signed-rank test analysis disclosed a statistically significant difference for the effect of housing condition on path length for day 1 ($p = 0.04$) within female rats, which was further confirmed by a large effect size for day 1 ($d = 0.93$). Furthermore, a statistically significant difference was not observed between male and female rats for the effect of housing condition on path length for day 1 ($p = 0.07$), day 2 ($p = 0.08$), day 3 ($p = 0.68$), and day 4 ($p = 0.39$). Cohen's d was computed and presented large effect sizes for day 1 ($d = 0.90$) and day 2 ($d = 0.96$) of the acquisition phase, in addition to small effect sizes for day 3 ($d = 0.27$), and day 4 ($d = 0.34$) of the acquisition phase.

5.1.2 The Effects of CELSI on Spatial Reference Memory in the Probe Test

To evaluate spatial reference memory, probe trial performance was measured using average time spent in the quadrant where the platform was once located (target quadrant) throughout the 30 s probe trial (D'Hooge & De Deyn, 2001). In the target quadrant, group housed

male rats averaged 9.30 s, socially isolated male rats averaged 11.54 s, group housed female rats averaged 10.39 s, and socially isolated female rats averaged 12.53 s (normalized data presented in Figure 4). Analysis by the Wilcoxon signed-rank test indicated no statistically significant differences for the effect of housing condition on average time spent in target quadrant by either male ($p = 0.23$), or female rats ($p = 0.13$). Cohen's *d* was computed and revealed a small effect of housing condition on average time spent in target quadrant within male rats ($d = 0.45$), and a moderate effect of housing condition on average time spent in the target quadrant within female rats ($d = 0.62$) (Table 3). Analysis by the Mann-Whitney U test revealed no statistically significant differences between male and female rats ($p = 0.85$) for the effect of housing condition on average time spent in the target quadrant. Calculation of Cohen's *d* revealed a small effect of housing condition on average time spent in the target quadrant during the probe trial ($d = 0.22$).

5.1.3 The Effects of CELSI on Swim Speed

To evaluate the motor function of the rats, swim speed was measured during the probe trial (Lindner, 1997; Vorhees & Williams, 2006). During the probe trial, group housed male rats averaged 26.51 cm/s, socially isolated male rats averaged 28.22 cm/s, group housed female rats averaged 28.10 cm/s, and socially isolated female rats averaged 29.35 cm/s (normalized data presented in Figure 5). Wilcoxon signed-rank test analysis indicated no statistically significant differences for the effect of housing condition on average swim speed by either male ($p = 0.19$), or female rats ($p = 0.49$). Cohen's *d* was calculated and confirmed no effect of housing condition on average swim speed for both male ($d = 0.46$) and female rats ($d = 0.31$). Mann-Whitney U test analysis found no statistically significant difference between male and female swim speeds ($p = 0.58$). Cohen's *d* was computed and also revealed no effect of housing condition on average swim speed during the probe trial ($d = 0.30$).

5.2 Stress-sensitive Biometrics Data

5.2.1 The Effects of CELSI on Stress-sensitive Biometrics in Male Animals

After seven weeks of CELSI, no statistically significant effects were observed upon body weight ($p = 0.92$), adrenal glands weight ($p = 0.56$), retroperitoneal fat pads weight ($p = 0.49$), liver weight ($p = 0.16$), and serum CORT level ($p = 0.56$) within male rats (Table 4). Additional analysis through computation of Cohen's *d* further established the absence of notable differences between male rats of the group housed and social isolation conditions, since effect sizes were small: body weight ($d = 0.40$), adrenal glands weight ($d = 0.17$), retroperitoneal fat pads weight ($d = 0.28$), and serum CORT level ($d = 0.39$). A moderate effect size was computed for the effect of CELSI on liver weight ($d = 0.58$) within male rats even though the *p* value did not pass the statistical significance threshold of 0.05.

5.2.2 The Effects of CELSI on Stress-sensitive Biometrics in Female Animals

Following seven weeks of CELSI, statistically significant effects of housing condition were not observed on body weight ($p = 0.49$), adrenal glands weight ($p = 0.38$), retroperitoneal fat pads weight ($p = 0.06$), liver weight ($p = 0.63$), and serum CORT level ($p = 0.50$) within female rats (Table 5). Notably, the data for block nine were identified as an outlier by Tukey's method (box and whiskers plot) and removed from the analyses. Computation of Cohen's *d* further indicated female rats from the social isolation condition did not differ from female rats of the group housed condition, given that effect sizes were small: body weight ($d = 0.39$), adrenal glands weight ($d = 0.17$), liver weight ($d = 0.07$), and serum CORT level ($d = 0.32$). However, the Cohen's *d* value for the effect of CELSI on retroperitoneal fat pads weight from female rats ($d = 0.65$) was found to be moderate, while the *p* value ($p = 0.06$) was almost at threshold for statistical significance.

5.2.3 The Effects of CELSI on Stress-sensitive Biometrics: Male-Female Comparison

No statistically significant differences in the effect of housing condition upon biometrics were found between male and female rats: body weight ($p = 0.80$), adrenal glands weight ($p = 0.80$), retroperitoneal fat pads weight ($p = 0.11$), liver weight ($p = 0.22$), and serum CORT level ($p = 0.84$) (Figures 6 and 7). Cohen's *d* was also computed and further indicated no effect of housing condition on body weight ($d = 0.04$) and adrenal glands weight ($d = 0.02$), since the effect sizes were very small. The calculated Cohen's *d* effect sizes for retroperitoneal fat pads weight ($d = 0.79$), liver weight ($d = 0.54$), and serum CORT level ($d = 0.60$) were moderate.

5.3 SDS-PAGE and Immunoblotting

5.3.1 Standard Curve Optimization Blot

Anti-PSD95, clone K28/43 (EMD Millipore) and mouse anti-Glial Fibrillary Acidic Protein (GFAP) monoclonal antibody, clone GA5 (EMD Millipore) were used with the hippocampal homogenate prepared from the male group housed rat from block three at a range of protein concentrations (5 μg, 10 μg, 15 μg, and 20 μg). The immunoblotting was done to determine the protein concentration that would produce a stable and unsaturated signal. The optimal sample loading concentration for each antibody was concluded to be 10 μg/μL.

5.3.2 The Effects of CELSI on PSD-95 Expression in the Hippocampus

The male values for blocks five and six were identified as outliers by Tukey's method (box and whiskers plot) and were excluded. After seven weeks of CELSI, a statistically significant effect of housing condition on PSD-95 expression ($p = 0.03$) in the hippocampus was detected within male rats. Cohen's *d* for the effect of CELSI on PSD-95 expression ($d = 3.57$) within male rats also revealed socially isolated male rats substantially differed from group housed male rats. In female rats, no statistically significant effect of housing condition on PSD-95 expression ($p = 0.95$)

in the hippocampus was found, and the negligible effect size of $d = 0.05$ also indicated that socially isolated female rats did not differ from group housed female rats. Between male and female rats, no statistically significant difference for an effect of housing condition on PSD-95 expression ($p = 0.14$) in the hippocampus was revealed. Additional analysis by calculation of Cohen's d suggested a moderate difference between the effect of CELSI on PSD-95 expression ($d = 0.69$) in the hippocampus of male and female rats.

5.3.3 The Effects of CELSI on GFAP Expression in the Hippocampus

Through Tukey's method (box and whiskers plot), block three's female group was determined to be an outlier, and therefore, it was excluded. Statistically significant differences were not observed for the effect of housing condition on GFAP expression in the hippocampus within either male ($p = 0.64$), or female rats ($p = 0.81$). Computation of Cohen's d for the effect of CELSI on GFAP expression within male ($d = 0.34$) and female rats ($d = 0.12$) additionally revealed that social isolation did not alter protein levels. Furthermore, a statistically significant effect of housing condition on hippocampal GFAP expression in male and female rats ($p = 0.96$) was not observed, and the absence of an effect of CELSI was reinforced by the small effect size ($d = 0.34$).

6.0 Discussion

The chronic, but mild, stress induced by the adverse experience of CELSI is believed to influence the brain's form and function by interfering with development of the nervous system and reducing the plasticity of neurons, in addition to affecting behaviours in adulthood in rodents (Heim & Nemeroff, 2001; Heim et al., 2004; Koike et al., 2009; Mirescu et al., 2004; Rapoport et al., 2005). CELSI is similar to human psychological stressors since it creates multiple behavioural effects comparable to those documented in humans who have experienced ELS, and it can elevate the occurrence of several brain disorders, behavioural abnormalities or impairments, such as social inhibition and problems with learning and memory, as well as psychopathology, including depression and anxiety, via its impact on nervous system development (de Kloet et al., 2005; Heim & Nemeroff, 2001; Shao et al., 2015; Weber et al., 2008). Young rats that experience CELSI also display a syndrome of changes in behaviour that can be studied to find possible treatments for the core symptoms of schizophrenia (Bianchi et al., 2006; Fone & Porkess, 2008; Gaskin et al., 2014; Jones et al., 2011; Weiss & Feldon, 2001; Wongwitdecha & Marsden, 1996). Therefore, to increase comprehension of the fundamental processes by which stress adversely impacts the brain and how CELSI is a risk factor in psychiatric disorders, the impact of CELSI on hippocampus-dependent learning and memory, stress-sensitive biometrics, changes in hippocampal cell number and structure, and sex-specific differences in each of these aspects, were analyzed (Blanchard et al., 2001; Gray et al., 2013).

6.1 Morris Water Maze Behavioural Data Analysis

6.1.1 Spatial Learning During the Morris Water Maze

Spatial memory performance is often founded on the capacity to encode, store, and recover mostly visual information concerning navigating a path and the positioning of objects (Postma et

al., 2004). Based on the results of rats with fimbria-fornix lesions, spatial learning performance in the MWM has been proposed to be separated into two processes, which include arriving at the platform and having knowledge of the platform's location (Whishaw et al., 1995).

Learning was measured by studying the performance of rats during the acquisition phase; in particular, by examining escape latency and path length (Blokland et al., 2004). Results demonstrated that rats of both housing conditions and sexes were able to improve their performance during the acquisition phase (Cevik et al., 2018; Gallagher et al., 2015). The *p* values for the effect of housing condition on escape latency on days 1, 3, and 4 of training within male rats and on days 2, 3, and 4 of training within female rats indicated no statistically significant differences. As well, the *p* values for the effect of housing condition on path length indicated no statistically significant differences for all days of training within male rats and for days 2, 3, and 4 of training within female rats. Since the *p* values provide information as to the probability that the detected difference amid two groups resulted from chance (statistical significance), Cohen's *d* effect sizes were computed to determine the substantive significance (the magnitude of effect) to more completely comprehend the findings (Sullivan & Feinn, 2012). Effect sizes for most training days showed a small effect of housing condition on escape latency, excluding the moderate effect size computed for day 2 within female rats. Moreover, statistically significant differences for the effect of housing condition on escape latency for day 2 ($p = 0.04$, $d = 0.73$) of training within male rats and for day 1 ($p = 0.03$, $d = 0.81$) of training within female rats were found and further established by the moderate and large effect sizes computed, respectively. In addition, a statistically significant difference for the effect of housing condition on path length for day 1 ($p = 0.04$, $d = 0.93$) of training within female rats was detected and confirmed by the large effect size calculated. Therefore, CELSI may have resulted in a difference in the spatial learning of male rats

on day 2, and of female rats on day 1 of the acquisition phase, such that socially isolated male and female rats had median escape latencies distinct from that of group housed male and female rats, respectively. More specifically, as depicted in Figure 2, socially isolated female rats appeared to take longer to escape (longer escape latency) relative to group housed female rats on day 1 of the acquisition phase, while socially isolated male rats appeared to escape faster (shorter escape latency) and, therefore, perform better, relative to group housed male rats on day 2 of the acquisition phase. Furthermore, statistically significant differences for both escape latency and path length within female rats on day 1 of the acquisition phase suggest CELSI may have caused the spatial learning of socially isolated female rats to distinctly vary from group housed female rats on day 1 of training, such that socially isolated female rats performed less well (longer escape latency and path length) than group housed female rats during the acquisition phase of the MWM.

Multiple studies have reported conflicting results on the effect of isolation on acquisition and retention of spatial learning during the MWM. Findings for days 1, 3, and 4 of the acquisition phase within male rats and days 2, 3, and 4 of the acquisition phase within female rats support those of Lapiz et al. (2001), Schrijver et al. (2004), and Quan et al. (2010) since these studies also detected no change in acquisition in the task when a fixed platform position was used. Furthermore, since these findings were observed in early adulthood, they also support those of Schrijver et al. (2002), who detected no difference in the performance of socially reared and isolation reared rats during the MWM in early adulthood. While impaired acquisition in the MWM for socially isolated rats has been suggested to result from the widely acknowledged impact of environmentally enriched cages for group housed rats as opposed to the impact of social isolation, the environment of all conditions, excluding the presence of other animals, remained the same (standard housing)

throughout the duration of the study (Schrijver et al., 2002). Thus, any difference in acquisition in my study may be attributed to housing condition, sex, and any interaction between the two.

As depicted in Figures 2 and 3, during days 1 and 2 of the acquisition phase, socially isolated female rats appeared to perform less well in the MWM compared to group housed female rats and, by days 3 and 4 of training, socially isolated female rats appeared to perform as well as group housed female rats. These findings suggest that CELSI-related stress significantly impaired and impaired, to a lesser degree, spatial learning in socially isolated female rats on days 1 and 2 of training, respectively. Despite the initial impairment, socially isolated female rats appeared to overcome their performance deficit by days 3 and 4 of training. Results for days 1 and 2 of the acquisition phase within female rats align with those of Hellemans et al. (2004), who found control rats performed superiorly to isolation-reared rats in the MWM and with those of Lu et al. (2003), who observed socially isolated male Sprague-Dawley rats exhibited an impaired performance compared to group housed male Sprague-Dawley rats in the MWM. Additionally, the general pattern of the deficit parallels that observed by Wade and Maier (1986), who detected that socially isolated male Sprague-Dawley rats were significantly impaired in spatial acquisition learning in the MWM, since they had longer escape latencies than group housed male Sprague-Dawley rats. In the present study, the initial impairment in spatial learning in socially isolated female rats suggests that any influences in the environment not present in the individual cages of socially isolated female rats may significantly be involved in preserving the readiness of rats to learn the escape response for the MWM (Wade & Maier, 1986). Notably, sensory deprivation due to social isolation may impact the maturation of cognitive neural pathways (Lapiz et al., 2003). Mechanisms linked to CELSI-induced deficits in spatial learning and memory may be related to heightened neophobia and functional changes linked to attention or emotion (Lapiz et al., 2003). Furthermore,

an elevated stress response in female rats subjected to CELSI may be interfering with the activation of processes that contribute to learning, as evidenced by the initial impairment in spatial learning observed (Lapiz et al., 2001; Wade & Maier, 1986).

As illustrated in Figures 2 and 3, on day 1 of the acquisition phase, socially isolated male rats appeared to perform as well in the MWM as group housed male rats and, by days 2 and 3 of training, socially isolated male rats appeared to perform moderately better in the MWM than group housed male rats. However, on day 4 of training, socially isolated male rats appeared to once again match the performance of group housed male rats in the MWM. As such, these findings seemingly indicate that stress induced by CELSI did not impair spatial learning in socially isolated male rats on day 1 of training, but, instead, provided a small advantage on day 2 of training. The small advantage appears to diminish by days 3 and 4 of training, as socially isolated male rats returned to a similar performance to group housed male rats. Overall, the observed pattern of enhanced spatial learning in socially isolated male rats aligns with that by Wongwitdecha and Marsden (1996), who found that socially isolated male Lister hooded rats performed better by exhibiting enhanced place learning relative to socially reared male Lister hooded rats in the MWM. Enhanced spatial learning in the MWM for socially isolated male rats may be attributed to modifications in central cholinergic neurotransmission brought on by the environment, since muscarinic receptor antagonists have been found to impair performance and, therefore, cognitive processes, in a variety of learning tasks (Burešová et al., 1986; Cozzolino et al., 1994; File et al., 1990).

Autonomic and endocrine stress responses, in addition to activation of the HPA axis, are the basis of how stress impacts learning and memory (D'Hooge & De Deyn, 2001). When CELSI-related stress generates a stress response and stress hormones are released from the adrenal glands, the process of forming memories and multiple physiological systems may be impacted and

impairments in cognitive functions may result (Cevik et al., 2018; Sandi, 1998). Once the HPA axis is activated and ACTH is released from the pituitary gland, CORT is secreted by the adrenal glands and can diffuse across the blood-brain barrier into the brain, where it acts by binding to MRs and GRs in the hippocampus (Cevik et al., 2018; Green & McCormick, 2013; Sandi et al., 1997). CORT's actions in the hippocampus can involve modifying two types of synaptic plasticity, including LTP in the hippocampus and primed burst potentiation (PBP), which are both considered to be factors in learning and memory (Bennet al., 1991; Diamond et al., 1992; Diamond et al., 1994; Kerr et al., 1994; Luine et al., 1996; Pavlides et al., 1993; Pavlides et al., 1995; Sandi et al., 1997). Stress levels of CORT may have restrained LTP in the hippocampus, while steroid concentrations similar to a basal physiological condition may have aided such potentiation (Sandi et al., 1997). Through electrophysiological studies on LTP and neuronal excitability, such concentration-dependent biphasic actions were found to be a consequence of differing functions of MRs and GRs on synaptic plasticity, as MRs aided, while GRs restrained potentiation in the hippocampus (Joëls & de Kloet, 1992; Pavlides et al., 1995; Sandi et al., 1997). Therefore, the potential exists that enhanced spatial learning in the MWM for socially isolated male rats may entail CORT-dependent increases of LTP and PBP (Luine et al., 1996).

6.1.2 Sex Differences in the Effect of CELSI upon Spatial Learning

In neuropsychiatric diseases, such as schizophrenia and depression, differences between sexes are especially evident (Conley & Rudolph, 2009; Vesga-López et al., 2008). In contrast to men, anxiety disorders and depression, are twice as frequent in women (Kessler, 2003). One common and key characteristic of these psychiatric disorders is their link to stress (Kendler et al., 2000). Sexual dimorphisms can be emphasized by sex hormones impacting the brain's functioning with respect to the activation and the response of the HPA axis (Patchev & Almeida, 1998; Rhodes

& Rubin, 1999; Young, 1998). The most often reported change corresponding to symptoms of mood disorders is an abnormality, or impairment in the regulation of the HPA axis (Fernández-Guasti et al., 2012; Pariante & Lightman, 2008; Spijker & van Rossum, 2012).

My analyses found no statistically significant differences between male and female rats for the effect of housing condition on either escape latency, or path length for each day of the acquisition phase. Notably, the *p* values for the effect of housing condition on escape latency during days 1 and 2 of the acquisition phase were 0.08 and 0.06 with large effect sizes of 0.88 and 0.98, respectively. The *p* values for the effect of housing condition on path length on days 1 and 2 of the acquisition phase were 0.07 and 0.08, with large effect sizes of 0.90 and 0.96, respectively. While the *p* values for days 1 and 2 for escape latency and path length suggest differences were not statistically significant, the *p* values are very near to the statistical significance threshold of 0.05, and the effect sizes computed are large; given this, the possibility exists that an effect of sex exists. Large *p* values and small effect sizes were computed for the effect of housing condition on escape latency and path length on days 3 and 4 of the acquisition phase, suggesting that no sex-related effects were present.

As depicted in Figures 2 and 3, socially isolated female rats had a deficit in learning during days 1 and 2 of the acquisition phase relative to control female rats. On days 3 and 4 of the acquisition phase, socially isolated female rats improved their performance, such that they performed similarly to control female rats. Furthermore, socially isolated male rats performed similar to control male rats on day 1 of the acquisition phase and performed moderately better than control male rats on days 2 and 3 of the acquisition phase. On day 4 of the acquisition phase, the performance of socially isolated male rats returned to a similar performance to control male rats. These findings suggest female rats are displaying a greater negative effect of CELSI relative to

male rats. In particular, CELSI may be causing an impairment in the acquisition of the water maze task during days 1 and 2 of the acquisition phase for socially isolated female rats. As well, CELSI may have provided an advantage in the acquisition of the MWM task during days 2 and 3 of the acquisition phase for socially isolated male rats relative to control male rats.

Moreover, socially isolated male rats performed better than socially isolated female rats on days 1 and 2 of the acquisition phase. However, by days 3 and 4 of the acquisition phase, socially isolated male and female rats performed similar to one another. Therefore, these findings seemingly exhibit an effect of sex evident on days 1 and 2 of the acquisition phase since male rats had a faster average escape latency and a shorter average path length compared to female rats. Additionally, the large effect sizes computed on days 1 and 2 of the acquisition phase demonstrate that the difference between the sexes in both the parameters of escape latency and path length were substantial.

Chronic stress exposure can modify performance on learning and memory tasks, such as the MWM, and, although studies on the lasting cognitive impacts of chronic stress once it concludes have mostly examined adult males, sex has been reported to be a significant factor in comprehending the effects of stress on learning and memory (Luine et al., 2007; Lupien & Lepage, 2001; McFadden et al., 2011). For example, sexually dimorphic effects on behaviour after stress have been reported in multiple learning paradigms involving rodents (Beck & Luine, 2002). In several studies, male rats and mice subjected to chronic stress, such as daily restraint, or various daily stressors, including chronic unpredictable stress (CUS), have been impaired in learning and memory tasks involving the use of spatial information (Conrad, 2010; Luine et al., 2017). In the MWM, Kitraki et al. (2004) observed delayed learning in adult male Wistar rats subjected to 21 days of chronic restraint stress relative to control male Wistar rats, while adult female Wistar rats

also subjected to 21 days of chronic restraint stress performed as well in the MWM as control female Wistar rats. McFadden et al. (2011) detected sex differences in their study on the influence of CUS for 10 days on adult male and female Sprague-Dawley rats. Specifically, in the MWM, adult female rats subjected to CUS had shorter path lengths during acquisition training relative to control adult female rats, while adult male rats subjected to CUS exhibited no difference in path length relative to control adult male rats (McFadden et al., 2011). Moreover, Isgor et al. (2004) noted juvenile male Sprague-Dawley rats subjected to variable social or physical stressors for 28 days, and tested one day following the last stressor, displayed longer escape latencies during the MWM relative to control juvenile male Sprague-Dawley rats. Additionally, Luine et al. (1994) detected impaired acquisition and performance in adult male Sprague-Dawley rats in the eight-arm radial maze, a spatial learning and memory task, following 21 days of daily restraint stress, and such impairments in performance did not inhibit learning because chronically stressed rats performed as well as control rats as testing proceeded. However, when the effect of chronic stress was investigated in adult female Sprague-Dawley rats subjected to 21 days of daily restraint stress, spatial memory was enhanced during performance in the eight-arm radial maze (Bowman et al., 2001). While my findings for an effect of sex on days 1 and 2 of the acquisition phase do not align with the findings of these studies involving multiple learning paradigms, it is important to note that these studies involved rodents subjected to forms of chronic stress as mainly adults. In my study, the perception of stress induced by CELSI during adolescence is different, despite also being chronic, and thus, the perception of stress for rats in my study varies from the perception of stress for adult rodents in these studies (Yorgason et al., 2016). As well, the tasks have distinct performance bases, since the MWM is aversive due to the potential of drowning and the radial arm maze is rewarding due to food reinforcement to finish the task (Luine et al., 2017).

The finding that socially isolated male rats performed better than socially isolated female rats on days 1 and 2 of the acquisition phase appears to align with the finding by Luine et al. (1996), who observed an enhanced performance in the eight-arm radial maze, though small and, yet, significant, in adult male Sprague-Dawley rats after 13 days of daily restraint stress. Luine et al. (1996) also noted that the impact on performance may have been reversible because later testing exhibited no variations amid the groups. The underlying mechanisms for the enhanced performance exhibited by rats following 13 days of restraint stress were not identified, but may be attributed to stress potentially assisting acquisition of the strategy for the maze, reference memory, short-term memory, or working memory (Luine et al., 1996).

The housing condition of subjects may confound comprehension of sex differences in the stress response induced by CELSI and, consequently, in sex differences in the effect of CELSI on MWM performance (Beck & Luine, 2002). Studies directly on, or related to sex differences in the effect of housing condition on learning in the MWM have not been published or are limited, respectively (McFadden et al., 2011). In particular, CORT may be a factor in sex differences in behavioural tests, like the MWM, after exposure to chronic stress, such as CELSI (McFadden et al., 2011). Kitraki et al. (2004) observed both male and female rats subjected to chronic restraint stress for 21 days displayed higher basal plasma CORT levels relative to controls, while Matuszewich and Yamamoto (2004) observed male rats subjected to CUS for 10 days did not display higher basal plasma CORT levels relative to controls. In general, female rats relative to male rats have increased basal CORT levels (Galea et al., 1997; Kitraki et al., 2004) and increased CORT increases brought on by stress (Galea et al., 1997; Verma et al., 2010). The variation in the amount of CORT circulating may bring about particular differences between the sexes seen in spatial memory tests and the effect of chronic stress in such behavioural tests (Kitraki et al., 2004).

Distinct acute stress reactions in females may also intervene in spatial learning, leading to sex differences in spatial learning in the MWM (Beiko et al., 2004). As previously noted, female rats relative to male rats exhibit elevated basal CORT as well as faster and increased CORT responses to novel stressors (Galea et al., 1997; Kant et al., 1983; Kitraki et al., 2004; Verma et al., 2010). In response to several different stressors, female rats also display increased plasma CORT faster compared to male rats in the initial five to 15 min of stress (Kant et al., 1983). Therefore, female rats relative to male rats may display increased acute stress responses early during training trials of the MWM when sex differences in the MWM most consistently take place (Frye, 1995; Perrot-Sinal et al., 1996; Warren & Juraska, 1997). As such, the seemingly evident effect of sex on days 1 and 2 of the acquisition phase, in which socially isolated male rats appeared to perform better than socially isolated female rats during the MWM, may have occurred due to sex differences in CORT release in response to stressors.

Sex differences in cognitive responses to stress induced by CELSI may also exist due to various strategies employed in learning concerning sex and stress and any interaction between the two (Luine et al., 2017). Sexes may vary in their dependence upon the hippocampal system (spatial learning) and the caudate system (stimulus-response) and the influence of stress on the use of these systems (Luine et al., 2017). Sex differences in the portion of rodents that display a tendency to use a stimulus-response as opposed to a spatial learning strategy exist, and, the portion varies upon subjection to stress (Luine et al., 2017). Schwabe et al. (2010) noted most male mice in a hole-board escape task tend to display a learned spatial strategy, but if stressed before training starts, a significant portion of them will learn a stimulus-response strategy. A consistent shift in neural processing has been hypothesized to potentially result from stress, and as a result, lead to a functional change in cognition from a spatial learning to a stimulus-response strategy (Schwabe et

al., 2008). Notably, Korol (2004) reported female rats displayed biases according to their phase of the estrous cycle on MWM performance. Specifically, Korol (2004) noted that at proestrus, when circulating ovarian hormones were increased, female rats (71%) were significantly more likely to employ a place as opposed to a response strategy. At estrus, when circulating ovarian hormones were decreased, female rats (70%) were significantly more likely to employ a response strategy (Korol, 2004). At diestrus, when circulating ovarian hormones were intermediate, female rats did not demonstrate any bias (Korol, 2004). Thus, during low estradiol phases of the habitually fluctuating cycle of female rats, non-spatial strategies tend to be employed and chronic stress may result in shifts in neural processing depending on the phase of the estrous cycle (Beck & Luine, 2010; Keeley et al., 2015). Luine et al. (2017) suggest that the resilience to stress females exhibit may depend on their use of spatial and response strategies, whereas males are mostly inclined to use spatial strategies. As such, a shift to response strategies may result due to stress and, in turn, lead females to exhibit resilience and males to not (Luine et al., 2017). However, these suggestions appear to conflict with the current study's finding that socially isolated female rats performed less well (not resilient) than socially isolated male rats in the MWM. Enhanced or impaired learning and memory after exposure to a stressor, such as CELSI, may, instead, be based on what is being learned and if it corresponds with the rat's natural biases (Beck & Luine, 2010; Luine et al., 2017).

The sex difference in the release of CORT may also be directly related to ambient estradiol levels (Beiko et al., 2004; Viau & Meaney, 1991). Alterations in HPA axis activity may intervene with the impacts of estrogen on performance during the MWM, including the effects of estrogen observed throughout the estrous cycle (Beiko et al., 2004). Additionally, elevated levels of naturally-occurring estradiol at proestrus or, when given exogenously, have been linked to inferior acquisition in female rodents during the MWM (Frye, 1995; Warren & Juraska, 1997). Some

variability exists in spatial learning performance in the MWM within female rats in the current study. Despite this, the computed SEMs and confidence intervals for escape latency and path length on all days of the acquisition phase suggests rats were not that different, or that any differences that were present, such as cycling levels of estradiol, were not that influential on spatial learning performance in the MWM. Consequently, the inferior performance of socially isolated female rats compared to socially isolated male rats subjected to CELSI observed in the current study may be attributed to elevated levels of estradiol during proestrus, as female rats may have been at that point of their cycle during testing.

Furthermore, females have low levels of estradiol circulating at adolescence (Luine et al., 2017). Consequently, socially isolated female rats may have also performed significantly less well than socially isolated male rats on days 1 and 2 of the acquisition phase due to an initial lack of resilience to CELSI-related stress (Luine et al., 2017). Also, while chronic stress at adolescence has not investigated cognition or both sexes, female Wistar rats relative to male Wistar rats subjected to chronic mixed-modality stress (isolation, restraint, and social defeat) in adolescence have exhibited increased depressive-like behaviour, such as less sucrose consumption and decreased struggling in the forced swim test, in adolescence as well as at adulthood (Bourke & Neigh, 2011; Luine et al., 2017; Rygula et al., 2005). The lack of an effect of CELSI on the behaviour of male rats in the study by Bourke and Neigh (2011) is unclear, but may be ascribed to the ongoing maturation of the hippocampus at adolescence (Luine et al., 2017). As such, socially isolated female rats may have performed less well in the water maze task on days 1 and 2 of the acquisition phase compared to socially isolated male rats due to increased depressive-like behaviour following CELSI, which delayed learning in the acquisition phase of the MWM.

6.1.3 Hippocampal Spatial Reference Memory in Each Sex and Between Sexes

The persistence of hippocampal-dependent spatial reference memory was measured by determining the time rats spent in the target quadrant throughout the probe trial (Blokland et al., 2004; Harburger et al., 2007; Markowska et al., 1993; Quan et al., 2010). Rats with a propensity to remain in the target quadrant for a greater time likely learned how to escape the maze through a strategy involving knowledge of the position of the platform based on cues in the setting, which indicates their spatial memory for the platform's position (Blokland et al., 2004).

As illustrated in Figure 4, both socially isolated male rats and socially isolated female rats appeared to spend more time in the target quadrant than control male rats and control female rats, respectively. The statistical analysis did not disclose any significant differences for the effect of housing condition on average time spent in the target quadrant during the probe trial for male rats and between the sexes. Due to the wide 95% CI and large SEM computed for male rats, no clear distinction could be made regarding a lack of difference, or an enhanced performance by socially isolated male rats relative to control male rats, or to socially isolated female rats in the probe trial, respectively. However, one conclusion that can be drawn is that an impaired performance in the probe trial was not displayed by socially isolated male rats compared to control male rats. Moreover, for female rats, the p value of 0.13 was relatively near to the statistical significance threshold of 0.05 and the effect size was moderate ($d = 0.62$). Consequently, there may be value in considering the prospect that socially isolated female rats spent more time in the target quadrant than control female rats during the probe trial. While an enhanced performance for socially isolated female rats relative to control female rats may not be confirmed, socially isolated female rats did not exhibit an impaired performance during the probe trial.

My findings are inconsistent with earlier work by Quan et al. (2010) and Ibi et al. (2008), who detected that socially isolated Wistar male rats remained in the target quadrant for less time relative to group housed Wistar male rats, and that saline-treated socially isolated male mice remained in the target quadrant for a much shorter time than saline-treated group housed male mice, respectively. Both Quan et al. (2010) and Ibi et al. (2008) suggested that their findings revealed rats subjected to CELSI experienced an impairment in the persistence of spatial memory. The results of Pisu et al. (2011), however, offer support for my findings, since average proximity to the target location during the probe trial was significantly reduced in socially isolated rats, indicating an enhanced spatial memory.

The potential that CELSI may have lead socially isolated female rats to spend more time in the target quadrant than control female rats during the probe trial in addition to the finding that socially isolated female rats did not exhibit an impaired performance relative to control female rats conflicts with the anticipated outcome that CELSI would impair the persistence of spatial memory and cause socially isolated female rats to perform less well (spend less time in the target quadrant) relative to control female rats during the probe trial. Since social isolation has been shown to adversely impact behaviours associated with cognition, my findings are a challenge to resolve (Ibi et al., 2008). In particular, CELSI induces stress in rats during a vulnerable time in development that, in turn, may lead to decreased hippocampal volume (due to changes in neurogenesis in the hippocampus) and decreased hippocampal LTP, which would be expected to negatively impact hippocampal-dependent memory (Gould & Tanapat, 1999; Ibi et al., 2008; Lu et al., 2003; Luener et al., 2006; Pisu et al., 2011; Roberts & Greene, 2003; Weiss et al., 2004; Westenbroek et al., 2004; Wongwitdecha & Marsden, 1996). The MWM is also a stressful occurrence, and CELSI in male rats has been shown to continue to increase plasma CORT concentration by impairing the

negative feedback regulation of the HPA axis (Greco et al., 1989; Hauger et al., 1990; Kant et al., 1983; Rivier & Vale, 1987; Serra et al., 2000; Serra et al., 2005).

6.1.4 Swim Speed in Each Sex and Between Sexes

To confirm that MWM results were inferred as indicative of spatial learning and memory, and were not confounded by sensorimotor deficits, the swimming ability of each housing condition group was measured (Vorhees & Williams, 2006; Vorhees & Williams, 2014). Escape latency is a measure partly dependent on swim speed, and does not solely denote acquisition of the platform position, since variations in swim speed may confound this index of learning (Contet et al., 2001; Vorhees et al., 2008).

Figure 5 depicts that average swim speed did not significantly differ between socially isolated male rats and control male rats, or between socially isolated female rats and control female rats during the probe test. Average swim speed also did not significantly differ between male and female rats, as established by the large *p* value and small effect size calculated. As such, control male rats, socially isolated male rats, control female rats, and socially isolated female rats were equivalent in their swimming ability, signifying that sensorimotor deficits were not the basis of any differences observed between the groups in spatial learning and reference memory during the MWM (Vorhees & Williams, 2006).

6.2 Stress-sensitive Biometrics Data Analysis

6.2.1 Effects of CELSI on Body Weight and Organ Weights

The effects of CELSI were studied on the weight of the body, adrenal glands, retroperitoneal fat pads, and the liver since the value of organ weights are often identified as overall signs of stress, toxicity, and metabolic dysfunction (Robb et al., 2017; Sellers et al., 2007). For body weight, statistical analyses showed no significant differences within male rats, within female

rats, and between male and female rats as a result of CELSI. Similarly, the effect sizes for the impact of CELSI on body weight within sexes and between sexes each displayed a small magnitude. The present study's findings accord with studies by Ryu et al. (2009), and Weiss et al. (2004), who reported social isolation did not influence body weight. However, other rodent studies have studied the effect of housing condition on body weight, and found that after social isolation, body weight either increased (Hellemans et al., 2004), or decreased (Nagy et al., 2002). The conflicting findings reveal the effect of social isolation housing on body weight may be contingent on variations in age, experimental design, as well as the rodents' features (Schipper et al., 2018).

Statistical analyses, including effect size measures, revealed no effect of CELSI within male rats, within female rats, and between male and female rats on the weight of adrenal glands. My results agree with previous studies that found post-weaning social isolation did not alter the weight of adrenal glands in comparison to group housed rats (Brain & Benton, 1979; Gamallo et al., 1986; Gentsch et al., 1981; Moore, 1968; Morinan & Leonard, 1980). However, my results conflict with other studies that found significantly higher relative and absolute adrenal gland weights for isolates (Syme, 1973) and that revealed gender differences, including that female isolates had significantly heavier weights of adrenal glands, while male isolates had similar non-significant increased weights of adrenal glands (Hatch et al., 1965; Holson et al., 1991). The lack of significant differences in the weight of adrenal glands within each sex and between sexes suggests that the basal adrenal activity of the rats did not differ across groups.

For liver weight, statistical analyses found no significant differences within male rats, within female rats, and between male and female rats due to CELSI. While the p value for the effect of CELSI on liver weight within male rats ($p = 0.16$) and between male and female rats ($p = 0.22$) was above the threshold for statistical significance, the precise confidence interval and

effect sizes do suggest that a larger sample size may allow for differences in liver weight within male rats (*d = 0.58*) and between male and female rats (*d = 0.54*) to be seen. Additionally, my results are not in line with previous literature that reported male C57BL/6J mice subjected to CELSI for 13 weeks had significantly elevated liver weight compared to the group housed mice (Sakakibara et al., 2012). Motoyama et al. (2009) detected male BALB/c mice subjected to CELSI for 30 days displayed a notable downregulation of the lipid metabolism-related pathway via the peroxisome proliferator-activated receptor alpha subtype, whereas the lipid biosynthesis pathway regulated by sterol regulatory element binding factor 1 was significantly upregulated, despite no alteration in body weight relative to controls. Thus, in the study by Sakakibara et al. (2012), CELSI was hypothesized to lead to increased weight gain and hepatic hypertrophy when CELSI extended for over 4 weeks, by initiating lipid biosynthesis and increasing energy accumulation, mainly in the liver. But, since the mean percent of respective group housed block average for liver weight in males is 92.53%, this mechanism may have not occurred. The finding in my study may have differed from the finding by Sakakibara et al. (2012) since subjects in my study were Sprague-Dawley rats, the length of CELSI differed (PND 21 to PND 70 in my study and PND 28 to PND 119 in the study by Sakakibara et al. (2012)), and mice in the study by Sakakibara et al. (2012) had a decreased volume of bedding to increase the feeling of isolation, while rats in my study had a standard form of housing. Notably, the neuroendocrine reaction to stress is largely controlled and modified by the liver through its anabolic actions, which make energy-abundant compounds, including glucose and lipids, to allow the body to adjust (Sapolsky et al., 2000).

6.2.2 Effect of CELSI on Retroperitoneal Fat Pads Weight

Statistical analyses detected no significant differences within male rats, within female rats, and between male and female rats for the effect of housing condition on retroperitoneal fat pads

weight. However, the moderate effect sizes within female rats (*d = 0.65*) and between male and female rats (*d = 0.79*) indicate that a greater sample size may have made such CELSI-related changes in retroperitoneal fat pads weight more apparent. Additionally, while both *p* values calculated for the effect of CELSI on the weight of retroperitoneal fat pads within female rats (*p = 0.06*) and between male and female rats (*p = 0.11*) did not reach statistical significance, both were quite near to the statistical significance threshold of 0.05. Although my results agree with the lack of a significant difference observed for the weight of retroperitoneal fat pads between male individually and group housed C57BL/6J mice (Nagy et al., 2002), they do not align with a consistent finding in the systematic review and meta-analysis by Schipper et al. (2018), which reported that significantly greater visceral adiposity in socially isolated animals is a consistent effect of social isolation. Social isolation has been reported to interfere with plasma glycemic and lipid profiles, the form, structure, and shape of adipocytes, and hepatic or adipocyte gene expression profiles associated with lipid metabolism without simultaneous alterations in body weight (Schipper et al., 2018). For example, the body composition of adult male C57BL/6J mice were profoundly impacted, without significant elevation in body weight, and adipocyte size increased in retroperitoneal fat pads partly because of adipocyte hypertrophy following social isolation (Sun et al., 2014). Given this, the lack of a clear effect of CELSI on retroperitoneal fat pads weight within female rats and between male and female rats was unexpected.

6.2.3 Effect of CELSI on Serum Corticosterone Level

Serum CORT levels were measured in male and female rats of both housing conditions to investigate the response of the HPA axis to stress resulting from CELSI. Statistical analyses revealed the absence of statistically significant differences within male rats, within female rats, and between male and female rats. As well, effect sizes for the effect of CELSI on serum CORT

levels were small for within male rats and within female rats, but moderate for between male and female rats. In agreement with my findings are those by Benton and Brain (1981) (ranging from seven to 196 days of social isolation), Cevik et al. (2018) (8 weeks of social isolation), Holson et al. (1991) (eleven weeks of social isolation), Misslin et al. (1982) (four weeks of social isolation), Moore (1968) (15 to 17 weeks of social isolation), and Morinan and Leonard (1980) (three weeks of social isolation), who each comparably reported that CORT levels of rats subjected to CELSI and control rats were unable to be identified as distinct from one another. Although the stress-producing effect of social isolation seems intuitive, my results along with many previous findings, do not offer support that social isolation will significantly increase CORT levels. Also, Hawkley et al. (2012) proposed that not identifying a rise in plasma CORT following chronic social isolation may be due to an adaptation to the experience of social isolation.

While the findings of the current study and multiple other studies have detected the absence of an effect of CELSI on serum CORT levels within male rats, within female rats, and between male and female rats, socially isolated rats and their unusual behavioural response to specific challenges have been linked to alterations in the endocrine stress response and, in the literature, different extents of social isolation, chronicity of isolation, ages at isolation, and test settings have been used, leading to discrepancies in the results (Hawkley et al., 2012; Serra et al., 2007). Gamallo et al. (1986) (six weeks of social isolation) and Serra et al. (2000) (30 days of social isolation) observed rats subjected to CELSI had increased plasma CORT levels. Consequently, Dronjak et al. (2004) suggested that the HPA axis is stimulated by chronic isolation in addition to no significant impact on initiating the actions of the SAS. Conversely, Holson et al. (1988) (10 weeks of social isolation), and Sánchez et al. (1998) (two months of social isolation) found that rats subjected to CELSI had reduced plasma CORT levels. Djordjevic et al. (2010a) and Djordjevic et

al. (2010b) suggest that reduced serum CORT levels following social isolation indicate a compromised systemic energy state resulting from isolation stress.

6.3 SDS-PAGE and Immunoblotting Data Analysis

6.3.1 PSD-95 Expression in the Hippocampus Following CELSI

The NMDA subtype of glutamate receptors intervenes in signal transduction through the influx of Ca^{2+} and by binding with cytoplasmic proteins that connect them to the cytoskeleton and to intracellular signal transduction pathways (Husi et al., 2000; Sheng & Pak, 2000; Yuste et al., 1999). NMDA receptor complexes are physically attached to an intricate network of signalling proteins, which are regulators of synaptic transmission and cytoskeletal proteins and therefore, the actions of NMDA receptors may control the structure and strength of dendrites and synapses, respectively (Husi et al., 2000; Husi & Grant, 2001). As such, modifications in the proteins involved directly with NMDA receptors may bring about the alterations, including in morphology and density, exhibited in spines as a reaction to afferent activity (Vickers et al., 2006). A member of the membrane associated guanylate kinase (MAGUK) family of proteins is PSD-95, which has been exhibited to attach to NMDA receptor 2 (NR2) subunits (Kornau et al., 1995; Vickers et al., 2006). One function of PSD-95 is to stabilize spine-filopodia and to create synapses, and in vitro studies of PSD-95 have exhibited overexpression of PSD-95 causes a surge in the size and number of dendritic spines on hippocampal neurons (El-Husseini et al., 2000; Prange & Murphy, 2001). Notably, the postsynaptic density, in which PSD-95 is abundant, is an extremely well-ordered cytoskeletal formation located next to the postsynaptic membrane of excitatory synapses that helps to structure and arrange receptors and associated proteins concerned with synaptic signaling (Cho et al., 1992; Kistner et al., 1993; Sans et al., 2000). In the hippocampus of mammals, and in several brain structures linked to memory formation, specific patterns of synaptic activity culminate in

enduring alterations in the effectiveness of transmission amid synapses (Migaud et al., 1998). The basis of mechanisms put forward to explain changes in synaptic strength are that the responsivity of postsynaptic transmitter receptors become altered, formerly silent receptors are activated, retrograde messengers are produced for the presynaptic terminal, dendritic spines are structurally altered, and transcription in the nucleus is initiated (Bear & Malenka, 1994; Bliss & Collingridge, 1993). Consequently, since social isolation may lead to major alterations in the structure of the brain and its neurochemistry, the impact of CELSI on the expression of PSD-95, a significant regulator of neuronal plasticity, and, therefore, neuronal cell number, was studied in rat hippocampus (Fone & Porkess, 2008; Vickers et al., 2006).

Expression of PSD-95 did not differ between control and socially isolated female rats, as suggested by the large *p* value of 0.95 and the small effect size of 0.05. However, hippocampal PSD-95 protein expression differed significantly between control and socially isolated male rats, as denoted by the small *p* value of 0.03 and the large effect size of 3.57. The finding indicates that hippocampal PSD-95 protein expression in CELSI rats was increased relative to control male rats, which suggests that CELSI-related stress is not likely to have reduced the number of neuronal cells in socially isolated male rats. Furthermore, in the hippocampus of socially isolated male rats, CELSI seems to have influenced the complexity of post-synaptic dendrites, which would affect the grouping and securing of NMDA receptors at the synapse (Zhang et al., 2012; Zhao et al., 2009). The anticipated adverse impacts of social isolation on hippocampal structure and synaptic plasticity, such as reduced spine density of hippocampal pyramidal neurons, less hippocampal synapses, as well as reduced survival of recently divided cells and neurogenesis in the dentate gyrus, may have not occurred (Ibi et al., 2008; Silva-Gómez et al., 2003; Varty et al., 1999).

The current finding agrees with an earlier report by Zhang et al. (2012) that found hippocampal PSD-95 protein expression in male C57BL/6 mice subjected to social isolation upon weaning for eight weeks was significantly upregulated in comparison to expression in control mice. Additionally, my finding bears similarity to work by Zhao et al. (2009), who found that, relative to control rats, hippocampal PSD-95 mRNA expression in male Sprague Dawley rats, who were socially isolated upon weaning for eight weeks, was significantly increased.

The finding of this study (and the noted earlier reports) that hippocampal PSD-95 protein expression was significantly increased in socially isolated male rats relative control male rats seems to conflict with long-established evidence that behavioural stress activates the HPA axis, which increases the level of glucocorticoids in the blood stream and causes harm to the survival of hippocampal neurons (Woolley et al., 1990). For example, Sapolsky et al. (1985) found that after 21 days of injections of excess CORT, the dendritic morphology of CA3 pyramidal neurons of male Sprague-Dawley rats appeared modified relative to control male rats due to atrophy of the apical dendritic tree and, in primates, Uno et al. (1989) observed degeneration of the hippocampus following prolonged social stress. Similarly, Jacobs et al. (2000) and Malberg and Duman (2003) revealed stress reduced cell proliferation in the hippocampus, while Westenbroek et al. (2004) observed chronic stress in socially isolated male rats reduced the number of newborn neurons in the granule cell layer. In addition, CELSI has also been found to reduce the dendritic length and the spine density of hippocampal pyramidal cells from the CA1 region in addition to the number of synapses in the hippocampus (Silva-Gomez et al., 2003; Varty et al., 1999). In the present study, a clear distinction was not apparent in serum CORT levels between socially isolated rats and group housed rats in both male and female rats. Consequently, the potential exists that the noted changes

regarding hippocampal neurons were not observed since CELSI did not induce the kind of modification necessary in serum CORT levels to observe such changes.

6.3.2 Sex Differences in PSD-95 Expression in the Hippocampus Following CELSI

Statistical analysis revealed that hippocampal PSD-95 protein expression did not statistically differ between male rats and female rats, even though the *p* value was computed as 0.14 (moderately close to the cut-off for statistical significance of 0.05) and the magnitude of the effect computed was moderate (*d = 0.69*). Accordingly, the possibility that a difference in hippocampal PSD-95 protein expression existed between the sexes following CELSI should be considered. Therefore, the potential direction of the effect of sex was male CELSI rats had a greater number of neuronal cells in the hippocampus relative to female CELSI rats.

The current findings accord with those by Juraska et al. (1985) and Juraska (1991). Juraska et al. (1985) observed a sex difference amid male and female Long-Evans hooded rats brought up upon weaning in an isolated environment, such that male rats had more dendrite per neuron compared to female rats. As well, differences were detected amid the sexes in dendritic tree size in granule cells from the dentate gyrus by measuring the amount of dentate granule cells of male and female Long-Evans hooded rats subjected to differential housing from weaning for one month – either group housing of same-sex rats in an environment with objects altered every day, or social isolation in average laboratory cages (Juraska, 1991). Specifically, the effect of sex for rats differed according to the housing condition such that socially isolated male rats exhibited more dendritic tree material of granule cells in the dendritic field (using the Sholl method) compared to socially isolated female rats, while group housed female rats exhibited more dendritic tree material of granule cells in the dendritic field compared to group housed male rats (Juraska, 1991). Juraska (1991) also analyzed the granule cell dendritic tree in castrated and sham-operated male Long-

Evans rats and detected comparable effects of sex to that of the dendritic tree of the intact male and female rats previously noted. Accordingly, Juraska (1991) observed that testosterone, which exerts its effects throughout development or near puberty, subdued the majority of the dendritic tree's developmental response to group housing and social isolation and contributed to a sexual dimorphism in the dendritic field of granule cells. Consequently, in the present study, testosterone may have subdued the impact of CELSI on the development of dendrites, resulting in an increased number of neuronal cells in the hippocampus of male rats, and, thus, increased hippocampal PSD-95 protein expression relative to female rats.

6.3.3 GFAP Expression in the Hippocampus Within and Between Male and Female Rats Following CELSI

Although GFAP, the major intermediate filament of astrocytes, is closely connected to the development of multiple diseases of the CNS (Messing & Brenner, 2003), the possible function of glia in relation to stress and the impacts of glucocorticoids have been largely uninvestigated (Jauregui-Huerta et al., 2010). As well, mounting evidence concerning the function of glia in brain remodelling linked to depression has shown alterations in neuronal cells and glial cells account for the neuropathology of primary mood disorders, such as depression (Harrison, 2002). While the exact mechanisms that depression takes to influence hippocampal volume remain to be found, multiple clinical studies implicate cortisol in them (Wennströmm et al., 2006). Also, evidence suggests that changes in the expression of astrocytic structural and functional proteins may be involved in the pathophysiology of schizophrenia (Bernstein et al., 2015). The gene expression of GFAP is controlled through glucocorticoids and, in adult brain, glucocorticoids may restrain the extent to which astroglia react (Nichols et al., 2005). Notably, a loss of neurons in specific regions of the pyramidal cell layer in the human hippocampus occurs alongside a rise in the quantity of

GFAP-immunoreactive astrocytes (Wolf et al., 1993). Thus, by assessing GFAP immunoreactivity, potential changes in the structure of the hippocampus resulting from elevated glucocorticoid levels can be investigated (Müller et al., 2001).

After CELSI, GFAP expression in the hippocampus did not differ by housing condition within male rats and within female rats according to statistical analyses, since the computed *p* values were much larger ($p = 0.64$ and $p = 0.81$, respectively) than the significance level of 0.05, and the effect sizes were small ($d = 0.34$ and $d = 0.12$, respectively). Also, hippocampal GFAP expression amid male rats and female rats did not differ following CELSI, as indicated by the large *p* value of 0.96 and the small effect size of $d = 0.34$. These results strongly suggest that any stress induced by CELSI did not affect the number of hippocampal astrocytes within either male, or female rats.

The lack of significant differences in hippocampal GFAP expression in the present data seemingly do not provide evidence to support reports from animal studies that gliogenesis in the hippocampus is diminished due to chronic stress (Czéh et al., 2007). In particular, the present data conflict with findings by Müller et al. (2001) that found patients with major depressive disorder (MDD) and treated with steroids exhibited a moderate reduction in GFAP-immunoreactivity in the CA1 and CA2 hippocampal subfields and a report by Czéh et al. (2006) that found psychosocial stress in adult male tree shrews lead to a 25% reduction in the number of hippocampal GFAP immunoreactive astroglia. As well, the current data do not support findings by Alonso (2000), who observed that male Sprague-Dawley rats treated with CORT for an extended period displayed significantly decreased generation of hippocampal glial cells, and by Wennströmm et al. (2006), who reported that decreased hippocampal gliogenesis brought on by CORT was impeded in male Wistar rats given electroconvulsive seizure treatment. Furthermore, in a study by Lambert et al.

(2000), a 30% elevation in hippocampal GFAP-immunoreactive astrocytes was observed in male Long-Evans rats following activity-stress for six days relative to control rats. As well, GFAP gene expression following extended exposure (three weeks) to CORT was identified to be elevated in astrocyte cultures (Jauregui-Huerta et al., 2010).

7.0 Conclusions and Future Directions

The current study revealed socially isolated female rats exhibited reduced retroperitoneal fat pads weight and a deficit in acquisition on days 1 and 2 of the acquisition phase, while socially isolated male rats exhibited an enhanced acquisition on day 2 and an increased expression of PSD-95, an important neuronal cell marker. Sex differences in acquisition appeared notable on days 1 and 2 of the acquisition phase, such that socially isolated male rats performed better relative to socially isolated female rats. As a whole, these data point to a greater negative effect of CELSI on female rats since retroperitoneal fat pads weight was likely reduced and an impairment in hippocampal-dependent spatial learning in the MWM was evident on days 1 and 2 in socially isolated female rats due to CELSI stress. Furthermore, in socially isolated male rats, stress induced by CELSI likely did not reduce hippocampal neuronal cell number and, instead, may have provided an advantage in hippocampal-dependent spatial learning in the MWM.

While some findings of the current study accord with earlier literature, there are several findings that conflict, such as increased retroperitoneal fat pads weight after social isolation, impaired spatial acquisition in the MWM in male rats subjected to CELSI, and adverse effects on hippocampal structure and synaptic plasticity following social isolation. Thus, since CELSI stress may significantly influence the brain's form, function, and neurochemistry, further study of its effect on hippocampal cell number and hippocampal-dependent spatial learning and memory is essential to elucidating possible mechanisms by which CELSI may alter neuronal plasticity and adult behaviours. In addition, the potential that CELSI enhanced acquisition in male rats, but impaired acquisition in female rats suggests the influence of CELSI on hippocampal-dependent behaviour may be sexually dimorphic. Future studies should also investigate the influence of sex on CELSI's impact on MWM performance, stress-sensitive biometrics, and hippocampal cell

number in both male and female rats to increase understanding of possible sex differences in how CELSI may alter hippocampal-dependent spatial learning and memory, and the possible role played by sex hormones, such as estrogen. Accordingly, additional research into the effect of CELSI and the effect of sex on CELSI on these aspects of research may clarify underlying mechanisms that can be used to diagnose or point to various neurological and psychiatric disorders, including schizophrenia, anxiety, and depression (Mumtaz et al., 2018). Additionally, it may allow for increased comprehension of sex differences in the biological response to the developmental stressor of CELSI, and to explicating sex differences reported in the occurrence of multiple human developmental disorders (Juraska et al., 1985; Pisu et al., 2016).

Day		Mean (% of Respective GH Block Average)	SEM	±95% CI of mean	N	*p* Value	Effect Size (*d*)
1	**Escape Latency**	101.99	7.32	85.43, 118.56	10	0.92	0.09
	Path Length	101.26	9.23	85.39, 127.14	10	0.77	0.21
2	**Escape Latency**	74.94	10.89	50.31, 99.57	10	0.04	0.73
	Path Length	79.77	13.70	48.79, 110.76	10	0.19	0.47
3	**Escape Latency**	77.17	14.92	43.43, 110.92	10	0.16	0.48
	Path Length	83.51	17.05	44.95, 122.08	10	0.43	0.31
4	**Escape Latency**	92.45	15.80	56.72, 128.18	10	0.63	0.15
	Path Length	106.87	18.92	64.07, 149.67	10	0.70	0.11

Table 1. Summary table for escape latency and path length data of socially isolated male animals from training days 1 to 4. CI, confidence interval; GH, group housed; SEM, standard error of the mean. Data are shown as % group housed block average matched to each social isolation block for male animals. Significance evaluated by *p* value (using the Wilcoxon signed-rank test) and Cohen's *d* value.

Day		Mean (% of Respective GH Block Average)	SEM	±95% CI of mean	N	*p* Value	Effect Size (*d*)
1	**Escape Latency**	127.56	10.72	103.32, 151.80	10	0.03	0.81
	Path Length	138.48	13.05	108.96, 167.99	10	0.04	0.93
2	**Escape Latency**	127.95	21.64	78.99, 176.90	10	0.70	0.41
	Path Length	134.97	21.68	85.92, 184.02	10	0.49	0.51
3	**Escape Latency**	93.40	16.71	55.60, 131.20	10	0.77	0.12
	Path Length	98.89	19.48	54.83, 142.94	10	0.85	0.02
4	**Escape Latency**	83.12	15.66	47.70, 118.55	10	0.38	0.49
	Path Length	86.97	18.56	44.99, 128.94	10	0.38	0.22

Table 2. Summary table for escape latency and path length data of socially isolated female animals from training days 1 to 4. CI, confidence interval; GH, group housed; SEM, standard error of the mean. Data are shown as % group housed block average matched to each social isolation block for female animals. Significance evaluated by *p* value (using the Wilcoxon signed-rank test) and Cohen's *d* value.

	Sex	Mean (% of Respective GH Block Average)	SEM	±95% CI of mean	N	*p* Value	Effect Size (*d*)
Probe Test	Male	154.41	38.27	67.83, 240.99	10	0.23	0.45
	Female	133.80	17.27	94.72, 172.87	10	0.13	0.62
Swim Speed	Male	109.74	6.69	94.62, 124.87	10	0.19	0.46
	Female	104.36	4.49	94.19, 114.52	10	0.49	0.31

Table 3: Summary table for average time spent in target quadrant during probe test and swim speed during probe test within male animals and female animals. CI, confidence interval; GH, group housed; SEM, standard error of the mean. Data are shown as % group housed block average matched to each social isolation block for male and female animals. Significance evaluated by *p* value (using the Wilcoxon signed-rank test) and Cohen's *d* value.

	Mean (% of Respective GH Block Average)	SEM	±95% CI of mean	N	*p* Value	Effect Size (*d*)
Body Weight	107.68	6.11	93.86, 121.50	10	0.92	0.40
Liver Weight	92.53	4.05	83.36, 101.70	10	0.16	0.58
Adrenal Glands Weight	96.98	5.76	83.95, 110.01	10	0.56	0.17
Retroperitoneal Fat Pads Weight	111.81	13.43	81.42, 142.20	10	0.49	0.28
Serum CORT Level	176.83	65.73	28.14, 325.52	10	0.56	0.39

Table 4: Summary table for the terminal biometrics of male animals. CI, confidence interval; GH, group housed; SEM, standard error of the mean. Data are shown as % group housed average matched to each social isolation block for male animals. Significance was evaluated by *p* value (using the Wilcoxon signed-rank test) and Cohen's *d* value.

	Mean (% of Respective GH Block Average)	SEM	±95% CI of mean	N	*p* Value	Effect Size (*d*)
Body Weight	108.51	6.98	92.73, 124.30	10	0.49	0.39
Liver Weight	101.35	6.05	87.66, 115.04	10	0.63	0.07
Adrenal Glands Weight	96.52	6.39	82.05, 110.98	10	0.38	0.17
Retroperitoneal Fat Pads Weight	84.81	7.41	68.06, 101.57	10	0.06	0.65
Serum CORT Level	84.37	16.10	47.25, 121.50	9	0.50	0.32

Table 5: Summary table for the terminal biometrics of female animals. CI, confidence interval; GH, group housed; SEM, standard error of the mean. Data are shown as % group housed average matched to each social isolation block for female animals. Significance was evaluated by *p* value (using the Wilcoxon signed-rank test) and Cohen's *d* value.

	Sex	Mean (% of Respective GH Block Average)	SEM	±95% CI of mean	N	*p* Value	Effect Size (*d*)
PSD-95	Male	112.76	1.40	108.64, 115.85	6	0.03	3.57
	Female	101.05	7.44	83.46, 118.64	8	0.95	0.05
GFAP	Male	92.69	7.52	74.91, 110.46	8	0.64	0.34
	Female	106.53	20.78	55.69, 157.37	7	0.81	0.12

Table 6: Summary table for PSD-95 and GFAP expression data from male animals and female animals. CI, confidence interval; GH, group housed; SEM, standard error of the mean. Data are shown as % group housed average matched to each social isolation block for male and female animals. Significance was evaluated by *p* value (using the Wilcoxon signed-rank test) and Cohen's *d* value.

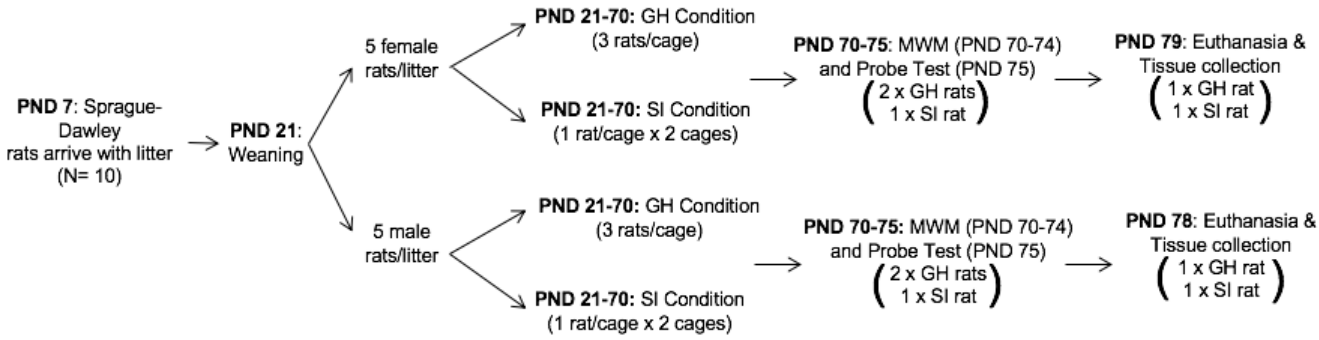

Figure 1: Experimental procedure for CELSI and behavioural testing. GH, group housed; MWM, Morris Water Maze; PND, postnatal day; SI, social isolation. Each week for a period of 10 weeks, the experimental procedure for CELSI, Morris Water Maze testing, euthanasia, and tissue collection was performed. To control for variability across litters, all SI data were standardized to the appropriate intra-litter GH average value.

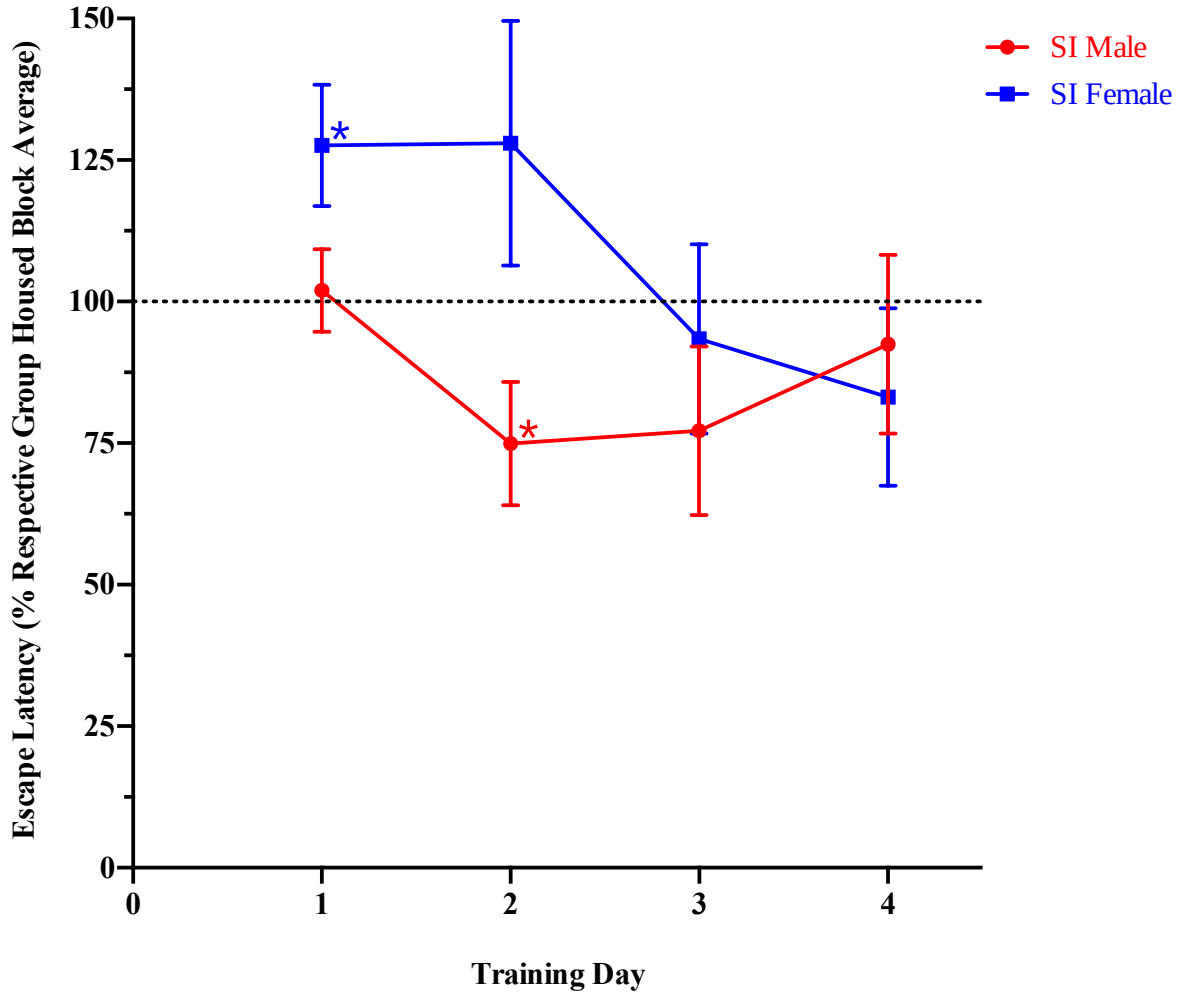

Figure 2: Percent respective group housed block averages for escape latency of male and female rats during acquisition phase. SI, social isolation. Data for escape latency, the time elapsed prior to the rat mounting the platform to escape the water, are displayed using block averages by taking the average value for escape latency of each socially isolated block as a percentage of the average value for escape latency of its respective group housed block. Escape latency for socially isolated male rats (N = 10) relative to group housed male rats (N = 10) was similar on day 1, shorter on days 2 and 3, and similar on day 4. Escape latency for socially isolated female rats (N = 10) relative to group housed female rats (N = 10) was longer on days 1 and 2, similar on day 3, and slightly shorter on day 4. The asterisk (*) shown on training days 1 and 2 indicate a statistically significant difference for the effect of housing condition on escape latency, such that the *p* value is less than 0.05 ($p<0.05$). Specifically, on training day 2, male rats had a significantly different effect of housing condition, with a significantly shorter escape latency for socially isolated male rats relative to group housed male rats (*p = 0.04, d = 0.73*). On training day 1, female rats had a significantly different effect of housing condition, with a significantly longer escape latency for socially isolated female rats relative to group housed female rats (*p = 0.03, d = 0.81*). An effect of sex on training days 1 (*p = 0.08, d = 0.88*) and 2 (*p = 0.06, d = 0.98*) appears to have occurred, such that male rats had an advantage in acquisition of the task during the acquisition phase of the MWM.

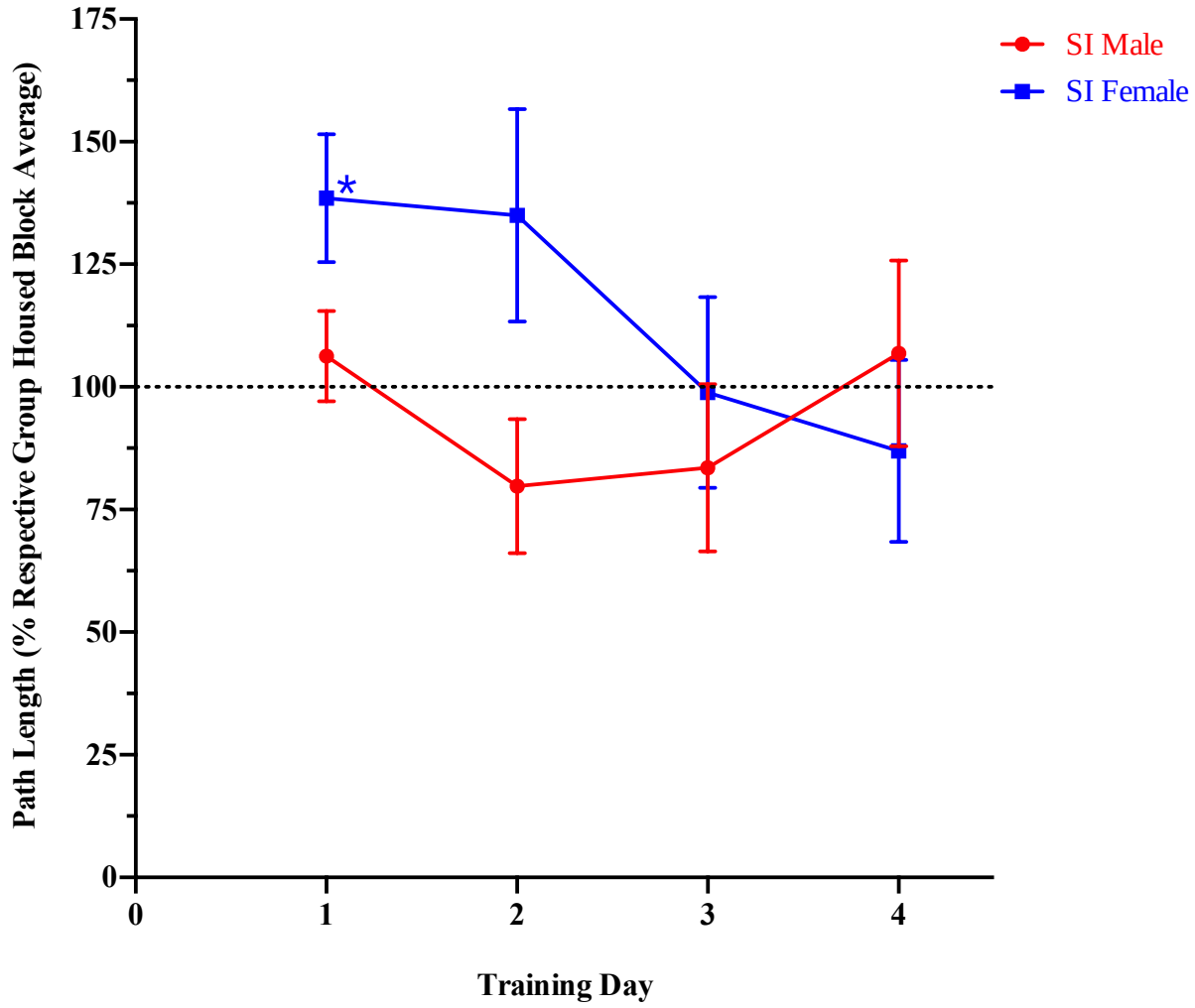

Figure 3: Percent respective group housed block averages for path length of male and female rats during acquisition phase. SI, social isolation. Data for path length, the distance travelled by the rat from start to mounting the platform to escape the water, are displayed using block averages by taking the average value for path length of each socially isolated block as a percentage of the average value for path length of its respective group housed block. Path length for socially isolated male rats (N = 10) relative to group housed male rats (N = 10) was similar on day 1, shorter on day 2, and similar on days 3 and 4. Path length for socially isolated female rats (N = 10) relative to group housed female rats (N = 10) was longer on days 1 and 2 and similar on days 3 and 4. The asterisk (*) shown on training day 1 indicates a statistically significant difference for the effect of housing condition on path length, such that the *p* value is less than 0.05 ($p<0.05$). Specifically, on training day 1, female rats significantly differed by housing condition, with a significantly longer path length for socially isolated female rats compared to group housed female rats ($p = 0.03$, $d = 0.81$). An effect of sex may have occurred on training days 1 ($p = 0.07$, $d = 0.90$) and 2 ($p = 0.08$, $d = 0.96$), such that male rats had an advantage in acquisition of the task during the acquisition phase of the MWM.

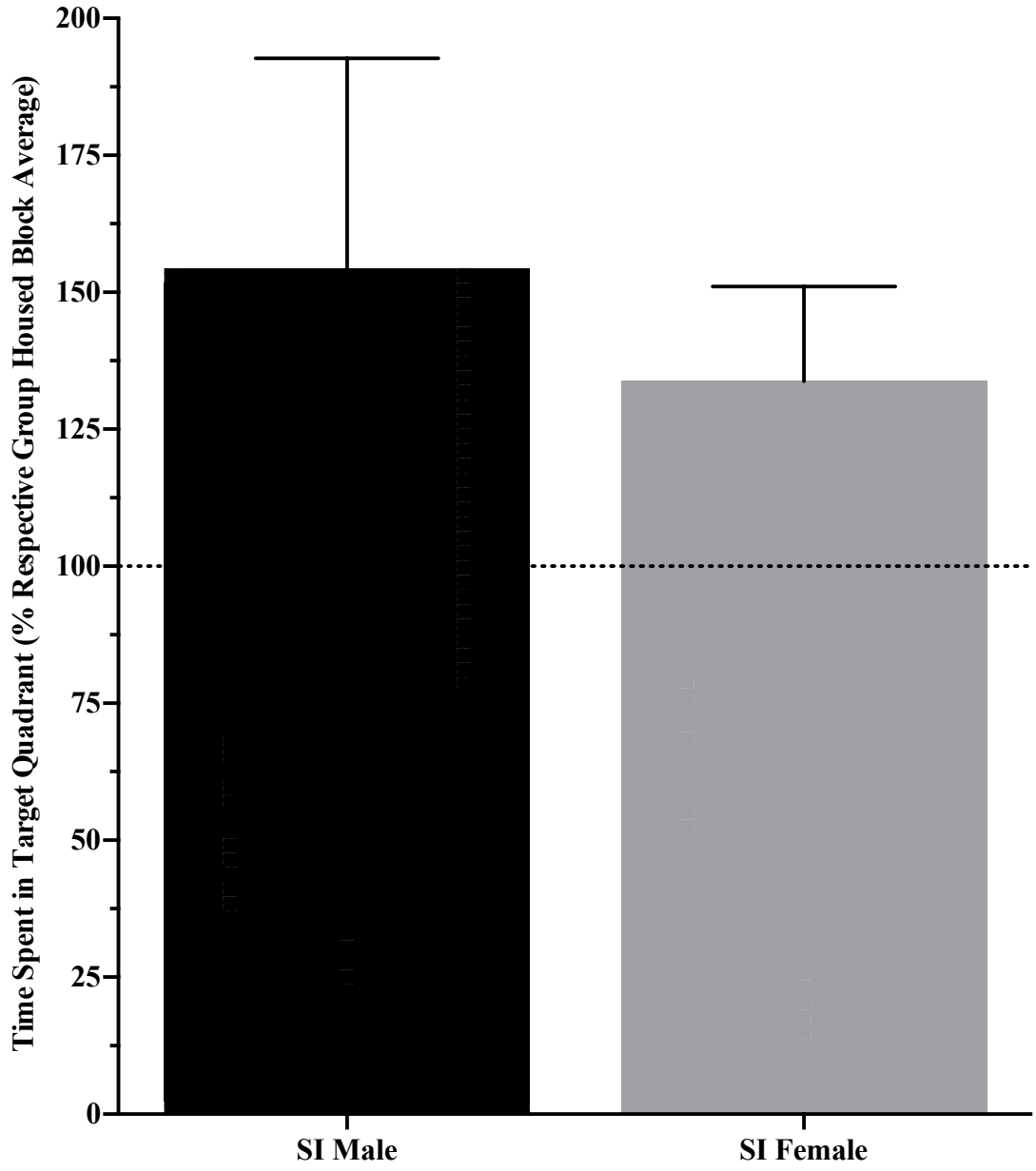

Figure 4: Percent respective group housed block averages for male and female time spent in target quadrant during the probe test. SI, social isolation. Data are displayed as the percent respective group housed block average value for time spent in target quadrant ± SEM (standard error of the mean). Time spent in target quadrant did not significantly differ by housing condition within male rats (group housed male rats (N = 10), socially isolated male rats (N = 10), $p = 0.23$, $d = 0.45$), within female rats (group housed female rats (N = 10), socially isolated female rats (N = 10), $p = 0.13$, $d = 0.62$), and between male and female rats ($p = 0.85$, $d = 0.22$).

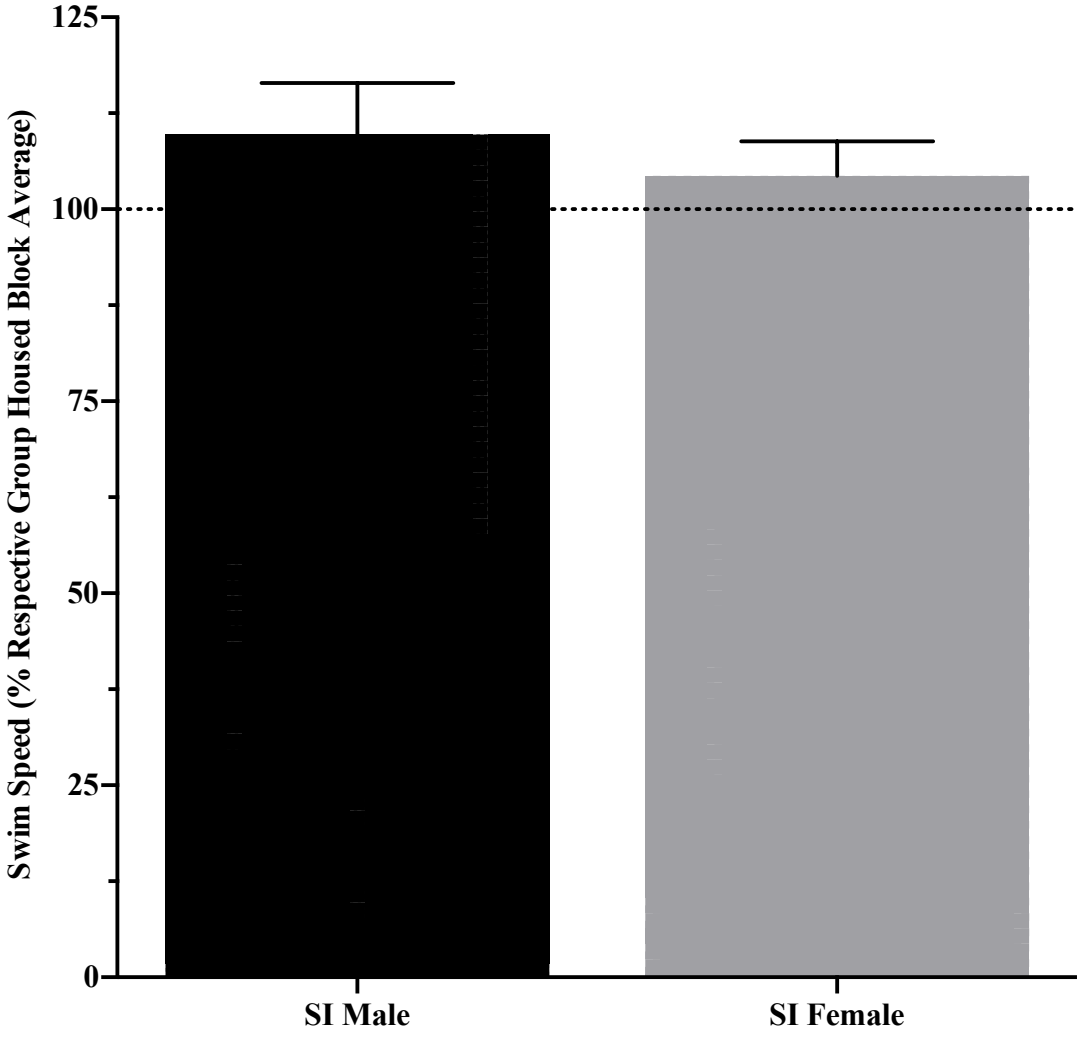

Figure 5: Percent respective group housed block averages for male and female swim speed during the probe test. SI, social isolation. Data are displayed as the percent respective group housed block average value for swim speed ± SEM (standard error of the mean). Swim speed did not significantly differ by housing condition within male rats (group housed male rats (N = 10), socially isolated male rats (N = 10), $p = 0.19$, $d = 0.46$), within female rats (group housed female rats (N = 10), socially isolated female rats (N = 10), $p = 0.49$, $d = 0.31$), and between male and female rats ($p = 0.58$, $d = 0.30$).

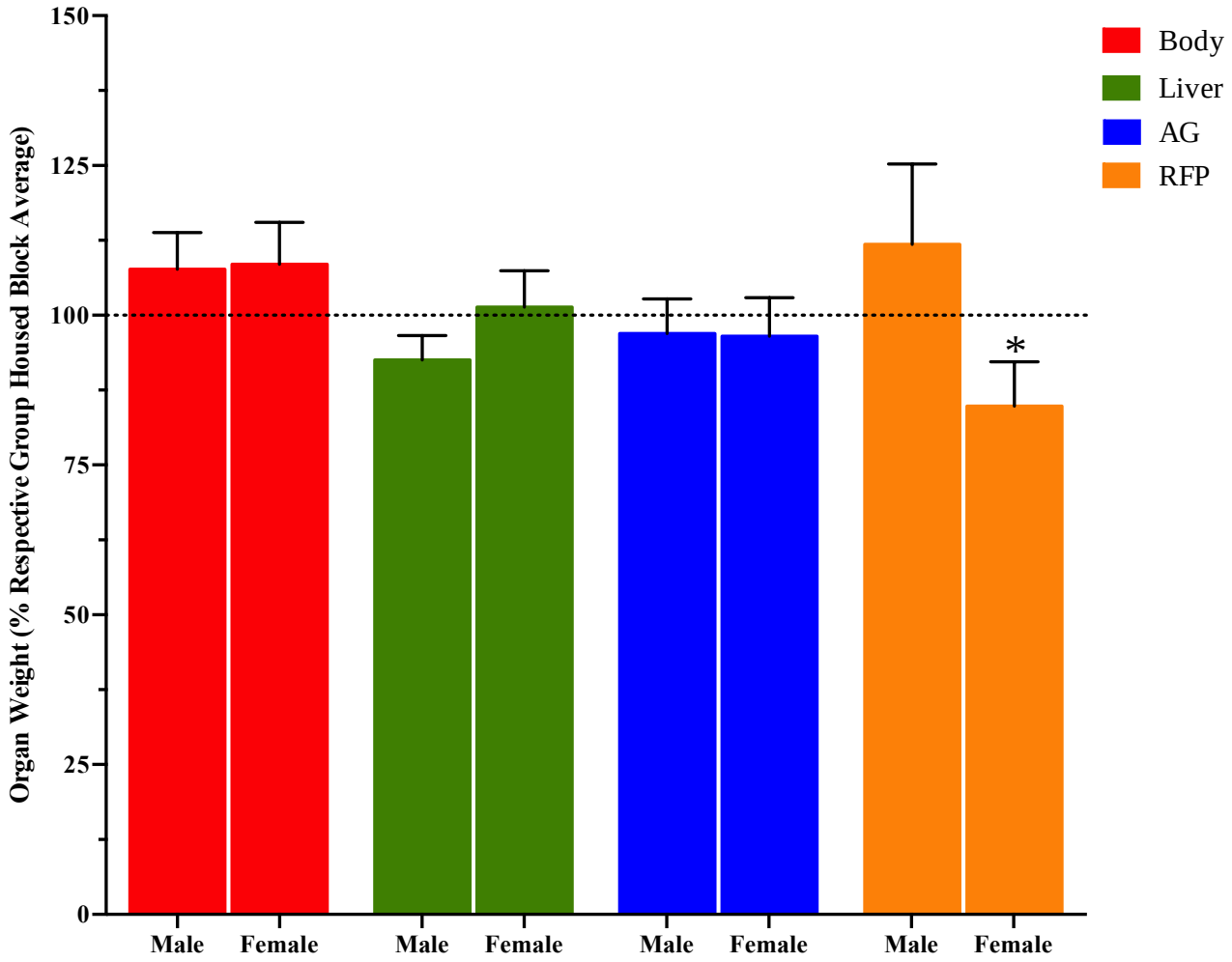

Figure 6: Percent respective group housed block averages for male and female body weight, liver weight, adrenal glands weight, and retroperitoneal fat pads weight. SI, social isolation; AG, adrenal glands; RFP, retroperitoneal fat pads. Data for SI groups are displayed as the percent respective group housed block average value for the organ weight ± SEM (standard error of the mean). Within male rats (group housed male rats (N = 10) and socially isolated male rats (N = 10)), body weight (*p = 0.92, d = 0.40*), liver weight (*p = 0.16, d = 0.58*), adrenal glands weight (*p = 0.56, d = 0.17*), and retroperitoneal fat pads weight (*p = 0.49, d = 0.28*) did not significantly differ by housing condition. Within female rats (group housed female rats (N = 10) and socially isolated female rats (N = 10)), body weight (*p = 0.49, d = 0.39*), liver weight (*p = 0.63, d = 0.07*), and adrenal glands weight (*p = 0.38, d = 0.17*) did not significantly differ by housing condition. For female rats, retroperitoneal fat pads weight (*p = 0.06, d = 0.65*) significantly differed by housing condition, with a significant reduction in retroperitoneal fat pads weight for socially isolated female rats relative to group housed female rats. Between male and female rats, body weight (*p = 0.80, d = 0.04*), liver weight (*p = 0.22, d = 0.54*), and adrenal glands weight (*p = 0.80, d = 0.02*) were not significantly different. Housing condition did appear to uniquely affect the weight of retroperitoneal fat pads between male rats and female rats (*p = 0.11, d = 0.79*).

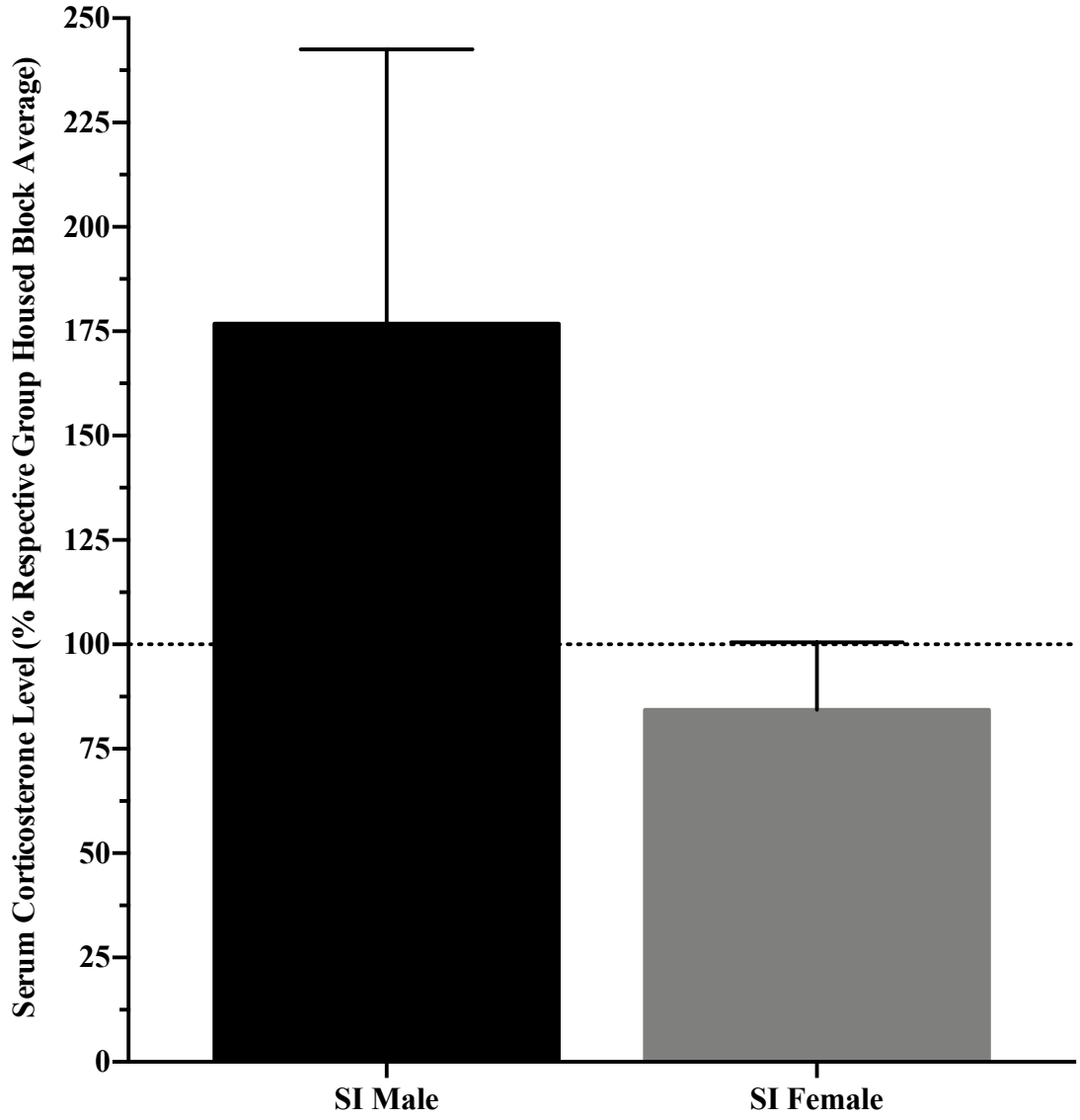

Figure 7: Percent respective group housed block averages for socially isolated male and female serum corticosterone levels. SI, social isolation. Data are displayed as the percent respective group housed block average value for serum corticosterone level ± SEM (standard error of the mean). Serum corticosterone level did not significantly differ by housing condition within male rats (group housed male rats (N = 10), socially isolated male rats (N = 10), *p = 0.56, d = 0.39*), within female rats (group housed female rats (N = 9), socially isolated female rats (N = 9), *p = 0.50, d = 0.32*), and between male and female rats (*p = 0.84, d = 0.60)*.

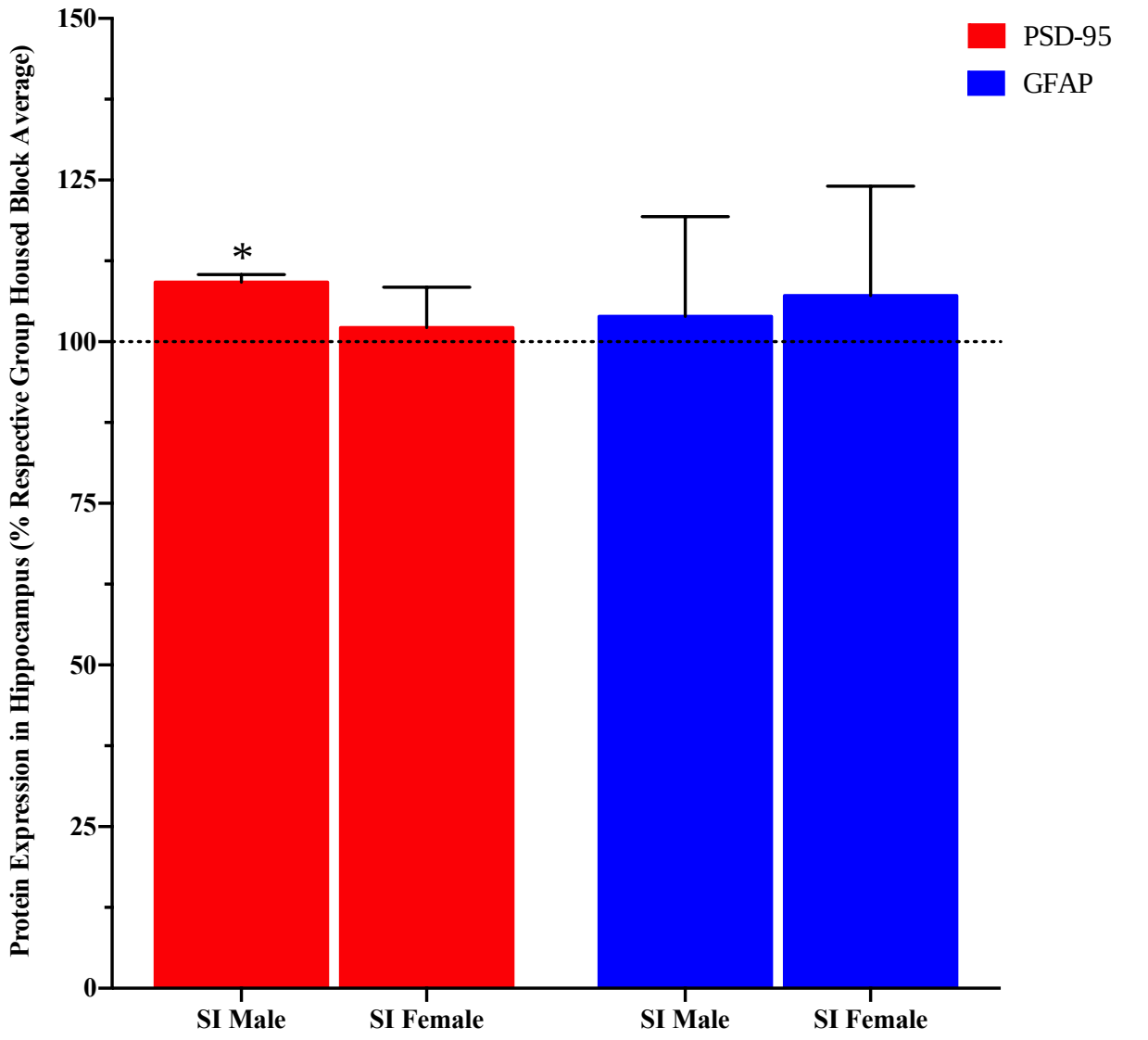

Figure 8: Percent respective group housed block averages for male and female PSD-95 and GFAP expression in the hippocampus. GFAP, glial fibrillary acidic protein; PSD-95, post-synaptic density protein 95; SI, social isolation. Data are displayed as the percent respective group housed block average values for PSD-95 and GFAP expression in the hippocampus ± SEM (standard error of the mean). PSD-95 expression in the hippocampus significantly differed within male rats [group housed male rats (N = 6), socially isolated male rats (N = 6), $p = 0.03$, $d = 3.57$], with a significant increase in PSD-95 expression in the hippocampus of socially isolated male rats relative to group housed male rats. No significant difference of housing condition on PSD-95 expression in the hippocampus was found within female rats [group housed female rats (N = 8), socially isolated female rats (N = 8), $p = 0.95$, $d = 0.05$]. The potential for a sex difference in PSD-95 expression in the hippocampus did exist between male and female rats ($p = 0.14$, $d = 0.69$). GFAP expression in the hippocampus did not significantly differ between male rats [group housed male rats (N = 8), socially isolated male rats (N = 8), $p = 0.64$, $d = 0.34$], within female rats [group housed female rats (N = 7), socially isolated female rats (N = 7), $p = 0.81$, $d = 0.12$], or between male and female rats ($p = 0.96$, $d = 0.34$).

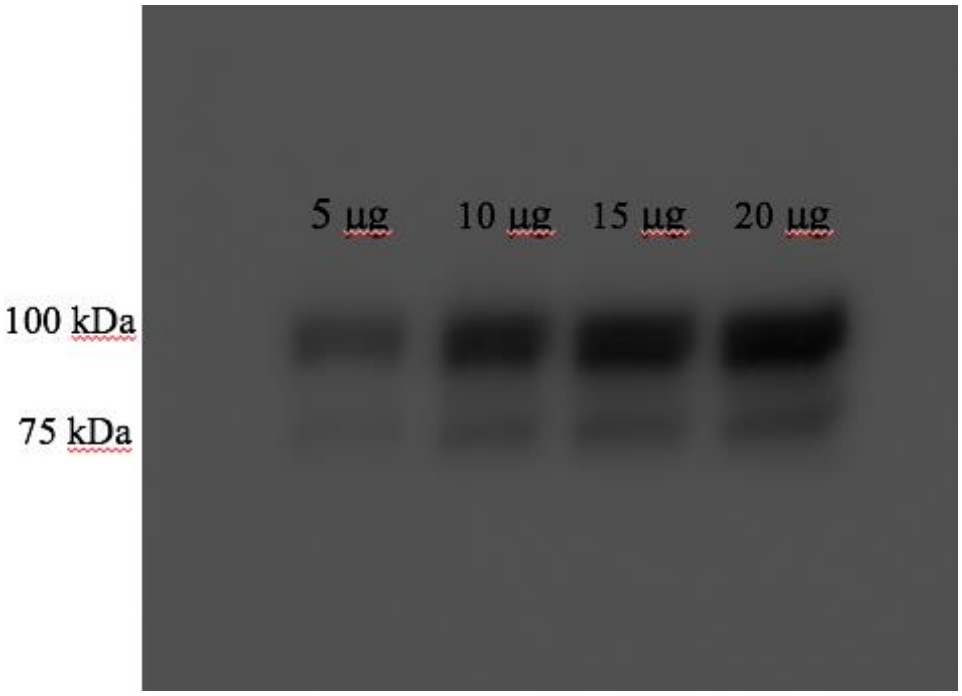

Figure 9: Standard curve optimization blot for PSD-95. To ascertain the optimal sample loading concentration for PSD-95 immunoblotting, 5 µg, 10 µg, 15 µg, and 20 µg of hippocampal whole homogenate from the male group housed rat of block three was used. The optimal sample loading concentration was concluded to be 10 µg/µL since it produced a stable and unsaturated signal.

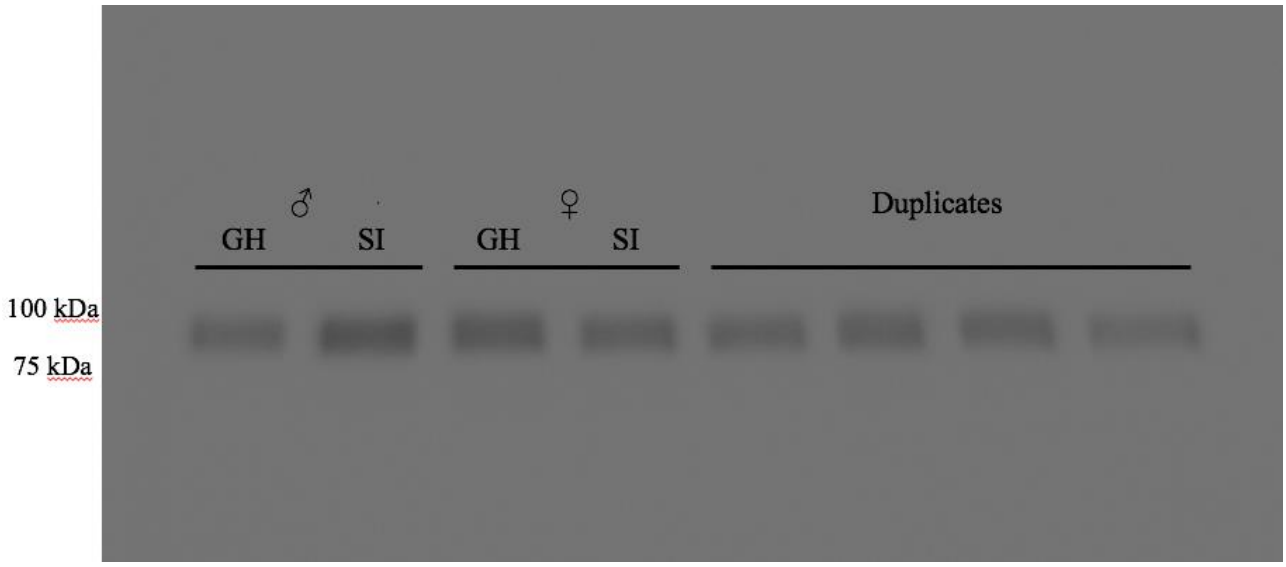

Figure 10: Representative blot of 95 kDa PSD-95 in hippocampus isolated from control and treatment conditions by sex. PSD-95 expression in hippocampal whole homogenates from block nine samples.

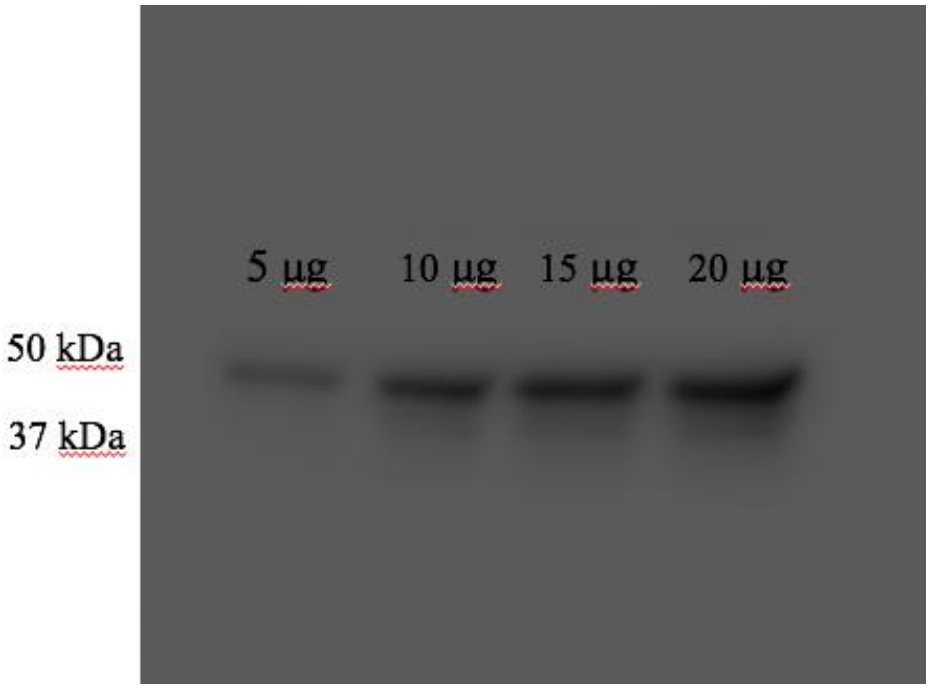

Figure 11: Standard curve optimization blot for GFAP. To ascertain the optimal sample loading concentration for GFAP immunoblotting, 5 µg, 10 µg, 15 µg, and 20 µg of hippocampal whole homogenate from the male group housed rat of block three was used. The optimal sample loading concentration was concluded to be 10 µg/µL since it produced a stable and unsaturated signal.

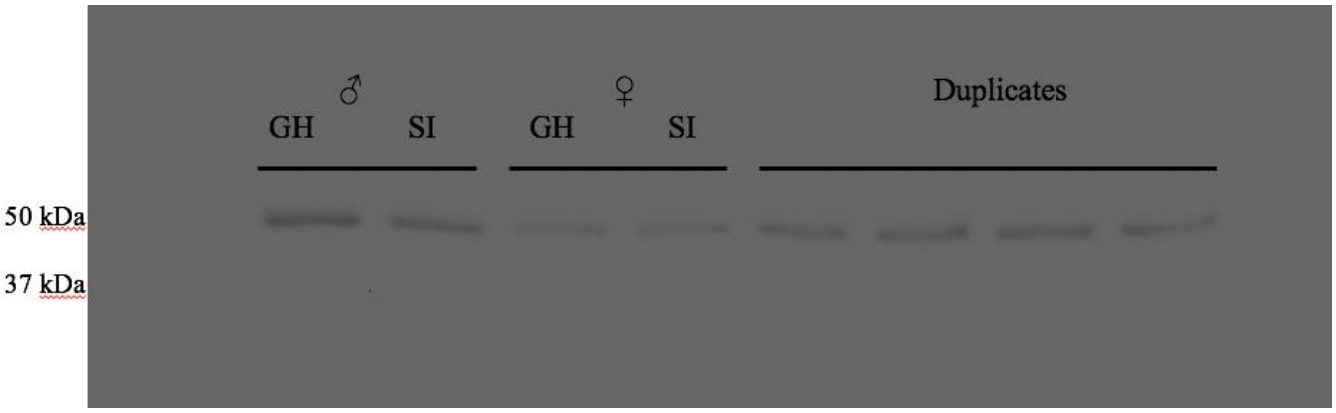

Figure 12: Representative blot of 50 kDa GFAP in hippocampus isolated from control and treatment conditions by sex. GFAP expression in hippocampal whole homogenates from block six samples.

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