Do primary and secondary psychosocial stressors vary in their effect upon plasticity-related proteins?

by

Adaora Chidinma Enumah

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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

The generous distribution of glucocorticoid receptors (GRs) is a primary reason for the widespread effects of stress on all biological systems. The prefrontal cortex (PFC), which is crucial in executive functioning, is one of the brain regions where GRs are most abundantly expressed, and as such the PFC is affected by numerous neuropsychiatric disorders associated with psychosocial stress. Glutamate receptors are responsible for mediating the vast majority of excitatory signals in the mammalian brain, including the PFC, and their modification is a neuroplastic change that may occur during stress. Although most of the stressors that have recently plagued humans have been more psychosocial than physical, most previous studies have focused on physical stress. With the above points in mind, the present study explored the effect of primary and secondary psychosocial stress on several important plasticity-related proteins in the PFC: the expression of several AMPA and NMDA receptor subunits and the phosphorylation of the major serine residues of GluA1 (a key AMPA receptor subunit). Notably, existing studies have tended to focus upon male rats, hence, this study aimed to explore sexually dimorphic changes.

From 6 litters, 4 male and 4 female Sprague-Dawley rats per litter were stratified by sex, and then randomly distributed into stress and control groups. In the stress group, each pair of rats was housed together in the same cage and were randomly assigned to receive either platform stress (PS), or bystander stress (ByS). Similarly, among each control animal group, rats were randomly allocated to either the platform control (PC), or bystander control (ByC) condition. The PS rats were placed on a small, elevated platform for 30 minutes twice daily for 5 days, and were returned back to their cages (which also housed the ByS rats) immediately after each stress exposure, while the PC animals were simply moved to another cage during the same period. All rats were sacrificed

approximately one hour after the final stress exposure, and the synaptoneurosome preparation technique used to enrich synaptic proteins from the dissected PFC samples; after this, proteins of interest were examined via Western blotting. In most cases, the general pattern of effect from both PS and ByS was an increase in AMPA and NMDA receptor subunit expression, although ByS tended to cause a more notable degree of change. In particular, ByS significantly increased the expression of GluA1 (77%) and GluN2A (87%) in male rats. As well, there was a significant difference in the ByS-related effects on pSer845-GluA1 levels between male and female rats (male phosphorylation levels were 51% greater). In summary, the psychosocial stressors applied appeared to selectively affect the plasticity-related proteins studied, with ByS appearing to have a more substantive effect overall, and male rats displaying more changes compared to female rats.

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Dedication

To God Almighty, for without His grace, I am nothing.

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List of Abbreviations

ACSF	Artificial Cerebrospinal Fluid
ACTH	Adrenocorticotropic hormone
AMPA(R)	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (receptor)
AVP	Arginine–vasopressin
BDNF	Brain-derived neurotrophic factor
ByC	Bystander control
ByS	Bystander stress
CAMKII	Calcium-Calmodulin-Dependent Protein Kinase II
CBG	Corticosteroid-binding globulin
CORT	Corticosterone
CREB	Cyclic adenosine monophosphate response element–binding protein
CRH	Corticotropin-Releasing Hormone
GABA	Gamma-AminoButyric acid
GLUR	Glutamate receptor
GR	Glucocorticoid receptor
GRIP	Glutamate receptor interacting protein
HPA	Hypothalamic-Pituitary-Adrenal
LC-NE	Locus coeruleus-norepinephrine system
LTP	Long-Term Potentiation
MDD	Major depressive disorder
MR	Mineralocorticoid receptor

NMDA(R)	N-methyl-D-aspartate (receptor)
PC	Platform control
PFC	Prefrontal cortex
POMC	Proopiomelanocortin
PS	Platform stress
PSD	Post synaptic density
PSNS	Parasympathetic nervous system
PVN	Paraventricular nucleus
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SN(P)	Synaptoneurosome (preparation)
TBS-T	Tris Buffered Saline with Tween-20

1. Literature review

1.1 Overview

The dynamic internal physiological equilibrium, *homeostasis*, which living organisms strive to maintain for their survival is constantly disrupted by internal and external forces often described as stressors (Chrousos & Gold, 1992). The effects of these stressors are counterbalanced by a number of offsetting forces, or adaptive responses that include various physical and mental changes that help to successfully re-establish homeostasis (Chrousos & Gold, 1992). Severe and prolonged use of such responses, according to Selye's "general adaptation syndrome", is responsible for the conglomerate of psychological and physiological features seen in any of a number of illnesses regardless of the nature of the causative agent (Selye, 1998).

1.2 Pathophysiology of stress

1.2.1 The general physiology of the stress response

Stress has a generalized effect on all body systems and exerts its influence on numerous processes within seconds (Habib, Gold & Chrousos, 2001). The processes stress can elicit include a wide variety of survival-promoting mechanisms such as mobilization of stored energy, gluconeogenesis, lipolysis, inhibition of processes leading up to energy storage, diminished appetite, re-direction of energy substrates to the large muscles, increased respiration, heart rate and cardiac output, elevated cerebral blood flow and perfusion, and regulation of the immune system (Chrousos & Gold, 1992; Habib et al., 2001). Taken together, these processes are adaptive responses mediated by various biological systems controlled by a central circuit and a

peripheral/effector limb (McEwen & Gianaros, 2011). The central circuit, housed in the hypothalamus and brain stem, is comprised of the parvocellular corticotropin-releasing hormone (CRH) and arginine–vasopressin (AVP) neurons of the paraventricular nucleus (PVN) of the hypothalamus, and the locus coeruleus (LC)–norepinephrine system (central sympathetic system) (Chrousos, 1992; Tsigos & Chrousos, 1994). The effector limbs include the hypothalamic-pituitary-adrenal (HPA) axis and the efferent fibers of the sympathetic system, which serve in the downstream control of all body processes during exposure to threatening stimuli (Gold et al., 1988a).

1.2.2 The Hypothalamic-Pituitary-Adrenal (HPA) axis

Activation of the HPA axis begins with the PVN of the hypothalamus secreting CRH, which is mainly responsible for regulating the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary that then moderates the secretion of glucocorticoids from the adrenal cortex (Gold et al., 1988a). Arginine vasopressin, a hormone also secreted by the PVN of the hypothalamus exhibits a strong synergistic activity with CRH in stimulating ACTH release, but tends to have poor secretagogue potential for ACTH on its own (Lamberts et al., 1984). Primarily, ACTH acts on the adrenal gland to regulate the secretion of glucocorticoids, adrenal androgens, and, to a lesser extent, mineralocorticoids from the zona fasciculata, zona reticularis, and zona glomerulosa, respectively (Gold et al., 1987; Habib et al., 2001). Further, glucocorticoid secretion may also be regulated by a number of hormones (e.g., AVP, angiotensin II and inflammatory cytokines) secreted by the adrenal gland, or circulating within the body regardless of whether neural signals are received from the autonomic nerves of the adrenal cortex (Hinson, 1990; Ottenweller & Meier, 1982).

Glucocorticoids are pleiotropic hormones, and they serve as the final effectors of the HPA axis by acting on a generous distribution of intracellular receptors (Habib et al., 2001; Tsigos & Chrousos, 2002). Cortisol and corticosterone are the most predominant glucocorticoids secreted by mammals; both steroid hormones are secreted in varying degrees and proportion in different species (Buckingham, 2006). The principal glucocorticoid in humans is cortisol, and in rodents is corticosterone, while mammals such as sheep, dog and pig produce both hormones in significant amounts (Buckingham, 2006). The effects of glucocorticoids in the central nervous system are mediated by two types of receptors; the glucocorticoid receptor type I (mineralocorticoid receptor, MR) and the glucocorticoid receptor type II (the classic glucocorticoid receptor, GR) (Habib et al., 2001). The MR is responsive to basal levels of stimulation while the GR is responsive to both basal and elevated levels of the hormone (Habib et al., 2001).

Glucocorticoids have a principal role in regulating the basal levels of activity in the HPA axis, and also in halting the stress response by acting at extrahypothalamic centres, the hypothalamus, and the anterior pituitary gland (de Kloet, 1991). To minimize the total exposure of tissue to elevated glucocorticoid levels, a negative feedback loop relays signals from glucocorticoids to ACTH secreting cells (corticotrophs) to inhibit stimulation of the adrenal cortex, which helps reduce the catabolic, immunosuppressive, and anti-reproductive effects of these hormones (Tsigos & Chrousos, 2002). In addition, the GR is involved in the negative feedback control of the HPA axis through the activation of an afferent GABAergic pathway to the PVN (Korte et al., 1993; Michelson et al., 1994).

1.2.3 Locus coeruleus-norepinephrine system (LC-NE)/sympathetic nervous system

The LC-NE/sympathetic nervous system is the second of the two principal components of the effector limb for the general adaptational response, the first being the HPA axis (Chrousos, Loriaux & Gold, 2013; Gold et al., 1988a, 1988b). The LC-NE system comprises the locus coeruleus and other noradrenergic cell groups found in the pons and medulla, and its activation results in the release of norepinephrine (NE) from highly complex interconnected neurons throughout the brain that are associated with the sleep-wake cycle, fear, anxiety, and eating behaviour (Chrousos & Gold, 1992; Tsigos & Chrousos, 2002). The brain relies mainly on the release of norepinephrine into the circulation by the LC-NE system because plasma norepinephrine is unable to cross the blood-brain barrier (Habib et al., 2001). Norepinephrine plays a critical role as the body's alarm system and is therefore important in stress responses to decrease neurovegetative functions (such as eating) and to increase autonomic and neuroendocrine functions, including activation of the HPA axis (Chrousos, 1998). As well, the sympathetic nervous system is known to exert its effect through peripherally located sympathetic nerves and the adrenal medulla in an adaptive response to stressful conditions (Chrousos & Gold, 1992).

1.2.4 Other central nervous system (CNS) components

In response to stress, the brain also activates three main central nervous system (CNS) elements that bring about specific actions aiding in stress management; for example, arousal, retrievability and analysis of information, and moderation of emotional responses (Chrousos, 1998; Chrousos & Gold, 1992). First, the mesocortical and mesolimbic dopamine systems, which

are activated by norepinephrine secretion from the LC-NE system (Roth et al., 1988; Tassin et al., 1979). The mesocortical system is thought to innervate the medial prefrontal cortex (mPFC), and in stressful conditions sends inhibitory signals resulting in a decline in the two main functions of the PFC: cognition and anticipatory response (Chrousos, 1998). On the other hand, the mesolimbic system has been linked to the nucleus accumbens and plays a role in motivation/reward (Chrousos & Gold, 1992). Second, the amygdala-hippocampal complex is also activated, and helps in the conditioning of fearful responses, retrieval, and emotional analysis of information pertinent to the stressor (Sakanaka et al., 1986). Third, the hypothalamic arcuate nucleus proopiomelanocortin (POMC) neuronal system, which induces opioid-receptor mediated, or stress-related analgesia and may affect emotional tone, is activated in turn by the activation of CRH neurons in the PVN (Charmandari et al., 2005; Chrousos, 1998; Yajima et al., 1986). The POMC also helps in the regulation of CRH neuronal activity and the LC-NE sympathetic system by sending projections to the PVN brain stem and other brain areas (Yajima et al., 1986).

1.3 Psychosocial stressors

The central circuitry, the effector limbs, and the other CNS elements are initiated when the body is faced with either of the two distinct types of stressors: physiological and psychosocial (Kogler et al., 2015). Physiological stress is associated with untoward emotional, or sensory experiences that have the potential for damage, or could pose a threat to the body (Peyron et al., 2000). The causes of physiological stress may vary from pain to hunger to injury. However, psychosocial stress is more subjective, and is often induced by the loss/ending of a social relationship, onset of interpersonal conflict leading to loss of control and predictability, and other social threats such as exclusion (Dickerson & Kemeny, 2004; Pryce & Fuchs, 2017).

Psychosocial stressors could be primary, or secondary based on the order in which they are experienced (Pearlin, 1989). Primary psychosocial stressors can be described as stress experienced directly from a particular event, or disaster (NHS, 2009; Pearlin, 1989). The stressor could be inherent in loss of a loved one, loss of occupation, or loss of living space/ territory, perhaps due to flooding. However, a secondary psychosocial stressor could be viewed from two perspectives. One could be a stressor that an individual experiences indirectly as a consequence of a primary stressor (Pearlin, 1989); for example, loss of valuable property occurring after the experience of flooding (the primary stressor). The other perspective, which will more directly shape the focus of this study, is stressors experienced by an individual because they share similar roles, or occupy a shared space with a person facing a primary stressor (Pearlin, 1989). People are not islands all by themselves, but have relationships with other individuals, sectors and institutions; hence, disruption in one area of an individual's life could pose problems in another owing to the interrelationships that exist (Pearlin, 1989). A befitting example would be loss of a job by one partner in a marriage, which could reasonably be expected to result in stress in the other partner (Pearlin & McCall, 1990).

Interestingly, animal models used in the study of stress may also be classified in a similar fashion. For example, animal models of physiological stress could take the form of electric foot shock, immobilization in a clear narrow tube (restraint stress), or forced swimming in cold water (Kolb et al., 2017). Psychosocial stress is often examined with a number of different models, and often times a mixture of these is used as a strategy to prevent habituation; for example, elevated platform stress, bystander stress, maternal separation stress, resident-intruder stress, and predator odour (Kolb et al., 2017). All these different models, except for bystander stress, are examples

where stress is directly applied to the animal(s) being studied (i.e., they are primary stressors). On the contrary, *bystander stress*, first described by Mychasiuk et al. (2011a, 2011b), provides a secondary psychosocial stress model where indirect stress is applied to the animals being studied by housing them in the same cage as an animal that has undergone direct (primary) stress. In other words, the animals being studied do not undergo any form of direct stress, which clearly differentiates this model from those involving primary psychosocial stressors. The indirect stress paradigm (bystander stress) was based on studies that have shown animals experience stress when forced to witness other animals in turmoil, and that the witness animals empathize with those in distress through altered vocalizations and pain behaviour (Langford et al., 2006; Mychasiuk, Gibb, et al., 2011).

Although the use of animal models in the study of stressors has been met with challenges, researchers have been able to make good deductions from animal models because of the similarity in elevated corticosterone (cortisol in humans) that is associated with activation of the HPA axis (Kolb et al., 2017). Notably, psychosocial stress paradigms have shown themselves to be more relevant in studying human conditions when compared to non-social, or physiological paradigms such as restraint stress (Slattery & Cryan, 2014). Laboratory animals that have been subjected to social stressors have exhibited various behavioural, physiological, and cellular changes that have a close resemblance with changes seen in humans exposed to acute and chronic social stressors (Pryce & Fuchs, 2017). For instance, rats exposed to five weeks of chronic social stress exhibited a reduced interest in gustatory reward, quite like the lack of interest exhibited by humans with major depressive disorder (MDD), and in both cases the behavioural change is reversed by administration of an antidepressant (Rygula et al., 2005, 2006). These animal stress models have

proven very useful in numerous studies, particularly since the effect of stress on humans has been linked with different psychiatric illnesses such as MDD, generalized anxiety, and post-traumatic stress syndrome (Fuchs et al., 2001).

1.3.1 Psychosocial stress and the PFC

As previously mentioned, corticosterone, which is the final effector of the HPA axis during stressful experiences, acts via the MRs and GRs (Habib et al., 2001; Tsigos & Chrousos, 2002). Although, the MRs and GRs are most abundant in the hypothalamic parvocellular neurons and corticotrophs of the pituitary gland, they are generally distributed throughout the brain (de Kloet et al., 1998). Brain structures such as the hippocampus, prefrontal cortex and amygdala have a high concentration of GRs, and as such are sensitive to stress (de Kloet et al., 2005; McEwen, 1999). Notably, of these three structures, the hippocampus has the highest concentration of GRs (Bianchi et al., 2005; McEwen, 2008), and for this reason many studies on stress have focused on this area of brain (McEwen, 2008). As well, the hippocampus controls several important functions, such as learning and consolidation of memory (de Kloet et al., 1998). However, while many effects of stress can be seen in the hippocampus, certain effects are unique to the PFC (Popoli et al., 2012).

The dorsolateral PFC (dlPFC) in primates is responsible for executive function that is described by Fuster (2015) as “the formation of coherent behavioural sequences towards the attainment of goals.” The basis of executive functioning is working memory, which refers to the ability to keep in mind, or call up a recent event without direct stimulation (Goldman-Rakic, 1995). The PFC also functions in behavioural changes and inhibition of unwanted actions geared towards better adaptation to a changing environment (Buschman & Miller, 2007; Thompson-Schill et al., 2002). Furthermore, the role of the PFC is very crucial in mitigating the effects of stress on

cognition and psychopathologies (Popoli et al., 2012). Carnevali et al., (2019) with the use of transcranial direct current stimulation to increase PFC excitability in healthy human subjects exposed to psychosocial stress, demonstrated the inhibitory role of dlPFC on the stress response system. The results of their study showed a reduction in the heart rate and anxiety level of the subjects with no effects on stress induced cortisol release (Carnevali et al., 2020). Similarly, there are animal studies that have shown the PFC plays an inhibitory role in regulating the HPA axis during response to stress (Gilabert-Juan et al., 2013; Herman et al., 2003). Impaired PFC function and plasticity have also been constant pathologic findings of numerous neuropsychiatric disorders including depression (Goto et al., 2010).

There has been some controversy regarding the similarity in the structural and functional homologues of areas in the dlPFC of primates and that of rat neocortex (Wise, 2008). For example, the granular layer of the PFC, which is characteristic of primate dlPFC, is thought to be absent in rats (Preuss, 1995; Wise, 2008). However, there are some areas of the rat PFC with agreeable similarities in cognitive function to the dlPFC in primates; these include the medial PFC (mPFC) consisting of the anterior cingulate (AC), prelimbic (PL), and infralimbic (IL) cortices (Kesner & Churchwell, 2011). Owing to this, studies on effects of stress on the PFC using rodents have focused on the mPFC (McEwen & Morrison, 2013). The mPFC exerts downstream regulation of autonomic and neuroendocrine functions (Thayer & Brosschot, 2005) via projections onto the amygdala and the brain stem (Sesack et al., 1989), while also influencing the HPA axis (Diorio et al., 1993) and the parasympathetic nervous system (PSNS) (Thayer & Sternberg, 2006).

1.4 Plasticity-related proteins

Plasticity refers to the ability of the brain to physically adapt to change and is the basis for some of the brain's most important functional abilities, such as learning and memory (Ramirez & Arbuckle, 2016). Synaptic plasticity relates to changes in neuronal connections at the synaptic terminals, which are the primary points of functional contact between most neurons (Ramirez & Arbuckle, 2016). Plasticity-related proteins at synaptic terminals in the PFC include the glutamate receptors [e.g., the N-methyl-D-aspartate receptor (NMDAR) and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR)]. Modifications in neuronal plasticity, such as alterations in signal transduction pathways are major ways by which stress can influence the brain (Duman, 2002; Duman et al., 2000).

1.4.1 Glutamate Receptors

Glutamate is the major excitatory neurotransmitter in the brain (Popoli et al., 2012), and is involved in numerous physiological functions including learning and memory (Bliss & Collingridge, 1993; Recasens et al., 2007). As well, glutamate receptors have been implicated in various neuropsychiatric disorders, including depression (MacHado-Vieira et al., 2009), schizophrenia (Gilmour et al., 2012), Alzheimer's disease (Hu et al., 2012), Parkinson's disease (Ahmed et al., 2011), and amyotrophic lateral sclerosis (Bogaert et al., 2012). Glutamate regulates synaptic signal transmission and plasticity through two broad categories of glutamate receptors, ionotropic (iGluRs) and metabotropic (mGluRs), which are widely dispersed affording glutamate its multiple intracellular functions (Pavlov & Mielke, 2012; Popoli et al., 2012). Importantly, the two receptor sub-types have key physiological differences. Ionotropic receptors are

transmembrane ion channels that open and close in response to ligand binding. Ionotropic receptors open quickly, but stay open only for a few milliseconds, hence, they are responsible for mediating all major fast excitatory signals in the CNS (Goodwani et al., 2017). In contrast, metabotropic receptors do not form channel pores, instead, signal transduction is mediated by a receptor-coupled G-protein, which activates intracellular processes through second messengers (Goodwani et al., 2017). Owing to the cascade of molecules required for the activation of the metabotropic receptors, a longer time is needed to transmit signals, but these receptors are able to continue signal transmission for a longer period (Goodwani et al., 2017).

Glutamatergic ionotropic receptors possess a common structure composed of four large subunits forming a central ion pore, but differ in distribution and function (Mayer & Armstrong, 2004; Ozawa et al., 1998). Based upon the relative selectivity to three synthetic agonists, iGLURs are mainly classified into three sub-types: AMPARs, NMDARs, and Kainate receptors (Pavlov & Mielke, 2012). These receptors have a cation-selective permeability to Na^{+}, K^{+}, differential permeability to Ca^{2+}, and blockade by Mg^{2+} (Mayer & Westbrook, 1987). AMPA receptors mediate fast excitatory neurotransmission at most synapses in the CNS (Ozawa et al., 1998; Ramirez & Arbuckle, 2016). On the other hand, NMDA receptors are uniquely characterized by a voltage-dependent block by Mg^{2+} at resting potential (Mayer et al., 1984). NMDAR channels open only when the membrane has been slightly depolarized by AMPAR activation, which causes the Mg^{2+} to be displaced and allows for the influx of Ca^{2+} that mediates various intracellular processes (Ramirez & Arbuckle, 2016).

AMPARs are a heterotetrameric complex of subunits GluA1-A4 (Man, 2011). The ability of this receptor to allow Ca^{2+} influx (Ca^{2+} permeability) is dependent on the presence/absence of

the GluA2 subunit in the receptor complex (Man, 2011). A majority of AMPARs contain GluA2, and thus are Ca^{2+} impermeable, allowing them to maintain low Ca^{2+} concentration intracellularly, which is crucial at basal levels of stimulation (Blanco-Suarez & Hanley, 2014; Shepherd, 2012). However, a fraction of GluA2-lacking AMPARs remain in the synapse of mature neurons, and their expression is dynamically regulated in basal, activated, and stressful conditions (Henley & Wilkinson, 2016; Wenthold et al., 1996). Similarly, the NMDARs are heterotetrameric complexes formed from a set of subunits, namely: GluN1, GluN2 (with known subtypes A-D), and GluN3 (with known subtypes A and B) (Collingridge et al., 2009). A functional NMDAR requires the assembly of two obligatory GluN1 subunits and either two GluN2 subunits, or a combination of one GluN2 and one GluN3 (Traynelis et al., 2010; Ulbrich & Isacoff, 2007). Besides this structural assembly, NMDARs require the simultaneous binding of glycine and glutamate to be activated. The extracellular segment of the GluN1 subunit provides a binding site for glycine, and the extracellular segment of the GluN2 and GluN3 subunits provide a binding site for glutamate (Pavlov & Mielke, 2012).

1.4.2 Expression of plasticity-related proteins

As mentioned earlier, AMPARs and NMDARs play a critical role in neuroplasticity, which is essential in various CNS processes such as learning and memory (Blanke & VanDongen, 2009; Henley et al., 2011; Kessels & Malinow, 2009). Thus, in a number of neurologic and pathological conditions, abnormal expression levels of AMPARs and NMDARs have been implicated (Blanke & VanDongen, 2009; Henley et al., 2011). As well, their reduced function has been linked to a decline in cognitive function, while their overstimulation has been shown to influence certain neurodegenerative illnesses (Blanke & VanDongen, 2009; Henley et al., 2011).

Yuen et al. (2009) observed that acute forced swim stress causes a 3-fold increased surface expression of the obligatory GluN1 subunit, and a 2-fold increased surface expression of the GluN2A and GluN2B accessory subunits of the NMDAR in the PFC (Yuen et al., 2009). However, while an increase in surface expression of these receptor subunits was observed, the total levels remained the same in both stressed and control groups (Yuen et al., 2009). Similarly, another study reported that acute swim stress caused no significant change in total expression levels of the GluN1 and GluN2B NMDA subunits in the PFC of both sexes, except for the GluN2A subunit which showed a 22% increase in males only (Fumagalli et al., 2009). Contrary to these findings, Cohen et al. (2011) reported a 47% decrease in expression of the GluN1 subunit in the post synaptic density of mice administered chronic corticosterone compared to the controls.

In addition, acute swim stress caused about a 2-fold increase in AMPAR GluA1 and GluA2 subunit surface expression in the PFC, but, as in the case of the NMDA subunits, there were no changes observed in the total expression levels of these AMPA receptor subunits (Yuen et al., 2009). In agreement, the findings from Fumagalli et al. (2009) showed no significant changes in the total levels of these AMPA receptor subunits for males and females. In addition to the observed increase in the surface expression of these receptor subunits, acute swim stress caused a prolonged potentiation of AMPAR and NMDAR-mediated signaling currents in the PFC (Yuen et al., 2009). Taken together, the results suggest that following acute stress, GR-mediated AMPAR and NMDAR trafficking to the postsynaptic plasma membrane induced prolonged potentiation of signaling currents, which likely contributed to enhanced working memory in the PFC for at least 24 h (Yuen et al., 2009).

By studying rats exposed to acute footshock stress, some authors have suggested that changes in the expression of ionotropic glutamate receptors in the PFC may be time-dependent (Bonini et al., 2016). For example, they observed a 30% decrease in the total levels of GluA2 subunits in animals sacrificed immediately after the stress procedure, but not two hours after, while no significant modifications were seen for GluA1. In addition, there was an increase by about 50% in the total expression of GluN1 and GluN2A NMDA subunits, but not in GluN2B, in animals sacrificed two hours after stress (Bonini et al., 2016). Their findings suggest that modifications in the glutamatergic system on exposure to acute stress occur early and are transient as no changes were observed in these receptor subunits in animals that were sacrificed 24 hours after the stress procedure (Bonini et al., 2016).

In the hippocampus however, there have been reports of increases of about 30% in the total protein expression of the GluN2A and GluN2B NMDA receptor subunits in the ventral hippocampus after exposure to chronic mild stress (Calabrese et al., 2012). Following chronic restraint stress, a 2-fold increase in GluN1 expression was found in the dorsal hippocampus (Pacheco et al., 2017). However, under similar conditions no changes were observed in GluN2A and GluN2B expression. (Pacheco et al., 2017). Further, Fumagalli et al. (2009) reported that female mice exposed to acute restraint stress exhibited a 24% increase in total expression levels of total GluA1 in the hippocampus compared to the unstressed group. However, male mice exposed to similar conditions showed no significant change in total GluA1. Similarly, another study observed no change in GluA subunits expression in the hippocampus of male rats after chronic restraint stress (Pacheco et al., 2017).

As expected, it appears from existing literature that changes in the expression of AMPA and NMDA glutamate receptor subunits vary depending upon which area of brain is being studied and the duration of stress application (that is, whether acute or chronic). Regardless, there is evidently sparse literature examining the expression of these proteins in the PFC, particularly in relation to either primary or secondary psychosocial stress. As well, studies that have been done on PFC using the physical stress paradigms have focused mainly on acute stress.

1.4.3 Phosphorylation of plasticity-related proteins

Interactions between proteins is a primary way by which a cell responds either directly, or indirectly to changes in its environment (Pavlov & Mielke, 2012). Phosphorylation is a form of post-translational modification that serves to regulate cellular distribution and molecular interactions of various proteins (Pawson & Scott, 2005). Phosphorylation, a process mediated by protein kinases and offset by protein phosphatases, involves the transfer of γ-phosphate from adenosine triphosphate to a serine, threonine, or tyrosine residue of a substrate (Pavlov & Mielke, 2012). Studies have shown that a predominant site for phosphorylation of NMDARs is at tyrosine residues, and that a balance between phosphorylation and dephosphorylation is required for proper functioning of the receptor (Pavlov & Mielke, 2012). Similarly, AMPA receptor phosphorylation is an important post-translational modification that governs its expression on the cell surface, channel properties and synaptic plasticity (Caudal et al., 2010). Owing to the importance of phosphorylation of these glutamate receptors in governing numerous channel properties, there has been a growing need to understand modifications resulting from stressful conditions, and how these changes go on to affect transmission of excitatory synaptic currents (EPSCs). Among the AMPA receptor subunits, GluA1 has been most extensively studied and characterized. From these

reports, it has been shown that the two major phosphorylation sites on the GluA1 subunit are the Serine 831 and Serine 845 residues which are phosphorylated by the action of PKC/CaMKII and PKA, respectively (Wang et al., 2005).

The phosphorylation of Ser831-GluA1 is thought to mediate plasticity by increasing ion conductance through AMPAR channels, and phosphorylation of Ser845-GluA1 is believed to facilitate the insertion of AMPAR at extra-synaptic sites, thus increasing receptor availability, which enhances long term potentiation (LTP) (Caudal et al., 2010). As well, pSer845-GluA1 increases the probability of channels opening and the amplitude of the EPSC (Wang et al., 2005). Ultimately, the effect of the phosphorylation of these two residues is thought to cause an increase in the strength of synaptic currents (Wang et al., 2005).

Reports from studies on the phosphorylation of AMPARs and NMDARs following stress have varied from an increase to a decrease to no significant change depending upon which subunit residues are being studied. Caudal et al. (2010) reported a 38.6% decrease in phosphorylation of Ser831-GluA1, and no change in pSer845-GluA1 in the PFC after the application of acute platform stress, suggesting a decrease in glutamatergic signal transmission in the PFC during occasions of acute stress. Interestingly, a report from Park et al. (2018) revealed quite similar findings, although their study involved animals that underwent a stressor that was physical and chronic (not psychosocial and acute stress, as was the case in the Caudal study). According to Park et al. (2018), the phosphorylation of pSer845-GluA1 in the mPFC was not significantly changed on exposure to chronic restraint stress, but there was a significant 30% decrease in Ser831-GluA1 in comparison to the controls (Park et al., 2018). However, a study done by Caudal et al. (2016) found that acute elevated platform (EP) stress resulted in an increase in the phosphorylation of Ser831-GluA1by

30% and pSer845-GluA1 by 20% in the mPFC. Further, the authors only reported an overall statistically significant rise in the phosphorylation of both GluA1 subunit residues when examining the collective findings from four brain regions including the mPFC, they did not state if these findings were statistically significant in the PFC alone. They went on to suggest that their earlier report (Caudal et al., 2010) was somewhat anomalous because previous studies done on the PFC and hippocampus mainly reported an increase (some were statistically insignificant) in both subunit residues after various types of stressful experiences (Caudal et al., 2016).

On the contrary, some studies have reported no significant change in the phosphorylation levels of both Ser845 GluA1 (Bonini et al., 2016) and Ser831 subunit residues in the PFC (Bonini et al., 2016; Fumagalli et al., 2009) after acute footshock and acute swim stress, respectively. As well, although the findings from Qi et al. (2009) show a marked increase in pSer831 and pSer845 in the PFC of animals exposed to acute EP stress, these results were statistically insignificant. However, Caffino et al. (2015) observed that acute swim stress caused a significant rise in pSer831-GluA1 by about 50% in the PFC. Notably, some of these studies utilized whole homogenates (Caudal et al., 2010, 2016; Qi et al., 2009) and others used crude synaptosomes (Caffino et al., 2015; Fumagalli et al., 2009), neither of which may offer the most accurate representation of the changes that have occurred at the synaptic region, especially the post-synaptic membrane.

In the hippocampus, there seems to be a consensus as to the direction of change seen in both pSer831 and pSer845 GluA1 subunits following varying stressful experiences. Previous studies have mainly reported an increase of both subunits. Murphy et al. (2014) reported that acute swim stress in rats caused about a two-fold increase in the phosphorylation of Ser845-GluA1

AMPAR subunit in the hippocampus. Some other studies done on hippocampus, show a two-fold increase in pSer831 (Fumagalli et al., 2011), and a 30% significant increase in pSer831 in male, but not female rats following acute restraint stress (Fumagalli et al., 2009).

1.5 Sex differences in stress

Stress-related disorders are influenced by sex, therefore, understanding sex differences that exist in various stress-related pathologies would prove beneficial in the development of appropriate interventions (Pinn, 2005). For example, women are at a higher risk of stress-related disorders such as generalized anxiety and PTSD compared to men (Stein et al., 2000; Weissman et al., 1988). As well, women are more than twice as susceptible to depression than men (Hyde et al., 2008).

There has been a growing body of work on sexual dimorphism in behavioural, morphological, and neurochemical changes to different brain areas resulting from stress (Garrett & Wellman, 2009). Conditioned fear, a behaviour mediated by the PFC, has been examined for sex differences (Miracle et al., 2006) by studying fear acquisition, extinction, and recall of fear extinction. Chronic stress impaired the recall of fear *extinction* in male rats (Baran et al., 2009; Miracle et al., 2006), but the recall of fear *acquisition* in female rats (Baran et al., 2009). As well, attentional impairments, a symptom of psychiatric illnesses associated with the PFC, have been studied. Liston et al. (2006) reported selective impairment of attentional set-shifting performance in male rats. Some other PFC-mediated behaviours, such as working memory and behavioural flexibility, were markedly impaired after corticosterone treatment in male rats (Cerqueira et al., 2005).

Notable sex-related morphological changes in the PFC and hippocampus following stress exposure have also been documented (Cook & Wellman, 2004; Magariños & McEwen, 1995). For instance, in the prenatal bystander stress model experiment conducted by Mychasiuk et al. (2011), stress-induced neuroanatomical changes in the PFC and hippocampus varied between male and female offspring. In particular, measurement of dendritic and neuronal cell morphological changes found the female offspring to be more susceptible to prenatal bystander stress than their male counterparts. Following *in utero* exposure to bystander stress, female offspring were affected in 13 of the 18 anatomical measures (dendritic branching, dendritic length, spine density and neuronal cell number) examined in the mPFC, OFC (Orbitofrontal Cortex) and hippocampus, while males were affected in 10. In the mPFC, female offspring showed an increase in all anatomical measures examined, suggesting an increase in number of excitatory synapses (Mychasiuk, Gibb, et al., 2011). On the contrary, the varying directional changes observed in these anatomical measures in the male offspring when considered collectively suggested that there was no change in the excitatory synapse number (Mychasiuk, Gibb, et al., 2011). Interestingly, while Mychasiuk et al. (2011) reported increased dendritic branching in both male and female offspring in the mPFC, Garrett & Wellman (2009) found a different outcome following another stress paradigm. In particular, they observed that chronic restraint stress shortened apical dendritic branch number and length in male rats, but caused an increase in the apical dendritic length of female rats (Garrett & Wellman, 2009).

With regards to sexual dimorphism in stress-related plasticity proteins, Lin et al. (2009) examined brain-derived neurotrophic factor (BDNF) and cyclic adenosine monophosphate response element–binding protein (CREB) levels in the PFC; importantly, these proteins are

implicated in neuronal plasticity in response to stress, and also play a role in mediating the effect of antidepressants. They found that the CREB and pCREB levels in the anterior cingulate and the dorsal PFC were reduced by chronic foot shock stress in male animals, but remained unchanged in female animals. An opposite effect was observed for BDNF, the levels were decreased in the prelimbic area in female rats, but not in male rats (Lin et al., 2009). Fumagalli et al. (2009) examined the expression and phosphorylation of AMPAR and NMDAR subunits, and calcium/calmodulin-dependent protein kinase II (αCaMKII) in male and female rats. Expression of the GluA1 subunit of the AMPAR, GluN2B subunit of the NMDAR, and αCaMKII remained unchanged in the PFC of both sexes following exposure to acute swim stress. However, in male, but not female rodents, there was a significant 22% increase in expression of the GluN2A subunit of the NMDAR induced by acute swim stress (Fumagalli et al., 2009). There was no significant sex difference in the effect of acute swim stress on phosphorylation of the noted subunits, but p-αCaMKII levels were significantly reduced by half in males, while females showed a significant decrease of about 20% (Fumagalli et al., 2009).

Some significant differences regarding plasticity- related proteins in male and female rodents have also been noted in the hippocampus. Fumagalli et al. (2009) reported a significant 24% increase in the total expression levels of GluA1 subunit in female rats, but not males. However, there were no noted sex differences in any of the other glutamate receptor subunits, or αCaMKII levels. As well, it was observed that acute swim stress increased the phosphorylation of pSer831-GluA1 by 30% in males, while the levels in females remained unchanged (Fumagalli et al., 2009).

Notably, there is a lack of literature specifically comparing male and female differences in plasticity proteins present at synaptic terminals, particularly those involved in signal transduction, such as receptors, transport proteins, and structural proteins. The existing literature that has examined changes imparted by platform stress, swim stress, or CORT on plasticity proteins involved in glutamatergic transmission has utilized only male Sprague-Dawley rats (Caudal et al., 2010; Cohen et al., 2011; Yuen et al., 2009). As well, there are no studies that have examined sex-related changes in these proteins induced by bystander stress.

The reasons/mechanisms behind sex differences following stress are not completely understood. However, one way in which sex differences may arise is through corticosterone (CORT) levels in the stress response. Corticosterone levels in the brain are largely determined by availability of corticosteroid-binding globulin (CBG) and, to a lesser extent, albumin (de Kloet et al., 1998). Due to the higher affinity of circulating corticosterone in the brain for CBG, it exhibits preferential binding to CBG over albumin (de Kloet et al., 1998). Interestingly, estrogen is a potent inducer of CBG synthesis; as a result, there is a reduced level of bioavailable CORT in female compared to male animals. (Galea et al., 1997; Minni et al., 2012; Siiteri et al., 1982). Research has shown that after undergoing one hour of restraint stress, male rats show a 7-10 fold rise in plasma corticosterone levels (Cook & Wellman, 2004; Watanabe et al., 1992) while female rats show just a 2-3 fold increase in CORT levels (Bowman et al., 2001; Luine, 2002).

2. Study Rationale

Although a few studies have examined the influence of physical stress, particularly swim stress, on the expression of some plasticity-related proteins, namely the GluA1 and GluA2 subunits of the AMPAR and GluN1, GluN2A and GluN2B subunits of the NMDAR (Fumagalli et al., 2009; Yuen et al., 2009), there are no studies that have examined the effect of primary, or secondary psychosocial stress on the expression of these iGLUR subunits in the PFC. As well, there is a paucity of studies that have examined the effect of platform stress (primary psychosocial stress) on the phosphorylation of the Ser831-GluA1 and Ser845-GluA1 subunits (Caudal et al., 2010, 2016; Qi et al., 2009). Further, sex differences resulting from changes seen in these plasticity-related proteins following primary, or secondary psychosocial stress are yet to be studied. As a result, the study aimed to examine the influence of platform stress and bystander stress on expression of GluA1 and GluA2 (AMPAR subunits), and GluN1, GluN2A and GluN2B (NMDAR subunits). In addition, effects of the stressors on phosphorylation of major serine residues of AMPAR subunits were explored. Finally, in each of the stress models, changes in protein expression and phosphorylation were compared across male and female rats.

In this study, I expected to answer the following questions:

1. Does platform stress (a primary stressor) induce changes in either the expression, or phosphorylation of key upstream plasticity-related proteins in the PFC?
2. Does bystander stress (a secondary stressor) induce changes in either the expression, or phosphorylation of key upstream plasticity-related proteins in the PFC?
3. Is there sexual dimorphism in the effect of psychosocial stressors on the identified plasticity-related proteins?

Based on what is currently reported in the literature, I hypothesized that platform stress and bystander stress will increase the expression of plasticity-related proteins, and phosphorylation levels of these proteins in the PFC will change - the direction of this change will depend on the receptor subunit residue being measured. Further, I hypothesized that the changes induced by platform stress will be significantly different from those caused through bystander stress. Lastly, I hypothesized that changes induced by psychosocial stressors will vary between male and female animals. Although, there is little research focused on sexual dimorphism in this area, based on previous studies on stress-related plasticity proteins such as CREB, p-αCaMKII, and NMDARs in the PFC, and given what is known about the higher bioavailability of CORT in males, I anticipated that the males would exhibit more pronounced changes in plasticity-related proteins.

3. Methods

3.1 Animals and Stress Procedure

All experiments involving male and female Sprague-Dawley rats followed procedures approved by the University of Waterloo animal care committee (please note the behavioural part of the study had already been completed, but is summarized below for context). All animals were maintained on a 12-hour reverse-light cycle in a temperature-regulated room (22ºC) and were allowed free access to standard rodent chow and water. A total of 48 Sprague-Dawley rats were used for this experiment, and were drawn from 6 litters. The rats in each litter were stratified by sex (4 male and 4 female rats per litter), and sub-groups were formed by randomly distributing the rats into stress and control groups. In a typical stress group, rats were randomly assigned to platform stress (PS) and bystander stress (ByS) while being housed together in the same cage. Similarly, among each control group, animals housed in the same cage were randomly allocated to either the platform control (PC), or bystander control (ByC) condition (Figure 1).

The PS rats were placed on an elevated Plexiform platform (92.5 cm tall, 18.5 x 18.5 cm) and exposed to bright light for 30 minutes twice daily for five days (9:00 to 9:30 and 15:00 to 15:30). As well, each time the PS rats were taken to undergo the stress procedure the PC rats were taken out and put in a cage in a separate room. At the end of each procedure, both PS and PC rats were placed back in their respective home cages with the ByS and ByC rats. Meanwhile, the ByS and ByC rats were left in their respective cages throughout the procedure. As previously mentioned, the idea behind the bystander stress paradigm is taken from previous studies that have shown rats empathize with cage mates in distress through modified vocalizations and behaviour (Mychasiuk, Gibb, et al., 2011; Mychasiuk, Schmold, et al., 2011). Hence, the ByS rats represent

our secondary psychosocial stress model and are assumed to experience stress indirectly by way of social interaction with the PS rats.

3.2 Brain Extraction and PFC Dissection

One hour after the last stress procedure on day 5, each rat was anaesthetized in a CO_2 induction chamber and then decapitated. Brains were extracted and immediately placed in chilled (4^oC) oxygen-rich (95% O_2: 5% CO_2) ACSF composed of: 124.0 mM NaCl, 3.0 mM KCl, 1.2 mM NaH_2PO_4/H_2O, 1.0 mM $MgSO_4/7H_2O$, 2.0 mM $CaCl_2/2H_2O$, 26 mM $NaHCO_3$, 10.0 mM D-Glucose, 10.0 mM HEPES buffer with a pH of 7.37-7.43 and osmolarity of 300-320 mOsm. The PFC was dissected as per standard procedure according to the atlas of Paxinos and Watson (1998). Precisely, after taking out the olfactory bulb, the PFC was dissected from approximately Bregma 4 mm to 2.7 mm. A mid-sagittal cut was made to separate the hemispheres, and the right hemisphere of the PFC was snap frozen in 1.5 mL Eppendorf tube with liquid nitrogen (N_2) and kept at -80°C for later use. The frozen PFC samples were used for this study.

3.3 Synaptoneurosome Preparation

Frozen samples were taken out of the -80°C freezer and left to thaw over ice to commence the synaptoneurosome preparation. Synaptoneurosomes (SN) were prepared from the PFC samples to enrich the synaptic terminals in order to study the proteins of interest.

Each snap-frozen PFC sample was placed directly into a 2 mL glass homogenising tube whilst on ice, and homogenized using 30 strokes with a total of 1000 μL of chilled, 1X modified Krebs buffer [containing 5 mL of 10X stock modified Krebs buffer (118.50 mM NaCl, 4.70 mM KCl, 1.18 mM $MgCl_2 \cdot 6H_2O$, 2.50 mM $CaCl_2 \cdot 2H_2O$, 1.18 mM KH_2PO_4), 24.90 mM $NaHCO_3$,

10.00 mM glucose at pH of 7.4] with a protease inhibitor cocktail (AEBSF, Aprotinin, Bestatin hydrochloride, E-64, Leupeptin hemisulfate salt, Pepstatin A) and sodium orthovanadate (Na_3VO_4) (stored at -20°C), and a Serine/Threonine phosphatase inhibitor (Sigma-Aldrich; stored at -4°C) added. After homogenization, samples were decanted into an Eppendorf tube and left on ice for ~10 min to permit gravity sedimentation of nuclear material. A small portion (100 µL) of the supernatant portion was aliquoted as whole homogenate (WH) to compare SNP to WH for quality control purposes. The two sets of 25 mm stainless-steel Millipore syringe filter units were assembled, and pre-wet using 1X Krebs buffer. Next, the remaining homogenate was passed through the filter units; first, a 25 mm filter unit holding three 100 µm pore size Millipore nylon net filters, and then another 25 mm filter unit holding one 5 µm pore size Millipore PVDF filter to obtain the filtrate. The filtrate was spun at 1000 x g for 15 min (at 4°C). The pellet (synaptoneurosome fraction) was re-suspended in 200 µL of 1X modified Krebs buffer with protease and phosphatase inhibitors, and Na_3VO_4 added. From the resuspended sample, a small fraction (20 µL) was used to measure the sample's protein concentration (section 3.4) and the the rest was aliquoted in 60 µL fractions into 0.5 mL microtubes. All samples were stored at -80°C.

3.4 Protein Assay

Following the completion of SNPs for all samples, a protein assay was completed to determine the protein concentration. The concentration of each sample was measured using a BioRad DC protein assay kit (BioRad Laboratories, Inc., CA, USA) and freshly prepared bovine serum albumin (BSA) standards of known concentration. Standards were prepared with different concentrations of BSA ranging from 1 to 7 µg/µL using Krebs buffer and varying volumes of the

BSA stock [5 mL of 1X Krebs buffer and 0.1 g of BSA]. A new 96 well microtiter plate was loaded in triplicate with 5 µL of each standard and lysate sample sequentially into the specified wells. Next, 25 µL of reagent A' [made from BioRad DC Protein Assay kit reagents: 20 µL of reagent S and 1 mL of reagent A] were 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 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. Thus, the concentration of protein in the samples was obtained by comparing the observed absorbance values against those measured in the BSA standards.

3.5 SDS-PAGE and Western blotting

3.5.1 Quality control blotting

Prior to commencing work on the main samples, some quality control steps were taken, which included immunoblotting using PSD-95 and GFAP antibodies on quality control blots that had practice samples (that is, these were samples not collected from the animals used for the main study). The importance of this step lies in ensuring the effectiveness of the SNP protocol in enriching synaptic terminals, therefore preventing waste of our primary samples. The PSD-95 protein, an important scaffolding component that regulates the localization of many receptors and channels at the synapse, is found at the post-synaptic density and as such is present in greater amounts in SNP compared to WH. On the other hand, Glial Fibrillary Acid Protein (GFAP) is an intermediate filament protein found in cytoskeleton and not concentrated at synaptic terminals; therefore, the protein is expected to be more evident in WH. Thus, immunoblotting for PSD-95

and GFAP provides positive and negative quality controls, respectively, to prove the effectiveness of the SNP protocol at enriching the synaptic region. Since the practice samples showed an enrichment of synaptic terminals (Figure 2a), the SNP was undertaken on the main study samples, and this quality control step was repeated on some randomly selected main samples (Figure 2b). Similarly, the results showed an enrichment of synaptic terminals, [illustrated after normalization to Ponceau by a higher optical density (OD) of PSD-95 in SNP compared to WH, and a far lower OD of GFAP in SNP than in WH].

3.5.2 Standard curve optimization blotting

In order the determine the optimal loading concentration for each sample, a standard optimization blot was prepared using the PSD-95 and GFAP antibodies. For the optimization blot, 5 µg, 10 µg, 15 µg, and 20 µg of PFC SNPs from one of our main samples was loaded sequentially into a 10% SDS gel, and immunoblotting was done (refer to figure 3). The 10 µg/µL was ascertained as the optimal loading concentration to be used for all samples since it produced a stable, clear, and unsaturated signal.

3.5.3 SDS-PAGE and Western blotting steps

The mixture of proteins in the samples was separated based on molecular weight with the use of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In each case, 10-well 10% SDS gels were freshly prepared, and the apparatus for electrophoresis was assembled. The PFC homogenate samples were removed from the -80°C freezer and left to thaw on ice. After the samples had thawed, the determined volume of each PFC sample and an equal volume of 2× sample buffer were added to a 0.5 mL Eppendorf tube. The mixture was vortexed for 1 s to 2 s,

centrifuged at 1000 × g for 30 s, and then put into a heating block at 95°C for 5 min. Next, the tubes were removed using forceps, vortexed for 1 s to 2 s and pulsed in the centrifuge.

Each group of animals comprised both male and female rats in the stress and control groups. For each of the groups, four gels were ran: two each for male rats and female rats. Protein samples (in order of platform control, platform stress, bystander control, bystander stress) and their duplicates were loaded into the 10% gels and electrophoresed at 200 V for about 1 h. On completion, the gels were removed from the apparatus and left to soak in a dish filled with chilled transfer buffer for 15 min. Subsequently, proteins on the gel were transferred unto pre-treated PVDF membranes using a sandwich assembly in chilled transfer buffer [composed of 25 mM Tris, 192 mM glycine, 20% (v/v) methanol] at 90 V for 120 min while refrigerated. To confirm whether the protein samples transferred properly, and as an internal control for loading variability, or transfer errors, PVDF membranes were incubated with Ponceau S stain for about 7 min, then de-stained by rinsing several times 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, each blot was rinsed in methanol for 1 min, left to dry, labelled, and kept at 4°C until used for immunoblotting.

3.6 Antibody probing steps

Prior to immunoblotting, the membranes were opened (using methanol 30 s, T1 water 2 min, and TBST 5 min), and incubated using gentle agitation with blocking buffer made of 5% BSA (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. Since there were two representative blots for each sex in every group, one membrane was incubated with a phosphorylation specific

Ser-831-GluA1 rabbit monoclonal antibody (*Cell Signaling Cat #75574S*) and the other with phosphoSer-845-GluA1 rabbit monoclonal primary antibody (*AbCam Cat #76321*) at concentrations of 1:1000 5% BSA/TBST at 4°C overnight. The next day, the primary antibody solutions were decanted, and 3 × 5 min washes were done using TBS-T. Following this, the membranes were incubated with mouse anti-rabbit IgG-HRP conjugated secondary antibody (*Santa-Cruz 1:5000*) for 1 h using gentle agitation. The secondary antibody solutions were then decanted, and 3 × 5 min washes were done using TBS-T. Next, blots were covered either with an enhanced chemiluminescence (ECL), *Immobilon Classico,* or ECL plus solution, *Immobilon Crescendo Western HRP Substrate* (Millipore) for 3-5 min. Blots were then positioned between a plastic wrap, and placed in the gel documentation system, *SNAPGENE*, to obtain densities of the protein bands for phosphoSer-831 GluA1 and phosphoSer-845 GluA1, by exposure at intervals from 30 s to 10 min, and the images were saved.

Following this, both membranes were stripped by incubating in 1X Re-Blot Plus stripping solution at room temperature for 25 min. Thereafter, the membranes were washed 3 × 5 min using TBS-T (this stripping protocol was validated to ensure that there was no carry-over of remnant signals by the application of secondary antibody alone on a test blot from which we had previously detected signals). Blocking buffer was reapplied to the membranes for 20 min before incubating both membranes with anti-total GluA1 antibodies (*Abcam Cat #183797, 1:500 5%NFDM/TBST*) at 4°C overnight. The corresponding secondary antibody was applied the next day, and the signals detected using the SNAPGENE software and saved.

A sequentially similar stripping and immunoblotting protocol was followed for all groups. Either Depending upon the blot being used, membranes were incubated with two of the following

antibodies: anti-total GluA2 rabbit monoclonal (*Cell Signaling Cat #13607S, 1:1000*), anti-total GluN1 rabbit monoclonal (*Millipore Cat #05432, 1:500*), anti-total GluN2A rabbit monoclonal (*Cell Signaling Cat #4205S, 1:1000*) and anti-total GluN2B rabbit monoclonal (*Cell Signaling Cat #4207S, 1:1000*) primary antibodies prepared in either 5% NFDM/TBST, or 5% BSA/TBST (as appropriate) at 4°C overnight. Mouse anti-rabbit IgG-HRP conjugated secondary antibody (*Santa-Cruz 1:5000*) was applied the next day, and the signals detected using the SNAPGENE software and saved. Densitometric analysis was done on all saved images using NIH ImageJ software.

3.7 Statistical analysis

All data extracted using the NIH Image J software were compiled in Microsoft Excel spreadsheets, and analysis was conducted using GraphPad Prism version 9 and SPSS version 25.

To analyze the effect of primary and secondary psychosocial stress on the expression, or phosphorylation of our proteins of interest, for all the groups, the optical density of the bands in each blot was measured and normalized to their respective Ponceau stain, or total GluA1 protein (in the case of the phosphorylated proteins). The duplicate on each blot was averaged and expressed as a percentage of the corresponding control group; that is, protein levels of the platform stress were expressed as a percentage of platform control, and bystander stress as a percentage of bystander control.

3.7.1 Outliers

To identify outliers, as well as understand the distribution and pattern of the data collected, exploratory data analysis was conducted on the pooled data stratified by stressor and sex for each of the proteins examined. Outliers in the dataset were excluded using Tukey’s method (box and

whiskers plot) in GraphPad Prism 9. Tukey's method is a non-parametric method of identifying outliers (that is, the analysis proceeds without the assumption of normally distributed data). In Tukey's method, the parameters used are the 25th percentile (lower quartile/Q1), the 75th percentile (upper quartile/Q3), and the interquartile range (IQR = Q3 – Q1) to construct the upper and lower fences. 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. Values that are not within the fences [Q1 – 1.5(IQR), Q3 + 1.5(IQR)] are considered outliers.

3.7.2 Testing for normality and homogeneity of variances

To analyze the data using parametric tests, certain assumptions need to be met. First, each sample group should be independent (a condition also required for non-parametric tests). For all groups, each sample was taken from a different rat brain and was prepared and tested separately, so the independence of each sample can be safely assumed.

The second assumption is that the data follow a normal distribution; this was tested using a combination of the Shapiro-Wilk test [appropriate for small sample sizes ($3 < N < 50$)] (D'Agostino et al., 1990) and Q-Q plots. Normality was further assessed by examining the skewness and kurtosis of the data collected for each protein of interest. If the data were normally distributed, the p value of the Shapiro-Wilk test would be insignificant ($p > .05$), and, on the Q-Q plot, points would tend to be close to a straight diagonal line. Ideally, skewness would rest between -1 and 1 (approximately symmetric to moderately skewed) and kurtosis (an indicator of height and sharpness of the central peak) would be close to 0.

The final assumption concerns homogeneity of variance, which is required because the Student's t-test compares the means between two groups, and, to do this, assumes that their

variances are equal. For all data sets that passed the normality test, this assumption was tested using the F-test (for equality of variance) with a p value < .05 indicating a significantly different variance between the two groups being compared. Where the results did not meet the assumptions of normality and/or homogeneity of variance, a non-parametric version of the corresponding parametric test was applied.

3.7.3 One-sample t-test/ Wilcoxon signed-rank test

For both sexes, in most groups of each plasticity-related protein of interest, the assumptions outlined in the previous sections were met, therefore the one sample t-test (a parametric test) was selected to conduct the first stage of the analysis. In the first stage, we aimed to compare the changes in the expression, or phosphorylation of these proteins caused by stress to the control group. Within each sex, data from the platform and bystander stress groups were expressed as a percentage of their respective controls [i.e., PS (% PC) and ByS (% ByC)] and the mean of these ratios was then compared to the theoretical control value of 100%. Results were considered statistically significant if $p < .05$. In cases where the data were not normally distributed, the Wilcoxon-signed-ranked test, the non-parametric version of the one-sample t-test, was employed.

3.7.4 Independent Student's t-test/ Mann-Whitney U test

To conduct the second and third stages of the analysis, an independent two-sample Student's t-test (two-tailed), or a Mann-Whitney U test (the non-parametric version of the Student's t-test) was performed.

The second stage of the analysis aimed to determine if there were any significant sex differences in the effect of either PS and/or ByS on the changes observed in the various protein

levels. The third stage of the analysis examined how the changes caused by PS (primary stress) differed from ByS (secondary stress), and was only conducted in cases where at least one of the stress groups was statistically different from its control group (first stage of the analysis). As well, in cases where the data were not normally distributed in at least one of the comparison groups, or equal variance was not ascertained, a Mann-Whitney U test was employed. Data are expressed as the mean ± standard error of mean (SEM).

3.7.5 Effect size

Effect size calculation is paramount in communicating the practical significance of scientific results. Cohen's d, one of the most widely used measures of effect size, was used. Cohen's d measures the standardized mean difference between two groups of independent observations (Lakens, 2013). Cohen's d was computed with an online calculator (Lenhard & Lenhard, 2016), and the reference values [small effect size ($d = 0.2$), moderate effect size ($d = 0.5$), and large effect size ($d = 0.8$)] were used for interpreting the biological importance of the standardized effect sizes (Lakens, 2013).

4. Results

4.1 SNP optimization results

Before commencing SNP on the main samples, practice PFC samples were used to optimize the protocol. Previously, our lab worked with hippocampal samples and used a 25 mm diameter filter holder for the first SNP filtration step and a 13 mm diameter filter for the second filtration step; however, this did not seem to work with the PFC samples, which were larger than the earlier hippocampal ones. As a result, the 13 mm filter holder was replaced with a 25 mm filter holder. Using the 25 mm filter holder provided more surface area and helped reduce how quickly the 5 µm pore PVDF filter became saturated, thereby decreasing the pressure created within the filter unit. The change in the filter unit holder was an important optimization step in the SNP because it determined the volume of filtrate that would be obtained.

Further, several modifications were made to the total volume of Krebs buffer used to homogenize the snap frozen samples and to re-suspend the SNP pellet. It was paramount to determine the optimal volume that ensured the samples were not too concentrated to pass through the filters (particularly the 5 µm pore PVDF filter, in which a lot of pressure can be generated), while also keeping the concentration of proteins in the samples in an ideal range of about 1-3 µg/µL. It was concluded that using a total volume of 1000 µL Krebs buffer to homogenize the samples, combined with using a 25 mm diameter filter unit worked well in overcoming any pressure during the filtration process. As well, resuspending the SNP pellet in 200 µL 1X Krebs buffer (with added protease and phosphatase inhibitors and Na_3VO_4) produced samples in the ideal protein concentration range. By going through this process, the SNP protocol outlined in the methods section was optimized for our main samples. Further, SDS-PAGE and Western blotting

was done on these practice samples as a quality control step to confirm that the SNP protocol was effective in enriching synaptic terminals (Figure 2a) prior to commencing work on our main samples.

4.2 Outliers

Following Tukey's method of identifying outliers, box and whisker plots were generated using GraphPad Prism (Figure 4). In each group, protein levels of platform stress were expressed as a percentage of platform control, and bystander stress as a percentage of bystander control. Data collected from all six groups for each protein of interest was also stratified by sex. In this study, two outliers were identified in the results from the male bystander stress group (pSer831-GluA1 and total GluN2A). Notably, we reviewed lab notes for technical reasons that might explain the outliers and found none. After taking into consideration our modest sample size for each sex (N = 6), and the possibility of every data point representing the variability that might occur in random sampling, we decided to retain all outliers (Yang & Berdine, 2016). In both cases noted above (pSer831-GluA1 and total GluN2A), retaining the outliers resulted in a non-normal distribution of the data, so non-parametric tests were used.

4.3 Normality and homogeneity of variance

All data sets were quantitatively assessed for normality using the Shapiro-Wilk test, and by graphical assessment using Q-Q plots. Further, skewness and kurtosis of the data sets were assessed. The results of the Shapiro-Wilk test for all *platform stress* data collected for the 6 plasticity-related proteins, showed that we could not reject the null hypothesis regarding normality in either male, or female rats. However, among the *bystander stress* data, we did reject the null

hypothesis for male pSer831-GluA1 ($p = .0037$), female pSer831-GluA1 ($p = .017$), male total GluA1 ($p =.035$) and male total GluN2A ($p = .0011$) (Tables 1-6).

Graphical assessment of Q-Q plots was done along with the Shapiro-Wilk test, and we would expect the points to lie close to a straight diagonal line if the data were normally distributed. According to the Q-Q plots (Figures 5-10) for each of the proteins categorized by stress type and sex, all data points were approximately aligned with a straight diagonal line, with the exception of male pSer831-GluA1 from the ByS group, female pSer831-GluA1 from the ByS group, male total GluA1 from the ByS group, and male total GluN2A from the ByS group (mainly due to the inclusion of outliers).

In addition, skewness and kurtosis were assessed (Tables 1-6). Skewness between -1 and 1, and kurtosis close to 0 were indicative of a normal distribution. The farther away the skewness values were from -1 and 1, the more indicative they were of a highly skewed distribution. Kurtosis clearly greater than 0, and less than 0 indicated a sharp peak and flat distribution, respectively. In general, the values from all data sets were within the ideal range, besides male pSer831-GluA1 ByS, female pSer831-GluA1 ByS, male total GluA1 ByS, and male total GluN2A ByS, which hovered between 4-5 for kurtosis, indicative of a relatively flat distribution, and 2 for skewness, indicating a left skewed distribution. The results from both statistical and graphical assessments were in general agreement, therefore the Student's t-test was applied to the normally distributed datasets, and the Wilcoxon-signed-ranked test (the non-parametric version of the one sample t-test) to those where the data were not normally distributed.

Homogeneity of variance between groups was tested using the F-test (for equality of variance). A significant p value ($p < .05$) indicates that the variance between the two groups was

unequal. For each protein, male and female rats were compared to determine if there were any sex differences resulting from exposure to either, or both stressors. As well, where there was a significant change in any of the plasticity-related proteins in at least one stress group compared to its control, PS was compared against ByS to determine if their impact was significantly different. For each comparison group (male/female, PS/ByS), the F-test showed no significant difference between the variances among the groups, except for the male and female pSer831-GluA1 ByS, male and female total GluA1 ByS, and male and female total GluN2A ByS (Tables 8-18). Hence, an independent two-sample Student's t-test was performed where equal variances between the comparison groups were confirmed, otherwise, a Mann-Whitney U test was performed.

4.4 Plasticity-related proteins

Platform stress and bystander stress does not significantly alter pSer831-GluA1 regardless of sex.

According to the immunoblot results, platform stress caused the phosphorylation levels of Ser831-GluA1 to increase slightly in male rats (% PC: 105 ± 17%) and decrease in female rats (% PC: 83 ± 15%). In the bystander stress group, there was an increase in pSer831-GluA1in both male (% ByC: 151 ± 37%) and female (% ByC: 117 ± 13%) rats compared to their respective controls (Figure 11). Regardless of these noticeable changes, there was no statistically significant effect of either PS, or ByS on pSer831-GluA1 in male ($t(5) = 0.34$, $p = .75$, $d = 0.14$ and $W = 15$, $p = .16$, $d = 0.56$, respectively) and female ($t(5) = 1.13$, $p = .31$, $d = 0.46$ and $W = 13$, $p = .22$, $d = 0.54$, respectively) rats (Table 7). In addition, there was no significant effect of sex on changes caused by either PS ($t(10) = 1.01$, $p = .34$, $d = 0.58$), or ByS ($U = 14$, $p = .59$, $d = 0.50$) on pSer831-GluA1 (Table 8).

Bystander stress substantially alters pSer845-GluA1 in a sex-specific manner.

The results of pSer845-GluA1, the other of the two major GluA1 subunit residues, showed both stress and sex-specific changes. Phosphorylation levels of Ser845-GluA1 barely changed following PS in male rats (% PC: 102 ± 17%), but increased in female rats (% PC: 125 ± 17%). Conversely, ByS caused an increase in pSer845-GluA1 in male rats (% ByC: 134.3 ± 20%) and a decrease in female rats (% ByC: 82.79 ± 12%) (Figure 12). There was no statistical, or major practical significant difference in the effect of PS on males and female rats ($t(5) = 0.11$, $p = .92$, $d = 0.05$ and $t(5) = 1.48$, $p = .20$, $d = 0.61$, respectively) compared to their respective control groups. However, although bystander stress did not cause a statistically significant effect on male and female rat phosphorylation levels ($t(5) = 1.71$, $p = .15$, $d = 0.70$ and $t(5) = 1.41$, $p = .22$, $d = 0.58$, respectively), Cohen's d showed a considerably large effect size ($d = 0.70$) between the male ByS and ByC rats (Table 9). Upon examining the influence of sex on the effect of PS, no significant difference was observed ($t(10) = 0.99$, $p = .34$, $d = 0.57$). Notably, bystander stress caused a significant change in pSer845-GluA1 that varied between sex, with a substantially large Cohen's d value ($t(10) = 2.19$, $p = .05$, $d = 1.27$) (Table 10).

Bystander stress increases total GluA1 expression in male and female rats.

In male and female rats, PS caused an increase in total GluA1 expression (% PC: 123 ± 36% and 110 ± 15%, respectively), which was not significant ($t(5) = 0.65$, $p = .55$, $d = 0.26$ and $t(5) = 0.65$, $p = .54$, $d = 0.27$, respectively). In contrast, ByS increased GluA1 expression in male (% ByC: 177 ± 35%) and female rats (% ByC: 130 ± 16%), with significant results (Figure 13). The effect of ByS on total GluA1 expression in male rats resulted in both statistical and practical significance ($W = 21$, $p = .03$, $d = 0.91$). Although in female rats, there seemed to be no statistically

significant effect of ByS ($W = 15$, $p = .16$), the large effect size ($d = 0.77$) shows that total GluA1 expression differed between female ByS and ByC rats (Table 11). In addition, there was no significant difference in the effect of PS ($t(10) = 0.34$, $p = .74$, $d = 0.20$) and ByS ($U = 11$, $p = .31$, $d = 0.70$) as a function of sex (Table 12). However, Cohen's d suggested that ByS caused a marginally large difference ($d = 0.70$) in total GluA1 expression between male and female rats. Since ByS resulted in a statistically significant difference in male rats, the effect of ByS was compared to PS. The results of the comparison showed no significant difference ($U = 10$, $p = .24$; $d = 0.63$), indicating that, with regards to total GluA1 expression, the effect of ByS did not differ from PS in male rats.

Platform stress and bystander stress does not significantly alter total GluA2 expression regardless of sex.

Among the PS rats, total GluA2 expression increased slightly in male rats (% PC: 113 ± 15%) and remained unchanged in female rats (% PC: 100 ± 9%). In the bystander stress group, there was an increase in total GluA2 in both male (% ByC: 111 ± 13%) and female (% ByC: 126 ± 21%) rats compared to their respective controls (Figure 14). There was no statistically significant effect of PS, or ByS on total GluA2 expression in either male ($t(5) = 0.86$, $p = .43$, $d = 0.35$ and $t(5) = 0.80$, $p = .46$, $d = 0.33$, respectively), or female ($t(5) = 0.011$, $p = .99$, $d = 0.01$ and $t(5) = 1.24$, $p = .27$, $d = 0.51$, respectively) rats compared to their respective control groups (Table 13). As well, there was no significant effect of sex on changes caused by either PS ($t(10) = 0.74$, $p = .48$, $d = 0.43$), or ByS ($t(10) = 0.61$, $p = .56$, $d = 0.37$) on total GluA2 (Table 14).

Bystander stress significantly increases total GluN2A expression in male rats while the effect of platform stress on total GluN2A varied largely between male and female rats.

In male rats, PS (% PC: 130 ± 18%) and ByS (% ByC: 187 ± 14%) caused an increase in total GluN2A expression. Conversely, in female rats, total GluN2A decreased after PS (% PC: 89 ± 61%), but increased following ByS (% ByC: 118 ± 18%) (Figure 15). There was no significant effect of PS, or ByS ($t(5) = 0.82$ $p = .45$, $d = 0.33$ and $W = 7$, $p = .56$, $d = 0.40$, respectively) on total GluN2A expression in female rats. However, male rats showed an effect of ByS on total GluN2A expression that was significantly different from ByC ($W = 21$, $p = .03$, $d = 0.58$), while the PS group did not significantly differ from PC ($t(5) = 1.65$, $p = .16$, $d = 0.67$) (Table 15). Interestingly, although the effect of PS was not different to a statistically significant degree between sexes ($t(10) = 1.81$, $p = .10$), there was a conspicuously large effect size ($d = 1.04$), indicating that PS influenced total GluN2A expression differently in male and female rats. In contrast, the effect of ByS did not seem to be significantly affected by sex ($U = 12$, $p = .39$, $d = 0.63$) (Table 16). Since, in male rats, ByS had a significant effect on total GluN2A, the effect of ByS was compared to PS. Further analysis showed that (in male rats) the effect of ByS on total GluN2B expression did not significantly differ from PS ($U = 16$, $p = .82$, $d = 0.52$).

Platform stress and bystander stress does not significantly alter total GluN2B expression regardless of sex.

The expression of total GluN2B increased in male rats (% PC: 124 ± 22%) and female rats (% PC: 119 ± 17%) after platform stress. Similarly, following bystander stress, total GluN2B expression increased in both male (% ByC: 203 ± 70%) and female (% ByC: 121 ± 20%) rats compared to their respective controls (Figure 16). There was no statistically significant effect of

PS on total GluN2B expression on either male ($t(5) = 1.08$, $p = .33$, $d = 0.44$), or female ($t(5) = 1.09$, $p = .33$, $d = 0.44$) rats. Additionally, ByS did not have any significant effect on total GluN2B expression in male ($W = 15$, $p = .20$, $d = 0.60$) and female ($W = 9$, $p = .34$, $d = 0.43$) rats compared to their respective control groups (Table 17). Further, there was no significant influence of sex on changes caused by either PS ($t(10) = 0.18$, $p = .86$, $d = 0.10$), or ByS ($U = 14$, $p = .59$, $d = 0.65$) on total GluN2B expression (Table 18).

5. Discussion

The glutamate receptors mediate the majority of excitatory neurotransmission in the mammalian brain, therefore, in occasions of stress they are likely to play an essential role in facilitating neuronal communications (Caudal et al., 2016; Popoli et al., 2012). In particular, the ionotropic glutamate receptors play a crucial role since they mediate fast excitatory signals leading to synaptic transmission within milliseconds (Goodwani et al., 2017). As well, during stressful episodes, many proteins respond to changes in the cellular environment by interacting with each other; one of the mechanisms governing such interactions is phosphorylation (Pavlov & Mielke, 2012). Importantly, studies have shown that the phosphorylation of a receptor subunit protein can alter signal transduction properties of the receptor; for example, pSer831-GluA1 may increase ion conductance through AMPAR channels, while pSer845-GluA1 facilitates the insertion of AMPAR at extra-synaptic sites. (Caudal et al., 2010; Wang et al., 2005). In addition, the expression of key plasticity-related proteins, such as those composing the glutamate receptors, at synaptic terminals determines several changes that occur downstream in the signal transduction process (Yuen et al., 2009). For the noted reasons, examining changes in the expression and phosphorylation of glutamate receptor proteins at central synapses after stress was the focus of the current project.

With the knowledge that AMPARs and NMDARs play a critical role in neuroplasticity (that is, they are important in learning and memory), and that these receptors have also been implicated in many neuropsychiatric conditions linked to psychosocial stress (Blanke & VanDongen, 2009; Henley et al., 2011), this study investigated how the experience of primary and secondary psychosocial stressors could change the phosphorylation and expression of several key plasticity-related proteins, and whether these changes varies by sex.

5.1 Impact of primary and secondary psychosocial stressors on plasticity-related proteins

In recent times, much of the stress that has plagued humans has been more psychosocial than physical. Psychosocial stressors, such as loss of a loved one, poor grades, or family conflicts, have been linked with anxiety, depression, schizophrenia, and other neuropsychiatric illnesses (Fuchs et al., 2001). In this study, primary psychosocial stress was modelled in rats using elevated platform stress, while secondary psychosocial stress was modelled using the bystander stress model as described by Mychasiuk, et al. (2011a, 2011b). In the primary psychosocial stress model, rats experienced the stressor directly, whereas with the secondary psychosocial stress model (bystander stress) the rats experienced stress by way of social interactions with a rat that had been directly stressed (i.e., with platform stress).

In this study, we investigated the effects of both types of psychosocial stress on key plasticity-related proteins from the family of glutamate receptors. The results demonstrated that, compared to platform stress, bystander stress caused a greater effect upon the phosphorylation and expression of many of these proteins. In particular, bystander stress caused a statistically significant increase in the expression of total GluA1 (77%), and total GluN2A in male rats (88%), but not in their female counterparts. Conversely, platform stress caused no statistically significant change in any of the proteins examined in either male, or female rats.

To give a more encompassing view of the results, Cohen's d effect sizes were computed to determine the practical significance of the findings. The effect sizes for bystander stress gave insights slightly different from the p-values, for the changes in AMPAR and NMDAR subunit proteins were moderate in all cases except pSer845-GluA1 ($d = 0.7$) in male rats and total GluA1 expression in male and female rats ($d = 0.91$, $d = 0.77$, respectively). Notably, the effect of

bystander stress on total GluA1 expression in female rats, which was statistically insignificant, crossed the threshold for practical significance (d = 0.77). In addition, although the results show a significant effect of bystander stress on GluN2A in male rats, the Cohen's d effect size was moderate (d = 0.58). On the other hand, platform stress only resulted in small and medium sized effects in the examined proteins, with the largest effect size (d = 0.67) noted when GluN2A expression levels in PS were compared to PC male rats.

Although we assumed that both PS and ByS would cause an increase in the expression of the plasticity-related proteins we chose to study, our results show that most changes were observed only with bystander stress. As well, we predicted a change (the direction would depend upon which residue is studied) in the phosphorylation of receptor protein subunit residues. In general, we assumed that the change caused by PS would be significantly different from ByS. However, once again, we observed that bystander stress seemed to cause the greatest degree of change.

The results suggest that the impact of stress from environmental interactions may alter these plasticity-related proteins more than and/or differently from what we envisaged. Interestingly, Mychasiuk and colleagues (2011) reported that the prenatal bystander stress effects they observed in the frontal cortex were different from the effects produced by post-natal stress stated in earlier reports. For example, Mychasiuk, Gibb, et al. (2011) found an increase in spine density in the mPFC of offspring born to bystander stress mothers, while Radley et al. (2008) reported a reduction in spine density in the medial PFC of rats exposed to repeated postnatal stress. Although, the comparison here might be between prenatal bystander stress and postnatal stress, it highlights how different stress types might exert their effects, and hence, we cannot assume that each stressor will cause the same sort of outcome.

In the existing literature, there has been a focus on how direct, physical stress affects proteins, with no studies having examined indirect psychosocial stress effects; as a result, comparing our findings to previous work is challenging. Considering that, intuitively, we would expect the impact of a direct stressor to be significantly greater compared to an indirect/secondary stressor, it is unclear why we observed the reverse to be the case. One possible explanation may be that our platform control rats were not the most ideal comparison group for the PS animals because the short bouts of social isolation and environmental change experienced when the rats were taken to a separate room during the platform stress procedure might have caused changes similar to what was seen in the platform stressed rats, thus reducing the degree of any possible differences.

5.2 Psychosocial stress and AMPAR phosphorylation

Serine 831 and Ser845 residues are the two major phosphorylation sites on the GluA1 subunit and have been the most extensively studied (Wang et al., 2005). Each residue plays a unique role in enhancing synaptic transmission and are thereby involved in mediating plasticity. Phosphorylation of Ser831-GluA1 increases ion conductance, while phosphorylation of Ser845-GluA1 facilitates insertion of AMPARs at extra-synaptic sites and their channel opening probabilities (Caudal et al., 2010; Wang et al., 2005).

Based on previous experimental findings, we anticipated that platform and bystander stress would cause an increase in the phosphorylation of Ser831-GluA1 and Ser845-GluA1 subunit residues. Our results indicate that platform stress does not significantly alter pSer831-GluA1, or pSer845-GluA1 in male and female rats. In addition, the present study shows that bystander stress caused a notable increase (34%) in pSer845-GluA1 in male rats (with a marginally large effect

size of d = 0.7), although the results were statistically insignificant. Interestingly, in female rats, bystander stress caused opposing changes in pSer831-GluA1 (17% increase) and pSer845-GluA1 (17% decrease); these changes were statistically insignificant, but displayed moderate effect sizes (d = 0.54 and d = 0.58, respectively).

With regards to pSer831-GluA1, the results of this study align with some previous work (Bonini et al., 2016; Fumagalli et al., 2009; Qi et al., 2009), but stand in contrast to the findings from Caffino et al. (2015) and Caudal et al. (2010) where a 50% increase and a 38% decrease, respectively, were reported. Further, the results of pSer845-GluA1 following PS in this study align with previous reports on acute stress effects where the findings were statistically insignificant (Bonini et al., 2016; Caudal et al., 2010). Bonini et al. (2016) reported no changes in pSer845-GluA1 in rats sacrificed immediately after acute footshock stress, while Caudal et al. (2010) reported that PS caused about a 10% increase in pSer845-GluA1. However, the large effect size seen in the male bystander stress group may infer that indirect stress increases pSer845-GluA1 levels, although the changes were statistically insignificant. Phosphorylation at Ser845-GluA1 has been reported to increase the likelihood that the channels will open, which would mediate synaptic currents, therefore, the pSer845-GluA1 increase induced by bystander stress predicts an increase in AMPA receptor currents (Bonini et al., 2016).

One possible reason for the variability observed between our results and previous work, particularly with pSer831-GluA1, could be attributed to type, duration, and intensity of stress. For example, Caudal et al. (2010) and Qi et al. (2009) exposed rats to acute platform stress (single 30 min exposure), while Caffino et al. (2015) and Fumagalli et al. (2009) utilized the acute swim stress procedure (single 5 min exposure). In our study however, the platform stress protocol we

employed might be best described as "sub-chronic" since the rats were exposed to stress for 5 days, clearly surpassing the upper bound for acute stress and well distant from what would be typically described as chronic stress. Our observations might therefore make a case for changes that might be observed in slightly prolonged, but not chronic, stress experiences.

Another consideration to keep in mind concerning the variability between our results and earlier reports is the time the animals were sacrificed. Since phosphorylation is reversible, observations may change in a time-dependent fashion. As suspected, various investigators have sacrificed experimental animals at different time points, ranging from almost immediately (Caudal et al., 2010) after a stressor, to 15 min after (Caffino et al., 2015; Fumagalli et al., 2009), to an hour after the stress procedure. For example, Qi et al. (2009) measured biochemical alterations in phosphorylation induced by platform stress over a time course, and reported about a 30% reduction in pSer831-GluA1 and pSer845-GluA1 levels in rats sacrificed 140 min compared to 60 min, and 60 min compared to 30 min following the stress procedure, respectively, however, these changes were not statistically significant. Similarly, Bonini and colleagues (2016) reported about a 20% decrease in pSer831-GluA1 and pSer845-GluA1 in the post-synaptic membranes when they compared the results of rats sacrificed immediately after acute footshock stress to those sacrificed 2 h after; these changes were also not statistically significant. As expected, from the reports of Bonini et al. (2016) and Qi et al. (2009), it is difficult to delineate the exact time point at which we might begin to observe a decline, but both reports certainly offer some useful information.

5.3 Psychosocial stress and AMPAR and NMDAR expression

In addition to phosphorylation of major GluA1 subunit residues, we also assessed the effect of primary and secondary psychosocial stress on AMPAR subunit expression. In this study,

platform stress slightly increased (10%) total GluA1 expression, but did not cause any obvious changes in total GluA2 expression in female rats. Among PS male rats, there was a 23% and 13% increase in total GluA1 and GluA2, respectively, however, these changes were not statistically significant and displayed small to moderate effect sizes. Interestingly, our results show that bystander stress may have different magnitude, but similar direction of effect, across AMPAR subunits in male and female rats. Amongst the bystander stress group, we observed 77% and 30% increases in total GluA1 expression in male and female rats, respectively. Only the result of the male group was statistically significant ($p = .03$) and displayed a large effect size ($d = 0.91$). In the female bystander stress group, a collective consideration of the p value and Cohen's d effect size best captures the pattern of the results. In particular, the large effect size ($d = 0.77$) between the female ByS and ByC groups is noteworthy, and indicates that a larger sample size may have allowed us to observe statistically significant results (Sullivan & Feinn, 2012).

To examine whether platform and/or bystander stress induces changes in the NMDAR subunits, we investigated the expression of total GluN2A and total GluN2B subunits, which are also known to play a critical role in synaptic plasticity. The general pattern of the results showed that PS and ByS led to an increase in total GluN2A and total GluN2B in both male and female rats, except for the effect of PS on GluN2A in female rats. Notably, PS caused total GluN2A expression to increase in male rats (30%), and decrease in female rats (11%), although these changes were not statistically significant and showed moderate ($d = 0.67$) to small ($d = 0.33$) effect sizes, respectively. Conversely, an increase in total GluN2B expression in both male (24%) and female (19%) rats was observed, although both were statistically insignificant and with only moderate effect sizes ($d = 0.44$, in both cases). Interestingly, bystander stress in male animals

appeared to cause a conspicuous 87% increase in total GluN2A and a two-fold increase in GluN2B, which was clearly different from female rats that showed an 18% and 21% increase in total GluN2A and GluN2B, respectively. Further, although among the male rats, the effect of ByS on total GluN2B was statistically insignificant and the effect size was moderate (d = 0.6), changes in total GluN2A were found to be statistically significant (p = .03) with a moderate effect size (d = 0.58).

We had hypothesized that both PS and ByS would cause an increase in expression of the AMPAR and NMDAR subunit proteins. According to the data, other than the PS female rats that showed no change in total GluA2 and a decrease in GluN2A expression, the trend in the results seem to align with our assumptions, but the difference was not statistically significant in all the groups. Notably, compared to PS, ByS caused more statistically and practically significant effects on AMPAR and NMDAR subunit expression, contrary to our expectations. Comparatively, the current study aligns with previous studies (Bonini et al., 2016; Fumagalli et al., 2009) that have consistently reported a significant increase (50% and 22% respectively) in total GluN2A expression in male rats exposed to acute stress, independent of a change in the other NMDAR subunits. Thus, we predict that there might be an increased sensitivity of total GluN2A to deviations away from homeostasis in male compared to female rats. Further, Fumagalli et al. (2009) reported that female rats showed no change in GluN2A, while an increase in total GluN2A expression was observed in male rats; similarly, in our results opposite directions of effect were noted in male and female rats after PS, which reflects how differently sex might influence total GluN2A expression.

The results of this study are quite insightful when contrasted against studies that have reported no change in the total expression levels of the AMPAR and NMDAR subunits after *acute stress* (Fumagalli et al., 2009; Yuen et al., 2009), but an increase in surface expression of AMPARs and NMDARs, which the authors suggested was responsible for the increase in glutamatergic signal transmission observed (Yuen et al., 2009). Collectively, it seems that while acute stress may mediate glutamatergic signal transmission by AMPAR and NMDAR surface trafficking, the current study suggests that somewhere within the spectrum of stress (ranging from acute to chronic, with varying intensities), we might begin to observe an increase in glutamatergic signal transmission backed by an increase total AMPAR and NMDAR expression. Further, although the general trend observed was an increase in AMPAR and NMDAR expression for PS and ByS, most of these changes were insignificant except for the ByS effect on GluA1 (for both male and female rats) and GluN2A (for male rats). Consequently, we hypothesized that the intensity of our stressors might not have been sufficient to observe an all-round significance.

Taken together, the data in this study suggest that following primary and secondary psychosocial stress, there tends to be increased expression of AMPAR and NMDAR subunits at synaptic sites. As it is known, ionotropic receptors mediate fast excitatory signal transmission, and, in particular, the AMPAR has been shown to initiate this process by allowing the membrane to depolarize to a threshold capable of displacing the Mg^{2+} blockade within the NMDAR and allowing for its participation in signal transmission. Therefore, it is not surprising that the present study reports mainly an increase in NMDAR subunits, alongside an increase in AMPAR expression after both types of stressful experiences.

5.4 Sex differences in the effect of stress

Clinically, stress-related disorders have been reported to be influenced by sex (Pinn, 2005), and this has been supported by various studies using animal model to examine sexual dimorphism in behavioral (Baran et al., 2009), morphological (Mychasiuk, Gibb, et al., 2011), and molecular changes (Lin et al., 2009). Studies that have examined the PFC for sex differences in plasticity-related proteins caused by stress are limited (Fumagalli et al., 2009; Lin et al., 2009), and, as far as we know, only one focused on the ionotropic glutamate receptors (Fumagalli et al., 2009). Further, none of these studies assessed the effect of primary, or secondary psychosocial stress, nor any type of secondary stress. Consequently, comparative analysis of our findings to the existing literature is limited.

In this study, one of our objectives was to determine if there are sex differences in the effect of primary and secondary psychosocial stress on certain key plasticity-related proteins. According to our results, in male rats, there was a general trend showing an increase in all the plasticity-related proteins studied (although only some of them were notable differences), while female rats showed less of an increase in many of the studied proteins and a decrease in a few others (pSer831-GluA1 and Total GluN2A in the PS group and pSer845-GluA1 in the ByS group).

An independent student's t-test, or its non-parametric equivalent (Mann Whitney U test) was used to explore for sex differences in the phosphorylation and/or expression of AMPAR and NMDAR subunits following psychosocial stress, and the Cohen's d effect size was measured. The results showed that amongst the bystander stress group, there was a significant difference between male and female rats in Ser845-GluA1 phosphorylation (mean difference = 51%) and total GluA1 expression (mean difference = 47%). In addition, PS caused a significant difference in total

GluN2A expression between the sexes (mean difference = 41%). However, statistically insignificant sex differences and small to medium effect sizes were observed in pSer831-GluA1, total GluA2 and GluN2B (PS and ByS), pSer845-GluA1 and total GluA1 (PS), and total GluN2A (ByS). Notably, an assessment of the between sex differences observed in pSer845-GluA1 and total GluN2A, where substantially large effect sizes were noted, revealed an opposite direction of effect in male and female rats. The ByS-related effect on pSer45-GluA1 was increased in male (34%) and decreased in female (17%) rats. Similarly, the PS-related effect on total GluN2A was increased in male rats (30%) and decreased in female rats (11%). On the other hand, the marginally large (d = 0.7) ByS-related effect size observed in total GluA1 was due to the higher rise in male (77%) compared to female (30%) rats.

Taken together, the effects of psychosocial stress were more pronounced in male than female rats, since the male rats showed statistical significance and larger effect sizes in many of the changes observed. Our results support the findings of Fumagalli et al. (2009) and Lin et al. (2009) that reported a higher susceptibility in male rats to stress-related changes in glutamate receptor subunits, and CREB levels respectively. Fumagalli et al. (2009) reported a significant 22% increase in expression of the GluN2A subunit of the NMDAR induced by acute swim stress in male rats, but not female rats. As well, p-αCaMKII levels were significantly reduced by half in males, while females showed a significant decrease of about 20% (Fumagalli et al., 2009). Lin et al. (2009) showed that pCREB levels in the anterior cingulate and the dorsal PFC were reduced by chronic foot shock stress in male rats, but remained unchanged in female rats.

Given that existing studies have reported females to be more prone to stress-related disorders, the results of the current study might be quite surprising, but contribute to a seemingly

growing body of evidence that males are more susceptible to stress effects on plasticity-related proteins. Moreso, the data from this study suggest that bystander stress may cause more pronounced sex-differences in these proteins. One possible explanation for the sex differences observed is the higher estrogen level in females; estrogen is a potent inducer of corticosteroid-binding globulin (CBG) synthesis, which is the preferential binding protein for CORT, hence reducing the free CORT available to exert effects in the neural system in female compared to male rats (Galea et al., 1997). Research has shown that after undergoing one hour of restraint stress, male rats show a 7-10 fold rise in plasma corticosterone levels (Cook & Wellman, 2004; Watanabe et al., 1992) while female rats show just a 2-3 fold increase in CORT levels (Bowman et al., 2001; Luine, 2002). Therefore, the downstream effects of stress (mediated by CORT) on glutamate receptors may be more pronounced in males owing to the higher bioavailable CORT.

6. Future Directions and Conclusions

In the current study, the general pattern revealed was an increase in the phosphorylation and/or expression of most plasticity-related proteins examined, following both primary and secondary psychosocial stress, with only a few being statistically, or practically significant (i.e., having a large effect size). In particular, secondary psychosocial stress seemed to affect AMPAR and NMDAR subunit proteins more than primary psychosocial stress. Although, various studies have shown that the effects of stress can differ depending upon its type, intensity, and duration, it is still a bit unclear why the effects of indirect, ByS would have outweighed direct, PS. In this study, PS was compared to PC, and ByS to ByC; these control groups could arguably not be the most ideal comparison because of the short bouts of social isolation (faced by both control groups) and the environmental change faced by the PC group (when they were moved away from their home cage while their cagemate underwent PS). Thus, further study on stress-related effects on protein expression (particularly, from PS) utilizing a cage control, or group-housed rats may be necessary (this new comparison group would not be moved, or undergo any social isolation). Moreso, further studies on the effects of primary and secondary psychosocial stressors may aid in unravelling possible mechanisms behind the findings of the current study.

In addition, subsequent studies should take into consideration the time of sacrifice for the rats and the sample preparation techniques used. For example, previous literature relevant to this study that reported statistically significant findings mainly used experimental animals sacrificed immediately, or 15 min after the stress procedure to measure phosphorylation. However, the present study utilized rats sacrificed an hour after the stress procedure, and found no statistically significant changes in the phosphorylation of the proteins assessed. Therefore, we predict that

studies completed with experimental animals sacrificed immediately to 15 min after the stress procedure may capture any possible time-dependent events, such as phosphorylation, that might occur. As well, unlike the current study, previous studies have mainly used whole cell homogenates and crude synaptosomal fractions, which might not clearly reveal changes at the synaptic terminals, hence, future studies should investigate these effects using the SNP technique.

Further, it appears that psychosocial stress may cause some sexually dimorphic changes, as opposing directional changes (not statistically significant, but with moderate to large effect sizes) were observed in the phosphorylated AMPAR subunits, and GluN2A expression in both the PS and ByS group. The possibility that male rats show greater susceptibility to stress-related changes in these AMPAR and NMDAR subunit proteins compared to female rats, as shown through both our results and previous work by others, should be further investigated. Currently, the existing studies in this area of research have primarily used male rats. In conducting further studies, using both sexes, and measuring basal and stress-induced CORT levels might reflect the relationship of CORT levels to the stressor applied in each sex, which may elucidate possible mechanisms underlying any sexual dimorphic changes.

7. Tables and figures

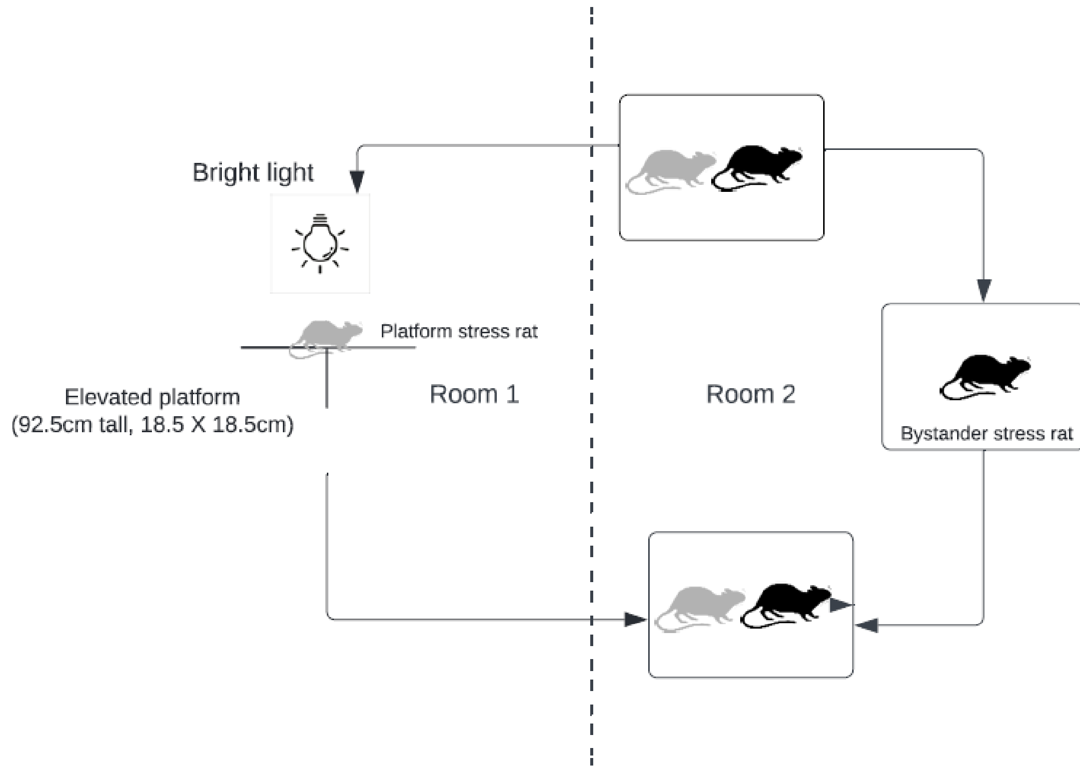


Figure 1. Schematic illustration of the Platform stress and Bystander stress protocols.

A PC rat was taken out of its home cage and put in a separate room each time the PS rat underwent the stress protocol (not shown in the image). On completion of PS, the PS rat was returned to its home cage (which housed the ByS rat) and the PC rat was placed back in its home cage (which housed the ByC rat).

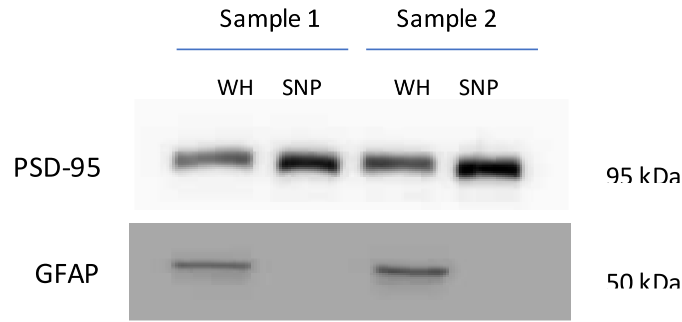


Figure 2a. Immunoblot results of SNP quality control step using practice samples.

Practice samples were used to confirm the procedure enriched synaptic terminals. The blots show PSD-95 (95 kDa) and GFAP (50 kDa) expression in whole homogenate (WH) and synaptoneurosome preparations (SNP). Given that the amount of PSD-95 was greater in the SNP, but the level of GFAP was lower, the results show that synaptic enrichment was achieved.

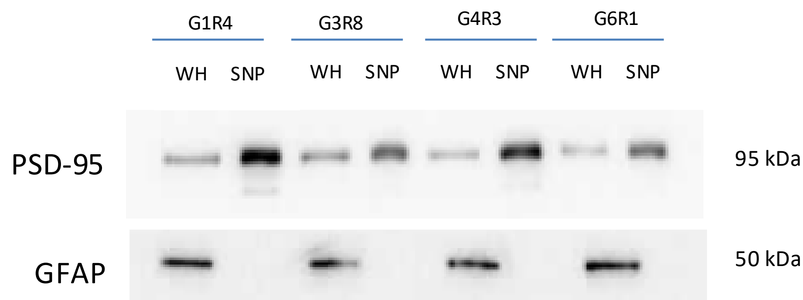


Figure 2b. Immunoblot results of the SNP quality control step on main samples.

Following validation of the SNP protocol using practice samples, SNP was carried out on the main samples. A quality control step was repeated on randomly selected main samples across the groups to ensure effectiveness of the SNP protocol. The blots show PSD-95 (95 kDa) and GFAP (50 kDa) expression in whole homogenate (WH) and synaptoneurosome preparations (SNP). The relative changes in both PSD-95 and GFAP show that synaptic enrichment was achieved.

G1R4, Group 1 rat 4; G3R8, Group 3 rat 8; G4R3, Group 4 rat 3; G6R1, Group 6 rat 1

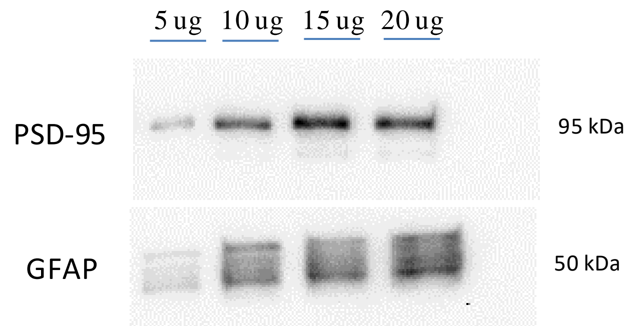


Figure 3. Standard curve optimization blot using PSD-95 and GFAP.
For the optimization blot, 5 µg, 10 µg, 15 µg, and 20 µg of PFC SNPs from one of our main samples was loaded sequentially into a 10% SDS gel, and immunoblotting was done using PSD-95 and GFAP antibodies.

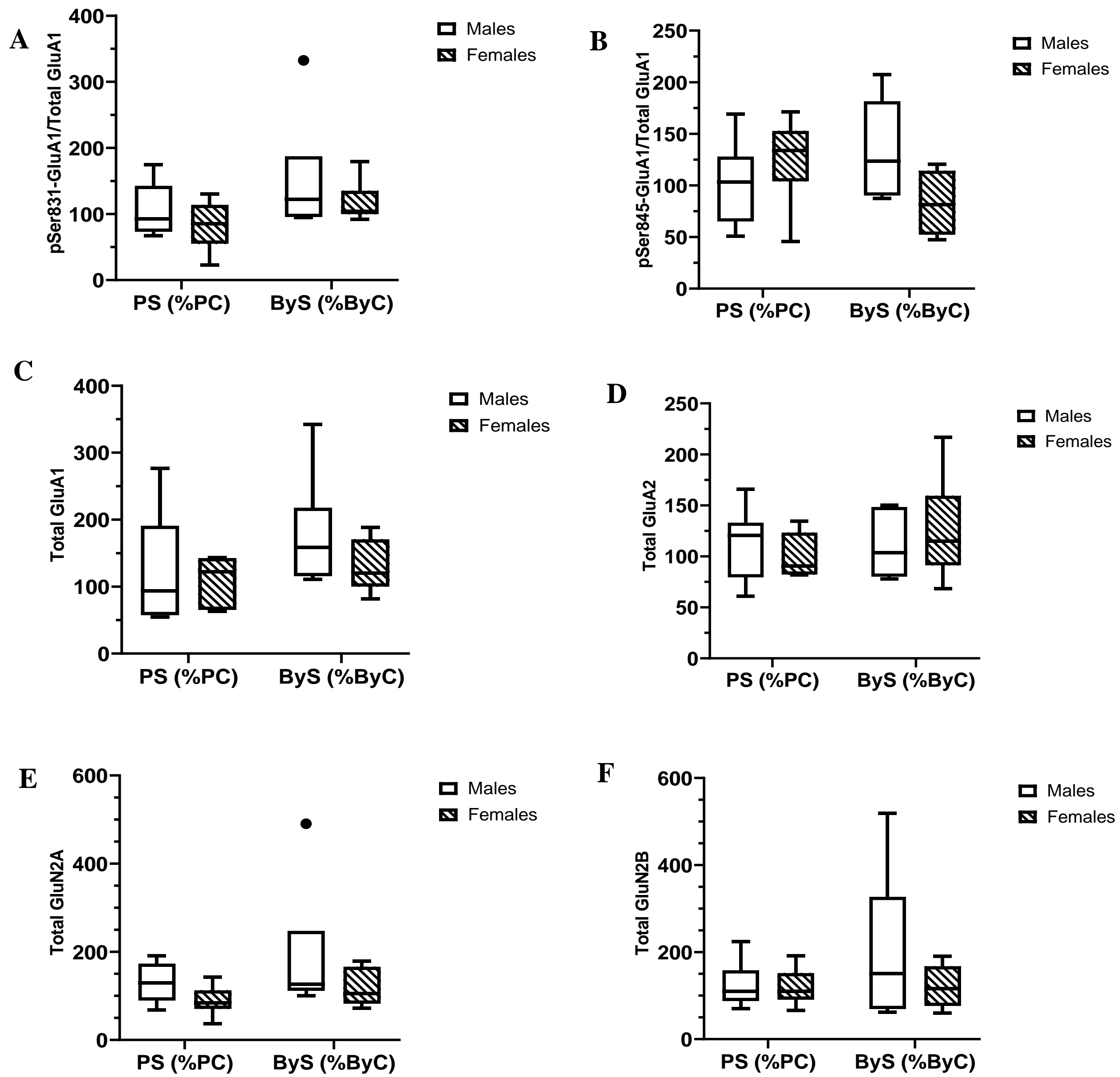
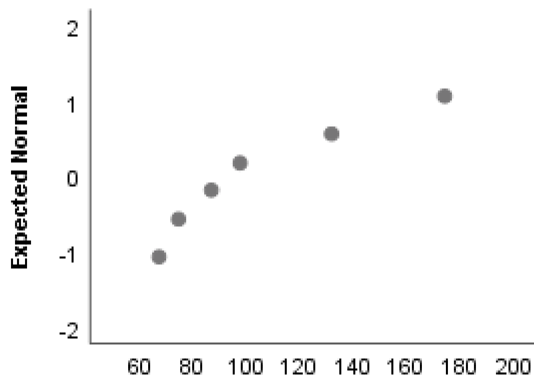
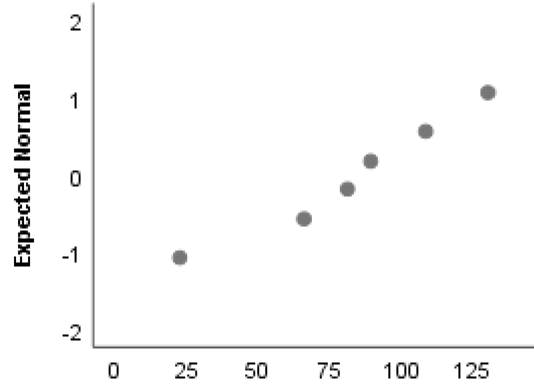
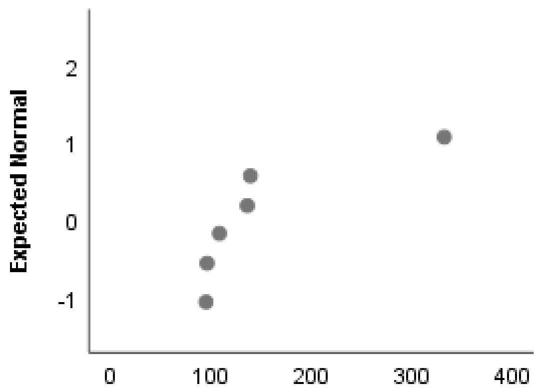


Figure 4. Box and Whiskers plots (Tukey's method) of the (A) pSer831-GluA1, (B) pSer845-GluA1, (C) total GluA1, (D) total GluA2 (E) total GluN2A, (F) total GluN2B immunoblot results for PS (%PC) and ByS (%ByC). Using Tukey's method, two outliers were identified, one among pSer831-GluA1 ByS male rats, and the other among the total GluN2A ByS male rats.







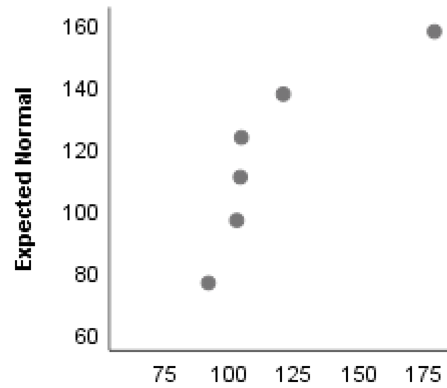
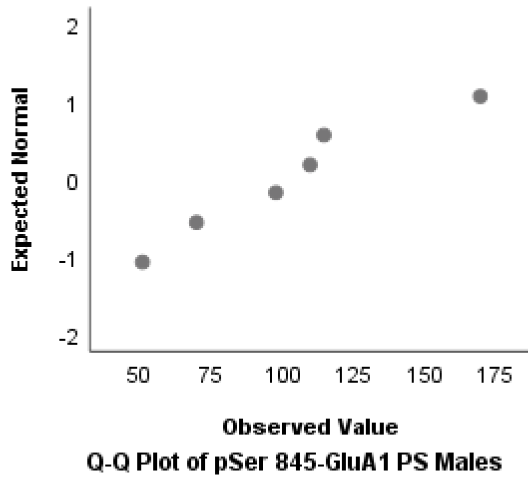
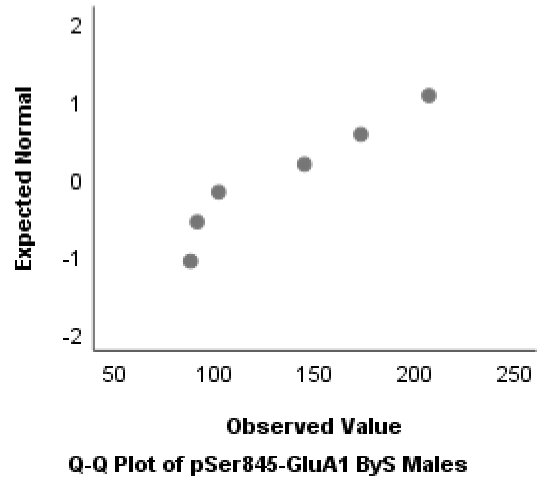
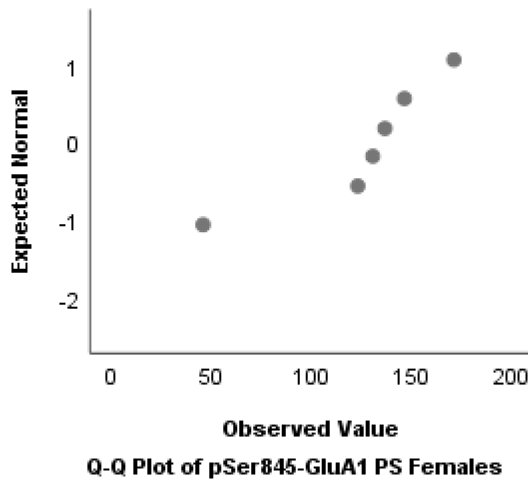


Figure 5. Q-Q plots of the pSer831-GluA1 immunoblot results.

The Q-Q plots are shown for PS and ByS (male and female). Normally distributed data will tend to fall close to a straight diagonal line.







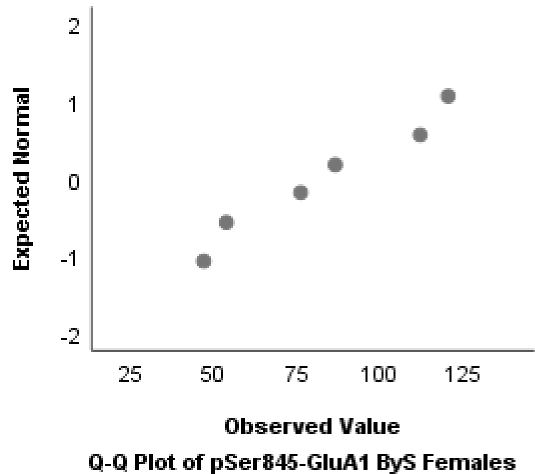
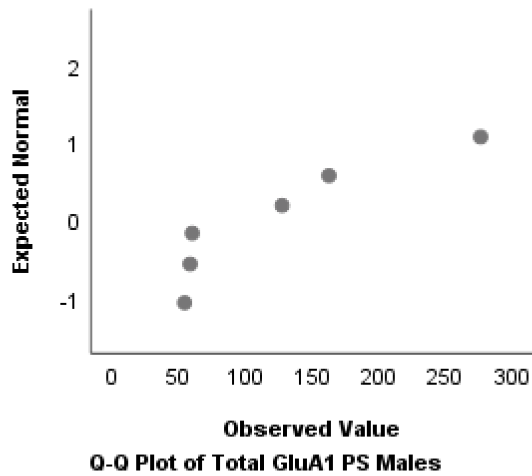
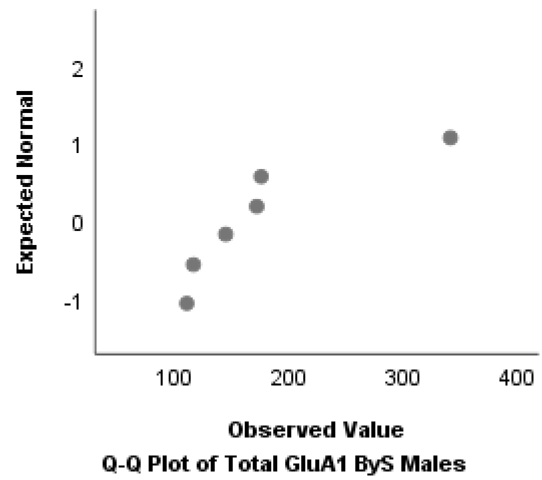
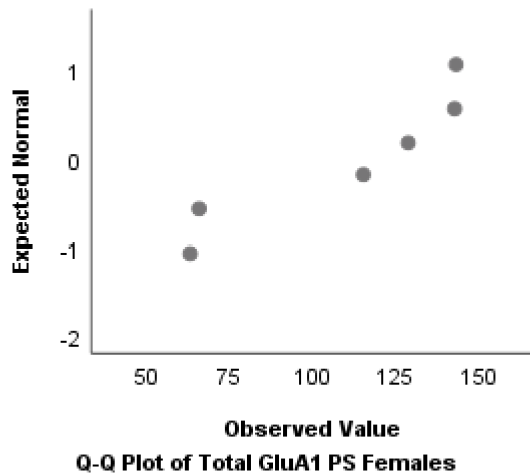


Figure 6. Q-Q plots of the pSer845-GluA1 immunoblot results.

The Q-Q plots are shown for PS and ByS (male and female). Normally distributed data will tend to fall close to a straight diagonal line.







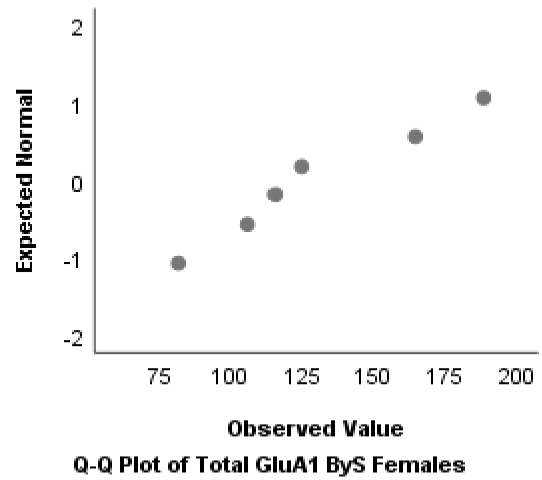


Figure 7. Q-Q plots of the total GluA1 immunoblot results.

The Q-Q plots are shown for PS and ByS (male and female). Normally distributed data will tend to fall close to a straight diagonal line.

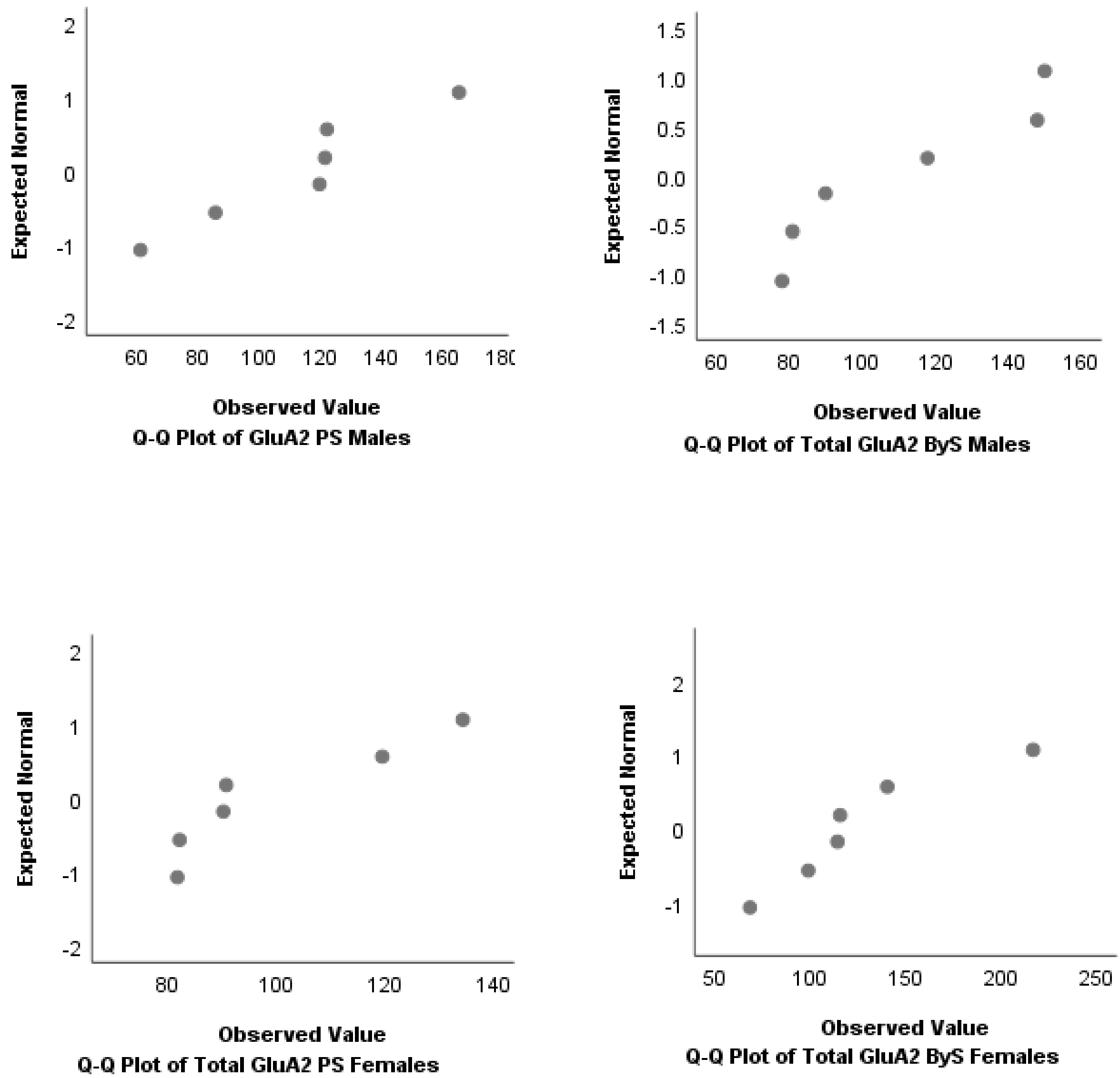


Figure 8. Q-Q plots of the total GluA2 immunoblot results.

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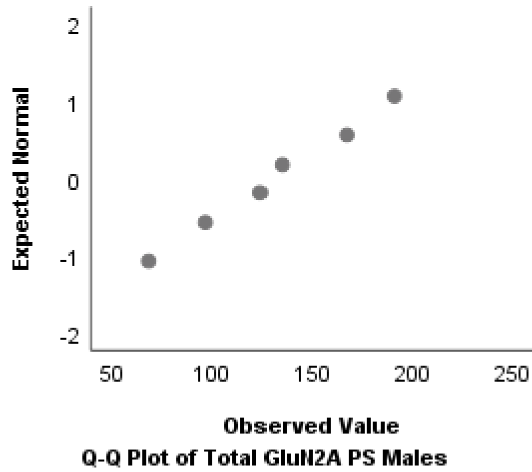
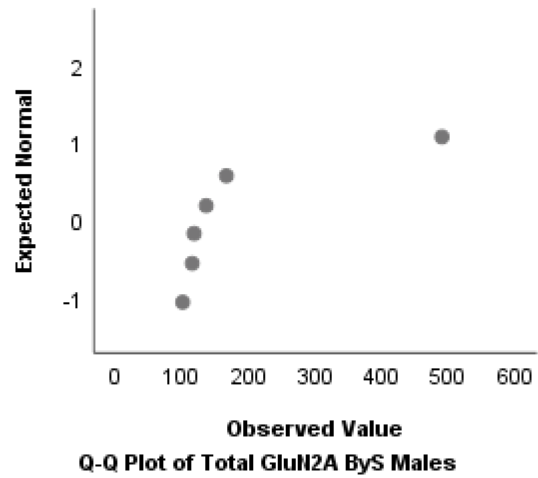
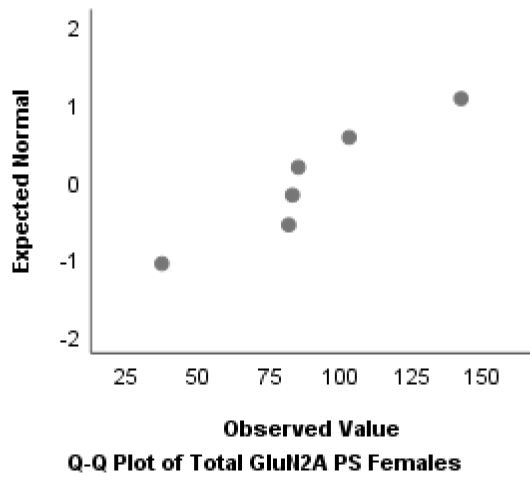






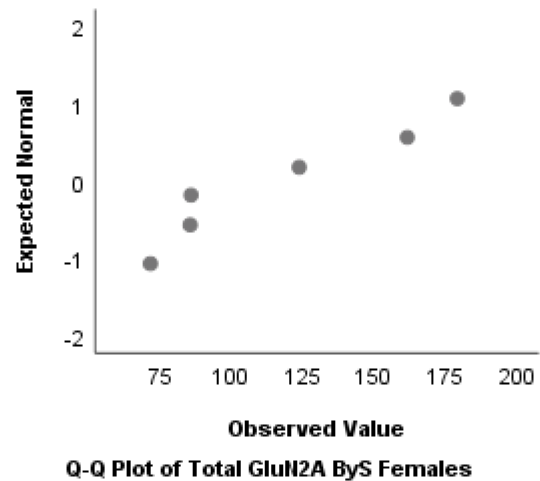
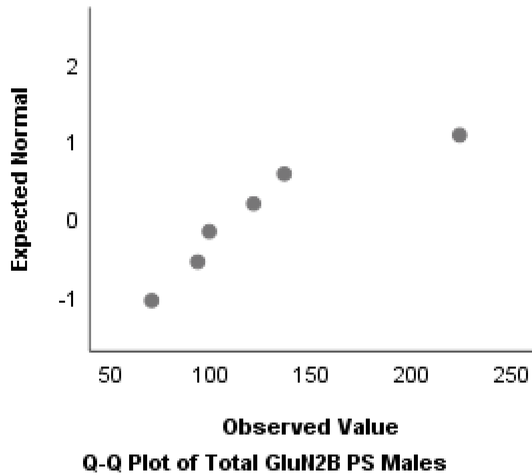
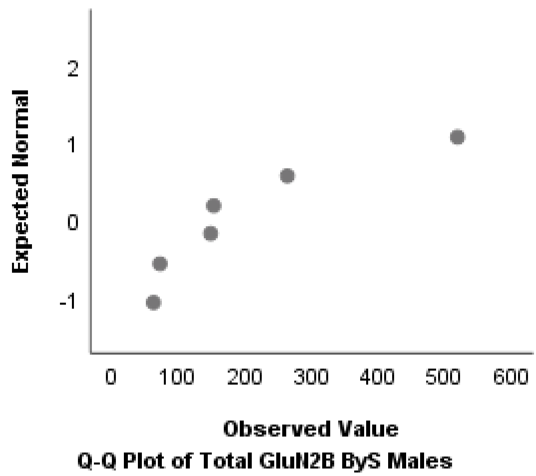
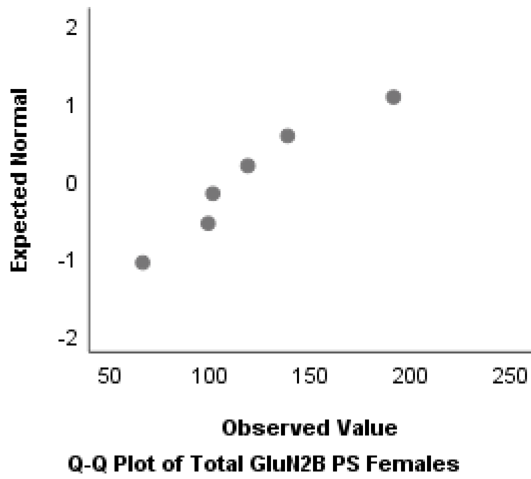


Figure 9. Q-Q plots of the total GluN2A immunoblot results.

The Q-Q plots are shown for PS and ByS (male and female). Normally distributed data will tend to fall close to a straight diagonal line.







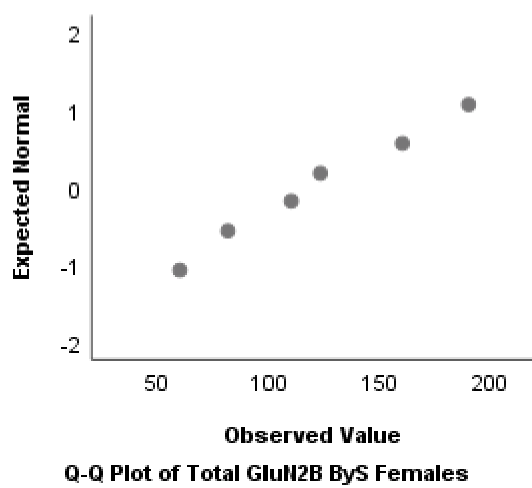


Figure 10. Q-Q plots of the total GluN2B immunoblot results.

The Q-Q plots are shown for PS and ByS (male and female). Normally distributed data will tend to fall close to a straight diagonal line.

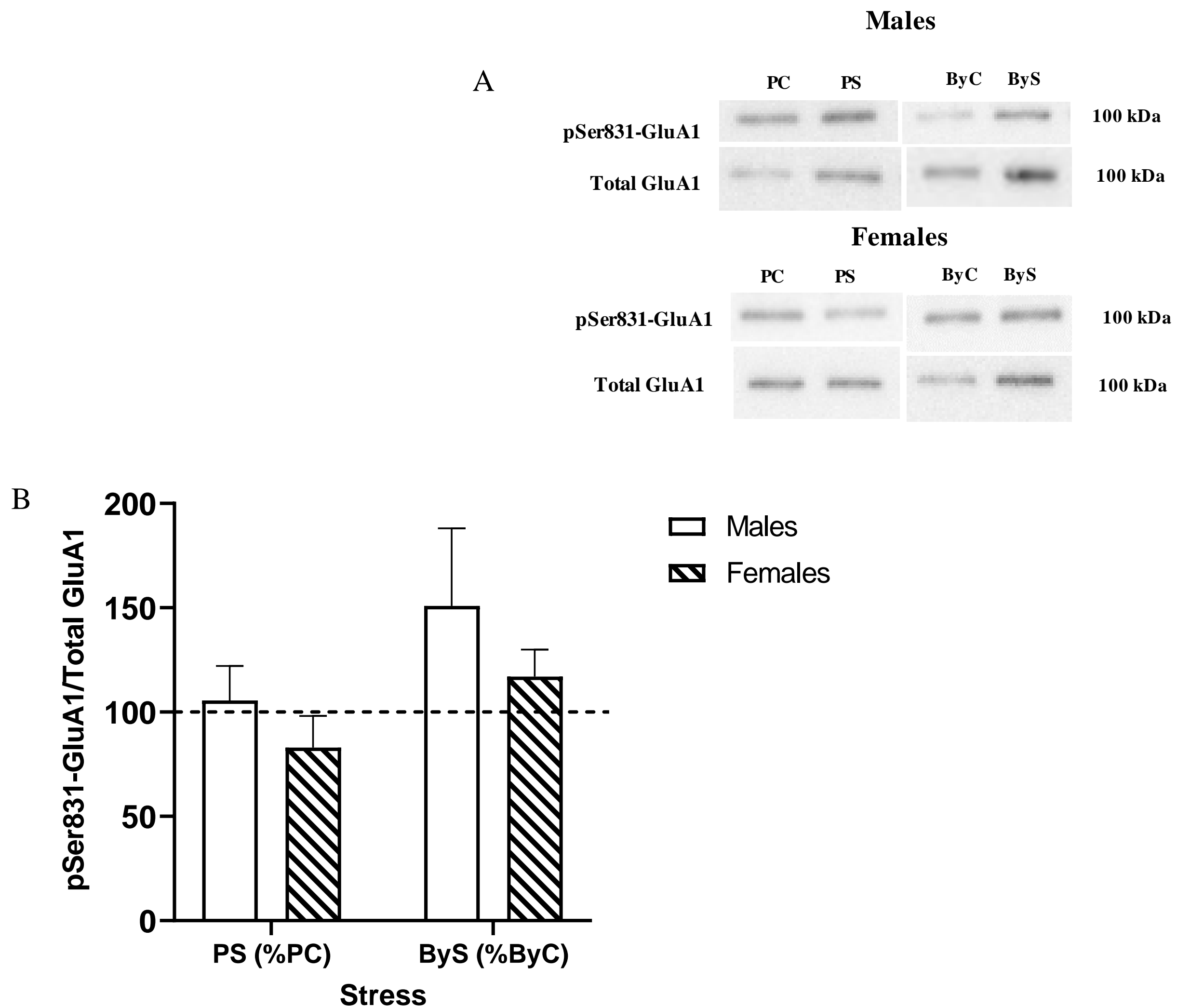


Figure 11. pSer831-GluA1 expression in platform and bystander stressed male and female rats. (A) Representative immunoblot images of pSer831-GluA1 expression in PS and ByS male and female rats, and their respective control groups. Although each sample was loaded in duplicate across the blots, in the interests of space, only a single lane is shown. (B) The summary graph presents the effect of PS and ByS on pSer831-GluA1 expression in male (N = 6) and female rats (N = 6). Each PS and ByS sample was taken as a percentage of their respective control group, PC and ByC. All data are expressed as mean ± SEM.

PS, platform stress; PC, platform control; ByS, bystander stress; ByC, bystander control

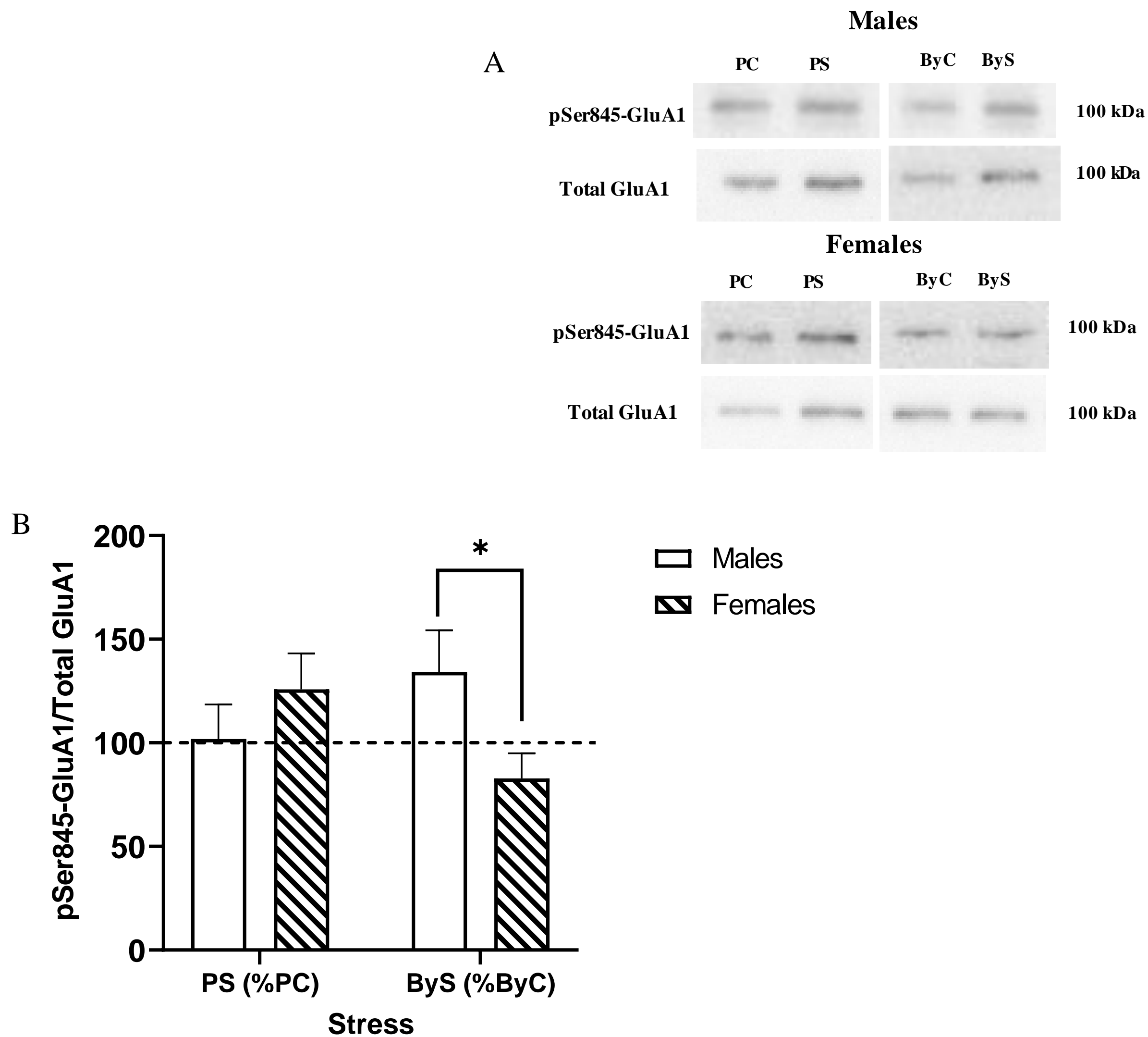


Figure 12. Total pSer845-GluA1 expression in platform and bystander stressed male and female rats. (A) Representative immunoblot images of pSer845-GluA1 expression in PS and ByS male and female rats, and their respective control groups. Although each sample was loaded in duplicate across the blots, in the interests of space, only a single lane is shown. (B) The summary graph presents the effect of PS and ByS on pSer845-GluA1 expression in male (N = 6) and female rats (N = 6). Each PS and ByS sample was taken as a percentage of their respective control group, PC and ByC. All data are expressed as mean ± SEM. *statistically significant p-value of < .05
PS, platform stress; PC, platform control; ByS, bystander stress; ByC, bystander control

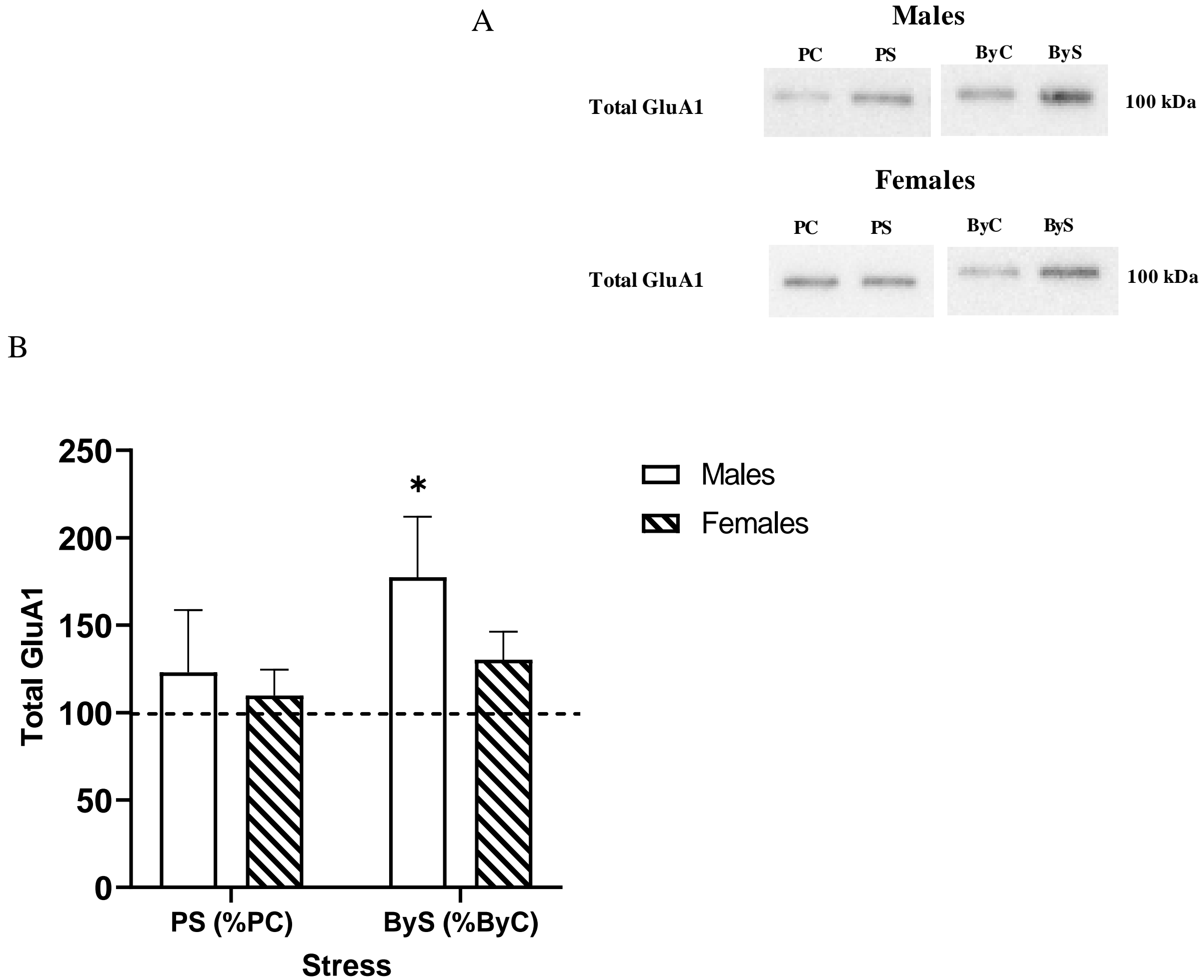


Figure 13. Total GluA1 expression in platform and bystander stressed male and female rats. (A) Representative immunoblot images of total GluA1 expression in PS and ByS male and female rats, and their respective control groups. Although each sample was loaded in duplicate across the blots, in the interests of space, only a single lane is shown. (B) The summary graph presents the effect of PS and ByS on total GluA1 expression in male (N = 6) and female rats (N = 6). Each PS and ByS sample was taken as a percentage of their respective control group, PC and ByC. All data are expressed as mean ± SEM. *statistically significant p-value of < .05

PS, platform stress; PC, platform control; ByS, bystander stress; ByC, bystander control

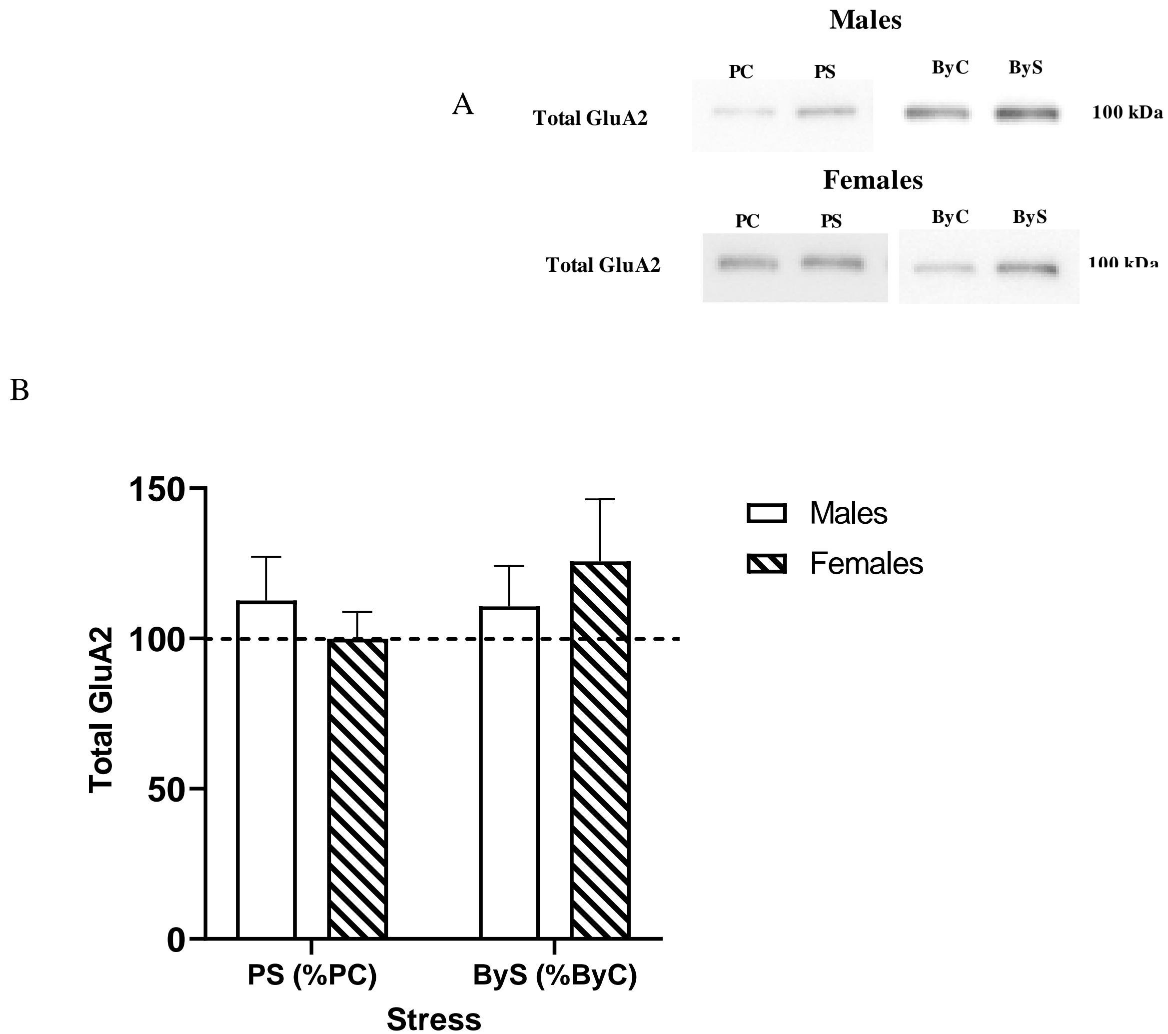


Figure 14. Total GluA2 expression in platform and bystander stressed male and female rats. (A) Representative immunoblot images of total GluA2 expression in PS and ByS male and female rats, and their respective control groups. Although each sample was loaded in duplicate across the blots, in the interests of space, only a single lane is shown. (B) The summary graph presents the effect of PS and ByS on total GluA2 expression in male (N = 6) and female rats (N = 6). Each PS and ByS sample was taken as a percentage of their respective control group, PC and ByC. All data are expressed as mean ± SEM

PS, platform stress; PC, platform control; ByS, bystander stress; ByC, bystander control

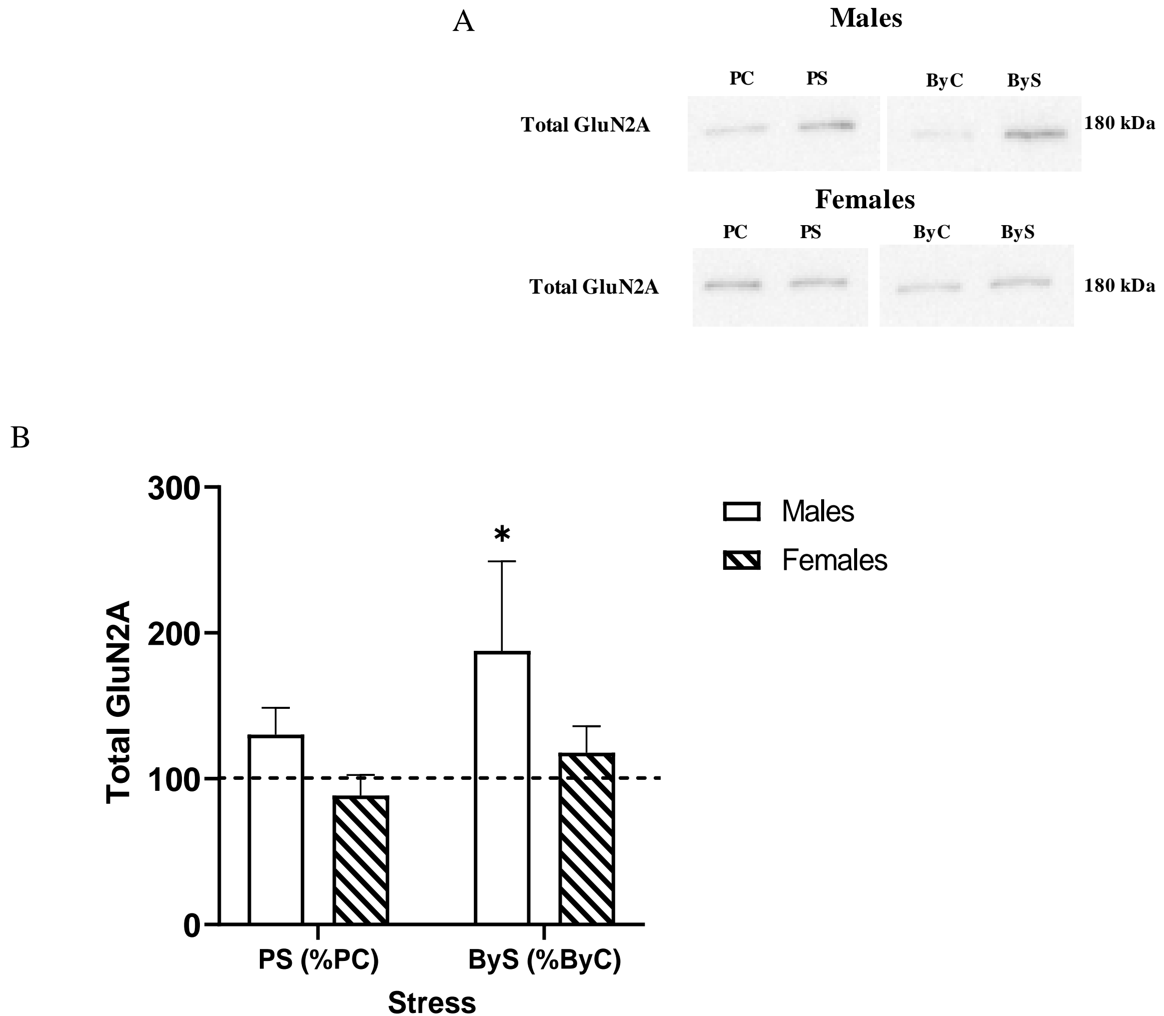


Figure 15. Total GluN2A expression in platform and bystander stressed male and female rats. (A) Representative immunoblot images of total GluN2A expression in PS and ByS male and female rats, and their respective control groups. Although each sample was loaded in duplicate across the blots, in the interests of space, only a single lane is shown. (B) The summary graph presents the effect of PS and ByS on total GluN2A expression in male (N = 6) and female rats (N = 6).

Each PS and ByS sample was taken as a percentage of their respective control group, PC and ByC. All data are expressed as mean ± SEM. *statistically significant p-value of < .05

PS, platform stress; PC, platform control; ByS, bystander stress; ByC, bystander control

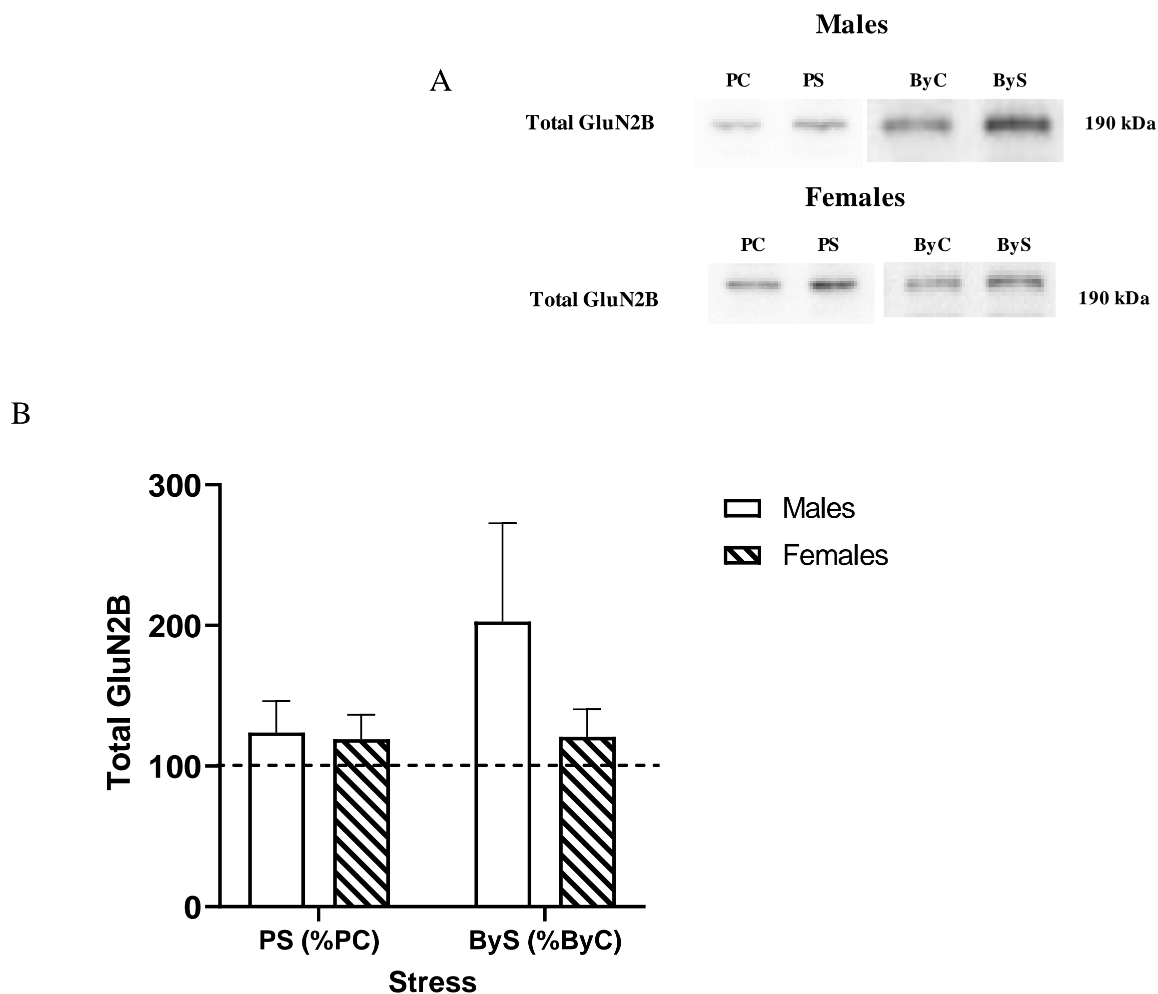


Figure 16. Total GluN2B expression in platform and bystander stressed male and female rats. (A) Representative immunoblot images of total GluN2B expression in PS and ByS male and female rats, and their respective control groups. Although each sample was loaded in duplicate across the blots, in the interests of space, only a single lane is shown. (B) The summary graph presents the effect of PS and ByS on total GluN2B expression in male (N = 6) and female rats (N = 6).

Each PS and ByS sample was taken as a percentage of their respective control group, PC and ByC. All data are expressed as mean ± SEM.

PS, platform stress; PC, platform control; ByS, bystander stress; ByC, bystander control

pSer 831-GluA1		Males	Females
PS	N	6	6
	Mean	105.55	82.96
	SEM	16.55	15.13
	SD	40.55	37.07
	Shapiro-Wilk test (p value)	.33	.93
	Skewness	1.13	-0.61
	Kurtosis	0.46	0.74
ByS	N	6	6
	Mean	150.91	117.01
	SEM	37.14	12.96
	SD	90.97	31.75
	Shapiro-Wilk test (p value)	**.004	**.017
	Skewness	2.21	2.02
	Kurtosis	5.07	4.29

Table 1. Assessment of normality and homogeneity of variance for the pSer831-GluA1 PS and ByS data for male and female rats.

Data were presented as % PC, or ByC, and the standard deviation (SD) and standard error of mean (SEM) were calculated for each group. The Shapiro-Wilk test with a p value > .05 was used as one measure to judge normality of the distribution. Skewness and kurtosis were examined to further assess normality.

**p value < .05, indicating a non-normal distribution

pSer 845-GluA1		**Males**	**Females**
PS	N	6	6
	Mean	101.84	125.81
	SEM	16.75	17.40
	SD	41.03	42.62
	Shapiro-Wilk test (p value)	.80	.16
	Skewness	0.63	-1.58
	Kurtosis	0.82	3.42
ByS	N	6	6
	Mean	134.25	82.79
	SEM	20.04	12.18
	SD	49.09	29.84
	Shapiro-Wilk test (p value)	.34	.61
	Skewness	0.59	0.11
	Kurtosis	-1.43	-1.75

Table 2. Assessment for normality and homogeneity of variance for the pSer845-GluA1 PS and ByS data for male and female rats.

Data were presented as % PC, or ByC, and the standard deviation (SD) and standard error of mean (SEM) were calculated for each group. The Shapiro-Wilk test with a p value > .05 was used as one measure to judge normality of the distribution. Skewness and kurtosis were examined to further assess normality.

Total GluA1		Males	Females
PS	N	6	6
	Mean	123	109.72
	SEM	35.62	14.92
	SD	87.24	36.55
	Shapiro-Wilk test (p value)	.12	.88
	Skewness	1.28	-0.66
	Kurtosis	1.18	-1.95
ByS	N	6	6
	Mean	177.36	130.18
	SEM	34.74	16.10
	SD	85.10	39.43
	Shapiro-Wilk test (p value)	**.035	.75
	Skewness	1.91	0.53
	Kurtosis	4.01	-0.77

Table 3. Assessment for normality and homogeneity of variance for the Total GluA1 PS and ByS data for male and female rats.

Data were presented as % PC, or ByC, and the standard deviation (SD) and standard error of mean (SEM) were calculated for each group. The Shapiro-Wilk test with a p value > .05 was used as one measure to judge normality of the distribution. Skewness and kurtosis were examined to further assess normality.

**p value < .05, indicating a non-normal distribution

Total GluA2		Males	Females
PS	N	6	6
	Mean	112.62	99.90
	SEM	14.64	8.92
	SD	35.87	21.84
	Shapiro-Wilk test (p value)	.66	.09
	Skewness	-0.042	1.03
	Kurtosis	0.35	-0.76
ByS	N	6	6
	Mean	110.71	125.67
	SEM	13.38	20.66
	SD	32.77	50.61
	Shapiro-Wilk test (p value)	.16	.38
	Skewness	0.35	1.28
	Kurtosis	-2.36	2.40

Table 4. Assessment for normality and homogeneity of variance for the Total GluA2 PS and ByS data for male and female rats.

Data were presented as % PC, or ByC, and the standard deviation (SD) and standard error of mean (SEM) were calculated for each group. The Shapiro-Wilk test with a p value $> .05$ was used as one measure to judge normality of the distribution. Skewness and kurtosis were examined to further assess normality.

Total GluN2A		Males	Females
PS	N	6	6
	Mean	130.25	88.62
	SEM	18.38	13.92
	SD	45.02	34.09
	Shapiro-Wilk test (p value)	.97	.61
	Skewness	-0.011	0.158
	Kurtosis	-0.83	1.66
ByS	N	6	6
	Mean	187.71	117.97
	SEM	61.27	18.17
	SD	150.08	44.51
	Shapiro-Wilk test (p value)	**.0011	.31
	Skewness	2.33	0.50
	Kurtosis	5.52	-1.89

Table 5. Assessment for normality and homogeneity of variance for the Total GluN2A PS and ByS data for male and female rats.

Data were presented as % PC, or ByC, and the standard deviation (SD) and standard error of mean (SEM) were calculated for each group. The Shapiro-Wilk test with a p value > .05 was used as one measure to judge normality of the distribution. Skewness and kurtosis were examined to further assess normality.

**p value < .05, indicating a non-normal distribution

Total GluN2B		Males	Females
PS	N	6	6
	Mean	123.97	119.04
	SEM	22.13	17.50
	SD	54.20	42.86
	Shapiro-Wilk test (p value)	.24	.75
	Skewness	1.52	0.85
	Kurtosis	2.73	1.21
ByS	N	6	6
	Mean	202.72	120.72
	SEM	69.82	19.73
	SD	171.03	48.33
	Shapiro-Wilk test (p value)	.10	.93
	Skewness	1.59	0.28
	Kurtosis	2.54	-0.95

Table 6. Assessment for normality and homogeneity of variance for the Total GluN2B PS and ByS data for male and female rats.

Data were presented as % PC, or ByC, and the standard deviation (SD) and standard error of mean (SEM) were calculated for each group. The Shapiro-Wilk test with a p value > .05 was used as one measure to judge normality of the distribution. Skewness and kurtosis were examined to further assess normality.

Stress	Sex	Mean (% ByC, or PC)	Percent change	n	T-statistic or W-statistic	p value	Effect Size (Cohen's d)
Platform	Males	105.5	5.55	6	t = 0.34	.75	0.14
	Females	82.96	-17.04	6	t = 1.13	.31	0.46
Bystander	Males	150.9	50.91	6	W = 15	.16	0.56
	Females	117	17.01	6	W = 13	.22	0.54

Table 7. Summary of the one-sample Student's t-test and Wilcoxon signed ranked test for pSer831-GluA1.

PS and ByS rats were compared to PC and ByC rats, respectively, in both male and female rats. Theoretical mean used for each comparison = 100.

Stress	Sex	F-statistic	T-statistic or U-statistic	p value	Cohen's d
Platform	Males Females	1.20 (p = .85)	t = 1.01	.34	0.58
Bystander	Males Females	8.21 **(p = .04)	U = 14	.59	0.5

Table 8. Summary of the two-tailed independent Student's t-test and Mann-Whitney U test for pSer831-GluA1.

Male and female rats were compared for significant differences in pSer831-GluA1 levels caused by PS and/or ByS.

**statistically significant result with p-value < .05. A significant p-value for the F-test indicated unequal variances between the groups, hence, a non-parametric test (Mann-Whitney U test) was performed.

Stress	Sex	Mean (% ByC, or PC)	Percent change	n	T-statistic	p value	Effect size (Cohen's d)
Platform	Males	101.8	1.839	6	0.11	.92	0.05
	Females	125.8	25.81	6	1.48	.20	0.61
Bystander	Males	134.3	34.25	6	1.71	.15	*0.7
	Females	82.79	-17.21	6	1.41	.22	0.58

Table 9. Summary of the one-sample t-test for pSer845-GluA1.

PS and ByS rats were compared to PC and ByC rats, respectively, in both male and female rats.

Theoretical mean used for each comparison = 100.

Cohen's d effect size measure was reported, *indicated where the effect size was approximately large (d = 0.8).

Stress	Sex	F-statistic	T-statistic	p value	Cohen’s d
Platform	Males Females	1.08 (p = .94)	0.99	.34	0.57
Bystander	Males Females	2.71 (p =.30)	2.19	**.05	*1.27

Table 10. Summary of the two-tailed independent Student’s t-test for pSer845-GluA1.

Male and female rats were compared for significant differences in pSer845-GluA1 levels caused by PS and/or ByS.

**statistically significant result with p-value < .05.

Cohen’s d effect size measure was reported, *indicated where the effect size was large (d = 0.8).

Stress	Sex	Mean (% ByC, or PC)	Percent change	n	T-statistic or W-statistic	p value	Effect size (Cohen's d)
Platform	Males	123	23	6	t = 0.65	.55	0.26
	Females	109.7	9.7	6	t = 0.65	.54	0.27
Bystander	Males	177.4	77.4	6	W = 21	**.03	*0.91
	Females	130.2	30.2	6	W = 15	.16	*0.77

Table 11. Summary of the one-sample t-test and Wilcoxon signed ranked test for total GluA1.

PS and ByS rats were compared to PC and ByC rats, respectively, in both male and female rats.

Theoretical mean used for each comparison = 100.

**statistically significant result with p-value < 0.05

Cohen's d effect size measure was reported, *indicated where the effect size was approximately large (d = 0.8).

Stress	Sex	F-statistic	T-statistic or U-statistic	p value	Cohen's d
Platform	Males Females	5.70 (p = .08)	t = 0.34	.74	0.2
Bystander	Males Females	4.66 (p = .12)	U = 11	.31	*0.7

Table 12. Summary of the two-tailed independent Student's t-test and Mann-Whitney U test for total GluA1.

Male and female rats were compared for significant differences on total GluA1 levels caused by PS and/or ByS.

Cohen's d effect size measure was reported, *indicated where the effect size was approximately large (d = 0.8).

Stress	Sex	Mean (% ByC, or PC)	Percent change	n	T-statistic	p value	Effect size (Cohen's d)
Platform	Males	112.6	12.62	6	0.86	.43	0.35
	Females	99.9	-0.10	6	0.011	.99	0.005
Bystander	Males	110.7	10.71	6	0.80	.46	0.33
	Females	125.7	25.67	6	1.24	.27	0.51

Table 13. Summary of the one-sample t-test for total GluA2.

PS and ByS rats were compared to PC and ByC rats, respectively, in both male and female rats.

Theoretical mean used for each comparison = 100.

Stress	Sex	F-statistic	T-statistic	p value	Cohen's d
Platform	Males Females	2.70 (p = .30)	0.74	.48	0.43
Bystander	Males Females	2.39 (p = .36)	0.61	.56	0.37

Table 14. Summary of the two-tailed independent Student's t-test for total GluA2.

Male and female rats were compared for significant differences on total GluA2 levels caused by PS and/or ByS.

Stress	Sex	Mean (% ByC, or PC)	Percent change	n	T-statistic or W-statistic	p value	Effect size (Cohen's d)
Platform	Males	130.2	30.25	6	t = 1.65	.16	0.67
	Females	88.62	-11.38	6	t = 0.82	.45	0.33
Bystander	Males	187.7	87.7	6	W = 21	**.03	0.58
	Females	118	17.97	6	W = 7	.56	0.4

Table 15. Summary of the one-sample t-test for total GluN2A.

PS and ByS rats were compared to PC and ByC rats, respectively, in both male and female rats.

Theoretical mean used for each comparison = 100.

**statistically significant result with p-value < .05

Stress	Sex	F-statistic	T-statistic	p value	Cohen's d
Platform	Males Females	1.75 (p = .56)	t = 1.81	.10	**1.04
Bystander	Males Females	3.09 (p = .30)	U = 12	.39	0.63

Table 16. Summary of the two-tailed independent Student's t-test for total GluN2A.

Male and female rats were compared for significant differences on total GluN2A levels caused by PS and/or ByS.

Cohen's d effect size measure was reported, *indicated where the effect size was approximately large (d = 0.8).

Stress	Sex	Mean (% ByC, or PC)	Percent change	n	T-statistic	p value	Effect size (Cohen's d)
Platform	Males	124	23.97	6	t = 1.08	.33	0.44
	Females	119	19.04	6	t = 1.09	.33	0.44
Bystander	Males	202.7	102.7	6	W = 15	.16	0.6
	Females	120.7	20.72	6	W = 9	.44	0.43

Table 17. Summary of the one-sample t-test for total GluN2B.

PS and ByS rats were compared to PC and ByC rats, respectively, in both male and female rats.

Theoretical mean used for each comparison = 100.

Stress	Sex	F-statistic	T-statistic	p value	Cohen's d
Platform	Males Females	1.60 (p = .62)	t = 0.17	.86	0.1
Bystander	Males Females	12.52 **(p = .02)	U = 14	.59	0.65

Table 18. Summary of the two-tailed independent Student's t-test for total GluN2B.

Male and female rats were compared for significant differences in total GluN2B levels caused by PS and/or ByS.

**statistically significant result with p-value < .05. A significant p-value for the F-test indicated unequal variances between the groups, hence, a non-parametric test (Mann-Whitney U test) was performed.

	Platform stress		Bystander stress	
	Males	Females	Males	Female
pSer831-GluA1	↑ (6%)	↓ (17%)	↑# (51%)	↑# (17%)
pSer845-GluA1	↑ (2%)	↑# (25%)	↑# (34%)	↓# (17%)
Total GluA1	↑ (23%)	↑ (10%)	↑* (77%)	↑# (30%)
Total GluA2	↑ (13%)	↔	↑ (11%)	↑# (26%)
Total GluN2A	↑# (30%)	↓ (11%)	↑* (87%)	↑ (18%)
Total GluN2B	↑ (24%)	↑ (19%)	↑# (103%)	↑ (21%)

Table 19. Summary of all findings

↑, ↓, ↔ signifies an increase, decrease, and no change respectively in the stressed compared to control group

* denotes p value < .05 + Cohen's d effect size of at least 0.5 in the stressed compared to the control group

# denotes Cohen's d effect size of at least 0.5 in the stressed compared to the control group

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