Examining the behavioural, glucocorticoid and neurochemical effects of single and repeated stressor exposure

by

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Abstract

Stressful experiences typically produce neuroendocrine and neurochemical effects that quickly resolve, but the mechanisms underlying these alterations may become sensitized upon subsequent stressor exposure. As stressors have been implicated as an instigator of anxiety-like states, we assessed whether acute and repeated stressors would result in behavioural, glucocorticoid and neurochemical changes, in the prefrontal cortex and hippocampus. Results of the present report are discussed in terms of their relevance to the progression of pathological outcomes, such as posttraumatic stress disorder. The present investigation demonstrated that repeated social defeat elicited hyperactivity in the elevated plus maze, which was attenuated by propranolol. Corticosterone levels were elevated following an acute stressor; however these elevations were blunted upon subsequent exposure. Catecholamine levels were elevated following acute and repeated stressor exposure. While an acute stressor resulted in increased prefrontal cytokine expression, repeated exposure elicited a desensitized cytokine response. Repeated exposure to a powerful stressor may promote characteristics reminiscent of PSTD.

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1. Introduction

Stressful events or traumatic experiences provoke multiple neurochemical and behavioural alterations (Anisman, Merali & Hayley, 2003). Typically, the neurochemical effects of stressors are relatively transient, being evident for only a few minutes or hours (Anisman, Zalcman, & Zacharko, 1993). It is thought that in humans, normalization of stressor-elicited neurochemical changes also occurs relatively quickly. However, it seems that there are a small proportion of individuals who fail to recover from stressor effects and are unable to regain and maintain homeostasis (Yehuda & Ledoux, 2007). This inability to recover can be overly taxing to adaptive neurochemical systems (allostatic overload), leading to the emergence of pathology (McEwen, 2000).

In addition to its immediate actions, a stressor can have proactive effects so that when an individual is faced with subsequent stressors, exaggerated behavioural and neurochemical responses are elicited (Anisman et al., 2003). This not only occurs when the stressors are similar, but is also evident even when the initial and the re-exposure stressor are different from one another (Anisman et al., 2003). In effect, it seems that a stressor can promote the "sensitization" of processes that regulate the neurochemical response, so that greater effects are elicited following subsequent encounters with an environmental challenge (Anisman et al., 1993).

When an organism develops a sensitized response to a stressor, pathological outcomes may result. Among these outcomes, depression and anxiety disorders are common and often co-morbid. It is important therefore to consider the underlying neurochemical and behavioural mechanisms of these disorders and how these processes are altered in the presence of stress. Paralleling the effects of stressors, depression and

anxiety disorders are associated with variations of several brain monoamines including, norepinephrine (NE) and serotonin (5-HT), as well as their receptors (Anisman et al., 2008). However, several other substrates have been implicated in these disorders. For instance, gamma amino butyric acid (GABA) functioning has also been implicated in anxiety disorders, and it appears that GABA moderates at least some of the behavioural and physiological response to stressors (Heim & Nemeroff, 2009). In this regard, benzodiazepine and other anti-anxiety treatments that act on the $GABA_A$ receptor are known to induce mild inhibition of neuronal firing which results in the reduction of anxiety symptoms. This would suggest that the GABAergic system might play a role in the pathophysiology of anxiety disorders and might even be involved in posttraumatic stress disorder (PTSD).

Taking these points into consideration, the present investigation will examine the behavioural and neurochemical changes that occur as a result of stressor exposure followed by re-exposure to either a social or physical stressor. It has only been in recent years that the focus of stress research has turned to psychosocial stressors as being particularly relevant in rodents, just as they are in humans. In part, the present investigation was undertaken to determine whether sensitized responses would be associated with a social stressor, as relevant data are presently unavailable in this regard. The second objective of this research was to assess the impact of the NE blocker, propranolol, administered prior to re-exposure to determine whether this treatment will attenuate the symptoms caused by stressor re-exposure. Propranolol, known to have anti-anxiety properties (Pitman et al., 2002; Ravindran & Stein., 2009; Vaiva et al., 2003) has found its way into the treatment of PTSD, and before assessing whether propranolol

affects the evolution of PTSD like effects, in the present study it was of interest to establish whether propranolol would attenuate the effects of stressor re-exposure.

1.1. Stress and the stress response

The term stressor is widely defined as a situation or event, negatively appraised by an organism, that elicits a biological response, termed the stress response (McEwen, 2000). The stress response is considered to be an effort made by an organism to adapt to environmental or psychological insults (McEwen, 2000). For research purposes there tends to be a distinction made between social stress and physical stress. Interestingly, both social stress and physical stress activate the hypothalamic-pituitary-adrenal (HPA) axis in similar ways, albeit via different pathways (Herman & Cullinan, 1997). Several monoamine neurotransmitters are essential for the regulation of the HPA axis, namely 5-HT, NE, and dopamine (DA). Alterations in HPA axis and amine functioning has been linked to psychological disturbances, such as depression (Anisman & Merali, 2002). The HPA axis has been explored using various types of stress, in varying contexts and with respect to its impact in the long and short term. Specifically, researchers have attempted to understand how stress can alter the HPA axis and more importantly the impact that stress has on the regulation of the HPA axis.

Herman and Cullinan (1997) characterize stressors as being either processive or systemic. Processive stressors are those which involve higher-order sensory processing. Whereas systemic stressors are those that involve physical insults leading to alterations of the circulatory, respiratory, and/or immune systems (Herman & Cullinan, 1997). These types of stressors can be further characterized as being either psychological

(psychogenic) or physical (neurogenic) (Anisman & Merali, 2002). Previously, it has been suggested that processive and systemic stressors promote neuroendocrine activity via different neuronal circuits (Herman, & Cullinan, 1997). Specifically, higher brain structures have been implicated in the processing of processive stressors as opposed to systemic stressors. The limbic system, mainly involved in processive stressors, sends afferent projections to the paraventricular nucleus (PVN) in order to activate the HPA axis. In contrast, it appears that systemic stressors circumvent the limbic system and stimulate the PVN directly. Interestingly, both processive and systemic stressors elicit similar neuroendocrine response irrespective of the neural circuitry involved. For instance, both types of stressors activate specific neurons in the PVN, which secrete corticotropin releasing hormone (CRH) as well as arginine-vasopressin (AVP). The combined expression of CRH and AVP trigger the release of adrenocorticotropic hormone (ACTH) from the pituitary gland, which stimulates the adrenal cortex to release glucocorticoids, such as corticosterone (cortisol, in humans) (Heim & Nemeroff, 2009; Leonard, 2005; Sapolsky, Romero, & Munck, 2000).

Typically, the stress response is believed to have an adaptive advantage, priming the organism to handle environmental or psychological insults. However, prolonged stressor exposure can become maladaptive to an organism as their resources for dealing with the stressor, become overly taxed. Under these circumstances, allostatic overload can lead to the development of physiological and psychological disorders (McEwen, 2000). HPA axis dysfunction caused by prolonged activation of the stress response has been noted with respect to psychological disorders, such as PTSD and major depression (de Kloet et al., 2006; Pariante & Lightman, 2008).

1.2. Post-traumatic Stress Disorder (PTSD)

The neurochemical and behavioural alterations due to stressor exposure frequently leads to depressive like states and depending on the stressor characteristics, might also promote PTSD. This disorder develops after exposure to a traumatic event, defined as a threatening experience that is accompanied by feelings of fear and helplessness (APA, 2000). In humans, the initial traumatic experience is usually followed by episodes of re-experiencing. The re-exposure or re-experiencing of a traumatic event can occur in several ways, including; re-experiencing the same trauma, experiencing a different trauma, ruminating about the trauma, or being faced with cues or reminders of the traumatic event. Moreover, as already indicated, previous research has demonstrated that re-exposure to stressors can lead to a sensitized stress response (Anisman et al., 1993; Tilders & Schmidt, 1999) which may be an integral feature in the progression of PTSD (Heim & Nemeroff, 2009). For humans, when an individual experiences a traumatic event, especially if the event is sufficient to provoke psychological trauma, it is often the case that the trauma is re-experienced or replayed cognitively. Severely traumatic events can be recalled years even decades later, and upon rumination of the event, if an individual does not employ adequate coping strategies or if the biological insults are too substantial, sensitization may occur, potentially resulting in PTSD like symptoms.

1.3. Stressor induced behavioural alterations

In addition to the neurochemical actions, stressful events can also trigger several behavioural alterations consistent with depressive and anxiety like disorders. Typically,

animal well-being is measured using specific, reliable tests of depression and anxiety; such as the elevated plus maze (EPM), and sucrose preference tests (SPT).

One of the most commonly used animal models of anxiety is the EPM. The EPM test is based on the premise that rodents display two types of naturally occurring yet conflicting behaviours; the avoidance of open spaces (avoiding open arms) and the propensity to explore novel environments (entering open arms) (Andreatini & Bacellar, 2000). The interpretation of these divergent behaviours is that exposure to novel stimuli (EPM test) can evoke both exploratory and fear behaviours, thus generating conflicting approach and avoidance behaviours in rodents. Higher plasma corticosterone concentrations were found in rats that were specifically confined to the open arms of the EPM compared to those confined to the closed arms (Pellow et al., 1985). These results suggest that exposure to the open arms is more stressful than exposure to the closed arms. The open arms of the maze therefore evoke greater fear and anxiety and result in augmented avoidance behaviour (Pellow et al., 1985). The anxiety level being measured in the EPM is generally expressed by the number of entries into and the length of time spent in the anxiety provoking open arms (Carola et al., 2002). Generally, if an animal enters more frequently or spends more time in the open arms, it is presumed that the animal is less anxious than their non-entering counterparts (Lister, 1987). Pharmacological compounds which provoke feelings of anxiety in humans, impact rodents in a similar fashion, as demonstrated in the EPM test, resulting in a reduction of entries into, and time spent on the open arms (Handley & Mithani, 1984) validating the use of the EPM in tests of anxiety.

Differing from the EPM, a conventional SPT is a method for measuring anhedonia (a depressive symptom) and the efficacy of anti-depressive pharmacological therapies (Willner et al., 1987). It is understood that rodents typically display a natural preference towards sweetened liquids. Under normal conditions, an animal will consume greater amounts of the sucrose containing liquid compared to regular drinking water. It is assumed that sucrose preference is a measure of interest and if an experimentally stressed animal loses interest for the sucrose, the resulting reduction indicates a lack of interest, referred to as anhedonia. Early research has reported reduced total sucrose consumption as well as suppression of sucrose preference following stressor exposure (Willner et al., 1987) and these results have consistently been replicated (D`Aquila et al., 1997; Gronli et al., 2005; Muscat & Willner, 1992; Papp et al., 1991). Moreover, Pijlman et al., (2003) demonstrated that when compared to controls, physically stressed animals exhibit a decrease in saccharine (sucrose) preference, whereas emotional stress caused only a minor increase in sucrose preference. Thus, the view was offered that physical stress results in decreased sucrose preference (anhedonia) whereas emotional stress appears to cause a marginal increase in sucrose preference and perhaps increased reward sensitivity (Pijlman et al., 2003).

1.4. Effects of social stress and physical stress: Similarities and differences

Animal models have revealed that social stressors like other stressors, leads to behavioural alterations including anxiety, defensiveness and helplessness (Blanchard et al., 2001). Social stressors also elicit altered brain neuronal transmission, and stimulate the serotonergic, noradrenergic and dopaminergic systems (Blanchard et al., 2001). Some

social stress models have primarily focused on the aggressive behaviour of rodents, often called social defeat stress. This particular stress paradigm focuses on the fighting behaviour of rodents, and examines the behavioural and neurochemical effects associated with dominance (winning) or submissiveness (defeat) (Merlot, Moze, Dantzer, & Neveu, 2003; Pizarro, et al., 2004). Social stress paradigms such as these have allowed researchers to examine the effects of social stress on behavioural and neurochemical systems in relation to depressive-like states. Social stressors consistently elicit behavioural changes in rodents including altered locomotor activity, anhedonia and increased anxiety-like behaviours (Bartolomucci, Palanza, Gaspani, Limiroli, Panerai, Ceresini, Poli, & Parmigiani, 2001). Socially defeated (submissive) animals display enhanced anxiety in the EPM test (Avgustinovich et al., 1997) and the open field (OF) test (Kudryavtseva et al., 1991). Moreover, 5-HT neurotransmission has consistently shown to be altered by social stress and 5-HT is believed to mediate many of the behaviours associated with social stress (Berton et al., 1998; Blanchard et al., 1991). Furthermore, re-exposing previously defeated rats to defeat threat elicits an increase in extracellular DA in the pre-frontal cortex (PFC) as well as the nucleus accumbens (Tidey & Miczek, 1996).

In contrast to social stressor paradigms, investigators have also examined the effects of physical stressors on behavioural and neurochemical systems in relation to depressive-like states. Like social stressors, physical stressors consistently elicit behavioural changes in rodents including increased anxiety in the EPM test (Albonetti & Farabollini, 1992; Pellow et al., 1985), decreased locomotor activity in the OF (Pijlman et al., 2003) and decreased sucrose preference (Pijlman et al., 2003; Plasnik et al., 1989).

Furthermore, like other types of stress, physical stress also causes various monoamine alterations, including increased brain DA levels (Sutoo & Akiyama, 2002), increased 5-HT levels (Maswood et al., 1998) and increased activity of noradrenergic neurons and subsequent release of NE (Sands et al., 2000). Of interest however, is that these studies have examined the immediate stressor effects and re-exposure effects but have failed however, to examine the potential protracted effects of delayed stressor re-exposure.

1.5. Stressor provoked neuropeptide alterations

Several neurobiological systems have been implicated in the pathophysiology of PTSD. These neurobiological systems include the HPA axis, as described earlier, as well as a network of brain regions which regulate fear and stress responses, namely the PFC, hippocampus, and amygdala (Heim & Nemeroff, 2009). The neuropeptide alterations caused by stress or trauma that occur within this network have been of particular interest with regard to specific pathological features of psychological disorders. Under normal conditions the stress response causes an increase in CRH, subsequently leading to elevated cortisol levels. The response to stress and resulting increase in cortisol is often observed in psychiatric disorders such as major depression (Parker et al., 2003).

Given the findings related to mild stressor exposure and the resulting increase in corticosterone levels, it is of interest to consider how stressor effects are different in psychiatric disorders. Studies examining the HPA axis alterations associated with depression indicated elevations in CRH (Newport & Nemeroff, 2000) and PTSD studies have found similar elevations in CRH (Baker et al., 1999). However, in contrast to elevated cortisol levels in depression, studies examining PTSD have reported decreased

cortisol levels in urine (Mason et al., 1986) as well as in saliva and plasma (Boscarino, 1996; Wessa et al., 2006). There appears to be inconsistent results across studies however, with some indicating elevated cortisol levels in adult PTSD patients (Lindley et al., 2004; Pitman and Orr, 1990; Young and Breslau, 2004). These cortisol level discrepancies have been attributed to differences in the timing of sampling as well as diverse sample populations, where features such as type of trauma, time since exposure and symptom severity are not consistent across the samples (Ravindran & Stein, 2009).

In an attempt to address these issues, Meewisse et al. (2007) conducted a meta-analysis in which they reported that lower levels of plasma and serum cortisol were found in patients with PTSD compared to healthy non-trauma exposed controls. However, the authors report no differences in basal cortisol levels when PTSD patients were compared to controls with prior trauma exposure (Meewisse et al., 2007). The authors suggest that similarities in cortisol levels among PTSD patients and trauma exposed individuals may be a result of trauma exposure rather than a diagnosis of PTSD itself (Meewisse et al., 2007).

1.6. Stressor induced neurotransmitter alterations

In addition to neuroendocrine effects, processive and systemic stressors also provoke a cascade of neurotransmitter alterations. These alterations are evident in NE, DA and 5-HT which are released at hypothalamic and extrahypothalamic sites, including the PFC, nucleus accumbens, and several amygdala nuclei (Anisman, Zalcman, & Zacharko, 1993). It is thought that stressor induced alterations of monoamine activity, may evoke certain behavioural pathologies such as anxiety disorders and depression,

especially with disproportionate and prolonged utilization of these monoamines (Anisman & Merali, 1999).

There are a growing number of studies which provide evidence for increased noradrenergic system activity in humans with PTSD (Ravindran & Stein, 2009). This increased activity generally is not observed under baseline or resting conditions, but rather is evident in response to a variety of stressors. This altered functioning suggests that there may be hyperactive noradrenergic functioning in PTSD patients. The NE system acts predominantly on the sympathetic nervous system. The cell bodies of many of the NE secreting neurons are found in the locus coeruleus (LC) of the brainstem. The LC is an important neural structure that has projections to several stressor sensitive areas of the brain, including the amygdala, hippocampus, thalamus and PFC (Vermetten & Bremner, 2002).

These brain areas are important in emotion, memory, and the stress response. In the presence of a stressor, the sympathetic nervous system is ordinarily activated, causing the release of NE from the LC. Typically, the release of NE produces various physiological responses, usually called the fight or flight response. However, there is a large body of evidence suggesting that NE functioning is altered in PTSD and it has been suggested that NE plays an important role in PTSD, being responsible for hyperarousal, anxiety, re-experiencing, and increased blood pressure (Krystal & Neumeister, 2009; Ravindran & Stein, 2009). Furthermore, changes of NE activity among several brain areas, important in the fear and stress response, have been observed after stressor exposure (O'Donnell et al., 2004).

In addition to NE dysregulation caused by stress, DA alterations have also been reported in individuals exposed to trauma or stressors (Friedman, 1994). Following several different types of stressors there have been reported brain increases in DA levels (Sasaki et al., 1998; Sutoo et al., 1991; Sutoo & Akiyama, 2002), metabolism (Inoue et al., 1994; Okuda et al., 1986; Sudha & Pradhan, 1995), and release (Doherty & Gratton, 1992; Funada & Hara, 2001; Imperato et al., 1991). Interestingly, PTSD inpatients showed higher urinary excretion of DA compared with both outpatients with PTSD and normal controls (Yehuda et al, 1992). In terms of cortical DA alterations, stress-induced alterations in mesolimbic DA functioning appears to affect the emotional responsivity to aversive experiences (Puglisi-Allegra, Kempf & Cabib, 1990).

The DA alterations associated with stressor exposure coincide with the behavioural disturbances, such as anhedonia and helplessness, observed in depression (Puglisi-Allegra et al., 1990). Moreover, an investigation using in vivo microdialysis indicated that DA levels in the nucleus accumbens and the PFC were elevated well above baseline both when the defeated animal was re-exposed to a reminder cue (empty resident cage) as well as a novel cage stressor (Tidey & Miczek, 1996).

Aside from the NE and DA stressor induced alterations, the central 5-HT system is an important component in the regulation of anxiety and the stress response (Chaouloff, 1993). Upon stressor exposure, animal models have shown that there is an upregulation of 5-HT_2 receptors and a downregulation of 5-HT_{1A} receptors (Heim & Nemeroff, 2009). It is believed that 5-HT neurons of the dorsal raphé, which project to the amygdala and hippocampus, exert stress inducing effects via 5-HT_2 receptors. In contrast, 5-HT neurons of the median raphé produce anxiolytic (stress-reducing) effects

via 5-HT_{1A} receptors (Heim & Nemeroff, 2009). Moreover, increases in 5-HT release, synthesis and turnover in the dorsal raphé have been reported in response to stressors (Chaouloff et al., 1999; Dunn, 1988).

Stressor provoked alterations in 5-HT activity occur in several brain regions, implicated in the pathophysiology of PTSD. Among the brain areas implicated in PTSD, altered 5-HT functioning has been noted in the amygdala (Parsey et al., 2006) and the PFC (Smith et al., 2006). The involvement of the serotonergic system in PTSD is also demonstrated by the efficacy of selective serotonin re-uptake inhibitors (SSRIs) in the treatment of many PTSD symptoms (Bisson, 2007; Ravindran & Stein, 2009) and is typically the first line of defence taken against symptoms of PTSD (Krystal & Neumeister, 2009). Taken together, upon stressor exposure changes to 5-HT functioning may facilitate the emergence of PTSD symptomatology, such as hypervigilance, increased startle, impulsivity and intrusive thoughts (Heim & Nemeroff, 2009).

1.7. Stressor induced brain cytokine mRNA expression alterations

Cytokines are potent protein molecules that are generally characterized to be involved in cellular activation and communication of the immune system. Cytokines are present in several brain structures as proteins and their respective receptors (Haas & Schauenstein, 1997). Aside from their well-established role in mediating the central components of the immune response, several reports have indicated that cytokines also play an important role in stress-related disorders, including depression and PTSD (Anisman & Merali, 2002; Hayley et al., 2003; Stam, 2007). In addition to neuropeptide and neurotransmitter processes, alterations to inflammatory immune activity have also

been implicated in the progression of stressor-related pathologies (Maes, 1995; Anisman et al., 2008; Dantzer et al., 2008). In this regard, previous research has examined the role that stressors play in alterations of plasma and brain concentrations of pro-inflammatory cytokines (signalling molecules between immune cells) without a present immune challenge (Maes et al., 1998; Bartolomucci et al., 2003). Aggressive social stressors have been associated with alterations to brain cytokine mRNA expression (Audet et al., 2010; Bartolomucci et al., 2003). To this end, aggressive social stressors have previously shown to affect inflammatory immune processes, including variations of pro-inflammatory cytokines, namely interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α in blood as well as stressor sensitive brain regions, including the PFC and hippocampus (Audet, et al., 2010). Given that previous research has shown that cytokines are influenced by stressors, including social stressors (Bartolomucci et al., 2003), and have been implicated in the progression of depressive illness such as anxiety and depression(Anisman et al., 2008; Dantzer et al., 2008), the present investigation was conducted, in part, to determine whether acute and repeated episodes of a psychosocial stressor comprising aggressive interactions would elicit plasma and brain cytokine mRNA expression, and whether these cytokine variations would be differentially influenced when compared to a physical restraint stressor. Moreover, the present investigation assessed whether social stressors would sensitize cytokine mRNA expression within the PFC upon a later social defeat challenge.

1.8. Sensitization and cross-sensitization effects of re-exposure

It is well known that stressful events have an impact on the neurochemical and behavioural systems of an organism and that these actions are immediate and often short lasting. Of particular interest however, is the notion that previous stressful events may influence the response to subsequent stressor experiences. It is clear from animal studies that exposure to stressors typically provoke physiological alterations that are short in duration. However, if an animal is re-exposed to the same stressor, the ensuing neurochemical changes occur more rapidly, perhaps because the neurochemical system has been "primed" by the previous stressor experience (Anisman & Merali, 1999; Anisman et al., 1993; Anisman & Sklar, 1979). While it appears that this process would be adaptive, the neurochemical changes associated with the stressor experience can have profound and lasting consequences that may ultimately lead to the development and progression of certain pathologies.

Stressor induced sensitization effects have been observed in monoamine activity in several brain regions, including increased NE release in the hypothalamus, hippocampus and amygdala, as well as increased DA release in the PFC (Anisman et al., 1993). Interestingly, these sensitization effects have not only been observed when the re-exposure session involves the same stressor, but cross-sensitization can occur when the re-exposure session is different from the initial stressor exposure (Anisman 1993; Tilders & Schmidt, 1999). Interestingly, the neurotransmitter alterations noted in PTSD patients and animal models of PTSD also seem to reflect a sensitization of biological systems, important in the regulation of the stress response and homeostasis (Geracioti et al., 2001).

1.9. The Proposed Research

Given the potential interaction between initial stressor exposure and re-exposure, the present investigation assessed whether social stressors can "prime" neurochemical systems, leading to increased vulnerability when exposed to subsequent stressors, reflected by anxiety and depressive-like symptoms. We also examined whether sensitization effects are dependent on re-exposure to the same social stress or if a dissimilar physical stressor can provoke these same changes. In effect, we are examining whether a cross sensitization effect occurs between two different types of stressors upon re-exposure. Previous reports have indicated that the rodent model of social defeat has the ethological relevance of examining social subordination (Malatynska and Knapp, 2005), and that social defeat has face validity in its ability to model the symptomatology of stress-related disorders like PTSD and depression (Avgustinovich et al., 2005; Martinez et al., 1998). As a result, for the present study, the first set of studies assessed the behavioural effects of a social defeat stressor (induced by 3 consecutive days of 15 min exposure to a dominant retired breeder) combined with a re-exposure stressor thirty days later. We were interested in determining whether these behavioural alterations occurred among both similar and dissimilar re-exposure stressors. Animals underwent two different behavioural tests including; the SPT, and EPM test under normal conditions. For testing done in the EMP, animals were tested two hours following re-exposure to capture the relatively immediate behavioural changes. Animals were tested again two weeks after re-exposure to determine whether the initial behavioural changes were sustained and to capture any protracted effects of re-exposure. For the SPT, animals

were tested over the course of a 24 hour period at two time points; immediately after re-exposure and again 2 weeks after re-exposure.

In a second set of experiments we examined blood plasma corticosterone levels as well as several brain monoamine levels in the hippocampus and PFC, three minutes after re-exposure. Specifically, brain levels of NE and 5-HT, and their metabolites, MHPG and 5-HIAA, were determined by HPLC in both the hippocampus and PFC and brain levels of DA and its metabolite DOPAC were determined by HPLC in the PFC. Furthermore, we also examined the expression of several genes associated with stress and the stress response, which included 5-HT receptors as well as pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) that have also been implicated in depression. In effect, we are examining the link between changes in the behavioural and neurochemical systems of stressor exposed animals to specific pathological features of PTSD, namely sensitization to stress and increased arousal. We predict that the behavioural, neuroendocrine and neurochemical changes will follow those associated with animal models of PTSD.

Finally, we assessed whether a β-blocker that acts as an anxiolytic drug (propranolol) would attenuate the effects of a previous stressor, when administered prior to the re-exposure of either the same stressor or a dissimilar stressor. Previous research has shown that during a learning task, when epinephrine is increased (either exogenously or endogenously) memory consolidation and fear conditioning is enhanced (Pitman et al., 2002). Therefore, if an excess of epinephrine during and soon after a traumatic event enhances consolidation of that traumatic memory (Pitman, 1989) there is a possible preventative opportunity by blocking β-adrenergic receptors, via propranolol administration. Propranolol is a non-selective beta blocker, that is, it blocks the action of

epinephrine and norepinephrine on both β_1- and β_2-adrenergic receptors (Orr et al., 2000). Prolonged states of adrenergic activation following trauma exposure has been linked to increased risk for PTSD and increased symptomatology related to PTSD, due to increased fear conditioning (Orr et al., 2000). Propranolol works to inhibit the actions of norepinephrine and studies have shown that individuals given propranolol immediately after a traumatic experience show less severe symptoms of PTSD compared to their respective control groups that did not receive the drug (Vaiva et al., 2003). Furthermore, a recent study indicated that β adrenoceptor blockade can disrupt re-consolidation of conditioned fear at very long intervals after the initial shock conditioning when re-exposed to a subsequent shock (Stam, 2007). Based on these findings, it lends support to the possibility that the conditioned elements in the psychiatric symptoms of PTSD patients may be treated by blocking noradrenalin action prior to a reminder or re-experience of the trauma. Based on these recent studies, it is believed that when propranolol is administered prior to re-exposure to similar stressors, it may lead to decreased physiological responding during re-exposure (Brunet et al., 2008).

Furthermore, administration of propranolol prior to re-exposure may block the consolidation of the traumatic memory, leading to decreased behavioural alterations (Brunet et al., 2008). By pairing the drug induced anxiolytic (calm) state and the re-experiencing of emotionally traumatic memories, the psychological turmoil that occurs may be less intense and an individual will experience decreased sensitization to the stressor as a result. The third study was conducted to assess whether the behavioural alterations associated with stressor re-exposure can be attenuated by administration of the β-blocker propranolol. To this end, we assessed behavioural outcomes in the EPM, of

mice that were treated systemically with propranolol prior to the re-exposure session. For this experiment, animals were tested 2 hours after exposure on the test day to determine the behavioural effects of propranolol administration prior to exposure on the test day.

2. Materials and Methods

2.1 Subjects

Naïve adult male CD-1 strain mice (6-8 weeks of age), obtained from Charles River Laboratories (St. Constant, Quebec), were used in each of the present studies. Animals were singly housed in 27 x 21 x 14 cm polypropylene cages. Mice were kept in a temperature (22°C) and humidity (63%) controlled room and maintained on a 12 hour light: 12 hour dark cycle (lights on at 08:00 hours). Animals were permitted approximately 1 week to acclimatize to the vivarium prior to becoming experimental subjects. All animals were given *ad libitum* access to food (Ralston Purina) and water. All experiments complied with the current guidelines set by the Canadian Council on Animal Care (CCAC) and were approved by the Carleton University Animal Care Committee. A schematic description of the experimental design for each of the 5 experiments is depicted in Figure 1.

Insert Figure 1 about here

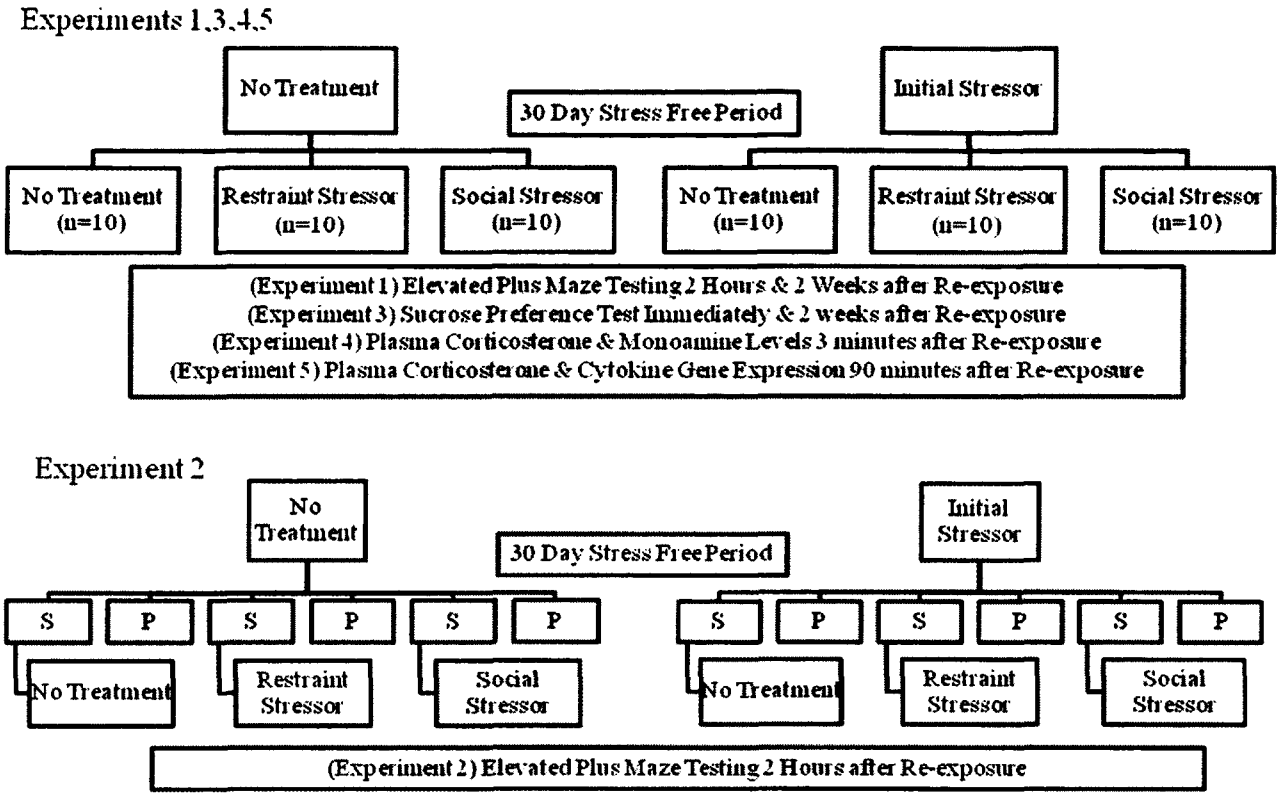

Figure 1. A schematic description of the experimental design for each of the 5 experiments. S = saline, P = propranolol.

2.2. Initial stressor exposure phase

All procedures were conducted between 0830 and 1300 h to minimize effects related to diurnal factors. During a social defeat session, a naïve mouse was individually introduced to the home-cage of a resident mouse (a male retired breeder) and direct interactions were permitted for 15 min. As wounds resulting from aggressive interactions could influence immune functioning (Merlot et al., 2003), the stressor procedure permitted physical contact, but injuries were limited by curtailing aggressive behaviours by lightly shaking or knocking the side of the cage. For ethical reasons, if aggression

escalated further, the mice were separated by a mesh partition that allowed them to see and smell each other, but physical interaction was prevented. After the 15-min session, the intruder mouse was returned to its home-cage. This stressor procedure was undertaken on each of three consecutive days (Initially stressed group; All Experiments). New pairs of ''intruder/resident'' mice were formed for each stressor session among the three consecutive days. Naïve mice that served as controls remained in their home-cages during the whole duration of the initial stressor phase (No stressor group).

2.3. Challenge test phase

In all experiments, after the initial stressor phase, mice of each condition remained undisturbed in their home-cages for thirty days, to ensure sufficient time had passed in order to examine any sensitization effects. Following the thirty day period of rest, animals were exposed to either another social defeat episode, a physical restraint challenge, or were not challenged (N = 10/group). On the day preceding this test phase, mice were brought to the testing area to permit adaptation to the new environment. On the test day, mice in the social stressor condition were confronted with a new retired breeder for 10 min (i.e., exactly as described in the initial stressor phase section). Animals in the restraint stressor condition were placed in a clear plastic baggie restraint for 10 min. Animals that received no stress on the test day remained undisturbed in their home-cages. Those mice that were not challenged on the test day were maintained in a separate room to preclude cues from stressed mice (e.g., pheromones during aggressive encounters) influencing non-stressed mice.

2.4. Elevated Plus Maze (EPM)

The EPM consisted of two open arms and two arms enclosed by 15 cm high walls. The EPM is predominantly used to assess anxiety-like behaviour in rodents (Pellow et al., 1985). The open arms are perpendicular to the closed arms, which intersect to form a maze in the shape of a plus sign (+). Each arm is approximately 30 cm in length and 5 cm in width. The intersection of the four arms creates a centre or middle section of the maze which is a 5cm x 5cm space. The EPM was elevated approximately 45 cm above the floor. Refuge by the animal is sought out by entering the closed arms while entry into the open arms indicates exploratory behaviour and lower anxiety. A video camera was mounted above the maze, allowing for recording and subsequent scoring of the trials.

Mice were placed in the testing room directly after exposure on the test day where they were permitted two hours of acclimatization to the room. Two hours after re-exposure, each mouse was placed into the back of a closed arm (facing away from the middle). The same closed arm for mouse entry was used across trials and testing days and the maze remained in the same position within the room, across trials and testing days. Mice were subsequently tested 2 weeks after exposure on the test day. The behaviour in the maze for each trial was monitored for 5 minutes and scored as follows: (1) frequency of entries in open arms (all four paws on an open arm); (2) time spent in the open arms; (3) frequency of entries in the closed arms; (4) time spent in closed arms; (5) latency to enter an open arm; and (6) frequency of stretch attenuations into open arms. Following each trial, the EPM was cleaned with 70% ethanol to avoid any scent distractions during subsequent trials.

2.5. Sucrose Preference Test (SPT)

The SPT has typically been used in rodents as a measure of anhedonia (Moreau et al., 1992; Plasnik et al., 1989). The decreased sensitivity to the rewarding aspect of a sweetened substance might reflect anhedonia in rodents, defined as the inability to experience pleasure, which is a core symptom of depression in humans (Willner, 1985). The SPT was conducted to determine whether the effects of stressor exposure would be enhanced given previous stressor exposure and whether sucrose preference is differentially affected by social (emotional) and physical stressors. The SPT was conducted over 24 hour periods on two separate occasions as follows; immediately after stressor re-exposure, and 2 weeks after re-exposure. In the SPT, mice had access to two 200 ml bottles which contain either tap water or a 2% sucrose solution. Intake volume of sucrose and water for each animal is determined based on the change in bottle weights, measured prior to consumption vs. after the 24 hour consumption period.

2.6. Experiment 1

To test the behavioural effects of stressor re-exposure under different re-exposure conditions, mice were randomly assigned to one of two separate conditions comprising either no treatment or an initial stressor treatment, consisting of three consecutive days of an aggressive social stressor regimen. The social stressor consisted of animals being exposed to a dominant, retired breeder (Charles River Laboratories, St. Constant, Que.) for 15 minutes on each of the three consecutive days. Mice were then returned to their home cage and after a 30 day stress-free period, they were further subdivided into three different conditions.

Mice received (a) no treatment, (b) re-exposure to a social stressor for 10 minutes or (c) exposure to a novel physical stressor (baggie restraint) for 10 minutes (N=10/group). The novel stressor (restraint stress) involved placing mice in a transparent, triangular shaped polyethylene bag, made from Fisherbrand specimen bags (01-816B), with a small hole at the end to allow mice to breathe. The bag was snug fitting and restricted movement of the mice. Two hours after re-exposure, animals were tested in the EPM for five minutes. Mice were tested again in the EPM 2 weeks after re-exposure to examine the residual effects of the treatment conditions. Animals were sacrificed using CO_2 following the testing at 2 weeks.

2.7. Experiment 2

To examine whether stressor induced behavioural alterations were attenuated by pharmacological intervention prior to re-exposure, mice were randomly assigned to the same conditions as noted in Experiment 1 (no treatment/initial social stress and subsequent re-exposure to no treatment/social stress/restraint stress); however, prior to receiving the re-exposure conditions, mice received either an intraperitoneal (*i.p.*) injection of propranolol (5mg/kg) or an equivalent dose of saline (vehicle). At this dosage, studies have found that propranolol does not affect locomotor and exploratory activity while still being effective at reducing the effects of predator stress on several behavioural measures of anxiety including: social interaction, hole board, light/dark box, elevated plus maze, and after re-activation of memory in an inhibitory avoidance procedure (Adamec et al., 2007; Przybyslawski et al., 1999). Propranolol was dissolved in 0.9% sodium chloride. Controls received an equivalent volume of 0.9% saline solution

in the same manner, controlling for potentially stressful effects of the i.p. injection. The drug/vehicle solutions were freshly prepared and injected at the same time of day in a volume of 1ml/kg body weight. Following the injection, mice rested for thirty minutes and were exposed to their assigned treatment conditions. To examine the immediate effects of propranolol administration prior to re-exposure on the test day, animals were tested in the EPM for five minutes, two hours after exposure on the test day. Animals were sacrificed using CO_2 following the testing.

2.8. Experiment 3

As in experiment 1, mice were randomly assigned to the same six conditions. The present experiment examined whether the combined stressor exposure can alter sucrose preference and whether these changes are dependent on the type of re-exposure stressor experienced. Immediately following stressor re-exposure, animals were placed back into their home cages and sucrose preference was tested over a 24 hour period. Sucrose preference was tested again 2 weeks after the final exposure on the test day, also over a 24 hour period. Animals were sacrificed using CO_2 following the testing at 2 weeks.

2.9. Experiment 4

In order to examine the neuroendocrine and neurochemical alterations as a result of stressor re-exposure, mice received the same experimental treatments described in Experiment 1. In a recent investigation, mice were sacrificed three minutes after single and repeated confrontations of social defeat and plasma corticosterone levels as well as 5-HT utilization within the PFC and hippocampus were increased in both submissive and

dominant animals (Audet and Anisman, 2009). Similarly, as previously reported, levels of corticosterone are increased shortly after an aggressive interaction (Bhatnagar et al. 2006; Keeney et al. 2001, 2006) and these elevations were not attributable to handling, cage transfer, or exposure to novelty. In order to test the immediate effects of single and repeated stressor exposure we chose to sacrifice animals 3 minutes following stressor exposure on the test day. Mice were decapitated three minutes after re-exposure, and both trunk blood and brain tissue was collected for plasma corticosterone and HPLC analysis.

2.10. Experiment 5

In order to determine alterations to brain cytokine mRNA expression, a parallel study was conducted following the same protocol described above in experiment 1. However, mice were decapitated 90 minutes after re-exposure and both trunk blood and brain tissue were collected for plasma corticosterone and qPCR analysis. This time point was selected based on an earlier study (Audet et al., 2010) showing that in defeated mice the mRNA expression of prefrontal pro- inflammatory cytokines was sufficiently elevated even after only 75 minutes as well as previous reports (Gibb et al., 2008, 2011) that showed that plasma corticosterone levels and prefrontal cytokine mRNA expression were markedly increased 90 min following exogenous LPS treatment.

2.11. Plasma Corticosterone Analysis

Blood plasma was analyzed to determine corticosterone levels in response to the three re-exposure conditions following initial treatment. Mice were brought into a separate room and sacrificed by rapid decapitation. Trunk blood was collected in tubes

containing 10μg EDTA, centrifuged for 8 min at 3600 rpm, and the plasma aliquoted stored at -80°C until analyzed. Blood plasma was collected 3 minutes after re-exposure and in a parallel study, collected 90 minutes after re-exposure, to examine changes to circulating corticosterone over time. Plasma levels were quantified using a commercial radioimmunoassay (RIA) kit (ICN Biomedicals Inc., CA.) according to the manufacturer's instructions. For each of two the studies involving plasma corticosterone analysis, corticosterone was determined in a single run to avoid inter-assay variability; the intra-assay variability was less than 10%. All plasma corticosterone determinations were completed by an Anisman Lab Research Associate, Dr. Jerzy Kulczycki.

2.12. Brain Dissection Technique

Following live decapitation, brains were quickly removed and placed on a stainless steel brain matrix (2.5 x 3.75 x 2.0 cm) situated on a block of dry ice. The brain matrix had a series of slots spaced approximately 500μm apart. Brains were sectioned into a series of coronal slices using razor blades. Brain sections were placed on glass slides resting on a bed of dry ice and, using the mouse brain atlas of Franklin and Paxinos (1997), the PFC and hippocampus were removed by micro-punch using hollow 16 and 20 gauge microdissection needles with a bevelled tip. The tissue punches were placed in 0.3M monochloroacetic acid containing 10% methanol and internal standards, and stored at -80°C for subsequent determination of monoamine and cytokine mRNA expression.

2.13. High Performance Liquid Chromatography (HPLC)

Levels of DA, NE and 5-HT, and their respective metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxy-4-hydroxyphenylglycol (MHPG) and 5-hydroxyindoleacetic acid (5-HIAA), were determined by HPLC. Tissue punches were sonicated in a solution derived from an initial mix of 500 mL of HPLC grade water, 5.0 mL methanol, 0.0186 EDTA and 14.17 g of monochloroacetic acid. Following centrifugation, 20 µl of supernatant was passed through a system consisting of M-600 pump (Milford, MA), guard column, radial compression column (5 m, c18 reverse phase, 8 mm· 10 cm) as well as three cell coulometric electrochemical detectors (ESA model 5100A) at a flow rate of 1.5 ml/min (1400–1600 psi). The mobile phase used for separation was made up of 1.3 g of heptane sulfonic acid, 0.1 g of disodium EDTA, 6.5 ml of triethylamine and finally 35 ml of acetonitrile. After filtering (using .22-mm filter paper) and degassing the mobile phase, the pH levels were adjusted to 2.5 using phosphoric acid. The height and area of the peaks were determined through a Hewlett-Packard integrator. The protein content of each sample was assessed using bicinchoninic acid in addition to a protein kit (Pierce Scientific, Brockville, Ont.), and a spectrophotometer (Brinkman, PC800 colorimeter). The lower limit of detection for the monoamines and metabolites was 5.0 pg/ml. All HPLC analyses were completed by the Anisman Research Associate, Dr. Jerzy Kulczycki.

2.14. Reverse transcription-quantitative polymerase chain reaction analysis

Brain tissue punches were homogenized using Trizol and total brain RNA was isolated according to the manufacturer's protocol (Invitrogen; Burlington, ON, Canada).

The total RNA was then reverse transcribed using Superscript II reverse transcriptase (Invitrogen; Burlington, ON, Canada). The resulting cDNA aliquots of this reaction were analyzed in simultaneous quantitative polymerase chain reactions (qPCR). Primers were purchased from Invitrogen Life Technologies (Burlington, ON, Canada). For qPCR, SYBR green detection was used according to the manufacturer's protocol (Stratagene Brilliant qPCR kit). A Stratagene MX-4000 real time thermocycler was used to collect the data. All PCR primer pairs used generated amplicons between 147 and 344 bp. Amplicon identity was verified by restriction analysis. Primer efficiency was measured from the slope relation between absolute copy number or RNA quantity and the cycle threshold using the MX-4000 software. All primer pairs had a minimum of 90% percent efficiency.

Primers that amplify glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were used as a control to normalize the data. GAPDH was used for normalization because this gene showed to be a stable housekeeping gene (Gutkowska et al., 2007). Although there was inter-subject variability in the cycle threshold (Ct) for GAPDH, there were no significant differences in the average Ct across the treatment groups ($p > .05$) within the PFC and hippocampus. To compensate for inter-individual variability that ordinarily exists within the assay, the relative expression of the RT-PCR products was determined. The expression of each gene of interest within the PFC and hippocampus was normalized by subtracting the cycle threshold (Ct) of GAPDH from the gene of interest Ct (ΔCt). The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008) was then used to convert ΔCt values to mRNA fold changes relative to the non-stressed-non-stressed control group (calibrator).

Primer sequences were as follows: Mus Synaptophysin, sense: GGA CGT GGT GAA TCA GCT GG, antisense: GGC GAA GAT GGC AAA GAC C; Mus GAPDH, sense: GGT CGG TGT GAA CGG ATT TG, antisense: TGC CGT TGG AGT CAT ACT G; Mus IL-1β, sense: TGT CTG AAG CAG CTA TGG CAA C, antisense: CTG CCT GAA GCT CTT GTT GAT G; Mus IL-6, sense: ACG GCC TTC CCT ACT TCA CA, antisense: TGC CAT TGC ACA ACT CTT TTC TC; Mus TNF-α, sense: CTC AGC CTC TTC TCA TTC CTG C, antisense: GGC CAT AGA ACT GAT GAG AGG G; Mus 5-HT1A (Htr1a) sense: CAT GGG CAC CTT CAT CC; antisense: TTG AGC AGG GAG TTG GAG TAG C, Mus 5-HT1B (Htr1b) sense: GTC AAA GTG CGA GTC TCA GAC G; antisense: ACA GAT AGG CAT CAC CAG GGA G.

2.15. Statistical Analyses

Data are presented as Means ± SEM. Behaviour in the EPM was analyzed using a 2 (initial stress) x 3 (re-exposure condition) repeated measures (2 hours & 2 weeks) analysis of variance (ANOVA) separately for each of the behavioural measures assessed. When propranolol was administered prior to re-exposure, EPM behaviour was analyzed using a 2 (initial stress) x 3 (re-exposure condition) x 2 (injection) between subjects ANOVA separately for each of the behavioural measures assessed. Monoamines (NE and 5-HT) and their respective metabolites (MHPG, and 5-HIAA) were analyzed independently in the hippocampus and PFC using a 2 x 3 between subjects ANOVA, considering both initial treatment and re-exposure condition. DA and its metabolite DOPAC were analyzed independently in the PFC using a 2 x 3 between subjects ANOVA, considering both initial treatment and re-exposure condition. Plasma

corticosterone was analyzed independently, also using 2 x 3 between-subjects ANOVA. Following the procedure described by Livak and Schmittgen (2001), the Ct values were converted to mRNA fold changes relative to animals in the control-control condition. The analyses of the mRNA fold changes for each gene, in the PFC comprised a 2 x 3 between subjects ANOVA in which, initial stressor and subsequent re-exposure condition served as between group variables. Follow up comparisons of the means comprising main effects and simple effects of significant interactions for all analyses were conducted through multiple comparisons with a Bonferroni correction to maintain the alpha level at 0.05.

3. Results

3.1. Experiment 1: Plus maze performance among stressed and non-stressed mice that either received prior social stressor exposure or not.

The behaviour of mice tested in the elevated plus maze was affected by both the type of re-exposure stressor and whether or not the animal had previously been exposed to an intense social stressor thirty days prior. The latencies to enter the open arms of the plus maze and the time spent in the open arms were relatively variable and did not differ as a function of initial stressor exposure or the type of stressor treatment administered upon the re-exposure day (Figures 2 & 3.). The latency to enter the open arms as well as the time spent in the open arms varied as a function of the within subjects factor Time (time of testing; 2 hours and 2 weeks), $F_{(1,54)} = 18.17, p < .001$ and $F_{(1, 54)} = 18.36, p < .001$ respectively. Follow up tests indicated that animals took longer to enter the open

arms of the plus maze ($p < .001$) and spent less time in the open arms ($p < .001$) 2 weeks after re-exposure compared to testing done 2 hours after re-exposure (Figures 2 & 3).

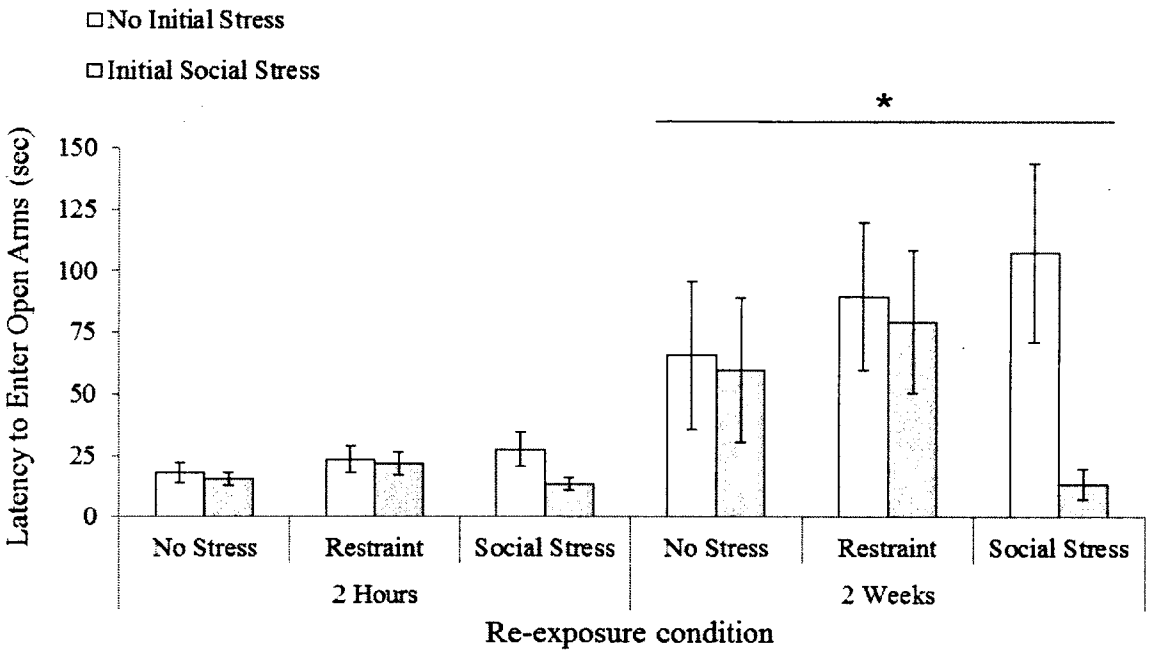

Figure 2. Latency to enter an open arm (Mean ± SEM) among initially stressed and non-stressed mice 2 hr and 2 weeks after re-exposure to no stressor, a physical stressor, or a social stressor. * $p < .001$ relative to animals tested 2 hours after re-exposure.

Insert Figure 3 about here

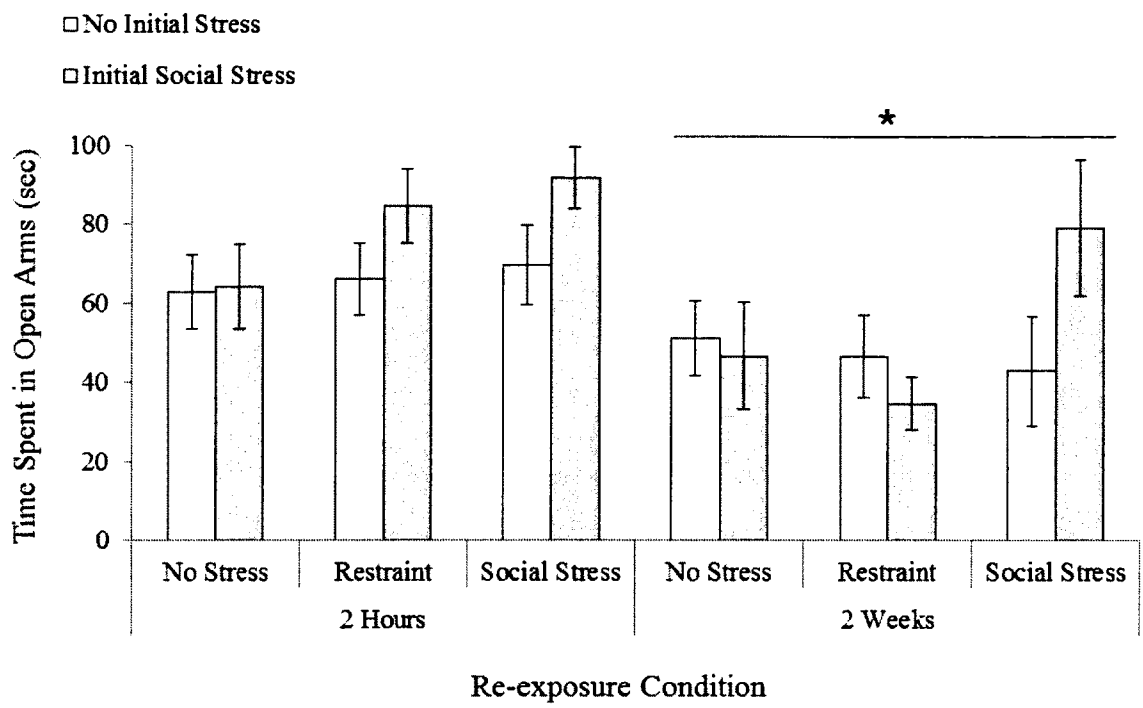

Figure 3. Time in open arms (Mean $\pm$ SEM) among initially stressed and non-stressed mice 2 hrs and 2 weeks after re-exposure to no stressor, a physical stressor, or a social stressor. * $p < .001$ relative to animals tested 2 hours after re-exposure.

The number of open arm entries varied as a function of the Time of Testing by Initial Exposure by Re-exposure interaction, $F_{(2,54)} = 4.08$; $p < .05$ (Figure 4). Analyses of the simple effects comprising this interaction indicated that among initially stressed mice that were re-exposed to a social stressor on the test day made *more* open arm entries 2 hours after re-exposure compared to mice that received no stress on the test day ($p < .05$), but not mice that received a physical stressor on the test day. Follow up tests also confirmed that these effects were sustained up to 2 weeks after re-exposure, and initially stressed mice that were re-exposed to a social stressor continued to make *more* open arm entries compared to both the control group and the physical restraint group, $p < .05$, and p

< .01 respectively (Figure 4). Interestingly, among animals that received a social stressor on the test day, animals that had been previously initially stressed made significantly *more* entries into the open arms compared to animals that had no previous stressor exposure, $p < .05$. Furthermore, when tested 2 weeks after exposure, initially stressed mice that subsequently received a physical restraint stressor made significantly fewer open arm entries compared to testing done 2 hours after exposure, $p < .05$.

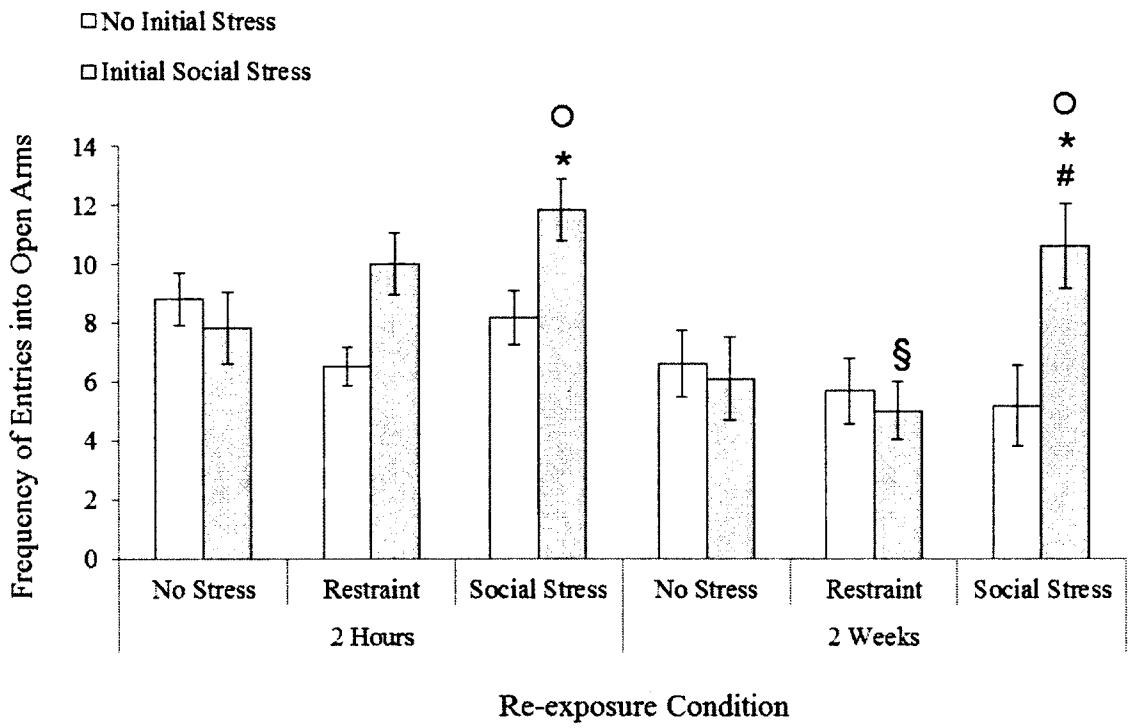

Figure 4. Frequency of open arm entries (Mean ± SEM) among initially stressed and non-stressed mice 2 hr and 2 weeks after re-exposure to no stressor, a physical stressor, or a social stressor. * $p < .05$ relative to similarly treated animals that received no stress at re-exposure. # $p < .01$ relative to similarly treated animals that received a restraint stressor at re-exposure. § $p < .05$ among animals that were initially stressed and that received a restraint stressor on the test day tested 2 weeks after exposure relative to testing done 2 hours after exposure. O $p < .05$ relative to animals that received a social stressor on the test day but that had no previous stressor exposure.

In contrast to the behaviour in the open arms, the frequency of entries into the closed arms varied significantly as a function of the Re-exposure Condition, $F_{(2, 54)} = 4.99$; $p < .01$, but not the Initial Exposure condition, $F < 1$. Follow up tests confirmed that animals that were exposed to a social stressor on the test day made *fewer* closed arm entries than animals that were exposed to no stressor on the test day, $p < .05$ (Figure 5). The frequency of entries into closed arms also varied as a function of the within subjects variable Time (time of testing; 2 hours & 2 weeks), $F_{(1,55)} = 13.90$, $p < .001$. Follow up tests indicated that animals made fewer entries into the closed arms 2 weeks after re-exposure on the test day compared testing done 2 hours after re-exposure, $p < .001$.

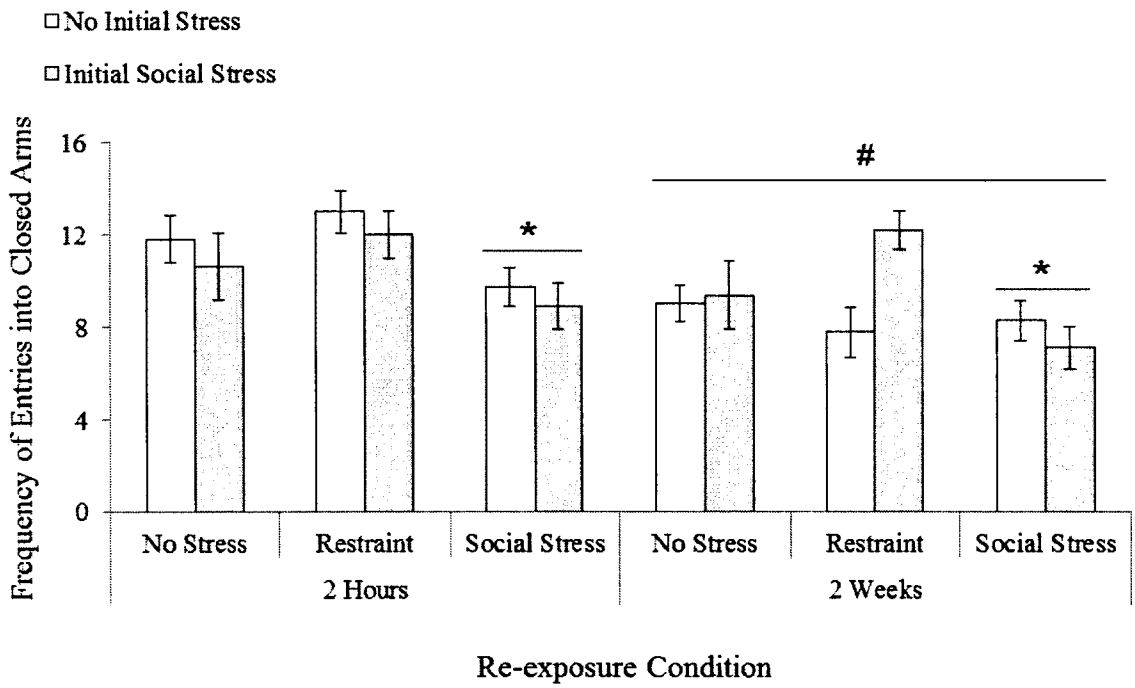

Figure 5. Frequency of closed arm entries (Mean ± SEM) among initially stressed and non-stressed mice 2 hr and 2 weeks after re-exposure to a social stressor, a physical stressor, or no stressor. * $p < .05$ relative to similarly treated animals that received no stressor on the test day. # $p < .01$ relative to animals tested 2 hours after re-exposure.

The time spent in the closed arms also varied as a function of Re-exposure Condition, $F_{(2, 54)} = 4.03$; $p < .05$, but not the Initial Exposure condition, $F = 1.18$, nor Time of Testing, $F < 1$. Follow up tests confirmed that animals that were exposed to a social stressor on the test day spent *less* time in the closed arms than animals that received no stress on the test day, $p < .05$ (Figure 6).

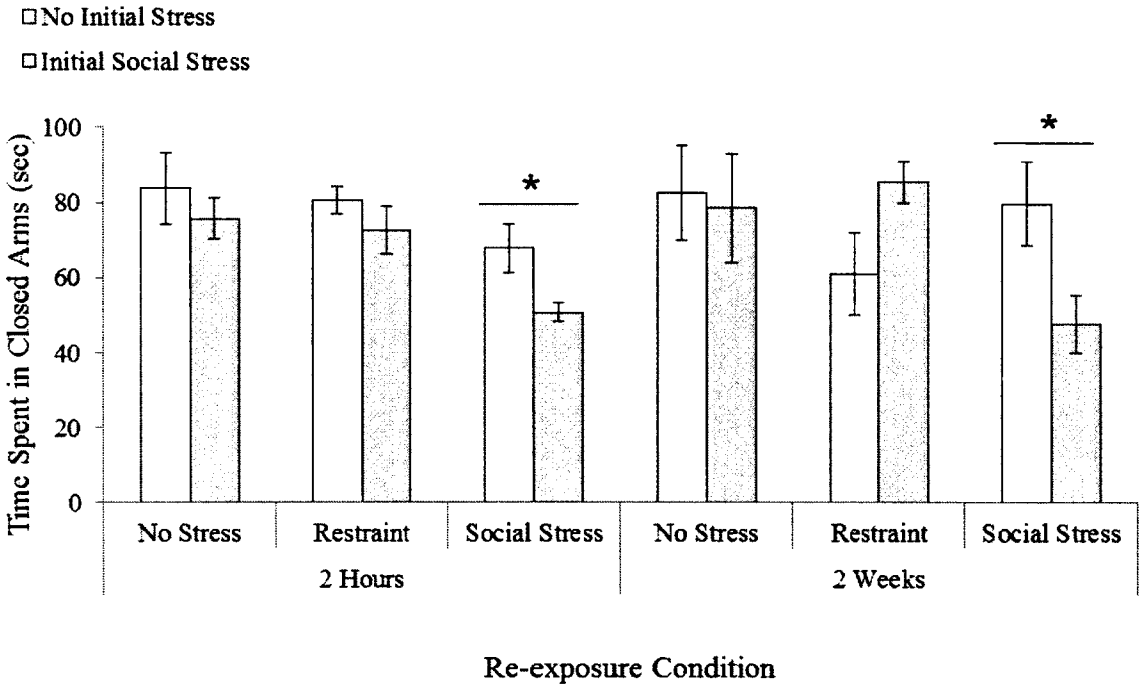

Figure 6. Time in closed arms (Mean ± SEM) among initially stressed and non-stressed mice 2 hr and 2 weeks after re-exposure to a social stressor, a physical stressor, or no stressor. * $p < .05$ relative to similarly treated animals that received no stress on the test day.

Both the Initial Exposure and Re-exposure conditions did not affect the number of stretch-attends directed at the open arms for any of the treatment groups, $p > .05$ (Figure 7). However, the number of stretch attends did vary as a function of the within subjects variable Time (time of testing; 2 hours & 2 weeks), $F_{(1,54)} = 78.56$, $p < .001$. Follow up tests indicated that animals made *fewer* stretch attends 2 weeks after re-exposure on the test day compared testing done 2 hours after re-exposure, $p < .001$.

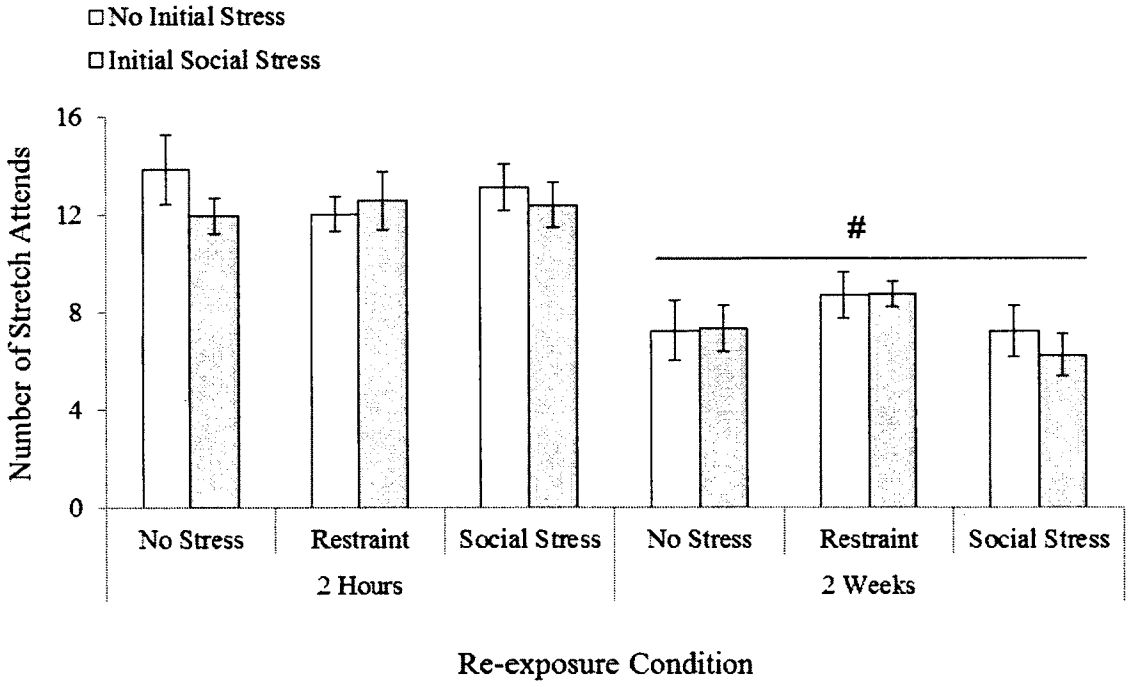

Figure 7. Number of stretch attends towards the open arms (Mean ± SEM) among initially stressed and non-stressed mice 2 hr and 2 weeks after re-exposure to a social stressor, a physical stressor, or no stressor. # $p < .01$ relative to animals tested 2 hours after re-exposure.

3.2. Experiment 2: Plus maze performance as a function of initial stressor exposure and re-exposure.

As in Experiment 1, the overall behaviour of mice in the EPM was affected by the type of stressor on the test day as well as whether the animal had previously been exposed to an intense social stressor or not. In the present experiment, the effects of receiving an injection prior to exposure on the test day also affected the animals' behaviour in the plus maze 2 hours after re-exposure on the test day. The latency to enter the open arms of the plus maze was relatively variable and did not differ as a function of Initial stressor exposure, Re-exposure treatment, or type of injection administered prior to exposure on the test day (Figure 8).

Insert Figure 8 about here

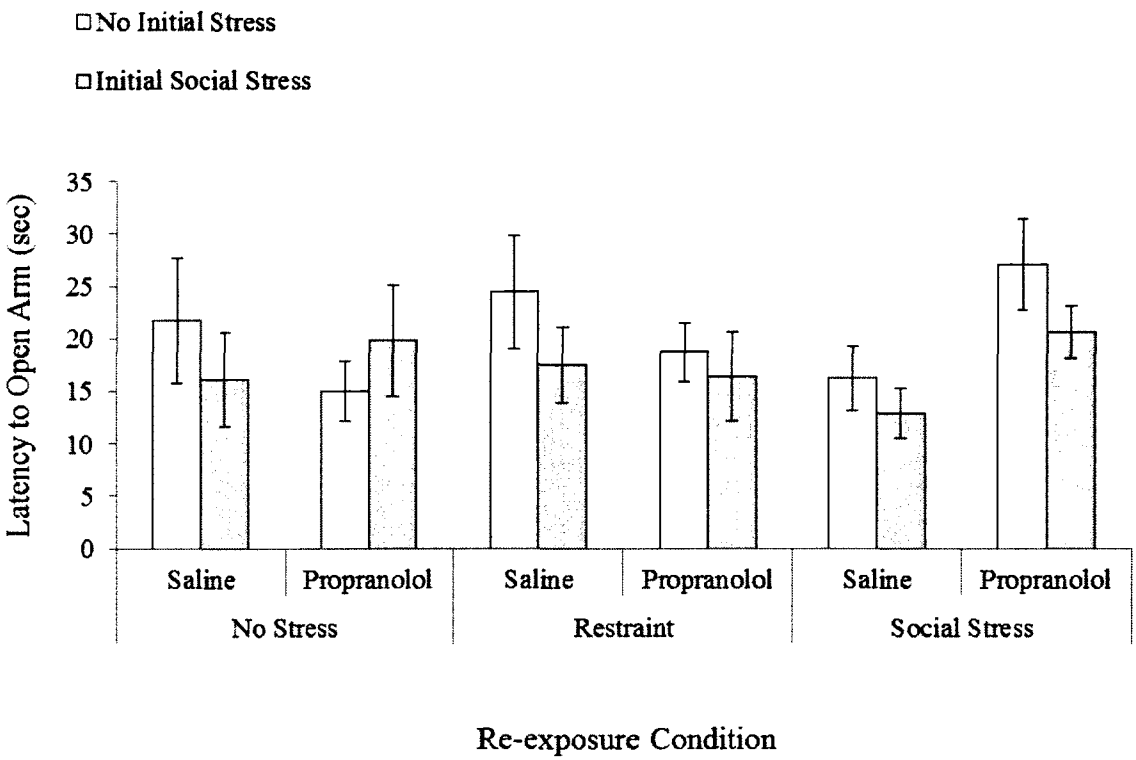

Figure 8. Latency to enter an open arm (Mean $\pm$SEM) among initially stressed and non-stressed mice 2 hrs after re-exposure to no stressor, a physical stressor, or a social stressor and who either received an injection of saline or propranolol.

In contrast, the number of entries into the open arms of the plus maze among mice that had initially received stressor exposure was greater than among animals that received no initial stressor exposure, $F_{(1, 114)} = 6.55$; $p < .05$ (see Figure 9). Entries into the open arms also varied as a function of the interaction between Re-exposure Condition and Injection treatment, $F_{(2,114)} = 3.91$; $p < .05$. As shown in Figure 9 and confirmed by multiple comparisons, among animals that were initially stressed and that were re-exposed to a social stressor on the test day, animals that received an injection of propranolol made significantly fewer entries into the open arms compared to animals that received an injection of saline, $p < .05$. Interestingly, among initially stressed animals that

were re-exposed to a restraint stressor, animals that received an injection of propranolol prior to re-exposure on the test day made significantly more entries into the open arms compared to animals that received a saline vehicle, $p < .05$ (Figure 9).

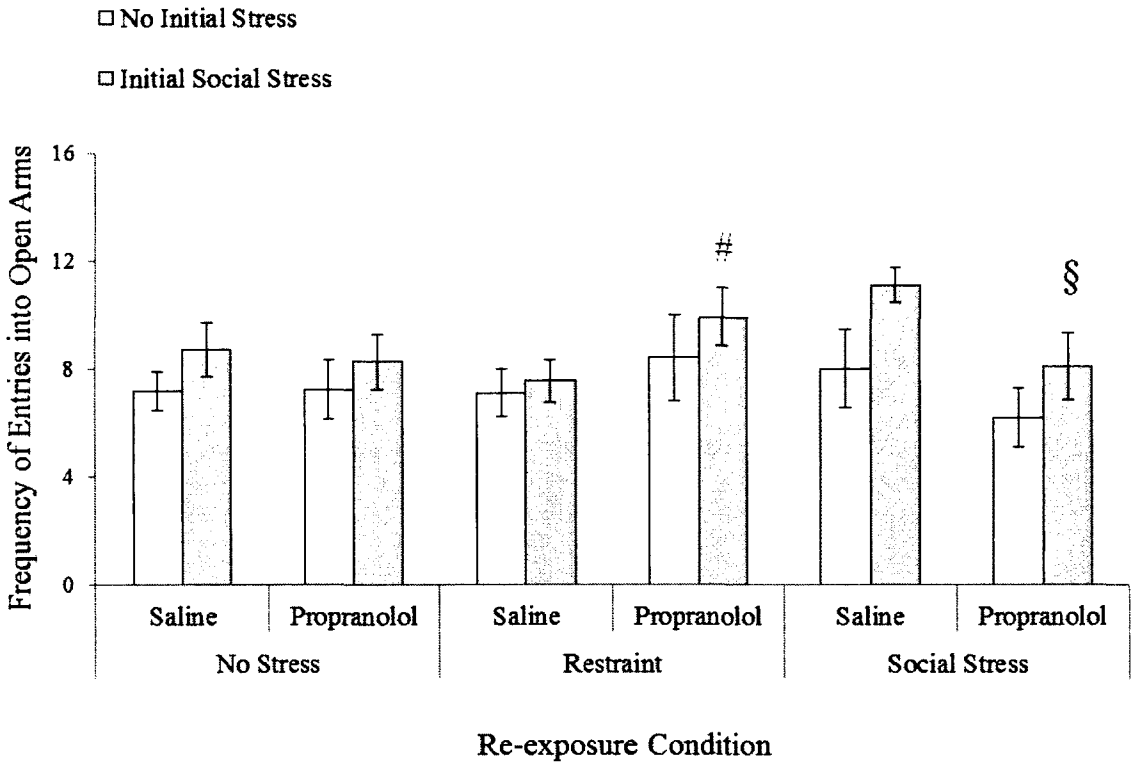

Figure 9. Frequency of entries into open arms (Mean ±SEM) among initially stressed and non-stressed mice 2 hrs after re-exposure to no stressor, a physical stressor, or a social stressor and who either received an injection of saline or propranolol. § $p < .05$ relative to initially stressed animals, re-exposed to a social stressor, that received a saline injection on the test day. # $p < .05$ relative to initially stressed animals, re-exposed to a restraint stressor that received a saline injection on the test day.

The amount of time spent in the open arms of the plus maze was relatively variable, but did vary as a function of the Initial Stressor treatment, $F_{(1,113)} = 25.2;3$ $p <$ .001. As shown in Figure 10, animals that received an initial stressor exposure spent significantly more time in the open arms of the plus maze compared to animals that were not initially stressed, $p < .001$. There was also a significant interaction between Initial stressor exposure and Re-exposure treatment condition, $F_{(2, 113)} = 9.21$; $p < .01$. Subsequent analyses of the simple effects that comprised this interaction revealed that, among initially stressed animals, those that received either a social or a physical stressor on the test day spent significantly more time in the open arms compared to animals that received no stress on the test day, regardless of the type of injection they received prior to exposure on the test day, p's $< .001$ (Figure 10).

Insert Figure 10 about here

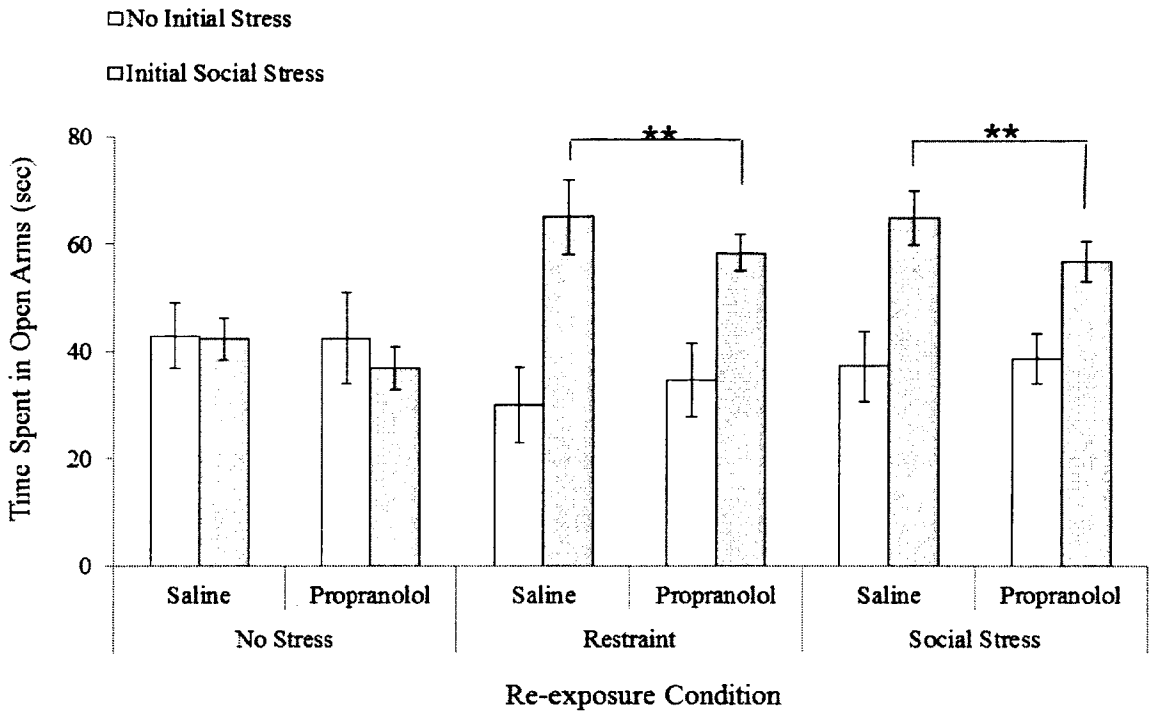

Figure 10. Time spent in open arms (Mean $\pm$SEM) among initially stressed and non-stressed mice 2 hrs after re-exposure to no stressor, a physical stressor, or a social stressor and who either received an injection of saline or propranolol. **p < .001 relative to similarly treated animals that received an initial social stressor but who received no stress on the test day, regardless of type of injection received (grey bars under no stress on the test day).

In contrast to the behaviour in the open arms, the number of entries into the closed arms did not differ as a function of initial stressor exposure, the type of stressor treatment on the test day, or the type of injection received on the test day (Figure 11).

Insert Figure 11 about here

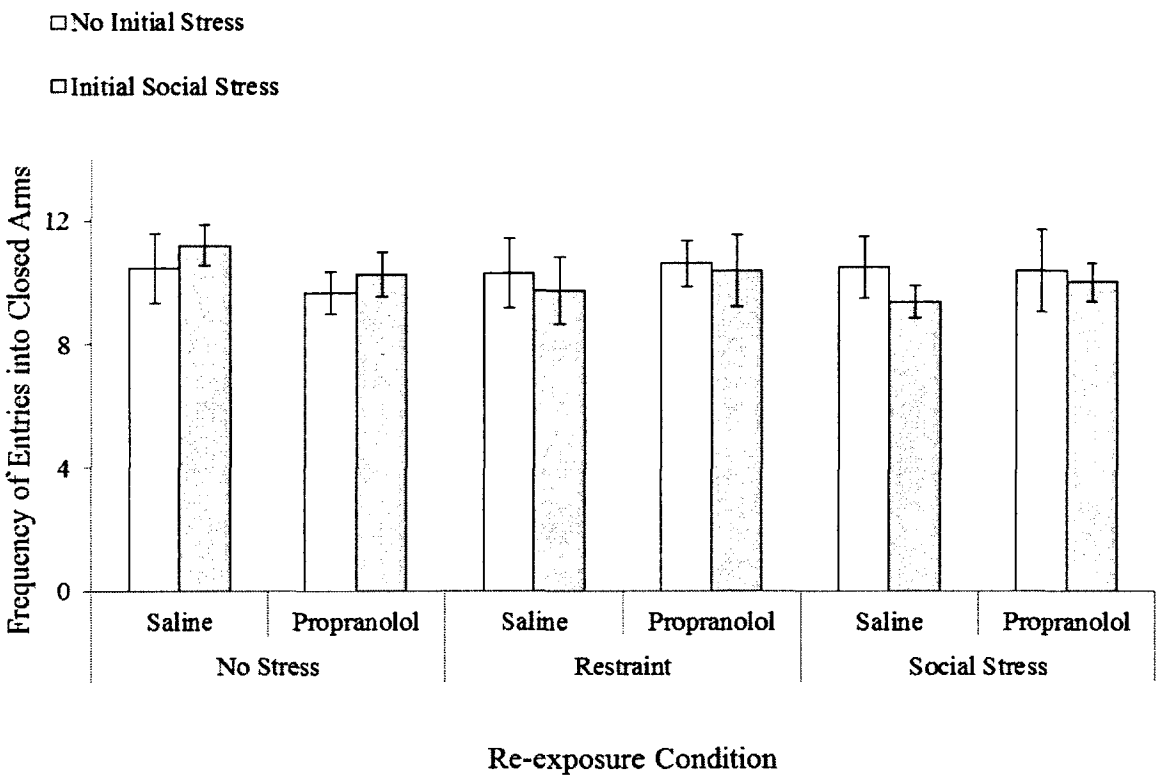

Figure 11. Frequency of entries into closed Arms (Mean $\pm$SEM) among initially stressed and non-stressed mice 2 hrs after re-exposure to no stressor, a physical stressor, or a social stressor and who either received an injection of saline or propranolol.

The time spent in the closed arms varied significantly as a function of the Initial Exposure as well as Re-exposure Treatment, $F_{(1,114)} = 6.50$; $p < .05$ and $F_{(1,114)} = 6.16$; $p < .01$ respectively. Given that initially stressed animals spent more time in the open arms, it is not surprising that animals that were initially stressed, spent significantly less time in the closed arms compared to animals that received no initial stress, regardless of the type of injection they received or the type of re-exposure treatment they received, $p < .05$ (Figure 12). Furthermore, animals that were exposed to a social stressor on the test day,

spent significantly less time in the closed arms compared to animals that received no stress on the test day, regardless of previous exposure or type of injection received, $p < .01$. There was also a significant interaction between Re-exposure condition and Injection treatment condition, $F_{(2,114)} = 4.70$ $p < .05$. Follow up comparisons revealed that among both initially stressed and non-stressed animals that later received no stress on the test day, animals that received an injection of propranolol on the test day, spent significantly less time in the closed arms compared to animals that received an equivalent saline injection, $p < .05$. Furthermore, among animals that were initially stressed and that received saline on the test day, animals that were re-exposed to a social stressor on the test day spent significantly less time in the closed arms compared to animals that received no stress on the test day, $p < .001$. Moreover, among animals that were initially stressed and that were re-exposed to a social stressor on the test day, animals that received an injection of propranolol spent significantly more time in the closed arms compared to animals that received a saline injection on the test day, $p < .05$ (Figure 12).

Insert Figure 12 about here

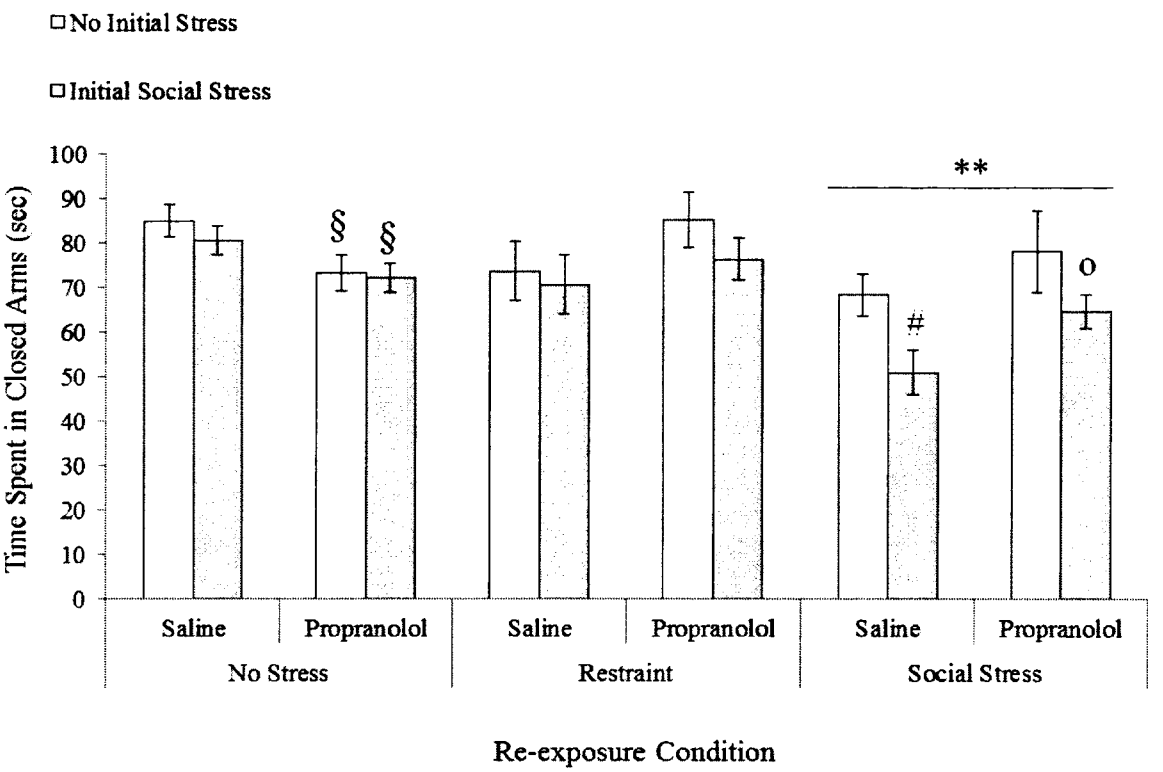

Figure 12. Time spent in closed arms (Mean ± SEM) among initially stressed and non-stressed mice 2 hrs after re-exposure to no stressor, a physical stressor, or a social stressor and who either received an injection of saline or propranolol. ** $p < .05$ relative to animals that received no stress on the test day. § $p < .05$ relative to similarly treated animals that received a saline injection on the test day. # $p < .001$ relative to similarly treated animals that received no stressor on the test day. O $p < .05$ relative to similarly treated animals that received an injection of saline on the test day.

The number of stretch attends towards the open arms did not differ as a function of initial stressor exposure, the type of exposure on the test day, or the type of injection received on the test day (Figure 13).

Insert Figure 13 about here

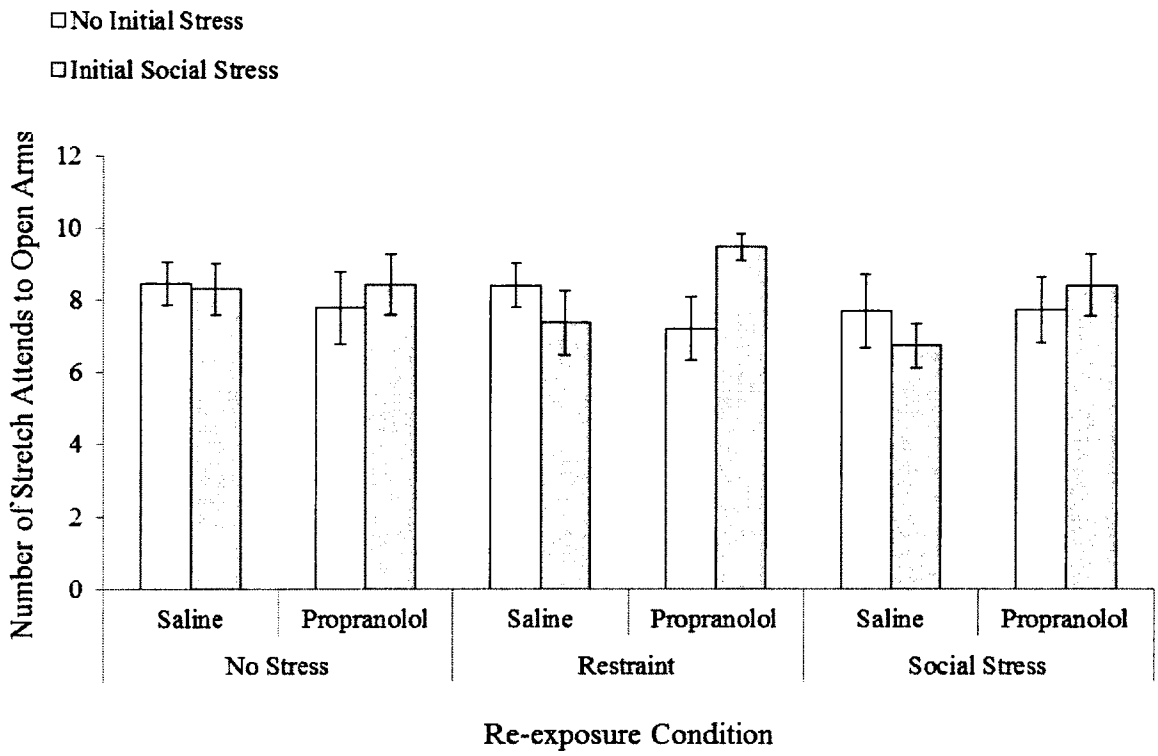

Figure 13. Number of stretch attends (Mean ± SEM) among initially stressed and non-stressed mice 2 hrs after re-exposure to no stressor, a physical stressor, or a social stressor and who either received an injection of saline or propranolol.

3.3. Experiment 3: Sucrose preference as a function of recent and earlier stressor exposure.

Sucrose consumption varied as a function of the Initial exposure by Re-exposure condition interaction, $F_{(2,48)} = 5.06$, $p = .01$, regardless of whether the testing was done immediately or 2 weeks after exposure on the test day. Analysis of the simple effects that comprise this interaction revealed that among animals that were initially stressed, animals that were re-exposed to a social stressor displayed significantly higher preference for sucrose compared to both non-stressed ($p < .05$) and animals that received a physical

restraint stressor on the test day ($p < .01$). Animals that received a physical restraint stressor exhibited a slight, non-significant decrease in sucrose preference (Figure 14).

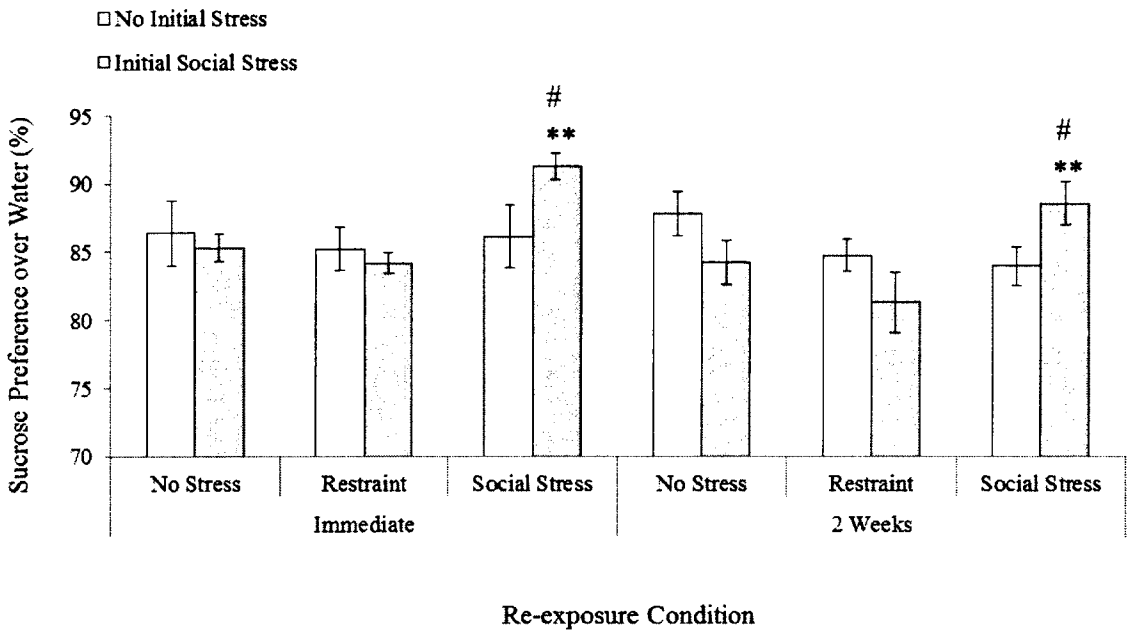

Figure 14. Sucrose preference (%Mean ± SEM) among initially stressed and non-stressed mice immediately and 2 weeks after re-exposure to no stressor, a physical stressor, or a social stressor. # $p < .05$ relative to non-stressed animals on the test day. ** $p < .01$ relative to animals that received a physical restraint stressor on the test day.

3.4. Experiment 4: Plasma corticosterone and central monoamine levels as a function of recent and previous stressor exposure.

Plasma Corticosterone: Levels of plasma corticosterone, determined three minutes after exposure on the test day, shown in Figure 15, varied as a function of Re-exposure condition, $F_{(2,60)} = 134.20$; $p < .001$. Follow-up analyses revealed that, circulating corticosterone levels were higher in animals that were exposed to either a physical or a social stressor on the test day, compared to animals that received no stress

on the test day (p's < .001) regardless of previous stressor exposure. Interestingly, animals that were exposed to a physical stressor on the test day also had higher circulating corticosterone levels than animals that were exposed to a social stressor on the test day, $p < .01$. In addition, there was a significant interaction between Initial exposure and Re-exposure treatment, $F_{(2,60)} = 3.52$; $p < .05$. Follow up tests confirmed that among animals that had no previous stressor exposure, animals that were exposed to either a physical or a social stressor on the test day had higher circulating corticosterone levels compared to animals that received no stressor exposure on the test day, $p < .001$. Furthermore, among initially stressed animals, animals that were exposed to a social stressor on the test day had significantly higher levels of plasma corticosterone than animals that received no stress on the test day, $p < .001$. Interestingly, among animals that were initially stressed, animals that were exposed to a physical stressor had significantly higher plasma corticosterone levels compared to both non-stressed animals and animals that were exposed to a social stressor on the test day, p's < .001 (Figure 15). Among animals that were exposed to a social stressor on the test day, animals that had undergone previous stressor exposure had significantly *lower* plasma corticosterone levels compared to animals that had not been previously stressed, $p < .01$.

Insert Figure 15 about here

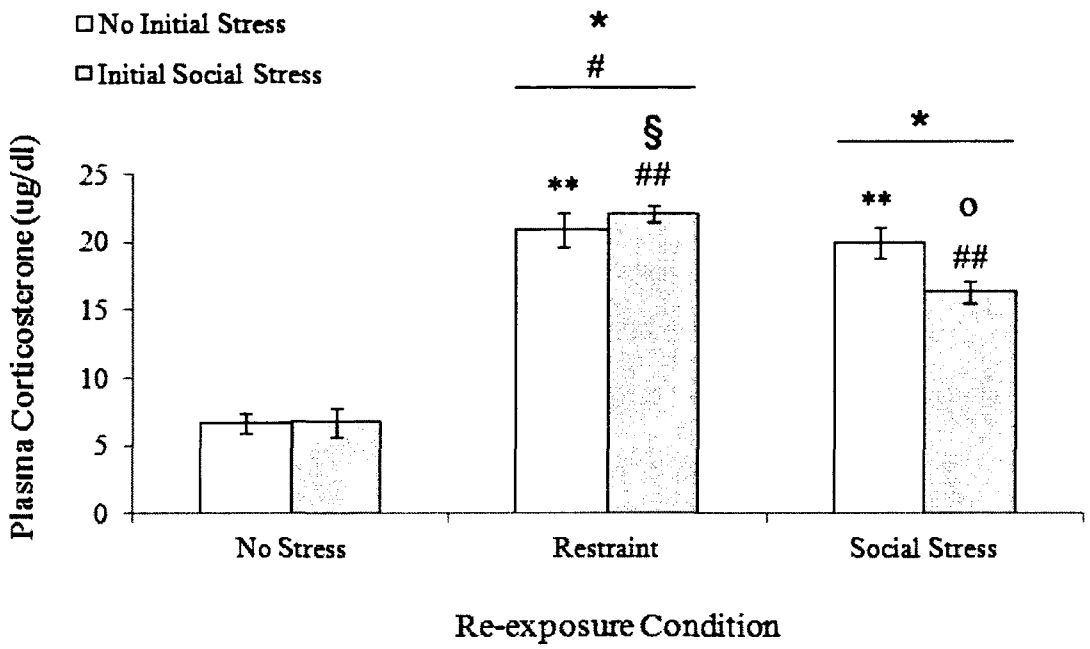

Figure 15. Plasma corticosterone levels (Mean ± SEM) among mice that either underwent initial stressor exposure or not and that were then re-exposed to a physical restraint stressor, social stressor, or no stressor on the test day. * $p < .001$ relative to non-stressed mice on the test day. # $p < .01$ relative to animals that received a social stressor on the test day. ** $p < .001$ relative to animals that had no previous stressor experience and that received no stressor exposure on the test day. ## relative to initially stressed animals that received no stress on the test day $p < .001$. § $p < .001$ relative to animals that were initially stressed and that subsequently received a social stressor on the test day. O $p < .01$ relative to animals that also received a social stressor on the test day but that had no previous stressor exposure.

Monoamine variations: In general, both physical and social stressors influenced central monoamine neurotransmission in similar ways. In the hippocampus, MHPG levels varied as a function of the Re-exposure condition on the test day, $F_{(2,58)} = 5.33$; $p < .01$, whereas NE levels in the hippocampus were unaffected in the treatment groups. As shown in Figure 16, and confirmed by follow up tests, MHPG levels were higher in

animals that were exposed to a social stressor on the test day compared to mice that received no stressor exposure on the test day ($p < .01$), regardless of previous stressor exposure. MHPH levels were not influenced in animals that received the restraint stressor compared to non-stressed or animals that received social stress on the test day (Figure 16).

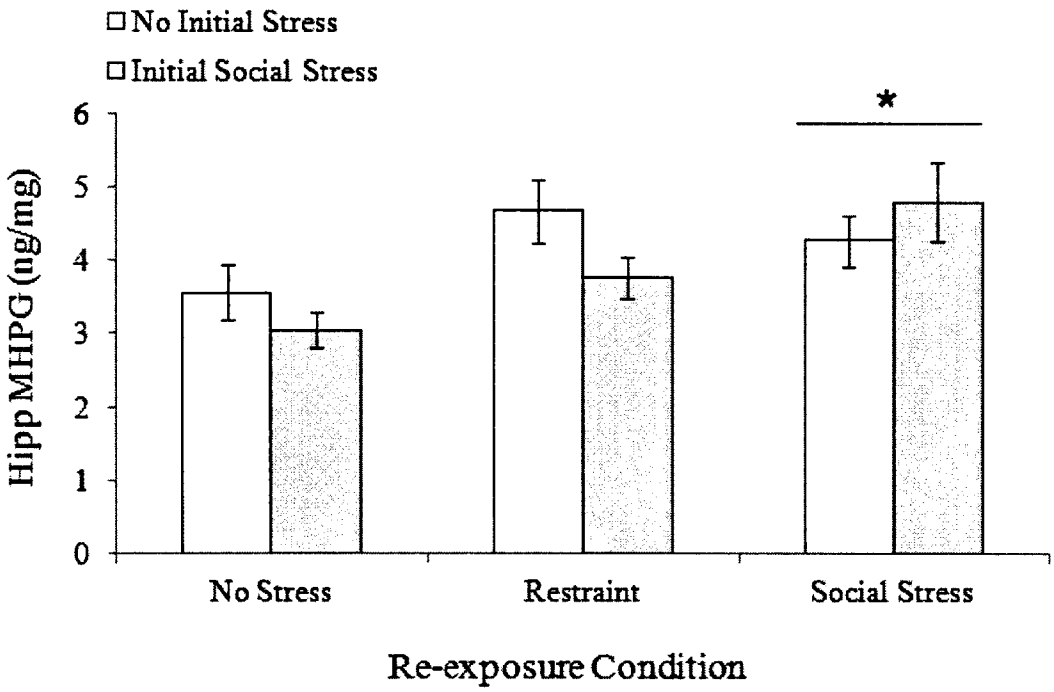

Figure 16. MHPG levels (Mean ±SEM) within the hippocampus among mice that either underwent initial stressor exposure or not and that were subsequently re-exposed to a physical restraint stressor, social stressor, or no stressor. * $p < .01$ relative to non-stressed mice at time of re-exposure.

In the PFC, MHPG levels varied as a function of the interaction between Initial exposure and Re-exposure, $F_{(2,60)} = 4.56$; $p < .05$, whereas PFC NE levels were unaffected in the treatment groups. Analysis of the simple effects that comprise this interaction indicated that among animals that had no previous stressor exposure, animals that were exposed to a physical restraint stressor on the test day had higher MHPG levels compared to animals that received no stress on the test day, $p < .01$, but not animals that received a social stressor (Figure 17). Furthermore, analysis of the simple effects of the interaction also revealed that among animals that received a restraint stressor on the test day, MHPG levels in the PFC were lower in animals that had been previously stressed and received a restraint stressor on the test day, compared to animals that had no previous stressor exposure, $p < .05$.

Insert Figure 17 about here

Figure 17. MHPG levels (Mean ±SEM) within the PFC among mice that either underwent initial stressor exposure or not and that were subsequently re-exposed to a physical restraint stressor, social stressor, or no stressor. * $p < .01$ relative to non-stressed mice at time of re-exposure. # $p < .05$ relative to mice that were physically stressed at re-exposure but that never received any initial stressor exposure.

In addition to MHPG alterations, levels of both hippocampal 5-HT and its metabolite, 5-H1AA, were influenced by Initial exposure treatment, $F_{(2,57)} = 6.16$ and $F_{(2,59)} = 4.82$; $p < .05$, respectively. As shown in Figure 18 and Figure 19, animals that received an initial social stressor 30 days prior to the test day had significantly lower levels of both 5-HT and 5-H1AA in the hippocampus compared to initially non-stressed animals, $p < .05$. Similar effects were not evident in the PFC with respect to alterations of either 5-HT or 5-H1AA.

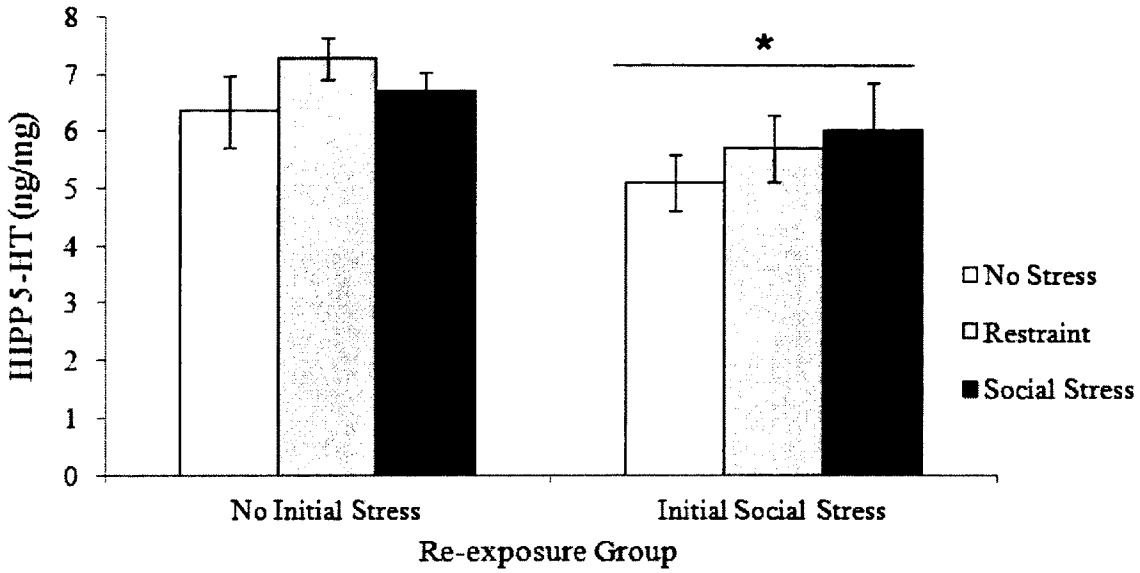

Figure 18. 5-HT (Mean ±SEM) within the hippocampus among mice that either underwent initial stressor exposure or not and that were subsequently re-exposed to a physical restraint stressor, social stressor, or no stressor. * $p < .05$ relative to non-stressed mice at time of initial exposure.

Insert Figure 19 about here

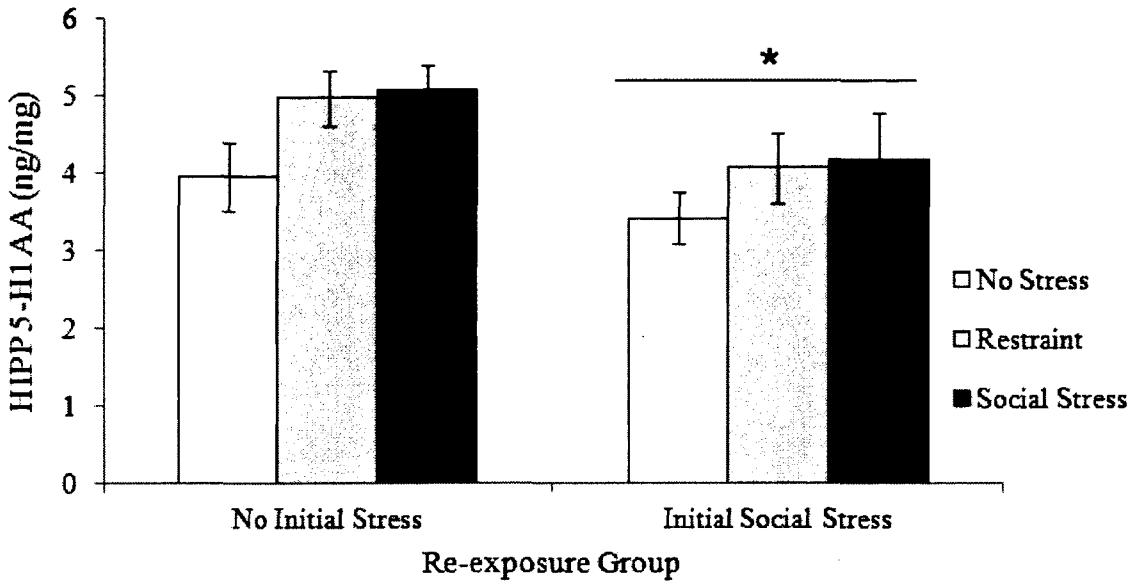

Figure 19. 5-H1AA (Mean ±SEM) within the hippocampus among mice that either underwent initial stressor exposure or not and that were subsequently re-exposed to a physical restraint stressor, social stressor, or no stressor. * $p < .05$ relative to non-stressed mice at time of initial exposure.

In the PFC, levels of both DA and its metabolite, DOPAC, varied as a function of Re-exposure condition, $F_{(2,58)} = 9.19$; $p < .001$ and $F_{(2,60)} = 7.86$; $p < .01$, respectively. As shown in Figure 20, and confirmed by multiple comparisons, DA levels were higher in animals re-exposed to either a social or a physical stressor compared to mice that received no stress at time of re-exposure regardless of previous stressor exposure, $p < .01$.

There was no difference in the levels of DA in the PFC between animals that received a social stressor compared to animals that received a physical stressor on the test day. As shown in Figure 21, and confirmed by multiple comparisons, DOPAC levels were higher in animals re-exposed to either a physical or a social stressor compared to

mice that received no stress at the time of re-exposure, $p < .05$ and $p < .01$ respectively. Similarly, there was no difference in the levels of DOPAC in the PFC between animals that received a social stressor compared to animals that received a physical stressor on the test day.

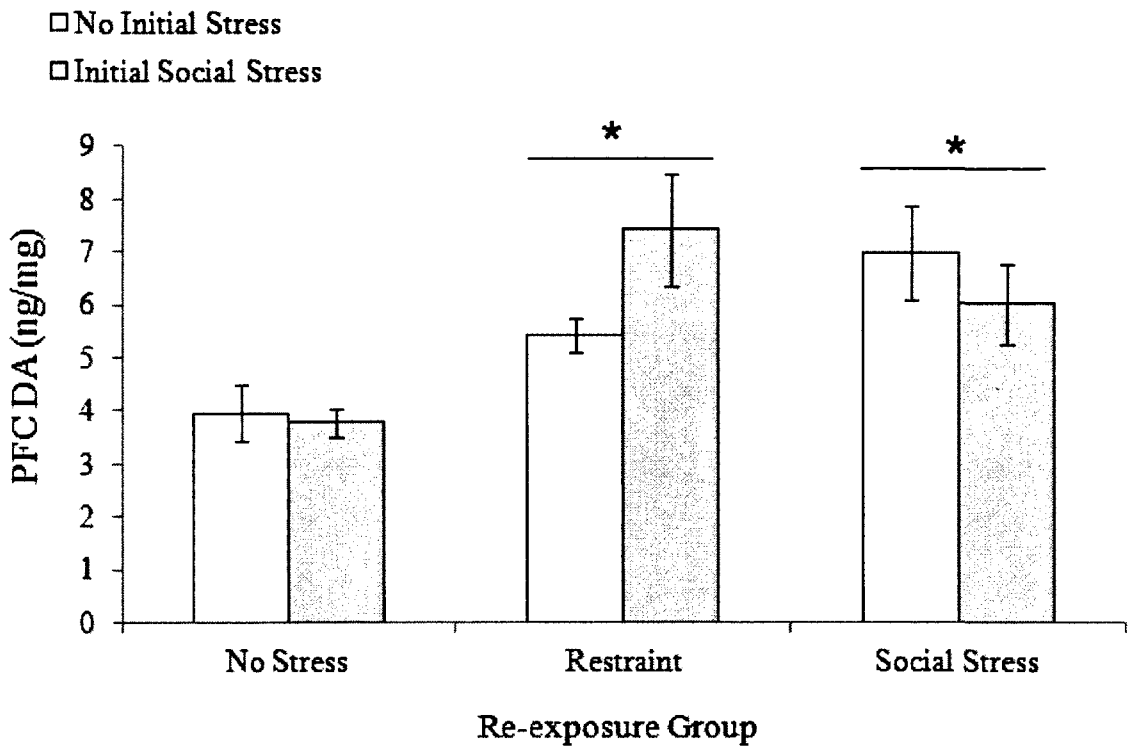

Figure 20. DA (Mean ± SEM) within the PFC among mice that either underwent initial stressor exposure or not and that were subsequently re-exposed to a physical restraint stressor, social stressor, or no stressor. * $p < .05$ relative to non-stressed mice at time of re-exposure.

Insert Figure 21 about here

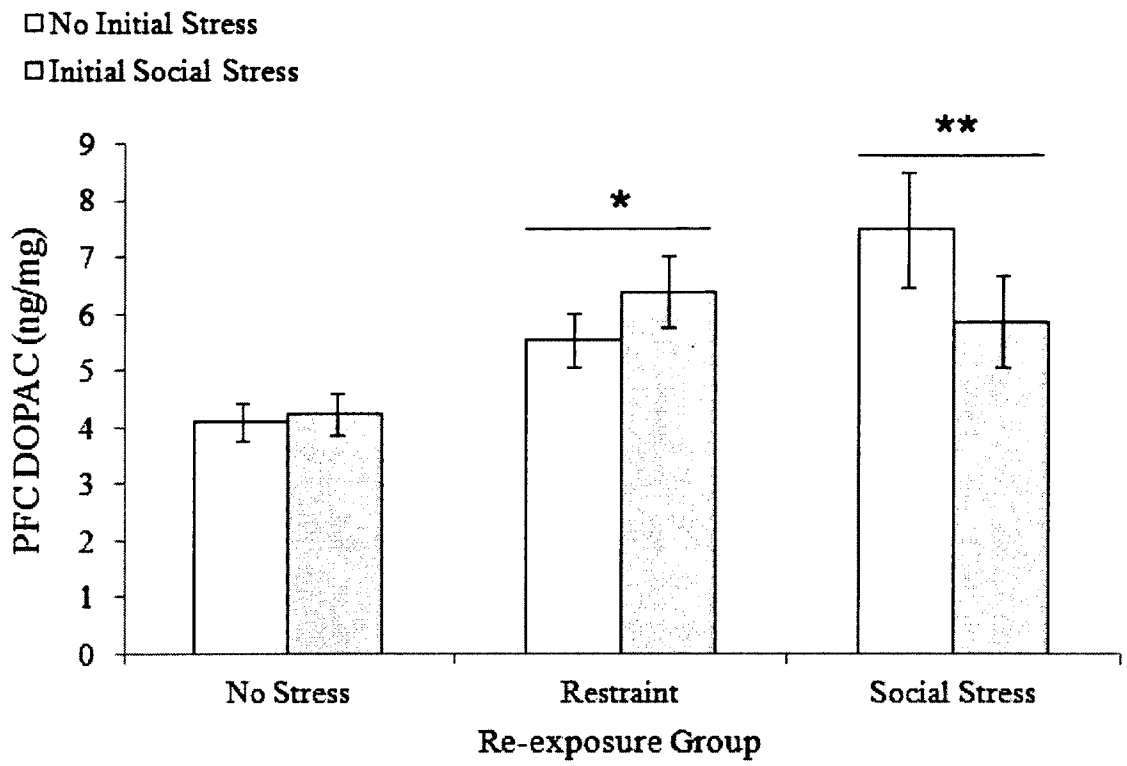

Figure 21. DOPAC (Mean ±SEM) within the PFC among mice that either underwent initial stressor exposure or not and that were subsequently re-exposed to a physical restraint stressor, social stressor, or no stressor. * $p < .05$ relative to non-stressed mice at time of re-exposure. ** $p < .01$ relative to non-stressed mice at time of re-exposure.

3.5. Experiment 5: Plasma corticosterone and mRNA expression as a function of recent and previous stressor exposure.

Plasma Corticosterone: Circulating corticosterone levels 90 minutes after exposure on the test day, shown in Figure 22, varied as a function of Re-exposure condition, $F_{(2,60)} = 26.22$; $p < .001$, but not the Initial Exposure condition, $F < 1$. Follow-up comparisons revealed that circulating corticosterone levels were significantly higher in animals that were exposed to a social stressor on the test day, compared to both animals

that received no stressor or animals that received a physical stressor on the test day (p <.001), irrespective of whether they had been initially exposed to a social stressor or not (Figure 22).

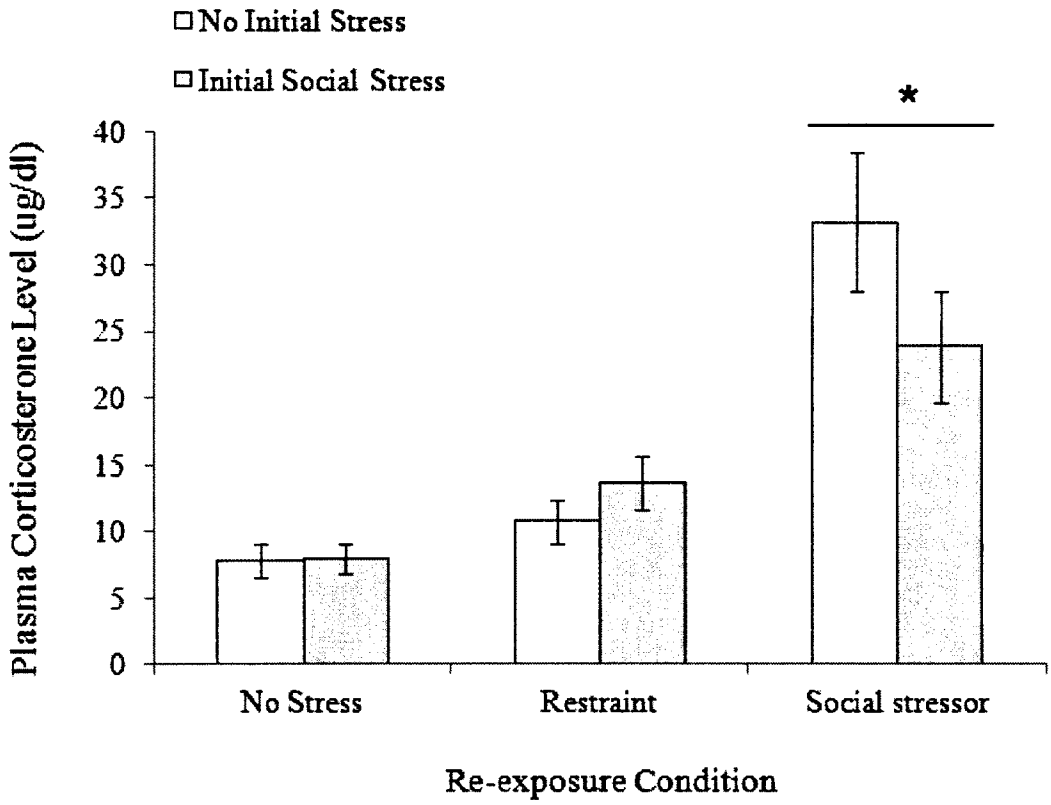

Figure 22. Plasma corticosterone levels (Mean ±SEM) among mice that underwent an initial stressor exposure or not and that were then re-exposed to a physical restraint stressor, social stressor, or no stressor. * $p < .001$ relative to animals that received no stress as well as animals that received a physical stressor on the test day.

Cytokine variations: In general, both physical and social stressors influenced central cytokine mRNA expression. Prefrontal mRNA expression of IL-1β was influenced by the Re-exposure Condition, $F_{(2,50)} = 3.55$, $p < .05$, but not by the Initial Exposure Condition, $F < 1$. Follow up tests indicated that IL-1β expression was higher in animals that were exposed to a physical restraint stressor on the test day compared to non-stressed animals, irrespective of whether they had received prior social stressor exposure or not, $p < .05$ (Figure 23). There was no difference between animals exposed to a restraint stressor compared to animals that received a social stressor on the test day, $p > .05$.

Figure 23. Fold changes of mRNA expression of IL-1β (Mean ±SEM) within the PFC among mice that either underwent initial stressor exposure or not and that were then re-exposed to a physical restraint stressor, social stressor, or no stressor. * $p < .05$ relative to animals that received no stress on the test day.

Cytokine mRNA expression of IL-6 within the PFC was affected by the Re-exposure Condition, $F_{(2,47)} = 11.01$, $p < .001$, but not the Initial Exposure Condition, $F < 1$. As shown in Figure 24 and confirmed by follow up tests, exposure to a social stressor on the test day resulted in higher expression of IL-6 compared to either a physical restraint stressor or no stressor, $p < .05$ and $p < .001$ respectively.

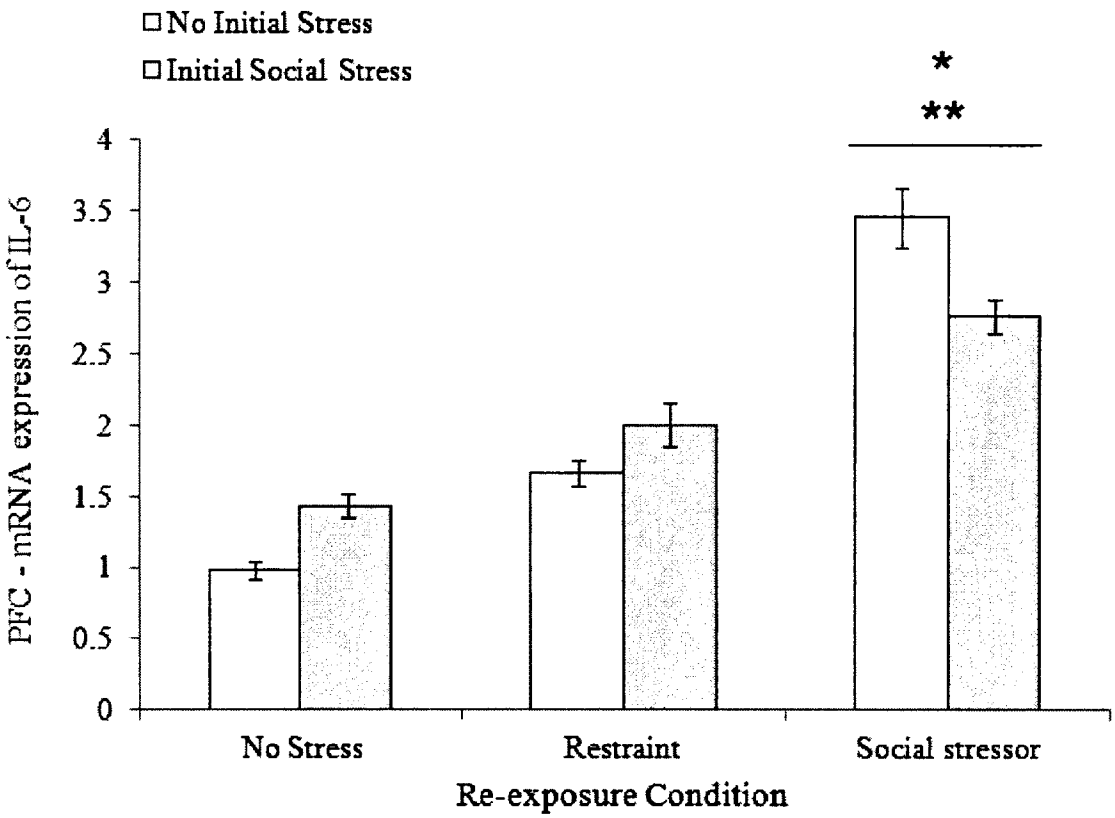

Figure 24. Fold changes of mRNA expression of IL-6 (Mean ±SEM) within the PFC among mice that either underwent initial stressor exposure or not and that were then re-exposed to a physical restraint stressor, social stressor, or no stressor. * $p < .05$ relative to animals that received a physical restraint stress on the test day. ** $p < .001$ relative to non-stressed animals on the test day.

Cytokine mRNA expression of TNF-α was significantly influenced by the Initial Exposure condition, $F_{(2,42)} = 27.38$, $p < .001$ as well as the Re-exposure condition, $F_{(2,42)} = 3.46$, $p < .05$. Follow up analyses indicated that animals that received an initial social stressor had significantly lower prefrontal TNF-α expression compared to mice that were not initially stressed, regardless of the type of stressor exposure they received on the test day, $p < .001$. Furthermore, animals that were exposed to a physical stressor on the test day had significantly higher prefrontal TNF-α expression compared to non-stressed animals on the test day, regardless of prior exposure to social encounters but not animals that were exposed to a social stressor, $p < .05$ (Figure 25).

In addition, prefrontal TNF-α expression varied as a function of the Initial Exposure by Re-exposure interaction, $F_{(2,42)} = 3.74$, $p < .05$. Analysis of the simple effects comprising this interaction confirmed that among initially stressed mice, increased TNF-α expression was observed in animals that were exposed to a physical stressor, compared to animals exposed to a social stressor ($p < .001$), but not in animals that received no stressor exposure on the test day. Further analyses revealed that among animals exposed to a social stressor on the test day, animals that had been previously exposed to a social stressor had significantly *lower* prefrontal TNF-α RNA expression compared to animals that had no previous stressor exposure, $p < .001$ (Figure 25).

Insert Figure 25 about here

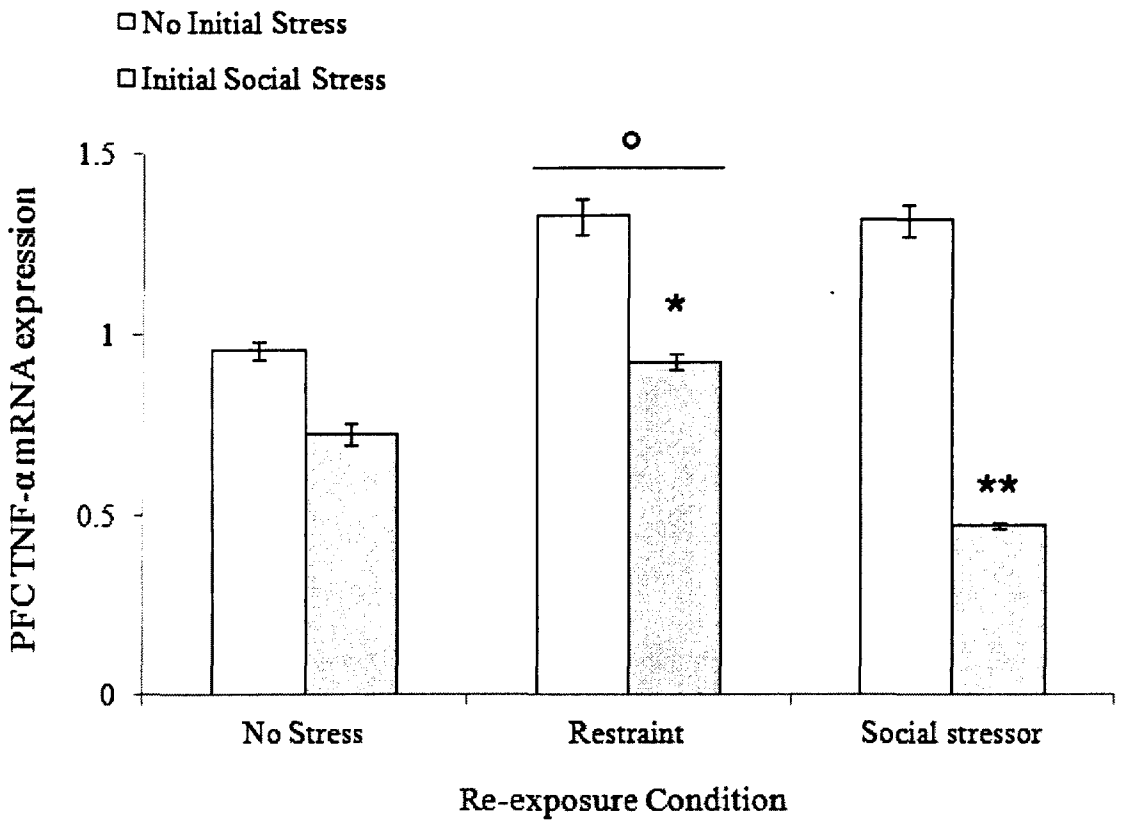

Figure 25. Fold changes of mRNA expression of TNF-α (Mean ±SEM) within the PFC among mice that either underwent initial stressor exposure or not and that were then re-exposed to a physical restraint stressor, social stressor, or no stressor. O $p < .05$ relative to animals that received no stress on the test day. * $p < .001$ relative to similarly treated animals that received a social stressor on the test day. ** $p < .001$ relative to animals that also received a social stressor on the test day but that had no previous stressor exposure.

Cytokine mRNA expression of the serotonin 5-HT1A receptor (Htr1a) within the PFC varied as a function of Re-exposure Condition, $F_{(2,51)} = 6.24, p < .01$ but not the Initial Exposure condition, $F < 1$. Follow up tests indicate that animals that were exposed to either a physical restraint stressor ($p < .01$) or a social stressor ($p < .05$) on the test day had significantly lower mRNA expression of Htr1a compared to animals that received no

stressor on the test day (Figure 26), irrespective of whether they had been previously exposed to a social encounter or not.

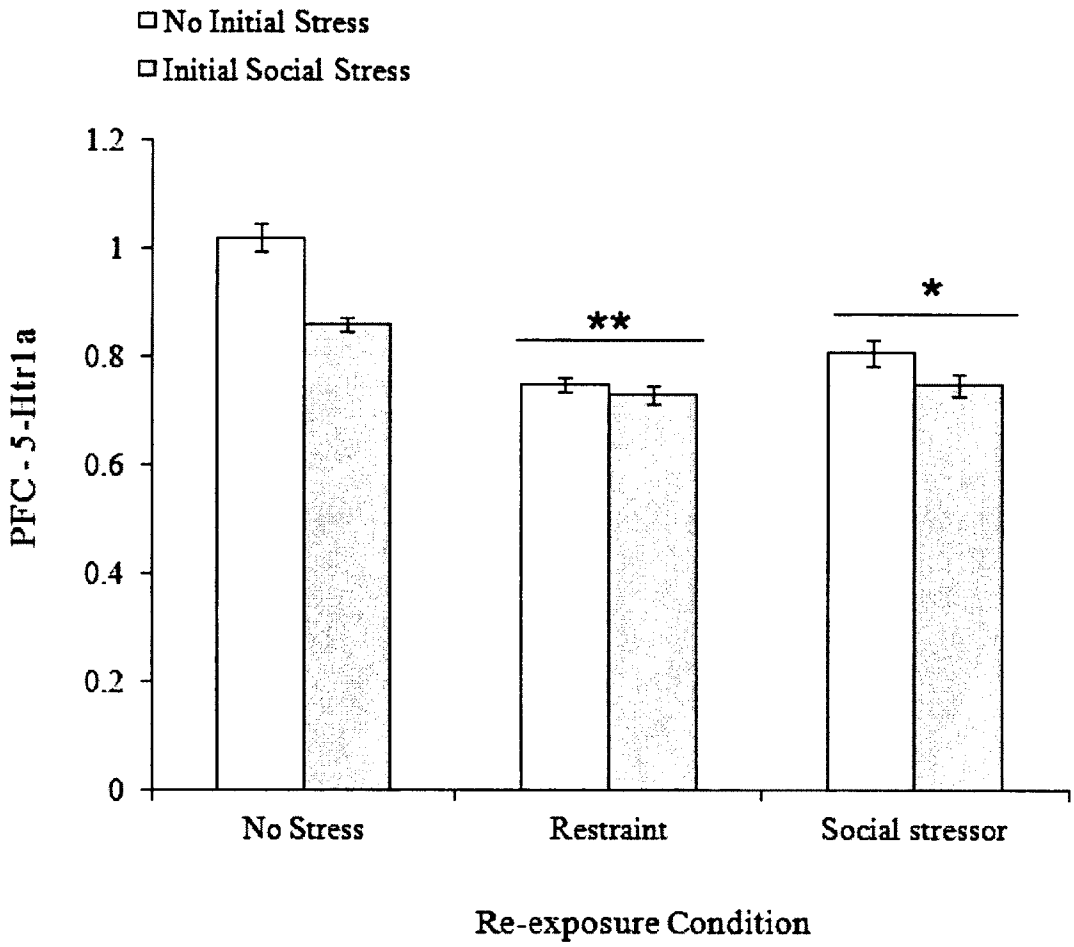

Figure 26. Fold changes of mRNA expression of Htr1a (Mean ±SEM) within the PFC among mice that either underwent initial stressor exposure or not and that were then re-exposed to a physical restraint stressor, social stressor, or no stressor. * $p < .05$ relative to animals that received no stressor on the test day. ** $p < .01$ relative to animals that received no stressor on the test day.

Prefrontal mRNA expression of the serotonin 5-HT1b receptor (Htr1b) also varied as a function or Re-exposure Condition, $F_{(2,52)} = 3.91$, $p < .05$, but not the Initial Exposure condition, $F < 1$. As shown in Figure 27 and confirmed by follow up tests, animals that were exposed to a physical restraint stressor had significantly lower expression of Htr1b compared to animals exposed to a social stressor on the test day ($p < .05$), but not compared to animals that received no stress on the test day, regardless of whether they received an initial stressor or not.

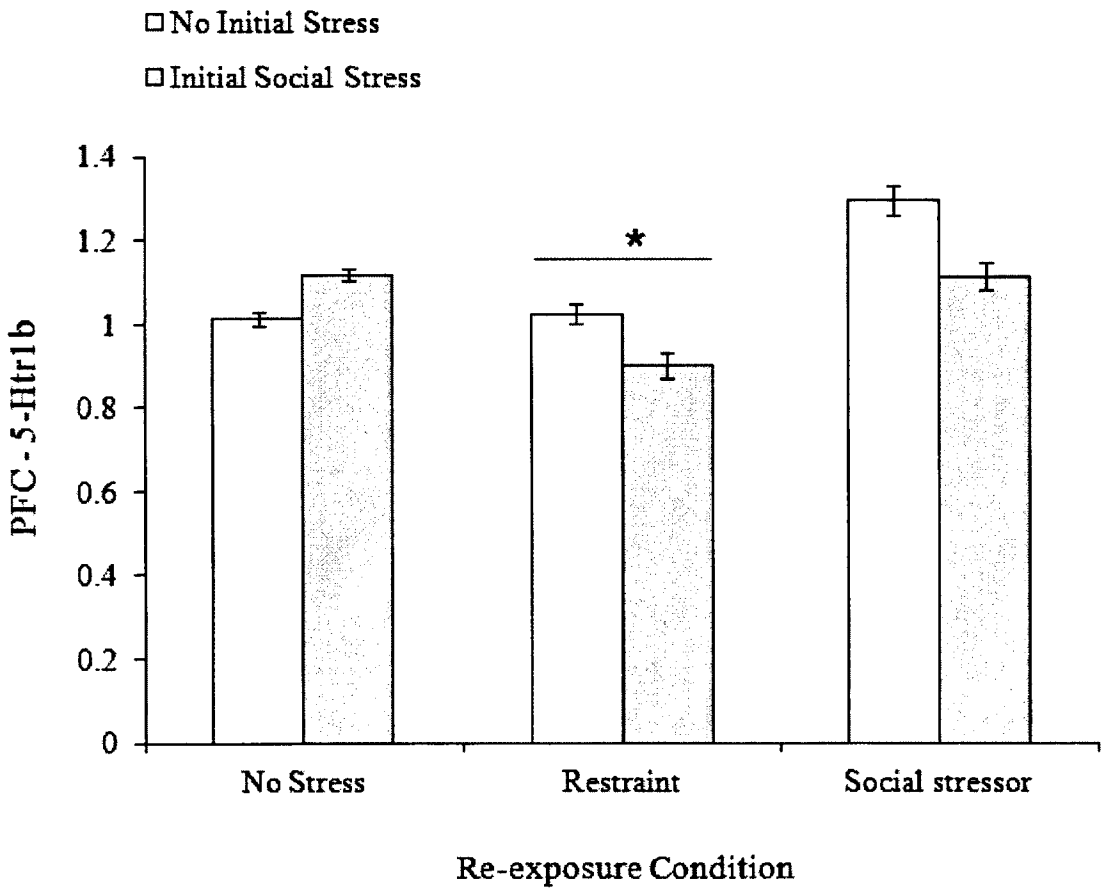

Figure 27. Fold changes of mRNA expression of Htr1b (Mean ±SEM) within the PFC among mice that either underwent initial stressor exposure or not and that were then re-exposed to a physical restraint stressor, social stressor, or no stressor. * $p < .05$ relative to animals that received a social stressor on the test day.

4. Discussion

4.1. Acute and prolonged behavioural effects of single and repeated stressor exposure

Most stressors reduce motor activity in rodents, and low levels of activity have been observed in defeated animals that were in persistent contact with a dominant animal (Bartolomucci et al., 2003; Rygula et al., 2005; Erhardt et al., 2009). However, in response to a distal predator, animals will engage in active, directed flight responses (Blanchard et al., 1998). In contrast to the effects of chronic stressors, in the present investigation, increased activity levels were evident following either a single or three aggressive social encounters. Interestingly, the behavioural excitation was especially marked among animals that had previously been exposed to the same (homotypic) stressor. These animals entered the open arms of the plus maze more frequently than animals that received only one agnostic bout with a dominant conspecific. In fact, among mice that were exposed to a social stressor on the test day, hyperarousal activity was only apparent in animals that had previously experienced the same stressor as opposed to a dissimilar physical stressor. This could potentially reflect flight related responses such as hyper-vigilance that are associated with desensitization of the HPA response to homotypic stressors, as previously observed by Belda et al., (2008). Behavioural excitation, in the form of hyperarousal, exhibited by animals that experience repeated stressor exposure has been associated with symptomatology that is reminiscent of those associated with PTSD psychopathology.

Among the symptoms observed in depression, anhedonia, an affective symptom related to the inability to experience pleasure, has been regarded as a distinctive sign correlated with the severity of depression (Snaith, 1992). Decreased intake of palatable

solutions, such as a water-sucrose solution, has been taken as a behavioural measure of anhedonia and a depressive-like state in rodents (Moreau et al., 1992; Willner, 1997) and stressors often give rise to a decreased preference for palatable chow or sucrose solutions. In experimental animal models, depressive-like behavioural alterations have been found to be caused by various types and degrees of stressors, such as immobilization stress (Armario et al., 1991), chronic social disruption (Wann et al., 2010a), and social defeat stress (Audet et al., 2011). Several studies have also shown that animals that are exposed to a stressor or repeated/chronic stressors, exhibited the core depressive symptom of anhedonia in the form of decreased preference for a sweetened sucrose solution (Harkin et al., 2002; Redei et al., 2001; Wann et al., 2010b).

It appears from the present report, however, that a single exposure to a social defeat stressor was not sufficient to cause a decrease in sucrose preference. However, in keeping with previous reports, animals that had undergone previous stressor exposure and subsequently exposed to a physical restraint stressor on the test day, showed a slight decrease in sucrose preference. Interestingly, however, in the present investigation repeated exposure to a social defeat stressor actually resulted in increased preference for sucrose. It is uncertain what might provoke this seemingly paradoxical effect. However, the possibility cannot be excluded that the stressor condition resulted in a mild depressive-like state and that animals consumed a palatable substance as a means of self-medicating. Indeed, the view has frequently been expressed that in humans and in animals, distress may encourage increased eating as a form of coping (Heatherton et al., 1991; Polivy et al., 1994; Spoor et al., 2007).

4.2. Stressor induced behavioural alterations attenuated by propranolol

It is well known that memories for stressful and emotional events become more strongly embedded than do memories for neutral events (McGaugh and Roozendaal, 2002). Intense emotional responses that accompany a traumatic event may help to explain why individuals with PTSD develop strong memories for the traumatic event or experience. Memories for traumatic events can persist for many years and even decades (Shalev et al., 1997). This type of 'over-consolidation' of stressful memories may produce emotional memories that are highly resistant to extinction (Orr et al., 2006; Wessa & Flor, 2007). The manipulation of stress hormones or neurotransmitters, which are central to memory consolidation, immediately following a traumatic event could potentially reduce an individuals' risk of developing anxiety disorders, such as PTSD. The facilitation of emotional memory consolidation has been shown to be influenced by adrenal hormones, i.e., catecholamines and glucocorticoids. These hormones are secreted after a stressful experience and appear to influence NE activity (McIntyre & Roozendaal, 2007).

Propranolol, a β-adrenergic blocker, works to inhibit the actions of NE, a neurotransmitter that has been found to enhance memory consolidation (McGaugh 2000, 2002). Previous reports demonstrated that administration of propranolol prior to or immediately after training inhibit the memory consolidation effects of NE (Cahill and McGaugh, 1996; Miranda et al., 2003; Salinas et al., 1997). Similarly, blockade of β-receptors with propranolol in animals also disturbed memory in spatial learning tasks (Cahill et al., 2000; Hatfield & McGaugh, 1999; Ji et al., 2003) and contextual fear conditioning in animals (Roozendaal et al., 2004). Furthermore, individuals who are

given propranolol immediately after a traumatic experience show less severe symptoms of PTSD compared to their respective control groups that do not receive the drug (Vaiva et al., 2003). There have certainly been an appreciable number of studies that have shown that drugs that diminish NE release or that block βNE receptors (such as propranolol) act antagonistically towards symptoms of PTSD (Krystal & Neumeister, 2009). The results of these studies are in keeping with the suggestion that increased NE is associated with hyperarousal, a common symptom of PTSD. The present study indicated that animals that underwent an initial stressor regime demonstrated greater exploratory behaviour in the plus maze by entering into the open arms more often as well as spending more time in the open arms compared to mice that were not initially stressed. Moreover, initially stressed animals that were re-exposed to an aggressive social encounter made the greatest number of entries into the open arms indicating behaviour reminiscent of hyperarousal/hyperactivity, which is consistent with findings from our first experiment.

It appears that repeated exposure to a stressor, whether physical or social, resulted in greater time spent in the open arms. Given that the open arms are known to be anxiety provoking areas of the maze for rodents, it is interesting that in the present investigation the animals that received the greatest amount of stressor exposure actually spent the most time in the open arms. Although animals that were repeatedly exposed to stressors spent more time and entered the open arms more frequently, they also spent less time and enter the closed arms less often, not necessarily indicating greater overall activity in the plus maze but instead revealing a proclivity to the open arms. Perhaps these animals are displaying a behavioural profile consistent with symptoms associated with PTSD rather than a depressive like illness. For instance, repeated exposure to intense stressors such as

the social stressor used in the present investigation may promote the emergence of symptoms such as hyperarousal, increased risk taking behaviours, and an exaggerated response to anxiety provoking situations, all of which are reminiscent of symptoms associated with PTSD.

Interestingly, the heightened exploratory behaviour was attenuated following an injection of propranolol prior to re-exposure in animals that were initially stressed and subsequently exposed to the same stressor. Furthermore, initially stressed animals that were re-exposed to a restraint stressor entered the open arms as often as their non-stressed counterparts. That is, these animals did not exhibit the apparent hyperarousal ordinarily elicited by an acute stressor. However, when this same group of animals was given an injection of propranolol prior to re-exposure the group exhibited increased exploratory behaviour. Under these circumstances, it appears that propranolol promotes entry into the open arms in anxious animals but curtails the hyperarousal and exploratory behaviour in over active mice that have undergone repeated stressor exposure.

4.3. Plasma corticosterone alterations

Psychosocial stressors are known to prompt neuroendocrine changes, and particularly hypersecretion of glucocorticoids. Studies using social stressor paradigms, such as social defeat, have consistently reported increased secretion of glucocorticoids and elevated circulating levels of stress hormones (ACTH and corticosterone) upon single exposure (Audet et al., 2011; Bartolomucci et al., 2001; Bhatnagar et al., 2006; Keeney et al., 2001; 2006;). In addition to having profound immediate effects, stressful events may promote a sensitized response to later challenges, believed to contribute to the

development and recurrence of various psychological disorders (Anisman et al., 2003). Given that stressor-related illnesses might be associated with corticoid changes (Gold and Chrousos, 2002); it was of interest in the present investigation to assess whether an initial intense social stressor experience would sensitize glucocorticoid responses upon subsequent challenges to the same emotional stressor (social stressor) versus a very different physical stressor (restraint stressor).

Consistent with previous reports (Audet et al., 2010; Keeney et al., 2006), the present investigation showed that plasma corticosterone levels were elevated following the social stressor exposure in otherwise naïve mice, even after a lengthy (90 min) interval of rest. Interestingly and potentially reflecting a desensitization of the glucocorticoid response, corticosterone elevations were reduced upon subsequent exposure to a social stressor, although not significantly so, in mice that had previously been exposed to a social stressor during the initial stressor phase. In a parallel study, after a relatively short interval (3 min) of rest, corticosterone levels were similarly elevated following both a social stressor and a restraint stressor exposure in otherwise naïve mice. However, corticosterone elevations were significantly decreased following re-exposure, in mice that had previously been exposed to a social stressor, clearly reflecting a desensitization of the glucocorticoid response.

It has been proposed that a repeated stressor experience may result in an HPA axis system adaptation, so that upon re-exposure to previously encountered stressors, a more rapid inhibitory modulation of the HPA axis system will occur. The down-regulation of this system may then result in a blunted stress response, thereby protecting the organism against the potential adverse effects of a hyper-corticoid response (Girotti et al., 2006).

As PTSD has been associated with diminished cortisol levels (Yehuda et al., 2009), the desensitization observed with respect to corticosterone secretion might be reflective of a PTSD profile as suggested earlier. Of course, the present investigation does not speak directly to this possibility and further investigation is needed to determine the exact mechanisms involved in this PTSD-like outcome.

4.4. Catecholamine variations provoked by single and repeated exposure to social and restraint stressors

Along with the behavioural and endocrine effects, it is understood that neurochemical alterations also occur in response to a stressor. Several reports have demonstrated variations in catecholamine concentrations in stressor sensitive brain regions as well as circulating plasma levels (Anisman et al., 1991; Anisman & Merali, 1999; Tannenbaum et al., 2002). Beyond behavioural and neuroendocrine changes, aggressive social interactions and restraint stressors provoke neurochemical changes like those elicited by relatively strong stressors. Several neurochemical changes are evident following stressor exposure that may act to reduce the physical or psychological impact on an organism (Maier & Seligman, 1976; Weiss & Simson, 1985). The type and severity of stressor exposure has certainly been shown to contribute to behavioural as well as neurochemical alterations associated with stressful events (Amat et al., 2008; Anisman et al., 1991; Anisman & Merali, 1999). For instance, acute stressor exposure typically results in the release of brain monoamines that exceed their synthesis, giving rise to an overall decrease in monoamine concentrations in stressor sensitive brain regions (Anisman et al., 1991, Stanford, 1995; Weiss et al., 1980). However, following repeated

stressor exposure, the amine reductions typically found after acute stressor exposure may not be present (Roth et al., 1988) perhaps resulting from the downregulation of autoreceptors (Tannenbaum et al., 2002).

The central 5-HT system is known to be involved in the regulation of stress and anxiety (Harvey et al., 2004) and several preclinical studies have reported an increase in 5-HT release and increased 5-HT synthesis and turnover in response to stressor exposure (Anisman & Zacharko, 1990; Audet & Anisman, 2009; Chaouloff et al., 1999). These stress induced alterations of 5-HT activity occur in several brain regions, which have been implicated in the pathophysiology of PTSD, including the amygdala and the PFC (Bruening et al., 2006; Gobert et al., 1998; Mitsushima et al., 2006).

Previous reports have indicated that acute social defeat in mice enhanced hippocampal 5-HT release (Keeney et al. 2006), and after repeated confrontations, tissue levels of 5-HT, its metabolite 5-hydroxyindoleacetic acid (5-HIAA), and tryptophan hydroxylase were also increased within several stress-related brain regions (Amstislavskaya & Kudryavtseva, 1997; Devoino et al. 2003). Furthermore, a recent study found that following aggressive confrontations 5-HT utilization as well as 5-HIAA accumulation was enhanced within the PFC and hippocampus (Audet et al., 2010). Similarly, exposure to strong uncontrollable stressors increased prefrontal 5-HIAA accumulation and resulted in a modest reduction in 5-HT levels, perhaps indicating that 5-HT utilization exceeded its synthesis (Anisman & Zacharko, 1990). In keeping with previous results reported above, the present investigation revealed that a single exposure to an initial social defeat stressor enhanced hippocampal 5-HT release and resulted in elevated tissue levels of hippocampal 5-HT and 5-H1AA.

As in the case of 5-HT, exposure to an acute immobilization stressor enhanced NE release in several stress-related brain regions, namely the amygdala, lateral bed nucleus of the stria terminalis, and prefrontal cortex (Morilak et al., 2005). Moreover, these researchers found that when an adrenergic antagonist was injected directly into these regions, the resulting release of NE facilitated several anxiety-like behaviours such as reduced open arm exploration in the EPM and reduced social interaction (Morilak et al., 2005). The present investigation found that hippocampal MHPG levels, but not NE levels, were higher in animals that received either a single or repeated exposure to a social stressor. Moreover, a single exposure to restraint stressor resulted in elevated levels of MHPG, but not NE levels, within the prefrontal cortex. Interestingly, repeated exposure to dissimilar stressors resulted in a desensitization of prefrontal MHPG levels. Animals that were initially exposed to an intense social stressor, and then later exposed to a physical restraint stressor had lower levels of prefrontal MHPG compared to animals that received an acute restraint stressor on the test day but that had no prior stressor exposure.

Similar to the NE and 5-HT variations, DA functioning may also contribute to the provocation of psychiatric disorders such as depression and PTSD and are particularly important in fear and anxiety responses. Over-activation of DA transmission has been found to exacerbate the fear response, and therefore create inhibition of an organism's ability to reduce the conditioned fear response. (Pezze & Feldon, 2004).While acute stressors appear to increase DA levels in the PFC (Finlay et al., 1995) and hippocampus (Pawlak et al., 2000), its relationship to clinical anxiety disorders as well as its role in PTSD is more complex and poorly understood. Nevertheless, it was suggested that a

causal role for PFC DA dysfunction exists regarding intrusive thoughts and diminished extinction of memories associated with the traumatic event (Morrow et al, 1999; Pezze & Feldon, 2004; Seamans & Yang, 2004). Several reports have revealed that acute stress increases DA activity in the PFC (see Roth et al, 1988). Our results confirm and extend these observations. In particular, levels of both DA and DOPAC were enhanced in PFC following either a single or repeated exposure to both an aggressive social encounter as well as a physical restraint stressor. The altered functioning of the DA system revealed in the present investigation appears to be relevant and consistent based on the accompanying behavioural disturbances demonstrated in the first experiment. The same group of animals that displayed behavioural changes also had accompanying changes in DA functioning within the PFC.

4.5. Cytokine alteration provoked by social stressors and restraint stressors

The underlying mechanisms responsible for various cytokine effects, in addition to their behavioural and neurological actions, are largely unknown. This said, a recent investigation proposed that although numerous pro-inflammatory cytokines (e.g. IL-1β, IL-6, TNF-α, IFN-α, etc.) are associated with a wide range of psychological and neurological disturbances, the involvement of specific cytokines may be linked to certain pathological outcomes, but not to others (Anisman et al., 2008). Likewise, it may be that certain stressor conditions (e.g., single versus repeated exposure) as well as the type of stressor experienced (i.e. psychogenic versus neurogenic) contribute to a particular pathological outcome over another. Therefore, alterations in central pro- and anti-

inflammatory cytokines might contribute to the comorbidities often reported in PTSD as well as anxiety and depressive illnesses.

Beyond their behavioural and neurotransmitter effects, stressors also influence brain cytokine mRNA expression (Bartolomucci et al., 2003; Deak et al., 2005; Anisman et al., 2008; Gibb et al., 2008) and protein levels (Nguyen et al., 1998). Repeated exposure to a social stressor results in enhanced prefrontal IL-1β mRNA expression (Audet et al., 2011), whereas a single social stressor challenge enhanced IL-6 and TNF-α mRNA expression within the PFC, without substantially affecting IL-1β. The present investigation revealed that an acute physical stressor markedly increased prefrontal mRNA expression of IL-1β, whereas both a single as well as repeated exposure to a social stressor increased prefrontal mRNA expression of IL-6. There appears to be a divergent pattern of pro-inflammatory immune activation between acute social and physical stressors. It appears that acute social stress enhances IL-6 and TNF-α but not IL-1β, whereas acute physical stress appears to enhance IL-1β and not IL-6 or TNF-α.

Consistent with previous reports (Audet et al., 2011), the present investigation indicated that expression of prefrontal IL-6 mRNA was markedly elevated (~3 fold) 90 min following a single social stressor challenge. Furthermore, animals that experienced a subsequent exposure to a social stressor showed a marked decrease in prefrontal IL-6 mRNA expression. However the magnitude of the decrease after subsequent exposure was considerably less pronounced compared to previous reports (Audet et al., 2011). The desensitization of plasma corticosterone and prefrontal IL-6 expression associated with repeated social stressor exposure were evident in response to a challenge on the test day that mimicked the initially experienced stressor. It appears that the endocrine and

neurochemical outcomes in response to the social and physical challenges operate through different mechanisms, and perhaps these findings suggest that the diminished corticoid and IL-6 response were part of an adaptation to the cognitive distress associated with the repeated social stressor experience.

Interestingly, the effects of repeated social stressor exposure on prefrontal mRNA expression of TNF-α contrasted with those previously reported (Audet et al., 2011). A recent investigation demonstrated that repeated social stressor exposure resulted in increased mRNA expression of TNF- α (Audet et al., 2011). The present investigation demonstrated, however, that upon subsequent challenge, animals exhibited a desensitized cytokine response and TNF-α mRNA expression was diminished. Of particular interest in the present investigation was the decrease of prefrontal TNF-α mRNA expression upon subsequent exposure to a social stressor. In effect, it appeared that the initial stressor exposure provoked a desensitization of TNF-α expression within the PFC that was apparent upon subsequent exposure to the same social stressor treatment. A desensitized IL-6 response in this same region was also elicited by the same treatment, however, not to the same extent. Given that central inflammatory immune processes has been implicated in the development of neuroaffective disorders (Anisman et al., 2008; Dantzer et al., 2008; Maes, 1995), a desensitization effect shown here is of particular interest, because it raises the possibility that desensitized prefrontal expression of the pro-inflammatory cytokine, TNF-α, in response to repeated stressor exposure may be one of the processes by which an organism can protect itself against the development and progression of certain psychopathologies.

Given the range and diversity of alterations in cytokine expression found with different stressor conditions, as well as across brain regions, it is difficult to attribute any particular biological disturbances to the specific behavioural discrepancies found among the stressor groups. Despite these caveats, a recent report revealed that mRNA expression of several pro- and anti-inflammatory cytokines were elevated among animals exposed to chronic mild stress relative to non-stressed animals (You et al., 2011). Therefore, if one accepts the premise that central cytokine variations contribute to the emergence of anxiety and depressive illness, then the present findings add to the notion that aggressive social interactions are a viable model to evaluate pathological outcomes and that stressor exposure results in marked cytokine variations among stressor sensitive brain regions.

4.6. Conclusion

Stressful experiences have been associated with the progression of depressive illness, and aggressive social encounters have been used to model this disorder in rodents (Koolhaas et al., 1997; Berton et al., 2006; Krishnan et al., 2007). In keeping with the understanding that inflammatory factors contribute to stressor-related pathological disturbances (Maes, 1995; Anisman et al., 2008; Dantzer et al., 2008), the present investigation showed that distinct behavioural changes, altered plasma corticosterone levels, and variations in catecholamine and brain cytokine expression were all triggered by stressful social defeat interactions and in some cases by physical restraint stressors.

Indeed, the present investigation demonstrated that behavioural excitation as well as a desensitized corticosterone response was especially marked among animals that had been previously exposed to the same (homotypic) stressor and the hyperarousal was only

apparent in animals that had previously experienced the same stressor as opposed to a dissimilar physical stressor. Interestingly, this heightened exploratory behaviour was attenuated following an injection of propranolol prior to re-exposure in animals that received repeated stressor exposure, indicating that propranolol might be a viable candidate for treatment of PTSD symptomatology following a traumatic event, or that it would be a useful pharmaceutical intervention given to patients soon after experiencing a trauma in an attempt to prevent symptoms of PTSD from emerging.

Moreover, our investigation revealed that either a single or repeated social defeat stressor resulted in elevated tissue levels of hippocampal 5-HT and 5-H1AA, elevated prefrontal and hippocampal MHPG levels, and elevated levels of prefrontal DA and DOPAC. However, repeated exposure to dissimilar stressors (heterotypic) resulted in a desensitization of prefrontal MHPG levels. With respect to cytokine variations, an acute physical stressor increased prefrontal IL-1β mRNA expression, whereas both a single as well as repeated social stressor exposure increased prefrontal IL-6 mRNA expression. It appears that acute social stress enhances IL-6 and TNF-α but not IL-1β, whereas acute physical stress appears to enhance IL-1β and not IL-6 or TNF-α. It seems that the endocrine and neurochemical outcomes in response to the social and physical challenges operate through different mechanisms. Taking into account the diminished corticoid and IL-6 response, perhaps these mechanisms are part of an adaptation to the cognitive distress associated with repeated stressor experience. Of particular interest, we found that TNF-α expression was diminished and a desensitized cytokine response occurred following subsequent defeat challenge. Taken together, these findings reveal a possible

model for evaluating PTSD as well as possible mechanisms involved in the provocation, treatment and prevention of the disease.

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