The effects of single prolonged stress, an animal model of PTSD, on novelty-seeking behaviours and alterations in the dopaminergic system in male and female rats

by

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Abstract

A common underlying behavioural tendency of post-traumatic stress disorder (PTSD) involves novelty-seeking behaviours, which have been related to impairments in the dopaminergic system. A validated rodent model of PTSD known as single prolonged stress (SPS) has been extensively used in PTSD literature, however, only one study has inquired into the effects of this stressor on dopamine-mediated behaviours. We therefore investigated dopamine-related novelty-seeking behaviours and extracellular dopamine (DA) release in response to SPS. Rats were exposed to SPS on Day 0 and then received either saline or 0.5 mg/kg, i.p. *d*-amphetamine (AMP) injection on Day 7 before being placed in a modified novel object exploration test (Experiment 1) or being subjected to *in vivo* microdialysis to detect extracellular DA release in the nucleus accumbens (NAcc) for 140 minutes (Experiment 2). Male and female animals were examined to further explore any sex differences within this stress model. SPS exposure in males significantly increased locomotor activity, time spent in center, time spent sniffing and touching the object, and number of approaches to the object. Female rats exhibited the same patterns of exploratory behaviours, however they appeared to display higher levels of each measure compared to males. SPS exposure in males significantly increased DA levels in saline-administered rats. The same trends were not found in female rats. AMP elevated DA release in both males and females, but did not augment SPS-induced DA release. These data suggest that exposure to SPS results in an increase in DA-mediated behaviours in male and female rats, and elevates basal DA release in the NAcc only in males. Thus, SPS may be a useful tool in further understanding neurobiological systems within PTSD.

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The effects of single prolonged stress exposure in males and females: novelty-seeking behaviours and alterations in the dopaminergic system

Post-traumatic stress disorder (PTSD) is a debilitating psychiatric disorder that emerges after direct or indirect exposure to a traumatic stressor. In Canada, the lifetime prevalence of PTSD is estimated to be 9.2%, affecting significantly more women (12.8%) than men (5.3%), with high rates of comorbidity for substance use disorders (SUDs) (Van Ameringen, Mancini, Patterson, & Boyle, 2008). Both PTSD and SUDs involve altered reward functioning, evidenced by deficits in reward anticipation, approach, and hedonic responses, and increases in novelty-seeking behaviours, which are all thought to result from dysfunctional dopamine (DA) transmission (Bardo, Donohew, & Harrington, 1996; Friedel, 2004; Harmer, Hitchcott, Morutto, & Phillips, 1997; Jentsch & Taylor, 1999; Nawijn et al., 2015; Richman & Frueh, 1996; Roberts, Koob, Klonoff, & Fibiger, 1980; Wang et al., 1997). Individuals with PTSD have also been shown to have increased levels of serum and urinary DA compared to healthy individuals (Glover et al., 2003; Hamner & Diamond, 1993; Yehuda et al., 1992). This hyperdopaminergic hypothesis has yet to be explored in animal models of PTSD, to further investigate the implication of DA on reward deficits within this disorder. Currently, a validated rodent model of PTSD known as single prolonged stress (SPS) has been extensively examined in the context of memory, cognition, and contextual fear (Eagle, Fitzpatrick, & Perrine, 2013; Knox, Nault, Henderson, & Liberzon, 2012). However, only one study has probed the effects of this stressor on dopamine-mediated behaviours – particularly its effects on sensitization to cocaine and cocaine self-administration (Eagle et al., 2015). There is evidently a gap in the literature exploring the effects of SPS on the dopaminergic system and other associated behaviours,

despite clinical evidence demonstrating hypersecretion of DA and dysfunctional reward functioning in individuals with PTSD.

Overview of PTSD and current animal models

According to the fifth edition of the Diagnostics and Statistics Manual of Mental Disorders (DSM-V), PTSD is categorized as a traumatic and stressor-related disorder. Individuals must experience at least one symptom from four symptom clusters: re-experiencing (recurrent memories or dreams related to the traumatic event), avoidance (evading external reminders of the event), negative cognitions and mood (distorted feelings and diminished interest), and arousal (novelty-seeking) (American Psychiatric Association & American Psychiatric Association, 2013). Clinical work has identified specific physiological and behavioural symptoms of PTSD, such as HPA axis abnormalities, diminished social functioning, exaggerated startle response, increased anxiety, impaired reward functioning, and enhanced fear conditioning (Grillon, Morgan, Southwick, Davis, & Charney, 1996; Meewissee, Reitsma, De Vries, Gersons, & Off, 2007; Pickett, Bardeen, & Orcutt, 2011). However, one of the most prevalent traits of individuals with PTSD is novelty-seeking (Bardo et al., 1996; Wang et al., 1997). Individuals with PTSD report significantly higher scores on scales for novelty-seeking, in which it is described as "the need for varied novel and complex sensations and experiences, and the willingness to take physical and social risks for the sake of such experience" (Wang et al., 1997). This fits into the high-risk hypothesis of comorbid PTSD and SUD, which posits that engaging in substance abuse is a tendency of PTSD individuals towards high-risk behaviours (Brady, Dansky, Sonne, & Saladin, 1998; Chilcoat & Breslau, 1998). Epidemiological studies have reported high comorbidity rates between PTSD and substance use disorders (SUDs), with an increasing amount of evidence suggesting that PTSD and SUDs share common

neurobiological components, of which dysregulation of DA systems appears to be the most vital mediator of novelty-seeking behaviours (Bardo et al., 1996; Kessler, Sonnega, Bromet, Hughes, & Nelson, 1995; Norman et al., 2012; Regier et al., 1990).

Animal models of PTSD. Animal models of PTSD are extremely beneficial in regards to investigating neural substrates and mechanisms involved in this psychopathology. A number of animal models have been proposed over the years in an endeavor to mimic the pathophysiological abnormalities and behavioural tendencies of PTSD. Paradigms used to elicit PTSD-related behaviours in animals include exposure to severe stressors such as inescapable electric foot shocks, underwater trauma, predators, or predator odours (Adamec, Blundell, & Burton, 2006; R. Adamec, Muir, Grimes, & Pearcey, 2007; Cohen et al., 2004; Shimizu et al., 2006; Wakizono et al., 2007). Behavioural responses to these stressors are reflective of those found in humans, comprising of anxiogenic responses, cognitive impairments, exaggerated startle responses, enhanced fear conditioning, and decreased social interaction. Only a few studies have demonstrated HPA axis abnormalities similar to those observed in PTSD (Cohen et al., 2006; Stam, 2007).

Among animal models of PTSD, the use of single prolonged stress (SPS) has been gaining legitimacy as a valid model of the disorder. SPS involves exposure to three stressors, beginning with two hours of restraint stress, immediately followed by 20 minutes of forced swim, and then 15 minutes of recuperation before exposure to ether until loss of consciousness (Liberzon, Krstov, & Young, 1997). Subsequently, SPS rats are typically isolated and undisturbed for a minimum of seven days. This period of isolation is necessary in order to emulate PTSD symptoms and allow from structural changes in the brain (Yamamoto et al., 2009).

SPS was first proposed as a rodent model of PTSD after male rats with previous SPS exposure exhibited enhanced negative feedback of glucocorticoid receptors, a prevalent neuroendocrinological abnormality observed in PTSD patients (Liberzon et al., 1997; Stein, Yehuda, Koverola, & Hanna, 1997; Yehuda et al., 1993). This paradigm also reproduces central PTSD phenotypes, such as increased acoustic startle responses, impaired learning and memory, and anxiogenic behaviours (Eagle et al., 2013; Pitman et al., 2012). SPS' ability to parallel current theories in PTSD research is significant. Various SPS studies have reported HPA axis alterations and hippocampal abnormalities, which are consistent with the human literature (Knox et al., 2012; Liberzon et al., 1997; Stein et al., 1997; Tyrka et al., 2008; Yehuda et al., 1993). In spite of this, recent technological advances and neuroimaging studies in humans have implicated other brain regions (i.e. striatum, NAcc) in PTSD, and have not yet been examined using SPS (Sailer et al., 2008). Furthermore, studies have consistently shown that sustained SSRI administration following SPS exposure successfully reduces PTSD-like symptoms, which is comparable to human studies (Harvey, Naciti, Brand, & Stein, 2004; Takahashi, Morinobu, Iwamoto, & Yamawaki, 2006). Together, SPS appears to be an appropriate animal model of PTSD, with considerable evidence supporting its reflection of human symptomology. However, there is still a lack of literature examining effects of this model on DA-related dysfunctions, such as novelty-seeking behaviours, which are prevalent in PTSD.

Stress and reward functioning in human and animal studies

Dopaminergic functioning and stress. A growing body of evidence has suggested that PTSD may involve a hyperdopaminergic state. Elevated urinary and serum levels of DA and increased striatal dopamine transporter (DAT) density have been observed in PTSD (Hamner & Diamond, 1993; Hoexter et al., 2012; Lemieux & Coe, 1995; Segman et al., 2002; Spivak et al.,

1999; Yehuda et al., 1992). Urinary DA concentrations have been correlated with severity of PTSD symptoms in both males and females, suggesting that hypersecretion of DA is associated with more severe manifestations of the disorder (Glover et al., 2012; Yehuda et al., 1992). Building on this, an *in vivo* single-photon emission computed tomography (SPECT) investigation observed elevated striatal DAT density in PTSD individuals (Hoexter et al., 2012). Together, these results propose dopaminergic hyperactivity as a characteristic of PTSD and may help explain the increased inclination of these individuals to engage in novelty-seeking behaviours, which are mediated by mesolimbic DA.

Animal research has also demonstrated that levels of DA and DA metabolites increase in certain brain regions in response to severe stress (Bliss & Ailion, 1971; Tidey & Miczek, 1996). This is evidenced in *in vivo* microdialysis studies, where various severe stressors (i.e. social defeat stress, tail shock) result in increased extracellular DA release in the prefrontal cortex (PFC) and NAcc (Tidey & Miczek, 1996). Stress has also been associated with locomotor behavioural sensitization and enhanced vulnerability to develop self-administration, which are both modulated by the dopaminergic system (Piazza, Deminiere, le Moal, & Simon, 1990). Psychostimulant treatment has consistently displayed a cross-sensitizing effect when combined with stress. This sensitization has been linked to several neurochemical alterations, such as an increase in accumbal DA release, a decrease in DAT sites at the NAcc core, and lower D3 binding sites in the NAcc shell (Brake, Zhang, Diorio, Meaney, & Gratton, 2004; Kalivas & Stewart, 1991). These findings support the idea of altered DA transmission and receptor sensitivity in response to severe stress, resulting in considerable physiological and behavioural effects.

Reward functioning. The reward pathway, often referred to as the mesolimbic dopaminergic pathway, is occupied primarily by DA neurons that project from the ventral tegmental area (VTA) to the hippocampus, amygdala, septum, and nucleus accumbens (NAcc) (Dunlop & Nemeroff, 2007). A functioning mesolimbic dopaminergic system is necessary for modulating reward processes, such as reward anticipation, behavioural approach, processing salience of stimuli, and experiencing pleasure (Horvitz, 2000; Liberzon, Luan, Decker, & Taylor, 2003). Numerous studies have found that the mesolimbic reward pathway is highly vulnerable to stress, resulting in deficits or enhancements of reward functioning, and increasing vulnerability to other perturbations, such as addiction. The stress and reward pathways are crucial in maintaining homeostatic processes and behavioural control (Arnsten, 1998). Clinical research has demonstrated that increasing levels of stress can result in maladaptive behaviour, deficits in impulse control, and distress (Anestis, Selby, & Joiner, 2007; Barkley, 1997; Fishbein et al., 2006; Tice, Bratslavsky, & Baumeister, 2001). In situations of high stress, studies have shown that prefrontal functioning diminishes while limbic-striatal activity increases, revealing that the brain's stress mechanisms target the reward pathway (Arnsten & Goldman-Rakic, 1998; Li & Sinha, 2008; Sinha, 2008).

In regards to reward anticipation, one study demonstrated that males with PTSD were less motivated to extend viewing time of attractive female faces (Elman et al., 2005). Similarly, males with PTSD reported lower expectancy and satisfaction with rewards from a Wheel of Fortune-type reward paradigm (Hopper et al., 2008). This reflects impairments in reward anticipation and motivation, which supports the findings that PTSD individuals display lower 'wanting' of a reward when more effort is required (Casada & Roache, 2005). Neuroimaging studies in both males and females demonstrated that individuals with PTSD are less aroused in

response to pleasant images, compared to healthy subjects, consistent with the emotional numbing symptom cluster (Spahic-Mihajlovic, Crayton, & Neafsey, 2005). Functional magnetic resonance imaging (fMRI) has also shown that PTSD patients have reduced bilateral striatal activations compared to healthy individuals (Elman et al., 2009). Based on these findings, it appears that PTSD is associated with impairments in reward anticipation. Additionally, studies using self-report questionnaires discovered that approach behaviours in response to rewarding stimuli were positively related to PTSD avoidance symptom severity (Contractor, Elhai, Ractliffe, & Forbes, 2013; Pickett et al., 2011). This suggests that individuals with PTSD engage in reward-seeking behaviours not due to increased positive affect, but as an avoidance strategy. This pattern of behaviour can be related to the concept of sensation-seeking or novelty-seeking (Contractor et al., 2013; Nawijn et al., 2015; Weiss, Tull, Viana, Anestis, & Gratz, 2012).

Novelty-seeking. PTSD individuals display an array of novelty-seeking behaviour, including substance abuse, antisocial behaviours, interpersonal aggression, binge eating, self-harm, and risky sexual behaviour (Cloitre, Koenen, Cohen, & Han, 2002; Galovski & Lyons, 2004; Holzer, Uppala, Wonderlich, Crosby, & Simonich, 2008; Jakupcak et al., 2010; Resnick, Foy, Donahoe, & Miller, 1989; Rosenberg et al., 2001). Individuals diagnosed with PTSD have a six-fold increased probability of committing suicide (Kessler, Borges, & Walters, 1999).

Psychophysiological studies have indicated that individuals with PTSD have heightened reactions in response to novel stimuli. Altered processing of novel stimuli in PTSD has been assessed using event-related electric brain potentials. Results from these studies show that PTSD patients are unable to filter irrelevant sensory stimuli and are overresponsive to novel stimuli (Karl, Malta, & Maercker, 2006; Scott P Orr, Metzger, & Pitman, 2002). This is consistent with hypervigilant behaviours, where individuals are highly sensitive to any external or internal

stimuli (Kimble et al., 2014). DA activity modulates emotional responses, and the dopaminergic dysregulation observed in PTSD could result in inappropriate reactions to novel stimuli (Rosenkranz & Grace, 2001). This is further supported by studies that have found that PTSD symptom severity is linked to increased reward-seeking/novelty-seeking (Contractor et al., 2013; Pickett et al., 2011). Thus, there appears to be a relationship between symptom severity, novelty-seeking, and DA dysfunction.

Novelty-seeking behaviours have consistently been linked to the mesolimbic DA pathway in animals. Novelty-seeking behaviours have been blocked by DA antagonists in a place preference test (Bardo, Neisewander, & Pierce, 1989) and by microinjections of DA antagonists into the NAcc in an open-field test (Hooks & Kalivas, 1995). In these cases, the diminished novelty-seeking observed was independent of any changes in locomotor activity. Depletion of mesolimbic DA structures via microinjections of 6-hydroxydopamine (6-OHDA) demonstrated a significant decrease in novel object exploration (Fink & Smith, 1980; Pierce, Crawford, Nonneman, Mattingly, & Bardo, 1990). This disruption in novelty-seeking was rescued and restored with apomorphine, a DA agonist (Fink & Smith, 1980). *In vivo* voltammetry measured DA-related electrochemical signals in the NAcc of rats in a free-choice novelty test, and showed a temporary increase in DA activity at the NAcc when the rat entered a novel compartment (Rebec, Christensen, Guerra, & Bardo, 1997). The results of these studies suggest that novelty-seeking is related to the positive incentive value of the novel stimuli and is mediated by mesolimbic DA, similar to processing of other reinforcing stimuli, such as food and drugs of abuse (Bardo et al., 1996; Pierce et al., 1990).

A number of studies have found that exposure to novelty induces an increase in accumbal DA release (Verheij & Cools, 2007, 2008). The D1 receptor also appears to play a role in

novelty-seeking, in which blockage of this receptor leads to disruptions in novelty-seeking behaviours in rats (Bardo et al., 1993). Individuals with higher impulsivity have lower levels of DA autoreceptors in the substantia nigra (SN)/ventral tegmental area (VTA), leading to elevated DA neuron firing and DA release following exposure to novel or rewarding stimuli (Buckholtz et al., 2010). Positron emission tomography (PET) is one method of investigation of DA receptors in the human brain. Researchers are able to use this technique to assess alterations in DA receptor levels in response to psychosocial stressors and behavioural tasks (Koepp et al., 1998). A PET study revealed that diminished D2/D3 autoreceptor availability in the SN/VTA area was correlated with higher impulsivity, whereas elevated prefrontal D2/D3 receptor availability was strongly associated with responsiveness to aversive/stressful stimuli (Kobiella et al., 2010). To date, there are no human studies exploring D2 and D3 receptor densities in PTSD individuals, however the previous data suggests that location and magnitude of DA autoreceptor availability could possibly mediate novelty-seeking behaviours in PTSD. Together, the accumulated evidence encourages preclinical research to explore the behavioural effects of elevated DA levels on reward functioning and novelty-seeking in PTSD.

Studies using the predator odour model of PTSD have shown varying results in regards to extracellular DA release. One microdialysis study illustrated an increase in DA release in male rats in the PFC within 5 min of exposure and the NAcc shell following 40 - 50 min of exposure. Interestingly, no effect on DA release was observed in the NAcc core (Bassareo, Luca, & Chiara, 2002). Other studies analyzing post-stress brain tissue found that predator odour did not result in an increase in DA metabolism in the NAcc, but resulted in an elevation in the PFC (Morrow, Redmond, Roth, & Elsworth, 2000; Wilson, Ebenezer, McLaughlin, & Francis, 2014). Intracranial self-stimulation (ISS) of the VTA, which is dependent on intact DA

neurotransmission, was not impaired in mice exposed to predator odour. However, there were impairments in the acoustic startle response. These results suggest that the anxiogenic effects (exaggerated startle response) following stressor exposure could be dissociated from the anhedonic effects (no difference in ISS). Thus, dopaminergic-mediated behaviours did not appear to be impaired by exposure to the predator odour stressor (Hebb, Zacharko, Gauthier, & Drolet, 2003). An inescapable shock stress resulted in reduced reward sensitivity and increased motivation to explore, which is similar to behavioural phenotypes of humans with PTSD (Shumake, Barrett, & Gonzalez-Lima, 2005).

Preclinical research has focused on the effects of severe stress on various neurochemical systems and behaviours. SPS has been extensively studied in regards to fear extinction responses, however there are gaps in the literature on its effects on specific neurotransmitters, such as DA, and behavioural effects associated with these systems. Currently, there is only one published study exploring the effects of the SPS model on dopaminergic-related behaviours. Eagle et al. (2015) demonstrated that SPS exposure induced a cross-sensitization with cocaine-related hyperlocomotion. There are no studies at this time examining novelty-seeking behaviours or directly evaluating DA function in this PTSD model.

Pilot work from our lab investigated the effects of SPS in male rats administered saline (SAL) or *d*-amphetamine (AMP) on exploratory behaviours in a modified novel object exploration (NOE) paradigm one week after the stressor. AMP increases DA release and induces dose-dependent hyperlocomotion in animals. Higher doses have been associated with stereotypy, thus a lower dose of 0.5 mg/kg was utilized in our pilot project to effectively induce hyperactivity without stereotypic behaviour (Arnt, 1995). A number of studies have concluded that exploratory and locomotor behaviours in animals are dependent on a functional mesolimbic

dopaminergic system (Fink & Reis, 1981; Fink & Smith, 1980; Mällo et al., 2007). Removal of mesolimbic DA neurons abolished exploratory behaviour in rats, but was rescued following systemic injections of a DA agonist (Fink & Smith, 1980). Thus, shifts in the modified NOE test can be linked to dopaminergic alterations. Approaching and exploring a novel stimuli is considered to reflect incentive salience and be motivating to animals, but neophobic tendencies typically conflict with this (Mällo et al., 2007). Stress or drugs of abuse can influence whether novelty-seeking or neophobia prevail in these behavioural tests.

Because the distinction between anxious behaviours (avoidance) and exploratory behaviours (novelty-seeking) when placing a rat in a novel environment may be problematic, it has been suggested to test in low-light conditions, include a habituation phase, and measure multiple behaviours to allow for a complete overview (Ray & Hansen, 2004). Typically, high levels of exploration have been related to high levels of novelty-seeking behaviours in other tests, such as the free-choice novelty test (Davis, Clinton, Akil, & Becker, 2008; Dellu, Mayo, Piazza, Le Moal, & Simon, 1993). Likewise, high exploration also predicts susceptibility to voluntary alcohol and drug intake (Galen, Hendersen, & Whitman, 1997; Johansson & Hansen, 2002). Thus, exploratory behaviours have been linked to both novelty-seeking and vulnerabilities to substance abuse.

Using the modified NOE conditions described above, SPS-exposed rats displayed more exploratory behaviours compared to control rats. These increased exploratory behaviours were comparable to those displayed by rats treated with AMP, suggesting that SPS could trigger an increase in dopaminergic activity similar to AMP. This preliminary work is the first to assess novelty-seeking behaviours – a predominant trait of PTSD – in animals. The heightened exploratory behaviours observed in SPS-exposed rats supports the high-risk hypothesis of

comorbid PTSD and SUD, in that SPS exposure promotes novelty-seeking and the tendency to engage in high-risk behaviours. Through this framework, it is proposed that SPS exposure results in a long-term dysfunctional increase in DA transmission, resulting in similar behavioural outcomes as AMP treatment.

Neuroendocrine abnormalities

In addition to neurochemical alterations, PTSD has been associated with modifications in of the HPA axis. The HPA axis is a vital biological system, responsible for managing the body's stress response. Individuals with PTSD have reportedly abnormal cortisol levels and alterations in the sensitivity of glucocorticoid receptors.

Cortisol. Cortisol, a glucocorticoid released from the adrenal cortex, is essential in terminating the biological stress response through negative feedback inhibition on the hypothalamus and pituitary. Typically, the level of cortisol secreted is a direct indicator of the magnitude of a stress response (Chrousos & Gold, 1992). Due to the considerable evidence linking high cortisol levels with increased stress responses and depression, researchers initially hypothesized that PTSD individuals would exhibit elevated cortisol levels (Yehuda, Teicher, Trestman, Levengood, & Siever, 1996). Unexpectedly, studies observed that urinary cortisol levels were lower in PTSD individuals compared to control subjects and patients with other psychiatric disorders (i.e. major depressive disorder, schizophrenia, and bipolar disorder) (Mason, Giller, Kosten, Ostroff, & Podd, 1986). To further support these findings, an epidemiological study of over 2000 Vietnam veterans revealed that levels of morning plasma cortisol in PTSD veterans were lower than in non-PTSD veterans, and that lower cortisol levels were associated with severity of combat trauma (Boscarino, 1996). Despite this, a systematic review of over 35 studies discovered that lower cortisol levels have only been found in PTSD

individuals exposed to physical or sexual abuse, and were more pronounced in females (Meewissee et al., 2007). Interestingly, reduced cortisol levels have been correlated with novelty-seeking behaviours and emotional withdrawal, which are consistent with the avoidance and arousal PTSD symptom clusters (Tyrka et al., 2008). Overall, evidence points towards hypocortisolemia as a defining characteristic of PTSD under specific conditions.

Despite exhibiting lower cortisol levels at baseline, individuals with PTSD display increased cortisol responsivity compared to healthy controls when exposed to a stressor. Cortisol levels in women with PTSD were 122% higher than non-PTSD controls during traumatic reminders (personalized trauma scripts), with severity of symptoms being highly predictive of cortisol levels (Elzinga, Schmahl, Vermetten, van Dyck, & Bremner, 2003). Alternatively, when a cognitive stressor was used, cortisol levels were not significantly different between PTSD and control groups (Bremner et al., 2003). The exaggerated cortisol response observed in only specific cases may be related to hyperarousal symptoms associated with PTSD. It is possible that individuals with PTSD experience periods of both hypocortisolemia and hypercortisolemia depending on the type of stressor they are faced with. In this theoretical framework, decreased basal cortisol levels in PTSD patients may compensate for states of elevated cortisol when exposed to stress.

Sex differences in severe stress

PTSD affects significantly more women than men in Canada, and thus research in females is critical, but lacking (Bangasser & Valentino, 2014; Van Ameringen et al., 2008). There are many reasons females are more vulnerable to PTSD, one being that women experience different types of trauma than men and its severity. A meta-analysis of PTSD studies revealed that adult or childhood sexual assault and abuse is significantly more reported by females,

whereas males typically report accidents, nonsexual assault, combat, injury, or witnessing death/injury as their history of trauma (Tolin & Foa, 2006). Sex differences are also seen in behavioural manifestations of the disorder. Males are more likely to exhibit higher levels of irritability, anger, verbal aggression, or violent behaviour (Bangasser & Valentino, 2014; Green et al., 1997). On the other hand, females are more likely to express higher anxious and depressive behaviours (Green et al., 1997). A study examining childhood physical and sexual abuse revealed that abused adolescent boys displayed higher levels of aggressive behaviour and higher probability of meeting diagnostic criteria for conduct disorder compared to abused girls (Darves-Bornoz, Choquet, Ledoux, Gasquet, & Manfredi, 1998; Livingston, Lawson, & Jones, 1993). The divergence of symptom patterns between males and females may also reflect the social expectations for each gender. Men and boys may view aggression and belligerence as a more acceptable response to a traumatic event, whereas, women and girls consider anxious and depressive behaviour to be more appropriate (Eagly, 2013). On other measures, such as emotional numbing, females are comparable to males with PTSD (Spahic-Mihajlovic et al., 2005).

Behavioural outcomes. It has been established that women with PTSD display more anhedonic behaviours and reward impairments compared to men (Green et al., 1997). Women are more likely to be victims of childhood trauma, which has been shown to result in dysfunctional reward functioning (Goff et al., 2012; Guyer et al., 2006). Women with PTSD reported lower positive emotions in response to pleasant imagery and elevated negative affect in response to both positive and negative imagery, compared to controls (Frewen et al., 2010). Similarly, women with PTSD rated the emotional expressions of happy faces as less positive than women without PTSD (Steuwe et al., 2012). An EEG study revealed that in response to

happy faces, women with PTSD displayed a slower P3 event related potential in frontal areas compared to trauma-exposed controls (Ehlers et al., 2006).

As previously established, diminished behavioural control is a common tendency among PTSD individuals. Women with PTSD display increased risk-taking behaviours compared to healthy female subjects, such as binge-eating, self-harm, and risky sexual behaviour (Cloitre et al., 2002; Holzer et al., 2008; Rosenberg et al., 2001). The range of impulsive behaviours differs from those more commonly observed in males, such as interpersonal aggression and substance abuse (Galovski & Lyons, 2004; Jakupcak et al., 2010). Impulsive behaviours are also related to increased approach or reward-seeking, and has been found to be mediated by emotional dysregulation in PTSD (Weiss et al., 2012). Controlled novelty-seeking behaviours in humans are less clearly understood. Sex differences tend to vary depending on the type of novelty tested (Feingold, 1994; Rosenblitt, Soler, Johnson, & Quadagno, 2001).

In nonstressed female animals, it has consistently been found that they exhibit more dopaminergic behaviours, such as novelty-seeking and exploratory behaviours, compared to males. Female animals have previously demonstrated elevated exploratory behaviours compared to males in various exploration tests (Hughes, 1968; Tropp & Markus, 2001). In a maze learning paradigm, females exhibited significantly higher exploratory activity compared to males (Joseph, Hess, & Birecree, 1978). This may be due to their overall higher activity levels (Archer, 1975). After stressor exposure, DA-mediated behavioural outcomes appear to vary between genders. Sucrose preference has been used to monitor DA-dependent hedonic behaviours. Chronic mild stress (CMS) exposure appears to decrease sucrose intake in both male and female rats (Dalla et al., 2008; Dalla, Antoniou, Drossopoulou, & Papadopoulou-Daifoti, 2005). In prairie voles, CMS elicits more robust impairments in sucrose intake in females compared to males (Grippo et al.,

2007). CMS also induced lower exploratory behaviours in a novel open field in females relative to males (Dalla et al., 2005).

Although the human PTSD literature has explored symptomology and neurobiology in both sexes, animal research is especially lacking. The majority of severe stress work has been conducted in male animals. However, there is growing evidence that severe stress can affect female animals differentially. Chronic restraint stress has been shown to cause deficits in extinction processes in male animals, consistent with PTSD animal studies. However exposure to this chronic stressor had the opposite effect in females, with stressed female animals exhibiting enhanced fear extinction (Baran, Armstrong, Niren, Hanna, & Conrad, 2009; Izquierdo, Wellman, & Holmes, 2006). Although studies are limited, researchers have proposed that there are complex estradiol-stress relationships that may mediate the variance in behaviours between males and females, given the link between high estradiol levels and enhanced extinction (Antov & Stockhorst, 2014; Lebron-Milad, Graham, & Milad, 2012). Increased corticosterone negative feedback has also been observed in females using a single electric footshock stressor followed by situational reminders, with responses comparable to the neuroendocrinological abnormalities observed in male SPS rats (Liberzon et al., 1997; Louvart et al., 2006). Together, these results indicate that severe stress has both similar and distinct impacts on females and male rats. This line of investigation warrants further research to explore the effects of severe stressors on female animals.

To date, there has only been one study testing females in the SPS model. Results demonstrated that females did not exhibit the same extinction-retention deficits sustained by SPS males, which conflicts with results from human studies as well. The authors suggest that this discrepancy may be due to differences between rodents and humans, and thus SPS may not be

the optimal PTSD model for female animals (Keller, Schreiber, Staib, & Knox, 2015). Due to the scarcity in female SPS literature, these results have not yet been replicated or disputed by other studies. Evidently, further work is needed in order to confirm these observations and explore other sex differences in the SPS model.

Neuroendocrine and neurochemical abnormalities. The literature on the neuroendocrinological and neurochemical profiles of women with PTSD have been established and are similar to those observed in men. Women with PTSD experience enhanced GR negative feedback (Stein et al., 1997). This has been replicated in female rats using an SPS model (Keller et al., 2015). Women also exhibit lower basal cortisol levels compared to men (Meewissee et al., 2007). Neurochemically, comparable hypersecretion of DA in urine have been found in females and males with PTSD (Lemieux & Coe, 1995; Meewissee et al., 2007). Furthermore, female PTSD patients also exhibit impairments in fear extinction, however these deficits have been associated with naturally low levels of estrogen (Glover et al., 2012). These studies support the emerging view that estrogen may play a protective role against the symptomology of PTSD, and that decreases in estrogen secretion may lead to more severe PTSD. Understanding the underlying neurobiological mechanisms of this disorder in both males and females is an advancement towards developing effective treatment strategies. In this context, establishment of an accurate animal model of PTSD is essential and would provide insight into this complex psychiatric disorder.

Female gonadal hormones impact many different neural circuits in both humans and animals. Numerous studies have suggested that estrogen and progesterone modulate DA activity in the striatum and NAcc. Estrogen elicits DA release via G-protein coupled receptors on DA terminals in the NAcc (Becker, 1999). In males, gonadal hormones do not affect DA activity to

the level observed in female rats. This intense estrogen-DA relationship results in sex differences of DA-mediated behaviours and DA transmission. These effects vary based on estrus cycle stage, inducing variations in basal extracellular DA release. Estrogen priming in ovariectomized female rats was found to cause a phasic increase in mesolimbic DA release, accompanied by significant increases in DA reuptake and clearance times (Thompson & Moss, 1994). Previous work has shown that estrogen is associated with increased AMP-induced sensitization and striatal DA release (Stöhr, Wermeling, Weiner, & Feldon, 1998). However, these effects fluctuate in response to stage of estrous cycle, where AMP-stimulated DA release in striatal tissue is lower in proestrus females compared to diestrus and estrus females (Becker & Ramirez, 1981). Furthermore, intraperitoneal injection of a D1 receptor agonist (SKF38393) rescues fear extinction deficits in low-estrogen female rats (Rey, Lipps, & Shansky, 2014). Together, these findings suggest that estrogen modulates DA activity at the NAcc and that there is a complex relationship between estrogen and DA. Studying female animals would aid in the establishment of key sex differences in DA neurotransmission.

When adding stress as a factor, there are various sex differences and estrus stage-dependent effects. Acute stress induced significantly elevated basal glucocorticoid levels in females compared to males (Shors, Pickett, Wood, & Paczynski, 1999). Unpredictable stress and restraint stress in regularly cycling female rats resulted in elevated corticosterone during all stages of the cycle, with proestrus rats exhibiting significantly higher levels than estrus and diestrus females (Pollard, White, Bassett, & Cairncross, 1975; Viau & Meaney, 1991). Proestrus involves elevated levels of estrogen secretion with minimal progesterone secretion in unstressed rats. It has been established that estrogen mediates corticosterone release, while progesterone seems to inhibit some of the estrogen-mediated glucocorticoid secretion (Bowman, Ferguson, &

Luine, 2002; Pollard et al., 1975). Together, these results propose that females are most vulnerable to stress exposure and neuroendocrine dysfunctions during proestrus. Stressors have also lead to disruptions in the estrus cycle, with studies suggesting that physical stressors result in major disturbances involving a constant diestrus state, while emotional stressors lead to relatively minor complications (Echandía, Gonzalez, Cabrera, & Fracchia, 1988; Pollard et al., 1975). In terms of DA neurotransmission, chronic mild stress showed decreased DA activity in the PFC in females, whereas no effects were found in males (Dalla et al., 2008). Further exploration into the link between estrogen, PTSD, and DA and its associated behaviours is necessary.

Objectives and Hypotheses

The current study has three major objectives. First, the effects of SPS on dopaminergic-related behaviours, such as novelty-seeking and exploratory behaviours, will be explored. Second, as elevated plasma and urinary DA concentrations (Mason et al., 1986; Yehuda et al., 1992) and increased cortisol response to stress (Elzinga et al., 2003) have been found in humans, DA activity in the NAcc and plasma corticosterone levels in response to a mild stressor will be characterized in SPS rats. An AMP-treated group was added to provide a positive control for behavioural and neurochemical outcomes. Finally, only a limited amount of work has examined sex differences in the SPS model, despite the high prevalence of PTSD in females. For this reason, behavioural, corticosterone, and NAcc DA activity resulting from SPS exposure will be examined in females as well.

Based on the prevalence of novelty-seeking in humans with PTSD and the involvement of DA in this response, it is hypothesized that increased novelty-seeking behaviours will be

observed in male and female SPS rats compared to rats not exposed to SPS. In conjunction with this and based on previous animal studies, it is hypothesized that AMP administration coupled with SPS exposure will result in a cross-sensitization of these behaviours in both male and female rats, reflecting the high comorbidity and overlapping mechanisms of PTSD and SUDs. Second, with the evidence of hyperdopaminergic states in PTSD, we hypothesize that SPS will result in elevated extracellular DA levels in the NAcc of both male and female rats, and that extracellular DA elevations will be more pronounced in rats injected with AMP. Finally, it is important to further characterize the SPS paradigm in female animals and determine whether it is an effective animal model of PTSD in females. A growing body of evidence within clinical research has suggested that low estrogen may be a factor in developing PTSD symptoms and that estrogen has the ability to modulate mesolimbic DA release, revealing a complex estrous-dopamine relationship. Taken together, it is hypothesized that female SPS rats will show diminished DA release and novelty-seeking behaviours compared to male SPS rats, due to the additional variable of estrogen.

To test these hypotheses, we examined the capacity of SPS to alter 1) behaviour in the modified novel object exploration (NOE) paradigm and 2) DA release at the NAcc using *in vivo* microdialysis in both male and female animals.

Method and Materials

Animals

Seventy-two male and seventy-two female Sprague-Dawley rats (200-225 g) were obtained from Charles River laboratories in St-Constant, Québec. Rats were housed in pairs in polycarbonate cages (10" x 14" x 7.5") for 1 week prior to single prolonged stress (SPS) exposure. Following SPS exposure, rats were individually-housed for the remainder of the study. A 12-hour light/dark cycle (lights on from 07h00 to 19h00) was maintained in the animal room, with temperature (22°C) and humidity (63%) kept constant, and food and water provided *ad libitum*. Vaginal swabbing (dorsal approach) was performed daily at 9h30 in females to confirm phase of estrous cycling which was defined using cytology results based on previous literature (Shansky, Rubinow, Brennan, & Arnsten, 2006). All experimental procedures were approved by the Animal Care Committee at the Royal's Institute of Mental Health Research and met the guidelines set out by the Canadian Council on Animal Care.

Summary of procedures

Forty male and forty female rats were used to assess behavioural and physiological effects in response to SPS. All rats were handled for 7 days prior to stress day, where half of the animals experienced the SPS procedure. All testing occurred at least 7 days following stressor exposure. Animals were randomly assigned to treatment groups (NoSPS+Sal, NoSPS+Amp, SPS+Sal, SPS+Amp) before testing. ASR was conducted in order to validate the SPS model in our rats. An exaggerated ASR is a common behavioural symptom of both PTSD and SPS (Grillon et al., 1996; Khan & Liberzon, 2004; S. P. Orr, Lasko, Shalev, & Pitman, 1995). Additionally, thirty-two male and thirty-two female rats were used for microdialysis to

evaluate extracellular DA content at the NAcc in all treatment groups. Female animals were swabbed daily for 14 days prior to Day 0.

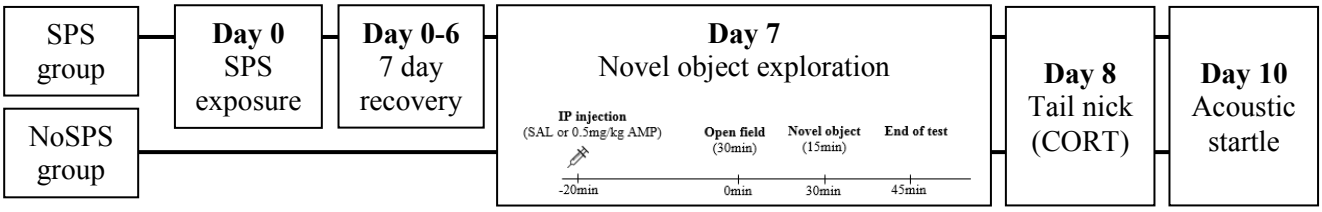

Figure 1: Experiment 1 procedure. Male and female rats underwent the following experimental procedures. Depending on treatment group, rats were or were not exposed to SPS on Day 0. NOE testing occurred on Day 7 following SAL or AMP pretreatment. Tail nicks for corticosterone sampling occurred the next day (Day 8), followed by acoustic startle response on Day 10.

Single Prolonged Stress (SPS)

Half of the rats from each cohort were exposed to the SPS procedure. First, rats experienced a 2-h physical restraint stress in plastic conical bags, where their noses faced a small hole at the tip and tape was used to secure the bag tightly around their tail to immobilize animals. Rats were then individually placed in vertical columns of water with a depth of 30 cm and underwent forced swim for 20 min (water temperature 22±2°C). Following this, rats were immediately removed from the water columns and placed back in their home cages to allow a 15-min recuperation period. Rats then underwent diethyl ether anesthesia via inhalation for 2-5 min until muscle tone was lost. Following this, rats were singly-housed and left undisturbed for 7 days. Control (No SPS) rats from each cohort did not undergo SPS exposure, but were transitioned to individual housing the same day. Daily vaginal swabbing was halted during the 7 days post-SPS and resumed on Day 7 prior to behavioural testing. This was based on previous protocol for SPS procedure in female animals (Keller et al., 2015).

Drugs and Injections

D-amphetamine (AMP) was dissolved in Krebs ringer buffered saline solution (SAL) consisting of 2.7 nM K^+, 145 nM Na^+, 1.35 nM Ca^{2+}, 1.0 nM Mg^{2+}, and 150 nM Cl^- ascorbate, with a pH of 7.4. SAL or AMP (0.5 mg/kg) were injected intraperitoneally 20 min prior to the modified NOE test (Experiment 1) and 5 min prior to the microdialysis procedure (Experiment 2). Control animals received a volume of SAL proportional to their weights.

Modified Novel Object Exploration (NOE) test

Exploratory behaviours reflect a properly functioning mesolimbic dopaminergic system, which can also be linked to reward functioning and novelty-seeking behaviours (Fink & Smith, 1980; Fink & Reis, 1981; Horvitz, 2000; Jentsch & Taylor, 1999). Twenty minutes after SAL or 0.5 mg/kg AMP injections, rats from Cohorts 1 (males) and 2 (females) were individually placed in the center of an open field and allowed to habituate to their new environment for 30 minutes. The open field measured 50cm x 50cm x 50cm, and was thoroughly cleaned with 70% ethanol between sessions. A novel object constructed of Duplo Lego blocks was then placed in the center of the open field and behavioural exploration was assessed for 15 minutes. The novel object was visually stimulating, with multiple colours and an irregular shape, with a sufficient height (10 cm) and width (7 cm) as to avoid rats climbing over or sitting on it.

Three modifications to the traditional NOE paradigm used by Lemos et al. (2012) were made to better serve the purpose of our experiment. First, time spent in the empty open field was increased from 15 min to 30 min to allow for thorough habituation to the testing arena. Second, the test was conducted on a single day rather than over multiple days with different novel objects each day to preserve the novelty aspect of the paradigm. Finally, additional measures have been

included to provide a wholesome perspective of the behaviours observed within this paradigm. Typically, researchers have focused on only one or a few of the following measures: center duration, number of approaches, locomotor activity, and total object contact time (Fink & Reis, 1981; Lemos et al., 2012). As outlined below, we examined all of these measures along with additional ones.

Exploration measures in the NOE test were divided into locomotor exploration and investigatory exploration (novelty-seeking behaviours), based on previous research (Fink & Reis, 1981; Lemos et al., 2012). Locomotor exploration encompassed center duration (time spent in center area) and locomotor activity (total distance travelled). Investigatory exploration included number of approaches to novel object (nose directed at object (< 2cm)), total object contact time (time spent touching the object with forepaws and sniffing), and latency to object. An additional measure of freezing time was also included to further explore anxiety-like behaviours within this paradigm. All sessions were videotaped and scored afterwards using computer software or manually. The Noldus computer software was used in order to objectively and consistently assess center duration, locomotor activity, and number of approaches. Total object contact time and freezing behaviour were evaluated manually using ODLog software. Latency to object was assessed manually as well. Locomotor activity and freezing behaviour was assessed over the full 45 minutes of the paradigm, and thus values were clustered into time bins of 15 minutes (i.e. 0-15 min, 15-30 min, and 30-45 min) to allow for a more accurate and time-dependent reflection of these behaviours. All other measures were evaluated during the last 15 minutes of the test when the novel object was present. Results from the pilot project conducted earlier in male rats were combined with the results of the current study, with all parameters and procedures kept identical.

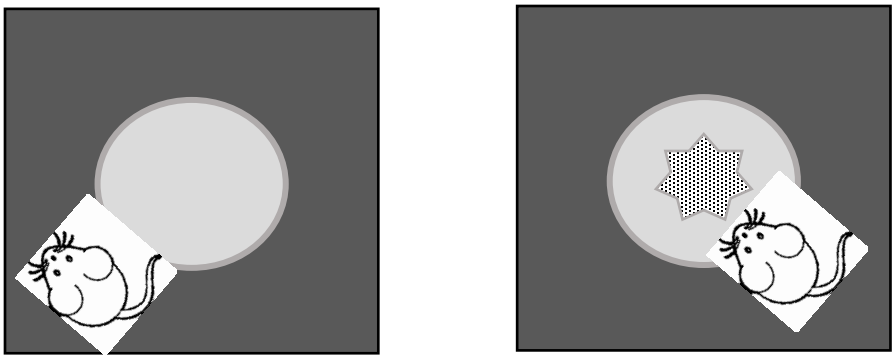

Figure 2. Summary of modified NOE paradigm. Rats were placed in an open field arena for 30 min to habituate to their environment, followed by placement of novel object (dotted star) in the center for an additional 15 min. Arena was divided into periphery (dark gray) and center (light gray) areas during analysis.

Body weight

Weights of rats were recorded on Day 0 and again on Day 7 at 9h30. This was done to assess any body weight gain differences between SPS-exposed and control rats.

Corticosterone sampling

Blood samples for corticosterone levels were obtained via tail nick 1mm from the tip of the tail. Samples were collected one day following the NOE test to allow for corticosterone levels to return to baseline. Collections were made at 0 min (baseline) and then 15 min, 30 min and 60 min following a mild restraint stress to examine stress reactivity in NoSPS and SPS exposed rats.

Acoustic Startle Response (ASR)

An ASR procedure adapted from those described previously was used (Kohda et al., 2007). An acrylic cylinder holder (9 cm in diameter and 20 cm in length) connected to a piezoelectric accelerometer (San Diego Instruments Inc., San Diego, CA, USA) was placed in a soundproof chamber (28.5×30.5×28.5 cm). A high-frequency loudspeaker inside the chamber produced both continuous background noise and acoustic stimuli. Ten days following SPS exposure, rats were placed in the holder for a 20-min startle session. One session consisted of 5 min of habituation followed by 15 min of 50-ms bursts of 90 dB, 105 dB, and 120 dB delivered in a randomized order 30 times every 30 s. Chambers were not illuminated during the procedure. The amplitudes of ASR converted to voltage were transferred to a computer using SR-LAB software (San Diego Instruments).

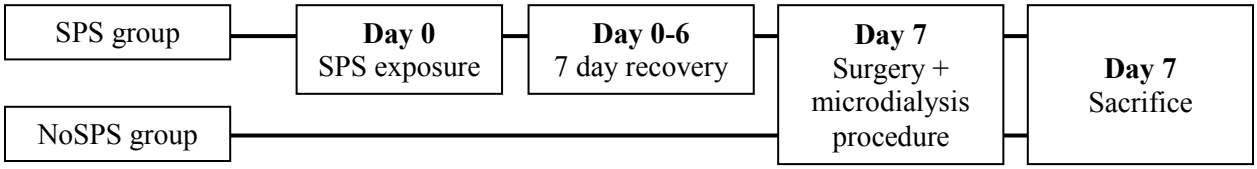

Figure 3. Experiment 2 procedure. Male and female rats underwent the following experimental procedures. Depending on experimental group, rats were or were not exposed to SPS on Day 0. On Day 7, surgeries were performed in preparation for microdialysis in anesthetized rats, with microdialysis following immediately after. Rats were then sacrificed and brains were collected after the procedure.

Surgery

Male and female rats designated for microdialysis procedures were anesthetized with isoflurane inhalant (1.5%) and placed into a stereotaxic frame (Kofp Instruments, Tujunga, CA). A central microdialysis probe (Bioanalytical Systems Inc, Lafayette, IN) was lowered into the right NAcc using the following coordinates relative to bregma, such that the active membrane

extended through the NAcc; AP +1.70, ML +1.60, and DV -7.2, angle at 10°. Probes were held in place by the stereotaxic arm during the experiment.

Microdialysis

Artificial cerebrospinal fluid (aCSF) was perfused through the probes at a flow rate of 1.5 µl/min, and sample collection began after a 2 h stabilization period. Dialysate samples were collected every 20 min for up to 5 h. Following this, three baseline samples were collected. Five minutes prior to the end of the third baseline sample, rats were injected with SAL or AMP (0.5mg/kg i.p.) and an additional seven samples were collected for up to 140 minutes post-injection. A summary of sample collection is outlined below in Figure 4.

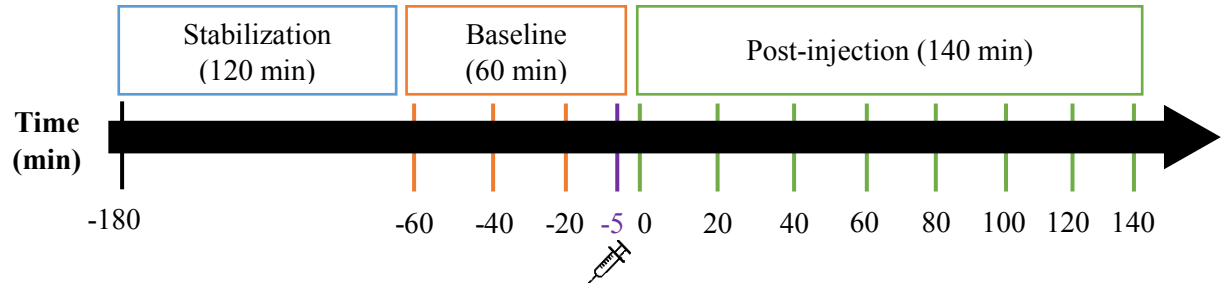

Figure 4. Dialysate sample collection. Following a 2 h stabilization period, baseline samples (orange lines) were collected every 20 minutes. At -5 minutes, a SAL or 0.5 mg/kg AMP (i.p.) injection was administered. Seven post-injection samples (green lines) were collected every 20 minutes.

Dialysate Sample Analysis

Immediately after collection, samples were acidified with 5 µL of glacial acetic acid. High performance liquid chromatography (HPLC; Agilent Technologies, Walbronn, Germany) was employed in order to analyze DA content of the dialysate samples. A 40 ul volume of each sample was injected via an autoinjector into the HPLC system. Separation of analytes was

achieved by a Kinetex C18 column (4.6 x 100 mm 2.6 μm, Phenomenex Inc.) which was calibrated at a flow rate of 0.5 mL/min with mobile phase consisting of the following (in mM): 90 sodium dihydrogen phosphate (monobasic), 1.7 1-octane sulfonic acid (sodium salt), 50 citric acid (monohydrate), 2 KCl, 50 mM EDTA, and 10% acetonitrile, with a final pH of 4.4.

DA elution was detected by a single cell electrochemical detector (Intro; Antec Leyden, Montreal, Canada) at 500 mV vs Ag/AgCl, a filter of 1 s, and a range of 1.0 nA/V. Quantification analysis of DA was performed by comparing their area under the curve to that of a known external standard (calibrated at 9 points, 1.0 through 250 pg, 40 μL injections) using computerized Agilent ChemStation chromatography data acquisition system (Agilent).

Histology

Following completion of microdialysis experiments, rats were euthanized and brains removed and rapidly frozen in ethanol. Brains were appropriately sectioned and mounted on gel slides to confirm injection sites and probe placements. Only animals for which all placements were accurate were included in the data analysis.

Statistical Analysis

All statistical analyses were conducted using IBM SPSS Statistics Version 20. Results from the modified NOE test were combined with those obtained from the pilot project, with an overall sample size of 63 rats. This was done after ensuring that all testing parameters (i.e. time of day, protocol, arena, treatments, pre-stress handling) were replicated and consistent with the pilot study. Center duration, number of approaches, total object contact time, and latency to object in the NOE test were analyzed using a series of two-factor analyses of variance (ANOVAs), with Stress (NoSPS or SPS) and Treatment (SAL or AMP) as between-group variables. Locomotor activity and freezing behaviour scores in the NOE test were clustered into

three time bins (0-15 min, 15-30 min, and 30-45 min) and analyzed using a mixed ANOVA, with Stress (No SPS or SPS) and Treatment (SAL or AMP) as the between-group factors, with Time as the within-subjects factor.

A mixed ANOVA design was used in order to further analyze ASR results. Sound (90 dB, 105 dB, 120 dB) was considered the within-subjects factor, while Stress (NoSPS or SPS) acted as the between-subjects factor.

Changes in body weight were assessed using repeated measures ANOVA, with Stress (NoSPS or SPS) as the between-subjects factor and Day (Day 0 or Day 7) as the within-subjects variable. For plasma corticosterone, an independent t-test was used in order to compare percentage of differences from baseline corticosterone in NoSPS and SPS-exposed male rats at each time point (15 min, 30 min, 60 min). Stress (NoSPS or SPS) acted as the only independent variable.

$$\%\ change\ from\ baseline\ CORT = 100 \times \frac{(absolute\ value\ - baseline\ at\ 0\ min)}{baseline\ at\ 0\ min}$$

Equation 1. Percentage change from baseline plasma CORT release. This equation was used to convert plasma CORT obtained via tail nick at every time point (15 min, 30 min, 60 min) following a mild restraint stress. This reflects the change in CORT release relative to each individual rat's baseline level.

In regards to microdialysis, both absolute values and percentage change from baseline values were used. A mixed repeated measures ANOVA was used in order to analyze the absolute values of extracellular DA content. DA level acted as the dependent variable, with Time (0, 20, 40, 60, 80, 100, 120, 140 min) as the within-subjects factor, and Stress (NoSPS or SPS) and Treatment (SAL or AMP) as between-subjects factors. Data was then converted into percentage

change using Equation 2. Three baseline measures were averaged for each animal and denoted as 100% of pre-injection DA levels. Post-injection sample values were subsequently expressed as a percentage of change from the baseline average value, and served as the dependent variable. A multivariate ANOVA was conducted in order to analyze percentage of DA change (from baseline average) with Stress and Treatment as between-group factors. Dependent variables included percentage difference from baseline accumbal DA release at seven time points (20 min, 40 min, 60 min, 80 min, 100 min, 120 min, and 140 min). Both absolute values and percentage change values were separately analyzed for main effects and interactions. Alpha level was set to 0.05 for all data. Data from male and female animals were analyzed separately.

$$\%\ change\ from\ baseline\ DA = \ 100 \times \frac{(absolute\ value\ - baseline\ average)}{baseline\ average}$$

Equation 2. Percentage change from baseline accumbal DA release. This equation was used to convert absolute values at every time point after injection. This reflects the magnitude of change in DA release relative to each individual rat's basal level.

Results

Experiment 1: Physiological and behavioural effects of SPS exposure in males and females

Physiological Measures

Weight. Figure 5A presents body weights on Day 0 (pre-SPS) and again on Day 7 (post-SPS) among male rats exposed to SPS and non-stressed rats. Results indicated that the interaction between Day and Stress, $F(1, 38) = 8.420$, $p = .006$, was significant. Follow-up analyses of the simple effects comprising the Day x Stress interaction revealed that weights did not differ on Day 0 ($p = .298$) between NoSPS and SPS rats. However, by Day 7 SPS-exposed rats exhibited significantly lower weight gain compared to NoSPS rats ($p = .005$).

Likewise, analysis revealed that the interaction between Day x Stress, $F(1, 37) = 21.636$, $p = .000$, was significant in female animals as well (Figure 5B). Follow-up analysis of the simple effects comprising this interaction showed that there was no significant difference in weights between NoSPS and SPS groups on Day 0 ($p = .372$). However, on Day 7 SPS rats had significantly lower body weight gain compared to NoSPS rats ($p = .002$).

Plasma corticosterone levels. Figure 6A shows percentages of plasma CORT changes from baseline (Time 0) at three different time points following exposure to a mild stressor (15min, 30min, 60min). Analysis revealed that SPS rats exhibited lower percentage of CORT changes from baseline compared to NoSPS rats at 15min [$t(27) = 2.701$, $p = .012$], 30min [$t(27) = 2.695$, $p = .021$], and 60min [$t(27) = 2.695$, $p = .012$].

Figures 6B and 6C display stress reactivity trends in female animals in both diestrus and estrus. Only rats in diestrus and estrus were included in analysis as they comprised the majority of the sample. Thus, one rat was excluded due to being in proestrus on the day of testing, and

two rats were excluded based on the IQR method of identifying outliers. An independent samples t-test showed that during diestrus, percentage change from baseline CORT was not significantly influenced by SPS exposure at 15min [$t(16) = 1.202$, $p = .247$], 30min [$t(16) = 1.279$, $p = .219$], or 60min [$t(16) = 1.844$, $p = .084$] after a mild stressor. A similar pattern was observed in estrus females as well, revealing no Stress effects on stress reactivity at any time points: 15min [$t(14) = 1.123$, $p = .280$], 30min [$t(14) = .974$, $p = .347$], or 60min [$t(14) = -.029$, $p = .977$]. Although effects failed to reach significance, graphical analysis showed that SPS-exposed rats in both diestrus and estrus displayed lower magnitudes of change from basal CORT compared to NoSPS rats. This trend of stress reactivity is comparable to what was discovered in males.

Estrous cyclicity. Daily vaginal swabbing confirmed that 39 female rats were cycling regularly and were thus used in subsequent behavioural testing. The average estrous cycle length between the NoSPS and SPS females did not statistically differ. The pre-stress average cycle length was 4.16±0.09 days for NoSPS animals and 4.14±0.09 days for SPS animals (mean ±S.E.M.), which are typical estrous cycle lengths for female rats (Goldman, Murr, & Cooper, 2007).

Behavioural Measures

Novel object exploration. Only one male rat (from NoSPS+Saline group) was excluded from all statistical analysis, due to experimental difficulties in the NOE paradigm, where the rat was able to move the novel object from the center, thus skewing results. For females, the original sample size of 39 rats was decreased to 28 rats, due to the following experimental and statistical reasons. Four rats (n=1 from each group) were excluded due to experimental difficulties in the NOE paradigm. Additionally, the IQR method revealed that one rat (from the NoSPS+Sal group)

was an extreme outlier, and thus was also removed from statistical analysis. Vaginal cytology revealed that among the remaining 34 rats, 28 rats were in diestrus and 6 in estrus on the day of behavioural testing. To prevent any confounds of estrus stage, only rats in diestrus were considered for statistical analysis, as they consisted of the majority of the sample.

Locomotor exploration: Center duration

Figure 7A presents the time spent in the center of the open field during the presentation of the novel object among SPS and NoSPS rats injected with AMP or SAL (something like this). Two rats (one from NoSPS+Sal and one from NoSPS+Amp) were excluded from analysis based on the interquartile range (IQR) method of identifying outliers (find REF **). The two factor ANOVA indicated no significant Stress x Treatment interaction, $F(1, 52) = 1.097$, $p = .300$, and no significant main effects of Stress $F(1, 52) = 3.290$, $p = .075$ or Treatment $F(1, 52) = 1.364$, $p = .248$ on center duration. Although results were not significant, the main effect of Stress suggested a trend for SPS-exposed rats to exhibit higher center duration times compared to non-stressed rats. Based on graphical analysis and the a priori hypothesis that SPS would induce enhanced exploratory behaviour, follow up simple effects comprising the Stress x Treatment interaction were conducted and revealed that SPS increased center duration times in SAL rats ($p = .053$) but not in AMP rats.

Figure 7B summarizes the center duration while the object is present among diestrus females in this behavioural paradigm. Two factor ANOVA indicated that time spent in center was influenced by Stress [$F(1, 24) = 6.080$, $p = .022$], Treatment [$F(1, 24) = 5.195$, $p = .032$], and the Stress x Treatment interaction [$F(1, 24) = 6.436$, $p = .018$]. Follow-up analysis of the simple effects comprising this interaction showed that AMP significantly increased center time

among NoSPS rats (p = .003) but not among SPS rats (p = .855), among which center duration was already increased due to stress (p = .003).

Locomotor exploration: Total distance travelled

In Figure 8A, total distance travelled during the full duration of the paradigm is displayed. Data was divided into 15 minute time bins to allow for a detailed overview of locomotor activity. The assumption of sphericity was violated according to Mauchly's test, Time $X^2(2) = 39.824$, p = .000, therefore Greenhouse-Geisser corrected tests are reported (ε = .654). AMP [$F(1, 54) = 6.430$, p = .014] and the Stress x Treatment interaction [$F(1, 54) = 4.379$, p = .041] significantly increased distance travelled. Follow up analysis of the simple effects comprising the Stress x Treatment interaction were conducted and revealed that AMP administration induced hyperlocomotion (p = .002) among nonstressed rats, but not among SPS rats in which locomotor activity was already increased by stress (p =.042).

In Figure 8B, total distance travelled by diestrus females during the full paradigm are displayed. The assumption of sphericity was violated according to Mauchly's test, Time $X^2(2) = 15.931$, p = .000, therefore Greenhouse-Geisser corrected tests are reported (ε = .673). A Stress x Treatment x Time interaction significantly affected distance travelled in diestrus females [$F(1.35, 33.67) = 3.602$, p = .054]. Follow up analysis of the simple effects comprising this interaction revealed that AMP-treatment significantly increased locomotor activity at all time points (see Table 1), except in the SPS group during the last 15 minutes where the novel object was present (p = .197), and SPS did not induce any changes to locomotor activity at any time points (Table 2).

Table 1

Summary of simple effects of AMP comprising the Stress x Treatment x Time interaction for total distance travelled in female animals

Stress	Time	Significance
No SPS	0-15 minutes	.002
	15-30 minutes	.012
	30-45 minutes (novel object)	.003
SPS	0-15 minutes	.004
	15-30 minutes	.000
	30-45 minutes (novel object)	.197

Table 2

Summary of simple effects of SPS comprising the Stress x Treatment x Time interaction for total distance travelled in female animals

Treatment	Time	Significance
Saline	0-15 minutes	.948
	15-30 minutes	.276
	30-45 minutes (novel object)	.108
Amphetamine	0-15 minutes	.939
	15-30 minutes	.670
	30-45 minutes (novel object)	.861

Investigatory exploration: Approaches to object

Two factor ANOVA revealed that SPS $F(1, 54) = 47.325$, $p = .009$, and AMP treatment $F(1, 54) = 3.881$, $p = .054$, significantly increased number of approaches to the novel object in male rats (Figure 9A). No significant Stress x Treatment interaction [$F(1, 54) = 1.630$, $p = .207$] was found, indicating that both SPS and AMP separately increased frequencies of approaching the novel object.

In regards to number of approaches to the novel object for female animals, there was no Stress x Treatment interaction [$F(1, 24) = 2.494$, $p = .127$]. However, based on graphical analysis and an a priori hypothesis that AMP administration and SPS exposure would increase approach frequency, follow-up simple effects revealed that AMP pretreatment increased number of approaches in NoSPS rats ($p = .017$), but not in SPS rats ($p = .144$) where approach frequency was already elevated due to SPS exposure ($p = .043$). Approach behaviour among females is summarized in Figure 9B.

Investigatory exploration: Total object contact time

Total object contact times among nonstressed and stressed rats administered SAL or AMP are displayed in Figure 10A. A two factor ANOVA revealed a significant main effect of Stress $F(1, 58) = 4.508$, $p = .038$, and Treatment $F(1, 58) = 7.851$, $p = .007$, with no significant Stress x Treatment interaction $F(1, 58) = .051$, $p = .822$. This indicates that SPS-exposed rats spent significantly more time sniffing and touching the novel object compared to control rats. Similarly, AMP-treated rats displayed higher total object contact times compared to SAL-treated rats. Together, these results suggest that SPS and AMP induce enhanced physical exploration behaviours (i.e. sniffing and touching of object) independently.

Figure 10B reflects the total time spent sniffing and touching the novel object displayed by female diestrus rats. A two factor ANOVA revealed a significant Stress x Treatment interaction [$F(1, 24) = 9.536$, $p = .005$]. Follow-up simple effects consisting of this interaction revealed that AMP administration increased total object contact time among No SPS rats ($p = .001$), but not among SPS rats ($p = .549$), among which stress exposure already enhanced object contact time ($p = .000$).

Latency to object

Figure 11A presents latency to object times among all male treatment groups. Two factor ANOVA revealed that the main effect of Treatment on the latency to approach the novel object was significant $F(1, 35) = 11.183$, $p = .002$, demonstrating that AMP-treated male rats took less time to approach the object compared to SAL-treated rats, regardless of stress exposure. The factor of Stress $F(1, 35) = .621$, $p = .436$, did not affect latency to object. Similarly, there was no significant Stress x Treatment interaction, $F(1, 35) = .439$ $p = .512$.

Latency to object times observed in female animals are displayed in Figure 11B. Two factor ANOVA revealed that Stress [$F(1, 24) = 6.876$, $p = .015$], Treatment [$F(1, 24) = 11.946$, $p = .002$], and the Stress x Treatment interaction [$F(1, 24) = 6.197$, $p = .020$] significantly decreased time to first approach the novel object. Follow-up simple effects consisting of the Stress x Treatment interaction demonstrated that AMP administration significantly reduced latency to object in the NoSPS group ($p = .000$) but not in the SPS group, in which decreased latency to object was already apparent ($p = .002$).

Freezing time

In regards to freezing time within the NOE paradigm, data was divided into three 15-minute time bins, which are presented in Figure 9. Mauchly's test of sphericity was violated, Time $X^2(2) = 41.206$, $p = .000$, therefore Greenhouse-Geisser corrected tests are reported ($\varepsilon = .591$). A Time x Treatment interaction significantly affected freezing behaviour, $F(1.18, 42.56) = .5.974$, $p = .015$. Follow up simple effects comprising this interaction revealed that during the first ($p = .872$) and second ($p = .136$) time bins, freezing time was not significantly influenced by Treatment. However, during the last time bin, in which the novel object was introduced, AMP

treatment significantly decreased freezing tendencies (p = .007). Freezing time was not significantly influenced by Stress $F(1, 36) = .004$, $p = .942$, or the Stress x Treatment interaction $F(1, 36) = 1.542$, $p = .222$.

Female freezing behaviour is presented in Figure 12B. Mixed ANOVA showed that freezing did not differ significantly between female groups at any of the time points. Freezing was not altered by Stress [$F(1, 24) = .337$, $p = .567$], the Stress x Treatment interaction [$F(1, 24) = .409$, $p = .528$] or any within-subjects effects. AMP appeared to decrease freezing, however, this effect did not reach significance [$F(1, 24) = 3.670$, $p = .067$].

Acoustic startle response test. As previously mentioned, three sound intensities, 90 dB, 105 dB, and 120 dB, were used in the ASR test. Startle amplitudes at each intensity between NoSPS and SPS groups are presented in Figure 10. Analysis revealed that the assumption of sphericity was violated according to Mauchly's test, Sound $X^2(2) = 16.265$, $p = .000$, therefore Greenhouse-Geisser corrected tests are reported ($\varepsilon = .619$). Results indicated that the startle amplitude was significantly affected by the intensity of the sound pulse, $F(1.24, 22.28) = 185.949$, $p = .000$, and exposure to SPS, $F(1, 18) = 11.863$, $p = .003$. Startle amplitude also significantly varied based on the Stress x Sound interaction, $F(1.24, 22.28) = 5.254$, $p = .025$. Follow up analysis of the simple effects comprising this interaction confirmed that within the NoSPS ($p = .000$) and SPS group ($p = .000$), ASR at each ascending sound intensity level significantly differed. It was also found that at 90 dB there was no significant SPS-enhanced ASR [$F(1, 18) = .56$, $p = .463$], suggesting that this sound intensity was ineffective in producing an exaggerated ASR in stressed rats. At both 105 dB [$F(1, 18) = 30.07$, $p = .000$] and 120 dB [$F(1, 18) = 5.39$, $p = .032$], SPS-exposed rats had significantly higher startle amplitudes

compared to NoSPS rats. As expected, stressed rats exhibited a significantly exaggerated ASR at the 105 dB and 120 dB level, compared to rats which were not exposed to SPS (Figure 10).

Female animals were divided into subgroups (diestrus and estrus) based on estrous stage on the day of ASR testing. Among females in diestrus, mixed ANOVA indicated that the assumption of sphericity was violated according to Mauchly's test, Sound $X^2(2) = 17.284$, $p = .00$, thus Greenhouse-Geisser corrected tests were used ($\varepsilon = .585$). As predicted, sound intensity significantly increased startle amplitude, $F(1.17, 17.55) = 34.103$, $p = .000$. SPS exposure [$F(1, 15) = .216$, $p = .649$] nor the Sound x Stress interaction [$F(1.17, 17.55) = .705$, $p = .434$], however, did not affect ASR in diestrus females,.

Analysis of the estrus subgroup revealed that the assumption of sphericity was violated according to Mauchly's test, Sound $X^2(2) = 5.980$, $p = .050$, therefore Greenhouse-Geisser corrected tests were reported ($\varepsilon = .690$). Startle amplitude was significantly affected by the intensity of the sound pulse [$F(1.38, 15.17) = 34.588$, $p = .000$] and the Sound x Stress interaction [$F(1.38, 15.17) = 4.943$, $p = .032$], but not by Stress [$F(1, 11) = 2.526$, $p = .140$]. Follow up simple effects consisting of the Sound x Stress interaction confirmed that at both 90 dB [$F(1, 11) = .22$, $p = .650$] and 105 dB [$F(1, 11) = .00$, $p = .960$] there was no significant SPS-enhanced ASR. At the highest sound pulse of 120 dB, SPS-exposed estrus rats had significantly higher startle amplitudes compared to NoSPS rats [$F(1, 11) = 4.86$, $p = .050$]. Thus, it appears that stressed rats in estrus exhibited a significantly exaggerated ASR at the 120 dB level, compared to rats which were not exposed to SPS, an effect that was not observed in diestrus females.

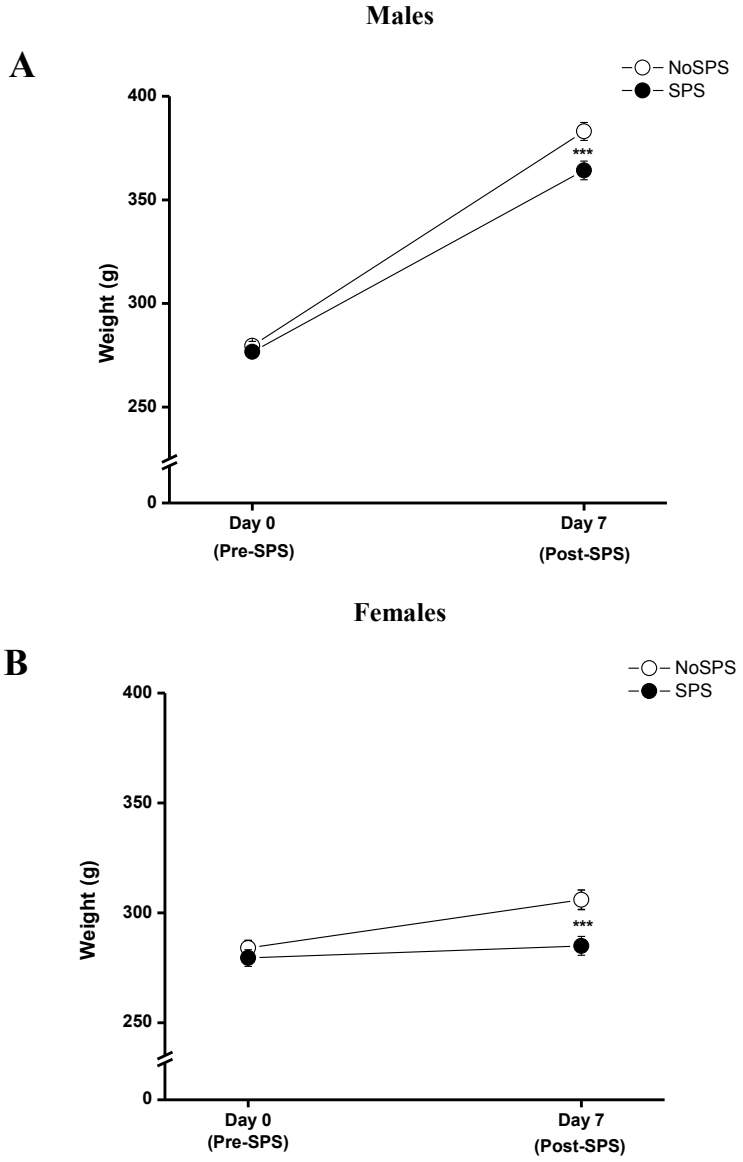

Figure 5. Effect of SPS exposure on body weight. A) In males, no significant differences were observed between groups on Day 0. SPS exposure significantly decreased weight gained on Day 7, compared to NoSPS rats. NoSPS rats (n=19) and SPS rats (n=20). B) An identical trend was observed in female rats, where SPS exposure resulted in lower weight gain than NoSPS rats.

Data represents mean (±SEM) weight (g) measured on Day 0 and Day 7.

*** $p < .005$ compared to the NoSPS group.

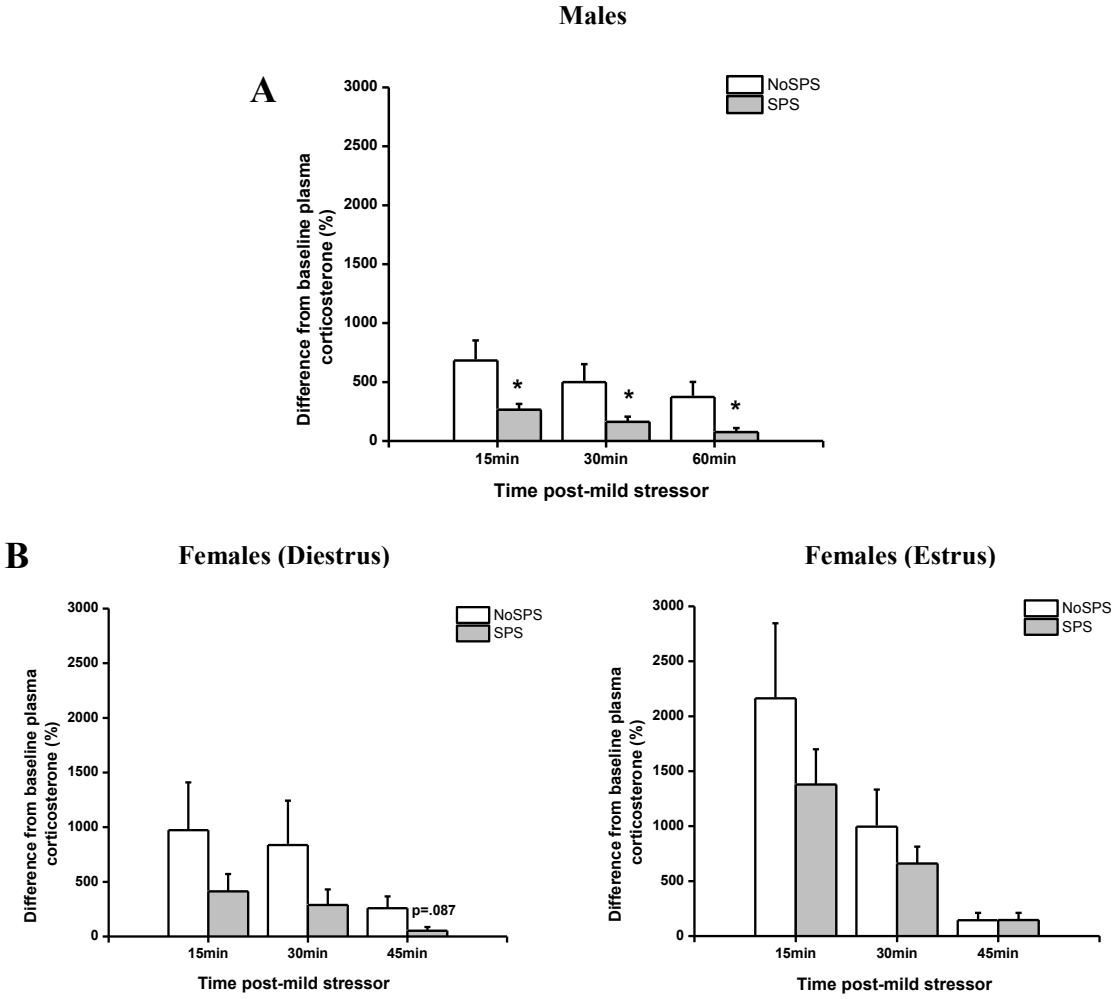

Figure 6. Effect of SPS exposure on percentage of plasma corticosterone (CORT) changes from baseline in rats exposed to a mild stressor. A) Male rats subjected to SPS had significantly lower percentages of plasma CORT changes at all time points after exposure to a mild stressor, compared to NoSPS rats. NoSPS rats (n=12) and SPS rats (n=17). B) Diestrus [NoSPS rats (n=9) and SPS rats (n=9)] and estrus [NoSPS rats (n=7) and SPS rats (n=9)]. females did not have significantly different magnitudes of change from baseline CORT at any time points. Data represents mean (±SEM) difference from baseline plasma corticosterone (%) measured at 0min, 15min, 30min, and 60min after a mild stressor. SPS = single prolonged stress.

*: $p < .05$ compared to NoSPS group.

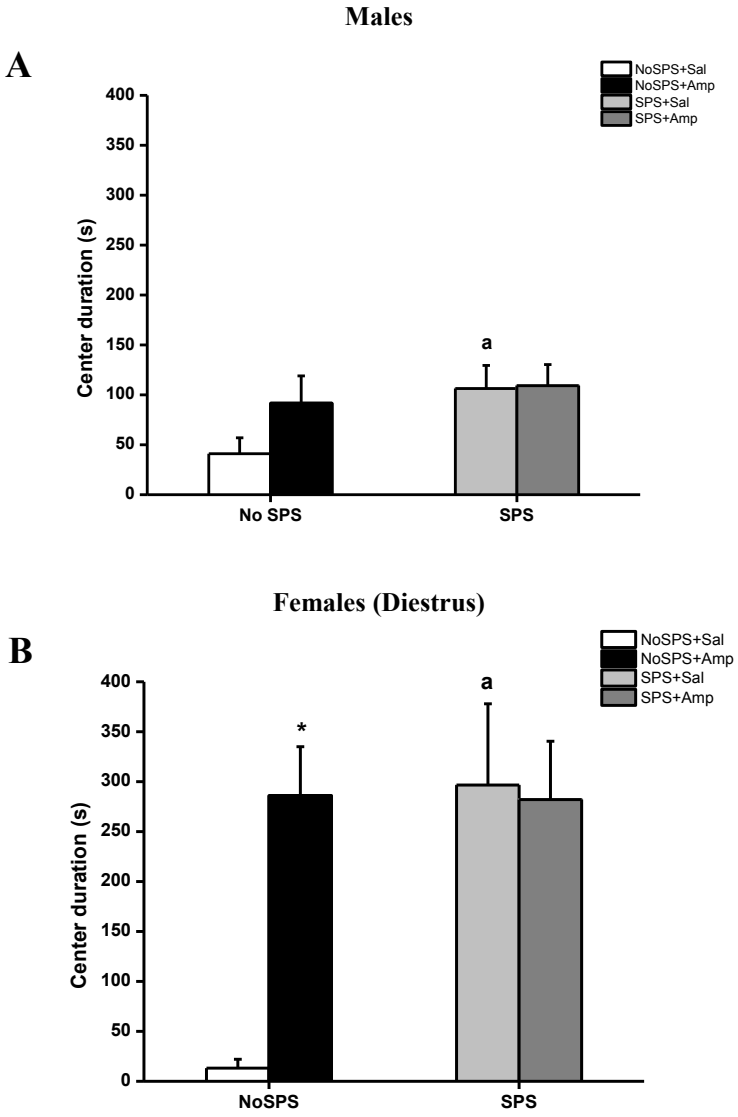

Figure 7. Effect of SPS exposure and AMP on center duration in the NOE paradigm. Time spent in the center of the arena was measured during the last 15 minutes of the paradigm where the novel object was present. A) SPS resulted in elevated center duration in male rats. NoSPS+Sal (n=13), NoSPS+Amp (n=15), SPS+Sal (n=16), and SPS+Amp (n=15) rats. B) SPS and AMP differentially enhanced time spent in center in female animals. NoSPS+Sal (n=7), NoSPS+Amp (n=7), SPS+Sal (n=6), and SPS+Amp (n=8) rats. Data represents mean (±SEM) center duration (s).

*: $p < .05$ compared to SAL matched counterpart; a: $p < .05$ compared to NoSPS matched counterpart.

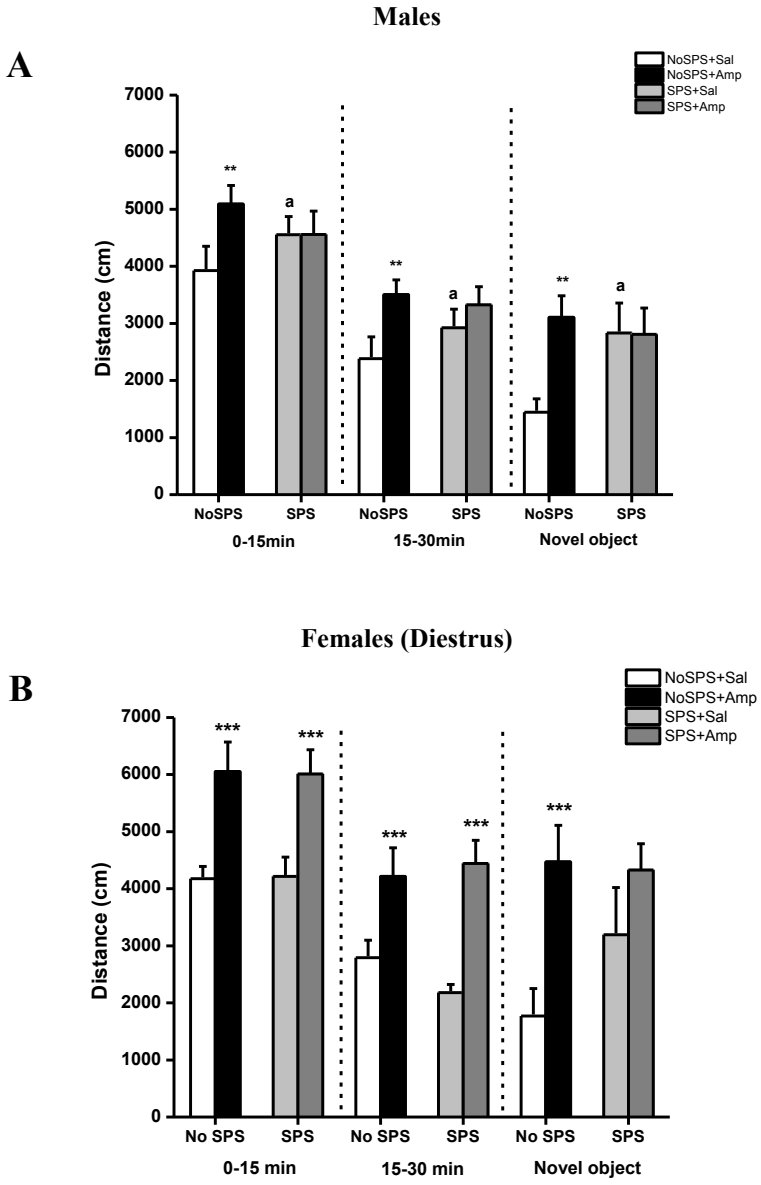

Figure 8. Effect of SPS exposure and AMP on total distance travelled in the NOE paradigm in male rats. Locomotor activity was measured as total distance travelled during 15 minute time bins. A) When the novel object was present, males displayed AMP-induced and SPS-induced hyperlocomotion at all time points. NoSPS+Sal (n=13), NoSPS+Amp (n=15), SPS+Sal (n=16), and SPS+Amp (n=15) rats. B) Female animals showed increased distance travelled due to AMP at all time periods except among SPS rats during the last 15 minutes. NoSPS+Sal (n=7), NoSPS+Amp (n=7), SPS+Sal (n=6), and SPS+Amp (n=8) rats. SPS = single prolonged stress. SAL = saline. AMP = amphetamine. NOE = novel object exploration. Data represents mean (±SEM) distance (cm) measured over a 15 min time bins.

**: $p < .01$ compared to SAL counterpart; ***: $p < .005$ compared to SAL counterpart; a: $p < .05$ compared to NoSPS counterpart.

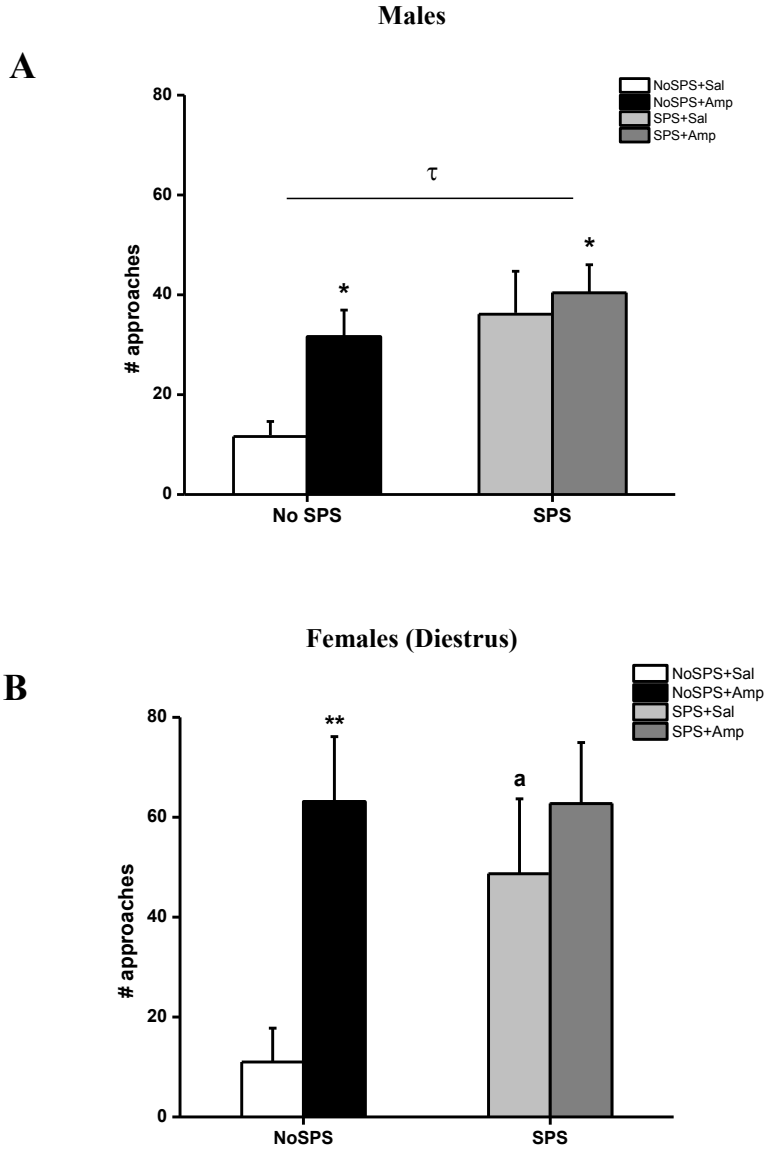

Figure 9. Effect of SPS exposure and AMP on number of approaches to object in the NOE paradigm. Number of direct approaches to the novel object were assessed over 15 minutes in male (A) and female (B) rats. SPS and AMP separately increased number of approaches in males [NoSPS+Sal (n=14), NoSPS+Amp (n=16), SPS+Sal (n=16), and SPS+Amp (n=16) rats] and diestrus females [NoSPS+Sal (n=7), NoSPS+Amp (n=7), SPS+Sal (n=6), and SPS+Amp (n=8) rats]. Data represents mean (±SEM) number of approaches.

*: $p < .05$ compared to SAL matched group; **: $p < .01$ compared to SAL matched group; τ: $p < .05$ between Stress groups. a: $p < .05$ compared to NoSPS matched counterpart.

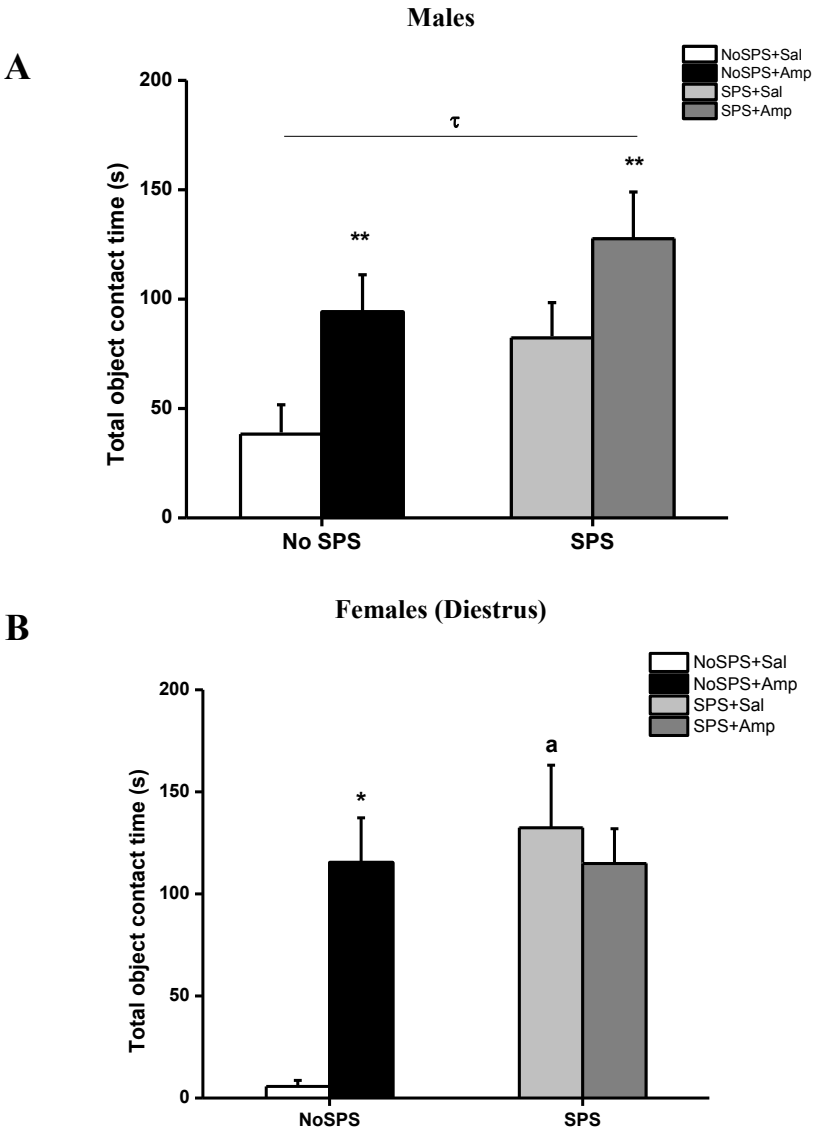

Figure 10. Effect of SPS exposure and AMP on total object contact time in the NOE paradigm. Total object contact time was defined as time spent sniffing and touching the novel object over 15 minutes. A) Male rats showed significant Stress and Treatment effects on increasing total object contact time. NoSPS+Sal (n=14), NoSPS+Amp (n=16), SPS+Sal (n=16), and SPS+Amp (n=16) rats. B) Female rats demonstrated an SPS- and AMP-enhanced effect of this measure. NoSPS+Sal (n=7), NoSPS+Amp (n=7), SPS+Sal (n=6), and SPS+Amp (n=8) rats. Data represents mean (±SEM) total object contact time (s). SPS = single prolonged stress. SAL = saline. AMP = amphetamine. NOE = novel object exploration.

*: $p < .05$ compared to SAL matched group; **: $p < .01$ compared to SAL matched group; τ: $p < .05$ between Stress groups. a: $p < .05$ compared to NoSPS matched counterpart.

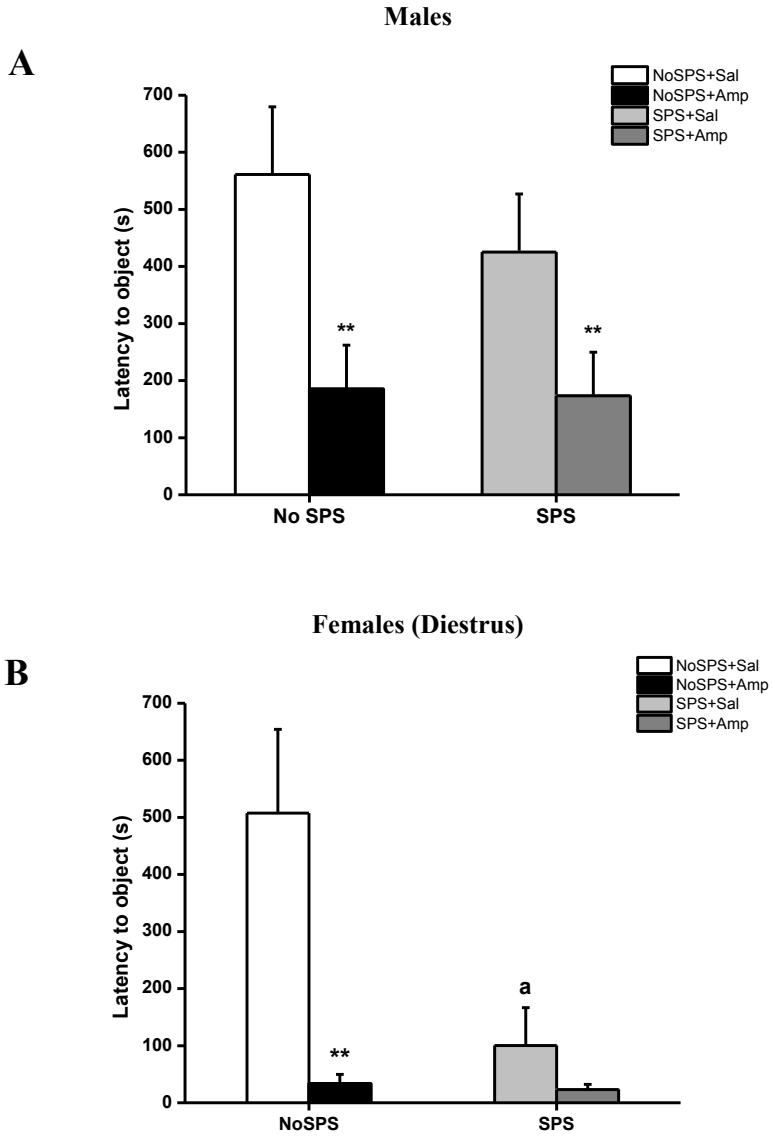

Figure 11. Effect of SPS exposure and AMP on latency to object in the NOE paradigm. Latency to object was assessed as the time taken to first approach the novel object after its placement in the arena. A) AMP significantly decreased latency times in male rats. NoSPS+Sal (n=14), NoSPS+Amp (n=16), SPS+Sal (n=16), SPS+Amp (n=16) rats. B) In diestrus females, AMP and SPS separately induced a decrease in latency to object times. NoSPS+Sal (n=7), NoSPS+Amp (n=7), SPS+Sal (n=6), and SPS+Amp (n=8) rats. Data represents mean (±SEM) latency to object (s). SPS = single prolonged stress. SAL = saline. AMP = amphetamine. NOE = novel object exploration.

**: $p < .01$ compared to SAL matched group; a: $p < .05$ compared to NoSPS matched counterpart.

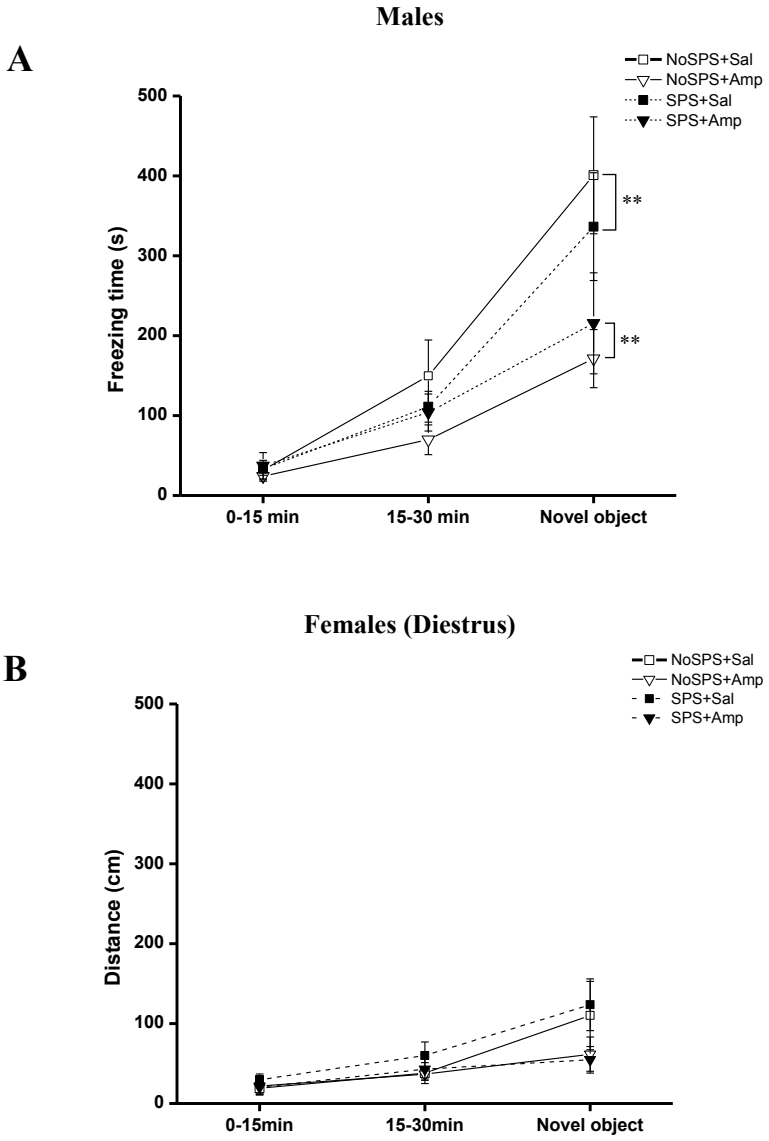

Figure 12. Effect of SPS exposure and AMP on freezing behaviour in the NOE paradigm. Time spent freezing was assessed and divided into 15min time bins. A) During the last 15 min, where the novel object is introduced, AMP-treated rats exhibited less freezing behaviour. NoSPS+Sal (n=9), NoSPS+Amp (n=10), SPS+Sal (n=10), SPS+Amp (n=10) rats. B) Freezing was comparable among all groups at all time points. NoSPS+Sal (n=7), NoSPS+Amp (n=7), SPS+Sal (n=6), and SPS+Amp (n=8) rats. Data represents mean (±SEM) freezing time (s). SPS = single prolonged stress. SAL = saline. AMP = amphetamine. NOE = novel object exploration.

**: $p < .01$ between treatment-matched groups.

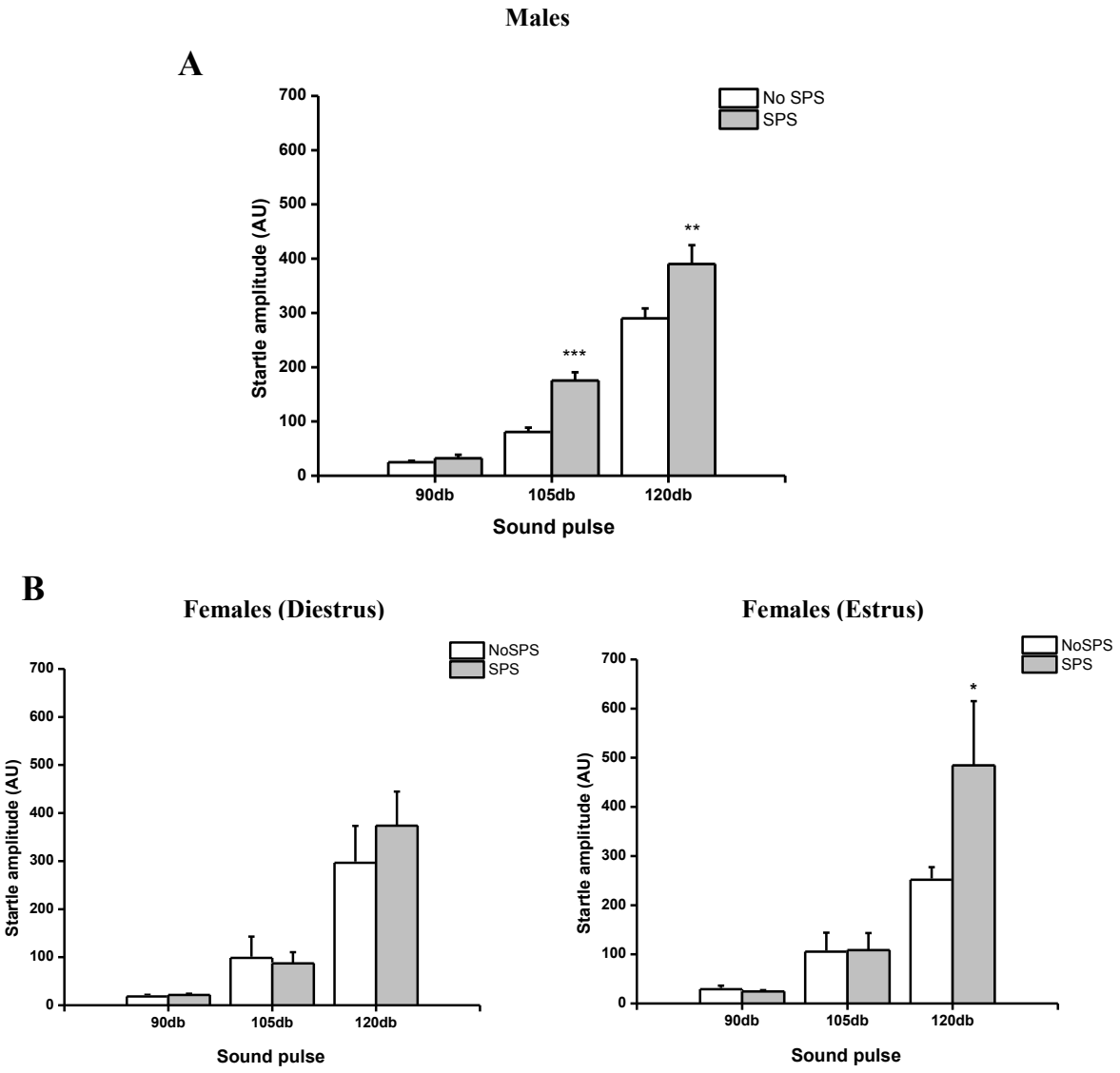

Figure 13. Effect of SPS exposure on acoustic startle response. Startle amplitude was assessed at 90dB, 105dB, and 120dB. A) Males displayed an SPS-induced exaggerated startle response at 105dB and 120dB, but not at the 90dB level. NoSPS (n=10) and SPS (n=10) rats. B) Diestrus females [NoSPS (n=10) and SPS (n=7) rats] exhibited no SPS-enhanced startle, however, rats in estrus [NoSPS (n=5) and SPS (n=8) rats] displayed an augmented response at the 120dB level. Data represents mean (±SEM) startle amplitude (AU). SPS = single prolonged stress. AU = arbitrary units.

*: $p < .05$ compared to NoSPS matched group; **: $p < .01$ compared to NoSPS matched group; ***: $p < .005$ compared to NoSPS matched group.

Experiment 2: Effects of SPS exposure on extracellular accumbal DA release in male and female rats

Figure 14A display the trends in extracellular DA release in males in response to SPS exposure and AMP administration. A mixed design repeated measures ANOVA revealed that AMP administration enhanced accumbal DA release in male animals [$F(1, 30) = 51.904$, $p = .000$]. SPS exposure [$F(1, 30) = 1.472$, $p = .235$] nor the Stress x Treatment interaction [$F(1, 30) = 3.090$, $p = .090$] had a significant effect on DA transmission in males. Although the Stress x Treatment interaction failed to reach significance, follow-up simple effects consisting of this interaction were conducted based on graphical analysis and the a priori hypothesis that SPS increases accumbal DA release. Analysis showed that SPS independently resulted in a constant stable increase in DA neurotransmission at the NAcc ($p = .039$).

Two female rats were excluded from analysis due to irregular cycling. Among the remaining females, those in the estrus stage on the day of microdialysis were included in analysis, as they comprised the majority of the sample (28 rats out of 38). Accumbal DA release over 140 minutes in estrus females is presented in Figure 14B. Similar to males, AMP resulted in augmented extracellular DA secretion at the NAcc [$F(1, 24) = 11.536$, $p = .002$]. DA release, however, was not affected by Stress [$F(1, 24) = .107$, $p = .747$] or the Stress x Treatment interaction [$F(1, 24) = .585$, $p = .452$].

Based on the current data, an additional analysis of baseline values between male and female rats was conducted using a two-way ANOVA. Baseline DA was not affected by Stress [$F(1, 58) = .632$, $p = .430$] or the Gender x Stress [$F(1, 58) = .175$, $p = .677$] interaction. However, it was found that females exhibited significantly higher basal levels of DA release at the NAcc relative to males, regardless of stress exposure [$F(1, 58) = 21.547$, $p = .000$].

Percentage change from baseline values of male animals are presented in Figure 15A. In regards to standardized percentage change values, MANOVA revealed that extracellular DA release at the NAcc was significantly influenced by AMP at every time point following the 20 minute mark: 40 min [$F(1, 30) = 23.099$, $p = .000$], 60 min [$F(1, 30) = 41.224$, $p = .000$], 80 min [$F(1, 30) = 35.221$, $p = .000$], 100 min [$F(1, 30) = 34.630$, $p = .000$], 120 min [$F(1, 30) = 22.929$, $p = .000$], 140 min [$F(1, 30) = 24.346$, $p = .000$]. Stress, however, did not enhance percentage change values at any time point: 20 min [$F(1, 30) = .159$, $p = .693$], 40 min [$F(1, 30) = .074$, $p = .788$], 60 min [$F(1, 30) = 2.148$, $p = .154$], 80 min [$F(1, 30) = .702$, $p = .410$], 100 min [$F(1, 30) = 1.068$, $p = .311$], 120 min [$F(1, 30) = .321$, $p = .576$], 140 min [$F(1, 30) = .362$, $p = .553$]. Based on graphical analysis and the a priori hypothesis that SPS elevates accumbal DA, follow-up simple effects comprising the Stress x Treatment interaction were conducted and demonstrated that SPS did not significantly influence the magnitude of change at any time points. However, in combination with AMP, SPS induced an increase in percentage change values at 60 min ($p = .019$), and almost significantly elevated DA levels at 80 min ($p = .061$).

The same patterns in DA activity were seen in estrus females (Figure 15B). AMP expectedly increased DA transmission at all time points after 20 minutes: 40 min [$F(1, 24) = 8.246$, $p = .008$], 60 min [$F(1, 24) = 19.632$, $p = .000$], 80 min [$F(1, 24) = 7.428$, $p = .012$], 100 min [$F(1, 24) = 5.183$, $p = .032$], 120 min [$F(1, 24) = 8.722$, $p = .007$], 140 min [$F(1, 24) = 5.443$, $p = .028$]. However, accumbal DA release was not influenced by SPS exposure or the Stress x Treatment interaction.

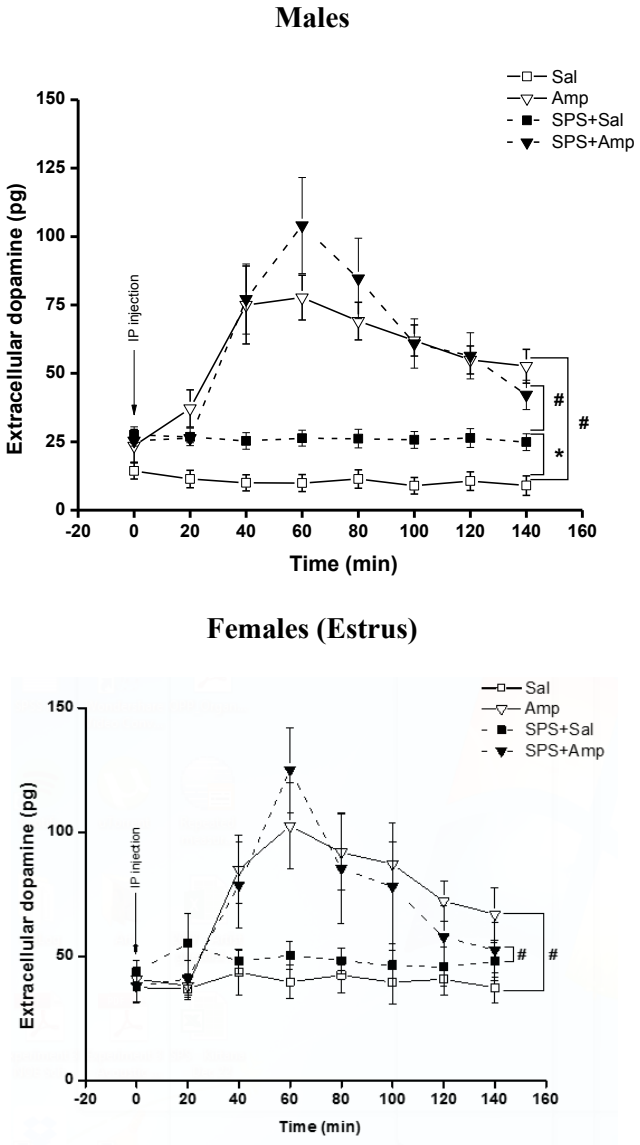

Figure 14. Effect of SPS exposure on extracellular accumbal DA release. DA release was assessed over 140 minutes following SAL or AMP (0.5 mg/kg i.p.) injection. A) In male rats, SPS induced an elevated stable level of DA release. AMP administration significantly increased DA quantities, irrespective of stress exposure. NoSPS+Sal (n=8), NoSPS+Amp (n=9), SPS+Sal (n=9), and SPS+Amp (n=8) rats. B) In estrus females, AMP significantly augmented DA release, regardless of stress. NoSPS+Sal (n=6), NoSPS+Amp (n=9), SPS+Sal (n=6), and SPS+Amp (n=7) rats. Data represents mean (±SEM) dopamine (pg).

*: $p < .05$ between treatment-matched groups; #: $p < .005$ between stress-matched groups.

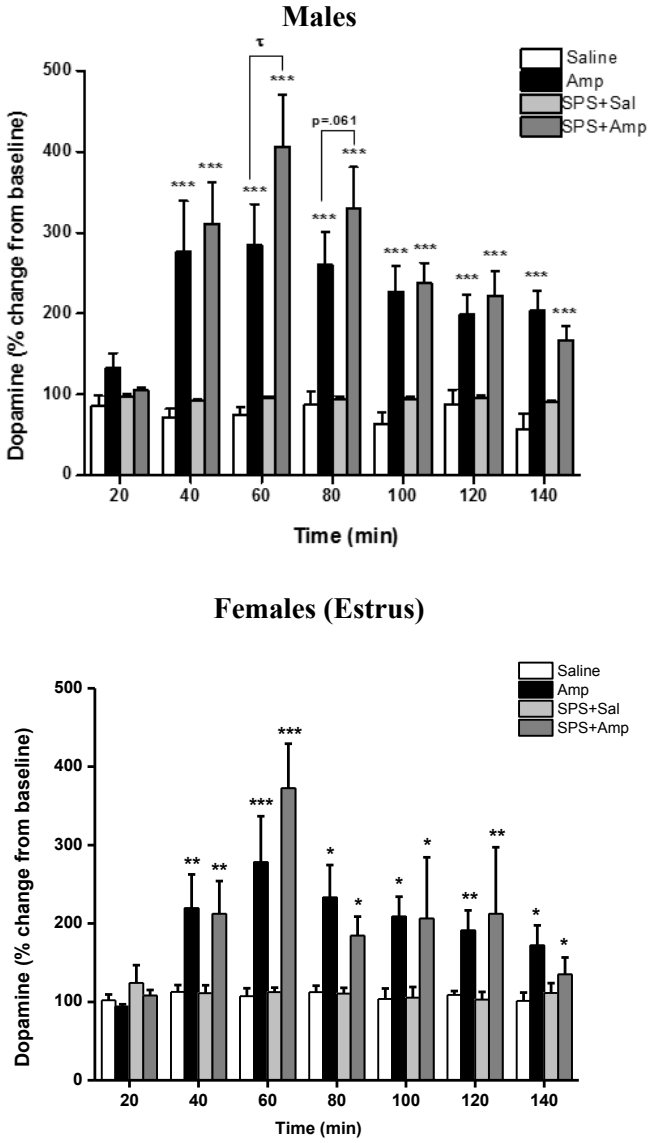

Figure 15. Effect of SPS exposure on percentage change from baseline accumbal DA. DA release was assessed over 140 minutes following SAL or AMP (0.5 mg/kg i.p.) injection. A) AMP increased percentage change from baseline DA at all time points following 20 minutes. At 60 minutes, male SPS rats treated with AMP displayed enhanced DA percentage change. NoSPS+Sal (n=8), NoSPS+Amp (n=9), SPS+Sal (n=9), and SPS+Amp (n=8) rats. B) In estrus females, AMP significantly augmented DA percentage difference, regardless of stress. NoSPS+Sal (n=6), NoSPS+Amp (n=9), SPS+Sal (n=6), and SPS+Amp (n=7) rats. Data represents mean (±SEM) startle amplitude (AU). SPS = single prolonged stress.

*: $p < .05$ compared to SAL counterpart; **: $p < .01$ compared to SAL counterpart; ***: $p < .005$ compared to SAL counterpart; τ: $p < .05$ between stress-matched groups.

Discussion

The purpose of this study was to further explore the SPS paradigm, an animal model of PTSD, in terms of physiological and behavioural effects in both males and female rats. A pilot study from our lab previously demonstrated that SPS exposure in male rats increased novelty-seeking behaviours in a modified novel object exploration (NOE) test. We postulated that this may be due to a dysfunction in the mesolimibic dopaminergic pathway in SPS rats, yielding a hyperdopaminergic state following exposure to the stressor. The first experiment aimed to replicate the behavioural results of the pilot study in both males and females. The current literature is sparse in regards to SPS in female animals, and thus the purpose of this experiment was to explore any behavioural or physiological gender differences within this stress model. Particularly, we were interested in investigating whether the same heightened novelty-seeking behaviours that were observed in males would be exhibited by females as well. Our results were consistent with our pilot study, revealing that SPS exposure increased various measures of exploratory behaviour in both male and female rats.

A validation test (i.e. acoustic startle response (ASR)) and physiological measures (i.e. body weight changes and plasma corticosterone changes) were added to the experiment in order to further characterize the SPS model and to examine any similarities or discrepancies with human PTSD studies. Results revealed that SPS-exposed male and female rats displayed an exaggerated ASR in comparison to NoSPS rats, which was expected based on previous literature and comparable to clinical PTSD results (Grillon et al., 1996; Khan & Liberzon, 2004). Male and female SPS rats also exhibited lower weight gain seven days after SPS exposure. Lower percentage change from baseline plasma CORT was observed in male SPS animals over the

course of 60 min following a mild stressor. This pattern was also observed in SPS females, but did not reach significance.

Our second experiment aimed to explore *in vivo* DA release at the NAcc in both males and females exposed to SPS. Based on our behavioural results, we hypothesized that SPS would alter the dopaminergic pathway resulting in elevated extracellular DA levels in the NAcc compared to NoSPS rats. Consistent with our hypothesis, results revealed that male rats exposed to SPS had higher baseline DA levels compared to non-stressed rats. This effect was not seen in estrus females. However, females had significantly elevated basal DA levels compared to males, irrespective of stress. AMP administration also increased DA neurotransmission in the NAcc in both males and females, as expected. There was no observed cross-sensitization of SPS and AMP on accumbal DA release in either males or females.

Behavioural and physiological effects of SPS

Validation of SPS. The augmented startle response is a well-characterized and predominant behavioural symptom of PTSD in both men and women, reflecting hyperarousal (Orr et al., 1995; Shalev, Orr, Peri, Schreiber, & Pitman, 1992). In line with previous research, our current findings demonstrate that SPS exposure results in an exaggerated ASR response (Khan & Liberzon, 2004). SPS males exhibited a significant increase in ASR compared to control animals at both 105dB and 120dB sound intensities. Females exposed to SPS that were in estrus on testing day displayed an exaggerated ASR at only 120dB. This hyperarousal response was not observed in diestrus females, suggesting that estrus stage may influence behavioural reactivity to stimuli. This is the first study to explore ASR in female animals using the SPS model, and confirms that elevated ASR extends to estrus females. Although it appears that this effect is stronger in males than females, we believe that this behavioural result adds to the

validity of SPS as an effective model of PTSD within female animals. Further work is needed to characterize ASR in other estrus cycle stages.

Physiological effects. Lower weight gain was observed in SPS male and female groups compared to rats that did not undergo the stressor. Animal studies have found that severity of the stressor can dictate food intake in rats, and thus influence changes in body weight (Marin, Cruz, & Planeta, 2007). Prior literature has neglected to assess the effects of SPS on body weight. However, other stress models have seen a similar trend in decreased weight gain. Acute severe stressors such as electric foot shock, immersion in cold water, and 2-h immobilization have all resulted in lower weight gain rates compared to control rats (Retana-Márquez et al., 2003). A study examining the effects of single exposure stressors (i.e. immobilization, lipopolysaccharide administration) on food intake demonstrated changes on feeding behaviour, resulting in lower body weights compared to unstressed groups on post-stress day 3 (Valles, Marti, Garcia, & Armario, 2000). In terms of human studies, about 14% of individuals experiencing anorexia nervosa are comorbid with PTSD. Clinical research reveals that among this subgroup, the traumatic event occurs prior to the onset of the eating disorder (Reyes-Rodríguez et al., 2011). Thus, previous exposure to a traumatic stressor seems to result in deficits in feeding behaviour in both animals and humans. To our knowledge, our results are the first to indicate that SPS exposure results in smaller weight gain compared to control rats.

We also found that the SPS group exhibited lower CORT level changes from baseline in males compared to NoSPS rats at 15min, 30min, and 60min following exposure to a mild stressor. Percentage changes in CORT relative to baseline in the SPS group clearly demonstrated a deficient stress response compared to nonstressed rats. Previous SPS studies have explored only one of the numerous neuroendocrinological abnormalities associated with PTSD –

enhancement of the negative feedback of the HPA axis. This has been achieved by examining the glucocorticoid fast feedback system and assessing CRF, ACTH, and CORT levels in response to a saline or cortisol pretreatment. Despite these advancements in characterizing HPA dysfunctions within the SPS model, these studies have failed to analyze simple differences in CORT level changes in response to a mild stressor. In terms of our results, SPS-exposed male rats exhibit a significantly smaller percentage increase in CORT compared to nonstressed rats, which is contradictory to clinical studies on cortisol reactivity in PTSD patients, in which PTSD individuals experience higher or comparable cortisol levels depending on the stress challenge (Bremner et al., 2003; Elzinga et al., 2003). This diminished cortisol reactivity pattern was also displayed in diestrus and estrus females, but was not significant. Further work is needed to establish specific differences on CORT reactivity related to estrogen levels within the SPS model.

Behavioural measures. Within the modified NOE paradigm, we have shown that male and female rats exposed to SPS displayed enhanced exploratory activity (novelty-seeking behaviours), which is dependent on a functioning dopaminergic system (Bardo et al., 1996; Fink & Smith, 1980). SPS exposure resulted in increased time in the center area, more approaches to the novel object, higher total object contact time, and reduced latency to object times compared to rats not exposed to SPS. This indicates that SPS exposure significantly promoted novelty-seeking behaviours. Locomotor activity was enhanced by SPS in males only. As expected, AMP-treated groups displayed the highest rate of exploratory behaviours in the NOE paradigm. Studies have continuously confirmed that low doses of AMP result in enhanced exploration in rats in novel environments (Arnt, 1995; Lipska, Jaskiw, Arya, & Weinberger, 1992; Russell & Pihl, 1978). Interestingly, the average center duration, locomotor activity, number of approaches, and

total object contact time of the SPS groups were comparable to that of the control group treated with AMP (0.5 mg/kg), suggesting that SPS exposure can promote novelty-seeking behaviours to the same degree as AMP in males and females. Based on our results, it can be inferred that SPS exposure induces a heightened dopamine-mediated novelty-seeking profile in both males and females.

Contrary to our predictions, the SPS effect was not further enhanced by AMP pretreatment in male or female rats, as it was already augmented by the stress exposure itself. A number of studies have previously found that severe stress can induce cross-sensitization to AMP and other psychostimulants (Nikulina, Covington, Ganschow, Hammer, & Miczek, 2004). Although cross-sensitization was not observed in the current study, higher exploratory activity has been previously linked to increased vulnerability in alcohol and substance abuse in rats (Galen et al., 1997; Johansson & Hansen, 2002). One speculation for the lack of cross-sensitization observed is due to a ceiling effect of the AMP dose (0.5 mg/kg) and the possibility that it does not allow for additional behavioural effects beyond the point already reached by SPS animals. Overall, the overarching trend remains that SPS exposure appears to increase novelty-seeking behaviours in both genders.

In regards to freezing behaviour, which commonly reflects anxiety-like tendencies in rodents, SPS exposure had no effect on this measure in both males and females. This suggests that SPS differentially affects anxious behaviours and exploration within this paradigm. SPS exposure has previously been shown to augment anxious behaviours, evidenced by increased contextual freezing in a contextual fear test and reduced percentage of open arm time and open arm entries in the elevated plus maze test (Harada, Yamaji, & Matsuoka, 2008; Imanaka, Morinobu, Toki, & Yamawaki, 2006; Takahashi et al., 2006). These effects are elevated when

rats are in the proestrus or estrus stage, compared to diestrus (Frye, Petralia, & Rhodes, 2000; Johnston & File, 1991; Zimmerberg & Farley, 1993). In the current study, it appears that SPS exposure had no effect on anxiogenic behaviours in the paradigm, but still enhanced novelty-seeking behaviours. This may be due to our attentiveness in reducing the aversion of the NOE test, by testing in low-light conditions and providing an appropriate length of habituation to the arena. This ensured that we primarily tested for novelty-seeking behaviours as opposed to anxiogenic behaviours. Therefore, our results suggest that the experimental conditions used for our modified NOE paradigm are ideal in assessing exploration behaviours, but other tests, such as the contextual freezing test and elevated plus-maze, should be used to more effectively examine anxiety in the SPS model.

Sex differences. Physiologically, very similar trends emerged between males and females following exposure to SPS. In regards to ASR, a significant exaggerated behavioural response was only observed in estrus females and not in diestrus females, suggesting that manifestations of PTSD-like symptoms in female animals is more likely in the estrus phase as opposed to the diestrus phase.

Interestingly, female animals displayed very similar exploratory behaviours as males in the modified NOE paradigm. In the current study, NOE results consisted of a diestrus only sample, based on majority. The diestrus phase involves the lowest levels of circulating estrogen and progesterone relative to the other stages and was expected to result in the lowest level of exploration, given that estrogen modulates DA activity. Female animals showed higher levels of exploration on all measures, compared to males. When exposed to SPS or administered AMP, females exceeded males on all measures, displaying considerably higher center duration, locomotor activity, approaches, total object contact times, lower latency to center times, and

reduced freezing behaviour. These data suggest that female animals were overall more curious and exploratory-driven in the paradigm, and that SPS exposure and AMP pretreatment were more effective in increasing these novelty-seeking behaviours in females compared to males. To date, this is the first study exploring DA-related behaviours in females exposed to SPS. It is possible that estrogen is upregulated in SPS females, leading to increased exploration. This theory, however, contradicts human work in which women with PTSD exhibit lower levels of estrogen. More likely is that female rats have previously been found to be more active than male rats and display higher levels of exploration and novelty-seeking behaviours overall, and may provide an explanation for their augmented activity relative to males (Joseph et al., 1978; Tropp & Markus, 2001). Further work is needed to establish the estrogen profile of females who have undergone SPS and explore any estrogen-mediated effects on DA-related behaviours.

The results demonstrated that AMP strongly influenced many novelty-seeking measures (i.e. center duration, total object contact, latency to center) to a higher level than males. This is in accordance with previous work that has shown that sensitization of AMP-induced behaviours are more prominent in female animals than males (Forgie & Stewart, 1993; Robinson, 1984; Robinson, Becker, & Presty, 1982; van Haaren & Meyer, 1991). Thus, our work suggests that there are sex differences in terms of the behavioural response to SPS and AMP, revealing that females are more susceptible to stress and psychostimulant sensitization.

The current results demonstrated that our initial hypothesis of females displaying lower exploration than males, was incorrect. On the contrary, female rats who underwent SPS and/or AMP treatment exhibited higher levels of exploration and novelty-seeking on all measures relative to males. In regards to AMP administration, previous animal work has shown that female animals display higher levels of AMP-induced locomotor activity and behaviours than males

(Camp & Robinson, 1988). However, we did not expect this to be paralleled in females exposed to SPS. The current results indicate that SPS exposure leads to the same level of sensitization seen in nonstressed females administered AMP. Our preliminary framework mistakenly presumed that female rats exposed to SPS would have decreased circulating estrogen levels, which is an observed characteristic of women with PTSD. This diminished estrous state would have subsequently lead to lower exploratory behaviours compared to male rats. Rather, it appears that this is not the case, and SPS exposure and AMP treatment both induce sensitized behaviours individually, and to a higher degree than in males. Estrogen modulates DA neurotransmission and DA-related behaviours by acting on membrane receptors at DA terminals (Becker, 1990). Previous work has demonstrated that acute stress induces constant enhancements in estrogen levels at all stages in female rats (Shors et al., 1999). With this in mind, it is hypothesized that SPS may produce a similar augmentation in estrogen secretion, which subsequently induces an increase in DA-mediated behaviours. Multiple studies have also confirmed that female rats are more susceptible to the effects of AMP and that estradiol levels have been linked to this sensitization (Becker, 1990; Camp & Robinson, 1988; Forgie & Stewart, 1993). Future work aiming to characterize circulating estrogen levels in SPS-exposed females, would help further elucidate sex differences in dopaminergic behaviours seen in the current study. Future studies should also test for behavioural variances among different cycle stages.

Apart from being an additional index of exploration, freezing behaviour is also a reliable measure of anxiogenic behaviours. AMP significantly reduced freezing behaviour in only males during presence of the novel object. While the novel object was present, freezing times for females were all under 150 sec, whereas male freezing times ranged from 170 sec to 400 sec over a 15 minute time span. Animal research reveals that female animals typically exhibit less

anxiogenic behaviours compared to males. This is apparent in the elevated plus-maze test, where females have higher locomotor activity and increased open arm time (Johnston & File, 1991; Zimmerberg & Farley, 1993). It has been suggested that estradiol modulates anxiety levels in female rodents, and thus anxiogenic behaviours are further decreased during the proestrus phase compared to estrus and diestrus (Marcondes, Miguel, Melo, & Spadari-Bratfisch, 2001). Thus, based on the observed freezing behaviour, the current data supports the notion that anxious tendencies were lowered in female animals, and were diminished with AMP pretreatment among males within this paradigm.

The animal results are distinct from the human literature, in regards to the fact that women with PTSD display more anhedonic and anxious behaviours compared to men (Feingold, 1994; Gray, 1971). However, women with PTSD also engage in more risk-taking and sensation-seeking behaviours relative to males (Cloitre et al., 2002; Holzer et al., 2008; Rosenberg et al., 2001). Previous work has suggested that novelty-seeking in animals can be related to sensation-seeking in humans (Dellu, Piazza, Mayo, Le Moal, & Simon, 1996). Thus, our results partially agree with clinical work, in that SPS exposure increased novelty-seeking in female rats relative to males, similar to women with PTSD; but the results on anxious behaviours (i.e. freezing time) within our paradigm conflict with the human literature, in that SPS-exposed females exhibited lower anxiety-like behaviours compared to males. This discrepancy may simply be the result of gender or species disparities. Another explanation arises from the choice of test used. The modified NOE is primarily a measure of novelty-seeking behaviours rather than anxious behaviours. Thus, other validated tests of anxiety, such as the elevated plus maze, should be used to further determine the anxious profile of SPS-exposed male and female rats.

Overall, our study is the first to explore sex differences in novelty-seeking behaviours in rats exposed to SPS. Key distinctions have been made in terms of the responses of males and females to AMP, SPS, and a combination of both. Although female rats were in diestrus (characterized by low estrogen secretion), females displayed similar exploratory behavioural trends as males, but to a higher degree, and also appeared less anxious in the paradigm. Future research should aim to further explore these fundamental differences and explore the effects SPS on estrogen and estrus cycle stage on ensuing novelty-seeking behaviours within the NOE paradigm.

Neurochemical effects of SPS

DA release. In males, microdialysis revealed that SPS resulted in overall higher DA levels compared to non-stressed males. This is the first study to examine neurochemical alterations in SPS-exposed animals, and suggests that the dopaminergic activity in the NAcc is augmented in males seven days post-SPS. This effect was not seen in estrus females, where SPS did not induce changes to DA release. AMP administration predictably increased DA release in both sexes, but was not significantly enhanced by SPS. SPS coupled with AMP resulted in non-significantly higher DA secretion in males at 60 minutes and 80 minutes following injection. Although results failed to reach significance, there is a possibility that SPS enhances the AMP-induced peak of DA release at the NAcc at certain time points.

SPS, however, did not have an effect on percentage change from baseline DA in male or female rats, indicating that the magnitude of DA increase was comparable between non-stressed and SPS groups. In males, the combination of SPS and AMP resulted in significantly higher percentage changes from baseline at 60 minutes, suggesting that SPS exposure amplifies the AMP-induced increase in DA release from baseline. It is intriguing to note, that although SPS-

induced DA release was much lower than AMP-induced DA release, they both generated comparable behavioural results in the NOE paradigm. In estrus females, SPS had no effect on the magnitude of change from baseline DA. No cross-sensitization patterns emerged either.

Sex differences. These results provide a fascinating insight into the neurochemical abnormalities associated with the SPS model. Given these findings, it is suggested that SPS produces a dopaminergic dysfunction in males, resulting in higher overall baseline DA levels and higher AMP-induced percentage increases from baseline at 60 minutes. Further work is required to assess whether this is due to a decrease or increase in dopaminergic receptor availability or whether another mechanism is involved. This dysfunction was not well-defined in female animals. Nevertheless, we observed higher baseline levels of DA in females compared to males, regardless of stressor exposure. This helps to explain why female animals were overall more active in our NOE test compared to males, however it does not isolate any stress differences. Behaviourally, SPS females exhibited very high exploratory behaviours, which was presumed to be related to increased DA at the NAcc. Our microdialysis results do not confirm this hyperdopaminergic state in stressed females. This may be due to a number of explanations. First, the effects of SPS or SPS in combination with AMP may have not been apparent simply due to the small group sizes (n=6-9). Another possibility is that estrus cycle stage may play a vital role, given that our behavioural results were in diestrus females and our neurochemical results were in estrus females. Although the cycle stage differs between our results, previous studies have found that AMP-induced DA secretion is comparable during diestrus and estrus (Becker & Ramirez, 1981). Despite this, it is possible that females are more vulnerable to SPS during the diestrus phase (e.g. exhibiting hyperdopaminergic-related behaviours) compared to the estrus phase (e.g. lack of elevated DA release at the NAcc). Alternatively, other DA-related alterations, such as

increased receptor density, in the brain may be responsible for the behavioural hyperactivity observed in females. It is also a possibility that SPS was unable to induce an observable change in females since basal levels were already extremely elevated, compared to levels in males.

Taken together, heightened novelty-seeking behaviours were only observed after exposure to SPS, indicating that this behavioural manifestation emerged due to some neurobiological alteration induced by the stressor. Based on the current results, it is speculated that basal changes in extracellular accumbal DA release are a result of SPS exposure, in males only. The increased activity of the mesolimbic dopaminergic system may alter processing of incentive salience and explain the preference for novel stimuli by SPS rats compared to nonstressed rats.

SPS-induced changes of extracellular DA release at the NAcc were not observed in estrus female animals. This however, does not rule out the hypothesis of a hyperdopaminergic state in female animals following SPS. Examining accumbal DA release during the diestrus phase would help better understand the augmented novelty-seeking observed in our first experiment with diestrus female animals. It is proposed that DA-related alterations, such as receptor density or DA activity while awake, may contribute to the behavioural manifestations in females. It is possible that differential mechanisms of novelty-seeking are observed in males and females. Nevertheless, replications of the current study should be conducted before gender distinctions in accumbal DA release can be accurately determined.

The results of the current study confirm the increased tendency of engaging in novelty-seeking behaviours by PTSD patients. These findings support the high-risk hypothesis of comorbid PTSD and SUDs, in which PTSD leads to higher novelty-seeking which subsequently increases vulnerability to substance abuse. The SPS model may provide a suitable animal model

for further examining this comorbidity. Potential treatments may focus on reducing the extreme novelty-seeking tendencies in order to prevent the potential development of an SUD in the future. Additional research is required in order to identify specific neurobiological mechanisms associated with this hyperactive exploratory and novelty-seeking response.

Limitations and Future Directions

In the current study, certain limitations must be acknowledged, involving estrus cycling and experimental procedures.

In keeping with our strict protocol of testing seven days following SPS exposure, it was difficult to ensure an appropriate group size in each estrus cycle stage for testing. For this reason some of our results only provide insight to females in one particular stage. In the future, it would be advantageous to have a larger sample size to examine DA-mediated behaviours and DA release in other cycle stages to understand the intricacies of the estrogen-DA relationship within this stress model. The proestrus stage would be fascinating to study, given that it involves the highest levels of estrogen, which is typically a protective factor against stress. It would be interesting to see whether SPS diminishes this protective role or whether estrogen remains unaffected. The long-term effects of SPS on estrus cyclicity would also provide vital information as to whether this stressor induces changes in the cycle length.

Due to the preliminary nature of our study, we opted to conduct microdialysis in anesthetized animals to provide an initial overview of the effects of SPS on DA release before delving deeper. Anesthesia can affect the excitability of neurotransmitters, and thus the results obtained in the current study should be taken with consideration (Di Chiara, 1990). With this in mind, the next step involves assessing DA release in freely-moving rats exposed to SPS and

within the NOE paradigm. A follow-up study could also assess DA receptor density in the NAcc to provide a wholesome view of the effects of SPS on the mesolimbic dopaminergic system in male and female animals.

As a result of our large sample sizes and staggering animals, CORT reactivity testing spanned approximately 2-3 hours. CORT levels follow a circadian rhythm, and time of day can significantly influence plasma CORT and responsiveness (Atkinson & Waddell, 1997). The procedure itself of tail nicking introduces uncontrollable stressors for the animal as well and is less than ideal (Thanos et al., 2009). The interpretation of the results related to CORT should be considered carefully.

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