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Intermittent swim stress effects on anxiety behavior

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INTERMITTENT SWIM STRESS EFFECTS ON ANXIETY BEHAVIOR

BY

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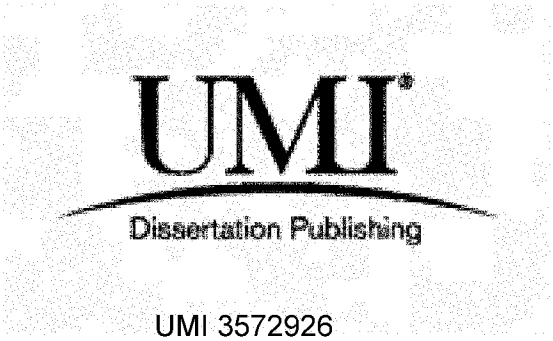

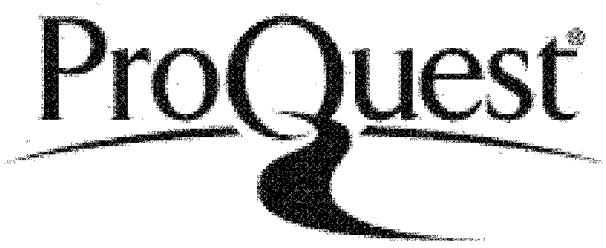

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DEDICATION

I would like to dedicate this work to my parents and other loved ones who have helped me throughout my academic career. Without you, none of this would have been possible.

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ABSTRACT

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University of New Hampshire May 2013

Millions of Americans are suffering from depression each year, leading to a significant number of individuals who seek treatment for their ailment. However, fewer than 50 percent of depressed individuals fully recover using current methods. The comorbidity between depression and anxiety could be a contributing factor in the lower rates of recovery. The demonstrated correlation between anxiety and depression has led to the term "anxious depression," which is associated with difficulty in coping, a poorer rate of recovery, and more severe symptoms of depression. The purpose of this dissertation was to expand on an existing animal model of depression (intermittent swim stress) and its possible relationship to anxiety. In the intermittent swim stress (ISS) model, animals experienced 100, 5 second trials of cold water swim stress, and subsequent behavioral and cellular mechanisms were assessed. Behavioral measures incorporated animal models of anxiety (i.e., open field test and juvenile social exploration), while serotonergic and noradrenergic neurons were assessed at the dorsal raphe nucleus and locus coeruleus, respectively, through immunohistochemistry techniques. Results indicated ISS-induced deficits were noted for social exploration, but not with the open field test. No apparent cellular

differences were revealed following the open field test, but this has yet to be investigated for juvenile social exploration. The anxiety effects produced by ISS support the trans-situational value of the model and also suggest ISS as a possible animal model of post-traumatic stress disorder. Future directions should assess cellular mechanisms following exposure to juvenile social exploration as well as explore the time course of the neural activity marker described in the experiments.

CHAPTER I

INTRODUCTION

Stress is affiliated with a variety of psychological conditions. In particular, stress is a common risk factor for depression and anxiety (Lapiz-Bluhm, Bondi, Doyen, Rodriguez, Be´dard-Arana, and Morilak, 2008; Revollo, Qureshi, Collazos, Valero, & Casas, 2011). Depression is a debilitating disorder that anyone can experience. An estimated 35 to 40 million Americans, or approximately 17.6 million Americans each year (Knol, Twisk, Beekman, Heine, Snoek, & Pouwer, 2006), will suffer from severe depression at some point during their lives (Kathryn, 2011; Weissmanm & Klerman, 1978). It is anticipated that this number will continue to climb, as depression is projected to be the second most disabling condition in the world by 2020 (Mathers, Fat, & Boerma, 2008). This expectation could be due to the fact that depression has a strong association with anxiety. The demonstrated correlation between anxiety and depression has led to the term "anxious depression" (Fava, Rosenbaum, Hoog, Tepner, Kopp, & Nilsson, 2000; Fava, Rush, Alpert, Balasubramani, Wisniewski, Carmin, et al., 2008; Fava, Uebelacker, Alpert, Nierenberg, Pava, & Rosenbaum, 1997; Simon, 2009; Van Valkenburg, Akiskal, Puzantian, & Rosenthal, 1984). Anxious depression is correlated with difficulty in coping, a poorer rate of recovery (Nelson, 2008), more severe symptoms of depression (Silverstone &

Salinas, 2001), but, in terms of the rate of relapse, anxious depression is similar to that of depressed patients without anxiety (Flint & Rifat, 1997).

Fewer than 50 percent of depressed patients experience a complete recovery using the current treatment methods (Berton & Nestler, 2006). This lack of effective treatment options is burdensome not only to those who suffer from depression, but also to those who must care for them (Mathers, Fat, & Boerma, 2008). As a result, it is critical to have a further understanding of the neural substrates involved in the comorbidity of depression and anxiety.

Depression

Symptoms. According to the American Psychiatric Association (2000), major depression is a psychiatric disorder characterized by: depressed mood; loss of interest in activities; significant change in weight; difficulty sleeping or thinking; restless activity; low energy; feelings of worthlessness, hopelessness, or guilt; and recurrent thoughts of death or suicide. These symptoms may be similar to other psychiatric disorders (e.g., anxiety disorders, bipolar disorder, and post-traumatic stress disorder). However, major depression can only be classified as such when several of the above symptoms are persistent for two weeks or longer, disrupt daily social- or work-related activities, and cannot be attributed to other causes (e.g., medical conditions such as hypothyroidism) or disorders. Depression is a common disorder that anyone could be susceptible to, but people with family members suffering from depression are at a greater risk of development. The genetic risk is approximately 40-50 percent greater than people with no family history of depression (Fava & Kendler, 2000; Weissman,

Wickramaratne, Nomura, Warner, Verdeli, Pilowsky, et al., 2005). Additionally, environmental factors, such as some form of trauma, disease, or life stress, can contribute to depression (Fava & Kendler, 2000; Nestler, Barrot, DiLeone, Eisch, Gold, & Monteggia, 2002); and, in general, women tend to be at a greater risk of depression (Kessler, McGonagle, Swartz, Blazer, & Nelson, 1993; Wade, Cairney, & Pevalin, 2002).

Relation to Anxiety. There is a noticeable relationship between depression and anxiety, as depression is often experienced by anxiety patients and vice versa. In fact, 62 percent of patients suffering from major depression also have high levels of anxiety (Boehnlein & Kinzie, 2007). Ultimately, anxiety and depression share several symptoms such as sleep problems (Eller, Aluoja, Vasar, & Veldi, 2006), feelings of worthlessness, and cognitive impairments to name a few — with stress as a common risk factor (Lapiz-Bluhm et al., 2008). This overlap between anxiety and depression is the result of similarities between neurobiological mechanisms, and the fact that both may respond to the same pharmacological treatments (i.e., serotonin- and norepinephrine-based drugs; Nutt, Ballenger, Sheehan, & Wittchen, 2002).

Neuropsychology of Anxiety. Gray (1982) initially outlined the idea of the "neuropsychology of anxiety." The neural and behavioral effects of anxiolytic drugs in animals can translate to the anxiety experienced by humans. The behavioral actions of anxiolytic drugs can be depicted through the behavioral inhibition system. The primary role of the behavioral inhibition system is to assess risk and increase risk aversion when situations of conflict present

themselves through a comparator, which compares actual and expected stimuli, known as the septo-hippocampal system. A conflict may be generated through signals of punishment, signals of non-reward, or novel or fearful stimuli. However, the behavioral inhibition system is implemented for resolution of the conflict to avoid negative or painful outcomes. The septo-hippocampal system is believed to encode various facets of anxiety. When there is a failure to handle an expected conflict (e.g., an unpredictable or fearful event) this system will activate the amygdala to produce a state of anxiety and increase avoidance tendencies. The administration of anxiolytics can help to resolve the conflict by modulating behavioral inhibition induced by fear, increasing arousal, and increasing attention. The septo-hippocampal system is indeed a vital component of the behavioral inhibition system, as anxiolytics impair the control of theta activity, the principle response to arousal, in the hippocampus. More importantly, a lesion to the septal or hippocampal regions leads to a significant reduction in functioning of the behavioral inhibition system (McNaughton & Gray, 2000).

Neurobiological Basis of Anxiety. In terms of the neurological aspects of anxiety, research has primarily focused on GABAergic, serotonergic, and noradrenergic neurotransmitter systems playing the most critical roles for anxiety-related disorders. GABAergic neurons, specifically $GABA_A$ receptors, are widely distributed throughout the brain and are believed to regulate anxiety/fear responses by inhibiting both the locus coeruleus and raphe nucleus — thereby suppressing neuronal firing. The locus coeruleus and raphe nucleus are where the majority of noradrenergic and serotonergic neurons are located, respectively.

Due to its inhibitory effects, GABA (gamma-aminobutyric acid) acts as a moderator by suppressing neuronal firing in the locus coeruleus and raphe nucleus (Dell'Osso, Buoli, Baldwin, & Altamura, 2010).

The noradrenergic neurons in the locus coeruleus project to the forebrain. Their role in anxiety could also be associated with their connection to stress, arousal, and fear (Aston-Jones & Cohen, 2005; Aston-Jones, Rajkowski, Kubiak, Valentino, & Shipley, 1996; Bremner, Kristal, Southwick, & Charney, 1996). When there is an increase in the production and release of norepinephrine, there is an elevation in anxiety levels. On the other hand, when neuronal firing in the locus coeruleus is depleted (Grimsley, 1995) or lesioned (Redmond, 1977; Redmond, Huang, Synder, & Maas, 1976), there is a significant reduction in anxiety-fear behavior. As for the serotonergic neurons in the raphe nucleus, there is a projection to areas throughout the brain such as the limbic system, hypothalamus, and bed nucleus of stria terminalis (BNST). Moreover, when there are elevated levels of serotonin, there is also a greater incidence for anxiety-related disorders (Dell'Osso, Buoli, Baldwin, & Altamura, 2010), and the serotonergic neurons of the dorsal raphe nucleus (DRN) are known to act as mediators in stressful situations. In fact, the DRN-BNST pathway is suggested to be important in mediating anxiety-related behaviors (Commons, Connolley & Valentino, 2003; Phelix, Liposits & Paull, 1992).

Corticotropin-releasing factor (CRF) acts as a physiological mediator of stress-related functions (Hammack, Richey, Schmid, LoPresti, Watkins, & Maier, 2002; Hammack, Schmid, LoPresti, Der-Avakian, Pellymounter, Foster, et al.,

2003; Price, Kirby, Valentino, & Lucki, 2002; Mo, Feng, Renner, & Forster, 2008; Rivier & Vale, 1983), as intracerebral administration of CRF yields similar results produced by stressors (Koob & Heinrichs, 1999). Moreover, blocking the CRF receptors greatly attenuates the release of serotonin during the stress response (Hammack et al., 2002, 2003; Price & Lucki, 2001; Mo et al., 2008), and reduces anxiety behaviors (Deak, Nguyen, Ehrlich, Watkins, Spencer, Maier, et al., 1999; Ising & Holsboer, 2007; Risbrough & Stein, 2006). This is an interesting notion, because the DRN receives extensive projections from the CRF neurons (Sakanak, Shibasaki, & Lederis, 1987). As a result, areas containing CRF receptors have been correlated with anxiety. CRF has two receptor subtypes: CRF type 1 receptor (CRF_1) and CRF type 2 receptor (CRF_2). CRF_1 receptors are mostly located in the amygdala, BNST, cerebral cortex, and brainstem, while CRF_2 receptors can be found mostly in the amygdala, BNST, lateral septum, and ventromedial hypothalamus (Chen, Brunson, Muller, Cariaga, & Baram, 2000; Van Pett, Viau, Bittencourt, Chan, Li, Arias, et al., 2000). The following will expand more on the roles of serotonin and norepinephrine as well as the brain areas involved for anxiety/depression.

Serotonin & Neural Innervations. The role of serotonin (5-hydroxytryptamine, 5-HT) in anxiety is complex, as it has various receptor subtypes, which can be located on either the presynaptic or postsynaptic membranes. These receptors can yield excitatory or inhibitory effects (Hoyer, Hannon, & Martin, 2002), which is also true of other monoamines (Knapp, Breese, Mueller, & Breese, 2001). Electrical stimulation of the dorsal

periaqueductal gray (DPAG) area induces anxious behavior, but stimulation of 5-HT_{1A} or 5-HT_{2A} presynaptic autoreceptors in the DPAG reduces anxiety. This suggests that the 5-HT nerve fibers in the DPAG may regulate anxiety behavior (Graeff, 2002). The 5-HT receptor subtypes in the DPAG may have unique characteristics, as genetic studies with rodents noted that the inactivation of 5-HT_{1A} (Gross, Zhuang, Stark, Ramboz, Oosting, Kirby, et al., 2002) and 5-HT_{2A} (Weisstaub, Zhou, Lira, Lambe, Gonzalez-Maeso, Hornung et al., 2006) postsynaptic receptors led to increased or decreased anxiety, respectively. Additionally, when 5-HT_{1A} receptors are active in the dentate gyrus of the hippocampus there is a decrease of anxiety behavior (Tsetsenis, Ma, Lacono, Beck, & Gross et al., 2007).

With regard to other brain areas associated with 5-HT, the basolateral amygdala receives serotonergic innervation from the DRN (Hale et al., 2008a; Fallow & Ciofi, 1992). Furthermore, there is noticeable activation of the basolateral amygdala when subjected to an anxiety-provoking stimulus (Hale et al., 2008a; Hale, Hay-Schmidt, Mikkelsen, Poulsen, Shekhar, & Lowry, 2008b). There is also activation of the 5-HT neurons in the DRN, a significant source of production of 5-HT in the brain, following anxiogenic or stressful stimuli derived from drug-induced anxiety (Christianson et al., 2008a) or uncontrollable stress (Grahn, Will, Hammack, Maswood, McQueen, Watkins, et al., 1999).

5-HT neurons in the DRN are sensitive to pharmacological compounds. The administration of anxiogenic compounds such as β-carbolines (Christianson, Paul, Irani, Thompson, Kubala, Yirmiya, et al., 2008a; Abrams, Johnson, Hay-

Schmidt, Mikkelsen, Shekhar, & Lowry, 2005) that bind to 5-$HT_{2A/2C}$ receptors (Grella, Teitler, Smith, Herrick-Davis, & Glennona, 2003), inverse benzodiazepine agonists (Maier, Busch, Maswood, Grahn, & Watkins, 1995a), 5-$HT_{2A/2C}$ receptor agonists, and even caffeine administration are associated with enhanced anxiety levels (Abrams et al., 2005). Whereas, anxiolytics such as 5-HT_{1A} agonists have been associated with attenuating anxiety levels (Christianson et al., 2008a). 5-HT_{1A} agonists such as 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT), ipsapirone, gepirone, and buspirone work by inhibiting the neuronal firing of the DRN (Fornal, Litto, Metzler, Marrosu, Tada, & Jacobs, 1994), which result in the anxiolytic responses (Blier, Piñeyro, Dennis, & DeMontigny, 1993).

The activation of the 5-HT neurons in the DRN from an uncontrollable stressor (e.g., inescapable shock) increases 5-HT levels in the DRN (Maswood, Barter, Watkins, & Maier, 1998), and also sensitizes these neurons for 24-72 hours. Later stimulation of the 5-HT neurons during further behavioral testing creates an exaggerated amount of 5-HT that is released in the DRN projection regions (e.g., areas associated with the limbic system; Amat, Matus-Amat, Watkins, & Maier, 1998). Inescapable shock (an uncontrollable stressor) seems to activate the DRN 5-HT neurons to a greater degree than escapable shock (a controllable stressor) in four ways: [1.] DRN lesions (Maier, Grahn, Kalman, Sutton, Wiertelak, & Watkins, 1993) or otherwise inhibiting the activation of the DRN (Maier, Grahn, & Watkins, 1995b) prevent inescapable shock-induced deficits, [2.] Blocking 5-HT_{2C} receptors in projection regions of the DRN (Christianson, Ragole, Amat, Greenwood, Strong, Paul, et al., 2010) prevent inescapable

shock-induced deficits, and [3] In the absence of any inescapable shock exposure, pharmacological activation (via β-carbolines) of the DRN 5-HT neurons induces inescapable shock deficits (Maier et al., 1995a), and [4] DRN lesions block the effects of anxiogenic drugs such as β-carbolines (Hindley, Hobbs, Paterson, & Roberts, 1985; Maier et al., 1995a). However, the DRN does not appear to be associated with the process of stress controllability or coping. Controllability (escapable shock) is believed to be regulated by the ventromedial prefrontal cortex (vmPFC; Amat, Baratta, Paul, Bland, Watkins, & Maier, 2005).

When the vmPFC is activated or inactivated during inescapable shock, it either prevents or facilitates, respectively, inescapable shock-induced deficits. The above observations indicate that the activation of the vmPFC is critical for the protective effects of controllability of electric shock (Amat et al., 2005; Amat, Paul, Watkins, & Maier, 2008). Furthermore, vmPFC plays a significant role in regulating the activity of the amygdala, a brain area known for emotion regulation such as anxiety (Jovanovic & Ressler, 2010). For people suffering from an anxiety disorder, such as post-traumatic stress disorder, higher levels of activity in the vmPFC correlate to higher levels of anxiety (Koenigs et al., 2008). In general, there appears to be mixed results for the role of the vmPFC with regard to the stress response (i.e., glucocorticoid release), as activity in the vmPFC has been shown to inhibit as well as enhance glucocorticoid release (Myers-Schulz & Koenigs, 2011), which could be due to the prelimbic and infralimbic subregions of the vmPFC (Baratta, Zarza, Gomez, Campeau, Watkins, & Maier, 2009).

Norepinephrine & Neural Innervations. Norepinephrine also plays a significant role in anxiety, as noradrenergic neurons in the LC project to a variety of areas throughout the brain (e.g., cerebellum, hypothalamus, amygdala, hippocampus, prefrontal cortex; Bishop, 2007). Exposure to different stressors (e.g., electric shock or conditioned fear) results in increased production of norepinephrine in the LC (Pacak & Palkovits, 2001), but activity in the LC does not appear to be dependent upon stressor controllability (McDevitt et al., 2009). Stress exposure also results in norepinephrine increases in the amygdala, hippocampus, and hypothalamus (specifically in the paraventricular nucleus; Pacak & Palkovits, 2001). The increase of norepinephrine in the paraventricular nucleus of the hypothalamus activates the sympathetic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis. This increases the release of glucocorticoids and adrenalin (or epinephrine), which can also result in increased anxiety behaviors (Koob, 1999).

Electrical stimulation of the LC induces anxiety, while lesioning this region prevents anxiety symptoms (Redmond, 1977; Redmond, Huang, Synder, & Maas, 1976). Within the LC, norepinephrine effects tend to be mediated through the presynaptic alpha-2 receptors and postsynaptic alpha-1and beta-1 receptors. When targeting the alpha-2 autoreceptor via an alpha-2 adrenergic antagonist, yohimbine, there is an increase in production in norepinephrine from the LC. This, in turn, facilitates symptoms of anxiety (Grimsley, 1995). However, an alpha-2 adrenergic agonist, clonidine, has been shown to inhibit functioning of the LC with the dose playing a vital role. These effects tend to be caused by

lower doses acting on presynaptic (alpha-2) autoreceptors (Solanto, 1998), and the diminished production of norepinephrine will thereby attenuate anxiety levels (Grimsley, 1995). Higher doses of clonidine are believed to act on postsynaptic receptors, are ineffective at inhibiting norepinephrine, and actually foster the release of norepinephrine (Ramos and Arnsten, 2007).

Traumatic stress such as some anxiety disorders (i.e., post-traumatic stress disorder) results in an exaggerated production and release of norepinephrine (Strawn & Geracioti, 2008). However, depression is not directly caused by either an increase or decrease of norepinephrine in the brain, as norepinephrine's more critical role is to act as a moderator for major inhibitory or excitatory neurotransmitters (e.g., gamma-aminobutyric acid and glutamate; Anand & Charney, 2007).

Serotonin & Norepinephrine Interaction. 5-HT and norepinephrine have a significant relationship with each other, as 5-HT projects to norepinephrine neurons and vice versa. Moreover, the projections of 5-HT neurons to norepinephrine neurons appear to be inhibitory. When there is a significant lesion of 5-HT neurons, the firing rate of norepinephrine neurons increase to approximately 70 percent above baseline in the locus coeruleus (Dremencov, El Mansari, & Blier, 2007). 5-HT's inhibitory nature on norepinephrine is further confirmed by depleting the availability of norepinephrine and administering selective-serotonin reuptake inhibitors (SSRIs). Inhibition of norepinephrine neurons via systemic administration substantially diminishes the rate of neuronal discharge for 5-HT in the DRN for the first few days (Svensson, Bunney, &

Aghajanian, 1975). Prolonged exposure to SSRI treatment has demonstrated a considerable decrease in the firing rate from norepinephrine neurons over a longer period of time (Blier, 2001).

Mirtazapine, which provides treatment for anxiety and depression, acts as an antagonist at both the 5-$HT_{2A/2C}$ and norepinephrine alpha-2 receptors (Kooyman, Zwart, Vanderheijden, Van Hooft, & Vijverberg, 1994; Millan, Gobert, Rivet, Adhumeau-Auclair, Cussac, Newman-Tancredi, et al., 2000). Chronic treatment with mirtazapine will cause an increase in the production of both 5-HT and norepinephrine; a smaller increase for norepinephrine and a more prominent increase for 5-HT. However, when the norepinephrine neurons are lesioned in the LC, the increase in the 5-HT activity produced by mirtazapine is no longer apparent (Blier, 2001). Furthermore, projections of norepinephrine neurons to 5-HT neurons tend to enhance the regeneration of serotonergic axons (Liu & Nakamura, 2006) suggesting an excitatory pathway. Ressler and Nemeroff (2000) have even suggested that there are both excitatory and inhibitory projections from the LC to DRN.

When selecting an antidepressant drug, for treatment of depression or an anxiety disorder, it is important to understand the functioning of both the 5-HT and norepinephrine systems. The effectiveness of antidepressants is thought to be due to a prolonged increase in the availability of the neurotransmitter at the synapse, which will decrease the number of receptors on the postsynaptic terminal (also known as down-regulation; Norman, 1999), as well as increases in brain-derived neurotrophic factor (BDNF) expressions and subsequent

neurogenesis in the brain (Jacobs, van Praag, & Gage, 2000; Malberg, Eisch, Nestler, & Duman, 2000). Additionally, in terms of more effective therapeutic treatment, if there is a deficiency in one neural system (i.e., 5-HT or norepinephrine) at times it may be more beneficial to treat the other system to reach the desired end result. In fact, depression that is associated with 5-HT depletion can be alleviated by enhancing the availability of norepinephrine. The cause of depression is far more complex than just focusing on the fluctuations of only 5-HT or norepinephrine levels. Dysfunctions in the brain associated with depression as well as anxiety disorders are likely modulated by different monoamine systems (Delgado, 2006; Delgado & Moreno, 2000). In an effort to investigate 5-HT or norepinephrine changes, in particular anatomical regions following exposure to a stressful stimulus, a variety of methods have been implemented (e.g., micro dialysis, autoradiography, or c-Fos). However, c-Fos is ideal because it allows an isolation of an anatomical area of interest.

c-Fos Immunohistochemistry. c-Fos is the protein product of an immediate early gene, and is a marker of neuronal activation. Following exposure to anxiogenic (e.g., β-carboline) or stressful stimuli, c-Fos is expressed in the DPAG (Lino-de-Oliveira, de Oliveira, Padua Carobrez, de Lima, del Bel, & Guimaraes, 2006), basolateral amygdala (Knapska, Radwanska, Werka, & Kaczmarek, 2007), DRN (Amat et al., 2005), prelimbic and infralimbic subregions of the vmPFC (Baratta et al., 2009), and LC (Webb, Patton, Landry, Mistlberger, 2010). The double-immunostaining protocol for c-Fos is an ideal technique for identifying which class of neurons is active, thereby allowing us to

investigate the 5-HT and norepinephrine systems. It is advantageous because it can provide greater clarity to different aspects of the cell (i.e., shape, size, and orientation of dendrites), which is not possible using the nuclear, Fos immunoreactivity technique (Peng, Chen, & Bentivoglio, 1995).

Animal Models of Depression

Use of Animal Models

Animal models are critical to understanding the various components of depression and to allow insight into novel drug discovery. Pre-clinical experiments allow researchers to have complete control over the experimental parameters of the subject (e.g., age, experiential history, sex, weight, food-intake, and environment), which can be quite difficult to accomplish in human models. Additionally, animal models have the potential to provide ground-breaking results, and can establish unequivocal cause-and-effect relationships. While research has been conducted on a variety of animals, rats have been a reliable and extensively used subject. The rat is an ideal animal specimen because its anatomical and physiological features parallel those of humans. However, one might wonder how depression can be elicited and objectively measured in animals?

Experimentally simulating depression has been discussed in great detail, as various authors have considered the advantages and disadvantages of animal models (Cryan, Markou, & Lucki, 2002; Henn, Edwards, & Muneyyirci, 1993; Porsolt, 2000). Ultimately, most authors accept a set of criteria devised by Mckinney and Bunney (1969). These guidelines indicate that the animal model includes: comparable symptoms to those experienced by humans; behavioral

endpoints that can be measured in an objective manner; induced depression to be remedied by treatments that could potentially be effective for humans (e.g., antidepressants); and procedures and results that can be replicated by other researchers.

A limitation of animal models is that it is difficult to examine all of the symptoms identified by the American Psychiatric Association (2000) in the diagnostic criteria of major depression. In particular, symptoms such as depressed mood, feelings of worthlessness, hopelessness, or guilt, and recurrent thoughts of death or suicide are impossible to evaluate — researchers cannot access what the animal is thinking during behavioral testing. However, through behavioral endpoints, it is possible to monitor other depressive behaviors such as anhedonia or low energy, significant change in weight, difficulty sleeping, cognitive impairments, social interaction deficits, and restless activity.

Forced-swim test. One of the most widely used models for screening novel antidepressants is the forced-swim test (FST), also known as the behavioral despair test (Porsolt et al., 1977). In this model, rats are placed in a cylinder filled with water. The rat is unable to make contact with the bottom of the cylinder with its feet (Borsini, Volterra, & Meli, 1986; Drugan, Skolnick, Paul, & Crawley, 1989), which forces the animal to swim. The rat swims in the apparatus for 15 minutes during the first session, and then 24 hours later the rat is forced to swim again for only 5 minutes. During the time spent in the cylinder on the second day, three distinct patterns of behavior are noted by the animal in the modified FST (Detke et al., 1995): swimming, climbing, and immobility (floating).

The behavioral pattern of immobility is of greatest interest, as this is a sign of behavioral depression in the animal. Researchers have questioned whether the rat is truly experiencing depression when the rat is immobile. Some tend to believe the immobile behavior expressed by the rat is rather a "functionally adaptive strategy" to cope with the inescapable stressor (Nishimura, Tsuda, Oguchi, Ida, & Tanaka, 1988). However, when rats are administered antidepressants they emerge from the immobile state to an active state of increased swimming or climbing based on the type of antidepressant given (Christianson, Rabbett, Lyckland, & Drugan, 2008; Detke, Rickels, & Lucki, 1995; Drugan, Macomber, & Warner, 2010; Lucki, 1997). Moreover, antidepressant drugs reverse immobility in rats selectively bred for low activity in the swim test (Weiss, Cierpial, & West, 1998; West & Weiss, 1998). The effect is not a result of general activation, as antidepressant-treated rats subsequently placed in an open field test do not show hyperactivity (Porsolt, Anton, Blavet, & Jalfre, 1978).

Learned Helplessness. Another widely used model of depression is that of inescapable shock/learned helplessness (Maier & Seligman, 1976; Henn et al., 1993; Weiss, Goodman, Losito, Corrigan, Charry, & Bailey, 1981). This phenomenon was first demonstrated with dogs exposed to inescapable electric shocks. The shocks interfered with the dogs' ability to escape in a shuttle-box task (Overmier & Seligman, 1967; Seligman & Maier, 1967). Soon after, researchers began testing the effects of learned helplessness on rats and noting similar results (Maier, Albin, & Testa, 1973; Weiss & Glazer, 1975). Although learned helplessness has been examined in numerous different species, the

process of its examination has remained largely consistent. The typical format for investigating this concept includes three animals that will be used at a time, following a triadic design, with two of the three animals receiving intermittent electric shocks and the third serving as the restrained control (therefore receiving no shock). Of the two animals being shocked, one animal has the opportunity to terminate the shock (escape), for both itself and the yoked-inescapable shock subject; typically accomplished by the animal pressing a lever or turning a wheel. The other animal will be shocked regardless of its actions (yoked), and thus, the highly stressful scenario of inescapable shock is created. After being exposed to inescapable shock, research has demonstrated that the animal does not attempt to avoid or escape the shocks if given the opportunity to escape. This behavioral outcome is indicative of that animal experiencing "learned helplessness" (Maier & Seligman, 1976). Animals exposed to inescapable, but not escapable, shock have exhibited other behavioral and physiological changes in addition to behavioral depression, including: "freezing behavior," which is an expression of fear (Maier, 1990), opioid-mediated stress-induced analgesia (Drugan, Ader, & Maier, 1985), anorexia (Dess, Choe, & Minor, 1998), learning deficits (Seligman & Maier, 1967), lower activity levels (Desan, Silbert, & Maier, 1988; Drugan & Maier, 1983), reduced food competition dominance (Rapaport & Maier, 1978), changes in conditioned place preference for drugs of abuse (Rozeske, Der-Avakian, Bland, Beckley, Watkins, & Maier, 2009), and anhedonia (Dess, Minor, & Brewer, 1989).

Learned helplessness may result from serotonergic activation (Amat et al., 1998) and noradrenergic inactivation. Specifically, in the dorsal raphe nucleus (Amat, Tamblyn, Paul, Bland, Amat, Foster, et al., 2004; Grahn et al., 1999; Maier and Watkins, 2005) and the locus coeruleus, respectively, as inescapable shock produces large increases in 5-HT (Amat et al., 1998) and decreases in norepinephrine (Weiss & Simson, 1986). Animals that are able to cope with the effects of stress (escape from the shock) do not display depleted levels of norepinephrine (Weiss et al., 1970; Weiss et al., 1981), nor do they show the sensitization of the DRN (Rozeske, Evans, Frank, Watkins, Lowry, & Maier, 2011) in comparison to yoked rats. On a pharmacological level, drugs that enhance the release of norepinephrine combat the effect of learned helplessness (Sherman et al., 1982), while drugs that actively deplete levels of norepinephrine produce many behavioral deficits comparable to learned helplessness (Anisman, Remington, & Sklar, 1979). Other studies have produced similar results when norepinephrine levels were enhanced (Petty, Kramer, Wilson, & Chae, 1993; Sherman & Petty, 1980). In terms of the role of 5-HT-based drugs, the administration of a 5-HT agonist in the DRN blocks the effects of inescapable shock (Maier et al., 1995b).

Intermittent Swim Stress (ISS). ISS has a component of learned helplessness, as it uses intermittent, inescapable stress exposure, and also a component of behavioral despair (or FST) by using water as the stressor. Water is a predominant part of the environment for many animals, and is a naturally occurring stressor for rats in comparison to the shock or restraint stress

mentioned previously (Russell, Towns, Anderson, & Clout, 2005). ISS is an effective stressor to induce behavioral depression such as enhanced immobility in the FST, interference with instrumental swim escape performance (Christianson & Drugan, 2005; Christianson et al., 2008b; Drugan, Macomber, & Warner, 2010), as well as increased latency to escape in the Morris water maze (MWM; Warner & Drugan, 2012). Unlike inescapable shock and continuous swim stress, or stressors that are sensitive to both acute SSRIs and norepinephrine selective reuptake inhibitors (NSRIs; Detke et al.,1995; Drugan et al., 2010; Maier & Watkins, 2005), ISS effects are sensitive to NSRI yet resistant to a variety of serotonergic manipulations — including SSRIs (Christianson et al., 2008b; Drugan et al., 2010). This difference suggests that the ISS effects are mediated by distinct neural systems that may lead to new insights into stress-related pathology and hasten novel drug discovery.

Reserpine-Induced Depression. This is considered as a pharmacological-based animal model of depression. Of the various pharmacological agents, the administration of reserpine, in particular, elicits signs of depression due to its depletion of monoamines. The importance of monoamines can be explained by the monoamine hypothesis, which is a controversial idea that depression is the result of the underactivity of the monoamines in the brain (Baumeister, Hawkins, & Uzelac, 2003).

Olfactory Bulbectomy. For this animal model, the olfactory bulb is surgically removed. While it is not clear how this procedure translates to depression, it has been suggested that the chronic sensory disruption

experienced by the animals can act as an intense, stressful experience (O'Neil & Moore, 2003)

Congenital Learned Helplessness. This is a genetic animal model of depression that incorporates selective breeding. In this model, rats are bred to be more or less prone to the effects of learned helplessness, (i.e., shuttlebox escape deficits) and thereby creating two categories of rats: congenitally learned helplessness and congenitally not learned helpless. For the congenital learned helpless group, these rats express a helpless phenotype even during escapable shock. For the non-congenital group, this strain of rats is resistant to the effects of learned helplessness even during inescapable shock (Henn & Vollmayr, 2005).

Flinders Sensitive Line Rats. Another genetic animal model of depression, these rats are selectively bred to be more sensitive to cholinergic agonists (acetylcholine). This paradigm parallels the cholinergic hypersensitivity experienced by depressed patients (Overstreet, Friedman, Mathé, & Yadid, 2005) who experience a heightened sensitivity to cholinergic agonists compared to normal controls (Janowsky, Overstreet, & Nurnberger, 1994; Risch, Kalin, & Janowsky, 1981).

Animal Models of Anxiety

In pre-clinical models of anxiety, the goal is to mirror the same symptoms, behavioral responses, biological mechanisms, and response to pharmacological treatments to those of human anxiety (Ramos, 2008). While there are genetic strains of anxious rats such as the Maudsley reactive rat (Broadhurst, 1960),

research predominately focuses on conditioned and unconditioned animal models of anxiety. Described below are samples of such models.

Conditioned Animal Models of Anxiety

Fear-Potentiated Startle. In this model, an animal will associate a neutral stimulus (e.g., a light), with an aversive stimuli (e.g., electric shock). Following exposure, the animal will then be presented with an intense sound, which will produce a startle response. This startle response is potentiated with the additional presentation of the formerly neutral, but now conditioned, stimulus. There are a variety of drugs that reduce fear-potentiated startle in rats such as alpha-2 adrenergic agonists (e.g., clonidine), opioid receptor agonists (e.g., morphine), benzodiazepine/$GABA_A$ agonists (e.g., diazepam; Davis, Falls, Campeau, & Kim, 1993), and selective 5-HT_{1A} receptor agonists (e.g., busiprone; Kehne, Cassella, & Davis, 1988). This type of cue-dependent fear has been reported to be exclusively reliant on the amygdala (Rogan & LeDoux, 1996), as NMDA (*N*-methyl-D-aspartate) antagonists, a type of glutamate receptor, injected into the amygdala extinguish fear-potentiated startle (Falls, Miserendino, & Davis, 1992; Miserendino, Sananes, Melia, & Davis, 1990).

Contextual Fear. The rat will experience an aversive stimulus (e.g., electric shock), but will not be exposed to a novel or cued stimulus. Later the rat will be placed back in the same context to assess if re-exposure to the same environment without the aversive stimulus will elicit fear (Luyten, Vansteenwegen, van Kuyck, Gabriëls, & Nuttin, 2011). SSRIs, 5-HT_{1A} receptor agonists (Inoue, Kitaichi, & Koyama, 2011) as well benzodiazepines (Harris &

Westbrook, 2001) that act to enhance the inhibitory effects of GABA (Haefely, 1990), attenuate the symptoms associated with contextual fear. Lesioning of the amygdala, hippocampus, or periaqueductal gray results in varying anxiety levels determined by the animals' "freezing" behavior. Inactivation of the amygdala and ventral, but not dorsal, periaqueductal gray reveal a reduction in freezing (or lower anxiety levels), whereas inactivation of the hippocampus produces a robust level of freezing only initially that is no longer apparent 24 hours later. This indicates there are both short- and long-term conditioned fear states (Kim, Rison, & Fanselow, 1993). Moreover, depleting the stress horomone (corticosterone) via an adrenalectomy in rats does not eliminate a contextual fear conditioning response immediately, but an alleviation of fear is noted 24 hours later (Pugh, Tremblay, Fleshner, & Rudy, 1997).

Vogel Thirst-Lick Conflict Test. For this apparatus, water-deprived animals are given a reward of water while simultaneously receiving an electric shock to the tongue on every 21st lick. Animals that receive anxiolytics will continue consuming the water. However, control animals (who receive no drugs) will avoid the aversive stimulus (Bourin, Petit-Demouliere, Dhonnchadha, & Hascoet, 2007). Benzodiazepines have been reliable anxiolytics for either male or females, whereas in some pharmaceuticals such as SSRIs, e.g., fluoxetine, are effective in producing anxiolytic-like effects, only in male rats. It has also been suggested that the serotonergic activity in the dorsal hippocampus mediates this conflict behavior (Matsuo, Kataoka, Mataki, Kato, & Oi, 1996).

Geller-Seifter Test. For the rat, a positive reinforcer (such as food) is obtained by performing an instrumental response (i.e., lever pressing). After the rat reaches a point where it is making consistent operant responses for the positive reinforcer, approximately seven trials later a negative reinforcer (i.e., electric shock) is added. The presentation of the positive reinforcer is simultaneously paired with electric shock to the rat, and, thus, creating a conflict between the positive and negative reinforcement (Geller, Kulak, & Seifter, 1962). 5-HT_{2C} receptor antagonists (Kennett, Pittaway, & Blackburn, 1994) and benzodiazepines (Geller, Kulak, & Seifter, 1962) have shown anxiolytic properties, and encouraged rats to tolerate more shocks to obtain more food. Furthermore, a serotonergic antagonist injected into the basolateral amygdala results in anxiogenic effects for this particular conflict paradigm (Hodges, Green, & Glenn, 1987).

Defensive Burying Test. The rat will be placed in a cage filled with sawdust bedding where the rat will receive an electric shock from a stationary electrified prod. Typically, after receiving a shock, the rat will exhibit a passive behavioral response (i.e., inactivity) followed by a vigorous burying behavior to move the sawdust bedding onto and subsequently covering the electrified prod. This defensive burying is only seen when a shock is administered, so it does not occur in the absence of shock (Pinel & Treit, 1978; Treit, Pinel, & Fibiger, 1981). Benzodiazepines have been effective in reducing the defensive burying behavior (Treit, 1990; Treit et al., 1981). Furthermore, lesions to either the dorsal premammillary nucleus, anterior hypothalamus, or the dorsal medial portion of

the ventromedial hypothalamus suppress unconditioned defensive behaviors (Canteras, 2002; Canteras & Swanson, 1992; Risold, Canteras, & Swanson, 1994).

Unconditioned Animal Models of Anxiety

Elevated-Plus Maze. The elevated-plus maze is an effective animal model of anxiety. The design of this maze is an elevated platform with four arms. Two of the arms have surrounding walls, while the other two arms are open and without walls. The arms are interconnected by a central platform. Time spent navigating the maze, and the number of entrances in the open arms, are commonly used as measures of anxiety because of rats' innate fear of novel, open spaces; while time spent in the closed arms is assessed as a measure of general motor activity (Ramos, 2008). SSRIs tend to induce, rather than ameliorate, behavior indicative of anxiety (Takeuchi, Owa, Nishino, & Kamei, 2010). Furthermore, the performance of rats in the elevated-plus maze is unaltered by norepinephrine-based drugs (i.e., desipramine; Drapier, Bentue-Ferrer, Laviolle, Millet, Allain, Bourin, et al., 2007), however, serotonin-norepinephrine reuptake inhibitors (SNRIs) have produced results comparable to anxiolytic compounds, such as benzodiazepines (Takeuchi, Owa, Nishino, Kamei, 2010).

Light-Dark Box. The light-dark box consists of two areas. The larger area has a white and brightly lit background, while the smaller area has a dark and black background. Exploration in the larger, illuminated white background is used as the measurement of anxiety (due to a rat's innate fear of exposure to

bright light; anti-phototropic). The assessment of anxiety is based on the time spent in the area, motor activity, and number of entries (Crawley & Goodwin, 1981; Ramos, 2008). On a similar note to the elevated-plus maze, SSRIs are generally inactive in reducing anxiety and actually facilitate anxiogenic-like responses (Bodnoff, Suranyi-Cadotte, Quirion, & Meaney, 1989). Benzodiazepines, which are typically effective in reducing anxiety, do not have an impact in the light-dark box for rats, but do show anxiolytic properties for mice (Ramos, Pereira, Martins, Wehrmeister, &Izidio, 2008). The alpha-2 adrenergic antagonist (yohimbine) exerts anxiogenic effects in the light-dark box (Fernandez, Misilmeri, Felger, & Devine, 2004). In general, the light-dark box is likely not a reliable marker for screening anxiolytic compounds in rats (Ramos et al., 2008).

Holeboard Test. Rats are placed in a wooden box with four smaller holes located in the floor. Infrared photocells are placed on the sides of the box to detect locomotor activity and rearing. Photocells are also distributed below the surface of the holes to measure the frequency and duration of head-dips by the rat. Changes in head-dipping by rodents are believed to be a marker of anxiety, as increased head-dipping is considered an exploratory behavior that the rats would perform during less anxious states (File & Pellow, 1985; Takeda, Tsuji, & Matsumiya, 1998). Benzodiazepines have produced a significant increase in such exploratory behavior (File & Pellow, 1985). SSRIs and SNRIs have been advantageous in exhibiting anxiolytic properties in the hole board test (Ishizuka, Abe, Tanoue, Kannan, & Ishida, 2010).

Novelty-Suppressed Feeding Test. In this animal model, rats are food deprived (not water-deprived) for 48hrs, and then placed in a novel environment with food where the latency to begin eating is recorded (Bodnoff, Suranyi-Cadotte, Aitken, Quirion, & Meaney, 1988; Bodnoff et al., 1989). Benzodiazepines are effective anxiolytics (i.e., reduce latency to eat the food), whereas either a NSRI or SNRI is not nearly as effective (Bodnoff et al., 1988).

Adult Social Interaction Test. Two adult male rats are placed in the same environment and the interaction between the rats (e.g., sniffing, following, or grooming the other rat) is scored. Importantly, only one score for the pair of rats is used, as the behavior of one rat influences the behavior of the other. However, if only one rat is treated (e.g., drug administration), then only that rat is scored. Animals that engage in more social interaction are less anxious, while decreased social interaction would illustrate the opposite effect. The highest rate of social interaction occurs between animals in a familiar environment with minimal lighting (File & Seth, 2003). Benzodiazepines have been effective in attenuating anxiety in this model (File & Pellow, 1984), whereas benzodiazepine receptor antagonists exhibit anxiogenic effects (File, Lister, & Nutt, 1982). Antidepressant drugs (e.g., SSRIs or tricyclic antidepressants) have been reported to have anxiogenic rather than anxiolytic responses (Bagdy, Graf, Anheuer, Modos, & Kantor, 2001; To, Anheuer, & Bagdy, 1999; To & Bagdy, 1999).

Juvenile Social Exploration Test. This test is similar to the adult social interaction test, but a key difference being the utilization of juvenile rats (28-32

days old). In this model, 24hr before the administration of a stressor (e.g., electric shock), the rat is taken from the vivarium and placed in a plastic tub cage with bedding that is free of food and water. The rat is given 60 min to become acclimated to the novel environment, then a juvenile rat is placed in the cage with the adult rat where the researcher will record exploratory behaviors exhibited by the adult rat (e.g., sniffing, pinning, or grooming the juvenile). Additionally, behavior is only recorded for the one adult rat in the tub cage. After a few minutes the juvenile is removed, and the adult rat is returned to its home cage. This initial social exploration (SE) test is used as a baseline procedure to screen for rats with any abnormal responses prior to the stressor, as non-stressed rats will spend a significant portion of time exploring a juvenile. Following the preliminary screening step, the adult male will be tested again for SE at a later time point post-stressor (Christianson et al., 2008a). This procedure is slightly modified from other versions of SE (Bluthe, Dantzer, & Kelley, 1992; Pollak, Orion, Goshen, Ovadia, & Yirmiya, 2000;Pollak, Ovadia, Goshen, Gurevich, Monsa, Avitsur, et al., 2000; Pollak, Ovadia, Orion, & Yirmiya, 2003). It has been illustrated as a successful animal model of anxiety, as the administration of an anxiogenic (e.g., β-carbolines) or anxiolytic (e.g., benzodiazepines) compound resulted in either reduced or increased SE, respectively (Christianson et al., 2008a).

This animal model of anxiety has three distinct advantages over adult social interaction: [1.] The juvenile test takes place in a familiar tub cage, so the anxiety experienced by the adult rats is not due to novelty. [2.] Aggressive behavior

is a far less common interaction with a juvenile compared to another adult rat (Blanchard, Wall, & Blanchard, 2003) leading to a clearer representation of anxiety. [3.] Typically in adult social interaction, the total amount of interaction time is involved with paired scoring for both adult rats, and, thus, requiring twice the number of rats and treatments to achieve the same statistical results.

Open Field Test. The open field test (Hall, 1934; Hall & Ballechey, 1932) is a widely adopted animal model of anxiety for rodents that typically utilizes an open-top square box (Belzung & Griebel, 2001; Prut & Belzung, 2003). When rats are experiencing anxiety, they do not explore new environments and hug the walls of the open field (a behavior known as thigmotaxis). The anxiety-related behavior is measured by the degree to which the rat avoids the center of the open field test (Christianson & Drugan, 2005; Walsh & Cummins, 1976). Behavioral responses in the open field can reveal signs of increased or attenuated anxiety levels when anxiolytic drugs (such as benzodiazepines and $GABA_A$ agonists; Prut & Belzung, 2003) and 5-HT_{1A} agonists, respectively, (Siemiatkowski, Sienkiewicz-Jarosz, Czlonkowska, Bidzinski, & Plaznik, 2000) are administered. However, behavioral measures are non-responsive to SSRIs (Durand, Berton, Aguerre, Edno, Combourieu, Mormede et al., 1999). Due to these tendencies, the open field test does appear to be a representative model of normal anxiety (i.e., similar to a daily stressor), but may not be representative of pathological anxiety associated with various anxiety disorders (Prut & Belzung, 2003).

All of these tests rely on the unconditioned avoidance of a threatening situation. Moreover, they all measure the conflict a rat has in the desire to explore new places, but also its natural fear of brightly lit or novel areas (i.e. neophobia). While all of the animal models would potentially be an effective measure of anxiety, the open field test is ideal in order to compare its effects to past work in our laboratory (Christianson et al., 2008b).

Specific Aims

Inescapable shock has been correlated with c-Fos expression in the DRN (Amat et al., 2005) and LC. However, exposure to either inescapable shock or escapable shock in the LC, while both producing elevated levels of c-Fos expression, shows no difference in the amount of c-Fos expressed. This suggests that the LC is not sensitive to stressor controllability for electric shock (McDevitt et al., 2009). Exposure to ISS is suggested to activate neural substrates differently compared to electric shock (Drugan et al., 2010; Warner & Drugan, 2012). Generally, the ISS model tends to be unresponsive to various 5-HT manipulations and 5-HT-based antidepressants (Christanson et al., 2008b; Drugan et al., 2010), while norepinephrine-based antidepressants have had favorable results in alleviating ISS-induced deficits (Drugan et al., 2010; Warner & Drugan, 2012).

The following experiments will explore the implications of serotonergic and noradrenergic neurons in the DRN and LC, respectively, following ISS exposure and its possible association with anxiety. The effects of ISS will also be evaluated, behaviorally, with two animal models of anxiety (i.e., open field test

and SE). Experiment 1 used double-labeled TPH and TH/c-Fos immunohistochemistry (IHC) in the brain to detect the neural activity in the DRN and LC, as well as the open field test to monitor anxiety behavior. However, increased anxiety-like behavior was not depicted with the behavioral analysis from the open field data. As a result, experiments 2 and 3 will address the same concerns as the preceding experiment, but will investigate a different behavioral endpoint for anxiety (i.e., SE) with experiment 2 being a preliminary experiment exploring the optimal time post-ISS to evaluate SE.

CHAPTER II

EXPERIMENT ONE

Method

Subjects

48 male Sprague-Dawley rats (SAS Derived, Charles River Labs, NY, USA) each weighing between 180-200 grams were used in the experiment. For the first week, animals were allowed the allotted time to acclimate to the vivarium. During that time the rodents were housed four to a cage, while food and water was provided *ab libitum*. The vivarium was maintained on a 12-hour light/dark cycle (6:00am to 6:00pm) with the light cycle beginning at 6:00am. All procedures were conducted during the first 6 hours of the light cycle. After the first day of procedures, animals were individually housed in tub cages and given food and water *ab libitum*. In addition, all procedures were reviewed and approved by the University of New Hampshire Institutional Animal Care and Use Committee (IACUC; APPENDIX A).

Apparatus

Intermittent Swim Stress. ISS was conducted in two Plexiglas cylinders (21cm diameter X 42cm height) with a ¼-inch galvanized wire mesh at the bottom of each cylinder that was suspended over a tank (28.6cm height, 80.6cm length, and 45.7cm width). The tank was filled with water that reached a depth of 20cm, with the water maintaining a temperature of 15±1°C (ice was consistently

added to ensure that the temperature remained constant). During the ISS treatment, a rat was placed in each cylinder and both cylinders were lowered (simultaneously) into the cold water where the rats were forced to swim. The cylinders remained in the water for 5 seconds, and then retracted to their original placement (12.7cm above the water). Over the duration of ISS, space heaters (two above and two in front of each cylinder) blew warm air (~36°C) to keep the rats warm in between swim trials. The swim stress apparatus was monitored by the means of a computer with med-PC hardware and software that controlled the movement of the cylinders on a variable interval-60sec schedule (Christianson & Drugan, 2005).

Open field Test. Open field tests were conducted in an open-top square plywood box (25cm height, 120cm length, and 120cm width) painted with flat black enamel. A cool white fluorescent lamp emitted 200–300 lux throughout the box. Open field test sessions were recorded with a video camera located directly over the center of the arena.

Procedure

On the first day of experimentation, rats were randomly assigned to one of six conditions: home cage control (HCC)/open field, confined control (CC)/open field, ISS/open field, HCC/HCC, CC/HCC, or ISS/HCC with 8 rats/group. ISS rats were exposed to 100-5sec forced swims in the cold water (15°C) on a variable interval (VI)-60sec schedule (range = 10 – 100sec) in a procedure that we have shown to produce behavioral depression (Christianson & Drugan, 2005). CC rats were placed in the same apparatus and put through the same

intermittent procedure, but in the absence of water. After being exposed to the swim stress, ISS rats were warmed under incandescent lamps (75W just above the cage top) for 30 min while CC rats were placed under lamps positioned 90cm above the cage to control for light exposure. Following the warming period, all rats were returned to the vivarium. The HCC rats were never exposed to the ISS apparatus to ensure that the CC animals were a reliable control group.

On day two, depending on the condition, rats would either experience the open field test or remain in the vivarium as a HCC. Rats were placed in the open field test for 10 min with the each rat initially being placed in the center of the open field arena. The frequency of the following behaviors was recorded: grooming (using paws or tongue to clean itself), rearing (standing on hind legs), and corner facing (time spent facing a corner of the box). Time spent in the outer and inner sections of the arena was recorded. The outer section of the box was defined as all of the squares on perimeter of the walls, which included the four corners (i.e., 20 of 36 squares). The remaining region of the arena (16 squares) was defined as the inner section or center. Locomotor activity was recorded as number of line crossings (all four paws crossing a line; Hale et al., 2008a). The experimenters making the behavioral assessments were blind to group membership.

Tissue Collection and Preparation

90 min after the behavioral testing (e.g., open field test) rats were perfused (Sartor & Aston-Jones, 2012). Prior to perfusions, all rats were injected with a mixture of 80 mg/kg of ketamine and 8 mg/kg xlyazine of the animal's body

weight for the anesthetic. The researcher would pinch a paw of the animal firmly to ensure there was no longer a pain reflex for the rat. If there was still pain reflex, supplemental doses of 0.10 ml of ketamine were administered as needed to ensure that the rat was heavily sedated. When rats were sedated, they were transcardially perfused with physiological saline (0.9% sodium chloride; pH 7.4) followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB; pH 7.4). Following the perfusion, brains were extracted from the skulls of the rats and stored in small glass containers with the fixative solution (4% paraformaldehyde in 0.1 M PB). 12-16 hours later, the brains were transferred into PB (2 X 12 h). After being in the PB, the brains were then stored in 30% surcrose in PB until they had sunk to the bottom of the glass containers. With the use of a rat brain matrix (RBM-4000C, ASI Instruments, Warren, MI, USA), each brain was sectioned into the forebrain and hindbrain at the caudal portion of the mammillary bodies, and stored at -80°C until further processing. The hindbrain, which included the midbrain raphe complex and locus coeruleus complex, was sliced into 30µm sections using a cryostat (Leica CM1900, Leica Microsystems Ltd., Buckinghamshire, UK). The sections were placed in 6 different wells containing a cryoprotectant solution (30% ethylene glycol, 20% glycerol in 0.05 M PB; pH 7.4) in a 24 well-culture plate. Each well contained a representative set of sections at 180µm intervals throughout the midbrain raphe complex. After the slicing was completed in the cryostat, the sections were stored at -20°C.

Immunohistochemistry: Tryptophan Hydroxylase (TPH) and c-Fos

A set of hindbrain sections, including the midbrain complex, was removed from one of the 6 wells in a 24-well tissue culture plate. The tissue sections from each animal were placed in a 12-well tissue culture plate where the tissue was free-floating in 1 ml solutions at room temperature, and gently shaken on an orbital shaker throughout the immunohistochemistry. All tissue underwent a double-immunostaining process using primary antibodies for the protein product of the immediate early gene, c-fos (rabbit anti-c-Fos polyclonal antibody, Cat. No. PC38, Lot No.D00109969, 1:3000; Calbiochem, EMD Chemicals, Gibbstown, NJ, USA) and TPH (sheep anti-TPH antibody, Cat. No. T8575, Lot No. 010M1152, 1:12,000; Sigma-Aldrich, St. Louis, MO, USA). Immunohistochemistry was run simultaneously for all rats in experiment 1 in order to limit variability in the staining process. All washes or rinses during the staining process were 15min each, unless otherwise noted. On the first day of the immunostaining, tissue was rinsed from the cryoprotectant twice in 0.05 M phosphate buffered saline (PBS), then placed in 1% hydrogen peroxide in 0.05 M PBS, and followed by two rinses in 0.05 M PBS. Then the tissue was washed in 0.05 M PBS containing Triton X-100 (PBST; 0.03%), and incubated overnight with rabbit anti-c-Fos antibody in 0.01% PBST with 0.01% sodium azide. 12-16 hours later, on day 2, the antibody was rinsed off twice in 0.05 M PBS, followed by a 90 min incubation in a biotinylated donkey anti-rabbit secondary antibody (Cat. No. 711065152, Lot No.104183, 1:500; Jackson ImmunoResearch, West Grove, PA, USA) in 0.05 M PBS. Then the tissue was rinsed twice in 0.05 M PBS, followed by a 90 min

incubation with an avidin-biotin-peroxidase complex (Elite ABC reagent, Cat. No. PK-6106, 1:200; Vector Laboratories, Burlingame, CA, USA). Tissue was then rinsed twice with 0.05 M PBS, followed by incubation in a peroxidase chromogen substrate (Vector SG; Cat. No. SK-4700; Vector Laboratories; diluted as recommend by vendor) in 0.05 M PBS for 32 min. Immediately after the chromogen reaction was complete, the tissue was rinsed in 0.05 M PBS two separate times to terminate the reaction. Tissue was washed in 1% hydrogen peroxide in 0.05 M PBS, followed by two rinses in 0.05 M PBS, and then incubated with sheep anti-TPH antibody in 0.1% PBST with 0.01% sodium azide overnight. 12-16 hours later, on day 3, the antibody was rinsed off with two washes in 0.05 M PBS, followed by a 90 min incubation in a biotinylated rabbit anti-sheep secondary antibody (Cat. No. PK-6106, 1:200; Vector Laboratories, Burlingame, CA, USA) in 0.05 M PBS. Then the tissue was washed twice in 0.05 M PBS followed by a 90 min incubation with an avidin-biotin-peroxidase complex (Elite ABC reagent, Cat. No. PK-6106, 1:200; Vector Laboratories, Burlingame, CA, USA). The tissue was rinsed twice with 0.05 M PBS, then placed in a peroxidase chromogen substrate solution consisting of 0.01% 3,3′-diaminobenzidine tetrahydrochloride (DAB) in 0.05 M PBS, with the reaction activated prior to use with 0.005% hydrogen peroxide, for 80 min. The result of the reaction was faint, so the tissue was rinsed twice in 0.05 M PBS, and incubated once more with sheep anti-TPH antibody in 0.1% PBST with 0.01% sodium azide overnight, increasing the concentration of TPH from 1:12,000 to 1:8,000. 12-16 hours later, on day 4, all steps were the same as day 3

(excluding primary antibody). Following a successful reaction in DAB, the tissue was washed twice in 0.05 M PBS to stop the reaction, and then placed 0.1 M PB with 0.01% sodium azide at 4°C. Immunostaining of c-Fos was a blue/black color localized to the nucleus, while immunostaining of TPH was an orange/brown color localized to the cytoplasm. Following immunostaining, the tissue was rinsed briefly in 0.15% gelatin in distilled water, then mounted on microscope slides (VistaVision, Cat No. 16004-390, VWR, West Chester, PA, USA). The mounted tissue was dehydrated through the use of a series of graded alcohols (70%, 95%, and 100% ethanol) and cleared with xylene. Cover slips were then secured on the slides using Entellan mounting medium (Electron Microscopy Science, Hatfield, PA, USA).

Immunohistochemistry: Tyrosine Hydroxylase (TH) and c-Fos

This process was virtually identical to the TPH protocol that was previously described with exceptions in two key areas. On day 2, the primary antibody that was used was TH (rabbit anti-TH, Cat. No. AB152, Lot No. 21030329, 1:8000; Chemicon, Billerica, MA, USA) and was incubated for 12-16 hours. On day 3, the secondary antibody that was used was a biotinylated donkey anti-rabbit secondary antibody (Cat. No. 711065152, Lot.No.104183, 1:500; Jackson ImmunoResearch, West Grove, PA, USA) in 0.05 M PBS for a 90 min incubation.

Results

Open Field

A One-way analysis of variance (ANOVA) revealed no significant differences between ISS and control rats for any of the behavioral measures (i.e, rearing, grooming, corner facing, time spent in the outer/inner sections and locomotor activity) in the open field test (p's > 0.05; Figures 1-6). The open field data was analyzed in the same format as Hale et al. (2008a) in terms of the grouping the data in five minute segments (i.e., 0-5 min and 5-10 min).

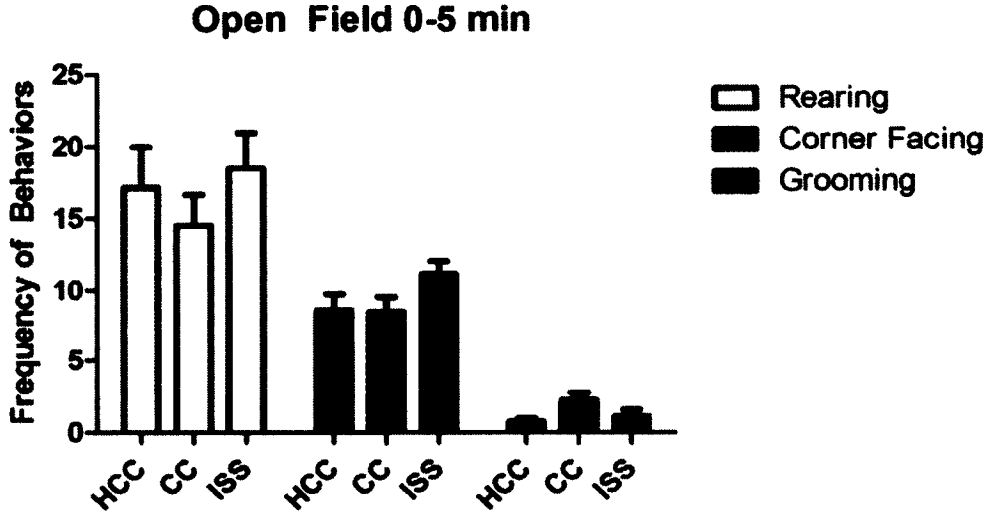

Figure 1. Mean (+/- SEM) frequencies of behavior in the open field (0-5 min).

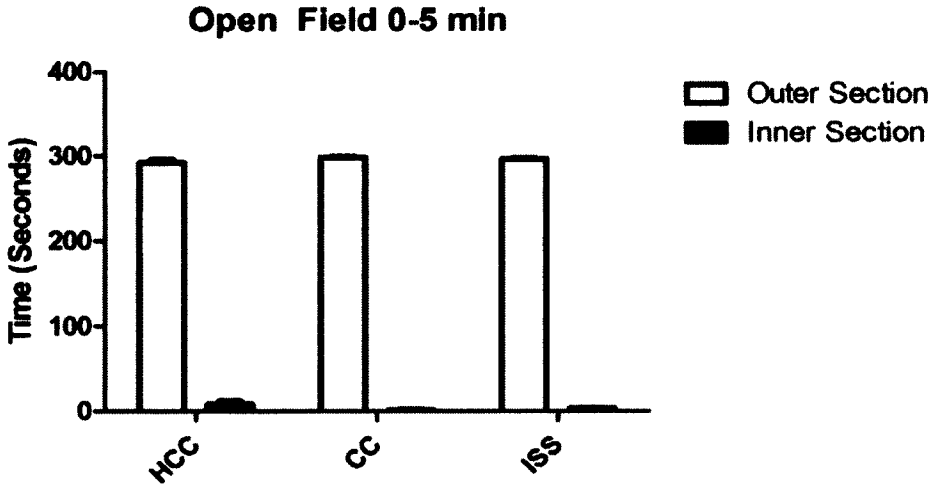

Figure 2. Mean (+/- SEM) time in sec spent in the outer or inner section of the open field (0-5 min).

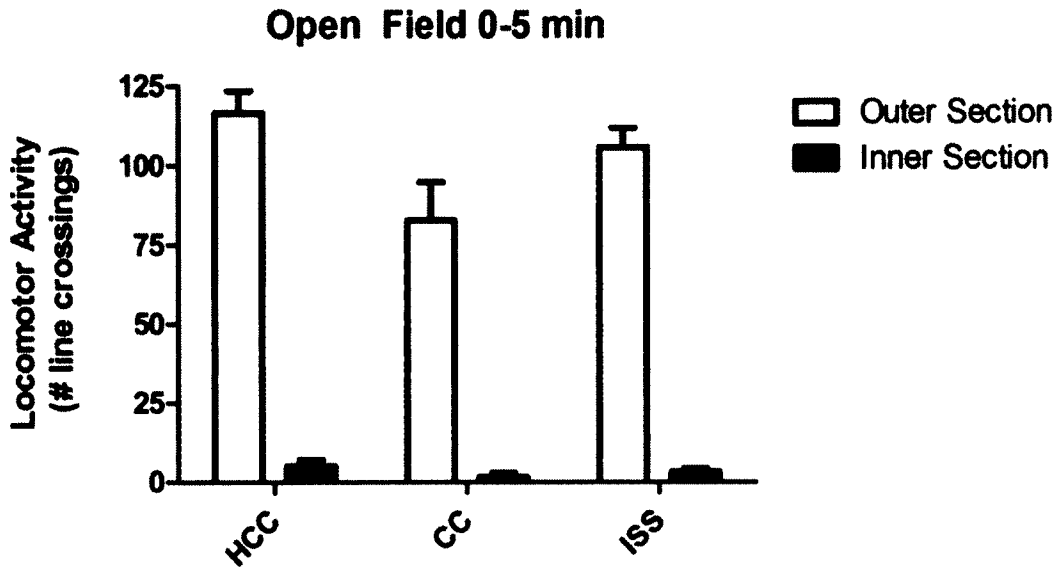

Figure 3. Mean (+/- SEM) number of line crossings in the outer or inner section of the open field (0-5 min).

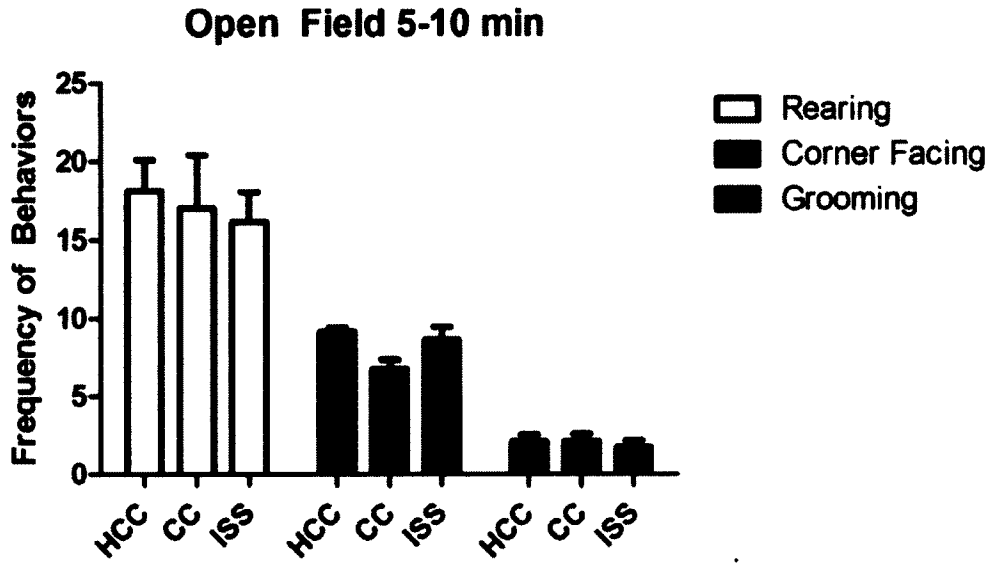

Figure 4. Mean (+/- SEM) frequencies of behavior in the open field (5-10 min).

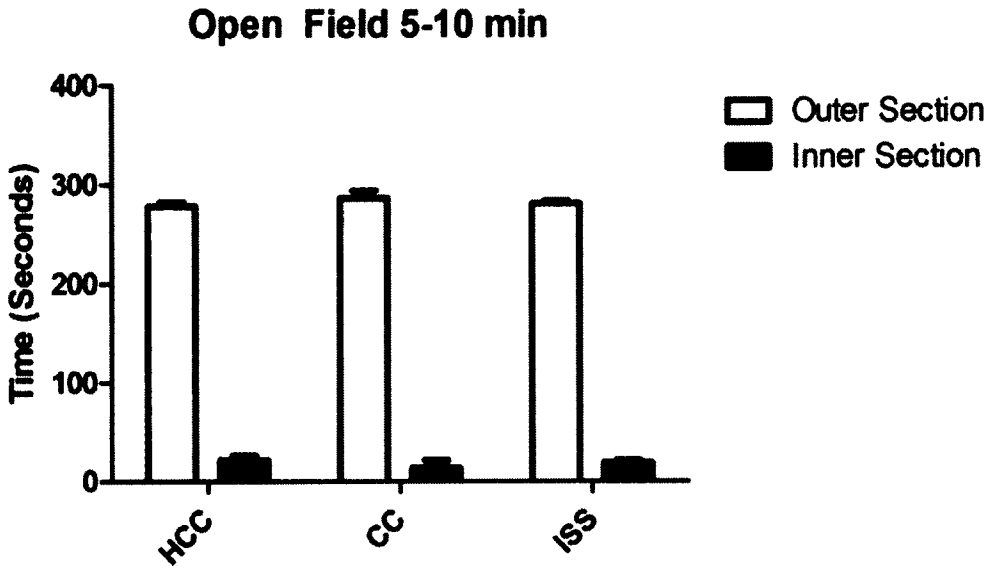

Figure 5. Mean (+/- SEM) time in sec spent in the outer or inner section of the open field (5-10 min).

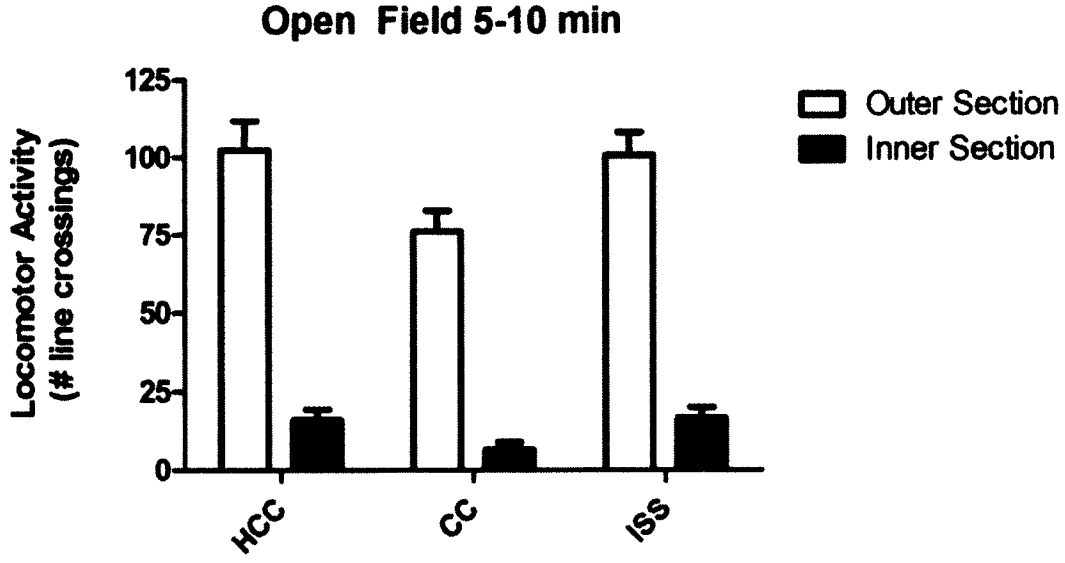

Figure 6. Mean (+/- SEM) number of line crossings in the outer or inner section of the open field (5-10 min).

Immunohistochemsitry for the DRN

Cell counting occurred in the dorsal part of the dorsal raphe nucleus (DRD) at -8.18mm Bregma (Paxinos & Watson 1997). The immunohistochemistry data for three rats was omitted due to the quality of the tissue. A Pearson's r revealed that the inter-rater reliability correlations were high for all three cell counts: $r(43)=0.93$ for c-Fos, $r(43)=0.90$ for TPH-stained neurons, and $r(43)=0.95$ for double-labeled neurons ($p<0.001$ for all counts). A repeated measures ANOVA was used with mean cell counts for c-Fos, TPH-stained (serotonergic) neurons, and double-labeled (presentation of c-Fos and TPH in same cell) as the within-subject factors and treatment as the between-subject factor. There was a significant difference for cell counts [$F(2,78) = 307.68$, $p < 0.001$], but there was not a significant effect for cell counts X treatment interaction [$F(10,78) = 0.570$, $p = 0.833$]. There was also no significant difference between the treatment groups [$F(5,39) = 0.527$, $p = 0.755$]. This indicated that the number of counts between c-Fos, TPH-stained neurons, and double-labeled neurons did differ. However, the cell counts did not differ among treatment groups (Figures 7 & 8).

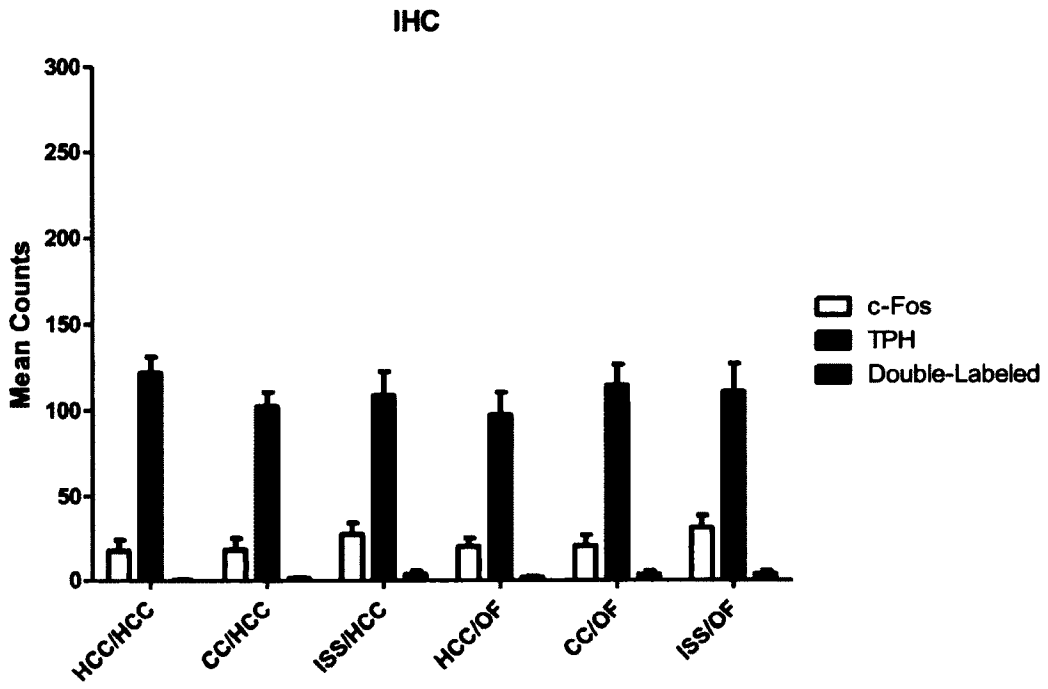

Figure 7. Mean (+/- SEM) counts for cells containing c-Fos, TPH, and both c-Fos and TPH (double-labeled) in the DRD (dorsal part of the dorsal raphe nucleus) at -8.18mm Bregma.

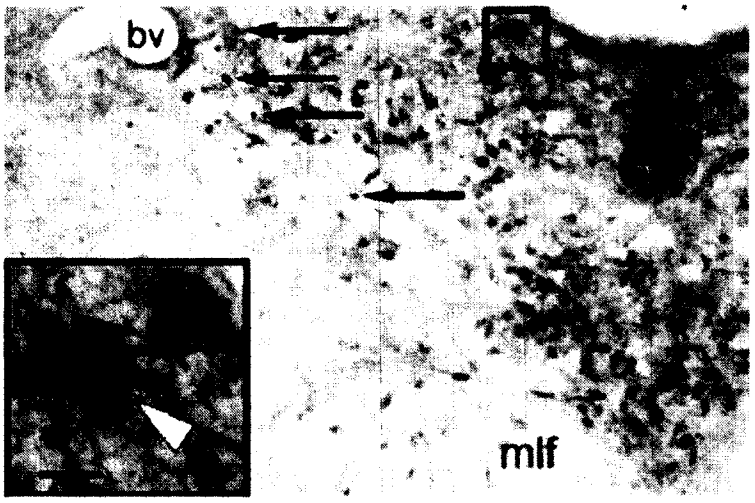

Figure 8. Photomicrograph representing a sample of c-Fos expression in serotonergic and non-serotonergic neurons in the mid-rostrocaudal dorsal raphe nucleus (-8.18mm Bregma). Black boxes indicate regions with higher levels of magnification in inserts in the lower left part of the panel. Black arrows illustrate examples of c-Fos (represented by a blue/black coloring of the nucleus), white arrowheads indicate TPH-stained cells (represented by brown/orange coloring of the cell body), and black arrowheads indicate the presentation of both c-Fos and TPH in the cell (double-labeled neurons).

Immunohistochemsitry for the LC

Cell counting occurred between –9.8 and –10.04 mm Bregma (Paxinos & Watson 1997). The immunohistochemistry data for six rats was omitted due to missing sections. A Pearson's r revealed that the inter-rater reliability correlations were high for all three cell counts: $r(40)=0.97$ for c-Fos, $r(40)=0.92$ for TH-stained neurons, and $r(40)=0.97$ for double-labeled neurons ($p<0.001$ for all counts). A repeated measures ANOVA was used with mean cell counts for c-Fos, TH-stained (noradrenergic) neurons, and double-labeled (presentation of c-Fos and TH in same cell) as the within-subject factors and treatment as the between-subject factor. There was a significant difference for cell counts [$F(2,72) = 940.94$, $p < 0.001$], but there was not a significant effect for cell counts X treatment interaction [$F(10,72) = 0.879$, $p = 0.557$]. There was also no significant difference between the treatment groups [$F(5,36) = 0.982$, $p = 0.442$]. This indicated that the number of counts between c-Fos, TH-stained neurons, and double-labeled neurons did differ. However, the cell counts did not differ among treatment groups (Figures 9 & 10).

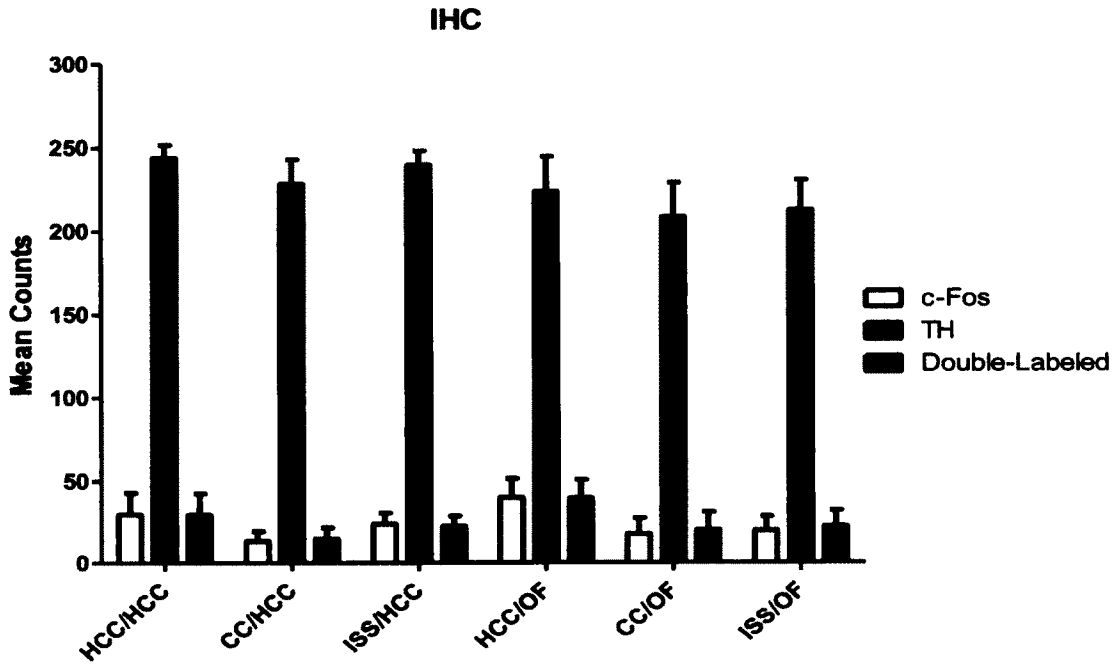

Figure 9. Mean (+/- SEM) counts for cells containing c-Fos, TH, and both c-Fos and TH (double-labeled) in the LC between –9.8 and –10.04 mm Bregma.

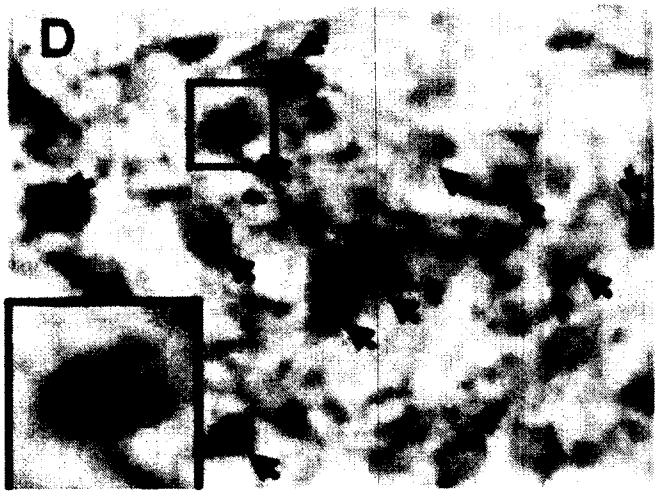

Figure 10. Photomicrograph representing a sample of c-Fos expression in noradrenergic and non-noradrenergic neurons. Black boxes indicate regions with higher levels of magnification in inserts in the lower left part of the panel. Black arrowheads indicate the presentation of both c-Fos and TH in the cell (double-labeled neurons).

Discussion

The non-significant results for the open field test could mean that exposure to the ISS model may better represent pathological anxiety. Indeed, if this is the case, Prut and Belzung (2003) have suggested that the open field test may not be a valid model to characterize pathological anxiety. In terms of the immunohistochemistry, the cell counting that occurred in the DRD at -8.18mm Bregma was chosen because it is densely populated with serotonergic neurons and has a strong association with anxiety. Expanding on the DRD's association with anxiety: it is innervated by structures in the forebrain (e.g., BNST), which control anxiety levels; plays a role in mediating emotional behavior; and is a key area that responds specifically to stress- or anxiety-provoking stimuli (Lowry et al., 2008). The selected sections between –9.8 and –10.04 mm Bregma were chosen because the largest portion of noradrenergic neurons are found in this rostrocaudal area (Dawe, Huff, Vandergriff, Sharp, O'Neill, & Rasmussen, 2001) and this area is innervated by amygdala, which is associated with fear and anxiety (Bishop, 2007).

The results obtained from the current experiment for both the c-Fos/TPH and c-Fos/TH protocols revealed no differences between the treatment groups. With the similar results between the stress and control conditions, this could mean that serotonergic neurons may not play as large of a role with regard to ISS — a consistent finding with our model (Christianson et al., 2008b; Drugan et al., 2010). However, NSRIs have been effective in alleviating ISS-induced deficits (Drugan et al., 2010), even though there was no difference at the cellular

level for noradrenergic neurons in the current experiment. The lack of cellular results could be due to when the assessment of c-Fos occurred (approximately 24h after ISS exposure), since c-Fos is a short-lived protein with a half-life of approximately two hours. This alludes to the importance of the time course associated with c-Fos, and could provide an opening for other longer lasting Fos measures (e.g., FosB or ΔFosB) as neural markers (Kovács, 1998).

CHAPTER III

EXPERIMENT TWO

Method

Contrary to experiment 1, SE was used as the dependent measure rather than the open field test (described below). The rationalization to use SE as a different behavioral endpoint to measure anxiety was derived from prior work by Christianson, Drugan, Flyer, Watkins, and Maier (2013) who found a significant difference in SE for rats exposed to a cold water continuous swim (19°C) with a 5 min swim duration. Differences in SE were noted at 1hr and 24hr post-stress. However, for our model, looking at the effects 1hr post-ISS may yield confounding results, as rats demonstrate hypothermic tendencies for at least 2hr post-ISS (Levay, Govic, Hazi, Flannery, Christianson, Drugan et al., 2006). With that known, experiment 2 was a pilot study to determine the optimal time point post-ISS (15°C; 100-5 sec swims) to evaluate SE.

Subjects

All rats were exposed to ISS. 16 male Sprague-Dawley rats (SAS Derived, Charles River Labs, NY, USA) were divided into two groups: No Context Re-Exposure and Context Re-Exposure with 8 rats/group. All procedures were reviewed and approved by the University of New Hampshire IACUC (APPENDIX B).

Apparatus

Juvenile Social Exploration. This consisted of a single, plastic tub cage (20.3cm height, 47.6cm length, and 25.4cm width) that contained wood shavings as bedding and a metal wire lid, but free of food and water. Test sessions were recorded with RT counter/timer version 2.1 (an open-source laboratory timer written by John Christianson, 2007) used by Christianson et al. (2008a).

Procedure

On days 1 (48hr pre-ISS; SE 1) and 2 (24hr pre-ISS; SE 2) of experimentation, rats underwent baseline tests for SE. Two baseline tests were administered to ensure that the amount of time the rats spent exploring the juveniles was consistent across multiple time points. For the test itself, adult (4 rats/cage) and juvenile (6 rats/cage) rats were group housed and were taken from the vivarium and placed in a separate room from either the vivarium or ISS room. All rats were given 60 min to become acclimated to the novel environment, then a juvenile rat (28-32 days old) was placed in a separate cage with an adult rat for three minutes where exploratory behaviors (e.g., sniffing, pinning, or grooming the juvenile) of the adult rat was recorded. After three minutes the juvenile was removed, and the adult rat was returned to its home cage (Christianson et al., 2008a).

Day 3 was the same as the first day of experimentation for experiment one with the exception of there being no CC condition, only ISS. Following ISS exposure, adult rats were individually housed (this was the housing condition for the remainder of the experiment). Juvenile rats always remained group housed

throughout the experiment. The adult rats were divided into two groups: No Context Re-Exposure and Context Re-Exposure. The first group would experience ISS, but was not re-exposed to the ISS apparatus at later time points. For the Re-Exposure group, those rats were re-exposed to the ISS apparatus for 10 minutes prior to each SE time point post-ISS. Regardless of the group, SE testing occurred at 3h (SE 3), 5h (SE 4), 8h (SE 5), and 24h (Day 4; SE 6) post-ISS. All of these SE tests were compared to the baseline measures—SE 1 and SE 2. The exact procedure described for SE on days 1 and 2 remained the same with the exception that the Re-Exposure group only experienced a 50 min acclimation to the SE room prior to testing. Important to note, is that all of the time points selected occurred during the light cycle.

Results

A Pearson's r revealed that the inter-rater reliability was high for all SE tests: $r(14)=0.99$ for SE 1-SE 6 ($p<0.001$). A one way ANOVA was conducted to determine the statistical significance between groups, and revealed a significant main effect [$F(11,95) = 2.713$, $p = 0.005$]. Post hoc Fisher's Least Significant Difference (LSD) tests indicated that the No Re-Exposure/SE 1 and SE 2, respectively, differed from SE 4 ($p = 0.005$; $p = 0.006$), SE 5 ($p = 0.015$; $p = 0.017$), and SE 6 ($p = 0.002$; $p = 0.003$). The same post hoc tests also revealed that the Re-Exposure/SE 1 and SE 2, respectively, only differed from SE 5 ($p = 0.016$; $p = 0.014$; Figure 11).

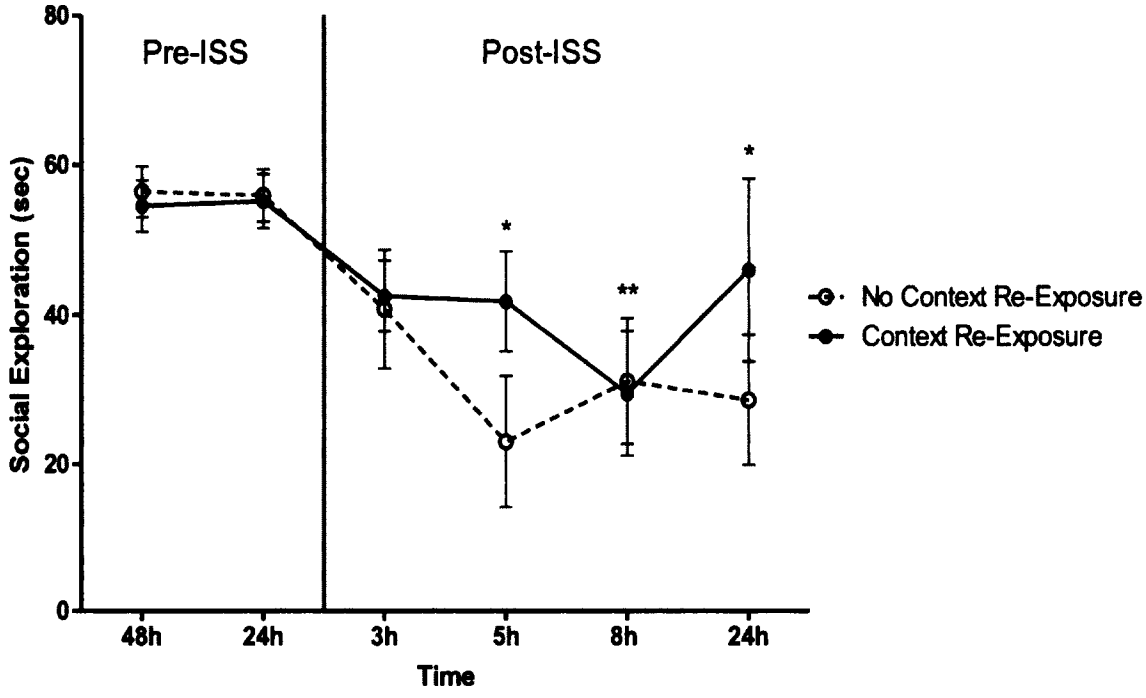

Figure 11. Mean (+/- SEM) time in sec of social exploration. All rats were exposed to ISS, but were divided into No Re-Exposure or Re-Exposure groups. There were 6 social exploration time points (48h pre-ISS = SE 1; 24h pre-ISS = SE 2; 3h post-ISS = SE 3; 5h post-ISS = SE 4; 8h post-ISS = SE 5; 24h post-ISS = SE 6). *Significant difference ($p<0.05$) from SE 1 and SE 2 for only the No Context Re-Exposure condition. **Significant difference ($p<0.05$) from SE 1 and SE 2 for both experimental conditions.

Discussion

Even though there was no discernible difference noted for the open field test in experiment 1, the results for the current experiment provide the first behavioral change of anxiety behavior in response to ISS exposure. Others have noted a reduction in SE 24h post-stress without re-exposure to the stressful environment (Christianson, Jennings, Ragole, Flyer, Benison, Barth, et al., 2011), and most studies have looked at the effects of juvenile SE 12 hours or later following stress exposure (Christianson et al., 2008a, 2011). However, because SE has never

been explored with regard to ISS, it was vital to assess multiple time points to ensure an effect was possible. The inconsistent results of the context re-exposure group was likely due to the fact that the 60 min acclimation period in the SE testing room was disrupted when the animals were placed back in the ISS chamber. So, in the subsequent experiment, no context re-exposure will occur. Since there was no statistical difference between SE 1 and SE 2, only one SE time point pre-stress will be used for future experiments.

CHAPTER IV

EXPERIMENT THREE

Method

With the results being much more consistent and reliable for the no context re-exposure group, I did not plan to pursue the context re-exposure condition for experiment 3. Moreover, the goal of the previous experiment was to select an optimal time point. A 24h SE time point seemed ideal for experiment 3 for a few reasons: [1.] This time point was consistent with our past work in our laboratory that has looked at the effects of ISS 24h later on various behavioral endpoints (Christianson & Drugan, 2005; Drugan, Eren, Hazi, Silva, Christianson, & Kent, 2005; Drugan et al., 2010; Warner & Drugan, 2012) [2.] This was the same time point that was investigated in experiment 1 [3.] Due to our general interest in serotonin and norepinephrine with our ISS model, there have been previous reports looking at the mechanistic functions of dorsal raphe nucleus and locus coeruleus at a 24h time point. At 24h post-uncontrollable stress, there was an increase in the firing rate of serotonin in the dorsal raphe nucleus (Rozeske et al., 2011) and a decrease in the firing rate of norepinephrine in the locus coeruleus (Pavcovich & Ramirez, 1991). [4.] In the small chance that the multiple SE tests affected performance 24h post-ISS, a 24 time point was measured again to provide a pure assessment of the results. This was helpful to ensure that fatigue or hypothermia was not a potential confound for the reduced SE times. The

methodology was identical to experiment 2 with the exception of the groups and only two SE time points were used.

Subjects

16 male Sprague-Dawley rats (SAS Derived, Charles River Labs, NY, USA) were divided into two groups: ISS and home cage control (HCC) with 8 rats/group. A CC group, as seen in experiment 1, was not included for this experiment. The reason being is that past results between the CC and HCC groups have been comparable (Christianson & Drugan, 2005), and most research involving an uncontrollable stressor only uses a HCC group (Christianson et al., 2008a, 2009, 2010). All procedures were reviewed and approved by the University of New Hampshire IACUC (APPENDIX C).

Procedure

Day 1, all adult and juvenile rats were group housed (4 rats/cage) and experienced SE 1 (24h pre-ISS). Day 2, only adult rats were individually housed and this was the housing condition for the remainder of the experiment. On this same day, for the ISS condition only, rats were exposed to the ISS apparatus. HCC rats remained in the vivarium during ISS sessions. Day 3, all rats experienced SE 2 (24 post-ISS).

Results

A Pearson's r revealed that the inter-rater reliability was high for all SE tests: $r(14)=0.99$ for SE 1 and SE 2 ($p<0.001$). A one way ANOVA was conducted to determine the statistical significance between groups and revealed a significant main effect [$F(3,29) = 4.757$, $p = 0.009$]. Post hoc LSD tests

indicated that ISS/SE 2 significantly differed from ISS/SE 1 ($p = 0.001$), HCC/SE 1 ($p = 0.011$), and HCC/SE 2 ($p = 0.018$; Figure 12). One rat was removed from the analysis because he failed to exceed 20 seconds of social exploration during the baseline testing.

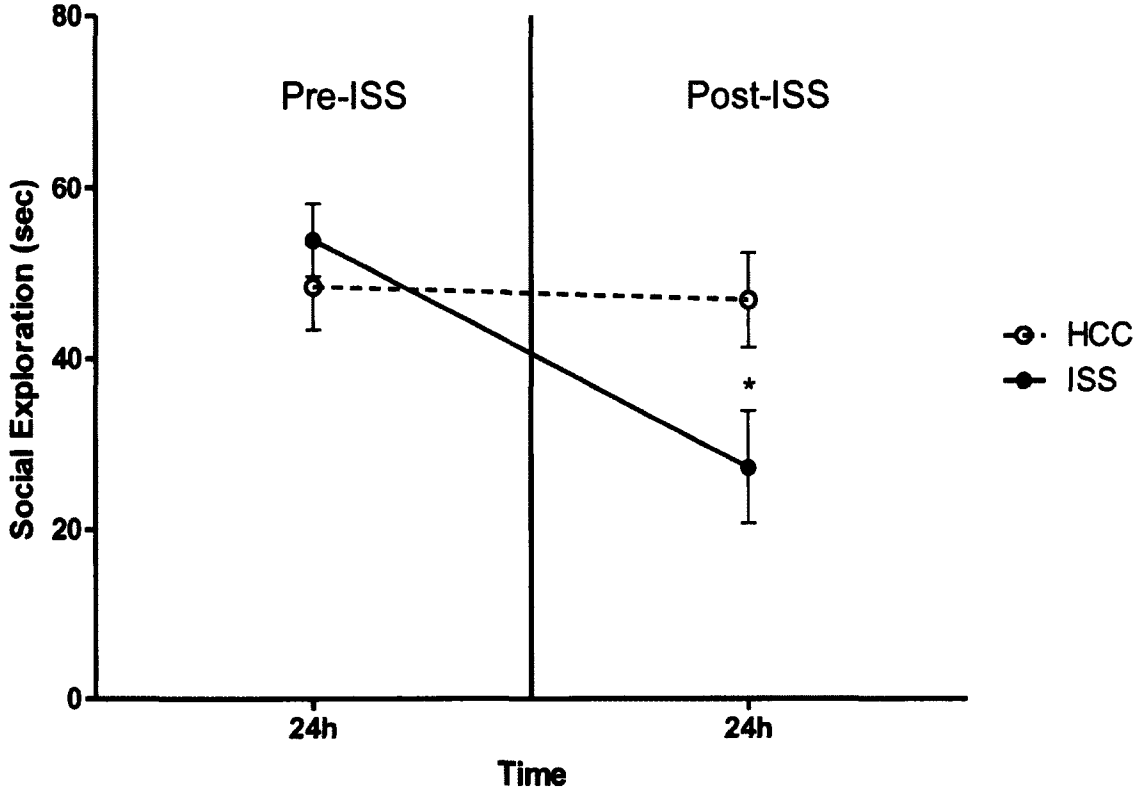

Figure 12. Mean (+/- SEM) time in sec of social exploration. There were 2 social exploration time points (24h pre-ISS = SE 1; 24h post-ISS = SE 2). * ISS group at SE 2 significantly differed ($p<0.05$) from all other conditions and SE time points.

Discussion

The present experiment replicates the reduction of SE following ISS exposure. Importantly, these results support the trans-situational value of the

ISS model. ISS-induced deficits have been noted for tasks assessing behavioral depression (Drugan et al., 2010), learning and memory (Warner & Drugan, 2012) and now anxiety. As stated earlier, this SE reduction 24h post-stress has been seen in other models (Christianson et al., 2008a, 2011).

CHAPTER V

GENERAL DISCUSSION

We have learned much since the ISS model was first described by Brown, Hurley, Repucci, and Drugan (2001). Researchers illustrated the feasibility of using a triadic design (i.e. escapable stress, yoked-inescapable stress and a non-stressed control) with the ISS model, which was previously only associated with the tailshock paradigm (Maier et al., 1986). Controllability over the stressor (i.e., escapable swim stress) was not a factor at 23°C (Brown et al., 2001), but was a factor at 30°C (Drugan et al., 2005) for ISS in regards to behavioral depression (or immobility) in the FST. At 30°C, rats exposed to inescapable swim stress experienced greater immobility compared to rats that experienced escapable swim stress (Drugan et al., 2005). The stressor appeared to have a more severe impact at 23°C since both inescapable and escapable stress groups showed a significant reduction for immobility in comparison to controls (Brown et al., 2001), and this ISS-induced deficit has since been replicated for inescapable stress using 15°C (Christianson & Drugan, 2005; Drugan et al., 2010). The impairments associated with ISS extend beyond immobility, as there have been instrumental (Christianson & Drugan, 2005) and spatial (Warner & Drugan, 2012) learning deficiencies as well. Furthermore, stress-induced analgesia noted for inescapable tailshock (Drugan et al., 1985; Maier, Davies, Grau, Jackson, Morrison, Moye, et al., 1980) was also observed for inescapable swim stress (Brown et al., 2001).

There have also been distinct differences between inescapable shock and swim paradigms. In response to alcohol, inescapable shock potentiated ataxic effects (Drugan, Coyle, Healy, & Chen, 1996) while inescapable swim either attenuated or had no influence on ataxia (Brown et al., 2001; Drugan, Wiedholz, Holt, Kent, & Christianson, 2007; Tayyabkhan, Mammola, & Drugan, 2002). In addition, there appear to be varying neurochemical systems regulating these different forms of inescapable stress. Learned helplessness resulting from inescapable shock is believed to derive from a serotonergic activation (Amat et al., 1998) and noradrenergic inactivation, (Amat et al., 2004), as inescapable shock produced large increases in 5-HT (Amat et al., 1998) and decreases in norepinephrine (Weiss & Simson, 1986). Fluoxetine, an SSRI, has been shown to alleviate the behavioral deficits imposed by inescapable shock (Valentine, Dow, Banasr, Pittman, & Duman, 2008). However, fluoxetine has had no impact on the behavioral deficits associated with ISS (Christianson et al., 2008; Drugan et al., 2010), while NSRIs (e.g., desipramine and reboxetine) have mitigated such behavioral deficits (Drugan et al., 2010; Warner & Drugan, 2012). As a result, the nature of the stressor can have a profound influence on both the behavioral and neurological outcomes.

A key characteristic of uncontrollable stress is that it is believed to be trans-situational, meaning once the subject is removed from the original stressful encounter, the resulting experience is capable of altering the subject's behaviors in different environments (Maier & Watkins, 2005). Results of the first experiment were inconsistent with this pattern as assessed via the open field

test. This open field result also differed from shock studies, which have revealed shock-induced behavioral deficits in the open field at 24h (Weyers, Bower, & Vogel, 2008) and 48h (Li, Yang, Yue, Liu, Yu, Wang et al., in press) post-shock stress. The difference in the behavioral outcomes of the open field between inescapable shock and swim was intriguing. Perhaps, inescapable shock is a more taxing stressor in comparison to inescapable swim, but both, as previously described, have resulted in various deficits, and there is also a noted elevation in corticosterone for animals exposed to both inescapable shock (Maier, Ryan, Barksdale, & Kalin, 1986) and swim (Drugan et al., 2005). Because the open field results are one of the few instances where inescapable shock and swim vary, the ISS apparatus may provide a better representation of pathological anxiety of which the open field may not validly measure (Prut & Belzung, 2003).

Later experiments provided justification of the trans-situational experience of ISS, and the first demonstration on anxiety behavior, as rats exposed to ISS had significant reductions in SE for both experiment 2 and 3. Although the trans-situational effects of ISS exposure have been suggested in the past using the FST (Brown et al., 2001; Christianson & Drugan, 2005; Drugan et al., 2005; 2010) and MWM (Warner & Drugan, 2012), the contextual similarity of water between all of the paradigms employed (i.e., FST, and MWM) could act as a cue for remembering the ISS paradigm. An ISS-induced deficit for the SE tests provides further validation for the trans-situational value of this particular uncontrollable stressor. The importance of this evidence is emphasized, as water did not serve as a contextual cue in this case, and this was also the first

behavioral measure of anxiety to reveal a significant impairment following ISS exposure. The SE reduction 24h post-stress is also comparable to the results seen for inescapable shock (Christianson et al., 2008a, 2011). Additionally, for both experiment 2 and 3, the ISS-induced deficit associated with SE occurred 24h after the stressor, which is consistent with other behavioral endpoints in our laboratory (Drugan et al., 2010; Warner & Drugan, 2012).

While we have investigated a variety of behavioral endpoints following exposure to ISS, we have yet to examine the cellular mechanisms associated with the resulting ISS paradigm. Experiment 1 was intended to shed light on this new area. However, no significant difference between any of the groups was identified when comparing the stress-induced activation of serotonergic neurons. This result was consistent with expectations based on previous pharmacological manipulations in our laboratory (Christianson et al., 2008; Drugan et al., 2010), and indicated that norepinephrine may cause the impairments associated with the ISS model (Drugan et al., 2010; Warner & Drugan, 2012). Results of experiment 1 also demonstrated that there was no significant difference in noradrenergic activity for rats. Importantly, baseline assessment (i.e., not being exposed to the open field) of ISS exposure in this experiment revealed no difference in neurological activation (with regard to serotonergic and noradrenergic activity) between the controls. A contributing factor to the lack of differences between groups may be due to the short-lived expression of c-Fos. In general, c-Fos is expressed in most cell types at all times in either low or undetectable levels and can become more readily transparent through exposure

to various stimuli (Eferl & Wagner, 2003). Following acute stress, the maximum amount of c-Fos protein is expressed at 1-3 hours, and gradually disappears from detection at 4-6 hours. (Cullinan, Herman, Battaglia, Akil, & Watson, 1995; Kovács, 1998; Kovács & Sawchenko, 1996). However, past studies have noted significant elevations in c-Fos exposure for both serotonergic and non-serotonergic cells in the dorsal raphe nucleus merely from exposure to the open field arena (Hale et al., 2008a). So, it was surprising that exposure to the open field arena 90 min prior to sacrificing the animals for experiment 1 did not yield some type of variation from the home cage condition.

Given this information, differences may still exist at the cellular levels for serotonergic and noradrenergic neurons resulting from ISS exposure, but different methods need to be employed upon further exploration such as assessing IHC with SE, assessing IHC shortly after ISS, or using a different Fos protein. The following expands on these three points. [1.] Investigation of the cellular mechanisms associated with SE exposure noted in experiment 3. Exposure to the open field for experiment one may not have provided a strong enough stimulus to elicit a disparity between experimental conditions. In both experiment 2 and 3, there was a noted SE reduction for rats exposed to ISS. As a result, the SE task appears to provide varying levels of anxiety for rats exposed to ISS or a control condition. It will be important to consider the neurological implications previously described with this particular anxiety-related measure, as the evaluation of c-Fos following juvenile social exploration has not been discussed in the literature. In taking this into consideration, data analysis will be

explored in the near future for the involvement of cellular mechanisms in experiment 3. [2.] The assessment of c-Fos must occur shortly after the rats have experienced the ISS condition to assess a baseline measure of the paradigm. Two hours following ISS exposure may be an ideal time to evaluate c-Fos in the paradigm, as the rats are no longer in a hypothermic state (Levay et al., 2006) and the maximum amount of c-Fos expression occurs 1-3 hours following a stressor (Cullinan, Herman, Battaglia, Akil, & Watson, 1995; Kovács, 1998; Kovács & Sawchenko, 1996). Two hours post-inescapable stress has also been used for tailshock to assess the protein product of c-Fos (Christianson et al., 2011). [3.] Implementing different neural markers (e.g., FosB and Fos-related antigens) to evaluate cellular mechanisms. FosB has a half-life of 9.5 hours following acute challenges, which would likely ensure protein expression upon further behavioral testing (e.g., social exploration) on the same day of the ISS procedure. A variant of FosB is ΔFosB. ΔFosB is able to maintain protein expression on a longer basis. Depending on the particular protein of ΔFosB, it can have a half-life at 28 h (Fos-related antigen-1) or 208 h (Fos-related antigen-2) in response to repeated stimuli (Kovács, 1998). Utilizing FosB or ΔFosB as a neural marker may appear to be a favorable option in assessing stress- or anxiety-related models that have larger gaps of time between testing in our laboratory for subsequent studies.

Conclusion

Overall, the experiments discussed provided further insight to the ISS paradigm. These experiments revealed the first behavioral ISS-induced deficit associated with an anxiety behavior (i.e., SE reduction), which supports the trans-situational value of our stressor. The experiments also validated the ISS model as an animal model of depression appropriate for examining the common comorbidity of anxiety and depression for people suffering from major depression (Wheeler, Blankstein, Antony, McCabe, & Bieling, 2011); and also suggests that the ISS paradigm is a model for post-traumatic stress disorder. As our laboratory has not previously investigated cellular mechanisms, the first experiment provided an enlightening initial step for subsequent experiments. It is now known that distinguishable c-Fos expression is not as readily present approximately 24h following exposure to ISS. As a result, future immunohistochemistry experiments must carefully assess the time course of various Fos proteins.

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APPENDICES

APPENDIX A

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04-Apr-2012

Drugan, Robert C
Psychology, Conant Hall
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IACUC #: 120302
Project: Intermittent Swim Stress Effects on Anxiety Behavior
Category: E
Approval Date: 28-Mar-2012

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category E on Page 5 of the Application for Review of Vertebrate Animal Use in Research or Instruction - *Animal use activities that involve accompanying pain or distress to the animals for which appropriate anesthetic, analgesic, tranquilizing drugs or other methods for relieving pain or distress are not used.*

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this project. Requests for extension must be filed prior to the expiration of the original approval.

Please Note:
1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. A Medical History Questionnaire accompanies this approval; please copy and distribute to all listed project staff who have not completed this form already. Completed questionnaires should be sent to Dr. Gladi Porsche, UNH Health Services.

If you have any questions, please contact me at 862-4629 or Julie Simpson at 862-2003.

For the IACUC,

Dean Elder, D.V.M.
Vice Chair

cc: File
Warner, Timothy

Appendix B

University *of* New Hampshire

Research Integrity Services, Service Building
51 College Road, Durham, NH 03824-3585
Fax: 603-862-3564

02-Jan-2013

Drugan, Robert C
Psychology, Conant Hall
Durham, NH 03824

IACUC #: 121102
Project: Optimal Anxiogenic Time Point Following Intermittent Swim Stress Determined by Social Exploration
Category: E
Approval Date: 13-Dec-2012

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category E on Page 5 of the Application for Review of Vertebrate Animal Use in Research or Instruction - *Animal use activities that involve accompanying pain or distress to the animals for which appropriate anesthetic, analgesic, tranquilizing drugs or other methods for relieving pain or distress are not used.*

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this project. Requests for extension must be filed prior to the expiration of the original approval.

Please Note:
1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. Information about the program, including forms, is available at http://unh.edu/research/occupational-health-program-animal-handlers.

If you have any questions, please contact either Dean Elder at 862-4629 or Julie Simpson at 862-2003.

For the IACUC,

Jill A. McGaughy, Ph.D.
Chair

cc: File
Warner, Timothy

Appendix C

University *of* New Hampshire

Research Integrity Services, Service Building
51 College Road, Durham, NH 03824-3585
Fax: 603-862-3564

14-Mar-2013

Drugan, Robert C
Psychology, Conant Hall
Durham, NH 03824

IACUC #: 130201
Project: Anxiogenic Effects of Intermittent Swim Stress at a 24h Time Point Determined by Social Exploration
Category: E
Approval Date: 21-Feb-2013

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category E on Page 5 of the Application for Review of Vertebrate Animal Use in Research or Instruction - *Animal use activities that involve accompanying pain or distress to the animals for which appropriate anesthetic, analgesic, tranquilizing drugs or other methods for relieving pain or distress are not used.*

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this project. Requests for extension must be filed prior to the expiration of the original approval.

Please Note:
1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. Information about the program, including forms, is available at http://unh.edu/research/occupational-health-program-animal-handlers.

If you have any questions, please contact either Dean Elder at 862-4629 or Julie Simpson at 862-2003.

For the IACUC,

Jill A. McGaughy, Ph.D.
Chair

cc: File
Warner, Timothy