The Role of Central 5-HT2C Receptors in Stress-Induced Anxiety and the Anxiolytic Effects of Exercise

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Abstract

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The Role of Central 5-HT2C Receptors in Stress-Induced Anxiety and the Anxiolytic Effects of Exercise
Thesis directed by Professor Monika Fleshner, Ph.D.

Stress-related mood disorders constitute some of the most common afflictions currently affecting public health. Despite past advances in clinical diagnostics, as well as the existence of pre-clinical animal models, new therapeutic discovery has been limited. Investigations into phenomena which reduce the incidence of stress-related psychiatric disorders, such as exercise, may reveal novel preventative and therapeutic options. While increasing evidence suggests a crucial role of the central serotonin (5-HT) system in the behavioral consequences of stress, a better understanding of the neurobiological substrates involved in the development and expression of anxiety and depression is necessary.

One relevant target in the etiology and treatment of stress-related mood disorders is the 5-HT_{2C} receptor (5-HT_{2C}R). The studies performed in this dissertation seek to elucidate the role of 5-HT_{2C}R in stress-induced anxiety, as well as the anxiolytic effects of exercise. In chapter 2, we delineate the role 5-HT_{2C}R in specific brain regions implicated in the expression of stress-induced anxiety behaviors. The results from these studies demonstrate that the involvement of 5-HT_{2C}R activation in stress-induced, anxiety-like behaviors is regionally specific.

In chapter 3, we identify the 5-HT_{2C}R a potential target for the anxiolytic effects of exercise. Physical activity has been associated with a reduction in the incidence and severity of psychiatric disorders such as anxiety. Similarly, voluntary wheel running can reduce anxiety-like behaviors in laboratory rats. The mechanisms underlying the anxiolytic properties of exercise, however, remain relatively unknown. We tested the hypothesis that voluntary wheel running prevents the behavioral consequences of 5-HT_{2C}R activation in the basolateral amygdala (BLA)

and dorsal striatum (DS). We found that voluntary wheel running reduced the behavioral effects produced by 5-HT_{2C}R activation and reduced levels of 5-HT_{2C}R mRNA in the both the BLA and the DS.

Overall, this dissertation extends previous work identifying the 5-HT_{2C}R as a relevant target in the treatment of stress-related anxiety disorders and implicates 5-HT_{2C}R in the anxiolytic effects of physical activity.

Table of Contents

List of Abbreviations

5-HT	Serotonin
$5\text{-HT}_{2C}\text{R}$	Serotonin 2C receptors
aCSF	Artificial Cerebrospinal Fluid
A/P	Anterior/Posterior
ANOVA	Analysis of Variance
BLA	Basolateral Amygdala
CeA	Central Amygdala
CNS	Central Nervous System
DA	Dopamine
DRN	Dorsal Raphe Nucleus
DS	Dorsal Striatum
DMS	Dorsal Medial Striatum
DLS	Dorsal Lateral Striatum
D/V	Dorsal/Ventral
FR-1	Fixed-ratio 1
FR-2	Fixed-ratio 2
GABA	Gamma-aminobutyric acid
HPLC	High pressure liquid chromatography
i.p.	Intraperitoneal
IP3	Inositol 1, 4, 5-triphosphate
mCPP	M-cholorphenylpiperazine
mPFC	Medial Prefrontal Cortex
M/L	Medial/Lateral
PLSD	Fisher's Protected Least Significant Difference
SEM	Standard Error of the Mean
SN	Substantia Nigra
SSRI	Selective Serotonin Reuptake Inhibitor

Figures and Diagrams

Chapter 1

Exercise, Stress, and 5-HT

The experiments included in this chapter
were conducted by P.V. Strong
in collaboration with J. Amat and S.F. Maier
under the supervision of M. Fleshner and B.N. Greenwood

Introduction

Stress-related mood disorders, including anxiety and depression, constitute some of the most common afflictions currently affecting American health. Despite past advances in clinical diagnostics, as well as the present existence of pre-clinical animal models to test the symptoms of these disorders, new therapeutic discovery has been limited. A better understanding of the neurobiological substrates involved in the development and expression of mood disorders may lead to improved therapeutic treatments. Additionally, investigations into phenomena which reduce the incidence of behaviors associated with stress-related mood disorders may reveal novel prophylactic and therapeutic manipulations.

Exposure to stressful life events has been identified as a primary casual factor in the development of mood disorders such as depression and anxiety (Kendler et al., 1999, van Praag, 2005). Symptoms of both anxiety and depression disorders include behaviors that can also be caused by exposure to stressful events, including social avoidance and exaggerated fear, as well as deficits in cognitive, learning, and memory processes. In rats, exposure to uncontrollable stress results in behaviors that resemble symptoms of human stress-related mood disorders (Maier and Watkins, 1998), such as reductions in social exploration, exaggerated conditioned fear, and interference with instrumental escape learning. Given the resemblance between the symptoms of human anxiety and depression and the stress-induced behaviors in rats, uncontrollable stress may be used to elucidate potential mechanisms underlying stress-related mood disorders.

The Role of the DRN and 5-HT in the Behavioral Consequences of Stress

Increasing evidence suggests a crucial role of the brain's serotonin (5-HT) system in the behavioral consequences of uncontrollable stress (Maier and Watkins, 2005). Consistent with the

idea that increases in 5-HT may be causal in disorders such as anxiety (Graeff et al., 1996), uncontrollable stress-induced behaviors are dependent upon hyperactivation (during stress) and sensitization (during later behavioral testing) of 5-HT neurons in a brainstem region rich in 5-HT neurons called the dorsal raphe nucleus (DRN). Briefly, uncontrollable stress hyperactivates 5-HT neurons in the DRN (Grahn et al., 1999). In turn, this hyperactivation triggers an exaggerated release of 5-HT within the DRN via local axon collaterals (Maswood et al., 1998, Amat et al., 2006) and may sensitize the neurons in the DRN through internalization of somatodendritic 5-HT_{1A} inhibitory autoreceptors (Riad et al., 2001, Riad et al., 2004). It is known that 5-HT_{1A} receptors in the DRN inhibit 5-HT neural activity and the release of 5-HT in DRN projection sites (Sprouse and Aghajanian, 1987, Bosker et al., 1997, Casanovas et al., 1997, Casanovas et al., 2000). Interestingly, these receptors are susceptible to presynaptic desensitization (Kennett et al., 1987, Rozeske et al., 2011a). Desensitization of the 5-HT_{1A} receptor in the DRN caused by uncontrollable stress removes presynaptic inhibitory input and may lead to 5-HT neuron sensitization and increased 5-HT activity (Petty et al., 1994, Amat et al., 1998a, b, Maswood et al., 1998). In fact, an exaggerated release of 5-HT in DRN projection sites is observed 24 hours after uncontrollable stress in response to moderate aversive stimuli such as behavioral testing procedures (Amat et al., 1998a, b, Bland et al., 2003, Christianson et al., 2010). While the specific mechanism by which DRN hyperactivation leads to later sensitization continues to be an important topic of investigation, we do know that both hyperactivation and sensitization of DRN 5-HT neurons are required for the development and expression, respectively, of the behavioral consequences of uncontrollable stress. Manipulations that block stress-induced 5-HT hyperactivation during stress or exaggerated 5-HT activity during behavioral testing 24 hours later also prevent or reverse the behavioral consequences of the stressor exposure (Maier et al.,

1993, Maier et al., 1994, Maier et al., 1995a, Maier et al., 1995b). Despite the knowledge that exaggerated 5-HT responses to relatively mild stimuli may underly the expression of many of the behavioral consequences of stressor exposure, treatment options for stress-related mood disorders are limited, and the relevant mechanisms to prevent these disorders remain elusive.

The Stress-Buffering Effects of Voluntary Exericse

Physical activity is one behavioral manipulation that can reduce the incidence of stress-related pyschiatric disorders. Human and animal literature indicate that exercise can constrain activation of the sympathetic nervous system in response to stress (Morimoto et al., 2000, Kramer et al., 2001, Kramer et al., 2002, Greenwood et al., 2003b, Beatty et al., 2005), attenuate mild stress-induced increases in stress hormones (Dishman et al., 1998, Droste et al., 2003), and reduce the incidence of stress-related disorders such as depression and anxiety (Dunn and Dishman, 1991, Martinsen and Morgan, 1997, Blumenthal et al., 1999, Fox, 1999, Biddle et al., 2000, Dunn et al., 2001, Salmon, 2001, Brosse et al., 2002, Suh et al., 2002, Blumenthal et al., 2007, Strohle, 2009, Strohle et al., 2009). In fact, the protective effect of exercise against depression and anxiety is equal to that of the protective effect of exercise against cardiovascular disease (Biddle et al., 2000).

Consistent with the stress-buffering effects of exercise in humans, rats allowed voluntary access to running wheels are protected against behavioral consequences of exposure to a variety of stressors. For example, wheel running elicits typical anxiolytic responses in the elevated plus maze, light / dark box (Binder et al., 2004), and acoustic and light-enhanced startle tests (Fox et al., 2008, Salam et al., 2008). Wheel running reduces stress-induced hyperthermia (Salam et al., 2008), blocks "behavioral despair" in the forced swim test (Solberg et al., 1999), and prevents the development of anhedonia produced by chronic mild stress (Solberg et al., 1999, Zheng et al.,

2006) or olfactory bulbectomy (Chambliss et al., 2004). Voluntary wheel running also prevents behaviors produced by uncontrollable stress (Greenwood et al., 2003a, Greenwood et al., 2005a, Rozeske et al., 2011b). In addition to *preventing* stress-induced behaviors, we have observed that wheel running can *reverse* long-lasting behavioral deficits (Greenwood et al., 2007). Finally, voluntary wheel running has been shown prevent the anxiogenic properties of acute SSRI administration in rats (Greenwood et al., 2008a), suggesting that exercise can protect against the anxiogenic effects of acute increases in 5-HT. Although the stress-buffering effects of exercise are clear, the underlying mechanisms remain unresolved. Identification of the specific mechanisms involved in the protective effects of exercise against stress-related mood disorders could reveal novel targets for treatment and prevention of those disorders.

Exercise, Stress Resistance, and Central Serotonergic Systems

Many adaptive neural changes produced by physical activity have been identified (Dishman et al., 2006, Ang and Gomez-Pinilla, 2007, Greenwood and Fleshner, 2011). It remains unclear, however, which of these adaptations are primarily responsible for the stress-buffering effects of exercise. Given that 5-HT has been widely implicated in stress-related mood disorders such as anxiety and depression (Graeff et al., 1996, Graeff et al., 1997, Anderson and Mortimore, 1999, Ninan, 1999, Graeff, 2004), it is plausible that changes in central 5-HT systems may be one of the links between exercise and its protective effects (Chaouloff, 1997, Greenwood et al., 2003a, Greenwood et al., 2005b). Wheel running may prevent the behavioral consequences of uncontrollable stress by producing plasticity at multiple sites within the central 5-HT system. The neuroplasticity produced by exercise could occur 1) in afferent regions which modulate DRN output such as the medial prefrontal cortex or the loceus coeruleus; 2) at the level of the

DRN itself via local changes in 5-HT neural activity; and/or 3) in DRN projection sites through changes in the expression or function of post-synaptic 5-HT receptors.

Our lab has long been interested in how plasticity within the DRN itself might mediate exercise-induced stress resistance. The protective effect of voluntary wheel running against the consequences of uncontrollable stress in rats could involve decreased DRN activation and 5-HT activity in response to stress, perhaps through an increase in 5-HT_{1A} inhibitory autoreceptor expression (Greenwood et al., 2003a, Greenwood et al., 2005a, Greenwood et al., 2005b) or a reduction in stress-induced internalization of $5HT_{1A}$ receptors. One potential mechanism by which wheel running may block the effects of uncontrollable stress could be through an increase in 5-HT_{1A} receptor mediated inhibition of 5-HT neurons in the DRN, thereby preventing hyperactivation and sensitization of the DRN and reducing exaggerated 5-HT release in DRN projection sites. Figure 1 illustrates how intra-DRN plasticity produced by 6 weeks of exercise may potentially contribute to exercise-induced stress resistance.

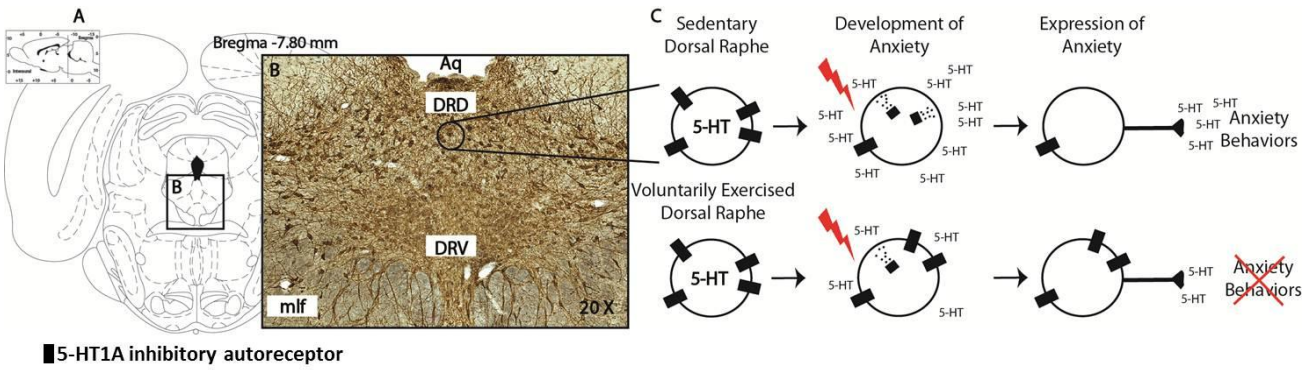

Figure 1. Intra-dorsal raphe nucleus (DRN) plasticity produced by 6 weeks of voluntary exercise may contribute to exercise-induced stress resistance. (A) Graphical reconstruction of the region of the DRN in the rat brain (adapted from Paxinos and Watson (Paxinos, 1998)). (B) Photomicrograph of a coronal section through the region of the rat DRN immunohistochemically labeled with an antibody against rat 5-hydroxytryptamine (5-HT). (C) Cartoon representation of the effect of uncontrollable stress on DRN 5-HT neurons in sedentary (top) and physically active (bottom) rats. Higher levels of 5-HT_{1A} mRNA in the DRN of physically active rats could lead to enhanced 5-HT_{1A} mediated autoinhibitory control over the activity of DRN 5-HT neurons, thereby preventing hyperactivation of the DRN during stressor exposure and limiting 5-HT release within the DRN. Constraint over the activation of DRN 5-HT neurons, or a simple increase in 5-HT_{1A} autoreceptor numbers, could limit potential 5-HT_{1A} internalization, thus preventing DRN sensitization after stress. Aq indicates cerebral aqueduct; DRD, dorsal aspect of the DRN; DRV, ventral aspect of the DRN; mlf, medial longitudinal fissure. (Figure and caption adapted from (Greenwood and Fleshner, 2011)).

The goal of the following preliminary experiment was to continue to explore the idea that exercise might prevent the stress-induced sensitization of 5-HT release in DRN projection sites upon exposure to subsequent mild stressors and thereby prevent the expression of anxiety-like behaviors.

Does Exercise Prevent Stress-Induced Sensitization of 5-HT in DRN Projection Sites?

We conducted a preliminary study to measure the potential effects of exercise on stress-induced sensitization of DRN 5-HT neurons using an in vivo microdialysis technique, whereby a probe was lowered into a specific brain region and extracellular levels of the 5-HT were quantified (Maswood et al., 1998, Amat et al., 2005). This data was obtained in collaboration with the laboratory of Dr. Steven Maier and under the direction of Dr. Jose Amat. These types of studies have previously indicated that during testing for the affomentioned behavioral consequences of uncontrollable stress, there is an exaggerated release of 5-HT in DRN projection

sites, including the basolateral amygdala (BLA) (Amat et al., 1998a, Christianson et al., 2010) and the dorsal striatum (Strong et al., 2011). The dorsal striatum (DS), a critical region for instrumental learning (Balleine et al., 2009), and the BLA, a key structure in fear and anxiety behaviors (LeDoux, 2003, Shekhar et al., 2005), stand out as likely candidates for being the proximal mediators of some stress-induced behaviors. For example, 5-HT activity in the BLA can produce social avoidance (Christianson et al., 2010) and increase fear (Campbell and Merchant, 2003, Strong et al., 2011); whereas 5-HT activity in the DS can interfere with instrumental learning (Mitchell et al., 2007, Tanaka et al., 2009, Strong et al., 2011). Thus, exaggerated 5-HT release seems to be a critical aspect of the mechanism by which uncontrollable stressors produce negative behavioral consequences.

We have previously investigated the effects of voluntary wheel running on the 5-HT system and the 5-HT system's response to uncontrollable stress. Interstingly, wheel running has the greatest stress-buffering effect on 5-HT neurons in the rostral most aspect of the DRN (Greenwood et al., 2003a, Greenwood et al., 2005b). This region contains 5-HT neurons which project to the BLA and the DS (Imai et al., 1986a, Imai et al., 1986b, Lowry, 2002, Lowry et al., 2005, Waselus et al., 2006). Given that hyperactivation of 5-HT neurons typically leads to sensitization of those same neurons, it is possible that wheel running prevents the sensitization of DRN 5-HT neurons that project to the BLA and the DS thereby preventing the exaggerated 5-HT release in the BLA and/or the DS and, ultimately, preventing the expression of stress-induced anxiety-like behaviors.

We hypothesized that wheel running would prevent the sensitization of extracellular 5-HT in the dorsal striatum (in response to mild stress) normally elicited by prior uncontrollable stressor exposure. Rats were divided into the following groups (Sedentary, No Stress, n=3; Exercise, No

Stress, n=6; Sedentary, Stress, n=2; Exercise, Stress, n=6). Following 4 weeks of voluntary wheel running or sedentary conditions, guide cannulae were implanted into the DS (A/P: -0.3mm; M/L: -4.2mm; D/V: -3.6mm from Bregma). After 2 weeks of recovery in their home cages, rats were exposed to uncontrollable stress or no stress conditions. Immediately after stress, rats were placed into microdialysis bowls and microdialysis probes were inserted into the guide, and artificial cerebrospinal fluid (aCSF) was perfused through the probes. Rats were left undisturbed in the microdialysis bowls overnight with access to food and water. Sample collection began the next morning (24 hours after stress) at 20 minute intervals (8 total samples). At the beginning of the 5^{th} sample each rat received 2, 5 second foot shocks (0.8mA) separated by 1 minute. 5-HT dialysates were analyzed with high-pressure liquid chromatography (HPLC) using methods previously described (Maswood et al., 1998, Amat et al., 2005).

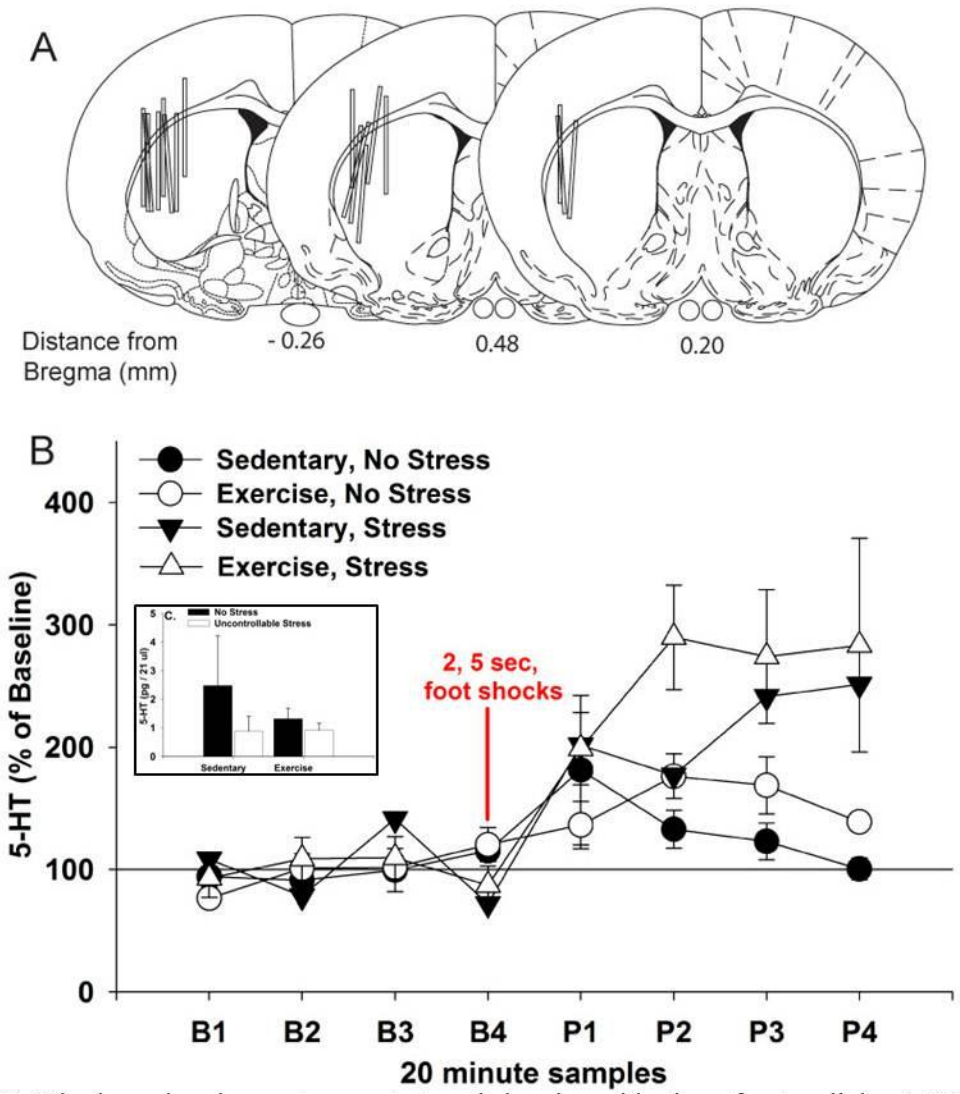

Figure 2. Wheel running does not prevent stress-induced sensitization of extracellular 5-HT in the dorsal lateral striatum (DLS). A. Graphic reconstruction of 3-mm dialysis probe placements within the striatum. B. Mean extracellular 5-HT in the striatum before and after 2 foot shocks. C. (inset) Basal 5-HT release in the dorsal lateral striatum. Rats were exposed to 6 weeks of voluntary wheel running or sedentary condition before receiving either uncontrollable stress or home cage control treatment. 5-HT is expressed as a percentage of the average of four baseline samples (B1 – B4). The 2, 5 sec, foot shocks were delivered immediately after the 4th baseline sample (B4) and dialysis continued for 4 samples after shock (P1 – P4). Illustrations were adapted from Paxinos and Watson and are listed in relationship to bregma in mm.

Consistent with prior reports (Amat et al., 1998a, Strong et al., 2011), we observed that in sedentary rats exposed to uncontrollable stress, 2 foot shocks elicited an exaggerated extracellular 5-HT response (Figure 2B). However, 6 weeks of prior wheel running failed to

prevent the sensitization of the extracellular 5-HT response in the DLS (Figure 2B). Additionally, there was no difference in basal 5-HT release in the DS (Figure 2C). Along with potential changes in areas upstream of the DRN, the stress-protective effects of exercise may rely upon changes in post-synaptic 5-HT receptor sensitivity or expression in the DRN projection sites. One post-synaptic target relevant to the etiology and treatment of stress-related mood disorders is the 5-HT_{2C} receptor (5-HT_{2C}R).

The 5-HT_{2C} Receptor

Presently there are seven known types of serotonin receptors with several distinctly identified subtypes (Hoyer et al., 1994, Barnes and Sharp, 1999, Hoyer et al., 2002). The structurally homologous 5-HT_2 family of receptors (5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C}) may represent a viable therapeutic option in a variety of central nervous system (CNS) disorders. The 5-HT_{2C}R is a trans-membrane, G protein-coupled receptor (Julius et al., 1988) first identified in the choroid plexus (Pazos et al., 1984). Ligand binding to the 5-HT_{2C}R initiates multiple second messenger cascades, including the inositol 1,4,5-triphosphate (IP3) pathway that leads to an increased release of intracellular calcium and strong modulation of multiple cellular responses (Conn and Sanders-Bush, 1986). The 5-HT_{2C}R is the only known G protein-coupled receptor to experience mRNA editing in order to produce multiple isoforms with varying levels of G-protein interactions (Burns et al., 1997). In fact, selective post-transcriptional editing of 5-HT_{2C}R mRNA may contribute to mood dysregulation, e.g. human depression and anxiety, through a decreased efficacy in G-protein interactions (Gurevich et al., 2002).

The 5-HT_{2C}R is distributed throughout the human and rat CNS (Molineaux et al., 1989, Pompeiano et al., 1994, Abramowski et al., 1995, Wright et al., 1995, Sharma et al., 1997, Pasqualetti et al., 1999, Clemett et al., 2000, Lopez-Gimenez et al., 2002, Li et al., 2003),

including areas implicated as mediators of stress-induced behavior such as the medial prefrontal cortex (mPFC), amygdala, dorsal striatum, periaqueductal gray and the DRN. The 5-HT_{2C}R is primarily located on GABAergic inhibitory interneurons (Huidobro-Toro et al., 1996, Serrats et al., 2005) and can modulate 5-HT (Boothman et al., 2006), (De Deurwaerdere et al., 2004), dopamine (Navailles and De Deurwaerdere, 2011) and hypothalamic-pituitary-adrenal (HPA) axis (Heisler et al., 2007a) activity.

The 5-HT_{2C}R has recently emerged as a relevant target in the treatment of mood disorders (Millan, 2005). Consistent with data implicating 5-HT_{2C}R activity in stress-related behaviors in humans (Murphy et al., 1989, Gatch, 2003, Millan et al., 2005, Olie and Kasper, 2007, Pjrek et al., 2007, Van Veen et al., 2007, Stein et al., 2008) and rodents (Bagdy et al., 2001, Burghardt et al., 2007, Heisler et al., 2007b, Greenwood et al., 2008b), we have observed that activation of the 5-HT_{2C}R with systemic administration of the 5-HT_{2C}R agonist CP809101 is sufficient to interfere with instrumental-escape learning and increase shock-elicited freezing (Strong et al., 2009). Moreover, systemic blockade of the 5-HT_{2C}R removes the deficit in escape learning (Strong et al., 2009) and social avoidance (Christianson et al., 2010) produced by prior uncontrollable stress. These data suggest that activation of 5-HT_{2C}R in specific projection sites of the DRN contribute to the expression of the behavioral consequences of uncontrollable stress. Christianson et al. (2010) recently demonstrated that 5-HT_{2C}R activation in the basolateral amygdala (BLA) is necessary and sufficient for the social avoidance produced by uncontrollable stress. Additionally, we have implicated 5-HT_{2C}R activation in the dorsal striatum in the expression of stress-induced interference with instrumental learning (Strong et al., 2011).

For the remainder of this dissertation, we will focus on the potential role of post-synaptic 5-HT_{2C}R. Specifically, we tested the hypothesis that 5-HT_{2C}R activation in specific brain regions,

the BLA and the DS, is necessary for anxiety-like behaviors produced by uncontrollable stress. We utilized site-specific administration of selective 5-HT_{2C}R antagonist and agonist compounds to investigate the involvement of 5-HT_{2C}R activation in discrete brain regions, the DS and the BLA, in deficits in shuttle box escape learning and exaggerated shock-elicited fear produced by uncontrollable stress. Additionally, we hypothesized that voluntary wheel running would prevent the behavioral consequences of 5-HT_{2C}R activation in the BLA and DS, specifically increased shock-elicited fear and interference with shuttle box escape learning. Overall, this dissertation extends previous work identifying the 5-HT_{2C}R as a relevant target in the treatment of stress-related anxiety disorders and implicates 5-HT_{2C}R in the anxiolytic effects of physical activity.

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Chapter 2

5-hydroxytrptamine 2C receptors in the dorsal striatum mediate stress-induced interference with negatively-reinforced instrumental escape behavior

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Abstract - Uncontrollable stress can interfere with instrumental learning and induce anxiety in humans and rodents. While evidence supports a role for serotonin (5-HT) and serotonin 2C receptors (5-HT_{2C}R) in the behavioral consequences of uncontrollable stress, the specific sites of action are unknown. These experiments sought to delineate the role of 5-HT and 5-HT_{2C}R in the dorsal striatum (DS) and the lateral/basolateral amygdala (BLA) in the expression of stress-induced instrumental escape deficits and exaggerated fear, as these structures are critical to instrumental learning and fear behaviors. Using in vivo microdialysis, we first demonstrate that prior uncontrollable, but not controllable, stress sensitizes extracellular 5-HT in the dorsal striatum, a result that parallels prior work in the BLA. Additionally, rats were implanted with bi-lateral cannula in either the DS or the BLA and exposed to uncontrollable tail shock stress. One day later, rats were with injected 5-HT_{2C}R antagonist (SB242084) and fear and instrumental learning behaviors were assessed in a shuttle box. Separately, groups of non-stressed rats received an intra-DS or an intra-BLA injection of the 5-HT_{2C}R agonist (CP809101) and behavior was observed. Intra-DS injections of the 5-HT_{2C}R antagonist prior to fear/escape tests completely blocked the stress-induced interference with instrumental escape learning; a partial block was observed when injections were in the BLA. Antagonist administration in either region did not influence stress-induced fear behavior. In the absence of prior stress, intra-DS administration of the 5-HT_{2C}R agonist was sufficient to interfere with escape behavior without enhancing fear, while intra-BLA administration of the 5-HT_{2C}R agonist increased fear behavior but had no effect on escape learning. Results reveal a novel role of the 5-HT_{2C}R in the DS in the expression of instrumental escape deficits produced by uncontrollable stress and demonstrate that the involvement of 5-HT_{2C}R activation in stress-induced behaviors is regionally specific.

Keywords: Uncontrollable stress, serotonin, anxiety, amygdala, learned helplessness, instrumental learning

Introduction

Despite the prevalence of stress-related psychiatric disorders such as depression and anxiety, available therapeutic options have limited success (Turner et al., 2008, Turner and Rosenthal, 2008, Markou et al., 2009) and little is known about how stress leads to the expression of these disorders. One factor contributing to the behavioral repercussions of exposure to a traumatic event is the nature of the experience, i.e. controllable stressors have less of an impact than uncontrollable stressors (Maier, 1976, Shapiro et al., 1996, Crombez et al., 2008). In fact, lack of perceived behavioral control over a stressful event may be critical to the development of stress-related disorders (Morgan et al., 2001). Exposure to an uncontrollable stressor not only alters behavior at the time of the stressful experience but also alters responding to subsequent aversive stimuli. Patients exhibiting symptoms of stress-related psychiatric disorders report difficulty engaging in goal-directed/instrumental behavior (Tull et al., 2007), have enhanced behavioral and neural fear responses (Rauch et al., 2000) and display exaggerated fear in response to a mild aversive stimuli (Jovanovic et al., 2009). Similarly, rats exposed to an uncontrollable stressor later have difficulty learning to escape from a simple instrumental shuttle box escape task (Maier, 1973, Jackson et al., 1980) and display reduced social exploration (Christianson et al., 2008) and exaggerated fear conditioning (Maier, 1990, Rau et al., 2005). Understanding the neurobiological mechanisms involved in the expression of uncontrollable stress-induced behavioral impairments is vital to the development of more effective therapeutic treatments of stress-related psychiatric disorders.

Serotonin (5-HT) has long been implicated in depression and anxiety (Graeff et al., 1996, Anderson and Mortimore, 1999, Blier and de Montigny, 1999, Ninan, 1999, Blier, 2001), and evidence supports a crucial role for 5-HT and the 5-HT_{2C} receptor (5-HT_{2C}R) in the development

and expression of the behavioral consequences of uncontrollable stress. Uncontrollable, relative to controllable, stress intensely activates 5-HT neurons in the dorsal raphe nucleus (DRN) (Grahn et al., 1999, Greenwood et al., 2003a, Amat et al., 2005, Takase et al., 2005). As a result of this hyperactivation, the DRN is left in a 'sensitized' state so that exposure to a subsequent aversive stimulus elicits a larger 5-HT response, both within the DRN (Maswood et al., 1998, Amat et al., 2005) and at DRN projection sites (Amat et al., 1998a, b, Christianson et al., 2010). DRN sensitization is critical for the expression of stress-induced behaviors because manipulations that increase DRN activity (Maier et al., 1995a) or rapidly increase 5-HT (Greenwood and Fleshner, 2008, Greenwood et al., 2008) mimic the instrumental-escape deficit and exaggerated fear produced by uncontrollable stress. Additionally, inhibition of the DRN abolishes the behavioral consequences of prior exposure to uncontrollable stress. (Maier et al., 1995c, Christianson et al., 2010).

The 5-HT_{2C}R has recently emerged as a relevant target in the treatment of neuropsychiatric disorders (Millan, 2005). Consistent with data implicating 5-HT_{2C}R activity in stress-related behaviors in humans (Murphy et al., 1989, Gatch, 2003, Millan et al., 2005, Olie and Kasper, 2007, Pjrek et al., 2007, Van Veen et al., 2007, Stein et al., 2008) and rodents (Bagdy et al., 2001, Burghardt et al., 2007, Heisler et al., 2007, Greenwood et al., 2008), we have observed that activation of the 5-HT_{2C}R with systemic administration of the 5-HT_{2C}R agonist CP809101 is sufficient to interfere with instrumental-escape learning and increase shock-elicited freezing (Strong et al., 2009). Moreover, systemic blockade of the 5-HT_{2C}R removes the deficit in escape learning (Strong et al., 2009) and social avoidance (Christianson et al., 2010) produced by prior uncontrollable stress. These data suggest that activation of 5-HT_{2C}R in specific projection sites of the DRN contribute to the expression of the behavioral consequences of

uncontrollable stress. Indeed, Christianson et al. (2010) recently demonstrated that 5-HT_{2C}R activation in the basolateral amygdala (BLA) is necessary and sufficient for the social avoidance produced by uncontrollable stress. The specific brain regions in which 5-HT_{2C}R activation contributes to the instrumental escape deficit and exaggerated fear produced by uncontrollable stress, however, remain elusive.

The dorsal striatum (DS), a critical region for instrumental learning (Balleine et al., 2009), and the BLA, a key structure in fear and anxiety behaviors (LeDoux, 2003, Shekhar et al., 2005), stand out as likely candidates for being the proximal mediators of the stress-induced instrumental-escape deficit and exaggerated fear. The DRN projects to both the DS (Yin et al., 2005, Corbit and Janak, 2010) and the BLA (Kim and Jung, 2006), where 5-HT can interfere with instrumental behaviors (Mitchell et al., 2007, Tanaka et al., 2009) and enhance fear (Campbell and Merchant, 2003), respectively. Twenty four hours after uncontrollable, but not equal controllable, stress, 2 brief foot shocks elicit a large increase in extracellular 5-HT in the BLA (Amat et al., 1998a). The current studies investigate whether a similar sensitized 5-HT release occurs in the striatum. Additionally, we utilized site-specific administration of selective 5-HT_{2C}R antagonist and agonist compounds to investigate the involvement of 5-HT_{2C}R activation in discrete brain regions, the DS and the BLA, in the deficit in instrumental escape learning and exaggerated fear produced by uncontrollable stress.

Experimental Procedures

Animals

Adult, male Fischer 344 rats (250-300g; N=161) were used in all behavioral experiments based on prior work optimizing stress-induced behaviors in this strain (Greenwood et al., 2003a, Greenwood et al., 2007, Strong et al., 2009). Similarly, adult, male Sprague-Dawley rats

weighing 250-300g (N=21) were used in microdialysis experiments based on our prior work optimizing the procedure in this strain (Amat et al., 1998a, b, Amat et al., 2005, Christianson et al., 2010). In accordance with our prior work on the role of 5-HT_{2C}R in stress induced behaviors (Greenwood et al., 2008, Strong et al., 2009), all rats were individually housed in Nalgene Plexiglas cages (45 x 25.2 x 14.7 cm) at a temperature of 22^{o} C. Lights were maintained on a 12:12 hour light/dark cycle. Animals had *ad libitum* access to food and water. All experimental protocols were approved by the University of Colorado Animal Care and Use Committee.

Stress Protocols

Controllable/Yoked-Uncontrollable Stress

The controllable/yoked-uncontrollable stress procedure was used in the microdialysis experiment and followed protocols known to produce differential effects of controllable vs. uncontrollable stress on brain and behavior (Amat et al., 1998a, Christianson et al., 2008, Christianson et al., 2010). Briefly, copper electrodes with electrode paste were wrapped around the tail and shocks were administered by a Precision Regulated Animal Shocker (Coulbourn Instruments, Allentown, PA). Rats were restrained in 14 x 11 x 17 cm (length by width by height) acrylic boxes with a wheel 7 cm wide and 9.5 cm in diameter located on the wall opposite the tail (Maier, 1990). Tail shocks were presented on a variable interval-60 second schedule (VI-60). Turning the wheel at the front of the chamber terminated each tail shock after a ¼ turn of the wheel. If the response was performed within 5 seconds of shock onset the response requirement doubled for the next trial until a maximum of 4 full wheel-turns was reached. If the response was made after 5 seconds but before 20 seconds the requirement was reduced by half and if no response was made by 30 seconds the shock terminated and the requirement was reset to ¼ turn. This procedure was used to ensure that the rat learned the

correct response rather than reflexively turning the wheel. In order to maintain escape behavior the shock intensity was 1.0 mA for the first 33 trials, 1.3 mA for the following 33 trials and 1.6 mA for the remaining 34 trials (Amat et al., 2005). A second rat, in the uncontrollable stress condition, was yoked to each controllably stressed rat, but had no control over shock termination and received exactly equal duration tail shock. Animals in the home cage control group remained in their cages. Rats were returned to their home cages following the termination of shock. The entire stress procedure lasted 2 hours and occurred from 0800-1000, 24 hours prior to microdialysis.

Uncontrollable Stress

In the intra-DS and intra-BLA microinjection experiments, only uncontrollable stress was administered. This uncontrollable stress procedure produces exaggerated shock-elicited freezing and interference with shuttle escape behavior that is indistinguishable from that produced by the yoked stress treatment. Briefly, 100, 5 second tail shocks were presented on a VI-60 schedule while the rats were restrained in acrylic tubes (23.4 cm length X 7 cm diameter). Shock intensity began at 1.0mA and was increased to 1.5mA after 1 hour. The entire shock session lasted for 1 hour and 50 minutes. Five second shocks were used because the controllable stress treatment maintains shocks at an average duration of 5 seconds (data not shown). Stress was performed between 0700 and 0900. Rats were returned to their home cages after stress.

In vivo microdialysis and quantification of striatal 5-HT

A uni-lateral dialysis cannula guide was implanted into the striatum (tip of guide targeted at +0.5 A/P, ±_3.0 M/L, and -4.6 D/V) (Atallah et al., 2007, Corbit and Janak, 2010) under inhaled isoflourane anesthesia (3% in O^2). A stylet was placed in the cannula to maintain patency. After the recovery period, rats were assigned to controllable stress, yoked-

uncontrollable stress or home cage treatment. Six hours after stress, rats were placed into microdialysis bowls and microdialysis probes (CMA 12, MW cut-off 20kD, 3mm) were inserted into the guide. Artificial cerebrospinal fluid (aCSF) was perfused through the probes using a CMA infusion pump at a flow rate of 0.2 μl/minute. Rats were left alone in the dialysis room with food and water for at least 15 hours on a light cycle that matched the vivarium. After 15 hours, the flow rate increased to 1μl/minute for a 90 minute equilibration period, at which time 8 samples were then collected at 30 minute intervals. Sample collection began 24 hours after the beginning of stress. Immediately prior to the onset of the 4th baseline sampling period rats were gently moved onto a grid floor surrounded by Plexi-glass walls located adjacent to the microdialysis bowl. The grid floor is identical to the grid floor of the shuttle box used in behavioral testing experiments. No change in extracellular 5-HT was observed during the 30 minute sampling period after the rats were moved from the dialysis bowls onto the grid floor (sample 4). At the onset of the 5th sampling period each rat received 2, 5 second foot shocks separated by 1 minute. This foot shock procedure is identical to the beginning of the fear/instrumental learning test. The rats remained on the grid floor for the remainder of the experiment as they would normally do during the shuttle box behavior test. At the beginning of the 5th sample each rat received 2, 5 second foot shocks (0.8mA) separated by 1 minute. This procedure is identical to the beginning of the fear/instrumental learning test described below. Here we sought to determine whether as a result of a sensitized DRN, rats in the uncontrollable stress group would exhibit greater extracellular 5-HT in the DS in response to foot shock than controllably stressed or non-stressed rats.

5-HT Quantification

Dialysates were immediately placed in an -80°C freezer until analysis. Samples were analyzed with high-pressure liquid chromatography (HPLC) using methods previously described (Amat et al., 2005).

Surgery

Under ketamine (0.75 mg/kg i.p.) and medetomidine (0.5 mg/kg i.p.) anesthesia, bilateral cannula (26 gauge, Plastics One, Roanoke, VA) were implanted as previously described (Greenwood et al., 2007). Cannula were aimed at either the DS: +0.5 A/P, ± 3.0 M/L, and -4.6 D/V from Bregma (Atallah et al., 2007) or the BLA: -3.0 A/P, ± 4.8 M/L, -6.2 D/V from Bregma (Christianson et al., 2010), based on the atlas by Paxinos and Watson (Paxinos, 1998). Atipamezole (0.5 mg/kg i.p.) was administered following surgery to reverse the effects of medetomidine. All rats were inoculated with .25 mL/kg (subcutaneous) penicillin (Combi-Pen, Agrilabs, St. Joseph, Missouri) immediately following surgery and allowed 7-10 days of recovery before any other manipulation occurred. Following experiment completion brains were sliced at 40 μm and stained with Cresyl Violet for cannula placement verification.

Drug Microinjections

Rats were subjected to either uncontrollable stress or home cage conditions 24 hours prior to drug microinjections. On the day of behavioral testing, a microinjector extending 0.5mm (intra-DS) or 1.0mm (intra-BLA) beyond the tip of the guide cannula was inserted. Drug doses and injection volumes were based on prior work examining the effects of these compounds on stress-induced behaviors (Strong et al., 2009, Christianson et al., 2010). The selective 5-HT_{2C}R antagonist SB242084 (Tocris Bioscience, Ellisvile, Missouri) was dissolved in 0.9 % sterile and sonicated to achieve a concentration of 50mM. SB242084 was administered intra-DS at a

volume of 1.0 μL/side and intra-BLA at a volume of 0.5 μL/side (Strong et al., 2009, Christianson et al., 2010). The selective 5-HT_{2C}R agonist CP-809191 (Tocris Bioscience) was dissolved in 0.9 % sterile saline and gently warmed at 45 ^{o} C for 10-15 minutes in a water bath to achieve at a concentration of 6mM and was administered intra- DS at a volume of 1.0 μL/side or intra-BLA at a volume of 0.5 μL/side (Strong et al., 2009, Christianson et al., 2010). Microinjections were made 15 minutes prior to behavioral testing. In the experiment involving pretreatment of the antagonist in the DS, SB242084 was administered 5 minutes prior to CP809101. In these experiments, pretreatment with SB242084 in the DS blocked the effects of the agonist, CP809101 (Fig. 3D/3E); therefore, a SB242084 pretreated group was not included in the intra-BLA agonist study. Additionally, prior work by our lab has shown that the behavioral effects of these compounds also are selective when injected systemically (Strong et al., 2009).

Behavioral Testing

Fear and instrumental learning were assessed sequentially in shuttle boxes (50.8cm x 25.4cm x 30.48 cm, Coulbourn Instruments, Whitehall, PA) using procedures previously described (Maier, 1990, Greenwood et al., 2003a, Takase et al., 2005, Greenwood et al., 2008, Strong et al., 2009). At the beginning of each session, rats were placed into shuttle boxes and allowed to explore for 5 minutes. During this 5 minute period (pre-shock period), fear behavior was assessed using a sampling procedure in which each rat was scored every 10 seconds as either freezing, defined as an absence of all movement except for that required for respiration, or not freezing. Rats then received two 0.8 mA foot shocks delivered through both sides of the grid floor. Foot shocks were terminated when the rat fully crossed over to the opposite side of the shuttle box (fixed ratio 1, FR-1). The latencies to cross were recorded (FR-1 latencies). Following the second FR-1 trial, shock-elicited freezing was observed for 20 minutes. Shock-

elicited freezing is a measure of fear conditioned to cues present in the shuttle box (Fanselow and Lester, 1988).

The post shock freezing period was followed by 25 fixed-ratio 2 (FR-2) escape trials. During FR-2 trials, rats were required to cross through the shuttle box door twice in order to terminate the foot shock (0.8 mA). An escape latency of 30 seconds was assigned if a correct escape response did not occur within 30 seconds, at which time the shock was terminated. Shocks occurred with an average inter-trial interval of 60 seconds and a single test session lasted approximately 1 hour. All behavioral tests occurred between 0900 and 1200 by an experimenter blind to treatment condition of the animals.

Statistical Analysis

Data were analyzed with analysis of variance (ANOVA) with stress and drug conditions treated as between-subjects factors and dialysis sample, fear or escape trial blocks as repeated measures. Extracellular 5-HT was converted to percentage of baseline by dividing each sample by the average of the first 4 samples X 100. Pre-shock freezing scores were averaged into 1 pre-shock score and shock-elicited freezing into 10, 2 min blocks. The two FR-1 trials were averaged into a single FR-1 latency score. FR-2 escape latencies were averaged into 5 blocks of 5 trials each. Average post-shock freezing and average FR-2 escape latencies were analyzed with ANOVAs that included an additional group of off-site controls when appropriate. Significant main effects and interactions were followed by Fisher's protected least significant difference (PLSD) post hoc analysis. Results were considered significant when $p<0.05$.

Results

Prior stressor controllability modulates extracellular 5-HT in the striatum in response to a subsequent stress challenge.

Rats were exposed to no stress, controllable stress or uncontrollable stress treatment and 24 hours later striatal extracellular 5-HT concentrations were determined by microdialysis and HPLC. Microdialysis probe placement is shown in Figure 1A. All rats in the controllable stress group learned to escape the tail shock and quickly reached the maximum response criterion and maintained escape behavior as observed previously (Amat et al., 2005). Twenty four hours later, 2 brief foot shocks were delivered after 4 baseline samples and dialysis continued for 4 samples after shock. Only rats with dialysis probes within the striatum were included; the resulting sample sizes were Controllable Stress, n = 8; Uncontrollable Stress, n = 7; No Stress, n = 6. Extracellular 5-HT was converted to percentage of baseline by dividing each sample by the average of the first 4 samples X 100 (Fig. 1B). As in prior studies (Christianson et al., 2010) stress did not influence basal 5-HT levels (data not shown). A large, but transient increase in extracellular 5-HT was found in response to foot shock in rats treated with prior uncontrollable stress, but not with controllable stress or home cage control groups. A repeated measures ANOVA revealed a significant effect of Stress, ($F(2, 18) = 4.183$; $p = 0.032$), Sample, ($F(7, 126) = 4.834$, $p < 0.001$), and the Stress by Sample interaction, ($F(14, 126) = 2.118$; $p = 0.015$). During the foot shock sample, there was significantly greater extracellular 5-HT in the DS of rats treated with prior uncontrollable stress compared to rats treated with prior controllable stress or home cage treatments (Sample P1; $p < 0.01$). Groups did not differ from one another during any other sample time. Within group comparisons between baseline and post-shock samples revealed a significant elevation in 5-HT from baseline only in the uncontrollable stress group; the extracellular 5-HT during the shock sample was significantly greater that the baseline samples

(Sample P1; $p < 0.05$). No other between-groups or within-groups comparisons reached significance; controllable stress and home cage control groups did not differ from each other or their baselines at any time.

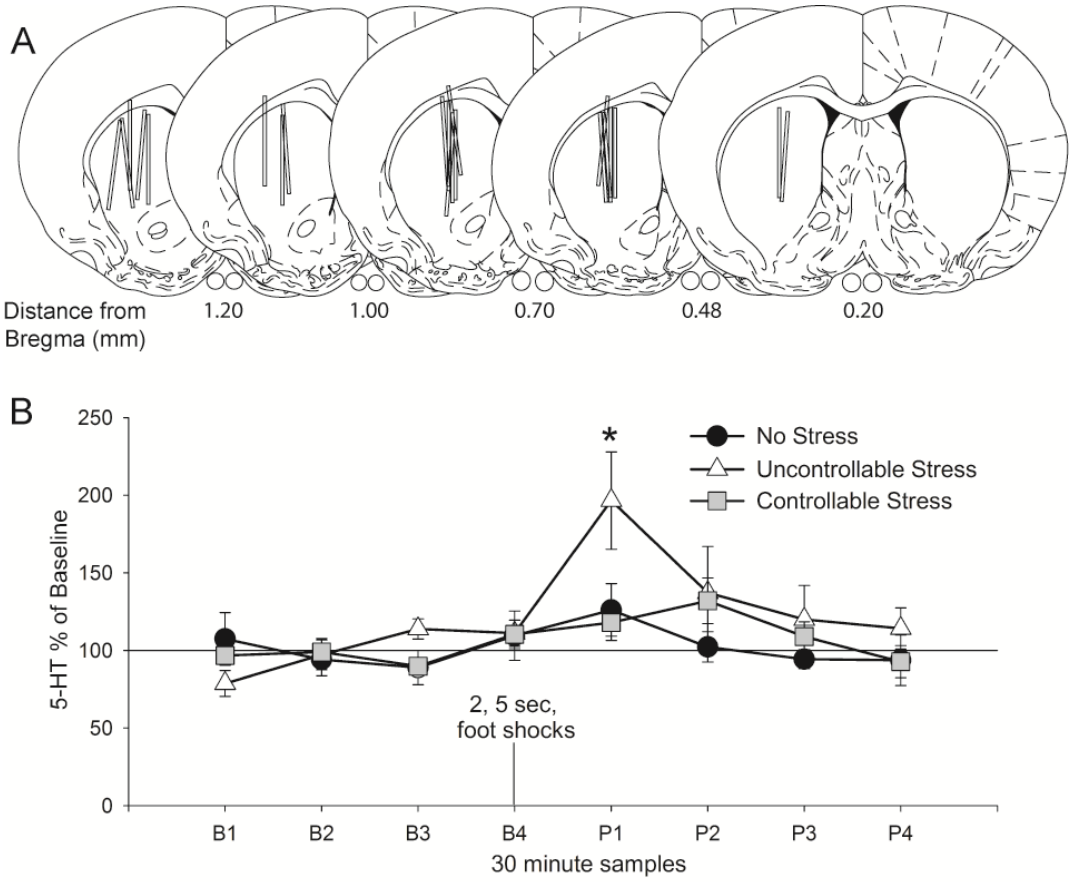

Figure 1. Prior stressor controllability modulates extracellular 5-HT in the striatum in response to a subsequent stress challenge. **(A)** Graphic reconstruction of 3-mm dialysis probe placements withing the striatum. **(B)** Mean extracellular 5-HT in the striatum before and after 2 footshocks. Rats received prior controllable stress, uncontrollable stress or homecage control treatment. 5-HT is expressed as a percentage of the average of four baseline samples (B1 – B4). The 2, 5 sec, footshocks were delivered immediately after the 4th baseline sample (B4) and dialysis continued for 4 samples after shock (P1 – P4). Data represent group means $\pm$ SEM. * $p<0.05$ relative to all other groups. Illustrations were adapted from Paxinos and Watson and are listed in relationship to bregma in mm.

Expression of the stress-induced escape deficit, but not exaggerated fear, is dependent upon 5-HT_{2C}R activation in the DS

The 5-HT_{2C}R antagonist SB242084 was injected into the DS and shock-elicited freezing (Fig. 2B/2C) and shuttle box escape (Fig. 2D/2E) behaviors were observed 15 minutes later. After exclusion of rats with misplaced cannula (n=10), group sizes were No Stress/Saline, n=7; No Stress/SB242084, n= 7; Uncontrollable Stress/Saline, n=7; Uncontrollable Stress/SB242084, n=15. Off-site controls were not included in this analysis because only one animal from the Uncontrollable Stress/SB242084 group had misplaced cannula. Cannula placements are shown in Figure 2A. Pre-shock freezing was minimal and did not differ between groups (Fig. 2B, pre-shock). Uncontrollable stress produced exaggerated shock-elicited freezing, regardless of intra-DS treatment with SB242084 (Fig. 2B). A significant main effect of uncontrollable stress on post-shock freezing ($F(1, 32) = 37.068$; $p < .0001$) was found, but neither the main effect of drug nor the interaction between stress and drug reached significance. Average post-shock freezing scores are shown in Figure 2C.

While no differences in FR-1 escape latency were observed across all groups and treatments (Fig. 2D, FR-1), uncontrollable stress did interfere with FR-2 escape learning behavior and this effect of uncontrollable stress was blocked by intra-DS administration of the 5-HT_{2C}R antagonist SB242084 (Fig. 2D). Reliable main effects of stress ($F(1, 32) = 29.310$; $p < .0001$), drug ($F(1, 32) = 10.066$; $p = .0033$), and time ($F(4, 128) = 3.638$; $p = .0077$) were found. Two-way interactions between drug and stress ($F(1, 32) = 9.907$; $p = .0036$), time and drug ($F(4, 128) = 5.532$; $p = .0004$), time and stress ($F(4, 128) = 2.614$; $p = .0383$), and the three-way interaction between time, drug and stress ($F(4, 128) = 5.322$; $p = .0005$) were all significant. Post hoc analysis revealed that during the first block of FR-2 trials, both stressed groups had significantly slower escape latencies compared to non-stressed groups. The stressed-

saline group differed from all other groups for the remainder of the trials. No other group differences were significant. Average FR-2 latency scores are displayed in Figure 2E.

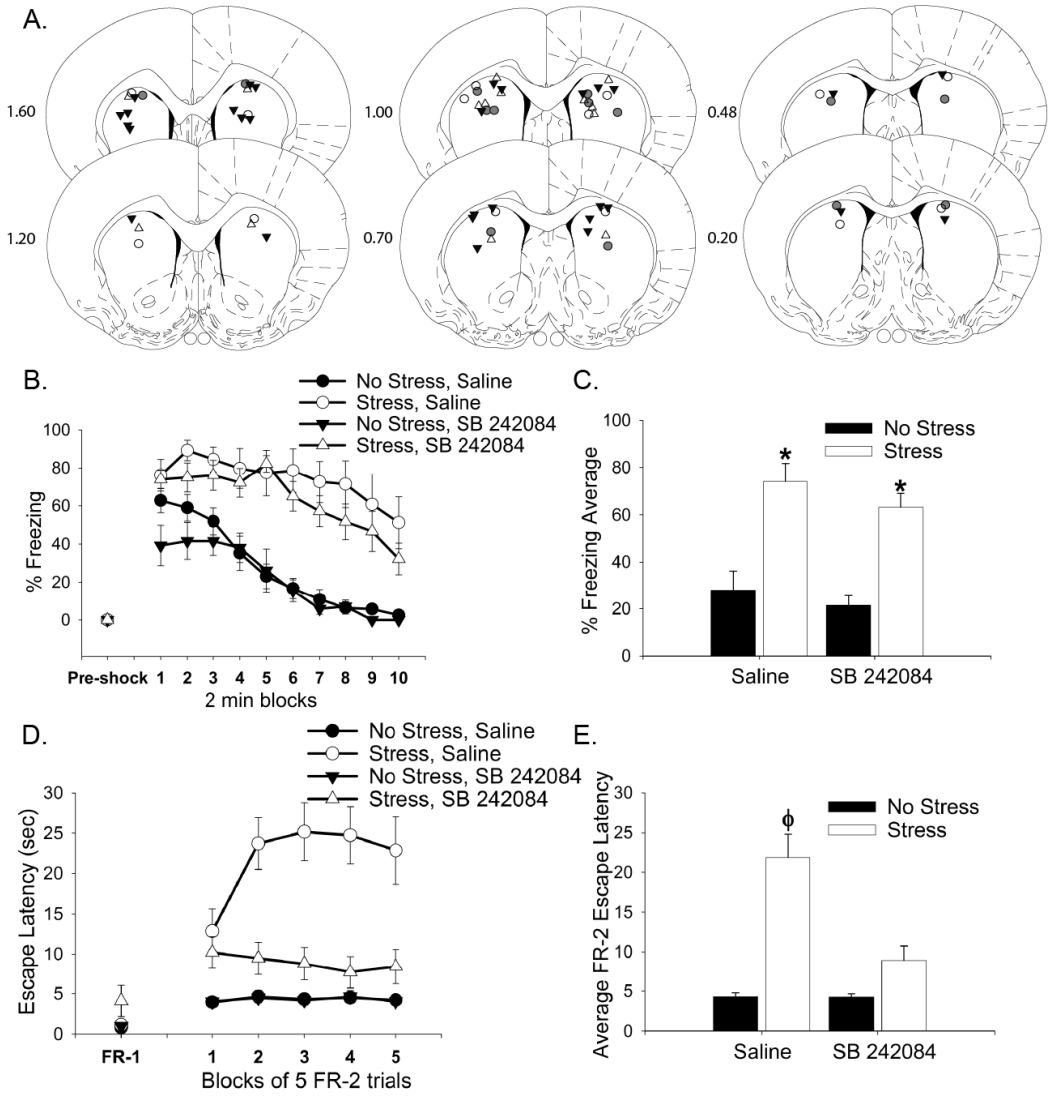

Figure 2. Expression of stress-induced escape deficits, but not exaggerated fear, is dependent upon 5-HT_{2C} receptor activation in the DS. Rats received uncontrollable stress or home cage treatment 24 hrs prior to behavioral testing. Fifteen minutes before behavioral testing, rats received intra-DS microinjections of either the 5-HT_{2C} receptor antagonist SB-242084 or saline. **(A)** Cannula tip placement within DS. **(B)** Freezing behavior presented in two minute blocks (pre-shock scores are not different and therefore overlap). **(C)** The mean percent shock elicited freezing for the entire 20-min observation period. **(D)** Shuttle box escape latencies for one block of 2 FR-1 trials (FR-1) and five blocks of 5 FR-2 trials (FR-2). The No Stress groups overlap. **(E)** The mean escape latency for all 25 FR-2 escape trials. Data represent group means $\pm$ SEM. * $p<0.05$ relative to no-stress groups; ϕ $p<0.05$ from all other groups.

5-HT_{2C}R activation in the DS is sufficient to interfere with escape learning without enhancing fear

The 5-HT_{2C}R agonist CP809101 was administered 15 minutes prior to behavioral testing, 5 minutes after injection of the 5-HT_{2C}R antagonist SB242084. Cannula placements are shown in Figure 3A. After exclusion of rats with misplaced cannula (n=4), group sizes were Saline, n=8; CP809101, n=8; SB242084 + CP809101, n=8. Rats with misplaced cannula that received CP809101 (n=2) were included as off-site controls (Fig. 3C/3E). The effect of the 5-HT_{2C}R agonist on freezing is shown in Figure 3B. Average post-shock freezing scores are shown in Figure 3C. Pre-shock freezing was minimal and did not differ between groups (Fig. 3B, pre-shock). There was a reliable main effect of time on freezing behavior ($F (9, 189) = 15.656$; $p < .0001$), but neither the main effect of drug treatment nor the interaction between time and drug were significant. Freezing behavior of rats that received injections of CP809101 through misplaced cannula (off-site control) was indistinguishable from that of all other groups.

No differences in FR-1 escape latency were observed across all groups and treatments (Fig. 3D, FR-1). Activation of the 5-HT_{2C}R in the DS prior to behavioral testing was sufficient to interfere with escape learning, and this effect was blocked by pre-treatment with the 5-HT_{2C}R antagonist SB242084 (Fig. 3D). Significant main effects of drug treatment ($F (2, 21) = 4.464$; $p = .0242$) and time ($F (4, 84) = 9.587$; $p = <.0001$) on escape behavior were found, but the interaction between time and drug was not significant. Average FR-2 latencies are displayed in Figure 3E. Off-site control rats behaved similarly to saline injected rats.

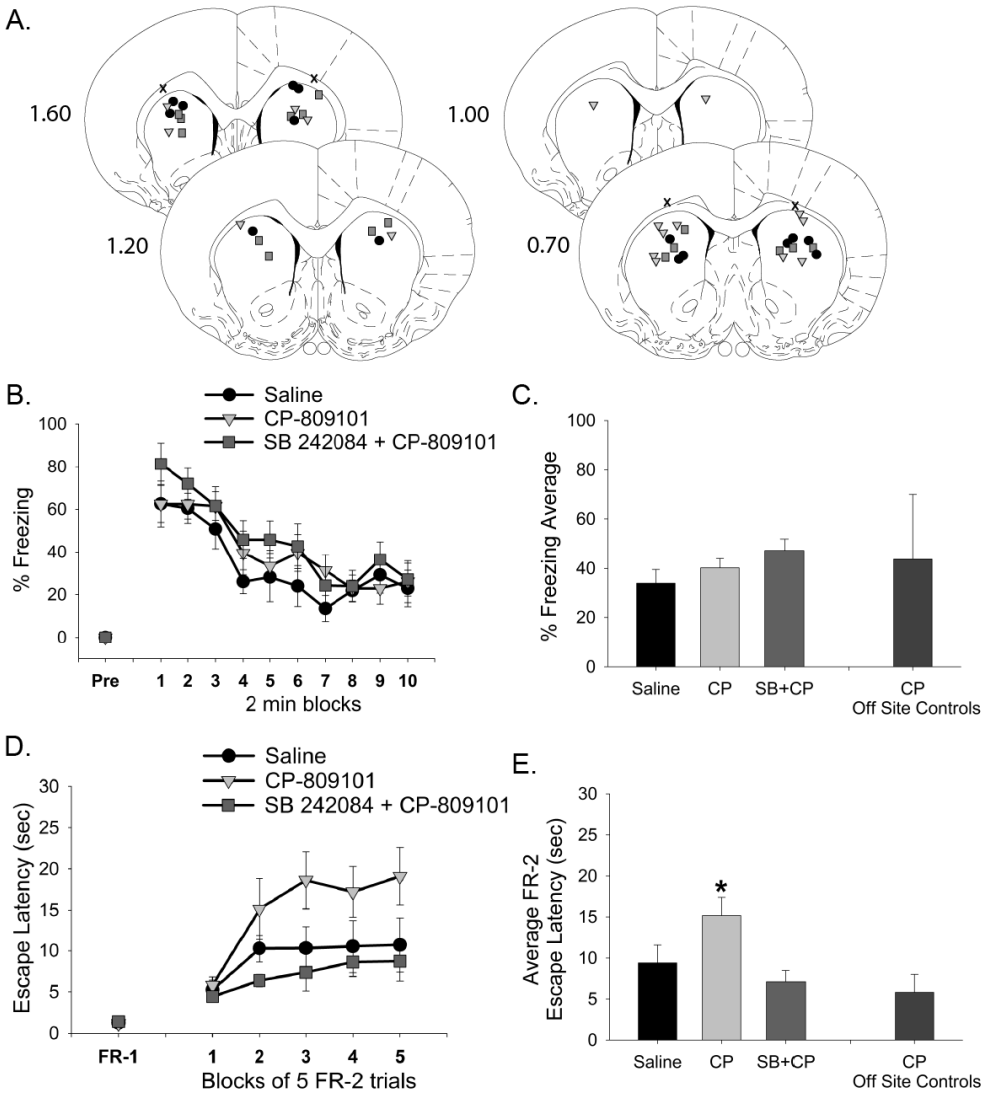

Figure 3. 5-HT_{2C} receptor activation in the DS is sufficient to interfere with shuttle box escape learning. Freezing behavior and shuttle box escape latencies were sequentially measured. Fifteen minutes prior to behavioral testing rats received intra-DS microinjections of either the 5-HT_{2C} receptor agonist CP-809101 or saline. In the antagonist+agonist group, intra-DS pretreatment with SB-242084 occurred 5 minutes prior to intra-DS administration of CP-809101. **(A)** Cannula tip placemnt within DS; off-site placements are denoted with an **x**. **(B)** Mean freezing behavior presented in two minute blocks (pre-shock scores are not different and therefore overlap). **(C)** The mean percent shock elicited freezing for the entire 20-min observation period. **(D)** Shuttle box escape latencies for one block of 2 FR-1 trials (FR-1) and five blocks of 5 FR-2 trials (FR-2). **(E)** The mean escape latency for all 25 FR-2 escape trials. Data represent group means $\pm$ SEM. * $p<0.05$ relative to all other groups.

5-HT_{2C}R activation in the BLA is not necessary for stress-induced exaggerated freezing but contributes to the expression of the escape deficit

The 5-HT_{2C}R antagonist SB242084 was injected in the BLA 15 minutes prior to assessment of shock-elicited freezing (Fig. 4B/4C) and escape behavior (Fig. 4D/4E). After exclusion of rats with misplaced cannula (n=9), group sizes were No Stress/Saline n=13; No Stress/SB242084, n=10; Uncontrollable Stress/Saline, n=15; Uncontrollable Stress/SB242084, n=10. Rats with misplaced cannula that received uncontrollable stress and SB242084 (n=5) were included as off-site injection controls. Cannula placements are shown in Figure 4A. No differences in pre-shock freezing were observed across all groups and treatments (Fig. 4B, pre-shock). A reliable main effect of stressor exposure on freezing behavior ($F (1, 44) = 13.688$; $p = .0006$) was found; however, no other main effect or interaction was significant. Average post-shock freezing scores are shown in Figure 4C. Freezing behavior of rats that were exposed to uncontrollable stress and injected with SB242084 through misplaced cannula (off-site control) was indistinguishable from that of all other stress groups.

While 5-HT_{2C}R blockade had no effect on conditioned fear, SB242084 reduced the stress-induced interference with escape behavior. No differences in FR-1 escape latencies were observed across all groups and treatments (Fig. 4D, FR-1), but significant main effects of drug ($F (1, 44) = 4.940$; $p = .0314$), stress ($F (1, 44) = 49.280$; $p = <.0001$) and time ($F (4, 44) = 29.967$; $p = <.0001$) were found. Significant two-way interactions were observed between time and drug ($F (4, 176) = 3.199$; $p = .0145$) and time and stress ($F (4, 176) = 13.969$; $p = <.0001$). The three-way interaction between time, drug and stress ($F (4, 176) = 4.564$; $p = .0016$) was also significant. Post hoc analysis showed that uncontrollable stress increased escape latency during all trial blocks regardless of treatment with saline or SB242084. 5-HT_{2C}R blockade in the BLA reduced stress-induced interference with escape learning during the last 3 trial blocks, i.e. the

stressed drug group was different from both the stressed saline group and both non-stressed groups. Average FR-2 latencies are displayed in Figure 4E. Off-site control rats behaved similarly to uncontrollable stressed, saline injected rats.

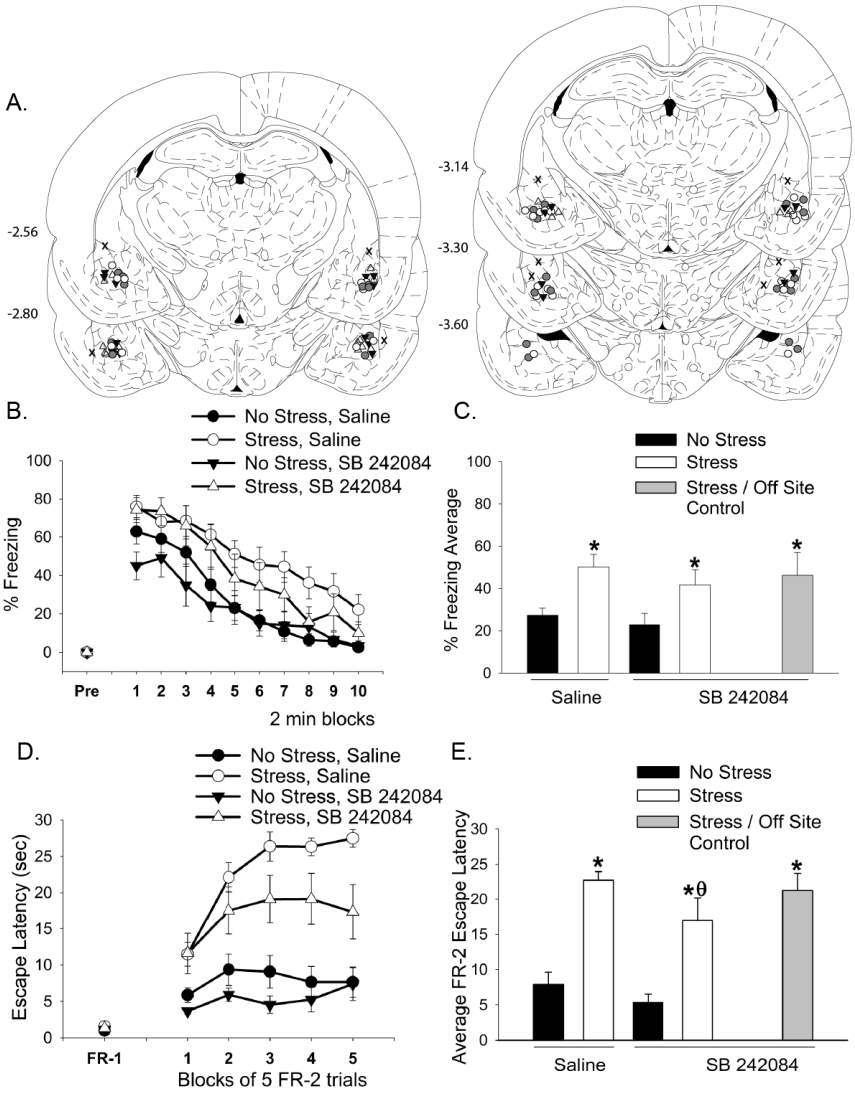

Figure 4. 5-HT_{2C} receptors in the BLA contribute to the stress-induced interference with shuttle box escape, but not exaggerated freezing. Rats received uncontrollable stress or home cage treatment 24 hrs prior to behavioral testing. Fifteen minutes before behavioral testing, rats received intra-BLA microinjections of either the 5-HT_{2C} receptor antagonist SB-242084 or saline. **(A)** Cannula tip placemnt within BLA; off-site placements are denoted with an **x**. **(B)** Mean freezing behavior presented in two minute blocks (pre-shock scores are not different and therefore overlap). **(C)** The mean percent shock elicited freezing for the entire 20-min observation period. **(D)** Shuttle box escape latencies for one block of 2 FR-1 trials (FR-1) and five blocks of 5 FR-2 trials (FR-2). **(E)** The mean escape latency for all 25 FR-2 escape trials. Data represent group means $\pm$ SEM. * $p<0.05$ relative to no-stress groups; θ $p<0.05$ from stress/saline group.

5-HT_{2C}R activation in the BLA is sufficient to enhance fear but not interfere with escape behavior

The 5-HT_{2C}R agonist CP809101 was administered 15 minutes prior to behavioral testing. Cannula placements are shown in Figure 5A. After exclusion of rats with misplaced cannula (n=16), group sizes were Saline, n=11 and CP809101, n=11. Rats with misplaced cannula that received CP809101 (n=12) were included as off-site injection controls (Fig. 5C/5D). The effect of the 5-HT_{2C}R agonist on freezing is shown in Figure 5B. Average post-shock freezing scores are shown in Figure 5C. Pre-shock freezing was minimal and did not differ between groups (Fig. 4B, pre-shock). Main effects of drug treatment ($F(1, 20) = 8.913$; $p = .0073$) and time ($F(9, 20) = 11.253$; $p<.0001$) on freezing behavior were significant but the interaction between time and drug was not significant. Freezing behavior of rats that received injections of CP809101 through misplaced cannula (off-site control) was indistinguishable from that of saline injected rats.

No differences in FR-1 escape latency were observed across all groups and treatments (Fig. 5D, FR-1). Activation of the 5-HT_{2C}R in the BLA prior to behavioral testing had no effect on escape behavior (Fig. 5D). A significant main effect of time ($F(4, 20) = 3.430$; $p = .0122$) on escape behavior was found, but neither the main effect of drug nor the interaction between time and drug were significant. Average FR-2 latencies are displayed in Figure 5E. Off-site control rats behaved similarly to saline injected rats.

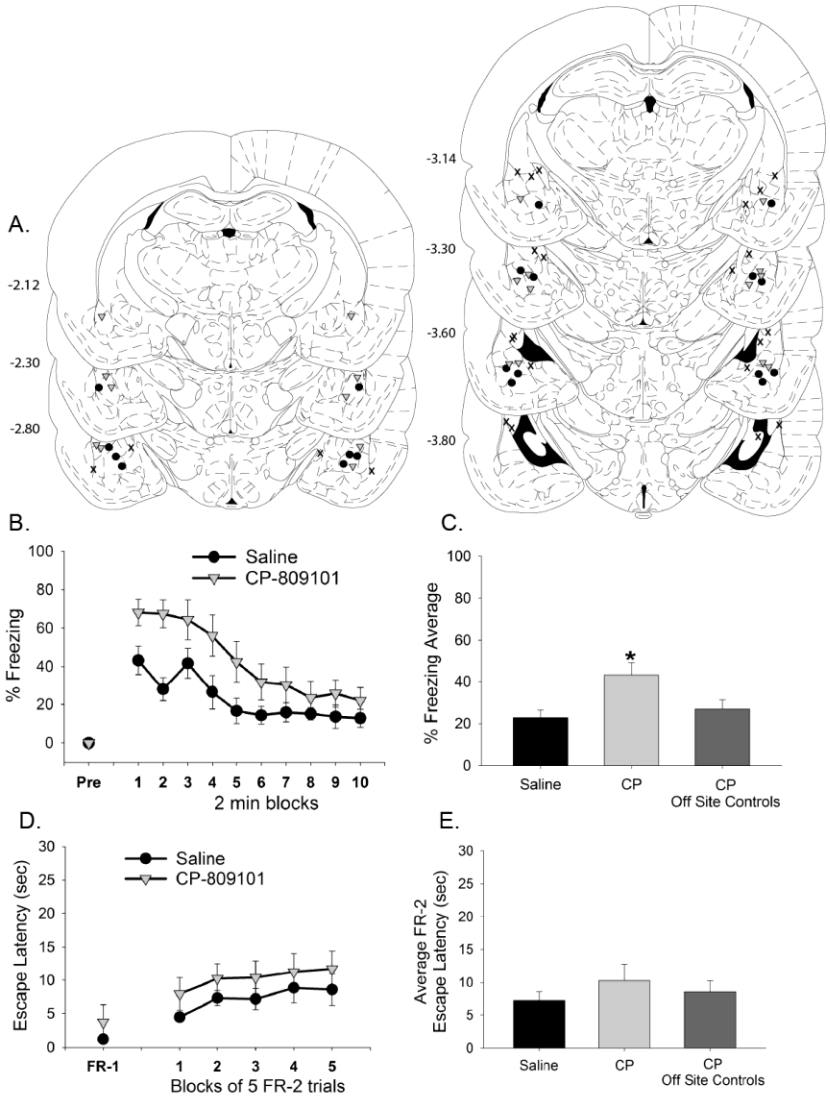

Figure 5. 5-HT_{2C} receptor activation in the BLA is sufficient to increase fear behavior but had no effect on shuttle box escape behavior. Fifteen minutes prior to behavioral testing rats received intra-BLA microinjections of the selective 5-HT_{2C} receptor agonist CP-809101 or saline. **(A)** Cannula placement within the BLA; off-site placements are denoted with an **x**. **(B)** Mean freezing behavior presented in two minute blocks (pre-shock scores are not different and therefore overlap). **(C)** The mean percent shock elicited freezing for the entire 20-min observation period. **(D)** Shuttle box escape latencies for one block of 2 FR-1 trials (FR-1) and five blocks of 5 FR-2 trials (FR-2). **(E)** The mean escape latency for all 25 FR-2 escape trials. Data represent group means $\pm$ SEM. * $p<0.05$ relative to saline or off-site control group.

Discussion

The ability to select the correct behavioral strategy in order to escape danger is vital to an organism's survival and can utilize instrumental learning mediated by the DS. These studies are the first to demonstrate that stress can interfere with instrumental escape behavior through a 5-HT_{2C}R-mediated mechanism in the DS. The present data indicate that 1) extracellular 5HT in the striatum during a mild stress challenge is sensitized by prior uncontrollable, but not controllable, stress, 2) 5-HT_{2C}R activation in the DS is necessary and sufficient for the expression of stress-induced deficits in instrumental learning, and 3) although 5-HT_{2C}R activation in the BLA is not necessary for enhanced post-shock freezing produced by uncontrollable stress, 5-HT_{2C}R activation in the BLA is sufficient to enhance fear and contributes to the stress-induced interference with instrumental learning.

The current results are exciting because they are the first to demonstrate that extracellular 5-HT in the dorsal striatum in response to a mild stress challenge is sensitive to prior stressor controllability. Uncontrollable stress led to the expression of exaggerated shock-elicited freezing and poor escape performance. These behaviors are thought to be mediated by hyper-activation and sensitization of 5-HT neurons in the DRN and subsequent exaggerated 5-HT release in DRN projection sites (Maier et al., 1995c, Amat et al., 1998a, b, Maswood et al., 1998, Grahn et al., 1999, Christianson et al., 2010). The DRN projection sites known to respond to a sensitized DRN now include the DS, where 2 foot shocks elicited an increase in extracellular 5-HT 24 hours after uncontrollable, but not controllable, stress. The increase in extracellular 5-HT in the striatum could be the result of a sensitized DRN based on work demonstrating that 1) the majority of 5-HT input to the DS originates in the DRN (Imai et al., 1986, Waselus et al., 2006), and 2) electrical stimulation of the DRN (McQuade and Sharp, 1997) and other stressors such as

forced swimming (Kirby et al., 1995) both increase extracellular 5-HT in the striatum. Although the use of two different rat strains in the dialysis and behavioral experiments (Sprague Dawley and Fischer 344, respectively) could negatively influence the ability to compare the two data sets, both Sprague Dawley and F344 rats display similar neurochemical (Grahn et al., 1999, Greenwood et al., 2003b, Greenwood et al., 2005, McDevitt et al., 2009) and behavioral (Greenwood et al., 2003a, Amat et al., 2005, Greenwood et al., 2005) responses to uncontrollable stress. We are therefore confident that the current observations apply equally to both strains.

5-HT_{2C}R activation in the DS is both necessary and sufficient for the deficit in instrumental escape behavior produced by uncontrollable stress. The escape deficits produced by the 5-HT_{2C}R agonist CP809101 were likely mediated by activation of DS 5-HT_{2C}R because the deficit was not observed in rats with misplaced cannula and was totally blocked by pre-treatment with intra-DS SB242084. The identified role of DS 5-HT_{2C}R in stress-induced interference with shuttle box escape is consistent with prior work indicating that stress interferes with instrumental tasks involving the DS in both humans (Schwabe and Wolf, 2011) and rodents (Dias-Ferreira et al., 2009) and that 5-HT_{2C}R in the DS are involved in stress-related behaviors. 5-HT_{2C}R knockout mice, for example, display a disruption in striatal function (Abdallah et al., 2009) and an anxiolytic phenotype in response to noxious stimuli (Heisler et al., 2007). Additionally, alterations of escape behavior produced by 5-HT_{2C}R manipulations in the DS occurred in the absence of changes in fear, supporting prior work (Maier, 1990, Maier and Watkins, 2005, Greenwood and Fleshner, 2008, Greenwood et al., 2010) indicating that the instrumental escape deficit produced by uncontrollable stress is separable from exaggerated fear and each occur via different mechanisms.

The current data extend our understanding of the repercussions of uncontrollable stress and indicate that the stress-induced shuttle box escape deficit is likely a consequence of interference with instrumental processes rather than simply a motor impairment. Although a motor deficit can certainly interfere with escape performance under specific laboratory conditions (Greenwood et al., 2008, Greenwood et al., 2010), the data suggest that the escape deficit measured using the current experimental protocol represent a stress-induced interference with instrumental learning processes. Importantly, the escape deficits produced by uncontrollable stress or intra-DS 5-HT_{2C}R agonist both develop over trials. This observation is consistent with the hypothesis of Jackson et al. (1980) that escape learning is predominantly an associative process and prior uncontrollable stress reduces sensitivity to correct escape contingencies as the result of subsequent associative interference (Jackson et al., 1980, Jackson and Minor, 1988).

Although 5-HT_{2C}Rs in the DS are clearly involved in the escape deficit produced by uncontrollable stress, the mechanism remains unknown. Dopamine (DA) in the striatum supports the association of an action with a particular outcome (Lex and Hauber, 2010) suggesting that 5-HT_{2C}R activation in the DS could interfere with instrumental process by interfering with DA activity in the DS. 5-HT_{2C}R activation can decrease DA neurotransmission in the DS (Alex et al., 2005). Additionally, activation of 5-HT_{2C}R in the striatum can increase the activity of inhibitory interneurons (Blomeley and Bracci, 2005, Blomeley and Bracci, 2009), and systemic blockade of 5-HT_{2C}R activation enhances neuronal activity in the striatum (De Deurwaerdere et al., 2010). It is possible that sensitized extracellular 5-HT in the striatum during exposure to aversive stimuli 24 hours after uncontrollable stress could interfere with instrumental escape learning through a DA-dependent mechanism and/or a net inhibitory effect on striatal output as a consequence of 5-HT_{2C}R activation.

It is of interest to note that distinct sub-regions of the DS mediate different aspects of instrumental behavior (Balleine et al., 2009). Goal-directed learning is initiated in the medial DS (Yin et al., 2005, Corbit and Janak, 2010), whereas habit learning is thought to be stored in the lateral DS (Yin et al., 2006). The fact that escape learning in the current paradigm occurs rapidly in non-stressed rats supports a role for the medial DS in this early stage of learning. The lateral DS, however, has been recently implicated in the acquisition of simple procedural learning (Yin, 2010), and could thus play a role in the acquisition of the shuttle box escape contingency. The cannula in the current studies were aimed at the border between the medial and lateral DS, but no differences in behavior were observed after comparing effects of drug injections between rats with cannula placements towards the extremes of either the medial or lateral DS. While the involvement of 5-HT_{2C}R in the DS in the behavioral consequences of uncontrollable stress remains an important and novel observation, the role of 5-HT_{2C}R in the discrete sub regions of the striatum warrants further study.

The current experiments also investigate the involvement of 5-HT_{2C}R activation in the BLA in stress-induced behaviors. Extracellular 5-HT in the BLA is sensitized 24 hours following exposure to uncontrollable, relative to controllable, stress (Amat et al., 1998a, Christianson et al., 2010). The BLA is involved in fear behavior (LeDoux, 2003), is critical for shock-elicited freezing (Kim et al., 1993), and has been implicated in stress-related psychiatric disorders such as anxiety (Graeff et al., 1996, Walker et al., 2003) and PTSD (Brunetti et al., 2010). In rodents, 5-HT_{2C}R activation in the BLA can elicit anxiety-like behaviors in the open-field test (Campbell and Merchant, 2003), potentiated auditory fear conditioning (Burghardt et al., 2007), and reduced social exploration (Christianson et al., 2010). 5-HT_{2C}R activation is also necessary for ethanol-withdrawal-induced anxiety (Overstreet et al., 2006). Here we demonstrate that 5-HT_{2C}R

activation in the BLA is sufficient to increase fear behavior (e.g. freezing) during shock-elicited fear conditioning, an effect similar to systemic administration of the 5-HT_{2C}R agonist (Strong et al., 2009). Despite these observations, 5-HT_{2C}R blockade in the BLA failed to prevent the expression of stress-induced exaggerated fear. This is consistent with the previous report that systemic administration of SB242084 also had no effect on this behavior (Strong et al., 2009). Importantly, the current data do not preclude a role for 5-HT in stress-induced exaggerated fear behavior as 5-HT is clearly necessary (Maier et al., 1993, Maier et al., 1995b, Maier et al., 1995c) and sufficient (Greenwood et al., 2008) for this stress-induced behavior.

5-HT_{2C}Rs in the BLA seem to be critical for the expression of some anxiety-like behaviors produce by uncontrollable stress but not others. Whereas, 5-HT_{2C}R activation in the BLA is necessary for the 5-HT-mediated potentiation of short-duration (3 min test), unconditioned, anxiety-like, behaviors such as stress-induced reductions in social exploration (Christianson et al., 2010), the current results suggest that BLA 5-HT_{2C}R may not be necessary for 5-HT-mediated sustained fear behaviors, such as the shock-elicited exaggerated freezing that is measured in the shuttle box test over a longer period (20 min). Indeed, while short-duration fear is mediated by the amygdala, the expression of sustained fear involves the bed nucleus of the stria terminalis (BNST) (Walker et al., 2003) and lesions of the BNST can prevent stress-induced exaggerated freezing (Hammack et al., 2004). It is possible, therefore, that while 5-HT_{2C}R activation in the BLA is sufficient to increase fear-like behavior per se, stress-induced exaggerated freezing may also require 5-HT activity in another region such as the BNST. Finally, a 5-HT receptor in the BLA other than the 5-HT_{2C}R may be responsible for exaggerated freezing following uncontrollable stress. The 5-HT_{2A} receptor is one candidate, as systemic 5-HT_{2A} blockade prevents stress-induced potentiation of acoustic startle (Jiang et al., 2009).

Surprisingly, while intra-BLA blockade of the 5-HT_{2C}R had no effect on stress-induced exaggerated freezing behavior, the antagonist did partially reduce the escape deficit produced by prior uncontrollable stress. These data could reflect a contribution of BLA 5-HT_{2C}R to the expression of the instrumental escape deficit. The DS receives inputs from the BLA (Kelley et al., 1982, Baldwin et al., 2000), and the BLA can play a role in acquisition of instrumental learning (Balleine et al., 2003, Wang et al., 2005, Lazaro-Munoz et al., 2010) and other forms of striatal-dependent learning (Graham et al., 2009). The BLA has been reported to drive striatal plasticity during the acquisition of instrumental learning (Popescu et al., 2007, Popescu et al., 2009); therefore, it is possible that exaggerated release of 5-HT in the BLA during escape testing (Amat et al., 1998a) could interfere with the BLA contribution to the processes supporting striatal-dependent instrumental learning through a mechanism involving the 5-HT_{2C}R. Additionally, activation of central amygdala (CeA)-dependent process can constrain the expression of instrumental behavior in the Sidman Avoidance task (Lazaro-Munoz et al., 2010). It is therefore possible that the partial 5-HT_{2C}R antagonist-mediated reduction in the stress-induced escape deficit was the result of unintended activation of 5-HT_{2C}R in CeA.

Conclusion

These experiments provide a more complete picture of the role of 5-HT_{2C}R in discrete brain regions, the DS and the BLA, in specific behavioral consequences of uncontrollable stress. The data reveal a novel role for the 5-HT_{2C}R in the DS in the expression of instrumental escape deficits produced by uncontrollable stress. The DS is implicated as an important DRN projection site mediating the effects of acute increases in 5-HT on learning deficits associated with uncontrollable stress and stress-related psychiatric disorders. 5-HT_{2C}R in the DS could, more generally, contribute to the expression of habit behavior produced by prior stressor exposure

(Schwabe and Wolf, 2011) and interference with goal-directed behavior present in patients exhibiting symptoms of post-traumatic stress (Tull et al., 2007). Finally, the results identify the DS as a brain region at which drugs targeting the 5-HT_{2C}R may increase therapeutic efficacy, perhaps through modulation of DA-mediated learning mechanisms.

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Chapter 3

5-HT_{2C} Receptors in the Basolateral Amygdala and Dorsal Striatum Are a Novel Target for the Anxiolytic Effects of Exercise

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Abstract

Physical activity has been associated with a reduction in the incidence and severity of psychiatric disorders such as anxiety. Similarly, in laboratory rodents, voluntary wheel running can reduce anxiety-like behaviors. The mechanisms underlying the anxiolytic properties of exercise, however, remain relatively unknown. One relevant pharmacological target in the treatment of anxiety is the 5-HT_{2C} receptor (5-HT_{2C}R). Consistent with data demonstrating the anxiogenic consequences of 5-HT_{2C}R activation in humans and rodents, we have previously reported that site-specific administration of the selective 5-HT_{2C}R agonist CP809101 in the basolateral amygdala (BLA) increases shock-elicited fear while administration of CP809101 in the dorsal striatum (DS) interferes with shuttle box escape learning. These findings suggest that activation of 5-HT_{2C}R in unique brain regions contributes to specific anxiety-like behaviors and may indicate potential brain sites involved in the anxiolytic characteristics of exercise. The current studies tested the hypothesis that voluntary wheel running prevents the behavioral consequences of 5-HT_{2C}R activation in the BLA and DS, specifically increased shock-elicited fear and interference with shuttle box escape learning. After six weeks of voluntary wheel running or sedentary conditions, the selective 5-HT_{2C}R agonist CP809101 was microinjected into either the BLA or the DS of adult Fischer 344 rats, and shock-elicited fear and shuttle box escape learning was assessed. Additionally, in-situ hybridization was used to determine if six weeks of voluntary exercise changed levels of 5-HT_{2C}R mRNA. We found that voluntary wheel running reduced the behavioral effects of CP809101 and reduced levels of 5-HT_{2C}R mRNA in the both the BLA and the DS. The current data extend previous work identifying the 5-HT_{2C}R as a relevant target for pharmaceutical discovery in the treatment of anxiety disorders and implicates 5-HT_{2C}R in the anxiolytic effects of physical activity.

Introduction

Physical activity is associated with a reduction in the incidence and severity of human psychiatric disorders such as anxiety (Strohle, 2009, Herring et al., 2010, Saeed et al., 2010). Similarly, voluntary exercise can reduce anxiety-like behaviors in laboratory rodents (Greenwood et al., 2003, Binder et al., 2004, Greenwood et al., 2005a, Fox et al., 2008, Greenwood et al., 2008, Salam et al., 2009, Vollert et al., 2011). The mechanisms underlying the anxiolytic properties of physical activity, however, remain unknown.

Many adaptive neural changes produced by physical activity have been identified (Dishman et al., 2006, Ang and Gomez-Pinilla, 2007, Greenwood and Fleshner, 2011). It remains unclear, however, which of these adaptations are primarily responsible for the anxiolytic benefits of exercise. Given that serotonin (5-HT) has long been implicated in anxiety (Graeff et al., 1996, Graeff et al., 1997, Anderson and Mortimore, 1999, Ninan, 1999, Graeff, 2004), it is plausible to propose that changes in central 5-HT systems, perhaps at the level of post-synaptic 5-HT receptors, could be one of the links between exercise and its anxiolytic effects (Chaouloff, 1997, Greenwood et al., 2003, Greenwood et al., 2005b). One relevant pharmacological target in the treatment of psychiatric disorders, including anxiety, is the 5-HT_{2C} receptor (5-HT_{2C}R) (Millan, 2003, Serretti et al., 2004, Millan, 2005). 5-HT_{2C}R agonists are known to produce anxiogenic profiles in humans (Charney et al., 1987, Murphy et al., 1989, Gatch, 2003, Millan et al., 2005, Olie and Kasper, 2007, Pjrek et al., 2007, Van Veen et al., 2007, Stein et al., 2008) and laboratory rodents (Bagdy et al., 2001, Campbell and Merchant, 2003, de Mello Cruz et al., 2005, Overstreet et al., 2006, Burghardt et al., 2007, Cornelio and Nunes-de-Souza, 2007, Christianson et al., 2010). Prior evidence also suggests that exercise may decrease sensitivity of 5-HT_2 receptors in humans (Broocks et al., 1999, Broocks et al., 2001) and rats (Dwyer and

Browning, 2000). In laboratory rodents, voluntary wheel running reduces anxiety-like behaviors that are dependent upon 5-HT_2 receptor activation. Fox and colleagues, for example, found that voluntary exercise reduced the anxiogenic effect of the non-selective 5-HT_2 receptor agonist metachlorophenylpiperazine (mCPP) on acoustic startle in mice (Fox et al., 2008). Additionally, we have observed that in rats, the anxiogenic effects of acute administration of the selective serotonin reuptake inhibitor fluoxetine can be prevented by both voluntary exercise and administration of the specific 5-HT_{2C}R antagonist SB242084 (Greenwood et al., 2008). Therefore, one mechanism underlying the anxiolytic effects of exercise may be a reduction in the expression, sensitivity and/or function of 5-HT_{2C}R.

The anxiogenic effects of 5-HT_{2C}R activation are brain-region dependent. Prior studies have reported increases in anxiety-like behavior, such as social avoidance and reduced exploration, produced by site-specific activation of 5-HT_{2C}R in the basolateral amygdala (BLA) (Campbell and Merchant, 2003, Christianson et al., 2010), a brain area classically implicated in fear and anxiety-like behaviors (Davis, 1992, LeDoux, 2003). More recently, we have observed that 5-HT_{2C}R activation in the BLA or the dorsal striatum (DS) increases anxiety-like behaviors such as shock-elicited fear and interference with shuttle box escape learning, respectively (Strong et al., 2011). In rats, exaggerated shock-elicited fear has been argued to represent anxiety-like behavior (Maier and Watkins, 1998, 2005) and can be blocked by administration of anti-anxiety drugs such as benzodiazepines (Maier, 1990). Additionally, cognitive dysfunction, such as disrupted processing of aversive stimuli and interference with goal-directed behavior, is often co-morbid with some types of anxiety disorders (Tull et al., 2007, Ferreri et al., 2011, Watkins, 2011). Deficits in shuttle box escape, a task in which the animal must learn to escape an aversive stimulus, may resemble aspects of the cognitive deficits associated with human anxiety

disorders (Maier and Watkins, 2005). Importantly, voluntary wheel running can prevent the development of these two anxiety-like behaviors following exposure to uncontrollable stress (Greenwood et al., 2003). Given the potential role of 5-HT_{2C}R activation in the BLA and DS in the expression of specific anxiety-like behaviors, it is possible that 5-HT_{2C}R in the BLA and DS could be a novel target for the anxiolytic effects of exercise.

The current studies investigated whether voluntary exercise could reduce anxiety-like behaviors produced by 5-HT_{2C}R activation. We hypothesized that voluntary wheel running would prevent exaggerated fear and interference with shuttle box escape learning produced by site-specific activation of 5-HT_{2C}R. The 5-HT_{2C}R agonist CP809101 was injected into either the BLA to produce exaggerated fear or the DS to interfere with instrumental escape behavior. Additionally, in-situ hybridization was used to investigate the effect of voluntary exercise on levels of 5-HT_{2C}R mRNA in the BLA and the DS.

Methods

Ethics Statement

All experimental protocols conformed to the NIH guide for the Care and Use of Laboratory Animals and were approved by the University of Colorado Institutional Animal Care and Use Committee (IACUC) protocol 1002.06.

Animals

Adult, male Fischer 344 rats (220-280g at the time of behavioral testing; N=116) were used in all experiments based on prior work optimizing 5-HT_{2C}-mediated behaviors in this strain (Greenwood et al., 2008, Strong et al., 2009, Strong et al., 2011). The rats were housed in a temperature- (22^{o} C) and humidity-controlled environment, were maintained on a 12:12 hour light/dark cycle, and had *ad libitum* access to food and water. Rats assigned to the sedentary

condition were individually housed in Nalgene Plexiglas cages (45 x 25.2 x 14.7 cm) lacking a running wheel. Rats assigned to 6 weeks of voluntary wheel running were individually housed in similar cages with attached running wheels. Animals were acclimated to these conditions for 1 week before any experimental manipulation. Wheels were rendered immobile with metal stakes during the acclimation period. Rats were weighed weekly.

Voluntary wheel running

Rats (6-7 weeks in age) were randomly assigned to either remain sedentary or allowed voluntary access to running wheels for 6 weeks, a duration of wheel running we have previously shown to prevent increased shock-elicited fear and deficits in shuttle box escape learning produced by exposure to uncontrollable stress (Greenwood et al., 2003, Greenwood et al., 2005a, Greenwood et al., 2008). At the start of each experiment, the wheels in the cages of the physically active rats were unlocked and these rats were allowed voluntary access to their wheels. Daily wheel revolutions were recorded digitally using Vital View software (Mini Mitter, Bend, OR, USA) and distance was calculated by multiplying wheel circumference (1.081 m) by the number of wheel revolutions.

Surgery

Rats underwent surgery during the fourth week of either voluntary wheel running or sedentary conditions. Under ketamine (0.75 mg/kg i.p.; Vedco, St. Joseph, MO, USA) and medetomidine (0.5 mg/kg i.p.; Pfizer, New York, NY, USA) anesthesia, bilateral cannula (26 gauge, Plastics One, Roanoke, VA, USA) were implanted as previously described (Strong et al., 2011). Cannula were aimed at either the DS: +0.5 A/P, ± 3.0 M/L, and -4.6 D/V from bregma (Atallah et al., 2007, Strong et al., 2011) or, separately, the BLA: -3.0 A/P, ± 4.8 M/L, -6.2 D/V from bregma (Christianson et al., 2010, Strong et al., 2011), based on the atlas by Paxinos and

Watson (Paxinos, 1998). Atipamezole (0.5 mg/kg i.p.; Pfizer, New York, NY, USA) was administered following surgery to reverse the effects of medetomidine. All rats were inoculated with 0.25 mL/kg (subcutaneous) penicillin (Combi-Pen, Agrilabs, St. Joseph, Missouri, USA) immediately following surgery and returned to their home cages. Behavioral experiments were conducted approximately 2-3 weeks after implantation surgery, by which time running behavior had returned to pre-surgical levels. Following experiment completion, brains were sliced at 40 μm and stained with Cresyl Violet for cannula placement verification. Misplaced cannula were excluded from analysis or used as off-site controls when appropriate.

Drug Microinjections

Microinjections of the selective 5-HT_{2C}R agonist CP809191 (Tocris Bioscience, Ellisville, MO, USA) were made 15 minutes prior to behavioral testing. On the day of behavioral testing, a micro-injector extending 0.5mm (intra-DS) or 1.0mm (intra-BLA) beyond the tip of the guide cannula was inserted. Drug doses and injection volumes were based on prior work examining the anxiogenic effects of CP809101 (Strong et al., 2009, Christianson et al., 2010, Strong et al., 2011). Specifically, CP809191 was dissolved in 0.9 % sterile saline and gently warmed at 45^{o}C for 10-15 minutes in a water bath at concentrations of 0.3mM, 2.0mM and 6.0mM. CP809101 was administered intra-DS at a volume of 1.0 μL/side or intra-BLA at a volume of 0.5 μl/side (Christianson et al., 2010, Strong et al., 2011). Prior work in our lab has shown that the behavioral effects of CP809101 can be blocked by pretreatment with the 5-HT_{2C}R antagonist SB242084 when the drugs are both injected systemically (Strong et al., 2009) or intra-cranially (Strong et al., 2011). Additionally, we have previously shown that saline injections (Strong et al., 2011), as well as the low dose of CP809101 (0.3mM) selected for these

experiments, have no effect on anxiety-like behaviors (Strong et al., 2009, Christianson et al., 2010).

Behavioral Testing

Behavioral testing was conducted following 6 weeks of voluntary wheel running or sedentary conditions. Fear and shuttle box escape behaviors were assessed sequentially in shuttle boxes (50.8cm x 25.4cm x 30.48 cm, Coulbourn Instruments, Whitehall, PA) using procedures previously described (Maier, 1990, Greenwood et al., 2003, Strong et al., 2011). At the beginning of each session, rats were placed into shuttle boxes and allowed to explore for 5 minutes. During this 5 minute period (pre-shock period), fear behavior was assessed using a sampling procedure in which each rat was scored every 10 seconds as either freezing, defined as an absence of all movement except for that required for respiration, or not freezing. Rats then received two 0.8 mA foot shocks separated by 1 minute and delivered through both sides of the grid floor. Foot shocks were terminated when the rat fully crossed over to the opposite side of the shuttle box (fixed ratio 1, FR-1). The latencies to cross were recorded (FR-1 latencies). Following the second FR-1 trial, shock-elicited freezing was observed for 20 minutes. Shock-elicited freezing is a measure of fear conditioned to cues present in the shuttle box (Fanselow and Lester, 1988). The post shock freezing period was followed by 25 fixed-ratio 2 (FR-2) escape trials. During FR-2 trials, rats were required to cross through the shuttle box door twice in order to terminate the foot shock (0.8 mA). An escape latency of 30 seconds was assigned if a correct escape response did not occur within 30 seconds, at which time the shock was terminated. Shocks occurred with an average inter-trial interval of 60 seconds and a single test session lasted approximately 1 hour.

All animals were scored for both freezing and escape behavior. All behavioral tests occurred between 0900 and 1200 by an experimenter blind to treatment condition of the animals.

In Situ Hybridization

In a separate experiment, rats were randomly assigned to either remain sedentary or allowed voluntary access to running wheels for 6 weeks. After 6 weeks, rats were sacrificed via rapid decapitation. Following previously published in-situ hybridization protocols (Greenwood et al., 2003, Greenwood et al., 2005b, Greenwood et al., 2011), brains were extracted, and frozen in isopentane cooled with dry ice (-20^{o}C; 4 minutes). Brains were stored at -80^{o}C prior to being sectioned at 10μm thickness with a cryostat. Slicing occurred at -21^{o}C, and rostral-caudal sections of the DS and BLA were collected and thaw-mounted onto poly-L-lysine-coated slides. Tissue sections were stored at -80^{o}C prior to use in single-label radioactive in-situ hybridizations. Before hybridization, sections were fixed in 4% paraformaldehyde for 1 hour, washed 3 times in 2X sodium saline citrate (SSC), acetylated with 0.25% acetic anhydride containing 0.1M triethanolamine for 10 minutes, and dehydrated in graded ethanol. The $5HT_{2C}R$ receptor plasmid construct was obtained from Dr. David Julius at the University of California, San Francisco. The $5HT_{2C}R$ probe is 555 base pairs long, spanning 1370-1925 (Accession # M21410). Customary transcription protocols were used to label the $5HT_{2C}R$ riboprobe with ^{35}S-UTP. Following completion of transcription, the riboprobe was mixed with 50% hybridization buffer comprised of 50% high-grade formamide, 10% dextran sulfate, 3X SSC, 1X Denhardt's solution, 0.2mg/mL yeast tRNA, and 0.05M sodium phosphate (pH 7.4). The $5HT_{2C}R$ riboprobe in hybridization buffer was applied directly to slides containing sections of DS and BLA. Slides were incubated overnight at 55^{o}C in humid chambers. The following day, slides were washed 3 times in 2X SSC, subjected to an RNase A (200 μg/ml) treatment for 1 hour, rinsed in graded concentrations

of SSC, washed in 0.1X SSC (65^{o}C) for 1 hour, and dehydrated in ethanol. After drying, slides were placed in light-tight autoradiography cassettes, and exposed to X-ray film (Biomax-MR) for 1 week.

Image Analysis for In Situ Hybridization

Levels of 5-HT_{2C}R mRNA were analyzed by computer-assisted optical densitometry following previously published protocols (Greenwood et al., 2005b, Greenwood et al., 2011). Brain section images were captured digitally (CCD camera, model XC-77; Sony, Tokyo, Japan), and the relative optical density of the x-ray film was determined using Scion Image Version 4.0 (Scion, Frederick, MD, USA). A macro was written that enabled signal above background to be determined automatically. For each section, a background sample was taken over an area of white matter, and the signal threshold was calculated as mean gray value of background +3.5 standard deviations. The section was automatically density-sliced at this value, so that only pixels with gray values above these criteria were included in the analysis. Results are expressed as mean integrated density, which reflects both the signal intensity and the number of pixels above assigned background (mean signal above background X number of pixels above background). Each subject's mean integrated density at a given level represents the average of three slices chosen for analysis between the following coordinates: DMS and DLS from +1.60 to +0.20 mm anterior to bregma; BLA and CeA from -2.56 to -3.30 mm posterior to bregma based on the atlas by Paxinos and Watson (Paxinos, 1998). Templates for each region were made to ensure that equivalent areas were analyzed between animals.

Statistical Analysis

Body weights were analyzed with repeated measures ANOVA. Pre-shock freezing scores were averaged into 1 pre-shock score and analyzed with ANOVA. Shock-elicited freezing scores

were collapsed into 10, 2 min blocks and analyzed using 2x3 (activity X drug), repeated measures ANOVA. The two FR-1 trials were averaged into a single FR-1 latency score and analyzed with ANOVA. FR-2 escape latencies were averaged into 5 blocks of 5 trials each and analyzed with 2x3 (activity X drug), repeated measures ANOVA. Average post-shock freezing and average FR-2 escape latencies were analyzed with ANOVAs that included an additional group of off-site controls when appropriate. Group differences in 5-HT_{2C}R mRNA expression in the DS, central amygdala (CeA), and the BLA were analyzed with ANOVA. Significant main effects and interactions were followed by Fisher's protected least significant difference (PLSD) post hoc analysis. Results were considered significant when $p<0.05$.

Results

Voluntary exercise reduces exaggerated fear produced by 5-HT_{2C}R activation in the BLA

In this experiment, rats remained sedentary or were allowed voluntary access to an in-cage running wheel for 6 weeks. Repeated measures ANOVA revealed significant main effects of time ($F_{(6, 234)} = 1323.117$; $p<0.0001$) and exercise ($F_{(1, 39)} = 358.978$; $p<0.0001$) and a reliable time by exercise interaction ($F_{(6, 234)} = 14.002$; $p<0.0001$) on body weight (Figure 1A). The sedentary and exercise rats had similar starting weights. The exercise rats, however, weighed less than the sedentary animals during weeks 3, 4, 5 and 6. Intra-BLA cannulas were surgically implanted during the fourth week of voluntary wheel running. Figure 1C shows the average daily running data pre- and post-surgery. As expected, surgery immediately decreased running distance. However, running distance resumed quickly and continued to steadily increase during the week after surgery.

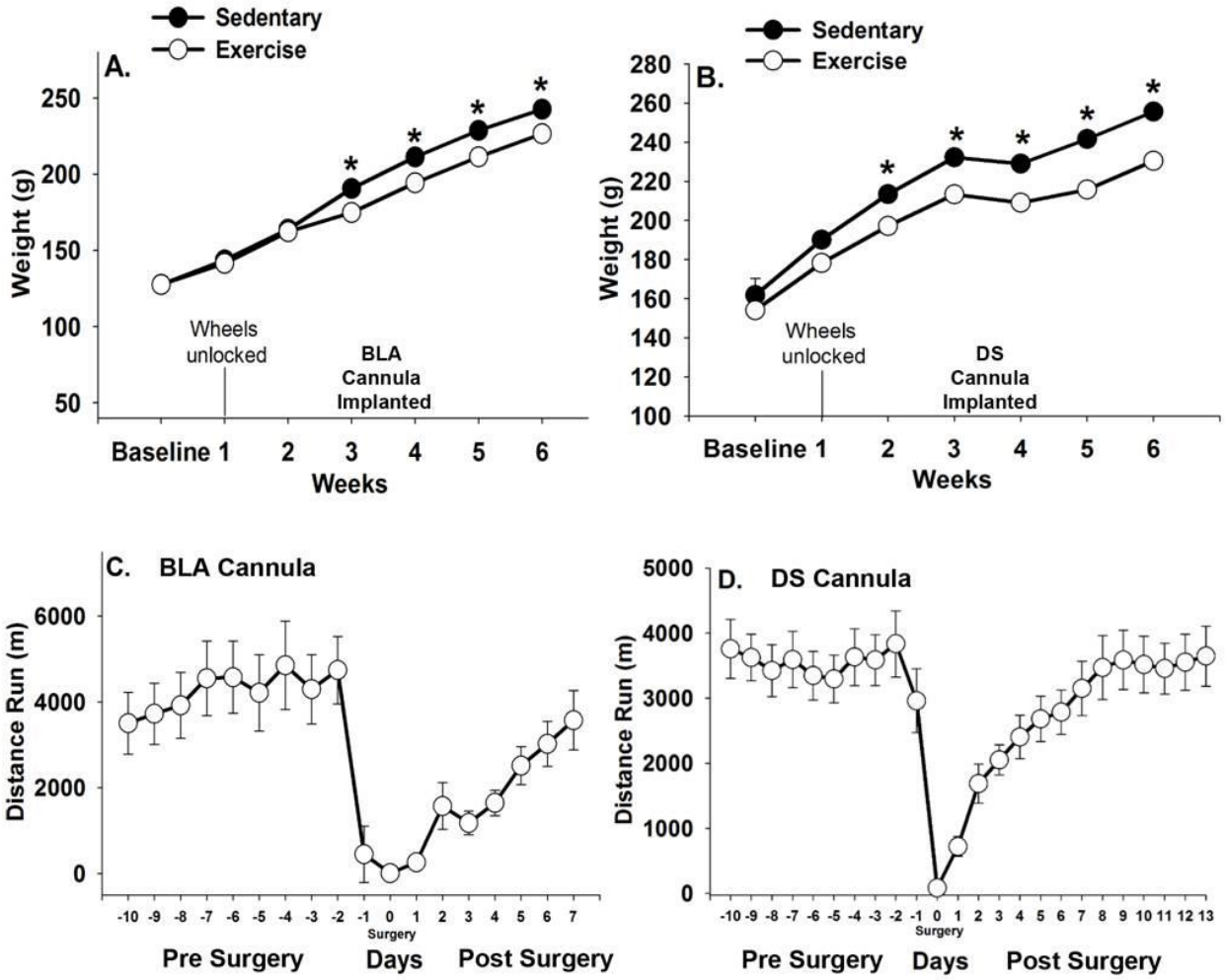

Figure 1. Adult, male Fischer 344 rats were allowed voluntary access to running wheels for 6 weeks (Exercise) or remained sedentary. Exercised rats underwent surgery to implant either intra-BLA or intra-striatal bilateral cannula between weeks 3 and 4. **(A, B)** Mean weekly body weight changes (grams) of physically active and sedentary rats. **(C, D)** The daily mean distance (meters) run pre- and post- cannula implantation surgery.

To determine whether 6 weeks of voluntary exercise reduces the anxiogenic effects of intra-BLA 5-HT_{2C}R activation, the 5-HT_{2C}R agonist CP809101 (0.3mM, 2.0mM and 6.0mM) was injected intra-BLA and shock-elicited freezing (Figure 2B/D) and shuttle box escape (Figure 2C/E) were observed 15 minutes later. Cannula placements are shown in Figure 2A. After exclusion of rats with misplaced cannula, group sizes were as follows: Sedentary, 0.3mM = 5; Sedentary, 2.0mM = 4; Sedentary, 6.0mM = 5; Run, 0.3mM = 4; Run, 2.0mM = 4; Run, 6.0mM = 4. Rats with misplaced cannula (N=9; Sedentary, 2.0mM = 2; Sedentary, 6.0mM = 2; Run, 2.0mM = 3; Run, 6.0mM = 2) were averaged and included as off-site controls (Figure 2D/E, off-site control).

Voluntary exercise increased the intra-BLA dose of CP809101 necessary to produce an increase in shock-elicited freezing behavior. The effect of the 5-HT_{2C}R agonist CP809101 on freezing behavior is shown in Figure 2B/D. Pre-shock freezing was minimal and did not differ between groups (Figure 2B, pre-shock). The main effects of time ($F_{(9, 180)} = 10.595$; $p<0.0001$) and drug ($F_{(2, 20)} = 3.637$; $p = 0.0450$), as well as the exercise by drug interaction ($F_{(2, 20)} = 4.542$; $p = 0.0236$), were all significant.

Post hoc comparisons revealed that the 0.3mM dose of CP809101 did not increase freezing in either the sedentary or exercise group, while the 6.0mM dose was sufficient to increase freezing in both the sedentary and run animals. Importantly, the 2.0mM dose of CP809101 administered into the BLA was sufficient to increase freezing behavior in sedentary, but not exercised, rats. Average post-shock freezing scores are shown in Figure 2D. Freezing behavior of rats that received injections of CP809101 through misplaced cannula (off-site controls) was indistinguishable from 0.3mM groups.

The effect of the 5-HT_{2C}R agonist CP809101 on escape behavior is shown in Figure 2C/E. Consistent with our prior observations, activation of the 5-HT_{2C}R in the BLA prior to behavioral testing had no effect on FR-1 or FR-2 escape behavior (Fig 2C). Average FR-2 latencies are displayed in Figure E. Off-site control rats behaved similarly to all other groups.

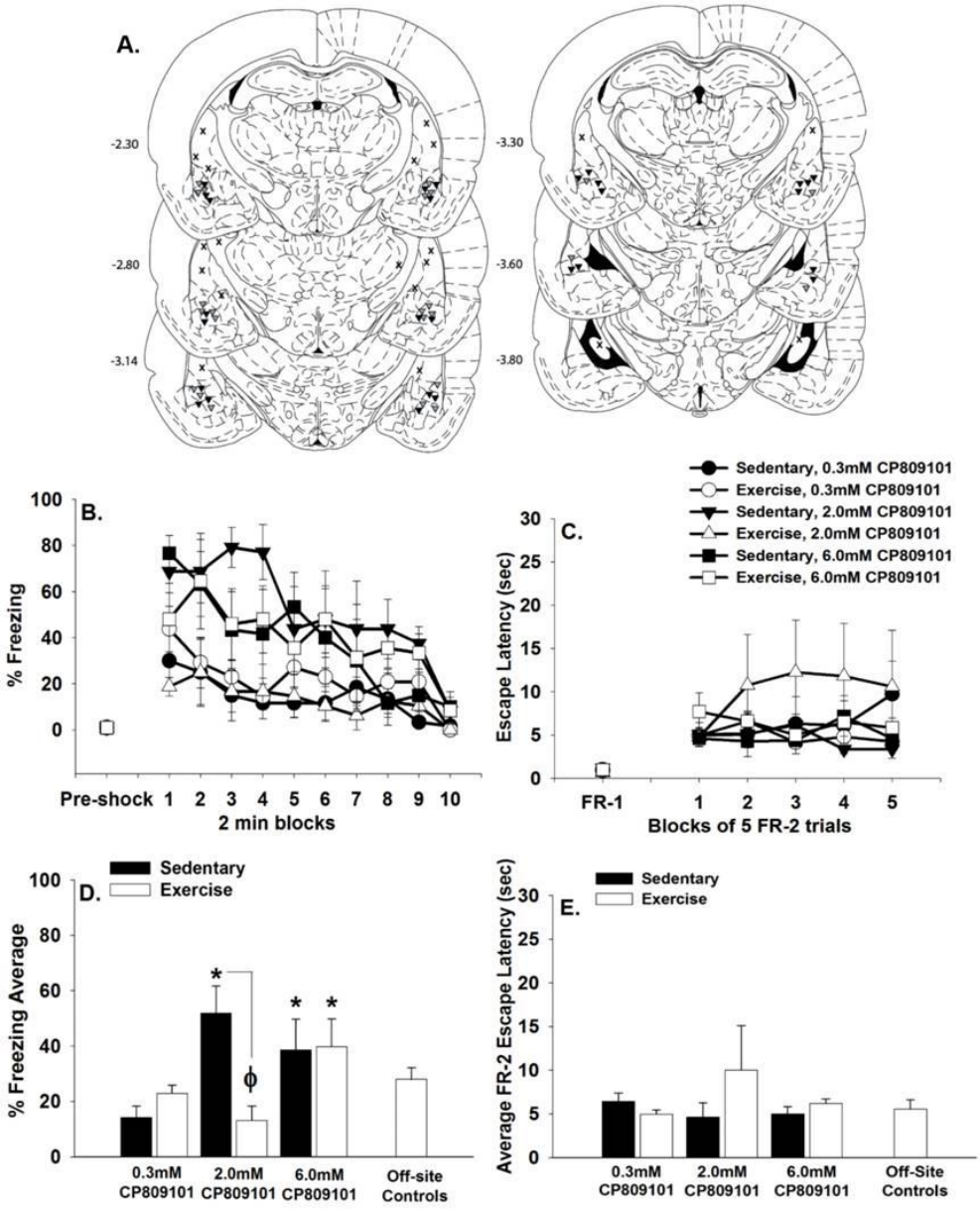

Figure 2. Six weeks of voluntary wheel running reduces the increase in fear behavior produced by 5-HT_{2C} receptor activation. Fifteen minutes prior to behavioral testing rats received, intra-BLA microinjections (0.5uL) of either the selective 5-HT_{2C} receptor agonist CP-809101 (0.3mM, 2.0mM or 6.0mM). **(A)** Cannula placement within the BLA; sedenatry rats are denoted with black triangles; exercise rats are denoted with gray triangles; off-site placements are denoted with an **x**. **(B)** Mean freezing behavior presented in two minute blocks (pre-shock scores are not different and therefore overlap). **(C)** Shuttle box escape latencies for one block of 2 FR-1 trials (FR-1) and five blocks of 5 FR-2 trials (FR-2). **(D)** The mean percent shock elicited freezing for the entire 20-min observation period. **(E)** The mean escape latency for all 25 FR-2 escape trials. Data represent group means $\pm$ SEM. * $p<0.05$ relative to 0.3mM groups and off-site control group; Φ $p<.05$ relative to 2.0mM sedentary group.

Voluntary exercise reduces shuttle box escape deficits produced 5-HT_{2C}R activation in the DS

To determine if voluntary wheel running prevents shuttle box escape deficits produced by intra-DS administration of CP809101, rats remained sedentary or were allowed voluntary access to an in-cage running wheel for 6 weeks. Weekly body weight data is shown in Figure 1B. Repeated measures ANOVA revealed significant main effects of time ($F_{(6, 402)} = 556.744$; $p<0.0001$) and exercise ($F_{(1, 67)} =44.254$; $p<0.0001$) and a reliable time by exercise interaction ($F_{(6, 402)} = 7.229$; $p<0.0001$) on body weight (Figure 1B). Sedentary and exercised animals had similar baseline body weights. After 2 weeks, rats allowed voluntary access to a running wheel weighed less than sedentary controls and this pattern continued for the duration of the experiment. Intra-DS cannulas were surgically implanted during the fourth week of voluntary wheel running. Figure 1D shows the average daily running data pre- and post-surgery. As expected, surgery immediately decreased running distance. However, running distance resumed quickly and continued to steadily increase during the week after surgery.

The 5-HT_{2C}R agonist CP809101 (0.3mM, 2.0mM and 6.0mM) was injected intra-DS and shock-elicited freezing (Figure 3B/D) and shuttle box escape (Figure 3C/E) were observed 15 minutes later. Cannula placements are shown in Figure 3A. After exclusion of rats with misplaced cannula, group sizes were Sedentary, 0.3mM = 7; Sedentary, 2.0mM = 12; Sedentary, 6.0mM = 11; Run, 0.3mM = 9; Run, 2.0mM = 15; Run, 6.0mM = 9. Rats with misplaced cannula (N=6; Sedentary, 2.0mM = 2; Sedentary, 6.0mM = 1; Run, 6.0mM = 3) were included as off-site controls (Figure 3D/E, off-site control).

The effect of the 5-HT_{2C}R agonist CP809101 on freezing behavior is shown in Figure 3B/D. Pre-shock freezing was minimal and did not differ between groups (Figure 3B, pre-shock). While there was a significant main effect of time ($F_{(9, 513)} = 36.546$; $p<0.0001$) on freezing

behavior, activation of the 5-HT_{2C}R in the DS prior to behavioral testing had no effect on freezing behavior, as in our prior work (Strong et al., 2011) (Fig 3B/C). Off-site control rats behaved similarly to all other groups.

The effect of the 5-HT_{2C}R agonist CP809101 on escape behavior is shown in Figure 3C/E. ANOVA revealed a significant main effect of exercise on FR-1 behavior (p=0.00340), i.e. exercise groups had slightly faster average FR-1 latencies than sedentary groups. However, neither the main effect of drug nor the interaction between exercise and drug were significant (Fig 3C, FR-1). Repeated measures ANOVA revealed significant main effects of time ($F_{(4, 228)}$ = 16.919; p=<0.0001) and drug ($F_{(2, 57)}$ = 3.476; p=0.0376) on escape behavior, as well as reliable time x drug ($F_{(4, 228)}$ = 2.384; p=0.0174) and time x drug x exercise ($F_{(8, 228)}$ = 3.232; p=0.0017) interactions. Post hoc analysis revealed that there was no difference between exercise and sedentary animals that received the sub-threshold dose of CP809101 (0.3mM). Importantly, the 2.0mM dose of CP809101 administered into the DS interfered with shuttle box escape learning in sedentary rats only, whereas physically active rats were resistant to the behavioral effects of that dose. Finally, the highest dose of CP809101 (6.0mM) was sufficient to interfere with escape learning in both sedentary and physically active rats. Average FR-2 latencies are displayed in Figure 3E. Off-site control rats behaved similarly to the sub-threshold dose (0.3mM) groups.

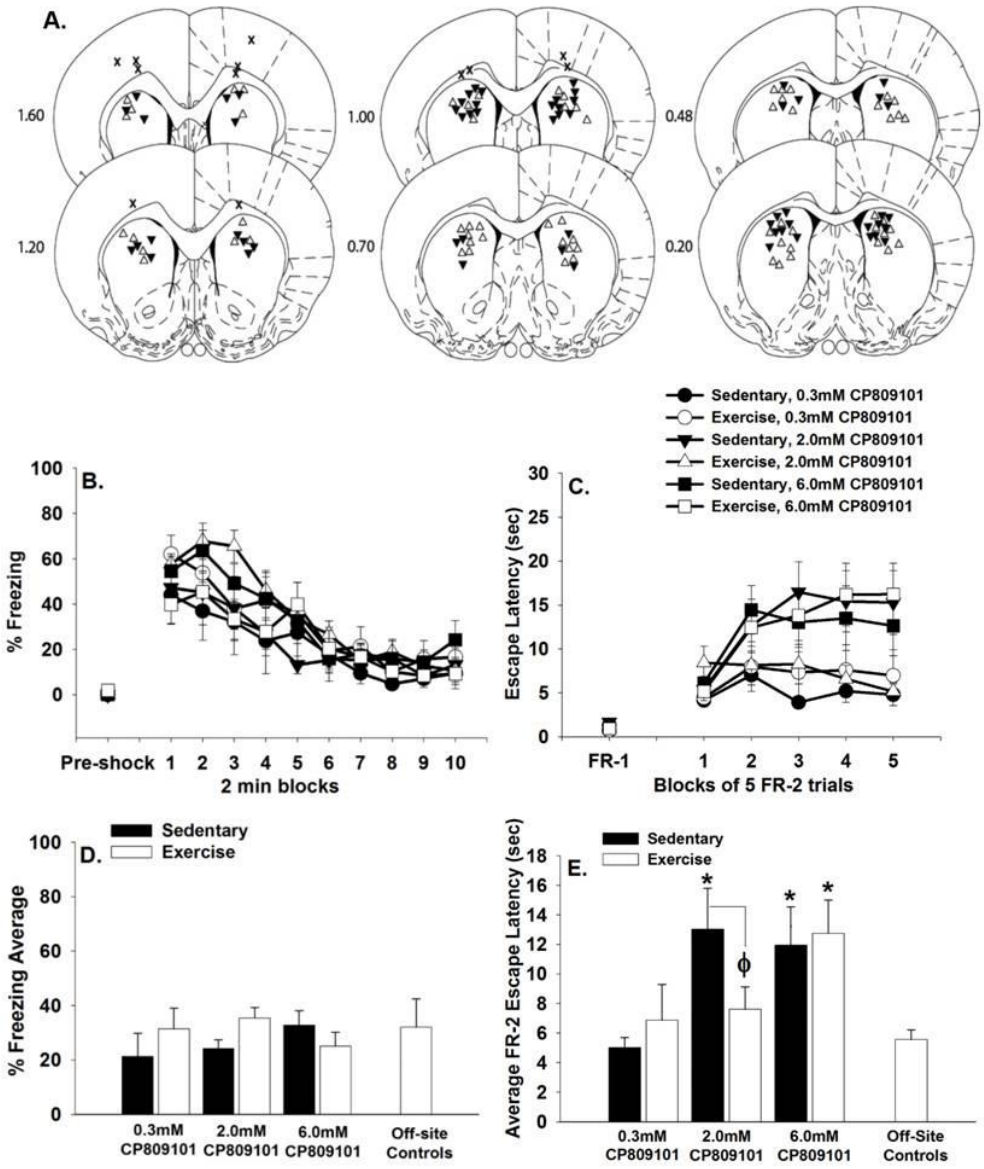

Figure 3. Six weeks of voluntary wheel running reduces the deficit in instrumental learning produced by 5-HT_{2C} receptor activation. Fifteen minutes prior to behavioral testing rats received intra-DS microinjections (1.0uL) of either the 5-HT_{2C} receptor agonist CP-809101 (0.3mM, 2.0mM or 6.0mM). **(A)** Cannula tip placemnt within DS; sedenatry rats are denoted with black triangles; exercise rats are denoted with white triangles; off-site placements are denoted with an **x**. **(B)** Mean freezing behavior presented in two minute blocks (pre-shock scores are not different and therefore overlap). **(C)** Shuttle box escape latencies for one block of 2 FR-1 trials (FR-1) and five blocks of 5 FR-2 trials (FR-2). **(D)** The mean percent shock elicited freezing for the entire 20-min observation period. **(E)** The mean escape latency for all 25 FR-2 escape trials. Data represent group means $\pm$ SEM. * $p<0.05$ relative 0.3mM groups and off-site control group; Φ $p<.05$ relative to 2.0mM sedentary group.

Voluntary exercise decreases levels of 5-HT_{2C}R mRNA in the dorsal medial striatum and both the basolateral and central amygdala

Rats were allowed voluntary access to running wheels (n=6) or remained sedentary (n=6) for 6 weeks to determine the effects of regular physical activity on 5-HT_{2C} mRNA levels. Average running distance steadily increased over the 6 week duration of the experiment (Figure 4B). Repeated measures ANOVA revealed significant main effects of time ($F_{(6, 114)} = 51.312$; $p<0.0001$) and exercise ($F_{(1, 19)} = 9.960$; $p = 0.0052$) on body weight (Figure 4A). After 1 week, exercised rats weighed less than sedentary rats for the duration of the experiment.

Six weeks of wheel running, compared to sedentary housing, resulted in a trend toward a significant reduction in 5-HT_{2C} mRNA levels (Figure 4C) in the dorsal medial striatum (DMS) ($F_{(1, 10)} = 4.886$; $p=0.0515$), the basolateral amygdala (BLA) ($F_{(1, 10)} = 5.317$; $p=0.0438$) and the central amygdala (CeA) ($F_{(1, 10)} = 11.563$; $p=0.0068$). No difference in 5-HT_{2C}R mRNA was observed in the dorsal lateral striatum (DLS; $F_{(1,10)} = 1.281$; $p=.2841$).

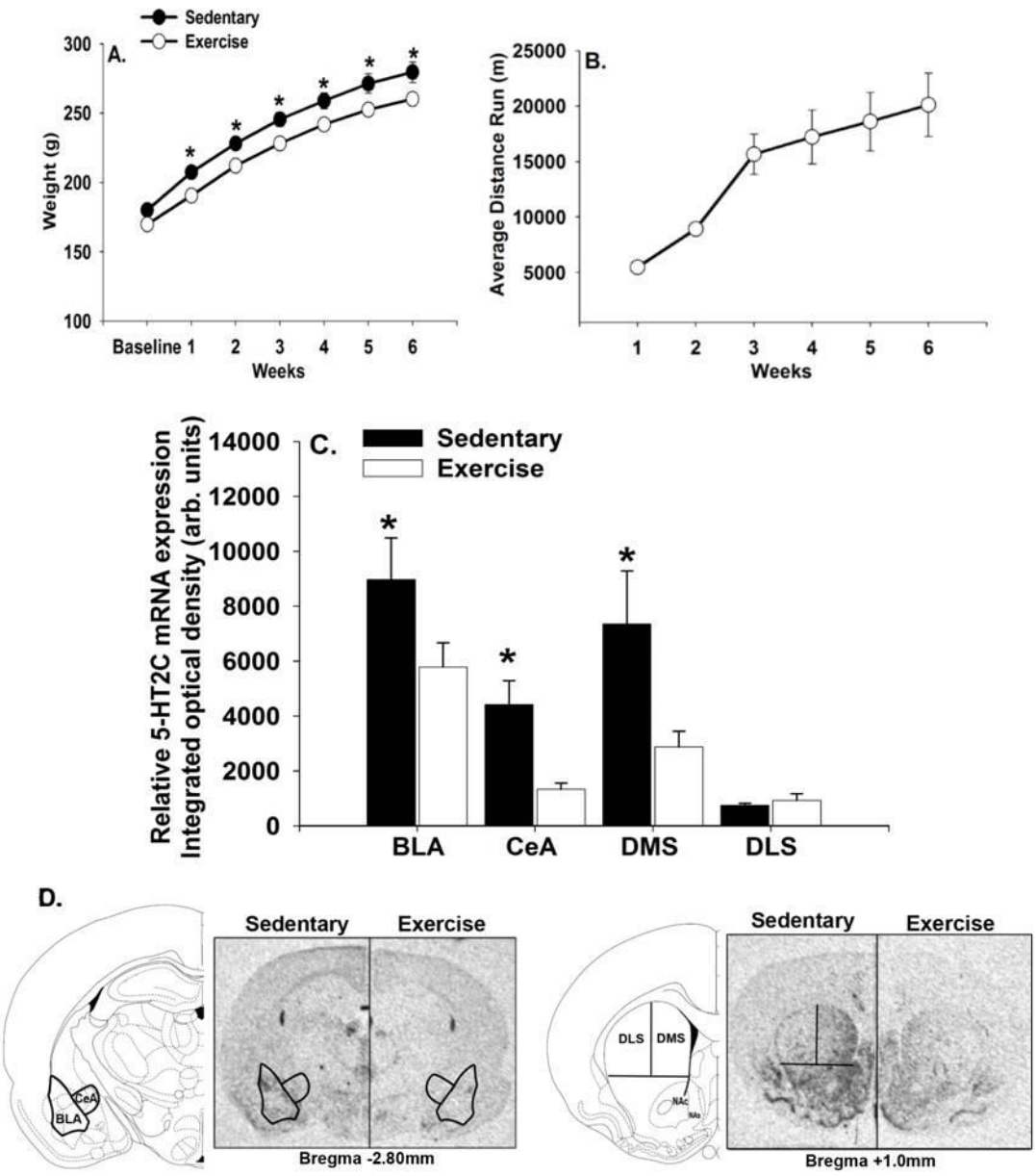

Figure 4. Six weeks of voluntary wheel running decreases 5-HT_{2C} mRNA expression. **(A)** Mean weekly body weight changes (grams) of physically active and sedentary rats. **(B)** The mean distance (meters) run each week. **(C)** Relative levels of 5-HT_{2C} receptor messenger ribonucleic acid (mRNA) in the basolateral amygdala (BLA), central amygdala (CeA), dorsal medial striatum (DMS), and dorsal lateral striatum (DLS) of sedentary rats or rats allowed voluntary access to running wheels for six weeks. **(D)** Representative autoradiographs showing in situ hybridization for 5-HT_{2C} mRNA in the BLA and CEA. **(E)** Representative autoradiographs showing in situ hybridization for 5-HT_{2C} mRNA in the DMS, and DLS. Values represent mean integrated density $\pm$ SEM. * $p \leq 0.05$ relative to respective exercise groups.

Discussion

The current data demonstrate for the first time that exercise can reduce anxiety-like behaviors produced by selective 5-HT_{2C}R agonists administered into discrete brain regions, and implicate 5-HT_{2C}R in the BLA as a potential target for the anxiolytic properties of exercise. Specifically, 6 weeks of voluntary wheel running increased the dose of intra BLA and DS CP809110 necessary to produce exaggerated fear and interference with escape learning, respectively. In-situ hybridization revealed that voluntary wheel running decreased the levels of 5-HT_{2C}R mRNA in brain regions implicated in these behaviors, including the BLA and the DS. These data add to our understanding of the neural pathways and mechanisms underlying the psychological and behavioral benefits associated with regular physical activity.

Prior work has shown that 5-HT_{2C}R agonist injections into the BLA increase anxiety-like behavior (Campbell and Merchant, 2003, Cornelio and Nunes-de-Souza, 2007, Christianson et al., 2010, Strong et al., 2011). Here we report that six weeks of voluntary wheel running was sufficient to reduce 5-HT_{2C}R agonist-induced exaggerated fear. Physical activity, therefore, may reduce the expression of some anxiety-like behaviors through a reduction in the expression, sensitivity or function of 5-HT_{2C}R in the BLA. Additionally, voluntary wheel running reduced levels of 5-HT_{2C}R mRNA in the BLA, suggesting that physical activity may reduce the behavioral consequences of 5-HT_{2C}R agonist administration via a reduction in transcription of 5-HT_{2C}R. Interestingly, voluntary wheel running also reduced 5-HT_{2C}R mRNA in the CeA, another area implicated in fear behavior (Campeau and Davis, 1995, Wilensky et al., 2006). The role of the 5-HT_{2C}R in the CeA in anxiety, however, remains relatively unknown. 5-HT_{2C}Rs are expressed throughout the amygdala complex, but 5-HT_{2C}R mRNA, as well as receptor density, appears to be greatest in the BLA (Li et al., 2003). Moreover, activation of 5-HT_{2C}R in the CeA

has no effect on the expression of some types of anxiety-like behaviors (Campbell and Merchant, 2003, Christianson et al., 2010). Instead, it appears that anxiety-like effects of 5-HT activity in the CeA are more likely mediated by 5-HT_{1A} receptors (Li et al., 2012). Further work is necessary to determine the functional role of a reduction of 5-HT_{2C}R mRNA levels in the CeA of physically active rats.

In addition to the BLA, the current data implicate the 5-HT_{2C}R in the DS as a target for the anxiolytic effects of exercise. The DS plays a critical role in instrumental learning (Yin et al., 2005, Yin et al., 2006, Yin, 2010), and the ability to select the correct behavioral strategy in order to escape danger is vital to an organism's survival. In the current behavioral paradigm, escape learning is a form of instrumental learning in which the rat must learn to successfully associate a specific action (running through the shuttle box door) with the desired outcome (termination of foot shock). Consistent with prior work (Strong et al., 2011), we observed that 5-HT_{2C}R activation in the DS is sufficient to interfere with escape learning. Here we report that exercise increased the dose of CP809101 necessary to interfere with shuttle box escape learning. Given that disrupted processing of aversive stimuli (Ferreri et al., 2011) and difficulty engaging in goal-directed behavior (Tull et al., 2007) are often co-morbid with anxiety disorders, the current results implicate physical activity as a potential therapeutic option for patients presenting with symptoms of anxiety and cognitive dysfunction.

The current data also indicate that voluntary wheel running has a selective effect on 5-HT_{2C} mRNA expression in different areas of the striatum. Voluntary wheel running reduced the expression of 5-HT_{2C}R mRNA in the dorsal medial, but not the dorsal lateral, striatum. It is widely accepted that distinct sub-regions of the DS mediate different aspects of instrumental learning. The early stages of instrumental learning are modulated by the dorsal medial striatum,

whereas the later stages of instrumental learning are modulated by the dorsal lateral striatum [for review see (Balleine et al., 2009, Lovinger, 2010)]. The fact that the acquisition of the escape contingency in the current paradigm occurs rapidly (within the first 5 training trials) seems to indicate a role for the medial DS. Therefore, it is possible that voluntary wheel running reduces 5-HT_{2C}R-mediated interference with escape learning through a reduction in 5-HT_{2C}R mRNA transcription in the medial DS. The mechanism by which exercise reduces levels of 5-HT_{2C}R mRNA, however, has yet to be determined.

It is also important to note that the 5-HT_{2C}R is known to undergo post-translational modifications. Increasing evidence suggests that the editing of 5-HT_{2C}R mRNA can lead to the expression of multiple 5-HT_{2C}R isoforms that have different G-protein activity and affinities for 5-HT (Burns et al., 1997, Wang et al., 2000), altered basal (Herrick-Davis et al., 1999) and constitutive activity (Niswender et al., 1999), as well as desensitized intracellular effects such as agonist-induced calcium release (Price and Sanders-Bush, 2000). These editing-induced changes in 5-HT_{2C}R function have been speculated to play a critical role in the etiology of anxiety and depression (Gurevich et al., 2002b, Gardiner and Du, 2006, Berg et al., 2008, Iwamoto et al., 2009). Additionally, 5-HT_{2C}R mRNA editing changes seem to occur after perturbations of 5-HT levels. Specifically, persistent increases in 5-HT neurotransmission, via drugs or SERT gene deletion, increase the occurrence of 5-HT_{2C}R mRNA editing events, while at the same time decreasing 5-HT_{2C}R responsiveness (Gurevich et al., 2002a, Barbon et al., 2011, Moya et al., 2011). Interestingly, acute bouts of physical activity can also increase central 5-HT (Chaouloff, 1994, Davis and Bailey, 1997). It is possible that there is an overlap between some of the underlying mechanisms of therapeutic drugs and exercise, such that persistent changes in 5-HT neurotransmission over extended periods of time, whether due to a regular drug regiment or

exercise program, can produce long-term plastic changes in the function of post-synaptic modulators of behavior such as 5-HT_{2C}R. In addition to changing mRNA levels in discrete brain regions, exercise may also affect 5-HT_{2C}R pre-mRNA editing events, which might contribute to our observed reduction in the expression of anxiety-like behaviors. The effect of regular voluntary exercise on the editing of 5-HT_{2C}R mRNA needs to be further explored.

In conclusion, physical activity reduces anxiety-like behaviors produced by 5-HT_{2C}R activation in discrete brain regions. The current data extend previous work identifying the 5-HT_{2C}R as a relevant target for pharmacological discovery, as well as shed light on potential mechanisms which underlie the anxiolytic effects of physical activity. Finally, our results further demonstrate the prophylactic and therapeutic potential of physical activity in the treatment of the behavioral symptoms of stress-related psychiatric disorders such as anxiety.

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Chapter 4

General Discussion

The experiments included in this chapter
were conducted by P.V. Strong
in collaboration with M.W. Hale, C.A. Lowry, J. Amat, and S.F. Maier,
under the supervision of M. Fleshner and B.N. Greenwood

Introduction

Implications of the data presented in each chapter of this dissertation are discussed in each respective chapter. However, a few final questions warrant some additional discussion. First, why does exercise constrain stress-induced increases in c-Fos in 5-HT neurons in the DRN but doesn't seem to prevent stress-induced sensitization in DRN projection sites? Second, what are some potential mechanisms by which 5-HT_{2C}R activation in the DS interferes with escape learning? Finally, we will present some preliminary data that we have recently collected as we try to get closer to understanding the underlying mechanisms by which stress and/or exercise may cause plastic changes in the DS and thereby change behavior.

Exercise and the DRN

Why does exercise constrain the stress-induced increase in c-Fos expression in the DRN but doesn't seem to prevent stress-induced sensitization?

In chapter 1, we presented preliminary data which seems to suggest that the stress-protective effects of exercise may not be due to constraint of stress-induced DRN activity, per se. These results are surprising given that our laboratory has previously shown that 6 weeks of voluntary wheel running is sufficient to constrain stress-induced activation of the DRN, as measured by c-Fos expression in the DRN of rats 90 minutes after exposure to an uncontrollable tail shock stressor (Greenwood et al., 2003). However, c-Fos induction has been shown to habituate to repeated exposure to a stimulus such as repeated restraint, repeated exposure to a novel environment, repeated immune stimulation, or repeated drug exposure (Umemoto et al., 1994, Curran et al., 1996, Struthers et al., 2005, Girotti et al., 2006, Kohman et al., 2010). After repeated exposure to a homo-typic stimulus, subsequent exposure to a novel stimulus can reveal a sensitization of c-Fos induction in a variety of brain areas (Weinberg et al., 2009). Long-term voluntary wheel running, however, may be capable of habituating the c-Fos response to novel

stressors, such as tail shock. Indeed, repeated exposure to voluntary wheel running or forced treadmill training seems to moderately habituate c-Fos induction in other brain regions such as the hippocampus (Lee et al., 2003, Clark et al., 2010). Additional work is needed to determine if indeed long-term voluntary wheel running habituates c-Fos induction in the DRN in our model and to determine how exercise produces stress-resistance, specifically at the level of activation and/or constraint of the DRN.

5-HT_{2C} Receptors and the Dorsal Striatum

What are some potential mechanisms by which 5-HT_{2C} activation in the striatum may interfere with instrumental escape learning?

In chapter 2, we presented data which indicated that 5-HT_{2C}R activation in the dorsal striatum was necessary for the expression of the stress-induced interference with instrumental escape learning. It is important to note that distinct sub-regions of the DS are thought to mediate different aspects of instrumental behavior (Balleine et al., 2009). Importantly, goal-directed learning of is initiated in the medial DS, whereas the long-term habit memory required to execute previously learned sequences is thought to be stored in the lateral DS (Yin et al., 2005a, Yin et al., 2005b, Yin et al., 2006, Corbit and Janak, 2010). Given that escape performance in our experimental paradigm is measured immediately after exposure to a footshock, it is possible that the medial DS modulates this early stage of the behavior. However, the lateral DS has also been recently implicated in the acquisition of simple procedural learning (Yin, 2010), and could also play a role in the acquisition of the shuttle box escape task.

Interestingly, we have recently observed that both the medial and lateral aspects of the dorsal striatum have increased expression of the immediate early gene zif268, both immediately and 2 hours after exposure to uncontrollable stress (Figure 1). Zif268 is an immediate early gene with a wide-spread neural distribution, and it has been used as a marker of neuronal activation

because of its rapid induction in acute stress paradigms (Worley et al., 1991, Rosen et al., 1992, Cullinan et al., 1995, Girotti et al., 2006). This data suggests that both areas of the striatum are stress-responsive and that a prior history of wheel running does not seem to affect zif268 induction, an perhaps neural activity in response to uncontrollable stress, in either region.

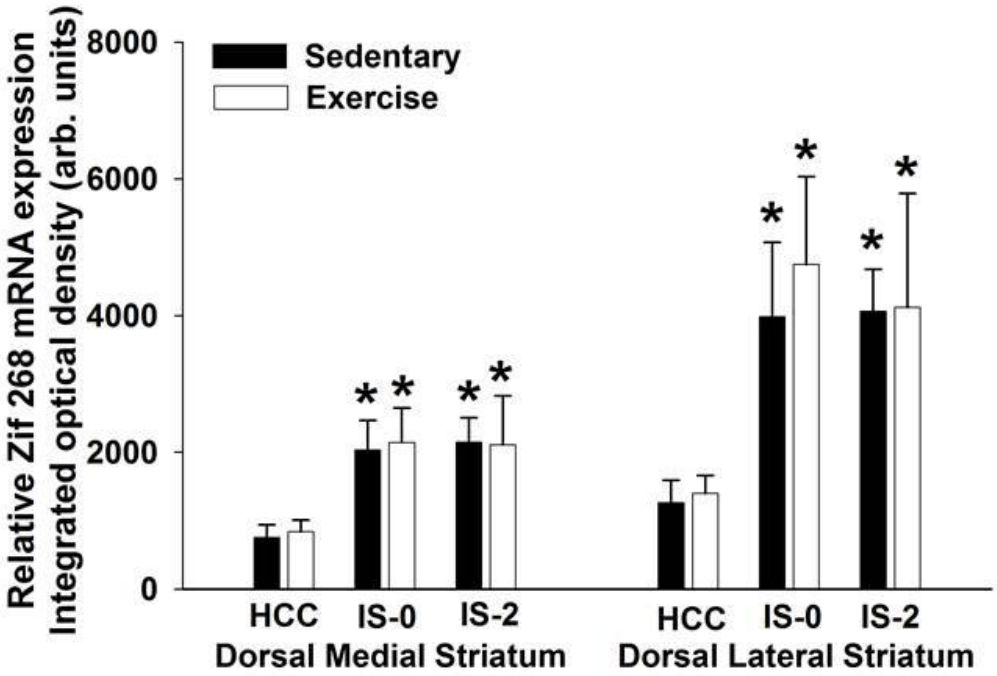
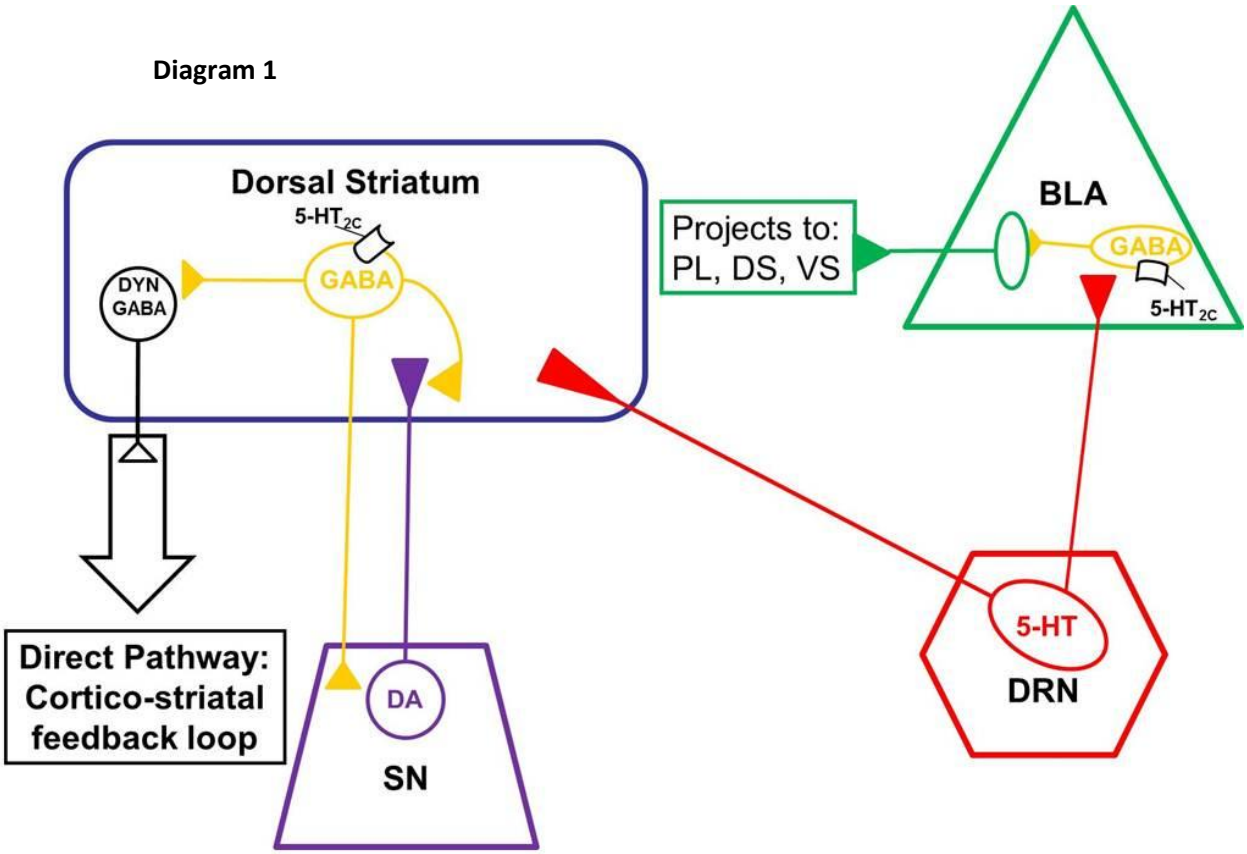


Figure 1. After six weeks of exercise (voluntary wheel running) or sedentary conditions, rats were exposed to uncontrollable stress or no stress. Immediately (IS-0) or 2 hours (IS-2) after uncontrollable stress, rats were sacrificed and brains were removed and sliced. I*n situ* hybridization analysis was conducted to determine mRNA levels of the immediate early gene zif268 in the dorsal medial and dorsal lateral striatum. Uncontrollable stress increased zif268 levels in both brain regions and this effect was still present 2 hours after stress. Six weeks of wheel running had no effect on basal or stress-induced expression. Groups were as follows: Sedentary/HCC=7; Sedentary/IS-0=7; Sedentary/IS-2=8; Exercise/HCC=8; Exercise/IS-0=8; Exercise/IS-2=8.
*** p<.05 relative to HCC groups.**

While it remains unknown which of these sub-regions is critical for shuttle box escape behavior, there are several plausible mechanisms by which 5-HT_{2C}R activation interferes with escape learning. For example, one important function of dopamine (DA) in the striatum is to support the stimulus-response association aspects of instrumental learning (Lex and Hauber, 2010), and 5-HT_{2C}R activation can decrease DA neurotransmission in the DS (Alex et al., 2005). It is possible, therefore, that an increase in extracellular 5-HT in the striatum after exposure to uncontrollable stress could interfere with a DA-dependent mechanism involved in escape learning (Diagram 1).

Diagram 1



1. DRN projects to DS and uncontrollable stress increases 5-HT in DS
2. 5-HT_{2C}R activation can decrease DA levels in DS; DA in the DS is released from terminals projecting from cell bodies in the substantia nigra (SN)
3. DA in the DS is necessary for instrumental learning associations
4. Deficits in escape learning could be the result of exaggerated 5-HT release in DS and subsequent activation of 5-HT_{2C} receptors located on GABA neurons leading to decreased DA release in DS or interference with DA-mediated processes.
5. BLA plays a role in encoding the value of instrumental outcomes. Exaggerated 5-HT release in the BLA may bind 5-HT_{2C} receptors on GABA neurons and inhibit BLA encoding, contributing to deficits in escape learning.

We have recently observed that 24 hours after uncontrollable stress, extracellular DA in the DS may be sensitized (Figure 2; preliminary data, 2 rats/grp). It seems that in the presence of a stress-induced increase in exaggerated extracellular 5-HT (page 13 and 36) in the DS, there may not be a simultaneous decrease in DA. Thus, 5-HT_{2C}R activation may not interfere with escape deficits through a decrease in DA release in the DS, per se, but may still interfere with learning processes modulated by DA. Further work is necessary to determine the interactions between 5-HT and DA in the DS in stress-induced escape deficits.

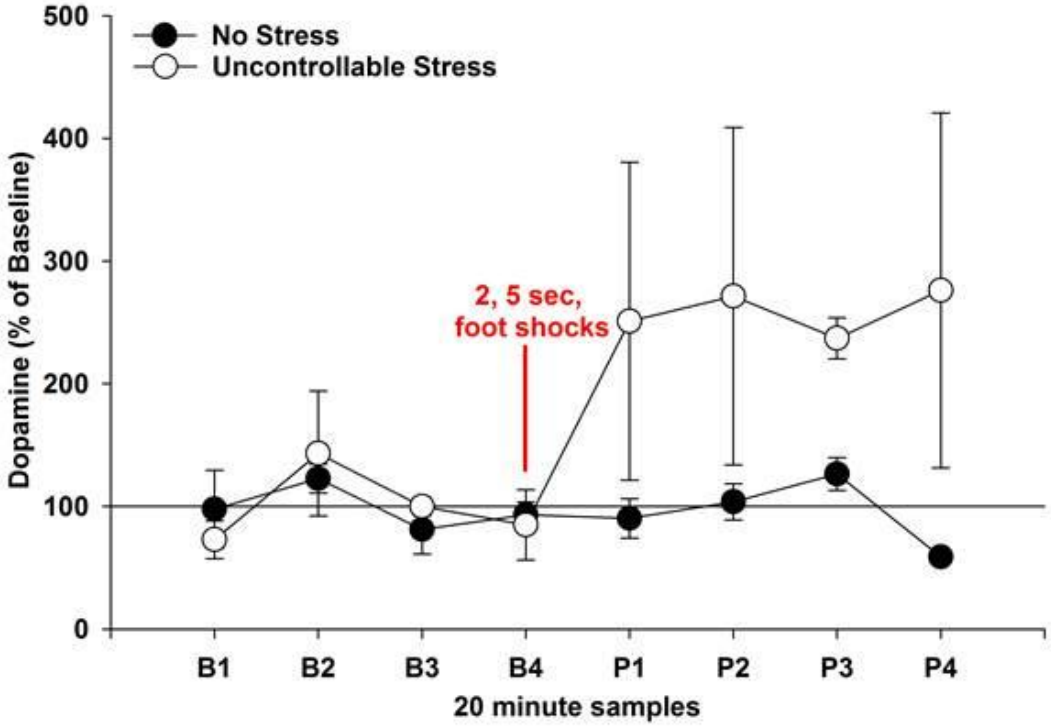


Figure 2. Uncontrollable stress may sensitize DA in the striatum. Mean extracellular DA in the striatum before and after 2 foot shocks. Rats received prior uncontrollable stress or home cage control treatment. DA is expressed as a percentage of the average of four baseline samples (B1 – B4). The 2, 5 sec, foot shocks were delivered immediately after the 4th baseline sample (B4) and dialysis continued for 4 samples after shock (P1 – P4).

Local inhibition of striatal output pathways can also interfere with the activation of cortical pre-motor circuits necessary for instrumental learning. The DS is mainly comprised of γ-amino butyric acid (GABA)-ergic cells, including a large number of projection neurons and a small, but highly influential, population of fast-spiking inhibitory interneurons, which are uniquely identified by their expression of parvalbumin (Kawaguchi, 1992, 1993, Bennett and Bolam, 1994, Kawaguchi et al., 1995). Located primarily in the lateral DS (Bennett and Bolam, 1994), these fast-spiking interneurons receive glutamatergic input from the cortex (Ramanathan et al., 2002) and project onto the output neurons of the striatum (Tepper and Bolam, 2004, Tepper et al., 2004). We have also recently verified that 5-HT_{2C}R receptors are co-localized with parvalbumin in the dorsal striatum using quad-color immunofluorescence (Figure 3).

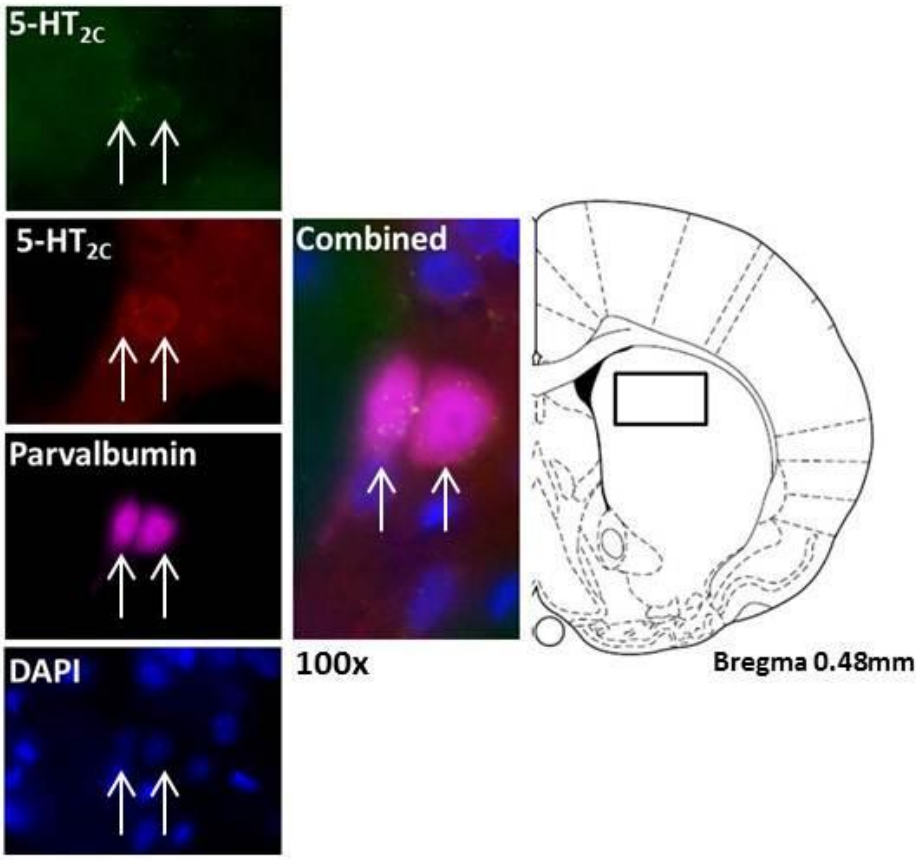


Figure 3. 5-HT2C receptors are co-localized with parvalbumin in the dorsal striatum. Two different antibodies for 5-HT2C receptors were used. DAPI is a nuclear stain used to verify location of cell bodies.

The firing properties and synaptic organization of the fast-spiking interneurons are such that these neurons are poised to mediate feed-forward inhibition of the DS (Koos and Tepper, 1999, Mallet et al., 2005). The presence of 5-HT increases the activity of fast-spiking interneurons and this excitation is inhibited by blockade of the 5-HT_{2C}R (Blomeley and Bracci, 2009). Additionally, activation of the 5-HT_{2C}R reduces overall c-Fos induction in the DS (De Deurwaerdere et al., 2010), perhaps via activation of fast-spiking interneurons. Stress-induced sensitization of extracellular 5-HT in the DS could interfere with instrumental escape behavior through DS 5-HT_{2C}R, potentially located on fast-spiking inhibitory interneurons, thereby inhibiting striatal activity and instrumental learning processes (Diagram 2). Further work is necessary to determine the exact mechanism by which 5-HT_{2C}R activation in the striatum interferes with escape learning.

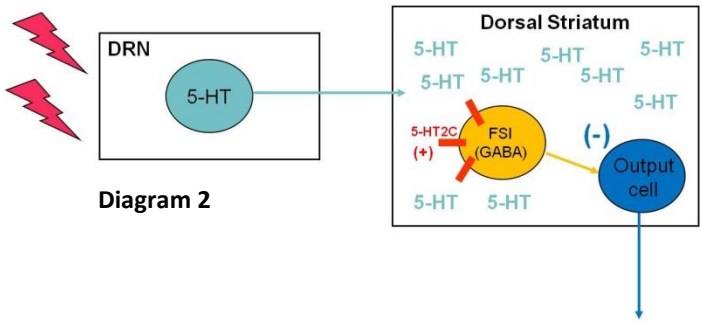


Diagram 2

Conclusions

The studies performed in this dissertation further elucidate the role of 5-HT_{2C}R in stress-induced anxiety, as well as the anxiolytic effects of exercise. We are the first to characterize the effects of stressor controllability and voluntary exercise on the 5-HT response to stress in the DS. Additionally, we delineated the novel role of 5-HT_{2C}R in brain regions implicated in stress-induced anxiety behaviors, specifically the BLA and the DS. Finally, we identified the 5-HT_{2C}R in the BLA and the DS as a potential target for the anxiolytic effects of exercise. Figure 4 depicts a diagram of potential brain areas involved in the stress-protective effects of exercise.

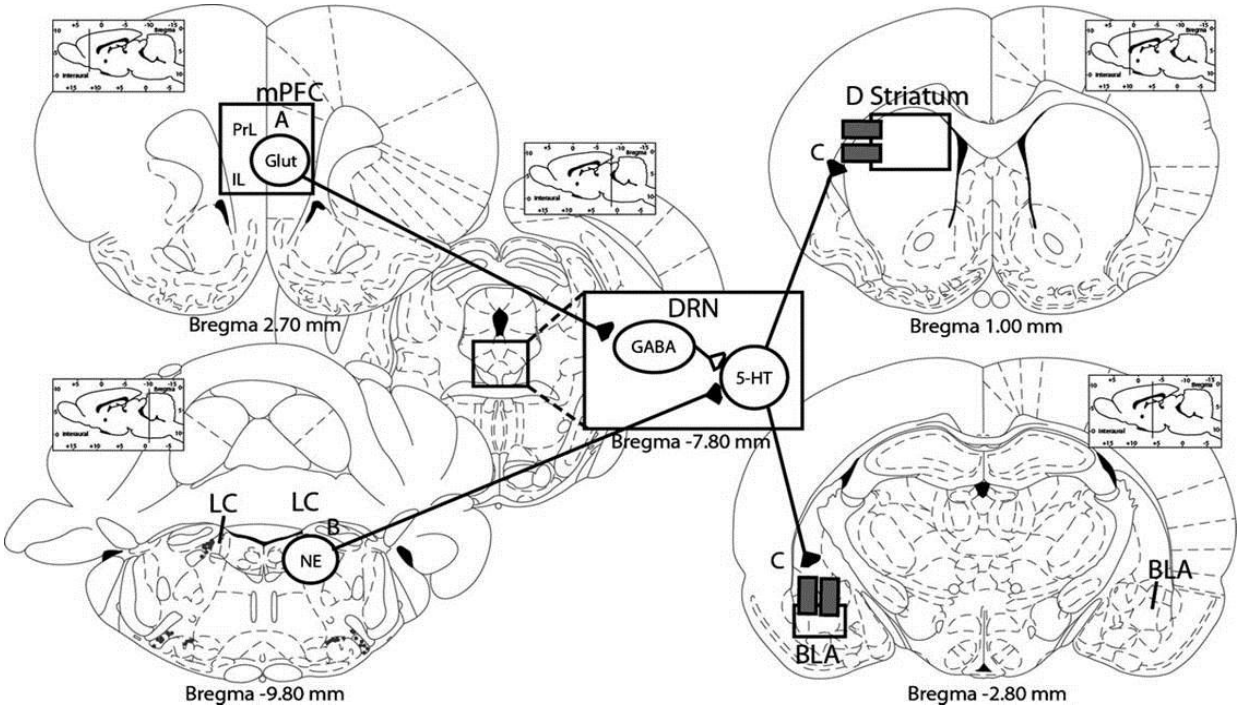


Figure 4. Plasticity produced by 6 weeks of voluntary exercise in the dorsal raphe nucleus (DRN) afferents and projection sites potentially contributing to exercise-induced stress resistance. A. Increased activation of the medial prefrontal cortex (mPFC) during stressor exposure could actively inhibit DRN 5-HT neurons. B. Constraint of norepinephrine (NE) neurons in adrenergic regions, such as the locus coeruleus (LC), could reduce excitation of DRN 5-HT neurons during stressor exposure. C. A reduction in the expression of 5-HT_{2C}R in DRN projections sites critical for the expression of stress-induced behaviors could reduce the behavioral impact of increase in 5-HT in these regions. BLA, basolateral nucleus of the amygdala; D Striatum, dorsal striatum; Glut, glutamate; IL, infralimbic cortex; PrL, prelimbic cortex. Brain diagrams adapted from Paxinos and Watson (Paxinos, 1998). (Figure and caption adapted from (Greenwood and Fleshner, 2011)).

The results of these studies may have far reaching impact as 5-HT_{2C}R activity in the DS has been identified as a key pharmacological target in other disorders such as Parkinson's disease, Huntington's disease, drug addiction, and obsessive compulsive disorder. Examination of the mechanisms by which factors such as exercise provide resistance against the behavioral consequences of uncontrollable stress could provide insight into the neurobiology of stress-related psychiatric disorders and perhaps lead to the identification of novel therapeutics or preventative strategies.

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