Trpc4 as a Gene Target for Addiction and the Effects of Stressor Controllability and Addiction on Anxiety and Depression in Rodent Models

By
Kristin Rasmus Burrow
B.A., University of Colorado Boulder, 2004

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Written by Kristin Rasmus Burrow

Has been approved for the Department of Psychology and Neuroscience

______________________________________________________
Dr. Marissa A. Ehringer (co-chair)

______________________________________________________
Dr. John K. Hewitt (co-chair)

______________________________________________________
Dr. Gregory Carey

______________________________________________________
Dr. Steven F. Maier

______________________________________________________
Dr. Jerry A. Stitzel

Date________________

The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in Psychology and Neuroscience.

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Burrow, Kristin R. (Ph.D., Behavioral Genetics and Neuroscience [Department of Psychology and Neuroscience])

Trpc4 as a Gene Target for Addiction and the Effects of Stressor Controllability and Addiction on Anxiety and Depression in Rodent Models

Thesis directed by Professor Marissa A. Ehringer, Ph.D.

Abstract:

Drug addiction, anxiety, and stress have several commonalities. One is that they all pose a substantial burden on individuals and society, both psychologically and financially. Another is that they have overlapping brain regions and/or neural pathways that are implicated in their etiologies. Consequently, these neuropsychiatric disorders are often exhibited concurrently or sequentially with one another. For these reasons, it is of clinical importance to better understand the mechanisms driving these disorders and how they interact with each other. The following studies aim to shed light onto these topics.

We first investigated the Trpc4 gene as potential target for drug addiction and anxiety. The Trpc4 gene was selected based on its expression pattern in the brain and its function. The gene is abundantly expressed in regions that receive extensive dopamine (DA) input from the brain's reward circuitry. These regions are highly involved in mood regulation and reward-seeking. The TRPC4 ion channel is the product of the Trpc4 gene, and is known for increasing neuronal excitability. These unique properties make Trpc4 an intriguing candidate for addiction and anxiety. Indeed, we found that rats lacking the Trpc4 gene showed an increase in anxiety-like phenotypes and a decrease
in cocaine-seeking compared to wild-type rats.

Next, we examined how repeated cocaine exposure and stressor controllability effect anxiety. Stress is a leading risk factor in developing such disorders as drug addiction and anxiety. However, it is known that the degree of control one has over a stressful situation is predictive of the outcome. Controllable stress has been shown to mitigate the negative effects induced by uncontrollable stress. We found that repeated cocaine exposure does not interfere with this phenomenon, even after a drug withdrawal period.

These studies and previously published studies on the effects of stressor controllability all used rat models. To further our understanding of these concepts, we next wanted to determine if a mouse could be used in order to take advantage of the multitude of transgenic strains available. However, based on our results, it appears that mice are not an ideal organism to study the effects of stressor controllability on neuropsychiatric disorders.
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Chapter One: Introduction

1.1 Cocaine Addiction

1.1.2 Significance to the general public

Nearly 1.4 million Americans meet the DMS-IV criteria for dependence or abuse of cocaine in 2008, costing some individuals their lives and the US government billions of dollars (Center for Behavioral Health Statistics). The total estimated cost of substance abuse in the United States is approximately $600 billion annually, this includes productivity and health- and crime-related costs (Fries et al., 2008). Drug-related crime accounts for more than half of the estimated costs of drug abuse and, in 2009, almost one million emergency room visits involved an illicit drug or a combination of drugs, with cocaine being responsible for nearly half of these incidents (Department of Health & Human Services, 2013). It is evident that drug abuse is a serious issue in the United States. Advancing our understanding of cocaine addiction can have a significant impact on both individuals and society as a whole. Developing more targeted and successful treatments could save lives and reduce the financial burden on taxpayers, communities, hospitals, and penitentiaries.

1.1.3 Neurobiology of addiction

The National Institute on Drug Abuse (NIDA) defines drug addiction as “a chronic, relapsing brain disease that is characterized by compulsive drug seeking and use, despite harmful consequences”. The “brain disease” categorization given to the definition of drug addiction is an important distinction. It is known that repeated
exposure to a drug can lead to long lasting structural and/or functional changes in the brain, many of which can result in self-destructive or harmful behaviors. For example, it has been shown that all classes of drugs of abuse, including cocaine, result in an increase in dopamine (DA) transmission in the brain’s reward pathway, which includes the ventral tegmental area (VTA), nucleus accumbens, amygdala and hippocampus (Carboni et al., 1989; Rowell et al., 1987; Wise, 1984). Additionally, simply a drug-related cue is sufficient to increase DA release (Ito et al., 2000; Volkow et al., 2006). It is theorized that the facilitation in DA transmission by both the drug itself and its cue-induced associations are responsible for the pathological drug-seeking behaviors and, ultimately, drug addiction (Adinoff, 2004; Dalley & Everitt, 2009; Volkow et al., 2004).

### 1.2 Anxiety

#### 1.2.1 Significance to the general public

Drug addiction and anxiety are often concurrently diagnosed. It has been reported that over 50% of individuals in inpatient substance abuse treatment centers report symptoms of general anxiety (Brown et al., 1999). Furthermore, findings from a study by Halikas et al. (1994) found that 76% of drug users in their sample reported having an anxiety disorder before becoming a regular user, indicating that anxious individuals may be using cocaine to self-medicate their symptoms. For the general population, generalized anxiety disorder (GAD) is considered as severe as depression with respect to social functioning, productivity, and healthcare costs (Wittchen & Hoyer, 2001). The disorder is estimated to affect 5.1% of the U.S. population aged 15 – 45 years old and is more commonly diagnosed in women than men. GAD sufferers also
report a high use of medication for their symptoms (Wittchen et al., 1994). Alarmingly, anxiety disorders are the most common disorders among 13 – 17 year old adolescents in the U.S. (Kessler et al., 2005), which is an extremely vulnerable period for drug use and experimentation, and may put these adolescents at a greater risk for drug abuse and addiction later in life. Due to the prevalence and potential consequences of anxiety symptoms, understanding the processes involved in the etiology of anxiety could significantly help in the development of more effective treatment options and work to minimize the severity of other disorders, such as drug addiction, that are often exhibited comorbidly.

1.2.2 Neurobiology of anxiety

Anxiety is generally defined by a sense of uncontrollability surrounding current or future events that present a possibility for negative outcomes for the individual or their loved ones (Barlow, 2003). Symptoms of anxiety can vary greatly and be both psychological and/or physical. Psychological symptoms include: anxious mood, tension, irritability, fears or phobias, insomnia, cognitive disruptions (such as forgetfulness or difficulty concentrating), and depression. Physical symptoms reported include: cardiovascular, gastro-intestinal, respiratory, and general autonomic symptoms (Hamilton, 1959). It is believed that the psychological symptoms of anxiety disorders are a result of an imbalance in neurotransmitter activity in the limbic system, which includes such brain regions as the hippocampus, hypothalamus, amygdala and nucleus accumbens (Martin et al., 2009). Note that many of these brain regions are also implicated in the reward pathway, which is involved in cocaine addiction, indicating that
neuronal activity in the overlapping regions may contribute to both addiction and symptoms of anxiety.

1.3 TRPC4 Channels

The family of canonical transient receptor potential (TRPC) channels have recently been identified as key players in effecting neuronal excitability. TRPC channels are a group of non-selective cation channels that consist of 7 members (TRPC1-7) and they effect neuronal excitability due to the channels’ unique properties, which ultimately result in the release of intracellular Ca\(^+\). They are tetrameric channels that contain three calmodulin sites and an inositol triphosphate (IP\(_3\)) site on the C-terminus of each subunit (Clapham et al., 2001; Ordaz et al., 2005) and are activated in response to Gq protein-coupled receptors (Schaefer et al., 2000). When Gq protein-coupled receptors are activated, it initiates the phospholipase C Beta (PLC\(\beta\)) pathway. Subsequently, this causes an increase in IP\(_3\), which then binds to the IP\(_3\) receptor located on the endoplasmic reticulum. This leads to the release in intracellular Ca\(^+\) and, ultimately, increases neuronal excitability (Zhu et al., 1996). See Figure 1.1 for a diagram of a TRPC channel.
As mentioned earlier, the TRPC family consists of 7 members. The TRPC4 channel is encoded by the $Trpc4$ gene and is one of the more abundantly expressed in the adult mammalian brain (Fowler et al., 2007). It is of particular interest to addiction and anxiety researchers due to its expression pattern. Previous findings by Fowler et al. (2007) show that TRPC4 channels are highly concentrated in corticolimbic regions, which include the lateral septum, hippocampus, prefrontal cortex (PFC), and amygdala. These regions receive extensive DA input from the brain’s reward circuitry. This circuitry is involved in dopaminergic signaling in the VTA, nucleus accumbens, PFC, amygdala, and hippocampal formation, and is pivotal in governing mood regulation, as well as reward-related decision-making, such as drug seeking. Furthermore, it has been shown
that repeated drug exposure alters this pathway by significantly enhancing dopaminergic activation (Henry et al., 1998). The presence of TRPC4 channels in this influential pathway, in addition to the critical role it plays in neuronal excitability and plasticity implies that these channels may play a pivotal role in regulating anxiety and serve to alter cellular excitability induced by cocaine exposure.

1.4 Stress

1.4.1 Significance to the general public

Also relevant to the development of drug addiction and anxiety is stress. Stress causes a severe burden on individuals and society, and it is well established that stress can have detrimental effects on an individual’s health (Cohen et al., 1995; Cohen et al., 2007; McEwen, 1998). It is not uncommon to come across reports in the media linking stress to a myriad of health complications, including hypertension, obesity, cancer, and diabetes, to name a few. The American Institute of Stress names stress as the most prevalent health issue, surpassing the common cold. A survey conducted by the Center for Disease Control found that 53.5% of people ages 18 and older had experienced adverse health effects due to stress in the past year and is responsible for an estimated $10 to $20 billion annual loss in industrial productivity (U.S. Department of Health and Human Services, 1990). Stress is an inevitable aspect of the human experience and, although individuals often recover from stressful events, it is evident that society would benefit from a clearer understanding of stress and its effects on behavior and the brain, and how best to mitigate its negative consequences.
1.4.2 Neurobiology of stress

Benson and Stuart (1992) define stress as “the perception of a threat to one’s physical or psychological well-being and perception that one is unable to cope with that threat”. Stress is often provoked by an adverse event and can lead to neurobiological and behavioral changes. Psychological disorders, such as anxiety, depression, and mood disorders frequently develop as a result of stress exposure (Bryant, 2010). Because the brain determines what is threatening, and therefore stressful, it is the fundamental organ of the stress response (McEwen, 2007). The stress response system includes interactions among multiple brain systems, namely the hypothalamus-pituitary-adrenal (HPA) axis, the hippocampus, amygdala, and prefrontal cortex.

1.4.2.1 HPA axis

When the brain detects a threat or stressful event, the HPA axis becomes activated. The HPA axis is a cornerstone of the stress system and culminates in the production of glucocorticoids. Upon the detection of threat, corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) are released from the hypothalamus. This causes the secretion of adrenocorticotropic hormone (ACTH) from the pituitary gland and the subsequent production of glucocorticoids and catecholamines (adrenaline and noradrenaline) by the adrenal cortex (Lupien, 2009). Once the detection of threat has dissipated, feedback loops throughout the stress system are triggered and the HPA axis returns to homeostasis.

Glucocorticoid receptors can be found in various brain regions, including the hippocampus, amygdala, and prefrontal cortex, making these brain regions pivotal in
mediating the HPA axis. Cortisol is the primary glucocorticoid in humans and it interacts with a number of major organ systems, including the autonomic nervous system, cardiovascular system, metabolism, and immune response. All of these systems interact with the HPA system and have been implicated in the physiological and behavioral response to stress (Lupien et al., 2009; McEwen & Gianaros, 2010).

1.4.2.2 Hippocampus

The hippocampus is known for its contributions to learning and memory function, however, it is also an important structure in the stress response by playing a key role in negative feedback regulation of the HPA axis (Herman et al., 2005). The hippocampus exhibits a high density of glucocorticoid receptors and, upon binding, cognitive processes are enhanced, thus affecting motivation and mood, excitability promotion, and are generally thought to be protective (McEwen & Gianaros, 2010). However, it should be noted that under conditions of chronic stress, in which excess amounts of glucocorticoids are present, a neurotoxic effect can occur, causing adverse effects on the hippocampus (McEwen, 2007; Seckl et al., 1991).

1.4.2.3 Amygdala

The amygdala is often associated with its role in fear detection and is another important brain structure implicated in the stress response. In contrast to the hippocampus, the amygdala is involved in activating the HPA axis by detecting and responding to environmental threats (Herman et al., 2005; McEwen & Gianaros, 2010).
1.4.2.4 Prefrontal cortex

The PFC is typically associated with top-down inhibition, which is consistent with its role in the HPA axis. Based on previous studies in rodents and humans, the medial PFC has been shown to be critical to the negative feedback loop under stress related conditions (Diorio et al., 1993; McEwen & Gianaros, 2010).

1.5 Stressor controllability

1.5.1 Significance to the general public

As mentioned previously, stress is often provoked by an adverse event and can impact brain function and structure. This makes stress a primary source of risk for a number of neuropsychiatric disorders, including anxiety, depression and drug abuse. However, many individuals exposed to an adverse or stressful life event do not develop these negative outcomes. Given the excessive burden that stress imposes on individuals and society as a whole, it is of clinical importance to better understand the mechanisms that promote stress resilience.

1.5.2 Neurobiology of stressor controllability

Multiple studies have shown that the degree of behavioral control an individual has over a stressor plays a crucial role on the impact of the event (Anisman et al., 1992; Seligman & Maier, 1967; Weiss et al., 1981). Studies in rats show that behavioral control over a stressor can mitigate negative stress-induced consequences, such as the development of anxiety or depression. For example, rats exposed to an uncontrollable stressor (IS = inescapable stress) show a significant decrease in social exploration time
compared to rats exposed to a controllable stressor (ES = escapable stress) in a juvenile social exploration test (Christianson et al., 2008). Furthermore, IS-exposed rats will self-administer significantly more oxycodone than ES-exposed rats in a conditioned place preference model (Der-Avakian et al., 2007). Finally, it has been shown that prior exposure to ES reduces subsequent contextual fear conditioning, while prior exposure to IS leads to a potentiation, indicating an exaggerated Pavlovian fear-response (measured by freezing behavior) following IS treatment (Baratta et al., 2007). In other words, uncontrollable stress produces behaviors in rats that resemble symptoms of depression and anxiety, while exactly equated controllable stress does not.

1.5.2.1 Dorsal raphe nucleus

These IS-induced behavioral consequences have been linked to functional changes in the brain. The dorsal raphe nucleus (DRN) is one brain region that has been highly implicated in this process. Previous studies show that the DRN exhibits greater serotonergic (5-HT) activation following IS exposure, when compared to ES exposure (Amat et al., 1998; Grahn et al., 1999; Maswood et al., 1998). Pharmacological activation of DRN 5-HT neurons produce the same sequelae of IS behaviors in the absence of stress (Maier et al., 1995). Importantly, 5-HT is a key neurotransmitter in mood regulation. It is a primary target of antidepressant medications and for the treatment of some anxiety disorders. A principle source of 5-HT neurons projecting to other areas of the brain is the DRN.

Upon exposure to an adverse event, the DRN receives input from several brain regions; namely the lateral habenula, locus coeruleus (LC), and the bed nucleus of the
striata terminalis (BNST), among others (Amat et al., 2001). These inputs lead to an increase in 5-HT neuronal activity in the DRN. Consequently, it is known that the DRN projections include the amygdala, nucleus accumbens, and the periaqueductal grey; all of these brain regions have been linked to such behaviors as anxiety and fear, drug reward, and escape responding, respectively. Likewise, these projection regions show elevated extracellular 5-HT levels following IS treatment but not ES treatment (Amat et al., 1998; Christianson et al., 2010). Therefore, behavioral consequences exhibited by IS-exposed rats, such as reduced social exploration, are likely DRN moderated and caused by the excess activation in 5-HT.

**1.5.2.2. Ventral medial prefrontal cortex**

Because the DRN is a brainstem nucleus, it is unlikely to possess the integrative and complex function of sensing controllability or, in other words, differentiating IS from ES. Previous studies show that the DRN receives a majority of its cortical input from the ventral medial prefrontal cortex (vmPFC) (Peyron et al., 1998). After some investigating, it is now known that the vmPFC is responsible for detecting control over a stressor and communicating this to the DRN. When the vmPFC senses control, it inhibits the 5-HT neurons in the DRN by way of glutamatergic pyramidal neuronal projections. These projections synapse onto GABAergic interneurons that then inhibit the 5-HT cells, thus mitigating the IS-induced behavioral changes (Amat et al., 2005; Jankowski & Sesack, 2004; Maier & Watkins, 2010). See Figure 1.2 for a schema of how stressor controllability modulates stress effects.
Figure 1.2: Schema of how stressor controllability modulates stress effects. Glut = glutamate, vmPFC = ventral medial prefrontal cortex, L. Habenula = lateral habenula, LC = locus coeruleus, BNST = bed nucleus of the stria terminalis, GABA = gamma aminobutyric acid, 5-HT = serotonin, DRN = dorsal raphe nucleus. Taken from Maier and Watkins (2010)

1.5.3 Animal model of stressor controllability

A well-characterized model for studying the stressor controllability phenomenon in rats is called the ES/IS tail-shock paradigm (Amat et al., 1998). It involves exposing one group of animals to ES, in which rats have behavioral control over terminating each of a series of tail-shocks by turning a wheel; another group experiences IS in which durations are yoked to those produced by the ES subjects, with no control over terminating the tail-shocks. Importantly, because the subjects are yoked, they are exposed to an identical shock pattern and intensity.

Following this treatment, the ES/IS stress groups can be compared both behaviorally and physiologically. An investigator can utilize any number of behavioral
tests to deduce stressor controllability effects on anxiety, depression, or drug-seeking phenotypes. Neurophysiological effects can also be measured to better understand the neural pathways involved in stress resilience.

The following studies examine the intersection of stress and drug addiction on anxiety and depression. In Chapter 2, we investigate the trcp4 gene as a possible influence on anxiety and drug addiction using a rat model of anxiety and self-administration, in addition to a number of histological and electrophysiological techniques. In Chapter 3, we examine how repeated cocaine exposure and withdrawal interact with stressor controllability in regulating anxiety-like behaviors. Our aim in Chapters 4 and 5 was to determine if the stressor controllability phenomenon that is so widely reported in rats and other mammals, could be transferred to a mouse model. Chapter 4 takes a behavioral approach, while Chapter 5 examines the neurophysiological changes that occur in mice following controllable or uncontrollable tail-shock. If successful, it would allow investigators to utilize the wide range of transgenic mice that are available to further our understanding of the effects of stress and resilience on neuropsychiatric disorders.
Chapter Two: Trpc4: A Possible Gene Target for Anxiety and Drug Addiction

2.1 Introduction

Canonical transient receptor potential (TRPC) channels are a group of non-selective cation channels that have recently gained attention for their involvement in neuronal excitability. This family of channels consists of 7 members (TRPC1-7) that can be activated in response to Gq protein-coupled receptor activation (Schaefer et al., 2000). Stimulation of Gq protein-coupled receptors activates phospholipase C Beta (PLCβ) producing elevations in inositol triphosphate (IP3) and intracellular Ca2+ (Zhu et al., 1996). TRPC channels contain three calmodulin sites and an IP3 site on the C-terminus of each subunit (Clapham, et al., 2001; Ordaz et al., 2005). Thus, intracellular signaling resulting from the stimulation of Gq protein-coupled receptors can enhance the activity of TRPC channels (Schaefer et al., 2000). These properties allow TRPC channels to play a pivotal role in responding to intracellular Ca2+ signaling, thereby affecting neuronal excitability.

TRPC4 channels, specifically, are co-localized with metabotropic glutamate type 1 receptors (mGluR1) (Phelan et al., 2012). Activation of mGluR1 receptors produce prolonged depolarizing potentials in lateral septal neurons and this effect is eliminated in trpc4 knock-out (KO) mice and rats (Phelan et al., 2012). Seizure-induced neuronal cell death is greatly reduced in trpc1/4 double-knockout mice in the pyramidal cell layers of the hippocampus and in the lateral septum suggesting an important role for TRPC4 channels in facilitating neuronal excitability (Phelan et al., 2012).
The TRPC4 channel is one of the two most abundant TRPC channel subtypes found in the adult mammalian brain (Fowler et al., 2007). Previous findings indicate that \textit{trpc4} mRNA is highly expressed in corticolimbic regions including the lateral septum, hippocampus, prefrontal cortex (PFC), and the amygdala (Fowler et al., 2007; Phelan et al., 2012). These regions receive extensive input from dopamine (DA) neurons in the ventral tegmental area (VTA) and are associated with the brain’s reward and emotion circuitry. This expression pattern, along with its ability to regulate neuronal excitability, raises the interesting possibility that TRPC4 channels may be important in regulating anxiety, learning and memory, and motivated behaviors.

In the present study we used a combination of behavioral, immunohistochemical and electrophysiological approaches to assess the functional role of the TRPC4 channels. To determine if these channels have an effect on anxiety-like behavior, we compared the activity of \textit{trpc4} KO and wild-type (WT) rats in a juvenile social exploration test. To test simple learning with natural rewards, we compared the performance of \textit{trpc4} KO and WT rats using a Y-maze discrimination task with water reward and lever-pressing acquisition with sucrose reward. To compare \textit{trpc4} KO and WT rats’ performance on more complex learning, we used two paradigms: a serial reversal shift paradigm with water reinforcement, in which the reward and non-reward cues switched between sessions; and a conditional reversal shift paradigm in which a tone cue signaled the switching of reward cues within sessions. Furthermore, to assess the role of TRPC4 channels in modulating cocaine infusion, we compared \textit{trpc4} KO and WT rats’ performance during a cocaine self-administration (SA) paradigm.
We used immunohistochemistry to assess the spatial distribution of Trpc4 expression in the adult rat brain. Finally, we used an electrophysiological approach to explore the functioning of TRPC4 channels in the VTA. Here, we measured directly the changes in cellular excitability in trpc4 KO compared to WT rats.

2.2 Materials and Methods

2.2.1 Animals

Trpc4 KO and WT genotyped littermates Fisher 344 rats (Transposagen, Lexington, KY) and trpc4 KO and WT genotyped littermates Long Evans rats, at least eight-weeks old (250-300 grams), were used in these studies. Juveniles were between 28 and 32 days old at the time of testing and were used only as stimuli for the Social Exploration tests. Fisher 344 trpc4 KO rats were generated using the Sleeping Beauty transposon system (Geurts et al., 2006). This line of animals was used in all self-administration experiments unless otherwise indicated. The Long Evans trpc4 KO animals were generated by backcrossing them with the Fisher 344 trpc4 KO rats. We then crossed the F1 generation back to the Long Evans background for 3 successive generations before using them for experimentation. Additionally, adult male Sprague-Dawley (SD) rats (Harlan Sprague Dawley, Indianapolis, IN, USA) and juvenile male rats of the same strain and source were used as a control strain in the Social Exploration tests.

Adult rats were housed in groups of two per cage, while juvenile rats were housed in groups of four per cage. All rats were allowed to acclimate to colony housing for at least 1 week. They were maintained on a 12-hour light/dark cycle (lights on at
7:00 am), and were given food and water ad libitum. All experiments were conducted in accordance with guidelines of the Institutional Animal Care and Use Committees at the University of Colorado at Boulder, Drake University, and the University of St. Thomas.

2.2.2 Animal genotyping and quantitative PCR

Three polymerase chain reaction (PCR) primers were designed and used as 20-24 oligonucleotide sequences (Eurofins MWG Operon, Ebersberg, Germany) to genotype rats. Reactions were carried out using Choice Taq Blue DNA polymerase (Denville Scientific Inc., Metuchen, NJ) in either a Techne Touchgene thermal cycler (Techne, Minneapolis, MN) or a BioRad C1000 thermal cycler. Ethidium bromide-stained agarose gels were photographed with a Kodak Gel Logic 200 UV transilluminator imager (Carestream Health Inc., Rochester, NY).

2.2.3 In situ hybridization and emulsion autoradiography

In situ hybridization (ISH) and emulsion autoradiography experiments were carried out by Cytochem Inc. (Montreal, QC Canada). S-labeled cRNA antisense and sense probes were freshly prepared and used. Rat sections were hybridized overnight at 55°C in 50% deionized formamide, 0.3 M NaCl, 20mM Tris-HCl (pH 7.4), 5 mM EDTA, 10 nM NaPO₄, 10% dextran sulfate, 1x Denhardt's solution, 50 µg/ml total yeast RNA, and 50-80,000 cpm/µl S³⁵-labeled probes. The tissues were then subjected to stringent washing at 65°C in 50% deionized formamide, 2x SSC, and 10 mM DTT, followed by washing in phosphate buffered saline (PBS) before treatment with 20 µg/ml RNAse A at 37°C for 30 minutes. After serial washes in 2x SSC and 0.1x SSC for 10
minutes at 55°C, the slides were dehydrated and apposed to x-ray film for 5 days. Following this step, the slides were covered with photographic emulsion (NTB Kodak) for 15 days and silver grains developed with D19 Kodak photographic solution.

To assess the extent of the knockout of the TRPC4 protein, in situ hybridization was conducted on coronal slices from trpc4 KO and WT rats. These experiments were carried out according to the methods described by Gold and Zachariou (Gold & Zachariou, 2004). Slide-mounted, fresh-frozen tissue sections were used. Halothane-anesthetized animals were decapitated and their brains removed and rapidly frozen on dry ice. Coronal, fresh-frozen sections were cut at 14 mm in a cryostat, thaw-mounted onto Superfrost Plus (Fisher Scientific, Pittsburgh, PA, USA) glass slides, and stored at -80°C until use. The sections were fixed in 4% paraformaldehyde, and washed in PBS, PBS+glycine, and 0.25% acetic anhydride in 0.1M triethanolamine. The sections were then dehydrated and delipidated in 50%, 75%, 95%, and 100% EtOH and 100% chloroform. Sections were hybridized for 18 hours at 60°C in hybridization buffer containing deionized formamide (40%), dextran sulfate (10%), 16 Denhardt’s solution, 46SSC, denatured and sheared salmon sperm DNA (1 mg/mL), yeast tRNA (1 mg/mL), dithiothreitol (10 mM), and either S35-labeled RNA probe (S35, Perkin Elmer, Waltham, MA, USA) or an identical, unlabeled RNA probe as a control. After hybridization, the sections were washed in sodium citrate buffer (SSC)+sodium thiosulfate and placed on film for 3–7 days before development.

2.2.4 Immunoblot procedure

Immunoblot experiments were carried out by Dr. Kurt Illig, a collaborator at the
University of St. Thomas in St. Paul, Minnesota. Homogenates were obtained as previously described (Fowler et al., 2007). Briefly, brain dissections were sonicated and the suspensions were boiled in 1% SDS lysis buffer. Protein concentrations were determined by BCA assay (Pierce, Rockford, IL, USA). Samples were loaded onto 10% SDS–PAGE gels (100 mg protein/lane). Gels were transferred to Hybond ECL nitrocellulose membrane (Amersham, Piscataway, NJ, USA) for immunoblotting. The membranes were blocked in 5% milk for 1 hour, then placed in primary antibody (TRPC4, 1:1000, gift from Dr. Bonanno; TRPC5, 1:400, Alomone, Jerusalem, Israel or SigmaAldrich, St. Louis, MO, USA; b-actin monoclonal, 1:1000, SigmaAldrich, St. Louis, MO, USA) while shaking for either 1 hour at room temperature or overnight at 4°C. The membranes were incubated in secondary antibody (GaRb, 1:8000 or GaM, 1:8000, Pierce, Rockford, IL, USA) for 1 hour at room temperature on a shaker and developed with enzymatic chemiluminescence (ECL, Amersham Biosciences, Piscataway, NJ, USA) reagent.

2.2.5 Immunohistochemistry

Immunohistochemistry experiments were also carried out by Dr. Kurt Illig at the University of St. Thomas. Animals were deeply anesthetized with sodium pentobarbital and transcardially perfused with 0.01M PBS (pH 7.4) followed by 4% buffered formaldehyde freshly depolymerized from paraformaldehyde. Brains were removed, post-fixed for approximately 24 hours and then cryo-protected in 30% sucrose in PBS. Brains were sectioned on a cryostat at 40μm. Sections were double-labeled for TRPC4 and tyrosine hydroxylase (TH) using fluorescence immunohistochemistry procedures.
adapted from those described previously (Iliig & Haberly, 2003). Briefly, sections were rinsed in 0.01M PBS followed by incubation in 0.03% H$_2$O$_2$ for 30 minutes at room temperature, then rinsed in a wash buffer containing 0.01M PBS, 2% bovine serum albumin (BSA), and 0.5% TX-100. Next, sections were incubated in a blocking buffer containing 0.01M PBS, 2% BSA, 0.5% TX-100 and 10% normal goat serum (NGS) for 1 hour at room temperature. Primary antibodies (mouse anti- TRPC4, 1:1500, NeuroMAB; rabbit anti-TH, 1:5000, Millipore) were added to the wash buffer, and sections were incubated overnight at room temperature. The following morning, sections were rinsed in wash buffer and incubated in fluorescence-tagged secondary antibody (1:500 goat-anti-rabbit IgG conjugated to AlexaFluor 488; 1:500 goat-anti-mouse IgG conjugated to AlexaFluor 594; both from Molecular Probes) at room temperature in the dark for 3 hours. Sections were rinsed 3 times with PBS and mounted on slides with ProLong anti-fade reagent containing DAPI (Molecular Probes). Omission of the primary antibody during processing eliminated all tissue staining. To determine the proportion of TH-labeled cells that were also labeled with TRPC4, material from 6 different WT animals were randomly sampled. A box measuring 100 microns on each side was placed in a random location in the VTA, and all cells within the box were categorized as TH-only or TH/TRPC4 double-labeled.

2.2.6 Social exploration test

Experimentally naïve male SD (n=8), trpc4 KO (n=12) and WT (n=15) rats were used in these experiments. The SD rats served as a strain control group. All social exploration tests were conducted in a quiet testing room as previously described.
(Christianson, et al., 2009). Each experimental adult rat was assigned its own cage (18x24x18 cm) and allowed to acclimate for 1 hour before testing. The social exploration test began when a juvenile rat of the same strain and source was placed into the cage of the experimental rat. The test lasted for 3 minutes. An observer blind to treatment conditions timed all exploratory behaviors initiated by the adult rat. Exploratory behaviors include: sniffing, grooming, pinning and sparring.

2.2.7 Simple Y-maze learning

Dr. William Klipec, a collaborator at Drake University in Des Moines, Iowa, carried out this simple learning task, in addition to the Serial and Reversal complex learning tasks described in the following section. Naïve trpc4 KO males (n=14) and females (n=18) and WT males (n=13) and females (n=28) were used in this experiment. The unequal numbers in each group were due to using all available KO and WT rats from three rounds of breeding. The rats were approximately 3 months old at the onset of the experiments and individually housed with 23 hours of water deprivation between sessions that were conducted five days per week. The experiment was conducted in four fully automated Coulbourn Y-maze Systems (Holliston, MA, USA) equipped with photocell activated doors and water dippers. Rats were trained to respond to a lighted water dipper, and then allowed to run through the Y-maze for 10 daily sessions. After fully entering an alley other than the starting alley, the door closed behind the rat. The lighted alley was randomly alternated across daily trials until 60 water reinforcers were earned. A correct choice resulted in access to a 4-second activation of the water dipper, followed by a 10-second delay before the door opened for the next trial. An incorrect
choice resulted in no water and a 20-second time-out in the incorrect alley before the
doors opened for the next choice trial. Both correct and incorrect choices were recorded
and the percent correct choices were determined for each session.

2.2.8 Serial reversal shift and conditional reversal shift learning

Experimentally naïve trpc4 KO male (n=4) and female (n=5) and WT male (n=5)
and female (n=5) rats were used in this experiment. The rats were approximately nine
weeks old at the onset of the experiments. The rats were individually housed with 23
hours of water deprivation between sessions that were conducted five days per week in
six fully automated Coulbourn operant chambers (Holliston, MA, USA) equipped with a
house light, retractable levers with cue lights, water dippers, tone generators and
speakers. For the serial reversal shift (SRS) protocol, the rats were trained to lever
press for water reinforcement (4-second access to water) and leaned out to a variable
ratio 6 (VR-6) schedule of reinforcement. They were subsequently trained to lever press
for water reinforcement (VR-6 schedule) when a light over the lever was on (S+), and to
not respond (extinction) when the light was off (S-). S+ and S- periods were 30 seconds
in duration and randomly alternated within sessions with a 5-second changeover delay
between periods during which the chamber was dark. Sessions were terminated after
60 reinforcements were earned. After one block of 14 days, S+ and S- were reversed
so that light off signaled reward and light on signaled extinction. The rats were trained
on this reversal for a second 14-day block, after which S+ and S- were again reversed.
Four 14-day blocks were followed by four 5-day blocks and three 3-day blocks. At this
point, using the same 19 rats, the protocol changed to a within session conditional
reversal shift (CRS) task. Here, procedures were identical to the SRS task except that within each daily session a 2kHz, 70 dB tone signaled S+ was light on and S- was light off, while the absence of the tone signaled the reversal of S+ and S-. For each rat, the session was terminated after 60 reinforcements had been earned. The rats were run 5-days each week for 17 sessions.

2.2.9 Sucrose self-administration

Experimentally naïve male *trpc4* KO (n=7) and WT (n=7) rats were used in this experiment. All self-administration procedures were performed in operant conditioning chambers (Med-Associates, St. Albans, VT, USA) equipped with two response levers, a sucrose hopper and an infusion pump system. Animals were food-deprived to 85% of free feeding weight and trained to lever-press for sucrose pellets on a fixed ratio 1 (FR1) reinforcement schedule for 5 days/week. A discriminative stimulus (cue light) was paired with sucrose delivery and illuminated above the corresponding lever. A correct lever response resulted in the delivery of a sucrose pellet (45g), termination of the cue light, and a 20 second time-out period during which responding produced no consequence. Throughout the session, inactive lever responses produced no consequence. The session was complete when the animal had administered 50 sucrose pellets. The latency (in minutes) to acquire 50 pellets was recorded as the dependent variable. Failure to reach criteria (50 pellets) within 120 minutes resulted in termination of the session. Animals completed 10 sucrose self-administration sessions, which was sufficient to establish stable baseline sucrose responding in all groups.
2.2.10 Catheter implant surgery

Following sucrose self-administration, rats were given ad libitum food. After 24-48 hours of free feeding, catheters were implanted into the jugular vein under halothane anesthesia (1-2.5%). During recovery, catheters were flushed daily with 0.1 mL heparinized saline to maintain patency. Rats were allowed 4-7 days to recover in their home cage before experimental procedures began.

2.2.11 Cocaine self-administration

The same group of Fischer 344 rats used in the sucrose self-administration experiment was used in the cocaine self-administration experiment, except for 2 trpc4 KO and 2 WT rats that failed the catheter verification test and were excluded from the analysis; this reduced each genotype group to n=7. After 4 to 7-days recovery from surgery, animals were trained to self-administer intravenous cocaine (0.5 mg/kg/100μL injection) on an FR1 schedule in daily 2-hour sessions for 5 days/week. Cocaine injections were delivered over 5 seconds and were concurrent with the illumination of a cue light above the active lever. Drug and cue delivery were followed by a 15 second time-out period in which the house light remained off and responding produced no consequence. Throughout testing, inactive lever responses produced no consequence. After approximately 10 cocaine self-administration sessions (until stable), animals were placed on a FR5 schedule in daily 2-hour sessions. Cocaine infusions and the light cue were delivered as previously described except that 5 lever presses were now required to initiate one cocaine infusion. Animals remained on the FR5 schedule for a minimum of 5 days to ensure a stable baseline was re-established. To examine strain differences,
we used the same cocaine self-administration procedures on two groups of Long-Evans (LE) x Fisher 344 hybrid *trpc4* KO (n=17) and WT (n=11) rats for five daily sessions on the FR1 schedule of reinforcement. In the LE hybrid group, 2 *trpc4* KO and 2 WT rats failed the catheter verification test and, thus, were excluded from the analysis. This reduced group size to n=15 and n=9 respectively. The LE hybrid rats were not trained on sucrose self-administration.

### 2.2.12 Catheter verification

At the completion of the cocaine SA experiments, catheter patency was tested to ensure that differences in self-administration were not due to catheter failure. Fatal Plus cocktail (390 mg/ml sodium pentobarbital, 0.01 mg/ml propylene glycol, 0.29 mg/ml ethyl alcohol, 0.2 mg/ml benzyl alcohol) was administered through the animal’s catheter at 0.2 mL/kg. Following Fatal Plus administration, catheter patency was confirmed if the animal responded immediately with muscle atonia and lethality. Animals with faulty catheters were excluded from the sucrose and cocaine SA experiments studies.

### 2.2.13 Ex vivo slice dopamine neuron electrophysiology

Dr. Jun-li Cao conducted all electrophysiology experiments at the University of Colorado in Boulder, whom was blind to the genotype. The Fisher 344 *trpc4* KO rats (n=5) WT rats (n=6) used for the ex vivo slice recordings were sampled from different litters. To minimize possible stress effects on the recordings, each rat was anesthetized with isoflurane immediately upon arrival in the experimental room. The brain was removed quickly and placed in ice-cold artificial cerebrospinal fluid (ACSF), which contained 128 mM NaCl, 3 mM KCl, 1.25 mM NaH$_2$PO$_4$, 10 mM D-glucose, 24 mM
NaHCO$_3$, 2 mM CaCl$_2$, and 2 mM MgCl$_2$ (oxygenated with 95% O$_2$ and 5% CO$_2$, pH 7.35, 295–305 mOsm). Acute brain slices (250 μm) containing the VTA were cut using a microslicer (VF-200, Precisionary Instruments; VT-1000, Leica Biosystems) in sucrose-ACSF, which was derived by fully replacing NaCl with 254 mM sucrose, and saturated by 95% O$_2$ and 5% CO$_2$. Slices were maintained in a bubbled holding chamber for 1 hour at 37°C. The recording external solution (flow rate 2.5 ml/min) was ACSF. Single unit extracellular recording was carried out by use of cell-attach configuration with a multiclamp 700B (Molecular Devices) using an ITC18 analog to digital converter and custom software (Cooper, DC) written in IgorPro (Wavemetrics) as described previously (Han, 2006; Krishnan et al., 2007). Recordings were obtained from VTA DA neurons identified by their location and well established electrophysiological criteria (Grace & Onn, 1989; Marinelli & White, 2000; Ungless, et al., 2001): regular spontaneous firing, action potential (AP) with triphasic waveforms (positive, negative, positive), and AP width (from start to trough) >1.1 ms. Recording pipettes (3-5 M) were filled with an internal solution containing 115 mM potassium gluconate, 20 mM KCl, 1.5 mM MgCl$_2$, 10 mM phosphocreatine, 10 mM HEPES, 2mM magnesium ATP, and 0.5 mM GTP (pH 7.2, 285 mOsm). VTA neurons were visualized via an upright Olympus BX-51 microscope using differential interference contrast illumination. The firing rate was recorded in the current clamp mode, and data acquisition and online analysis of firing rate.

2.2.14 Drugs
Fatal-Plus was obtained from Vortech Pharmaceuticals, LTD (Dearborn, MI, USA). Cocaine hydrochloride was obtained from Sigma-Aldrich (St. Louis, MO, USA). Cocaine was dissolved in sterile-filtered physiological saline (0.9%).

### 2.3 Results

#### 2.3.1 Generation of \(trpc4\) KO rats

The Sleeping Beauty (SB) gene-trap transposon method was used to create the \(trpc4\) KO animals (Geurts et al., 2006). The SB method uses cut-and-paste transposable elements to generate heritable loss-of-function mutations. Figure 2.1A shows the location of the \(Trpc4\) gene on the rat genome and where the transposon was inserted. By inserting the SB transposon into the first intron of the \(Trpc4\) gene, the full-length protein product can be deleted. Using primers for the \(trpc4\) KO and wild-type alleles, we were able to confirm the deletion using PCR and gel electrophoresis (Figure 2.1B).
Figure 2.1: A. Schema of the Trpc4 gene in the rat genome and the Sleeping Beauty (SB) gene knock-out system. The Trpc4 gene is located on chromosome 2 of the rat genome, between 143.35 Mb and 143.49 Mb. The Sleeping Beauty transposon was inserted into the first intron of Trpc4, therefore creating a complete knock-out of the coding sequence. B. Ethidium bromide-stained agarose gel visualizing the 905 bp marker for the WT allele and the 510 bp marker for the trpc4 KO allele. To genotype the animals, a 1.5% agarose gel electrophoresis was used.

2.3.2 Spatial distribution of trpc4 expression

The localization of Trpc4 in the adult rat brain is consistent with previous findings (Fowler et al., 2007). In collaboration with Cytochem Inc. (Montreal, QC Canada), we performed x-ray film autoradiography for gross anatomy resolution and emulsion autoradiography for cellular resolution. Figure 2.2 shows results from the x-ray film autoradiography. Detectable levels of trpc4 mRNA labeling were observed in forebrain regions such as the septal nuclei, hippocampus, habenula, hypothalamus and amygdala. The immunoblots and in situ hybridization experiments (Figure 2.2C-D)
revealed that the most robust labeling was detected in the lateral septum, CA1 subregion of the hippocampus, the substantia nigra and the lateral habenula. Compared to the WT samples, there was complete elimination of trpc4 in the PFC, lateral septum, striatum, hippocampus and cerebellum in the trpc4 KO samples.

Results from the emulsion autoradiography (Figure 2.3) indicate the possibility that Trpc4 expression may be concentrated in glutamate neurons, suggesting that TRPC4 channels may play a role in synaptic plasticity in those regions. No labeling occurred in astroglia or oligodendroglia. Given that the brain’s reward pathway is highly modulated by dopaminergic projections to these limbic regions and the prefrontal cortex, we also investigated whether trpc4 mRNA and TRPC4 protein could be located in DA neurons in the VTA. Using fluorescence immunocytochemistry in the VTA, we observed that TRPC4-labelled cells were relatively sparse, and were relatively uniformly distributed throughout the VTA (i.e., no clusters of cells). Moreover, double-labeling for TRPC4 and tyrosine hydroxylase (TH) revealed that TRPC4 was found only on a subpopulation (mean = 36.74% ±3.2 SE) of TH-expressing cells in the VTA of WT rats (Figure 2.4A-D); TRPC4 labeling was not observed on any cells that did not express TH. No TRPC4 labeling was observed on TH-expressing cells in trpc4 KO rats (Figure 2.4E). Elimination of the primary antibody in wild-type animals abolished all immunolabeling (Figure 2.4F). These findings suggest that, within the VTA, TRPC4 channels are found exclusively on TH-expressing neurons.
**Figure 2.2: Trpc4 expression in the adult rat brain.** A. X-ray film autoradiography exposed for 5 days, showing trpc4 mRNA distribution pattern in the septal region of the adult rat brain coronal section. Strong signal was evident in the lateral septum (LS) and low-level labeling in the cortex (Cx), lateral preoptic area (LPA) and the bed nucleus of the stria terminalis, medial division (BSTM). The caudate nucleus (CPu) was unlabeled. No labeling occurred in white matter structures anterior commissure (ac) and corpus callosum (cc). B. Coronal brain section in the hippocampus region. High-level trpc4 concentration was found in CA1 and CA2, whereas moderate level labeling occurred in CA3 and dentate gyrus (DG). High level of Trpc4 gene expression was observed in the lateral habenula (LHb). Much lower levels of labeling were seen in the basomedial amygdaloid nucleus (BM), premammillary nucleus (PMV) and arcuate nucleus (Arc). C. In situ hybridization showing the distribution of trpc4 mRNA in the prefrontal cortex (PFC) and LS of the WT rat (left panel) and its complete absence in the trpc4 KO rats (right panel). D. WT and KO TRPC4 immunoblots showed the highest protein expression in the LS and cerebellum (CER), moderately expressed in the PFC and hippocampus (HIP), and minimally expressed in the striatum (STR). An immunoblot showing the complete elimination of trpc4 protein in the trpc4 KO compared to the WT rat.
Figure 2.3: *Trpc4* expression in the adult rat brain. **A.** Emulsion autoradiography exposed for 15 days to the antisense probe (as), showing the presence of *trpc4* mRNA in the hippocampus (Hi) and substantia nigra pars reticulata (arrows). The area contained in the box is shown at higher magnification in (D). **B.** Emulsion autoradiography showing *trpc4* labeling in the lateral septum (SL), with no labeling in the striatum (St) or corpus callosum (cc), and moderate labeling in the cortex (Cx). **C.** Brain hippocampal region with strong labeling in the CA1 area (see inlayed box for example). Less labeling occurs in the area CA2 and CA3, and dentate gyrus. **D.** Fragment of the area in the substantia nigra (boxed area in (A)) showing labeling in neurons (arrows). Magnifications (A, B and C) x10 and (D) x 400.
Figure 2.4: Expression of TRPC4 protein in the VTA. Results of immunocytochemical studies showing TH and TRPC4 protein expression in the medial portion of the VTA in WT and trpc4 KO animals. Tissue from WT animals was triple-labeled for DAPI (blue; A), TH (green; B) and TRPC4 (red; C). D. Composite of DAPI, TH and TRPC4 labeling in the VTA of a WT rat. Cells in the VTA displayed only TH (arrows) or both TH and TRPC4 (arrowheads). E. Double-labeling for TH and TRPC4 in a trpc4 KO rat; note the lack of any staining for TRPC4. F. Section in a WT animal without primary antibody incubation, illustrating that TH and TRPC4 immunolabeling is not a result of nonspecific secondary antibody staining.
2.3.3 Social exploration test

For the past 25 years, social interaction tests have been used reliably to assess anxiety-like behaviors in mice and rats. Furthermore, they have been shown to be sensitive to the effects of anxiogenic and anxiolytic drugs (Christianson et al., 2008; File & Seth, 2003). On average, healthy naïve rats will explore for about 80 seconds per 3-minute trial (Christianson et al., 2011). A significantly below-average score on these tests indicates an increase in anxiety-like behavior in the rodent. Results from our social exploration trials (Figure 2.5) show that trpc4 KO rats exhibited the least amount of exploratory behaviors with a mean exploration time of 54 seconds (SEM ± 2). This is significantly less (**p<.001) than trpc4 WT rats, which explored for 81.63 seconds (SEM ± 2.45) on average, and SD rats, which had a mean exploration time of 93.19 seconds (SEM ± 4.66). Interestingly, the mean exploration time of trpc4 WT rats is significantly less than the SD rats (ǂp<.05). However, based on the literature previously cited, the mean exploration time of the trpc4 WT rats remains within the “normal” range.
Figure 2.5: A. Bar graph of the mean (+SEM) social exploration time (s) for Sprague-Dawley (SD), trpc4 WT (+/+)) and trpc4 KO (-/-) adult rats. The SD rats (n=8) were the most exploratory. The (+/+) rats (n=15) explored significantly less than the SD rats († p<.05). The (-/-) rats (n=12) explored significantly less than both the (+/+)) and SD rats (**)p<.001. B. Screenshot of the adult trpc4 KO rat with the juvenile rat stimulus. Error bars = SEM.

2.3.4 Maze learning

Figure 2.6 shows the learning curves for trpc4 KO and WT rats across 10 days of training on the Y-Maze discrimination training paradigm. Surprisingly, deletion of the Trpc4 gene had no apparent effect on the rate of acquisition or terminal performance on this simple learning task. All groups reached asymptotic performance by the fourth day.
A 2x2x10 split plot ANOVA revealed a significant effect for training days ($F_{9,621} = 236.2$, **$p \leq 0.001$), but no significant interactions for training days with sex ($F_{9, 621} = 1.43, p > 0.17$), or genotype ($F_{9, 621} = 0.61, p > 0.80$). Additionally, the three-way interaction of days, genotype and sex was not significant ($F_{9, 621} = 1.07, p > 0.38$). While the female rats made significantly more errors (88.5% correct) than male rats (90.6% correct) over the 10 sessions ($F_{1, 69} = 5.20, ^*p \leq 0.03$), these errors occurred mostly in the first four days, and only in the second group of the three that were run. When we saw these errors in the second group, we ran the third group to be sure no substantial and systematic sex difference existed across the groups. There were no differences between trpc4 KO and WT rats ($F_{1, 69} = 0.14, p > 0.71$) and the genotype by sex interaction was not significant ($F_{1, 69} = 0.15, p > 0.75$).

**Figure 2.6:** The trpc4 KO and WT rats show no difference in Y-Maze Discrimination Learning. WT males (n=13) and females (n=28) and trpc4 KO males (n=14) and females (n=18) were trained to run through the Y-maze toward a lighted dipper at the end of one arm of the alley, with the lighted position randomly alternated across 60 daily trials for 12 days. The single error bar off data shows the minimum difference required for significance using Tukey’s HSD test.
2.3.5 Serial reversal shift learning

Since there were no effects on the simple Y-maze learning task, we used the more complex serial reversal shift task, which required discrimination learning and strategic switching, to compare *trpc4* KO and WT rats. Figure 2.7 shows the acquisition of the multiple VR6 (EXT) lever pressing discrimination task with reversals of S+ and S- across multiple training blocks. The figure shows the first 3 days and last 3 days of each 14-day block and every day for the remaining blocks. For *trpc4* KO and WT rats across the 11 blocks a 2x2x2 split plot ANOVA of the percent of total responses on the first and last day of each block, during the “light on” components, showed a significant effect of blocks ($F_{21,315} = 95.65, p < 0.001$). Inspection of Figure 2.7 shows that both groups acquired the discrimination gradually in the first two 14-day blocks but performed the reversal very quickly by the onset of the 5-day blocks. There were no significant differences between *trpc4* KO and WT rats ($F_{1,15} = 0.02, p > 0.90$), or sex differences ($F_{1,15} = 0.74, p > 0.41$) and there was no genotype by sex interaction ($F_{1,15} = 2.33, p > 0.15$). Moreover, there were no interactions between genotype and blocks ($F_{21,315} = 1.12, p > .33$), sex and blocks ($F_{21,315} = 0.99, p > .48$) or genotype by sex by blocks ($F_{21,315} = 0.42, p > .99$). Accordingly, the absence of main effects of genotype cannot be attributed to masking by higher order interactions. Surprisingly, both groups acquired the reversal strategy equally well with no deficit due to the deletion of the *Trpc4* gene.
Figure 2.7: The trpc4 KO and WT rat performance on a serial reversal shift paradigm. The trpc4 KO (n=9) and WT (n=10) rats were trained on a simple discrimination task to lever press for water reinforcement (VR-6 schedule) when a light over the lever was on (S+) and not respond (extinction) when the light was off (S-). S+ and S- were repeatedly reversed between sequential blocks. Four 5-day blocks and three 3-day blocks followed four blocks of 14 days. The figure shows the mean percent of total responses to the light on during the first and last 3 days of each 14-day block and every day for the remaining blocks. The single error bar off data shows the minimum difference required for significance using Tukey’s HSD test.

2.3.6 Conditional reversal shift learning

The conditional reversal shift task required switching the response strategies within sessions based on a contextual cue (tone). Here, we analyzed the percent of correct responses when light on was S+ and light off was S+ as signaled by the presence or absence of a tone cue within sessions and across the 17 sessions (see Figure 2.8). Overall, correct responding was reduced from about 79% correct responses on the serial reversal shift task to about 68% correct responses on the conditional
reversal shift task. A 2x2x2x17 split plot ANOVA comparing the trpc4 KO and WT performance across the sessions revealed no significant differences during the conditional reversal shift task ($F_{1, 15} = 0.36$, $p > 0.56$). There were also no differences in overall correct responding across sessions ($F_{16, 240} = 1.01$, $p > 0.44$), and no genotype by session interaction ($F_{16, 240} = 1.18$, $p > 0.29$). Additionally, there were no differences between male and female rats ($F_{1, 15} = 0.35$, $p > 0.56$) or sex by genotype interaction ($F_{1, 15} = 0.34$, $p > 0.57$). There was no difference in correct responding between the tone on and tone off ($F_{1, 15} = 0.27$, $p > 0.61$), and none of the higher order interactions between genotype and sex with the tone cue or sessions were significant. As in the serial reversal shift task, both groups performed the conditional reversal shift strategy equally well with no deficit due to the deletion of the Trpc4 gene.
2.3.7 Sucrose self-administration

Here we sought to establish a baseline of sucrose SA against which we could compare cocaine SA, and to use a second natural reward (food) to extend the previous experiments that used water. The number of minutes to acquire 50 pellets in the trpc4 KO and WT rats was subjected to a 2x10 split plot factorial ANOVA which revealed a significant reduction in time to acquire the pellets across the ten sessions ($F_{9, 144} = 37.73 \ p \leq 0.001$). However, there were no differences between trpc4 KO and WT rats ($F_{1, 16} = 0.08 \ p > 0.78$), or interaction between genotype and time to acquire pellets ($F_{9,}$
As shown in Figure 2.9, both the trpc4 KO rats and their wild-type counterparts acquired 50 sucrose pellets at similar rates over the course of training. By day 7, both trpc4 KO and controls reached stable baseline responding, averaging between 16 and 26 minutes to acquire 50 pellets. Clearly, compared to WT controls, trpc4 KOs do not differ in their ability to learn to self-administer a natural reinforcer, sucrose, on a FR1 schedule. These findings are consistent with the previous results showing no differences between trpc4 KO and WT rats in simple and complex learning tasks, using water as a reinforcer. Taken together, these findings suggest there is no association between TRPC4 ion channels in rats and their ability to learn or perform tasks when using natural reinforcers.

Figure 2.9: The trpc4 KO and WT rats show no difference in their ability to self-administer sucrose. The graph shows the average minutes for the trpc4 KO (n=9) and WT (n=9) rats to acquire 50 sucrose pellets in the allotted time period (120 minutes). Error bars = SEM.
2.3.8 Cocaine self-administration

We next sought to determine whether trpc4 KO and WT rats would differ in cocaine self-administration. Figure 2.10A presents the mean number of infusions for trpc4 KO and WT rats across 5 days for the three groups. To compare the Fisher 344 rats SA on the FR1 and FR5 schedules of reinforcement, a 2x2x5 split plot factorial ANOVA was conducted with repeated measures on schedules and days comparing number of cocaine infusions by trpc4 KO and WT rats. The ANOVA revealed a significant overall reduction in infusions by the trpc4 KO rats ($F_{1,12} = 4.84, p = 0.048$) with no significant differences between schedules ($F_{1,12} = 0.38, p = 0.55$), or interaction between schedules and genotype ($F_{1,12} = 0.96, p = 0.35$). Additionally, there was no significant difference in infusions across days ($F_{4, 48} = 0.50, p = 0.73$), or days by genotype interaction ($F_{4, 48} = 0.98, p = 0.43$). None of the higher order interactions approached significance. Figure 2.10B shows the significant reduction in infusions during the FR1 schedule ($p < 0.01$ using Tukey’s HSD test) and the FR5 schedule ($p < 0.05$ using Tukey’s HSD test) for the trpc4 KO rats compared to the WT controls.

To compare the Fisher 344 to the LE hybrid strains on the FR1 schedule of reinforcement, a 2x2x5 split plot factorial ANOVA was conducted with repeated measures on days, comparing genotype (trpc4 KO and WT) and strain (Fisher 344 and LE Hybrid) across the 5 days of training. The analysis revealed a significantly reduced number of cocaine infusions by the Fisher 344 compared with the LE Hybrids across genotypes ($F_{1,34} = 5.26, p < 0.03$), with no significant interaction between genotype and strain ($F_{1,34} = 0.56, p > 0.47$). There was a significant overall reduction in cocaine infusions in the trpc4 KO rats across strains ($F_{1,34} = 8.83, p < 0.005$). There were no
significant differences in infusions across days ($F_{4, 136} = 0.95, \ p > 0.43$) or interactions of
days with genotype ($F_{4, 136} = 0.16, \ p > 0.96$), days with strain ($F_{4, 136} = 0.58, \ p > 0.68$) or
days by genotype by strain ($F_{4, 136} = 1.72, \ p > 0.15$). Overall, the LE Hybrids self-
administered more cocaine than the Fisher 344 rats, but the reduction in cocaine self-
administration in the trpc4 KO rats was significant in both strains. Figure 2.10C shows
the significant reduction in mean cocaine infusions by the trpc4 KO compared to WT for
the Fisher 344 rats ($p < 0.05$, using Tukey’s test). Based on results from the Fisher 344
rats, we had predicted a decrease in infusions for the trpc4 KO LE Hybrid rats. Figure
2.10C also shows the significant reduction of mean cocaine infusions by the trpc4 KO
compared to WT for the LE Hybrid rats ($t_{34} = 1.86, \ p < 0.05$). These results show that,
unlike when using natural rewards, the trpc4 KO rats show reduced cocaine self-
administration, and the effect was generalized across two strains of rats.
Figure 2.10: The trpc4 KO rats self-administer less cocaine than WT rats on a fixed-ratio 1 (FR1). Fisher 344 trpc4 KO (n=7) and WT (n=7) rats were tested first on an FR1 and then an FR5 reinforcement schedule, while the LE hybrid trpc4 KO (n=15) and WT (n=9) rats were tested on an FR1 reinforcement schedule. A. This panel shows the mean number of infusions for trpc4 KO and WT across 5 days for the three groups. B. Panel B shows the significant reduction in infusions during the FR1 schedule (**p < 0.01 using Tukey’s HSD test) and the FR5 schedule (*p < 0.05 using Tukey’s HSD test) for the trpc4 KO rats compared to the WT controls. C. Panel C compares the Fisher 344 to the LE hybrid strain on the FR1 schedule of reinforcement. Error bars = SEM.

2.3.9 Dopaminergic cell firing rates in the VTA

Spontaneous firing rates recorded in the VTA in n=68 trpc4 KO DA neurons were significantly lower than in n=57 WT DA neurons (Figure 2.11; t_{118} = 2.45, **P < 0.01). This reduction in spontaneous firing rate in trpc4 KO animals appears to be due to a
selective loss of cells that have a high rate of spontaneous firing (i.e., cells with spontaneous firing rates greater than ~2 Hz). This population of cells represent approximately 33% of the total number of DA cells recorded in WT animals (Figure 2.11A). This correlates with our finding that approximately 35% of TH-expressing cells in the VTA also express TRPC4 protein (see “Spatial distribution of trpc4 expression” section above). Taken together, these findings suggest that TRPC4 channels function to increase excitability of DA neurons in the VTA in WT animals, and that the reduction in spontaneous firing rate in trpc4 KO VTA DA neurons may be due to a lack of TRPC4 channels.

Figure 2.11: Dopaminergic cell firing rates in the ventral tegmental area (VTA) in both WT and trpc4 KO rats. A. The cumulative probability of baseline dopaminergic cell firing rates in the VTA for WT and trpc4 KO animals. Note that approximately 1/3 of cells obtained from WT animals have firing rates above 2 Hz. Fewer than ~10% of cells in trpc4 KO animals display firing rates above 2 Hz. B. The bar graph shows the average baseline firing rates, measured in Hz, from 50 dopaminergic cells of both WT and trpc4 KO animals. Error bars = SEM.
2.4 Discussion

The robust corticolimbic expression of TRPC4 indicates involvement in regulating the brain’s response to stress and anxiety (Kessler, et al., 2005). On the cellular level, TRPC4 is localized on pyramidal glutamatergic neurons (Fowler et al., 2007), thus effecting synaptic activity within these brain regions. For example, the lateral septum (LS) contains receptors for neurotransmitters (including serotonin, corticotropin-releasing factor and vasopressin) involved in anxiety, stress and social bonding, (Sheehan, et al., 2004). Multiple studies have shown that there is increased neural activity in the LS in animals exposed to anxiety-provoking situations (Campeau & Watson, 1997; Senba, et al.; 1993). The LS receives input from the amygdala and PFC, in addition to strong glutamatergic inputs from the hippocampus, all of which contain high to moderate TRPC4 expression. Based on these findings, we began our research by testing Trpc4 as a causal gene mutation for social anxiety.

After creating a trpc4 KO rat and confirming the gene deletion via PCR and gel electrophoresis, we tested these animals against controls in the juvenile social interaction test. We found that trpc4 KO animals spent significantly less time exploring than their wild-type counterparts and SD rats. Furthermore, trpc4 WT rats explored significantly less than SD rats. These findings indicate that TRPC4 is playing a fundamental role in social anxiety, most likely by supporting cellular processes in specific regions of the brain associated with regulating anxiety, such as the PFC and amygdala. This is the first evidence showing that the presence of TRPC4 effects anxiety-like behaviors in rats and may be a critical regulator of neural activity that occurs with anxiety-provoking stimuli.
One limitation to our investigation into the effects of TRPC4 on anxiety is the fact that our behavioral output was based on only one test - the juvenile social exploration test. Although this test has been well validated in rats (Amat et al., 1998; Christianson et al., 2010), assessing anxiety-like behaviors using multiple animal models would strengthen our argument. Such models may include the elevated plus/zero maze, the open field test, fear-conditioning, and/or the light/dark exploration test, all of which have been used extensively to measure anxiety-like behaviors in rodents.

Next, we examined the Trpc4 gene as a possible link in understanding the neurological processes behind drug addiction. To do this, we compared the behaviors of trpc4 KO rats to their wild-type counterparts in a cocaine self-administration animal model. We found that rats lacking the Trpc4 gene self-administered significantly less cocaine infusions than the WT rats. This supported our hypothesis, which was based on TRPC4 expression and involvement in intracellular Ca\(^2+\) signaling.

Given what we know about the spatial distribution of TRPC4 receptors, we hypothesized that these channels may also play a role in regulating motivation and learning and memory. To identify a role for the TRPC4 channels in behavior we used a series of learning tasks that involved brain regions where TRPC4 channels are expressed. We expected to find learning deficits in the trpc4 KO rats in the Y-maze learning task using water reward. Since we found no differences in learning using this simple task we employed a more complex serial reversal shift task with water reinforcement to compare the trpc4 KO and WT rats. This task required the rats to detect the change in reinforcement contingencies on the first day of the reversal, alter their behavior, and utilize the reversed cues across sessions until the next cue reversal.
occurred. Once again, the trpc4 KO and WT rats learned this task equally well and performed identically across many reversals. Even when a contextual cue (tone) was used to signal the reversal within sessions in the conditional reversal shift paradigm, we found no difference in performance between trpc4 KO and WT rats. These results were not limited to water reinforcement, since the trpc4 KO and WT rats also showed no differences in the acquisition of lever pressing for sucrose pellets. While the absence of a learning or performance deficit using natural reinforcers was unexpected, it is of particular importance in the context of our results showing reduced cocaine SA in trpc4 KO rats compared to WT rats. Taken alone, the reduced cocaine SA could be attributed to a deficit in learning or performance in trpc4 KO animals. However, since neither of these deficits was exhibited on tasks using water or sucrose reward, these findings suggest that they are specific to cocaine SA.

One limitation of the gene knockout approach is that any particular behavior is likely influenced by more than just one gene, and compensatory mechanisms at the molecular, cellular or systems level, make it difficult to identify or interpret a specific phenotype. When a phenotype is observed it suggests an important role for the gene of interest in that behavior or cellular function. Consistent with this, we found no differences between trpc4 KO and WT rats across a number of simple and complex learning tasks. We also found no differences in the rate of acquisition or asymptotic performance when using either water or sucrose reward. These results suggest that, despite robust expression throughout the corticolimbic brain regions, TRPC4 channels do not play a critical role in these behaviors. In contrast, we found a deficit in cocaine SA in the trpc4 KO rats. Since these deficits did not occur when using water or sucrose
as a reward, they cannot be attributed to general motivational, non-associative factors or learning deficits.

There are, however, paradigmatic differences between organisms working for natural rewards versus internally delivered rewards, such as intracranial self-stimulation (ICSS) or drug SA. Natural rewards require a consummatory response, such as approaching the dispenser and taking in the food or water. These responses have been studied extensively in the literature on sign tracking (Hearst & Jenkins, 1974). They result in chewing the lever when food is the reward and licking the lever when water is the reward, but not when ICSS is the reinforcer (Peterson, et al., 1972). These conditioned responses, which may add to the operant pressing responses, are missing in ICSS and drug SA paradigms.

Another procedural difference is that all the rats in the food and water reward experiments were tested in a deprivation state, while animals in the cocaine self-administration studies were not. Severe food restriction, such as 30% of free feeding weight, is associated with reduced basal dopamine levels in the terminal areas (Pothos et al., 1995a; Pothos et al., 1995b). Conversely, moderate stress at the level of food restrictions used in the experiments we are reporting has been shown to increase burst-firing of dopamine neurons (Branch, et al., 2013) or firing rate of dopamine neurons (Marinelli, et al., 2006). Accordingly, moderate food restriction could plausibly have increased firing rates in the trpc4 KO rats, acting as a compensatory mechanism that was not available to the cocaine SA rats, which were not food deprived. The mechanism for the increased dopamine firing rate in food-deprived animals is not fully understood and could reflect both network and intrinsic processes. The extent to which TRPC4
channels are involved in modulating the intrinsic excitability of dopamine neurons would likely influence the balance of excitation/inhibition responsiveness of VTA dopamine neurons at the network level. Clearly, further research is needed to clarify the role that differences in consummatory behavior and deprivation, and their possible interaction with the missing TRPC4 channels, might have played in the experiments conducted.

It is known that heritable traits associated with different genetic backgrounds influence the propensity for animals to self-administer cocaine (Ruiz-Durántez, et al., 2006). Therefore, it is possible that gene interactions in the Fisher 344 genetic background of the WT and KO rats influenced the phenotype we observed in the trpc4 KO rats. However, the reduced cocaine SA observed in the trpc4 KO rats was not unique to the Fisher 344 genetic background because we replicated the KO phenotype on the outbred LE hybrid background. Since the Fisher 344 rats had prior experience with lever pressing for sucrose pellets, the apparent escalation during the first three days of SA in the LE hybrids was plausibly due to their learning to lever press efficiently during the early sessions. Compared to learning via natural rewards, these results further suggest an important role for the Trpc4 gene in regulating cocaine intake.

The level of spontaneous DA neuronal activity output from the VTA has been causally implicated in cocaine SA. However, the intrinsic ion channel or other plasticity mechanisms responsible for such activity have yet to be identified (Wong, et al., 2013). Two findings in our research provide the first evidence that the TRPC4 channel may modulate spontaneous activity in DA neurons, and may influence cocaine self-administration. First, we found that although trpc4 mRNA is sparsely distributed in the VTA (consistent with previous findings - Phelan, et al., 2012), double-
immunocytochemical labeling indicates that TRPC4 channels are selectively expressed in a subpopulation of TH-positive neurons in the VTA (Figure 2.4).

Second, we found that DA cells in the VTA of trpc4 KO animals have reduced spontaneous activity, due to a lack of cells with high rates of spontaneous activity (Figure 2.11). This population of cells (i.e., cells with spontaneous firing rates greater than \(~2\) Hz) makes up about 1/3 of DA cells recorded from the VTA, which matches closely with the approximately 1/3 of TH-expressing cells in the VTA that also express Trpc4.

Taken together, these findings suggest a critical role for TRPC4 channels in regulating spontaneous activity on DA neurons in the VTA, which may regulate an animal's self-administration of cocaine. Given the important role for DA in cocaine reinforcement, the presence of TRPC4 channels in a select subpopulation of DA neurons may further indicate a circuit-specific modulation of DA activity. Since deletion of the Trpc4 gene does not impair learning that involves natural rewards, but does reduce cocaine self-administration, these data demonstrate a novel role for the TRPC4 channel in cocaine SA and suggest that functional TRPC4 expression may be an important determinant of DA activity and susceptibility to cocaine addiction or resistance to cocaine SA. Overall, the selective expression and functional role of the TRPC4 channels on a subpopulation of DA neurons, plus the lack of global changes to a variety of behaviors, suggest the potential for a novel class of pharmacological agents that target TRPC4 ion channels for more effective treatment of a variety of dopamine disorders.
Chapter Three: Investigation of a Drug by Stressor Controllability Interaction on Anxiety-like Behaviors in Rats

3.1 Introduction

It has been shown that the degree of behavioral control an individual has over a stressor plays a crucial role on the impact of the event (Anisman et al., 1992; Seligman & Maier, 1967; Weiss et al., 1981). Studies in rats show that behavioral control over a stressor can mitigate negative stress-induced consequences, such as the development of anxiety or depression. For example, rats exposed to inescapable stress (IS, uncontrollable) show a significant decrease in social exploration time compared to rats exposed to escapable stress (ES, controllable) in a juvenile social exploration test, indicating an increase in anxiety-like behavior following IS treatment, but not ES treatment (Christianson et al., 2008). In other words, uncontrollable stress produces behaviors in rats that resemble symptoms of anxiety, while exactly equated controllable stress does not. These behavioral changes have been linked to functional changes in the brain induced by exposure to IS. Most notable is the cross-talk between the ventral medial prefrontal cortex (vmPFC) and the dorsal raphe nucleus (DRN) that occurs during a stressful situation. Previous studies reveal that the DRN shows greater serotonergic (5-HT) activation following IS exposure, when compared to ES exposure (Amat et al., 1998; Grahn et al., 1999; Maswood et al., 1998). Enabling control over the stressor that occurs during ES is sufficient for the vmPFC to inhibit the 5-HT neurons in the DRN, thus mitigating the IS-induced behavioral changes (Amat et al., 2005). Furthermore, in the study previously cited by Seligman and Maier (1967), the
investigators show that rats previously exposed to an ES episode are protected against the behavioral impacts of later IS exposure. This phenomenon, termed “behavioral immunization”, persisted for over one week following the initial ES treatment. These finding indicate that individuals who experience control over an adverse event may develop resilience against future uncontrollable stressful events.

In a related paradigm, cocaine use disorders frequently co-occur with stress-related disorders (Mantsch et al., 2014). Furthermore, brain circuits involved in stress become increasingly sensitive with regular cocaine use, leading to symptoms of cocaine withdrawal (i.e., an increase in negative moods and displeasure) when not taking the drug (Büttner, 2012; Wolf, 2010). Previous research indicates that repeated exposure to cocaine can cause an increased risk for anxiety, depression, panic attacks and increased irritability (Goldstein et al., 2009; Riezzo et al., 2012). Thus, it is of clinical importance to determine if the degree of stressor controllability impacts the development of anxiety (positively or negatively) following exposure to cocaine; or if cocaine interferes with the “behavioral immunization” effects following an ES event.

Here we investigated the effects of cocaine on stressor controllability in rats. There are two aims to this study: In Aim 1 we considered the effects of stressor controllability on anxiety-like behaviors following repeated cocaine exposure. Aim 2 examined the effects of cocaine withdrawal on the “behavioral immunization” phenomenon. We used the juvenile social exploration test to measure anxiety-like behaviors in rats. Social interaction tests have been used reliably to assess anxiety-like behaviors in mice and rats for the past 25 years. Additionally, they have been shown to be sensitive to the effects of stressor controllability and anxiogenic and anxiolytic drugs.
(Christianson et al., 2008; File & Seth, 2003).

To address Aim 1, subjects were first trained to self-administer cocaine for one week. This was followed by a juvenile social exploration test to serve as the baseline measure. Rats were then exposed to one trial of ES and another juvenile social exploration test, which occurred 24 hours after ES exposure. The change in social exploration time before and after ES exposure was analyzed to determine whether the cocaine use effected anxiety-like behaviors following ES.

For Aim 2, the same rats remained in their home cages for one week following the last juvenile social exploration test in Aim 1; this served as the cocaine withdrawal period. Another juvenile social exploration test was run to provide a new baseline measure for social interaction time. Rats were then exposed to one trial of IS and another social exploration test, occurring 24 hours later. The change in exploration time before and after IS treatment was compared to determine if cocaine withdrawal interfered with the “behavioral immunization” effect.

To control for all possible variables, subjects were separated into four treatment groups. Group 1 (n = 6): the experimental group received cocaine during the self-administration period and was initially exposed to ES in Aim 1, followed by later exposure to IS in Aim 2. Group 2 (n = 4): received cocaine during the self-administration period but was initially exposed to IS in Aim 1 and IS again in Aim 2. This group served as the ES treatment control. Group 3 (n = 6): received saline during the self-administration period and was initially exposed to ES in Aim 1 and IS in Aim 2. This group served as the cocaine control group. Group 4 (n = 6): remained in their home cage during the self-administration period and was exposed to ES during Aim 1 and IS
during Aim 2. This group served as the home cage controls for the drug treatment. See Figure 3.1 for a schema of the experimental timeline.

![Experimental Timeline Schema](image)

**Figure 3.1:** Schema of the experimental timeline for the investigation into a drug by stressor controllability interaction on anxiety-like Behaviors in rats.

### 3.2 Materials and Methods

#### 3.2.1 Animals

All subjects in this study were male, Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN, USA). All experimental rats were adults, between 55 and 85 days old. Additionally, juvenile rats were used as stimuli in the juvenile social exploration tests and were between 28 and 32 days old. All rats were housed at the Institute for Behavioral Genetics at the University of Colorado Boulder. Upon arrival in Boulder, rats were allowed an acclimation period of at least 2 weeks prior to testing. All
rats were housed in pairs, maintained on a 12-hour light/dark cycle (lights on at 7:00 am), and given food and water *ad libitum*. All procedures were approved by the University of Colorado Institutional Animal Care and Use Committee, in accordance with National Institute of Health guidelines.

3.2.2 Catheter implant surgery

After 2 weeks of acclimation time to their home colony, catheters were implanted into the jugular vein under halothane anesthesia (1-2.5%). During recovery, catheters were flushed daily with 0.1 mL heparinized saline to maintain patency. Rats were allowed 4 to 7 days to recover in their home cage before experimental procedures began.

3.2.3 Cocaine self-administration

After 4 to 7-days of recovery from surgery, animals were trained to self-administer intravenous cocaine (0.5 mg/kg/100μL injection) or saline on an FR1 schedule in daily 2-hour sessions for 7 days; or they remained in their home cage and served as controls. Cocaine or saline injections were delivered over 5 seconds and were concurrent with the illumination of a cue light above the active lever. Drug/vehicle and cue delivery were followed by a 15 second time-out period in which the house light remained off and responding produced no consequence. Inactive lever responses produced no consequence throughout testing. After each daily session, rats were returned to their home cage.
3.2.4 Social exploration test

All social exploration tests were conducted in a quiet testing room as previously described (Christianson, et al., 2009). Each experimental adult rat was assigned its own cage (18x24x18 cm) and allowed to acclimate for 1 hour prior to testing. The social exploration test began when a juvenile rat of the same strain and source was placed into the cage of the experimental rat. All juvenile rats used were naïve to the experimental adult rat for each trial. The trials lasted for 3 minutes. An observer blind to treatment conditions timed all exploratory behaviors initiated by the adult rat. Exploratory behaviors included: sniffing, grooming, pinning and sparring.

3.2.5 Stressor controllability paradigm

The stressor controllability paradigm for these experiments followed the same protocol used widely in previous experiments (Amat et al., 1998). Rats in each drug treatment group were exposed to either escapable shock (ES) or inescapable shock (IS). Subjects were restrained in a small, Plexiglas shock box chamber containing a wheel (Med Associates, St Albans City, VT, dimensions: 14 x 11 x 17 cm). The wheel was rendered immobile during IS. The tail protruded from the back of the chamber and was taped in place using athletic tape. Two copper electrodes augmented with electrode paste were attached to the tail. Rats received shocks in yoked pairs (ES and IS) by a Precision Regulated Animal Shocker (Coulbourn Instruments, Allentown, PA). The treatment consisted of 100 trials with an average inter-trial interval of 60 seconds. Shocks began simultaneously for both rats in a pair and terminated for both whenever the ES rat met a response criterion. Initially, the shock was terminated by a quarter turn
of the wheel. When each of three consecutive trials is completed in less than 5 seconds, the response requirement was increased by one-quarter turn. Subsequent latencies under 5 seconds increased the requirement by 50%, up to a maximum of four full turns. If the requirement was not reached in less than 30 seconds, the shock was terminated and the requirement was reduced to a single quarter turn. Shock intensity was 1.0mA for the first 33 trials, 1.3mA for the following 33 trials and 1.5mA for the remaining trials. This procedure was used to ensure that the ES rats learned an operant response. Rats were returned to their home cage following the final shock.
3.3 Results

3.3.1 Aim 1: Change in social exploration time following cocaine addiction and ES/IS

After one week of cocaine self-administration treatment, we compared the difference in social exploration time before and after stress treatment to examine the effects of cocaine and stressor controllability on anxiety-like behavior. Interestingly, no significant differences were found in social exploration time before or after the ES/IS tail shock paradigm (Figure 3.2). In other words, social exploration time did not differ based on drug treatment or stress treatment.

![Change in Social Exploration Time Following Addiction and ES/IS](image)

**Figure 3.2:** Bar graph showing the change in social exploration time compared to baseline measure after 1 week of drug treatment (cocaine self-administration, saline or HC controls) and ES/IS tail-shock. Error bars = SEM.
3.3.2 Aim 2: Change in social exploration time following withdrawal and IS

To examine the effects of cocaine withdrawal and subsequent inescapable stress on anxiety-like behavior, we compared the difference in social exploration time before and after IS exposure following one week of cocaine withdrawal (Figure 3.3). No significant differences were found in behavior for the rats that were exposed to ES treatment first, regardless of whether they self-administered cocaine or saline. However, the rats that were exposed to IS first, showed a significant decrease in social exploration time compared to their baseline measure following the cocaine withdrawal period (paired t-test: p<0.05).

Figure 3.3: Bar graph showing the change in social exploration time compared to baseline measure after 1 week of drug withdrawal (cocaine, saline or HC controls) and IS tail-shock. Error bars = SEM.
3.4 Discussion

The research reported here is the first study to investigate the effects of cocaine on stressor controllability and “behavioral immunization” in rats. The aims of this study were based on three previously reported findings. The first is that the degree of control over a stressor is an important predictor in the outcome of the stressful event (Anisman et al., 1992; Seligman & Maier, 1967; Weiss et al., 1981). In other words, if an individual has behavioral control over an adverse event (ES), he/she is at a decreased risk for developing neuropsychiatric disorders that have been linked to exposure to an IS event, such as anxiety, depression, and/or drug abuse. The second finding is that previous experience with ES can reduce the risk of developing IS-induced behavioral changes in the future. This phenomenon is referred to as “behavioral immunization” (Seligman & Maier, 1967). The third finding is that cocaine use disorders often occur concurrently with stress disorders, and that repeated cocaine use can cause neural stress circuits to become increasingly more sensitive, which can lead to symptoms of withdrawal. Considering these findings, we investigated the effects that cocaine has on stressor controllability and anxiety-like behaviors in rats (Aim 1), and how cocaine withdrawal may or may not interfere with the “behavioral immunization” effect (Aim 2). To test this, we used the juvenile social exploration test to measure anxiety-like behaviors in rats, which has reliably been shown to be sensitive to the effects of stressor controllability in rats (Christianson et al., 2008).

Results from Aim 1 indicated that neither repeated cocaine exposure or stress treatment had an effect on anxiety-like behaviors in rats. Rats that were exposed to ES after one week of cocaine self-administration exhibited no difference in social
exploration time when measured before and after the stress treatment. This is consistent with previous studies that show that rats exposed to ES behave similarly in the juvenile social exploration test as home cage, stress naïve control rats (Christianson et al., 2008, 2010). However, the fact that these results remained consistent after one week of cocaine exposure is interesting. This indicates that repeated cocaine exposure, intended to mimic addictive-like behavior, did not interfere with the protective, behavioral effects induced by ES exposure. Not surprisingly, similar results were found for rats that self-administered saline instead of cocaine, and rats that were used as home cage controls.

While previous research shows that ES-exposed rats and home cage control rats show no significant differences in anxiety-like behaviors in the juvenile social exploration test, rats exposed to IS show a significant increase in anxiety-like behaviors. However, based on our results from Aim 1, this is not the case when the subject is exposed to cocaine for one week. We found that rats exposed to IS after one week of cocaine self-administration showed no difference in anxiety-like behaviors when measured before and after stress treatment. This original finding may indicate that cocaine use offers a protective effect against the behavioral outcomes of an uncontrollable stressful event. This could be explained by neuroadaptations that have been reported following cocaine exposure in animals. Specifically, chronic cocaine exposure leads to profound changes in glutamate (an excitatory neurotransmitter) neurotransmission within the brain’s reward pathway (Schmidt & Pierce, 2010; Wolf, 2010). When stimulated, the reward pathway (also known as the mesolimbic pathway) releases dopamine into the nucleus
accumbens and increases one’s perception of pleasure. These cocaine-induced neuroadaptations may override any IS-induced behavioral changes.

Aim 2 examined the effects of cocaine withdrawal on the “behavioral immunization” phenomenon previously reported. We were able to replicate this phenomenon in the home cage control group and the saline control group. As expected, previous exposure to ES in Aim 1 was protective against the effects of later IS exposure. No changes in anxiety-like behaviors were found in these two control groups when tested before and after IS treatment.

Analysis of the experimental group, which received ES after one week of cocaine self-administration in Aim 1, also showed no difference in anxiety-like behaviors following IS treatment in Aim 2. It appears the period of cocaine withdrawal did not interfere with the “behavioral immunization” effects. This indicates that the “behavioral immunization” phenomenon is more robust than symptoms of cocaine withdrawal.

Conversely, rats exposed to cocaine and IS in Aim 1, displayed a significant increase in anxiety-like behaviors following another exposure to IS in Aim 2. The potential protective effects of cocaine exhibited in results from Aim 1 appeared to diminish following a period of cocaine withdrawal. Furthermore, this group lacked prior exposure to ES, leaving them vulnerable to the IS-induced behavioral changes. This finding further supports the robustness of the “behavioral immunization” phenomenon.

There are several limitations to this study. One is the small sample size. Another is the fact that only one behavioral test was used to measure anxiety-like behaviors. This study was meant to serve as a preliminary investigation into the effects of cocaine on the stressor controllability model. Although the results are intriguing, verification of
these findings will require future studies that involve larger sample sizes and use multiple behavioral outputs.

Chapter Four: Investigation of Stressor Controllability on Anxiety-like and Depressive-like Behaviors: A New Mouse Model?

4.1 Introduction

Exposure to adverse events is one of the leading risk factors for developing a neuropsychiatric disorder such as anxiety or depression, or an increased risk for drug-abuse and addiction. However, not all individuals who are exposed to an adverse or stressful event end up developing such disorders. In other words, some individuals are more resilient than others following a stressful event. It has been shown that the degree of behavioral control in which an individual has over the stressor is pivotal to the impact of the event (Anisman et al., 1992; Weiss et al., 1981). In rats, it has been shown that behavioral control over a stressor can mitigate these stress-induced consequences. For example, rats exposed to inescapable stress (IS, uncontrollable) show a significant decrease in social exploration time compared to rats exposed to escapable stress (ES, controllable) in a juvenile social interaction test, indicating an increase in anxiety-like behavior following IS treatment (Christianson et al., 2008). Furthermore, IS-exposed rats show a significantly greater preference for oxycodone than ES-exposed rats in a conditioned place preference model (Der-Avakian et al., 2007). Finally, prior exposure to ES reduces subsequent contextual fear conditioning, while prior exposure to IS leads to a potentiation, indicating an exaggerated Pavlovian fear-response (measured by
freezing behavior) following IS treatment (M. V. Baratta et al., 2007). In other words, uncontrollable stress produces behaviors in rats that resemble symptoms of depression and anxiety, while exactly equated controllable stress does not.

A well-characterized model for studying this phenomenon in rats involves exposing one group of animals to ES, in which rats have behavioral control over the terminating each of a series of tail-shocks by turning a wheel; another group experiences IS in which durations are yoked to those produced by the ES subjects, with no control over terminating the tail-shocks. This exposure is followed by a number of behavioral tests that model anxiety and/or depression. The purpose is to compare responses between the two stress groups and a control group that remains in their home cages and receive no shocks. This stressor controllability phenomenon has been well characterized in the rat but not in the mouse.

Here we conducted an original study on the effects of stressor controllability in mice. We employed the same ES/IS tail-shock paradigm used in rats, followed by three behavioral tests (the open field test, the elevated zero maze, and the forced swim test) to assess anxiety-like and depressive-like behaviors. Our general aim in this study was to provide an important new mouse model to study stress-related disorders, where many genetic and genomics tools are more developed compared to the rat. Currently, the number of transgenic mice available is far more abundant than in rat, allowing a researcher to investigate a multitude of genetic and gene product effects in relation to stressor controllability. In addition, this model may be used to study novel strategies for the treatment and prevention of stress-induced anxiety, depression and drug-abuse, such as alcohol or nicotine intake following the acute stressor controllability paradigm.
A pilot study was first conducted using only IS and HC control mice to determine if, in fact, a stress effect could be found in mice using the three tests mentioned above as the behavioral output. The pilot study results were promising, so we followed up with a thorough investigation of the stressor controllability effect on anxiety-like and depressive-like behaviors in mice using ES, IS and HC controls. Figures 4.1A and B show the timelines and sample sizes for both the pilot study and the final study. It should also be noted that C57xDBA F1 mice were used in this study to minimize potential confounding results caused by behavioral differences among mouse strains, and to eliminate the risk of potential floor or ceiling effects in behavioral output. Previous experiments comparing mouse strains show C57 mice to be highly active, while DBA mice are moderately active (Crawley et al., 1997).
**Figure 4.1A:** Timeline of experimental procedures and sample sizes for the pilot study.

**Days 1-7:** Acclimation period in home cage (58 mice: 25 males and 33 females)

**Day 8:** Stress Treatment
- 29 mice (13 males, 16 females) experience **Inescapable Shock**
- 29 mice (12 males, 17 females) remain in their **homecage** and serve as controls

**Day 9:**
1) Open Field Test
2) Elevated Zero Maze Test

**Day 10:**
1) Forced Swim Test

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**Figure 4.1B:** Timeline of experimental procedures and sample sizes for the final study.

**Days 1-7:** Acclimation period in home cage (96 mice: 48 males and 48 females)

**Day 8:** Stress Treatment
- 32 mice (16 males, 16 females) experience **Escapable Shock**
- 32 mice (16 males, 16 females) experience **Inescapable Shock**
- 32 mice (16 males, 16 females) remain in their **homecage** and serve as controls

**Day 9:**
1) Open Field Test
2) Elevated Zero Maze Test

**Day 10:**
1) Forced Swim Test
4.2 Materials and Methods

4.2.1 Animals

Adult male and female C57xDBA (B6D2F1/J) mice bred at Jackson Laboratories (Bar Harbor, ME USA) were used for all final experiments in this study. The pilot study used adult male and female C57xDBA F1 mice, which were bred in-house at the Institute for Behavioral Genetics. Mice were housed at the Institute for Behavioral Genetics at the University of Colorado Boulder. All mice ordered from Jackson Laboratories were allowed an acclimation period of at least 2 weeks prior to testing. All mice were housed in pairs based on sex and shock treatment group. They were maintained on a 12-hour light/dark cycle (lights on at 7:00 am) and given food and water ad libitum. Mice were between 60 and 100 days old at the time of testing. All behavioral testing was done within the first 6 hours of the light cycle. All procedures were approved by the University of Colorado Institutional Animal Care and Use Committee, in accordance with National Institute of Health guidelines.

4.2.2 Stressor controllability paradigm

The stressor controllability paradigm for these experiments followed the same protocol as described in Chapter 3 and has been used widely in previous experiments with rats (Amat et al., 1998). After two weeks of acclimation time to their home-cages, mice were exposed to either escapable shock (ES), inescapable shock (IS) or remained in their home cage (HC) and served as controls. Mice in the ES or IS groups were restrained in a small, Plexiglas shock box chamber containing a wheel (Med Associates, St Albans City, VT, dimensions: 7.3x13.9x8.9 cm). The wheel was rendered immobile.
during IS. The tail protruded from the back of the chamber and was taped in place using athletic tape. Two copper electrodes augmented with electrode paste were attached to the tail. Mice received shocks in yoked pairs (ES and IS) by a Precision Regulated Animal Shocker (Coulbourn Instruments, Allentown, PA). The treatment consisted of 100 trials with an average inter-trial interval of 60 seconds. Shocks began simultaneously for both mice in a pair and were terminated for both whenever the ES mouse met a response criterion. Initially, the shock was terminated by a quarter turn of the wheel. When each of three consecutive trials was completed in less than 5 seconds, the response requirement was increased by one-quarter turn. Subsequent latencies under 5 seconds increased the requirement by 50%, up to a maximum of four full turns. If the requirement was not reached in less than 30 seconds, the shock was terminated and the requirement was reduced to a single quarter turn. Shock intensity was 1.0mA for the first 33 trials, 1.3mA for the following 33 trials and 1.5mA for the remaining trials. This procedure is used to ensure that the ES mice learn an operant response. Mice are returned to their home cage following the final shock.

4.2.3 Open field test

The open field test (OFT) was administered 24 hours following the stress treatment, after 1 hour of acclimation time in the testing room. The OFT is a commonly used method to measure anxiety and willingness to explore in rodents (Gould, et al., 2009; Hart, et al., 2010). The “open field” is a brightly illuminated square arena (60 x 60 cm square, 20.3 cm high). It is erected with acrylic plastic with walls to prevent escaping, and is equipped with multiple infrared beams to automatically track movement.
(Med Associates, Inc., St. Albans, Vermont). The floor was virtually divided into two areas: a center zone (11 cm from the wall) and an outer zone. Parameters of interest included the total distance traveled, time ambulatory, and the time spent in the center zone. Mice were tested individually for 5 minutes. Upon completion, each mouse was returned to its home cage.

4.2.4 Elevated zero maze

The elevated zero maze (EZM) test was carried-out approximately 1 hour following the open field test. Mice were acclimated to the testing room during this 1-hour interval. The test apparatus is a circular, black, elevated platform (30 cm inner diameter, 5 cm wide, 109 cm above ground) with two opposite enclosed quadrants and two open quadrants. This test is a modification of the elevated plus maze model of anxiety without the ambiguity of interpreting time spent in the center square of the traditional design, while allowing uninterrupted exploration (Shepherd, et al., 1994). Closed quadrants have clear acrylic walls (28.5 cm high), while the open quadrants have only a slightly raised Plexiglas lip. This design ensures approximately the same level of lighting in all quadrants while creating a border for measuring thigmotactic behavior (Cook, et al., 2001; Lister, 1987).

Anxiety-like behaviors were measured using both an automated system (AccuScan Instruments, Inc., Columbus, OH) and manually-scored observations. Automated measures include the time spent in the open quadrants versus the closed quadrants, activity count, and distance travelled. Manually-scored measures include the number of entries into an open quadrant, and the number of head dips, or “stretch
attend postures” (SAPs), which are define as a mouse stretching his/her head out of a closed arm into an open quadrant. Mice were tested individually and placed into the same closed quadrant at the start of each 5-minute test session. Between tests, the apparatus was wiped out with water to minimize odor cues from the previous subject. Water was used instead of bleach or ethanol to reduce any behavioral changes that may occur due to the odors these solvents leave behind. Upon completion, each mouse was returned to its home cage.

4.2.5 Forced swim test

Due to its anxiogenic nature, the forced swim test (FST) was the last of the behavioral tests carried out. It was performed 48 hours following stress treatment. This test, based on how long a mouse attempts to escape by swimming, has been used widely as a measure of depression or despair; more time spent not swimming is interpreted as increased despair (Petit-Demouliere, et al., 2005; Porsolt, et al., 1977).

The FST consists of a clear, acrylic, circular container measuring 45 cm high and 20 cm in diameter. The container was filled about halfway with room temperature water (23°-25° C). After approximately 1 hour of acclimation time to the testing room, the test mouse was gently placed in the water for 6 minutes, in which video tracking software (EthoVision by Noldus, The Netherlands) recorded its movements. All results from the pilot study were obtained using data from the video tracking software. For the final study, videos were scored manually by two blind observers to verify two measures: the latency to stop swimming and the total time spent not swimming. This was in addition to analyzing the video tracking software. Results did not differ when using the manually-
scored data versus the video tracking software data in terms of significant differences between the stress groups. However, upon visual observation of the FST, the manual data seemed to be a more accurate representation of mouse behavior in terms of time.

4.2.6 Estrous phase determination

To determine the stage of estrous that female mice were in during the behavioral tests, vaginal smears were collected and examined microscopically. Samples were collected using urethral cotton-tipped swabs moistened with saline, then transferred immediately onto a glass slide. The proportion of leukocytes and cornified and non-cornified epithelial cells was used to differentiate the estrous cycle stage. Estrous stages were characterized as follows: proestrus consisted predominately of nucleated epithelial cells; estrous consisted of only cornified epithelial cells, not containing a nucleus; diestrus, which also included the metestrus stage, consisted of a predominance of leukocytes with the potential for nucleated epithelial cells and/or cornified cells being present.

A standard mouse estrous cycle is about 4 days long (Caligioni, 2009). However, because cycle length can vary slightly from mouse to mouse, vaginal samples were taken for 8 consecutive days. This provided ample data to accurately determine the estrous cycle phase during behavioral testing for each mouse. The first sample was collected 1 to 3 hours after the FST (the last behavioral test), and then roughly at the same time of day for all subsequent collections (between 1pm and 3pm). By waiting to begin sample collection until all behavioral tests had concluded, we avoided any potential confounding handling effects. Furthermore, by collecting 8 days of estrous
cycle data, we could accurately estimate which phase a mouse was in for the previous behavioral tests. Please note that estrous cycle data were only collected for the finals, not for the pilot study.

4.3 Results

4.3.1 Pilot Study

4.3.1.1 Open field test

The behavioral output measures in which we were interested in the OFT include: the average distance traveled, the average time spent ambulatory, and the average time spent in the virtual center zone. Surprisingly, the stress condition had no effect on any of these measures among males or females (Figure 4.2). No significant differences were observed in the average distance traveled (males: $P = 0.85, F_{1,23} = 0.04$; females: $P = 0.23, F_{1,31} = 1.53$), the average time spent ambulatory (males: $P = 0.72, F_{1,23} = 0.13$; females: $P = 0.2, F_{1,31} = 1.76$), or the average time spent in the virtual center zone (males: $P = 0.1, F_{1,23} = 3.0$; females: $P = 0.46, F_{1,31} = 0.57$).

![Figure 4.2: Open field test results from the pilot study. A. Average distance traveled, measured in cm. B. Average locomotor activity, measured in seconds spent ambulatory. C. Average time spent in the virtual center zone, measured in seconds. Error bars = SEM.](image-url)
**4.3.1.2 Elevated zero maze**

For the EZM, four important behavioral output measures were identified: average activity count (measured by beam breaks), average latency to enter an open quadrant, average time spent in open quadrants, and the average number of open entries (i.e., the number of times a mouse crossed into an open quadrant). The results are shown in Figure 4.3. No significant differences were found between the stress groups among the females (average activity count: $P = 0.37$, $F_{1,31} = 0.84$; average latency to enter an open quadrant: $P = 0.32$, $F_{1,31} = 1.02$; average number of open entries: $P = 0.75$, $F_{1,31} = 0.12$; average time spent in open quadrants: $P = 0.38$, $F_{1,31} = 0.80$). However, the male mice exposed to IS showed a significant decrease compared to the HC controls in the average activity count ($P < 0.01$, $F_{1,23} = 14.12$), the average number of open entries ($P < 0.01$, $F_{1,23} = 8.37$), and the average time spent in open quadrants ($P < 0.01$, $F_{1,23} = 13.97$). No difference was found in the latency to enter an open quadrant among males, however ($P = 0.55$, $F_{1,23} = 0.37$). These results are interesting in that they may indicate that males are more sensitive than females to the effects of stress in this particular behavioral measure.
Figure 4.3: Elevated zero maze results from the pilot study. A. Average number of entries into an open quadrant. B. Average activity count, measured in beam breaks. C. Average time spent in open quadrants, measured in seconds. D. Average latency to enter an open quadrant for the first time, measured in seconds. Error bars = SEM.

4.3.1.3 Forced swim test

The FST was the final behavioral test conducted. Three output measures were used to assess depressive-like behaviors; they include: the average distance moved, the average time spent immobile (not swimming), and the average latency to stop swimming. This last measure represents how long the mouse will swim before “giving up”. Results are shown in Figure 4.4. Here we see that females exposed to IS stopped swimming significantly sooner than their HC counterparts ($P<0.01$, $F_{1, 31} = 10.57$), but no significant differences among males were found for this measure ($P = 0.2$, $F_{1,23} =$
1.76). For the other two measures, no significant differences were observed among males or females. Results for average time spent immobile are shown in Figure 4.4B (males: P = 0.89, F_{1,23} = 0.02; females: P = 0.2, F_{1,31} = 1.70), and results for the average distance moved are shown in Figure 4.4C (males: P = 0.71, F_{1,23} = 0.14; females: P = 0.12, F_{1,31} = 2.63) and Although more studies are needed, these results may indicate that females are more sensitive than males to the effects of stress when measuring depressive-like behaviors, as opposed to anxiety-like behaviors.

![Figure 4.4: Forced swim test results from the pilot study. A. The average latency to stop swimming for the first time, measured in seconds. B. Average time spent not swimming, measured in seconds. C. Average distance moved, measured in cm. Error bars = SEM.](image)

4.3.2 Final Study

4.3.2.1 Open field test

The behavioral output measures we used in the OFT were the same as the pilot study: the average distance traveled, the average time spent ambulatory, and the average time spent in the virtual center zone. Not surprisingly, the stress condition had no apparent effect on any of these measures, which replicates results from the pilot study. We did, however, find sex differences, indicating that females were significantly
more active/less anxious than males in all three measures (average distance traveled: \(P<0.001\), time ambulatory: \(P<0.001\), time spent in the center zone: \(P<0.05\)). Therefore, our results are separated by sex.

All statistical analyses utilized a between subjects ANOVA. Figure 4.5A shows no significant differences in average distance traveled among male and female stress groups (Males: \(P = 0.36, F_{2,47} = 1.05\); Females: \(P = 0.09, F_{2,45} = 0.61\)). Figure 4.5B shows no significant group differences in the average time spent ambulatory among males (\(P = 31, F_{2,47} = 1.19\)). Among females, we did find a difference among stress condition (\(P < 0.05, F_{2,45} = 3.44\)); however, following Tukey’s post-hoc analysis, it failed to reach significance (HC-IS: \(P = 0.07\), IS-ES: \(P = 1.0\), IS-HC: \(P = 0.07\)). Figure 4.5C shows results for the average time spent in the virtual center zone. Again, no significant differences among stress conditions were found (Males: \(P = 0.5, F_{2,47} = 0.77\); Females: \(P = 0.7, F_{2,45} = 0.367\)). These results are not particularly surprising, given that we observed no stress difference in the pilot study.

Figure 4.5: Open field test results from the final study. A. Average distance traveled, measured in cm. B. Average locomotor activity, measured in seconds spent ambulatory. C. Average time spent in the virtual center zone, measured in seconds. Error bars = SEM.
4.3.2.2 Elevated zero maze

For these experiments, we used the same four behavioral output measures used in the pilot study (average activity count, average latency to enter an open quadrant, average time spent in open quadrants, and the average number of open entries). In addition, we included one other measure - the average number of stretch attend postures (SAPs), which refers to when a mouse stretches its body from a closed quadrant into an open quadrant in an exploratory manner.

Between subjects ANOVAs were run for all statistical tests. Figure 4.6 separates the results by sex; this is due to significant sex differences found among three of the behavioral measures (average activity count: \( P = 0.01 \), average number of open entries: \( P < 0.001 \), and average time spent in open quadrants: \( P < 0.001 \)), with females being more active/less anxious than males in all three. No significant stress group differences were found in the behavioral measures, except for the average number of SAPs. Figure 4.6A shows the average activity count measure (Males: \( P = 0.88, F_{2,47} = 0.126 \); Females: \( P = 0.3, F_{2,45} = 1.25 \)). Average latency to enter an open quadrant is shown in Figure 4.6B (Males: \( P = 0.62, F_{2,44} = 0.49 \); Females: \( P = 0.68, F_{2,41} = 0.4 \)). Figure 4.6C shows results for the average time spent in the open quadrants (Male: \( P = 0.84, F_{2,47} = 0.18 \); Females: \( P = 0.91, F_{2,45} = 0.09 \)). Results for the average number of open entries is shown in Figure 4.6D (Males: \( P = 0.55, F_{2,47} = 0.611 \); Females: \( P = 0.75, F_{2,45} = 0.3 \)). Lastly, Figure 4.6E shows there is no significant group difference in the average number of SAPs among males (\( P = 0.28, F_{2,47} = 1.28 \)). However, among females, the HC mice showed significantly greater SAPs than mice exposed to the IS condition (IS – HC: \( P < 0.05 \)). Unfortunately, we did not find a significant ES or IS effect (IS – ES: \( P = 0.48 \), HC –
ES: P = 0.23). Furthermore, these results do not replicate results found in the pilot study, where male mice exposed to IS showed a significant increase in anxiety-like behaviors when compared to controls in several measures.

**Figure 4.6**: Elevated zero maze results from the final study. **A.** Average activity count, measured in beam breaks. **B.** Average latency to enter an open quadrant for the first time, measured in seconds. **C.** Average time spent in open quadrants, measured in seconds. **D.** Average number of entries into an open quadrant. **E.** Average number of “stretch attend postures”. Error bars = SEM.
4.3.2.3 Forced swim test

As in the pilot study, the FST was the final behavioral test conducted. The same three output measures were used to assess depressive-like behaviors, with the addition of one more measure - the average time spent being highly mobile (as determined by a threshold set by the software tracking system). Between subjects ANOVAs were used for the statistical analysis.

Significant sex differences were found among two of the measures: average distance moved (P < 0.001) and average time spent highly mobile (P<0.001). Interestingly, unlike in previous behavioral tests used to measure anxiety-like behaviors, the males were significantly more active than the females in this test. However, unlike the pilot study, no significant differences were found between stress treatment groups. Results for the average distance moved are shown in Figure 4.7A (Males: P = 0.62, F$_{2, 48}$ = 0.49; Females: P = 0.41, F$_{2, 45}$ = 0.92). Figure 4.7B shows the average time spent not swimming (Males: P = 0.65, F$_{2, 47}$ = 0.44; Females: P = 0.87, F$_{2, 45}$ = 0.14). Figure 4.7C shows results from the average time spent highly mobile (Males: P = 0.47, F$_{2, 47}$ = 0.76; Females: P = 0.65, F$_{2, 45}$ = 0.44). The average latency to stop swimming is shown in Figure 4.7D (Males: P = 0.7, F$_{2, 47}$ = 0.36; Females: P = 0.78, F$_{2, 45}$ = 0.26).
4.3.2.4 Estrous cycle effects

Between subjects ANOVAs revealed that estrous phase had no significant effect on behaviors in the OFT or the EZM. However, estrous phase influenced two behavioral measures in the FST: distance moved and the duration spent being highly mobile (Figure 4.8). In both measures, mice in the proestrous phase showed significantly less depressive-like behaviors than mice in estrous or diestrous phases (Distance moved: \( P < 0.01, F_{2,39} = 5.098 \); duration spent highly mobile: \( P < 0.01, F_{2,39} = 5.371 \)).
4.4 Discussion

Previous studies have shown that the degree of control individuals have over a stressful event can greatly influence their risk for developing untoward behavioral consequences, such as anxiety, depression, or drug-seeking (Anisman et al., 1992; Weiss et al., 1981). This phenomenon has been studied extensively in rats using the ES/IS trail-shock paradigm employed in the current study. Results have reliably shown that rats exposed to uncontrollable stress exhibit an increase in anxiety-like and drug-seeking behaviors, while rats exposed to controllable stress behave similarly to shock-naïve control rats (Jose Amat et al., 1998; M. V. Baratta et al., 2007; Christianson et al., 2008). However, when we tested this phenomenon in mice, we were unable to replicate these findings. Based on our results, the stressor controllability phenomenon does not seem to exist in mice as it does in rats and other species.
For this final study we employed three behavioral tests (OFT, EZM, and FST) that have been used extensively to assess anxiety-like and/or depressive-like behaviors in mice. We found no significant differences among the stress groups in any of the three behavioral tests, except for the average number of SAPs in the EZM test, in which both ES and IS animals showed increased anxiety-like behavior compared to HC controls. However, we failed to show a stress effect, which renders this finding irrelevant to our original hypothesis. We can make numerous speculations as to why this was the case. The first and most obvious reason being that the stressor controllability phenomenon simply does not hold true for mice. This does not discredit previous studies showing that the phenomenon exists in other species. Behavioral differences among mice and rats can vary greatly, depending on treatment and test conditions. Furthermore, while both mice and rats may be categorized as rodents, their genetic profiles and neuroanatomies differ, which can lead to vastly different behavioral manifestations.

The second possibility for our null findings may involve the timing of the behavioral tests. Because mice and rats differ in their neuroanatomies and gene expression, they may also exhibit temporal differences in neurotransmission following a certain event. In the previously cited studies in rats, behavioral tests were carried out at least 24 hours after stress treatment. We followed the same protocol here. However, there may be no ideal time-frame in which to capture robust behavioral changes caused by the stressor controllability paradigm in mice. Perhaps differences can be seen in mice only between 1 and 24 hours following stress treatment. More studies are needed to determine the ideal time-frame, if one exists, for studying this phenomenon in mice.
In addition to the biological and behavioral differences exhibited between mice and rats, we must consider the validity of the tests used in this study. While it would be ideal for an animal model to perfectly reflect a particular phenomenon under investigation, it is rarely the case that one model can account for the complexities present in a neuropsychiatric disorder. Therefore, an animal model of anxiety may not be able to encompass all of the features and symptoms of the disorder, but rather generate a state of anxiety that could best reflect the disorder (Lister, 1990). While the OFT, EZM and FST have all been validated under one condition or another as a reliable measure for anxiety or depression, these particular tests may not capture the features or symptoms of anxiety that are caused by inescapable and/or escapable tail-shock in mice. This is most likely the case for the OFT. We found no differences among the stress groups for males or females in either the pilot study or the final study. Perhaps a different set of behavioral tests would better capture the behavioral changes associated with stressor controllability in this particular species.

Finally, it is important to point out the origins of the mice used for the pilot study and the final study. As mentioned earlier, the mice used in the pilot study were bred in-house at the Institute for Behavioral Genetics, requiring no need for extensive relocation prior to testing. Conversely, the mice used in the final study were bred at Jackson Laboratories, requiring transport from Bar Harbor, ME to Boulder, CO prior to testing. The change was due to time constraints on completing the experiments. In the pilot study, males exposed to IS showed a significant increase in anxiety-like behaviors compared to HC controls in the EZM; females exposed to IS showed a significant increase in depressive-like behavior compared to HC controls in the FST. These results
were not replicated in the final study. Although both sets of mice were of the same strain, perhaps we were unable to replicate the pilot data due to the potential effects of transportation-induced stress in the final study animals. It is possible that the stress endured from transportation mimicked uncontrollable stress, thus rendering later exposure to controllable tail-shock invalid. In other words, perhaps the effects of the “uncontrollable” stress caused by transporting the animals could not be rescued by later controllable stress exposure. This may also explain why the HC control mice showed no difference in behavior from the ES or IS mice in the final study.

A previous study, conducted here at the Institute for Behavioral Genetics, may shed some light. The study examined the effects of locomotor activity and anxiety-like behaviors in mice lacking the beta3 nicotinic receptor subunit; this study faced a similar dilemma (Booker et al., 2007). Their original findings showed that B3 null mutant mice exhibit significantly less anxiety-like behavior in an Elevated Plus Maze than the wild-type or heterozygous mice. When the researchers attempted to replicate these findings, the effect was no longer present. The only difference between the two studies was the origin of the mice. For the original study, mice were obtained from the Salk Institute, thus requiring shipment from La Jolla, CA to Boulder, CO for testing. The follow-up study used mice bred in-house. The researchers hypothesized that the stress of shipping had an effect on the B3 null mutant mice. To test this, they created a shipping simulator by placing mice that were bred in-house on a gentle rocker prior to testing. Following this treatment, the behavioral effect previously found in the original study reemerged. Although another study has shown that shipping has no subsequent effects on behavior in multiple test paradigms (Crabbe, 1999), including the elevated plus
maze, previous exposure to stress (even mild) may cause a more nuanced effect than earlier thought.

Estrous cycle data were collected and analyzed for the final study. We found that estrous phase had an effect on behavior only in the FST, in which female mice in proestrous displayed significantly less depressive-like behaviors than mice in diestrous or estrous. These findings are consistent with the literature in that progesterone is known to surge during the proestrous phase and has been shown to be somewhat anxiolytic (Bitran et al., 1995; Fernández-Guasti & Picazo, 1990). However, when a linear regression model was run to account for estrous phase and stress treatment, no differences were found. In other words, estrous cycle did not change our original results. However, it is important to point out that estrous cycle data were only collected during the final study and not the pilot study, however. It would have been interesting to have this data available for the pilot study, given that significant stress treatment differences were found among females in the FST. It is also interesting to point out that estrous phase had no effect on behaviors in the OFT or the EZM, where no differences among stress groups were found in females in the pilot study. This may indicate that females are more sensitive to the effects of stress when measuring depressive-like behaviors than anxiety-like behaviors, and perhaps estrous cycle may play a role in this. However, further studies would be needed to make this conclusion.
5.1 Introduction

As reported in previous chapters, an individual (human or rat) exposed to an adverse or stressful event can be at risk for developing a variety of negative behavioral consequences, such as anxiety, depression, or drug-abuse. However, it is generally recognized that the degree of behavioral control over an aversive experience is a potent variable in determining the behavioral and psychological impact of the event (Charney, 2004; Shalev et al., 1998; Weiss et al., 1981). Much of the research conducted on this phenomenon used rats in a “stressor controllability” paradigm, as described in previous chapters and in the literature (Amat et al., 1998; Baratta et al., 2007; Grahn et al., 1999). In this paradigm, one group of subjects is exposed to escapable stress (ES) and has behavioral control over terminating a series of tail-shocks by turning a wheel; another group of subjects is exposed to inescapable shock (IS) and has no control over the tail-shocks. This group is yoked to the ES subject and, thus, receives an identical shock pattern and intensity. When utilizing this paradigm in rats, researchers find that animals exposed to IS exhibit significantly more anxiety-like behaviors than their ES-exposed counterparts in a number of behavioral tests, such as the juvenile social exploration test (Christianson et al., 2008) and fear conditioning paradigms (M. V. Baratta et al., 2007).

To grasp how stressor controllability effects behavior, we must have an understanding of the neurotransmitters and neural pathways involved. Serotonin (5-HT)
is a key neurotransmitter in the control of mood. It is a primary target of antidepressant medications, and also for the treatment of some anxiety disorders. A principle source of 5-HT neurons projecting to other areas of the brain is the dorsal raphe nucleus (DRN). Previous studies show that IS-induced behaviors have been linked to an increase in 5-HT neuronal activity in the DRN, which is not exhibited in ES-exposed rats (Grahn et al., 1999). Consequently, it is known that the DRN projections include the amygdala, nucleus accumbens, and the periaqueductal grey; all of these brain regions have been linked to such behaviors as anxiety and fear, drug reward, and escape responding, respectively. Furthermore, these brain regions have shown elevated extracellular 5-HT levels following IS treatment but not ES treatment in previous studies (Amat et al., 1998; Christianson et al., 2010). Therefore, behavioral consequences exhibited by IS-exposed rats, such as reduced social exploration, are likely DRN moderated and caused by the excess activation in 5-HT. However, because the DRN is a brainstem nucleus, it is unlikely to possess the integrative and complex function of sensing controllability or, in other words, differentiating IS from ES. It is now known that the ventral medial prefrontal cortex (vmPFC) detects control over a stressor and inhibits the 5-HT neurons in the DRN by way of glutamatergic pyramidal neuronal projections from the vmPFC to the DRN. These projections synapse onto GABAergic interneurons that then inhibit the 5-HT cells (Jankowski & Sesack, 2004; Steven F. Maier & Watkins, 2010).

The neurobiological consequences of stressor controllability have been characterized extensively in rats, but not in mice. Here we conducted an original study to determine if the same over-activation of 5-HT neurons in the DRN that is evidenced in rats also occurs in mice exposed to IS, but not ES. We used the same stressor
controllability paradigm and immunohistochemical technique for these experiments as was used with rats in the previously cited research articles. Although we were unable to replicate the behavioral effects of stressor controllability in mice (see Chapter 4), it is important to determine if the neurobiological effects of the phenomenon are still occurring. If so, it may indicate that the stressor controllability effect does, in fact, exist in mice but is not evidenced behaviorally in the open field test, elevated zero maze test, or the forced swim test. Results from this study will shed light on how stressor controllability effects mice neurobiologically and how best to study its effects in the future.

5.2 Materials and Methods

5.2.1 Animals

Adult male (ES: n=6, IS: n=6, HC: n=6) and female (ES: n=6, IS: n=6, HC: n=4) C57xDBA (B6D2F1/J) mice bred at Jackson Laboratories (Bar Harbor, ME USA) were used in this study. Mice were housed at the Institute for Behavioral Genetics at the University of Colorado Boulder. All mice ordered were allowed an acclimation period of at least 2 weeks prior to testing. All mice were housed in pairs based on sex and shock treatment group. They were maintained on a 12-hour light/dark cycle (lights on at 7:00 am), and given food and water ad libitum. Mice were between 60 and 100 days old at the time of experimentation. All procedures were approved by the University of Colorado Institutional Animal Care and Use Committee, in accordance with National Institute of Health guidelines.
5.2.2 Stressor controllability paradigm

The ES/IS tail-shock stressor controllability paradigm followed here was identical to the procedures described in Chapters 3 and 4 and has been used widely in previous experiments with rats (Jose Amat et al., 1998). After two weeks of acclimation time to their home-cages, mice were exposed to either escapable shock (ES), inescapable shock (IS) or remained in their home cage (HC) and served as controls. Mice in the ES or IS groups were restrained in a small, Plexiglas shock box chamber containing a wheel (Med Associates, St Albans City, VT, dimensions: 7.3x13.9x8.9 cm). The wheel was rendered immobile during IS. The tail protruded from the back of the chamber and was taped in place using athletic tape. Two copper electrodes augmented with electrode paste were attached to the tail. Mice received shocks in yoked pairs (ES and IS) by a Precision Regulated Animal Shocker (Coulbourn Instruments, Allentown, PA). The treatments consisted of 100 trials with an average inter-trial interval of 60 seconds. Shocks began simultaneously for both mice in a pair and terminated for both whenever the ES mouse met a response criterion. Initially, the shock was terminated by a quarter turn of the wheel. When each of three consecutive trials was completed in less than 5 seconds, the response requirement was increased by one-quarter turn. Subsequent latencies under 5 seconds increased the requirement by 50%, up to a maximum of four full turns. If the requirement was not reached in less than 30 seconds, the shock was terminated and the requirement was reduced to a single quarter turn. Shock intensity was 1.0mA for the first 33 trials, 1.3mA for the following 33 trials and 1.5mA for the remaining trials. This procedure was used to insure that the ES mice learn an operant response. Mice were returned to their home cage following the final shock.
5.2.3 Tissue preparation

One hour following the last tail shock, mice were anesthetized with sodium pentobarbital (65 mg/kg). They were then transcardially perfused with ice-cold physiological saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were post-fixed in the 4% paraformaldehyde solution for 24 hours, then transferred to 30% sucrose solution and stored at 4°C until ready for sectioning. Immediately before sectioning, brains were flash-frozen in ice-cold isopentane. DRN sections (30 µm) were sliced in a -20°C cryostat and collected in 12-well plates containing cryoprotectant solution (pH 7.2). Plates containing the free-floating DRN slices in cryoprotectant were then stored at 4°C until ready for immunohistochemistry (IHC) staining.

5.2.4 Immunohistochemistry

Staining for Fos and 5-HT was conducted sequentially in the mouse DRN sections, using the same protocol as previously described in Baratta et al. (2009). The Fos staining was conducted first using the avidin-biotin-horseradish (ABC) peroxidase (ABC) method. After a series of washes in phosphate-buffered saline (PBS, 0.01M), sections were incubated in 0.9% hydrogen peroxide solution for 35 minutes in order to reduce residual blood which may cause background interference. Following another series of PBS washes, sections were incubated for 24 hours at room temperature (RT) with Fos primary antibody (1:15,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in a blocking solution containing 1% normal goat serum (NGS), 0.25% Triton-X and 0.1% sodium azide. Following the Fos primary antibody incubation and another series
of PBS washes, sections were incubated for 2 hours in a biotinylated goat anti-rabbit secondary antibody (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in the same blocking solution as described above. After a series of PBS washes, slices were incubated for 1 hour at RT in ABC solution. This was followed by several washes in 0.1 M PB solution. The slices were then exposed to a solution containing 3,3′-diaminobenzidine (DAB), cobalt chloride, nickel ammonium sulfate, ammonium chloride, and glucose oxidase in PB. This solution reacted with the slices for 8 to 10 minutes before termination with multiple PBS washes. Slices were then incubated in blocking solution for 35 minutes at RT before being further processed for 5-HT. Slices were incubated in the 5-HT primary antibody (1:10,000; ImmunoStar, Hudson, WI, USA) for 48 hours at -4°C. Following a series of PBS washes, they were incubated in a non-biotinylated goat anti-rabbit secondary antibody (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 2 hours at RT. This was followed by a series of PBS washes and a rabbit peroxidase anti-peroxidase (PAP) soluble complex (1:500; Sigma-Aldrich, St. Louis, MO, USA) incubation for 2 hours at RT. Finally, slices went through a series of 0.1 M PB washes and another exposure to the DAB solution containing only glucose oxidase and 0.1 M PB. This exposure lasted 8 to 10 minutes and yields a yellowish-brown reaction as opposed to the dark grey or black reaction product obtained from the Fos reaction. After the final series of PBS washes, slices were floated onto gelatin coated slides and allowed to air dry overnight. They were then dehydrated with progressive ethanol baths, de-fatted with Histoclear, and cover-slipped with Permount.
5.2.5 Image analysis

Slide-mounted tissue was examined on a Leica BM IL LED microscope (Leica Microsystems Inc., Buffalo Grove, IL, USA) and photographed using the Leica LAS V4.4 software. Tissue was analyzed by two observers blind to animal treatment conditions. The number of 5-HT stained nuclei and 5-HT/Fos double-labeled nuclei were counted on each slice containing the DRN. Neurons displaying the yellowish-brown cell-bodies were identified as 5-HT, while Fos-stained nuclei were identified by dark-grey or black ovoid particles. The percentage of 5-HT cells expressing Fos (i.e., double-labeled cells) was used for the analysis.

5.3 Results

Figures 5.1 and 5.2 show results from the IHC analysis. Figure 5.1 shows the average number of 5-HT cells found in the DRN for each stress group. No differences were found between groups ($F_{2,28} = 0.499, P = 0.612$). This is to be expected given that stress treatment should not effect the number of 5-HT cells in a given brain area, and indicates that the 5-HT antibody staining was consistent throughout samples. Figure 5.2 shows results for the percentage of 5-HT/Fos double-labeled cells. An ANOVA, followed by Tukey’s post hoc test, revealed that mice exposed to ES exhibit a significantly greater percentage of 5-HT/Fos double-labeled neurons in the DRN compared to HC control mice ($F_{2,28} = 9.311, P<0.001$). Although differences were observed among all three groups (ES, IS, and HC), with HC having the least amount of double-labeled cells and ES mice the most, no differences proved to be statistically significant between ES-exposed mice and IS-exposed mice ($P = 0.098$), nor between IS-exposed mice and HC
controls ($P = 0.141$). Furthermore, no significant sex differences were found ($F_{1,28} = 1.188$, $P = 0.285$).

**Figure 5.1:** Immunohistochemistry results showing the average number of 5-HT cells in the mouse DRN. No differences were found between stress groups. Error bars = SEM.

**Figure 5.2:** Immunohistochemistry results showing the percentage of 5-HT/Fos double-labeled cells in the mouse dorsal raphe nucleus, as compared to serotonin-only expressing cells. **A.** Males and females combined. **B.** Separated by sex. Error bars = SEM.
5.4 Discussion

Previous studies in rats show that exposure to uncontrollable stress (IS) leads to significantly greater levels of 5-HT activation in the DRN compared to ES-exposed rats or HC controls. Subsequently, this leads to an increase in extracellular 5-HT in the DRN and its projection regions (i.e., amygdala, nucleus accumbens, periaqueductal grey) (Amat et al., 1998; Grahn et al., 1999). The IS-induced increase in extracellular 5-HT in these projection regions is believed to cause the behavioral changes exhibited following IS exposure. It is known that the detection of control that occurs with ES is sensed in the vmPFC and, consequently, leads to the inhibition of the DRN 5-HT neurons via glutamatergic neuronal projections (Jankowski & Sesack, 2004; Steven F. Maier & Watkins, 2010). This explains why ES-exposed rats do not exhibit the same negative behavioral consequences as the IS-exposed rats. However, we were unable to replicate these findings in mice.

In the current study, ES-exposed mice showed the greatest increase in 5-HT activation in the DRN compared to HC control mice. No significant differences were found between IS-exposed and ES-exposed subjects, or between IS-exposed and home cage control subjects. This is contradictory to existing literature in the rat. On the other hand, it does not entirely contradict the behavioral data presented in Chapter 4.

In Chapter 4, we investigated the effects of stressor controllability on anxiety-like and depressive-like behaviors in mice. Unlike in rats, we found no stress effects, controllable or uncontrollable, on behavior. In other words, there were no differences in anxiety- or depressive-like phenotypes among the ES, IS, and HC groups. This could partially be explained by the neurobiological results obtained in the current study,
showing no differences between the ES and IS groups or between the IS and HC controls. If the substantial differences in DRN 5-HT activation is not evidenced between the groups, we would not expect to see differences in anxiety- or depressive-like phenotypes. However, given that the ES-exposed mice showed greater amounts of 5-HT activity in the DRN than the HC mice, we would expect to see an increase in anxiety-like and/or depressive-like behaviors in the ES group compared to HC controls. Still, this was not the case.

There may be several explanations for these perplexing results. One explanation may involve the timing of tissue collection after stress treatment. For this study, mice were perfused and brains collected 1 hour after the last tail-shock was administered in the stress paradigm. This may not be the ideal time-frame to capture robust 5-HT neuronal activity in the DRN following stress. Previous studies using rats, collected brain tissue 2 hours after the final tail-shock (Baratta et al., 2009); hence, a 1 hour post-stress time point for tissue collection in mice seemed reasonable. However, upon review of the literature, previous researchers investigating 5-HT activity in the mouse DRN collected brain tissue at later time points. One study waited 2 hours after the stress treatment (Brooks et al., 2014), while another waited 24 hours following acute restraint stress (Issler et al., 2014).

Another explanation may be due to mouse strain differences in response of 5-HT transmission to stressors and to 5-HT receptor activation. It has been reported that exposure of DBA/2 mice to a range of stressors reduced 5-HT levels in the amygdala, hippocampus, and mesocortex and increased 5-HT in the frontal cortex (Jones et al., 1996; Shanks et al., 1991; Wimer, et al., 1973); whereas exposure of C57BL/6 mice to
stress increased 5-HT levels in the hypothalamus and mesocortex (Jones et al., 1996). Furthermore, it has been shown that fluoxetine, a selective serotonin reuptake inhibitor (SSRI) that is commonly used to treat depression, is only effective at decreasing depressive-like behaviors in the forced swim test in two inbred strains of mice: DBA/2J and BALB/cJ. The drug was not effective at reducing immobility (i.e., depressive-like behaviors) when tested in 5 other inbred strains, including C57BL/6 mice (Lucki et al., 2001).

A C57xDBA cross strain was used in our study. This strain was chosen in an effort to minimize confounding effects due to inherent mouse strain differences and to avoid potential ceiling or floor effects in the behavioral studies. Given that no data has been published on the 5-HT stress response of this particular hybrid strain, it is difficult to predict the actions of 5-HT neurons in the DRN following ES/IS tail-shock. However, the contradictory activity reported in 5-HT transmission and 5-HT receptors following stress among the two strains may explain our somewhat puzzling results.

Based on results from this study and the behavioral data reported in Chapter 4, it appears that the stressor controllability phenomenon is not as robust in the C57xDBA mouse strain as portrayed in rats. To make any conclusive statements as to why this may be the case, more experiments are needed.
Chapter Six: Conclusions

The studies presented here provide insights into the mechanisms involved in and the consequences of drug abuse and stress, and how best to study these disorders in the future. More specifically, we explored a gene of interest (Trpc4) as a potential target for anxiety and addiction, and shed light on the effects of repeated cocaine exposure and stress on anxiety-like and depressive-like behaviors in rats. We also attempted to determine if the ES/IS tail-shock paradigm could be utilized to study the stressor controllability phenomenon in a mouse model.

In Chapter 2, we designed and bred a trpc4 KO rat, which was then used to study the gene’s effects on anxiety, learning, and cocaine intake. This gene was designated based on its physiological attributes and its expression pattern in the brain. The Trpc4 gene codes for the TRPC4 channel, which is a non-selective canonical ion channel that has been linked with increasing neuronal excitability and synaptic plasticity by way of enhancing intracellular Ca+ release (Clapham et al., 2001; Schaefer et al., 2000). It is known that repeated cocaine exposure leads to long-term changes in synaptic plasticity in dopamine (DA) neurons (Ungless et al., 2001). Furthermore, Trpc4 expression in the adult rodent brain is highly concentrated in corticolimbic regions, which include a network of structures that receive dopaminergic innervation (Fowler et al., 2007). This suggests an association with motivation- and reward-related behaviors, in addition to regulating the brain’s response to stress and anxiety. Here we tested Trpc4 as a causal gene mutation, and as a possible link, in understanding the neurological processes behind drug addiction.
To assess Trpc4’s role in anxiety, we tested trpc4 KO rats against controls in a juvenile social exploration test. We found that trpc4 KO animals spent significantly less time interacting with the juvenile mouse than their WT counterparts and Sprague-Dawley control rats. This is the first evidence showing that the presence of Trpc4 affects anxiety-like behaviors in rats and these data indicate that TRPC4 ion channels are playing a fundamental role in anxiety, most likely by supporting cellular processes in specific regions of the brain associated with regulating anxiety.

Next, we aimed to determine if Trpc4 was playing a role in reward-seeking behaviors. To do this, we compared trpc4 KO rats to their WT littermates in a cocaine self-administration model. We found that the rats lacking the Trpc4 gene self-administered significantly less cocaine infusions than the WT rats. To verify that the trpc4 KO rats did not suffer any learning impairments that could interfere with their ability to self-administer a drug, we first trained them to self-administer sucrose prior to the cocaine trials. Additionally, we ran a separate cohort of rats through a battery of tests to measure learning and memory. No differences were found between the KO and WT rats in their ability to self-administer sucrose, nor in their learning or memory. Based on these data, we hypothesized that the lack in TRPC4 channels in areas of the brain associated with drug addiction decreased the cocaine-induced synaptic plasticity in DA neurons.

While these findings linking Trpc4 to addiction and anxiety disorders are interesting, they pose a challenge in terms of developing potential treatment options, given that Trpc4 expression is protective against anxiety-like behaviors but a risk for drug-seeking behaviors. It is important for further studies to identify possible upstream
or downstream effects of the gene’s expression to fully understand the mechanisms causing the differences in behaviors. In doing so, one may discover a more targeted approaches to treating the different disorders.

The study reported in Chapter 3 had two specific aims. Aim 1 was to investigate the effects of cocaine on the stressor controllability phenomenon. Aim 2 was to determine if cocaine withdrawal interfered with behavioral immunization that occurs during IS when the individual had previous exposure to ES. To do this, we used a rat model of cocaine self-administration and the juvenile social exploration test to measure anxiety-like behaviors following the ES/IS tail-shock paradigm.

Results from Aim 1 show that repeated cocaine exposure has no effect on anxiety-like behaviors following controllable stress. Rats that were exposed to ES showed no differences in behavior regardless of whether they received cocaine self-administration, saline self-administration, or remained in their home cage to serve as controls. Interestingly, repeated cocaine exposure diminished the negative behavioral effects of uncontrollable stress. The group of rats that received cocaine self-administration showed no evidence of IS-induced anxiety-like behaviors following uncontrollable stress. It seems that the repeated exposure to cocaine may be protective against the behavioral consequences of IS.

Results from Aim 2 show that cocaine withdrawal does not interfere with behavioral immunization. No difference in anxiety-like behaviors were found in the rats that had previous exposure to ES and went through the cocaine withdrawal period. This indicates that the behavioral immunization effect is robust enough to overcome the negative side-effects associated with cocaine withdrawal. However, the protective
effects of cocaine exposure following IS treatment evidenced in Aim 1 had completely disappeared following the withdrawal period. Anxiety-like behaviors significantly increased in the rats that had previous exposure to IS and went through cocaine withdrawal.

In Chapter 4, we set out to determine if the effects of stressor controllability could be transferred to a mouse model. If so, researchers would have the ability to study the effects of stress on a number of disorders, such as anxiety, depression, and drug abuse, by utilizing the plethora of mutant and transgenic mice that are more widely available compared to rats. We used the same ES/IS tail-shock paradigm that has been used extensively in rats. This was followed by a sequence of behavioral tests (the open field test, the elevated zero maze test, and the forced swim test) used to measure anxiety- and depressive-like phenotypes in mice. Unfortunately, we were not able to replicate the stressor controllability effects that have been reported in rats. Mice that were exposed to ES, IS or remained in their home cage to serve as controls showed no differences in anxiety- or depressive-like behaviors.

There are several possibilities for why this study yielded null results. The simplest and most obvious reason is that the stressor controllability phenomenon reported in humans and rats does not exist in mice. However, it is also possible, that the behavioral tests used in our study did not accurately capture the behavioral effects of stressor controllability in mice. Finally, the fact that the mice used in the final study had been shipped across the country may have interfered with the final results. Evidence has been reported showing mice that had been shipped prior to testing exhibit a difference in some behaviors (i.e., anxiety-like behaviors in the elevated plus maze) compared to
mice that were bred in the testing facility (Booker et al., 2007). Due to the discrepancy between the pilot study results and results from the final study, replication experiments should be carried out using all in-house bred mice.

In Chapter 5, we investigated the neurobiological effects of stressor controllability in mice. Given that we were unable to show behavioral effects of stressor controllability in mice in Chapter 4, we wanted to know whether mice experienced similar neurobiological changes following the ES/IS tail-shock paradigm as evidenced in rats. Studies in rats show that subjects exposed to IS will have greater 5-HT activation in the DRN compared to ES-exposed subjects or HC controls (Grahn et al., 1999). The increase in extracellular 5-HT in the DRN and its projection regions lead to the IS-induced behavioral consequences (i.e., anxiety, depression, drug abuse) (Amat et al., 1998). Results from this study would help us determine if the lack of behavioral changes following ES/IS tail-shock reported in Chapter 4 is due to a discrepancy in the neurobiology of stress in mice, or if we were simply unable to accurately capture the behavioral effects of stressor controllability using our study design.

Based on results from these experiments, it would seem that the neurobiology of stressor controllability in mice differs from that of the rat. We showed that mice exposed to ES exhibited significantly more 5-HT neuronal activation in the DRN compared to HC controls. However, there were no statistically significant differences between the ES and IS groups, or the IS and HC groups. Clearly, this is not representative of the rat data. However, these data are somewhat representative of the behavioral data collected in Chapter 4, in that no differences were found among the IS and HC groups and the ES
and IS groups. On the other hand, it does not explain why ES-exposed mice did not exhibit an increase in anxiety- or depressive-like behaviors compared to HC controls.

It is difficult to make any conclusive statements regarding the stressor controllability phenomenon in mice. Based on the behavioral data collected in Chapter 4 and the neurobiological data reported in Chapter 5, it appears that it is not as robustly evidenced in mice as it is in rats. However, given that the experimental mice may have experienced some shipping-induced stress, and the lack of experimentation regarding 5-HT transmission in the C57xDBA hybrid mouse strain, more experiments are needed to fully elucidate the effects of stressor controllability on mice.
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