DEVELOPMENT, VALIDATION, AND CHARACTERIZATION OF A NOVEL PRECLINICAL ANIMAL MODEL OF SOCIAL FAMILIARITY-INDUCED ANXIOLYSIS

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Social support is a powerful therapeutic against fear and anxiety and is utilized in many psychotherapies. The concept that a familiar or friendly presence helps a person learn to overcome anxiety has been well-known for decades, yet, the basic neural mechanisms that regulate this psychosocial learning remain unknown. A first step towards elucidating these basic mechanisms is the development of a valid preclinical animal model. However, preclinical behavioral models exploring the use of a social presence in reducing anxiety have not been fully characterized. Therefore, it was our goal to identify a useful way in which to study the mechanisms of how a social presence can induce anxiolysis (the reduction of anxiety). We accomplished this goal by characterizing and validating a preclinical model, as well as demonstrating that the model was capable of measuring deficits in rats given a mild traumatic brain injury. To this end, we identified an existing, but uncharacterized model, the social interaction-habituation model, as an effective model of social familiarity-induced anxiolysis (SoFiA), which demonstrates socially enhanced safety learning, or psychosocial learning. We find that as social familiarity develops across time, anxiolysis develops. We identified that the use of a Bright Light Challenge is a useful anxiogenic stimulus to use during SI-habituation training. The anxiolysis acquired following SI-habituation testing is partner specific, and can be blocked by an inhibition of the medical prefrontal cortex, while it can be enhanced by D-cycloserine. We found that this model identified deficits in SoFiA acquisition in rodents exposed to a mild traumatic brain injury, which, in humans, has been linked to psychosocial deficits. This work is a step in creating ways in which we can study and better understand the regulatory processes of emotions mediated by social behavior.

Anantha Shekhar, M.D., Ph.D., Chair

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LIST OF ABBREVIATIONS

5HT	5-hydroxytryptamine
5HTT	5-hydroxytryptamine transporter
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAr	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	Analysis of Variance
AP	Anterior/Posterior
AP5	(2*R*)-amino-5-phosphonovaleric acid; (2*R*)-amino-5-phosphonopentanoate
BLA	basolateral amygdala
BLC	Bright Light Challenge
bmTBI	Blast-induced mild traumatic brain injury
BNST	Bed Nucleus of the Stria Terminalis
BSA	Bovine serum albumin
CA2	cornu amonis
CBT	Cognitive Behavioral Therapy
cm	centimeter
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CRF	Corticotropin-releasing factor
CRH	Corticotropin-releasing Hormone
DCS	D-cycloserine
DNQX	6,7-dinitroquinoxaline-2,3-dione
DV	Dorsal/Ventral
EPM	Elevated Plus Maze
fMRI	functional magnetic resonance imaging
fmol	femtomol
g	gram
GABA	*gamma*-Aminobutyric acid
GAD 67	Glutamate decarboxylase 67
gSAD	generalized Social Anxiety Disorder
H	height
HPA	hypothalamic–pituitary–adrenal axis
IC	intracranial

IL	infralimbic
L	length
min	minute
ML	Medial/Lateral
mm	milliliter
mRNA	messenger Ribonucleic acid
mTBI	mild traumatic brain injury
NAc	nucleus accumbens
NMDA	*N*-Methyl-D-aspartic acid
NMDAr	*N*-Methyl-D-aspartic acid receptor
nl	nanoliter
OF	Open Field
ORX	Orexin
ORX1r	Orexin 1 receptor
ORX-A	Orexin-A
PFC	prefrontal cortex
PL	prelimbic cortex
pmol	picomol
PTSD	Post-traumatic Stress Disorder
SAD	Social Anxiety Disorder
SC	subcutaneous
SEM	Standard error of the mean
SI	Social Interaction
SoFiA	Social Familiarity-induced Anxiolysis
TBI	Traumatic brain injury
Tukey's HSD	Tukey's Honest Significant Difference
UCN	Urocortin
µm	micrometer
vmPFC	ventral medial Prefrontal Cortex
VTA	ventral tegmental area
W	width

Chapter 1: Main introduction

Social Support and mental health

Humans have a fundamental drive to socially interact, form social attachments to others and be a part of a social group (Baumeister & Leary, 1995). This drive likely stems from the importance of social interactions to our health and well-being. Indeed effective social functioning helps in coping with stress and is necessary for survival; ensuring access to protection, resources and mates (Adolphs, 2001; Amodio & Frith, 2006; Strodl & Schausberger, 2012). Additionally, a positive social environment and the existence of social support have strong positive influences on mental health maintenance and recovery (Chinman et al., 2014; Meyer-Lindenberg & Tost, 2012).

Social support imparts many benefits to individuals, and humans seek out social connections throughout life, starting early in development by forming attachments to caregivers, which provide a sense of safety (Eisenberger et al., 2011). Contact with another socially familiar person, either physical or implied, has been shown to alleviate anxiety caused by stress inducing stimuli such as threatening words or a shock (Conner et al., 2012; Eisenberger et al., 2011). This was also demonstrated by evidence that perception of pain and emotional distress to the threat of a painful stimulus is reduced when the subject is in contact with or viewing a picture of a familiar person compared to an unfamiliar person (Coan, Schaefer, & Davidson, 2006; Eisenberger et al., 2011).

Social support is important throughout life, and can help a person overcome maladaptive emotions to face a feared event or other situation (Coan et al., 2006; Eisenberger et al., 2011). Peer support systems can aide in the ability of patients to cope with or recover from some mental illnesses (Chinman et al., 2014). For example, the support from a social network can provide benefits such as help in preventing depression in people with high numbers of life stressors (Kaplan et al., 1994). Additionally, social support is protective against the deleterious effects of stress and is one of the best predictors of positive treatment outcome for multiple mental illnesses (Carter et al., 2012; Chinman et al., 2014; Dour et al., 2014; Meyer-Lindenberg & Tost, 2012). Anxiety is a common psychological ailment in humans that is characterized by feelings of worry and fear, often without any direct threat present. Anxiety can become pathological when these feelings become disruptive to everyday activities. Given the high lifetime prevalence of anxiety disorders (28.8%), in the adult American population, (Kessler,

Chiu, Demler, Merikangas, & Walters, 2005), it is important to better understand the interaction of social support and both normal and pathological levels of anxiety.

Social support from a familiar source as a means to alleviate anxiety is a core component of psychotherapies used on humans such as Cognitive Behavioral Therapy (CBT). In therapy, the social interactions between the patient and therapist or with the patient and their peers within a group are vital, creating a network of social support. Within these psychotherapies, the goal is directed towards specifically learning to reduce anxiety or fear, which is a form of safety learning. The success of the therapy is linked to the bond between the patient and the therapist, which is at the core of these behavioral and cognitive therapies. For anxiety, the subject's perceived alliance with the therapist (the patient-therapist relationship) is integral to the success of the treatment, and strengthens with increasing number of sessions as safety learning occurs (Crits-Christoph, Gibbons, Hamilton, Ring-Kurtz, & Gallop, 2011; Hersoug, Hoglend, Gabbard, & Lorentzen, 2013; Jaycox, Foa, & Morral, 1998; Martin, Garske, & Davis, 2000). Clients with high levels of perceived social support, or attachment styles favoring secure perception of social support, increases positive outcomes of psychotherapy compared to low perception of social support (Dour et al., 2014; Levy, Ellison, Scott, & Bernecker, 2011; Price, Gros, Strachan, Ruggiero, & Acierno, 2013). Even in cases of extreme pathological anxiety such as PTSD, the most effective therapies are exposure-based interpersonal or group therapies, and success of these treatments are dependent on perceptions of social support (Price et al., 2013). Beyond the source of social support, the capacity for anxiolysis is also dependent in part on the context and frequency of the socially supportive encounters. Social support from peers with anxiety had greater anxiolytic quality than social support from staff, mediated in part by the context of shared anxiety (Chinman et al., 2014; Hundt, Robinson, Arney, Stanley, & Cully, 2015).

Although the utilization of social support to regulate behavior is a known concept, and is a fundamental aspect to human social interactions, the underlying mechanisms of the regulatory processes of this are not well known. It is important that we understand these mechanisms, understanding the mediation of emotion regulation through social cues can help advance treatments for people who suffer from deficits in social/emotional regulation. A systematic way to study this process has, to our knowledge, not been fully developed, and so in order to understand how humans are able to utilize social support to overcome maladaptive psychological behaviors such as anxiety, animal models representing this are needed. We cannot model exactly all of the nuanced social interactions and behaviors between a patient and

their therapist, however the preclinical model presented within this thesis attempts to provide a way to study an aspect of this important therapeutic interaction, and to emulate the social enhancement of safety learning that occurs within some of these therapies.

Animal modeling

Animal model importance and validation

The preclinical model of social familiarity-induced anxiolysis that is used in this thesis needs to first be validated in order to establish it as a model of socially-enhanced safety learning. Animal modeling is the primary preclinical mode of scientific inquiry into pathological and normal processes of physiology and behavior (van der Staay, Arndt, & Nordquist, 2009). Specifically, it is through animal modeling that we elucidate the basic physiological mechanisms of various disease states. Studying humans is not always possible for logistic and ethical reasons, and cells are not able to demonstrate the complex interactions that occur within systems such as the brain, making animal models a necessary surrogate to human research, providing valuable information that is otherwise unattainable. Therefore, there is a need for reliable, efficacious preclinical animal models, which are needed for the development of treatment strategies for various diseases. My research is focused specifically on the utilization of a preclinical animal model to better understand the basic neural mechanisms of socially-enhanced safety learning. Knowledge gained through systematically investigating the underlying mechanisms that regulate psychological processes will provide insights into disease states of psychopathologies. This knowledge will allow us the opportunity to develop targeted therapies for patients.

Animal model validity

In order for an animal model to be successful, key features are needed; which are that the model must be reliable, replicable, and valid (van der Staay et al., 2009). An animal model that is reliable will produce results that are reproducible across time and among different laboratories with minimal variation between subjects (Salome et al., 2002; van der Staay et al., 2009). Additionally, there are various types of validity used to demonstrate an animal model is truly representative of a human condition. The ones that I will focus on are face, predictive, and construct (Kalueff & Tuohimaa, 2004). Face validity is when the phenotype that is presented in the animal model is similar to the behavioral and physiological phenotype in humans. Predictive

validity is when effects seen in humans with pharmacological agents have the same effects in the animal model. Construct validity relates to the underlying mechanism of the disease-state in the animal model, and we see construct validity when the mechanism and etiology of the disease in the animal model is similar to the disease state within humans (Kalueff & Tuohimaa, 2004).

Rodent models used to observe anxiety-like behavior

Rodents are a useful model organism for measuring anxiety-like behavior. Tests of rodent behavior are widely used, and take advantage of easily observable behaviors. For example, environment exploration tests take advantage of creating a conflict between the rodents' natural aversion to unknown and potentially dangerous environments and a natural drive to explore novelty (Ohl, 2003). One such test that utilizes this conflict is the Open Field (OF) test, a validated test of anxiety-like behavior in which the exploration behavior is assessed by quantifying the time spent within the designated zones of the arena or the number of entries into each zone (File, 1980; File & Hyde, 1978; Maciag et al., 2002; Prut & Belzung, 2003). Within open, exposed spaces, rats have a tendency to move along the more protected areas and stay nearer to the walls, a behavior termed thigmotaxis (Ohl, 2003). The behavior is interpreted as representing higher levels of anxiety-like behavior when the exploration of the middle and center zones is reduced compared to controls (Ohl, 2003).

The Elevated Plus Maze (EPM) is a test which utilizes environmental exploration and is a validated test of anxiety-like behavior in rats and mice (Hogg, 1996; Pellow, Chopin, File, & Briley, 1985). The closed arms are intended to create the enclosed space, while the open arms are exposed and elevated, creating a potentially dangerous environment. The EPM again takes advantage of the preference rats demonstrate towards enclosed spaces over open exposed ones, while also taking advantage of the drive for rats to explore novelty (Montgomery, 1955). Therefore, the key behaviors observed within the EPM are the amount of time the rats spend in the open and closed arms and the number of entries into the open arms (Kumar, Bhat, & Kumar, 2013). Increased anxiety-like behavior is determined by a decrease in the amount of time the animals spend exploring the open arms compared to controls. Additionally, pharmacological agents that decrease anxiety will increase the amount of time spent in the open arms relative to controls (Pellow et al., 1985).

Social exploration/interaction between rats occurs when a conspecific (member of the same species) partner is present, and the behaviors typically seen of the rodent is to physically interact with the conspecific through sniffing, climbing over or under, and leaning against. Rats typically prefer socially interacting, however rats can avoid interactions or show aggression towards the partner, such as biting or mounting. Tests take advantage of the normal rat behavior to prefer interaction, making these interactions the basis for the Social Interaction (SI) test, which is a well-established and validated test for anxiety (File, 1984; A. Shekhar, 1994). Anxiety-like behavior within this test is measured by the amount of time, in seconds, that the rat will engage in social interaction with a conspecific partner, with the amount of time interacting inversely related to the relative level of anxiety-like behavior being expressed, as compared to controls or baseline. The SI test is unique in its structure as it is an anxiety test that utilizes a partner rat in a social setting where the test rat must recognize social cues (friendly versus hostile, novel versus familiar) and adapt behavioral output to the partner rat.

Rodent models demonstrate social preference

Rodents are very social creatures, making them a useful model organism for observing social behavior. Rodents have a strong propensity to socially interact with other conspecifics. Rats show a place preference for a chamber paired with a conspecific partner, and in single housed rats, the partner paired chamber is more rewarding than a chamber paired with the reward activating drug amphetamine (Yates, Beckmann, Meyer, & Bardo, 2013). Rats also demonstrate a stronger conditioned place preference for a chamber where they were allowed to have full physical contact and social interaction with a conspecific versus only partial contact such as through bars or limited odor, vision, auditory and vibration contact with a conspecific (Kummer et al., 2011; Peartree et al., 2012). This demonstrates that social contact is rewarding in rats, and full physical social contact is preferred (Peartree et al., 2012). Additionally, rats are capable of forming social memories. Rats show a preference for a chamber containing a novel conspecific over a familiar conspecific presented 30 minutes or 24 hours previously (Gur, Tendler, & Wagner, 2014). A familiar juvenile resident intruder is recognized up to 24 hours later, and will receive less social investigation than a novel intruder rat, which is indicative of social recognition (Moura, Meirelles, & Xavier, 2010).

It is important to note that not all social contact is utilized as a positive reinforcement, as some rodent models involve social contact as a way to induce stress and fear behavior. These

negative social contact models include social defeat, social instability, and social fear conditioning, (Blanchard, McKittrick, & Blanchard, 2001; I. Toth & Neumann, 2013; I. Toth, Neumann, & Slattery, 2012). These tests involve using a social presence in a negative way, to instill fear and avoidance behaviors. These types of social interactions are not what we are attempting to capitalize on, and instead want to focus on the use of social presence as a positive reinforcement and a source of safety learning in most contexts.

Given the preference that rats have towards social contact, they make a good model organism for observing the effects that social contact can have on reducing different behaviors such as fear or anxiety-like behavior. I am interested in understanding how a social presence can be used to specifically reduce anxiety, and by observing the effects of social contact on anxiety-like behavior in rodents; a better understanding of the underlying mechanisms of the regulation of anxiety through social cues can be gained.

Modeling social exposure to reduce anxiety-like behavior

Social Buffering

Social Buffering is phenomena in which social animals are protected, or buffered, against experiences of distress in the presence of conspecific animals (Kikusui, Winslow, & Mori, 2006). The social buffering effect is characterized specifically as a reduction in stress, fear and anxiety behavior responses to a stressor while in the presence of a social conspecific (Davitz & Mason, 1955; Kiyokawa, Takeuchi, & Mori, 2007; Latane, 1969; Terranova, Cirulli, & Laviola, 1999). This response happens without prior training, and is the consequence of the presence of a conspecific, regardless of whether the conspecific is familiar. Additionally, these social buffering responses are conserved as the presence of a conspecific reduces anxiety-like behavior and normalizes neuroendocrine responses across multiple species including, rodents, birds, fish, pigs, sheep and primates (Detillion, Craft, Glasper, Prendergast, & DeVries, 2004; Galhardo, Vital, & Oliveira, 2011; Glasper & Devries, 2005; Hennessy, Kaiser, & Sachser, 2009; Hennessy, O'Leary, Hawke, & Wilson, 2002; Hostetler & Ryabinin, 2014; Kanitz, Hameister, Tuchscherer, Tuchscherer, & Puppe, 2014; Kikusui et al., 2006; Kiyokawa et al., 2007; Lieberwirth & Wang, 2016; Sachser, Durschlag, & Hirzel, 1998; Terranova et al., 1999). The effectiveness of social buffering is species selective, meaning that social buffering effects will not occur in the presence of a related species (da Costa, Leigh, Man, & Kendrick, 2004; Kiyokawa, Takeuchi, Nishihara, & Mori, 2009).

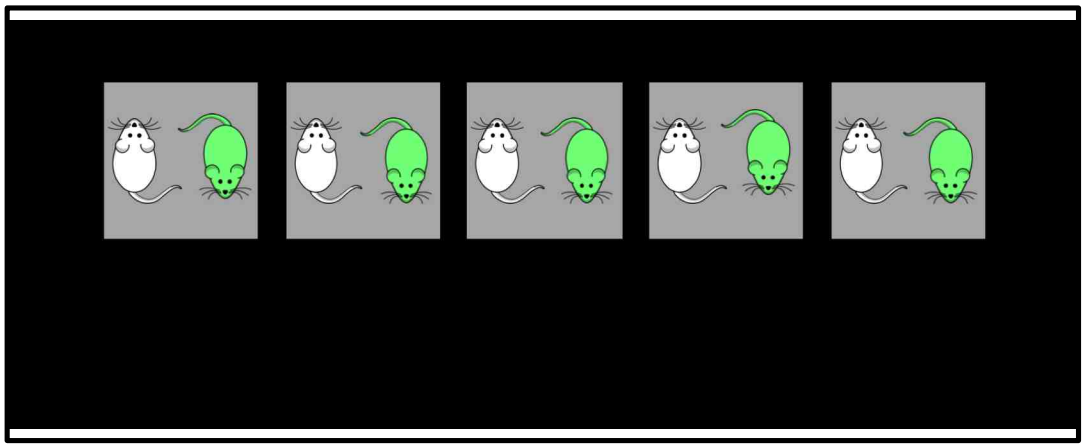
Social buffering, in preclinical models, is effective against multiple anxiogenic cues and stressors including, novelty exposure, social defeat and fear conditioning (Kiyokawa et al., 2007; Nakayasu & Kato, 2011). Often social buffering effects are measured through the stress response, which is defined in the context of the effect a conspecific has on the hypothalamic-pituitary-adrenal (HPA) axis in response to a stress-inducing stimuli or event (Hennessy et al., 2009; Kanitz et al., 2014; Lieberwirth & Wang, 2016; Terranova et al., 1999). The HPA axis is often evaluated through elevations in blood cortisol level in response to stressors such as fear conditioning (seen in rats), or to novelty exposure (seen in rats and piglets), which are reduced by the presence of a conspecific (Kiyokawa et al., 2007; Sachser et al., 1998; Terranova et al., 1999). Behavioral observations are often observed through induction of fear conditioning or exposure to novelty, and the effect of social buffering in the presence of a conspecific is seen as reduction of the fear behavior expression such as freezing (Davitz & Mason, 1955). For example, Davitz and Mason demonstrated in 1955 that fear conditioning through a light-shock pairing elicited less freezing behavior to the light when the rat was paired with another rat during the conditioning compared to when the rat experienced the conditioning alone (Davitz & Mason, 1955). Additional social buffering outcomes have been observed as well, such as a facilitation in wound healing in pair-housed monogamous mice or socially housed hamsters compared to socially isolated housing (Detillion et al., 2004; Glasper & Devries, 2005). Additionally, the presence of a conspecific in prairie voles prevents alcohol relapse-like behaviors compared to socially isolated counterparts (Hostetler & Ryabinin, 2014).

Physiological evidence for the presence of a conspecific to reduce fear during a social buffering experiment demonstrated that fear conditioning to a tone/shock pairing led to cellular activation measured by cFos expression increases (a measure of cellular activation) in the Infralimbic cortex as well as the Central Amygdala, Basal Amygdala, and Basolateral Amygdala (Kiyokawa et al., 2007). Rats pair-housed or fear conditioned with a conspecific had less fear behavior (freezing) and more cellular activation of these brain regions than rats housed alone and fear conditioned alone (Kiyokawa et al., 2007). This implicates the presence of a conspecific in affecting the neurological regulation of fear responses.

Social Familiarity

The social buffering effect demonstrates that a social presence can act as an important source of anxiety reduction; however, another level of social buffering can occur, and this is

when the conspecific is familiar. The presence of a familiar conspecific can produce an even greater reduction in anxiety and HPA axis activation; an effect that has been observed in multiple species including rats, cichlids and humans (Galhardo et al., 2011; Hennessy et al., 2002; Kikusui et al., 2006; Terranova et al., 1999). For example, the presence of a socially familiar partner increases environment exploration in male cichlid fish (Galhardo et al., 2011). Rats also demonstrate greater social buffering responses (reduced freezing and HPA axis activation) in the presence of a familiar versus unfamiliar partner. In the SI test in rats, a study found additional reductions in corticosterone levels when the partner was familiar versus unfamiliar (Terranova et al., 1999). In fear conditioning in rats, the presence of a familiar conspecific rat reduces freezing in response to a tone-paired shock more than an unfamiliar rat (Kiyokawa, Honda, Takeuchi, & Mori, 2014).



A unique behavioral protocol in which social familiarity is utilized to reduce anxiety-like behavior is termed Social Interaction-habituation (SI-hab), which was first described by Truitt and colleagues (Truitt et al., 2007). This SI-hab testing paradigm consists of giving rats a SI test every day for several consecutive days. The basic procedural protocol is shown in **Figure 1.1**. Previously, this procedure was used on a group of rats that were made persistently anxious, demonstrating elevated levels of anxiety-like behavior in the SI test. However, following the SI-hab testing, with the same partner each day in the SI test, the rats eventually developed anxiolytic-like behavior (Truitt et al., 2007). Specifically, with the implementation of the SI-hab protocol, a reduction in anxiety-like behavior was observed; the SI times eventually increasing significantly from the first SI-hab day, and were no longer significantly reduced from baseline by the 4^{th} and 5^{th} day of testing (Truitt et al., 2007). The repeated exposures to the same partner possibly produced a learned effect of an association of the socially familiar partner as a source

of anxiolysis, leading to the reduction in anxiety-like behavior. This is supported by follow-up testing in which a novel partner introduction led to a reversion to the previously increased anxiety-like behavior, seen as a significant reduction in SI time from the previous testing day with the familiar partner (Truitt et al., 2007).

The SI-hab testing paradigm has advantages which permits using the same conspecific partner repeatedly to investigate the regulation of anxiety-like behavior by social familiarity (Truitt et al., 2007). This testing procedure is a type of safety learning using social cues, where the partner rat becomes a safety cue following the formation of familiarity. Across the days tested, behavior changes can be observed, as the SI times increase with repeated exposures to that familiar partner. The novel partner challenge led to a significant decrease in SI time, indicating that the reduction in anxiety that was observed was likely partner-specific (Truitt et al., 2007).

The previous study investigating the effect of SI-hab had utilized rats made to display the persistent anxiety-like phenotype through a process called Urocortin (UCN) priming, first developed by Shekhar and colleagues. UCN is a neuropeptide that acts as a Corticotropin Releasing Factor (CRF) receptor 1 and 2 agonist (T. J. Sajdyk, Schober, Gehlert, & Shekhar, 1999a). A single high dose of UCN (100 fmol) injected directly into the basolateral amygdala induces a single episode of increased anxiety-like behavior in male rats in the SI test, seen as a significant reduction in the SI times compared to control injections (T. J. Sajdyk et al., 1999a). When UCN is injected 3-5 times at sub-anxiogenic doses (6 fmol), a persistent behavioral phenotype emerges, and rats will express increased anxiety-like behavior in the SI test, which has been replicated several times and shown to last for as long as 5 weeks after treatment (T. J. Sajdyk & Gehlert, 2000; A. Shekhar, Sajdyk, Gehlert, & Rainnie, 2003; Truitt et al., 2007).

The effect of social familiarity on the UCN primed rats was done without all of the necessary controls completed at that time, leaving a few questions unanswered. For example, how specific is the social familiarity in overriding the anxiety produced by the UCN priming? In other words, will the testing of the SI-hab lead to the same anxiolytic effect if the partner is a novel partner in each test? The novel partner challenge leads to a decrease in SI time, indicating that the novel partner was not anxiolytic. However, to ensure that the anxiolysis observed in the SI-hab test was purely partner-specific and a consequence of the social familiarity development, controlling for social familiarity must be done.

The UCN priming procedure induces anxiety-like behavior, and we have seen that the SI-hab paradigm can overcome this UCN-induced anxiety-like behavior. However, the UCN priming procedure itself has a few key drawbacks, preventing us from utilizing this stimulus to move forward in the study of how anxiety is regulated. Reasons for this are that the priming procedure involves several drug injections that lead to persistent changes in behavior as well as the neuronal architecture within the BLA. Specifically, UCN priming leads to increased excitability of the BLA caused by a reduced inhibitory tone (Rainnie et al., 2004), which could lead to anxiety-like behavior as an artificial construct, and any mechanisms that regulate that behavior could lack the intrinsic value that we are seeking to understand. I would like to explore other ways in which to induce anxiety-like behavior in a way much like the UCN priming, in that it is consistent and reliable, and I explore alternative anxiogenic stimuli in the first chapter.

My *main hypothesis* for this thesis is that the SI-hab paradigm is a valid way to model social familiarity-induced anxiolysis. To further develop the SI-hab protocol that was previously described, I began by identifying an anxiogenic stimulus to induce anxiety-like behavior within this protocol. I then applied the anxiogenic stimulus to the SI-hab testing paradigm to test the validity of the procedure as a preclinical model of social familiarity-induced anxiolysis. I then followed up with observing any deficits in the SoFiA model in rodents that had undergone an induced pathological state.

This goal was addressed via the following specific aims:

Specific AIMS

Aim 1: Identify the optimal procedure for inducing and measuring anxiety-like behavior to study social familiarity-induced anxiolysis.

Aim 2: Validate and further characterize the model of Social Familiarity-induced Anxiolysis (SoFiA) as a model of psychosocial learning.

Aim 3: Utilize the model of SoFiA to detect psychosocial learning deficits in a pathological animal model.

Chapter 2: Identifying the optimal procedure for inducing anxiety-like behavior to investigate social familiarity-induced anxiolysis

Introduction

Goal of the chapter

The SI-hab testing paradigm has been demonstrated as a useful test to investigate the effects of social familiarity on anxiety behavior across time. My goal in this chapter is to identify an optimal anxiogenic stimulus to use with the SI-hab testing paradigm. The previous work utilizing the SI-hab testing paradigm used UCN priming as the anxiogenic stimulus to initiate anxiety-like behavior. However, this process of initiating anxiety has some drawbacks. UCN priming involves a pharmacological manipulation, which potentially lacks ethological translation, and requires a surgical procedure that demands precise targeting of a specific brain area. Elevated anxiety-like behavior from the UCN priming procedure results from the effects of the UCN on the BLA, the mechanism of which results from an interoceptive stimulus. UCN priming does not allow the opportunity to study the mechanism of anxiety regulation; any mechanisms used to overcome the UCN induced anxiety may not reflect true anxiety regulation that occurs naturally due to the introduced interoceptive stimulus. Therefore, this chapter will focus on identifying an anxiogenic stimulus that fits the criteria below.

Initiating anxiety-like behavior within the SI-hab test

The anxiogenic stimulus used for the SI-hab testing must meet a set of specific criteria that would allow us to effectively study anxiety regulation within the SI-hab testing paradigm. In addition to being ethological, the anxiogenic stimulus should meet the following criteria: 1) reliably induce measurable increases in anxiety-like behavior, 2) implemented in a repeatable manner, and 3) efficacious within multiple tests of anxiety.

My first criterion for the anxiogenic stimulus is that it must reliably induce measurable increases in anxiety-like behavior. Here, reliability means that the stimulus will induce anxiety-like behavior across several cohorts of animals with minimal variability in behavior. Also, the stimulus must induce measurable changes in anxiety-like behavior, meaning the presence of the stimulus produces significant increases in the anxiety-like behavior compared to control conditions (absence of the stimulus). My second criterion is that the anxiogenic stimulus must produce anxiety-like behavior in a repeatable manner. A primary characteristic of the SI-hab

testing paradigm is that it is repeated across several days, with each test separated by 24 hours. Behavioral changes that occur in response to the anxiogenic stimulus should remain consistent across repeated exposures in the absence of other influencing factors. For example, this criterion is essential in order to isolate the effects of social familiarity on producing anxiolytic effects within this SI-hab paradigm. The third criterion is that the anxiogenic stimulus must be efficacious across multiple tests of anxiety. Specifically, an anxiogenic stimulus that is effective within only the SI test and no other tests of anxiety-like behavior may not be inducing anxiety, but rather an artifact of the specific test being employed. Therefore, in order to demonstrate that we are measuring anxiety-like behavior, the anxiogenic stimulus of choice must be robust and transferable to other tests such as the Elevated Plus Maze (EPM) or the Open Field (OF) tests, which are discussed below.

Along with the essential criteria that are required characteristics of the anxiogenic stimulus, I have also identified additional criteria that would make the testing procedures more refined. The most useful anxiogenic stimulus is one that is easy to administer. Meaning the stimulus would not require extensive prior training for the animal, should not involve invasive surgeries or other potentially painful procedures that would require recovery time. Lastly, the anxiogenic stimulus would preferably be ethological. This means that it is a stimulus that potentially could be encountered in the wild and elicits a reaction without conditioning to attain a potentially natural reaction from the rodents, providing a look into behavior that is not isolated to laboratory conditions (Campos, Fogaca, Aguiar, & Guimaraes, 2013).

Putative anxiogenic stimuli

There were a number of anxiogenic stimuli available, and I chose stimuli based on past evidence as well as according to the above criteria. Stimuli that would affect locomotion and prevent the ability to socially interact were immediately excluded from consideration. Food and water deprivation are ethological stimuli that rats often encounter in the wild, however we avoided these stimuli in favor of stimuli that had already been utilized in conjunction with the Social Interaction test. Therefore, I aimed to focus on stimuli that would not obstruct the social interaction behaviors to ensure that the rats had an opportunity to develop social familiarity. Within this chapter I explore the anxiogenic properties of Orexin, Restraint, and a Bright Light Challenge.

Orexin

The neuropeptide Orexin (ORX) is implicated in the induction of anxiety behavior (Li et al., 2010). ORX is generally anxiogenic, eliciting anxiety-like behaviors within multiple species including the hamster, mouse, and rat (Avolio, Alo, Carelli, & Canonaco, 2011; Johnson et al., 2010; Li et al., 2010; Plaza-Zabala, Martin-Garcia, de Lecea, Maldonado, & Berrendero, 2010; Suzuki, Beuckmann, Shikata, Ogura, & Sawai, 2005). ORX is generally agreed to be anxiogenic, however it was found to be anxiolytic in rats tested in a paradigm of acoustic startle responses when infused into the cerebral ventricles (Singareddy, Uhde, & Commissaris, 2006). Several brain areas receive ORX neuronal inputs originating from the hypothalamus, and many are involved in the modulation of anxiety, such as the ventral tegmental area, the paraventricular nucleus, amygdala and bed nucleus of the stria terminalis (Avolio et al., 2011; Li et al., 2010; Moorman & Aston-Jones, 2010b; Peyron et al., 1998). Exposure to anxiogenic stimuli leads to activation of ORX neurons and increased ORX gene expression, while anxiolytic drugs block the increased activation of ORX neurons in response to anxiogenic stimuli (Ida et al., 2000; Panhelainen & Korpi, 2012; Plaza-Zabala et al., 2010).

Dense Orexin-A (ORX-A) projections are found in an anxiety-related locus, the bed nucleus of the stria terminalis (BNST), making this area of interest to target with ORX to induce anxiety-like behavior (Johnson et al., 2010; Nambu et al., 1999; Peyron et al., 1998). The BNST is a neuronal structure associated with fear and anxiety behavior, as it has been implicated in regulating anxiety-like responses as well as the regulation of the HPA-axis, which is involved in the stress response (Davis, 1998, 2006; Forray & Gysling, 2004; Lee & Davis, 1997; Sink, Walker, Yang, & Davis, 2011; Sullivan et al., 2004; Treit, Aujla, & Menard, 1998). Activation of BNST CRF receptors with Urocrotin induces anxiety-like behavior specifically in the SI test but not the EPM, and activation of the BNST with GABA synthesis inhibitors or optogenetic activation increased anxiety-like behavior in the EPM and OF tests (S. Y. Kim et al., 2013; Lee, Fitz, Johnson, & Shekhar, 2008; T. Sajdyk, Johnson, Fitz, & Shekhar, 2008). Inhibition of the BNST through glutamate antagonists and optogenetic inhibition decrease anxiety-like behavior in the EPM, OF and SI tests, and inhibiting the ventral lateral BNST during a stressor exposure prevents reductions social exploration (reduced fear behavior) (Christianson et al., 2011; S. Y. Kim et al., 2013). ORX injections into the BNST as an anxiogenic stimulus does not fit my outlined criteria of not using a stimulus that required surgery. However, the utility of this stimulus was explored based on the prior findings that ORX was elevated in human patients. Patients with panic

disorder who were experiencing anxiety were found to have increases in Orexin concentrations within their cerebral spinal fluid compared to people without panic disorder and people with panic disorder with comorbid major depressive disorder (Johnson et al., 2010), suggesting some construct validity for the use of ORX as an anxiogenic stimulus. We therefore attempted to study the anxiogenic role of ORX in the BNST, injecting the ORX-A unilaterally directly into the BNST as a preliminary study.

Restraint

Restraint is a potential anxiogenic stimulus to use because it can induce anxiety-like behavior in rodents and represents an ethological stimulus (danger of being trapped). An exposure to restraint induces anxiety-like behaviors, and the response is associated with the length of time of the exposure, the amount of time after the exposure and if the exposure is given acutely or chronically (several exposures). A 30-minute acute exposure to a restraint stimulus has been used to induce increases in anxiety-like behavior in the SI test when given 0, 30, 60 or 90 minutes, but not 120 minutes prior to behavior testing (T. J. Sajdyk et al., 2008). A significant reduction (compared to controls) in the percentage of time spent in the open arms of the EPM was found 24 and 48 hours after rats were exposed to restraint, but not at shorter time points (Padovan & Guimaraes, 2000). Another study found that 48 hours after a 2 hour restraint stress, rats demonstrated increased conditioned freezing behavior to a fear conditioned context compared to controls (Cordero, Venero, Kruyt, & Sandi, 2003). Restraint can be administered chronically as well, with restraint application daily for multiple days (Campos et al., 2013). For example, a study found that five days of repeated exposures to a 90-minute restraint in rats led to significant reductions in social interaction time (compared to controls) following the last day of restraint exposure (Doremus-Fitzwater, Varlinskaya, & Spear, 2009). The ability to induce anxiety-like behavior in the SI test following multiple exposures to restraint provides the potential for us to utilize the restraint stimulus within the multi day SI-hab testing paradigm. In particular, the timeline used by Sajdyk and colleagues establishes a target time in which to administer the restraint for use as an anxiogenic stimulus within the SI-hab testing paradigm (T. J. Sajdyk et al., 2008).

The Bright Light Challenge

Previous studies have used a stimulus of bright illumination under which behavior testing was conducted. This bright illumination exposure in the OF test has previously been

shown to increase anxiety-like behavior in rats (Bouwknecht et al., 2007; Igarashi & Takeshita, 1995). File and colleagues used a brightly lit testing arena during the SI test and found that the rats had reduced SI times compared to rats tested in low lighting, but only when the testing arena was unfamiliar (File, 1984). Additionally, a study found that bright illumination was not effective for inducing anxiety-like behavior in Wistar rats in the EPM test (Becker & Grecksch, 1996). With these mixed results in mind, I asked whether a modified procedure of the implementation of bright illumination would be more effective, and so I hypothesized that a modified Bright Light Challenge procedure would effectively induce anxiety-like behavior. The modifications involve introducing the bright light to the animals through a sudden onset of the bright light at the initiation of the test. We do this with no prior habituation to the bright light, and instead habituate the rats to dim red lighting prior to testing. We call this modified procedure a Bright Light Challenge (BLC), which is described in detail in the methods section. I explored this stimulus further to see if it fits the criteria for the anxiogenic stimulus.

Rodent models of anxiety-like behavior within this chapter

Social Interaction test

The SI-hab testing paradigm that I aim to use consists of several Social Interaction (SI) tests. Rats are a gregarious species and thus have a natural propensity to be in social contact with other rats. The SI test takes advantage of this propensity for social contact and has been validated as a test of anxiety-like behavior in male rats (File, 1980, 1984; File & Hyde, 1978). Latane and colleagues described the need for rats to socially interact through full physical contact. This, they argue, is due to their tendencies to spend time rubbing up against each other rather than merely looking at each other (Latane & Glass, 1968). The SI test employs social interaction between two rats, and the SI time is quantified as the amount of time the test rat spends interacting with the partner, which is the dependent variable of anxiety behavior. SI time is inversely related to the relative amount of anxiety-like behavior being expressed, meaning that relative decreases (from baseline) in SI time is interpreted as an increase in anxiety, and vice versa. The SI test provides many advantages for our purposes, as it utilizes a partner presence, where the test rat must recognize social cues (novel versus familiar) and adapt anxiety-like behavioral responses to the partner.

Other specific tests of identifying anxiety-like behavior

Many tests measuring anxiety-like behaviors exist for rodents, and there are some more commonly used that provide useful tools in research, allowing for comparisons between experiments. Although I utilize the SI test as the primary measure of anxiety-like behavior, I additionally implement other common tests of anxiety-like behavior. As essential criteria, the anxiogenic stimulus should unequivocally induce anxiety-like behavior within the SI-hab test, but to increase confidence in the stimulus, additional tests are needed.

Open Field test

The Open Field (OF) test is a validated test of anxiety-like behavior that takes advantage of the natural aversion of rodents to open spaces (Hall, 1934). The behavior in this test is assessed by the amount of exploration the animal does within the arena. Specifically, the exploration behavior in the OF is assessed by quantifying the amount of time the animal spends in or the number of times the animal enters into the three designated zones of the arena; the outer, middle and center zones. Rats are safest nearest the walls and thus will spend more time near the outer walls when they feel threatened. The behavior is interpreted as increased levels of anxiety when the exploration of the middle and center zones are reduced compared to controls (Ohl, 2003). Additional locomotion behavior can be assessed in this test to ensure that total movement is not affected by treatments.

Elevated Plus Maze

The Elevated Plus Maze (EPM) is a validated anxiety test commonly used in rats and mice (Pellow et al., 1985). This test consists of an elevated apparatus made up of two walled (closed) and two non-walled (open) arms. The EPM takes advantage of creating a conflict between the drive to explore novelty and the aversion to being in exposed, and in this case heightened, spaces (the open arms). The measurement of anxiety behavior within the EPM is the amount of time the rat spends in the open arms versus the closed arms and the number of entries the rat makes into the open arms, which are both reduced compared to controls in rats expressing increased levels of anxiety-like behavior (Kumar et al., 2013).

Methods

Common Techniques

Some common techniques that are used throughout this thesis are outlined below. Specific behavioral manipulations within these paradigms are specified within each chapter.

Elevated Plus Maze

Prior to testing rats within the Elevated Plus Maze (EPM), rats were carried into a staging area within their home cages and allowed to habituate to the room under dim red lighting for a minimum of 30 minutes. The EPM was performed similarly as described previously (Lee et al., 2008; Walf & Frye, 2007). The EPM is a black Plexiglas apparatus (Hamilton Kinder, San Diego, CA) that consists of two open arms and two closed arms each 50.17 cm long and 10.8 cm wide that extend from a square center platform that is 11 cm^2. The closed arms have walls that are 40.01 cm high on each side. The entire apparatus is elevated 100 cm above the ground on aluminum legs attached to an aluminum base. For the test, rats were first introduced to the apparatus by placing them on the center platform facing one of the open arms. Testing time is 5 minutes and test sessions were video recorded from above and parameters quantified using ANY-maze version 4.81 Software from Stoelting for experiment 2 and scored by two independent scorers for experiment 5. The apparatus was thoroughly wiped down between animals.

Open Field

Prior to testing in the Open Field (OF), rats were carried into a staging area within their home cages and allowed to habituate to the room under dim red lighting for a minimum of 30 minutes. The testing apparatus consists of a painted Plexiglas open-top square box with dimensions 91.44 cm L x 91.44 cm W x 30.48 cm H. The box floor is demarcated into three zones, the outer ring, a middle ring and the center of the box. For the initiation of the test, the rats were placed into the center of the OF box alone and allowed to explore the environment with no closed areas to escape into during the 5-minute test session. The duration of freezing behavior during the test was quantified using ANY-maze version 4.81 Software from Stoelting. Additionally, the amount of time the rats spent in each of the designated outer, middle and center zones within the OF were measured. The apparatus was thoroughly wiped down between animals.

Bright Light Challenge Stimulus

The Bright Light Challenge (BLC) consists of an abrupt transition from dim red light (40-watt red light, 1 lux) to bright white fluorescent lighting (>488 lux at the approximate eye level of the rats) at the initiation of the behavior test. Prior to the BLC, rats were kept in the dimly lit (red lighting) staging room within their home cages for at least 30 minutes prior to the behavioral test/challenge. Immediately following the simultaneous placement of the test and partner rats into the testing arena the induction of the BLC was initiated by abruptly switching from the dim red lighting to bright white lighting by turning on the lights of the room with additional overhanging lights aimed at the open field arena. The lights remained on for the duration of the 5-minute testing session and were turned off at the cessation of the test.

Social Interaction Test

The Social Interaction (SI) test was originally described and validated as a test of anxiety-like behavior by File and colleagues (File, 1980; File & Hyde, 1978). The test itself has been since modified by our lab and utilized for nearly two decades as a validated measure of anxiety-like responses (Rainnie et al., 2004; T. J. Sajdyk & Gehlert, 2000; Sanders & Shekhar, 1995; A. Shekhar, 1994; A. Shekhar & Katner, 1995; A. Shekhar, Keim, Simon, & McBride, 1996; Truitt et al., 2007). The SI box itself is a painted Plexiglas Open Field test box. 24 hours prior to any behavioral SI testing, all rats underwent a 5-minute individual habituation to the SI box in dim red lighting. Baseline SI testing with an unfamiliar partner rat was obtained at least 48 hours prior to initiation of treatment or additional SI tests. On the day of testing, rats were carried into a staging area within their home cages and allowed to habituate to the dim red lighting for a minimum of 30 minutes prior to any testing. Just before SI testing, the experimental rat and the partner rat were both carried into the testing room within their home cages. SI testing consisted of placing the experimental rat into the SI box simultaneously with a partner rat for a 5-minute test session. To eliminate the possibility of the partner establishing dominance and affecting the amount of social interaction time, the two rats were age, weight and sex matched (Wesson, 2013).

During social contact, rats engage in exploration of each other through sniffing, the most common body areas being the face, flank and anogenital area of the partner rat (Wesson, 2013). Therefore, SI time was measured as the amount of time, in seconds, that the experimental rat spent engaging in non-aggressive physical investigation (sniffing) of the

conspecific partner. Aggression, climbing over and avoidant behaviors were not scored as social interaction time, although none of the experimental or partner rats displayed aggressive or avoidant behaviors in these studies. SI time is inversely related to anxiety-like behavior, and so expression of anxiety-like behavior is defined as a significant decrease in SI time when compared to baseline or control SI times. Likewise, anxiolysis is defined as a significant increase in SI time. Partner initiated contact or investigation was independent of the scored SI time; thus, SI times are independent of the partner's behavior. Partner rats were used for a maximum of two sessions in a single day, and these sessions were separated by at least 30 minutes. Each session was video recorded from above and subsequently scored using ODlog for Mac OS X version 2.6.1 by Macropod Software by a treatment blind observer. Only one test was performed within the testing room at a time, and the behavior box was thoroughly wiped down with a disinfectant cleaner between testing sessions. All SI testing occurred between 09:00-13:00 (during the rat's light period).

The Social Interaction-habituation testing paradigm

The Social Interaction-habituation (SI-hab) testing paradigm was performed similarly as described previously (Truitt et al., 2007). This testing paradigm consists of repeating the SI test across several consecutive days (typically 5-6). Specifically, the rat is first given a baseline test under non-anxiogenic conditions (dim red lighting), and 48 hours later, Day 1 of the SI-hab testing begins. The SI tests are then repeated, with tests separated by 24 hours and performed each day at approximately the same time. Variables within this testing paradigm can be altered such as the partner type (familiar vs. novel), pharmacological interventions or the presence of an anxiogenic stimulus before or during testing to initiate alterations in anxiety behavior.

Restraint Stimulus

The restraint stimulus was given to rats for 30 minutes followed by a 30-minute rest time in their home cage prior to any additional behavior testing. The restraint stimulus is performed by placing a rat into a plastic decapicone and securing the outside of the cone with tape. The rat was unable to move his legs or body but his nose was exposed at the end of the cone to ensure access to air. During the restraint stimulus, the rat within the decapicone was placed within their home cage and kept under dim red lighting. Following the cessation of the stimulus, the decapicone was removed and the rat placed back into the home cage and left undisturbed until behavior testing.

Animals

Adult male Wistar rats or adult male Sprague Dawley rats (Harlan Laboratories, Indianapolis, IN) between 300-350 g were used for behavioral experiments as indicated for each experiment. Upon arrival to the facility, the animals were individually housed in a temperature-controlled room (22°C), and kept on a 12-hr light-dark cycle (lights on at 07:00) with free access to food and water. Rats were handled daily for a minimum of 3 days prior to any behavior testing. Cages were changed weekly. All cage changes occurred after behavior testing and a minimum of 20 hours before the next day's behavior testing. All procedures were performed according to NIH Guidelines for the Care and Use of Laboratory Animals (NIH Publication no. 80-23, revised in 1996) and according to the guidelines of the Indiana University Purdue University at Indianapolis Institutional Animal Care and Use Committee.

Surgical Techniques for intracranial drug delivery

For intracranial drug delivery, each rat was fitted with a unilateral guide cannula directed towards the BNST. Rats were anesthetized by placing them in a Plexiglas box connected to an isoflurane system (MGX Research Machine, Vetamac, Rossville, IN). The animals were then placed on a stereotaxic instrument (Kopf Instruments, Tujunga, CA) with the incisor bar set at -3.3 mm and kept under a constant flow of 3-4% volume isoflurane through a Plexiglas nose cone. Rats were implanted unilaterally as described previously (Lee et al., 2008). Specifically, a small incision was made on the top of the head and the skin pulled back. Using a stereotaxic arm, a stainless steel microinjection guide cannula (26 gage) was lowered, directed towards the BNST coordinates (AP -0.24 mm, ML ±1.4, DV -6.8) or septum (AP -0.24 mm, ML ±0.8, DV -5.2) according to the brain atlas of Paxinos and Watson (Paxinos & Watson, 2005). Three 2.4 mm stainless steel screws were secured to the skull and dental cement was used to secure the guide cannula and seal the skin around the incision. Once dry, the cannula was sealed with dummy cannula inserts (Plastics One, Roanoke, VA). All rats were given a minimum of 4 days recovery prior to behavioral testing.

Drugs/Compounds

Drugs were delivered unilaterally through an injector cannula (33 gauge) fitted to extend 1 mm beyond the guide cannula. Infusions into the BNST were a total volume of 100 nl delivered over 1 minute, and injectors were left in for an additional 1 minute to allow for

diffusion. The drugs used were Orexin A (catalog #1455; Tocris) 300 pmol/100 nl [within the dose range used in ORX-A measures of anxiety (Li et al., 2010)], where Vehicle is 1% bovine serum albumin (BSA), which increases the efficiency of peptide delivery (T. J. Sajdyk, Schober, Gehlert, & Shekhar, 1999b), AP5 (catalog #A8054; Sigma), 10 pmol/100 nl with Vehicle 0.9% saline, CNQX (catalog #C239; Sigma), 250 pmol/100 nl with Vehicle 0.9% saline (Doses used to block anxiety/panic like behaviors in rats) (Johnson & Shekhar, 2006), and DNQX (catalog #D0540; Sigma), 250 pmol/100 nl with Vehicle 0.9% saline.

Histology

After the conclusion of the experiments in which rats had guide cannula surgery, the rats were sacrificed and the location of injection sites were determined using Nissl-stained 30µm coronal sections through the BNST at 5x magnification. Data from rats with injection sites outside of the intended region of interest were not included in behavioral analysis, except when the medial septum was targeted.

Statistics

All data were analyzed using Prism 6.0 Software (La Jolla, CA) and all data are presented as mean ±SEM. The dependent variable values were compared (e.g. comparison to baseline) using a paired t-test when only baseline and treatment were compared within a single group or between two groups with only one time point. Comparisons from a single treatment group over multiple days were made using repeated measures (RM) one-way ANOVA, whereas comparisons between two groups over multiple days were made using a RM two-way ANOVA. In the presence of significant main effects, post-hoc pairwise comparisons were conducted using Dunnett's multiple comparison tests to compare back to baseline or control day of testing, and Tukey's honestly significant difference tests were used for pairwise comparisons of a challenge day with other days within treatment groups (or across days regardless of group when main effect of day was observed in the absence of an interaction); comparisons between treatment groups for a given day were made using Bonferroni's test or Fisher's least significant difference test (where noted). The confidence level for significance in all tests was set at $p \leq 0.05$.

Specific Experimental Procedures

Experiment 1. SI-hab testing with a Novel Partner

Figure 2.1. The SI-hab testing paradigm was implemented for a group of Wistar rats (n=22). The SI test was repeated daily for 5 consecutive days under dim red lighting with a novel partner used each day.

Experiment 2. Behavioral testing under Dim Light or the Bright Light Challenge

Figure 2.2. A. Wistar rats were tested in either the BLC (n=11) or under dim red light (control, n=11) conditions in a single SI test. **B-C**. Following habituation to the dim red lights of the room, another group of Wistar rats were tested in the Open Field (OF) test. The rats were placed into the OF box alone and then given either the BLC (n=5) or dim red light (n=3) during the 5-minute test session. The amount of time the rats spent in each of the designated outer, middle and center zones within the OF were measured using ANY-maze version 4.81 Software from Stoelting. **D.** The duration of freezing behavior during the OF test was also quantified using ANY-maze. **E-F**. Another cohort of Wistar rats were given the EPM under either the BLC (n=8) or under dim red light (control, n=8). Scoring of the open arm entries and open arm time was done with ANY-maze. **G**. Testing on a cohort of Sprague Dawley rats was done at a testing facility at Purdue University. Rats were tested in the SI test under dim red light as Baseline, followed 24 hours later with SI testing under either dim red light (control, n=3) or under the BLC (n=3).

Experiment 3. The SI-hab test under the BLC

Figure 2.3. The SI-hab testing was done for a single group of Wistar rats (n=22). Following Baseline testing in dim red lighting with a novel partner, 48 hours later, the SI test was repeated daily for 5 consecutive days under the BLC with a novel partner each day.

Experiment 4. Repeated Restraint stimulus

Figure 2.4. Following the 30-minute rest time after the restraint stimulus, rats (n=6) were then given a 5-minute SI test in dim red lighting with a novel partner. This procedure was repeated for 6 days.

Experiment 5. Orexin injections into the BNST

Figure 2.5. B. The EPM test was performed 30 minutes following a unilateral injection of ORX-A (300 pmol/100 nl, n=7) or vehicle (1% BSA, n=7) into the BNST of Sprague Dawley rats. C**.** Forty-eight hours after baseline testing, Sprague Dawley rats were injected unilaterally into the BNST with ORX-A (300 pmol/100 nl, n=7) or vehicle (1% BSA, n=5) followed 30 minutes later by the SI test under dim red light with a novel partner. **D.** Observations of the SI times were separated out in animals in which the ORX-A injections went into the BNST (n=8) and those that were directed into the septum (n=9).

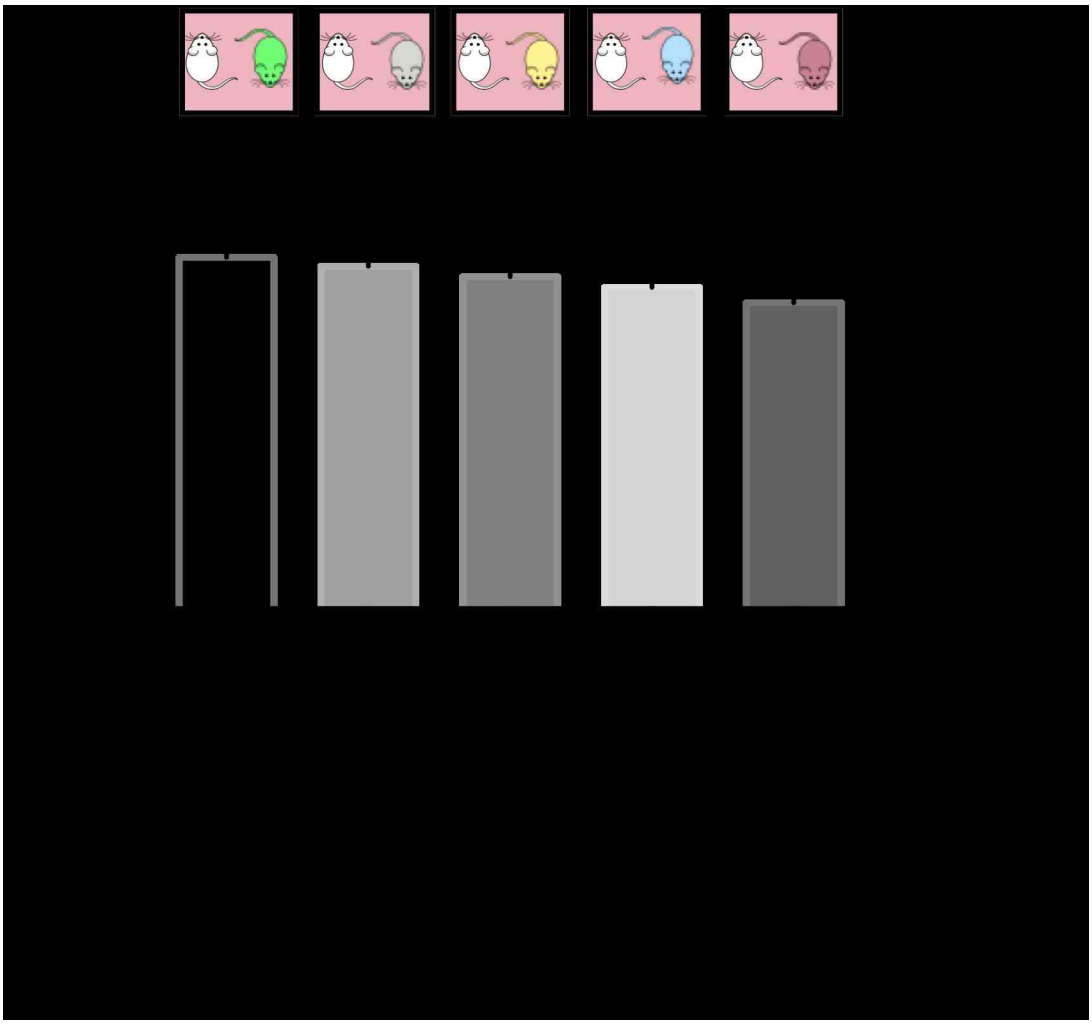
Experiment 6. Glutamate antagonist injections into the BNST prior to Orexin injections

Figure 2.6. A. AP5 (10 pmol/100 nl), CNQX (250pmol/100nl) or vehicle (0.9% saline in 100nl) was injected into the BNST 10 minutes prior to a unilateral injection of ORX-A (300 pmol/100 nl) through the same guide cannula. SI tests were performed 30 minutes following the last drug injection (ORX-A) into the BNST. The SI test was performed under dim red lighting with a novel partner. Between injections, rats were replaced into their home cages and kept in a separate staging area. The partner rats were untreated and did not undergo surgery, and so the test rat was clearly distinguished from the partner rat by the visible presence of the guide cannula head cap. Baseline and test sessions were done under dim red light and video recorded from above. The videotaped sessions were scored at a later time by an investigator blinded to treatment conditions. This process was repeated until all rats received all three pre-ORX treatment conditions; the order of the pre-ORX treatments was counterbalanced (n=11). Repeated SI test sessions were always separated by at least 48 hours and always involved a novel partner. **B.** Two subsets of rats were injected with CNQX (n=5) or AP5 (n=5) 10 minutes prior to a vehicle injection (1% BSA), followed 30 minutes after with the SI test in dim red lighting. **C**. This procedure was repeated with an additional set of rats (n=9) with DNQX (250pmol/100nl) pre-injections followed by vehicle (1% BSA) or ORX-A injections as well as vehicle-ORX-A injections. Injections were given as a counterbalanced design.

Results

The Social Interaction Test is reliable across repeated testing.

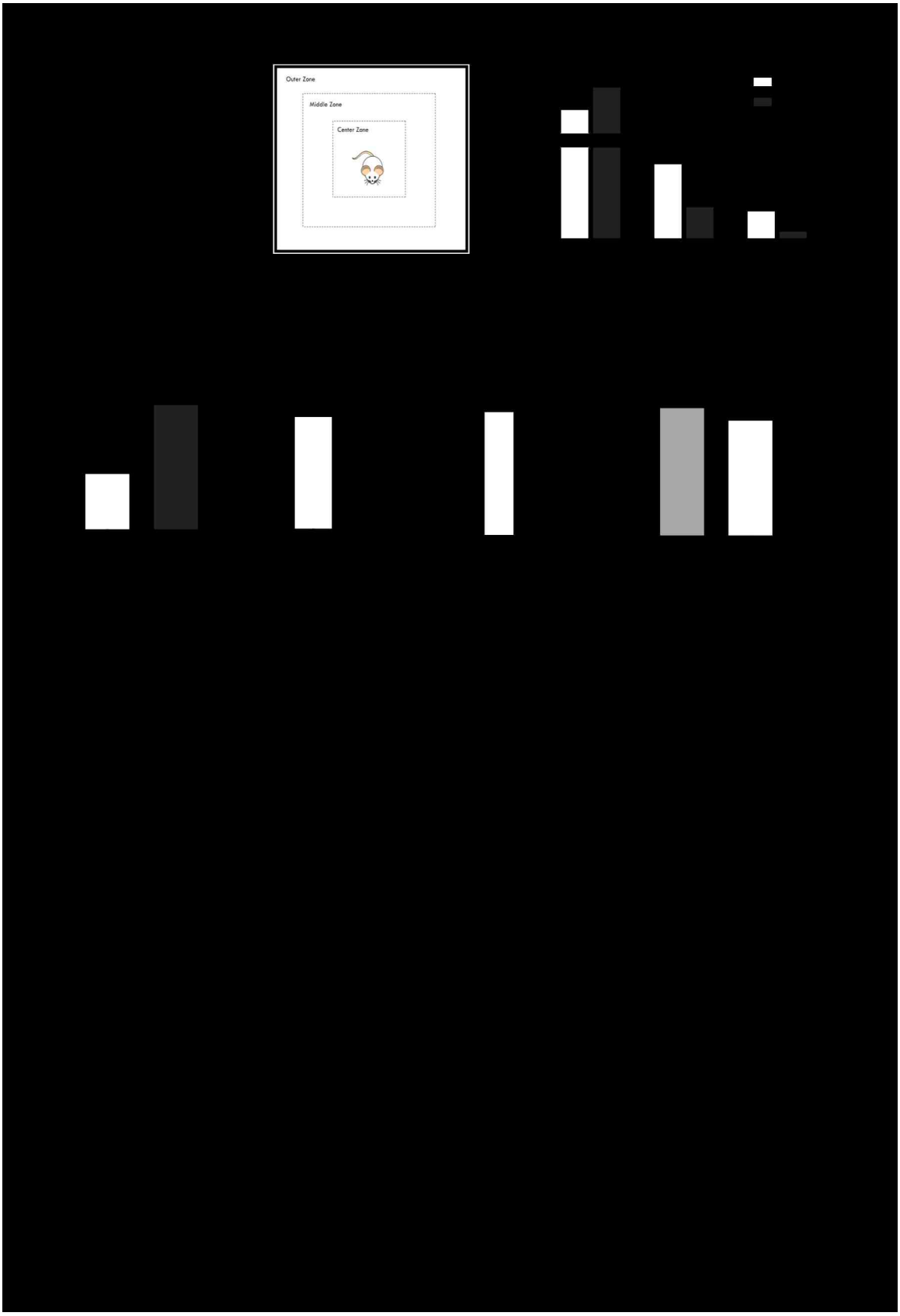
To demonstrate the reproducibility of the SI test under baseline conditions, we observed several cohorts of rats that underwent repeated SI testing under dim red lighting. In order to investigate how anxiety-like behavior changes over time, it is essential that the rodent not habituate to the test itself. Therefore, repeating the test under baseline conditions (absence of the anxiogenic stimulus and with a novel partner) should not produce significant changes in anxiety-like behavior across time. Rats (n=22) were given the 5-minute SI session in dim red lighting with a novel partner for 5 consecutive days (Figure 2.1). There was no significant effect of day for these individuals, (RM one-way ANOVA $F_{4,84}$=2.358, p=0.06). The mean SI times did not differ across the 5 test days, demonstrating that repeating the SI test under baseline conditions does not induce significant behavioral changes.



The Bright Light Challenge induces anxiety-like behaviors.

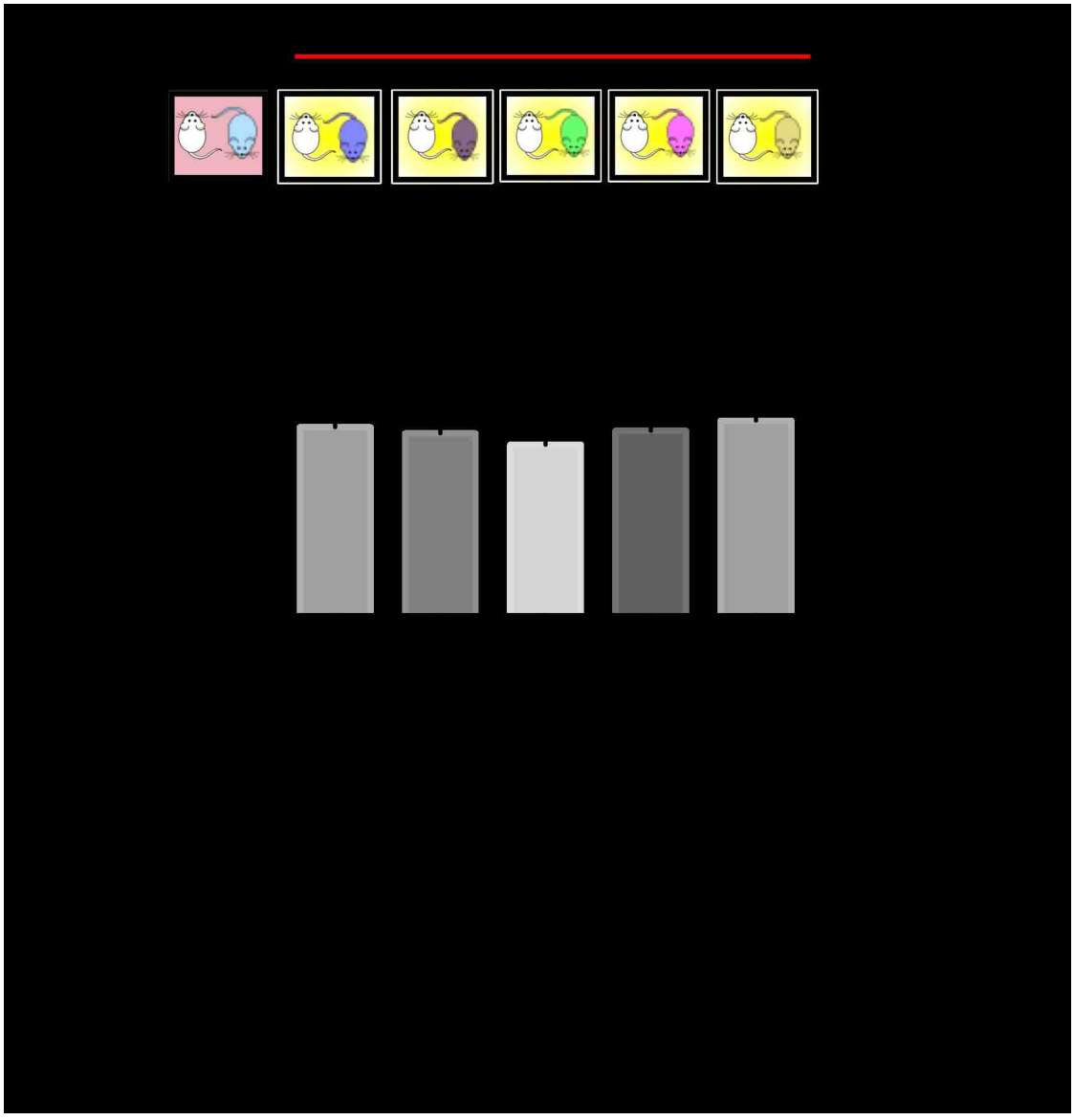
To determine the ability of the Bright Light Challenge (BLC) to induce anxiety-like behavior, we tested its efficacy within the SI test. Wistar rats were divided into two groups, the BLC group (n=11) and the Dim control group (dim red lighting) (n=11). The two groups were given a SI test with a novel partner rat, with the lighting condition as the independent variable (Figure 2.2A). BLC-exposed rats had significantly reduced SI time compared to Dim light exposed rats [SI time (mean ± SEM) Dim= 21.45 ± 1.10 and BLC= 12.84 ± 1.08; two-tailed unpaired t-test t_{20} = 5.57, p<0.0001]. The BLC is thus an anxiogenic stimulus capable of inducing anxiety-like behavior within the SI test. An additional cohort of rats was tested in a 5-minute Open Field (OF) (Figure 2.2B diagram) test either under BLC (n=5) or Dim red (n=3) lighting conditions. The amount of time the animals spent in each of the three designated zones, the outer, middle, and center zones, were scored through the ANY-maze automated video tracking software. The BLC rats spent more time than the Dim red light group in the outer zone (unpaired t-test, t=4.63, df=6, p=0.0036) and significantly less time in the middle (unpaired t-test, t=3.95, df=6, p=0.075), and center zones (unpaired t-test, t=4.83, df=6, p=0.0029) (Figure 2.2C). Additionally, within the OF test, the amount of time the rats spent freezing during the test session was greater in the BLC group compared to the Dim red light group (unpaired t-test, t=2.165, df=6, p=0.037) (Figure 2.2D), demonstrating fear-like behavior within this test. In an additional experiment, a cohort of rats were tested in the Elevated Plus Maze (EPM) in either dim red light (DIM, n=3) or BLC (n=4). The number of open arm entries was significantly lower for the BLC rats compared to the Dim rats (unpaired two-tailed t-test, t=2.66, df=5, p=0.0446,) (Figure 2.2E). However, the open arm time was not significantly different between the two light groups (unpaired two-tailed t-test, t=0.049, df=5, p=0.963) (Figure 2.2F).

To test the robustness of the BLC, we tested an additional strain of rats, Sprague Dawley (n=6), in the SI test under the BLC in a small pilot study (Figure 2.2G). First, rats were given a SI test in dim red light as a baseline test. 24 hours later, half of the rats were given the SI test with a novel partner under the Dim red lighting (n=3), while the other half were tested under the BLC (n=3). SI time decreased in the presence of the BLC. There was a significant effect of light (RM one-way ANOVA $F_{2,14}$=10.14, p=0.0019), with a significant reduction in SI time in the presence of the BLC from Baseline (Tukey's multiple comparisons test, p=0.0021) and Dim light conditions (Tukey's, p=0.0114). Collectively these results suggest that the BLC is an effective and reliable anxiogenic stimulus.


Outer Zone
Middle Zone
Center Zone

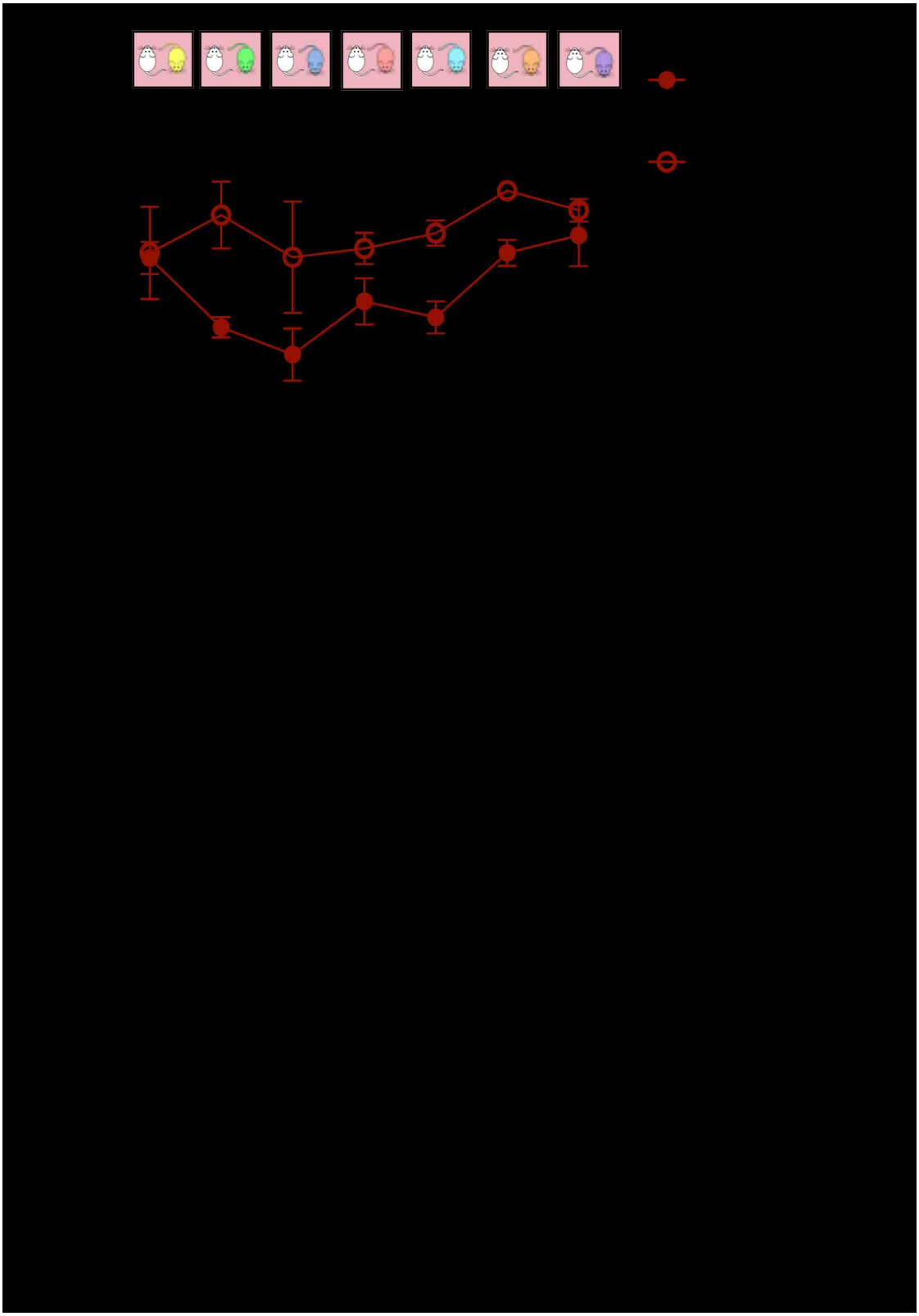
The BLC consistently induces anxiety-like behavior

To determine if the BLC would lead to anxiety-like behavior consistently across several repeated exposures, Wistar rats (n=22) were first given a baseline SI test under dim red lighting. 48 hours later, the rats were given a SI test under the BLC, which was then repeated for 5 consecutive days with a novel partner paired in each test (Figure 2.3). There was a main effect of Day (RM one-way ANOVA, $F_{5,105}$=14.63, p<0.0001), in which the rats had a significant decrease in SI time from baseline across all five test sessions under the BLC (Tukey's multiple comparisons test, Baseline vs Day 1-5 p<0.01). The behavior remained consistent across the 5 test sessions under the BLC, indicating that there was no habituation to the BLC that occurred within this time frame.



Anxiety-like behavior induced by restraint was inconsistent following multiple exposures.

To determine if the restraint stimulus could be used as an anxiogenic stimulus for use with the SI-hab test, rats (n=6) were given a 5-minute SI test (baseline conditions) following 30 minutes of restraint and a 30-minute rest within the home cage, for 6 days (Figure 2.4). The response to the restraint stimulus was unexpected, as some of the rats did not demonstrate a typical increase in anxiety-like behavior following the restraint exposure. In an attempt to parse out the responses seen, the rats were separated into two groups based on the change in SI time between the baseline test and the first SI test following restraint. The two groups were Responders (n=3, displayed reduction in SI time following restraint) and the Non-responders (n=3, no reduction in SI time following restraint). There was a main effect of time (RM two-way ANOVA $F_{6,24}$=4.24, p=0.0048), but not a main effect of group nor an interaction. Between the two groups of rats, the responder group had a significantly lower SI time than the non-responder group following the first restraint exposure (Bonferroni's multiple comparisons, $p<0.05$), with no other differences across any other test days. Additionally, within the Responders group, the rats demonstrated a habituation to the repeated exposures, having a significantly greater SI time on Day 6 compared to Day 1 of testing (Dunnett's multiple comparisons test, $p<0.05$), while the Non-responders did not have any significant differences across the days tested.

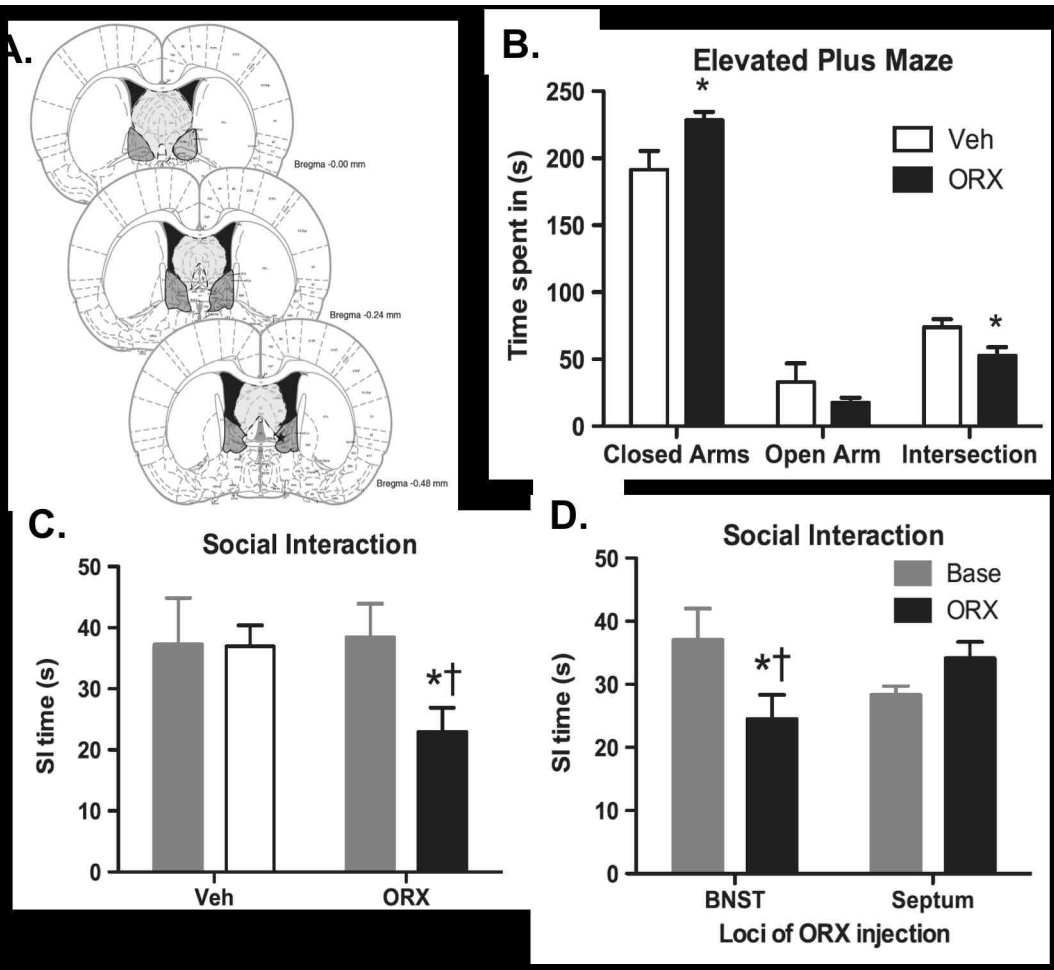


Orexin in the BNST leads to increased anxiety-like behavior

To determine if Orexin-A in the BNST was sufficient to induce anxiety-like behaviors, ORX-A (300pmol/100nl, n=7) or vehicle (1% BSA 100nl, n=7) was injected unilaterally into the BNST through implanted guide cannula. The injection sites are shown in Figure 2.5A. Thirty minutes following the ORX-A or vehicle injections, the anxiety-like behavior was evaluated within the EPM (Figure 2.5B). Rats given unilateral ORX-A injections spent more time in the closed arms (paired t-test, t=2.42, p=0.032) and less time in the intersection area (t=2.46, p=0.030) compared to rats that received unilateral vehicle injections. No significant differences between injection groups were observed for time spent in open arms (t=1.08, p=0.303) or distance traveled in the closed arms [vehicle (mean ± SEM) 1144±55 vs. ORX-A 989±114 mm; t=1.22 p= 0.246].

To determine if ORX-A in the BNST would lead to increased anxiety-like behavior in the SI test, unilateral injections were given directly into the BNST 30 minutes prior to SI testing with a novel partner (Figure 2.5C). Rats receiving unilateral BNST injections of ORX-A (300 pmol/100 nL, n=7) had greater anxiety-like responses in the SI test compared to rats receiving unilateral BNST injections of vehicle (1% BSA 100 nL, n=5). The ORX-A, but not vehicle injections into the BNST significantly reduced SI time compared to baseline (t=4.20, p=0.006). Furthermore, ORX-A injected rats had significantly lower SI times compared to vehicle-injected rats (t=2.54, p=0.030).

To determine if the ORX-A anxiogenic-like effect is selective to the BNST, rats that received injections that fell outside the BNST and into the septum were explored for anxiety-like behavior within the SI test (Figure 2.5D). In a separate cohort, rats that received ORX-A injections into the BNST (300 pmol/100 nL, n=8) but not those that went into the Septum (300 pmol/100 nL, n=9) had significantly reduced SI times compared to baseline (t=2.84, p=0.025). Additionally, rats injected with ORX-A into the BNST had significantly lower SI times compared to rats injected with ORX-A into the septum (t=2.14, p=0.049). The reduction in SI time induced by ORX-A injections was selective to the injections made into the BNST and not the Septum.


A.
Bregma -0.00 mm
Bregma -0.24 mm
Bregma -0.48 mm
B.
Elevated Plus Maze
Time spent in (s)
250
200
150
100
50
0
*
*
Veh
ORX
Closed Arms
Open Arm
Intersection
C.
Social Interaction
SI time (s)
50
40
30
20
10
0
*†
Veh
ORX
D.
Social Interaction
SI time (s)
50
40
30
20
10
0
*†
Base
ORX
BNST
Septum
Loci of ORX injection

Glutamate receptor antagonists effect on ORX-A anxiety behavior induction

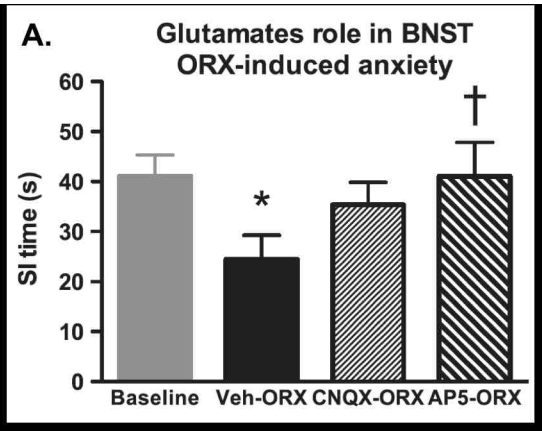
We investigated the extent to which anxiety-like behaviors induced by BNST injections of ORX-A requires interaction with NMDA or AMPArs. A possible mechanism of ORX-A induced anxiety involves an interaction between ORX and glutamate. Glutamate is reportedly co-localized and is co-released with ORX in terminals of ORX neurons (Henny, Brischoux, Mainville, Stroh, & Jones, 2010). ORX reportedly potentiates glutamate's excitatory postsynaptic responses elsewhere in the central nervous system, and this potentiation has been demonstrated to be necessary for ORX's induction of behavior changes (Borgland, Taha, Sarti, Fields, & Bonci, 2006; Moorman & Aston-Jones, 2010a). ORX has additionally been demonstrated to potentiate glutamate responses in other brain regions via interactions with glutamate receptors [N-methyl-D-aspartate-type receptors (NMDAr) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic-acid-type receptors (AMPAr)]. In order to demonstrate this relationship, we pre-injected the BNST with a NMDA receptor antagonist AP5. This was done to determine if we could block the increase in anxiety-like behavior in the BNST. AP5 has been shown to block fear-potentiated startle acquisition but not expression when injected into the BLA (Campeau, Miserendino, & Davis, 1992). Also, previous reports show that an AP5 injection into the BLA blocks increases in anxiety-like behavior caused by the previously described UCN priming (Rainnie et al., 2004).

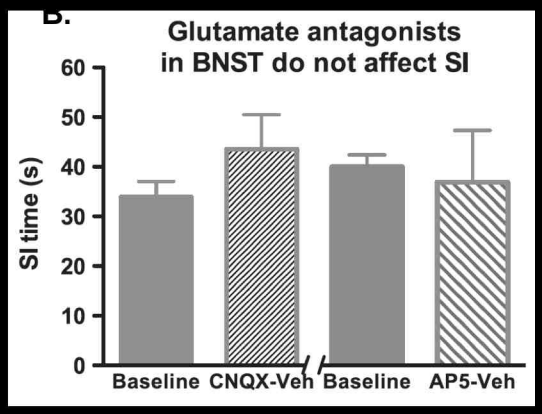
CNQX and AP5 affect the ORX-A influences in the BNST differently. To determine the mode of action ORX has within the BNST, rats (n=11) were injected unilaterally into the BNST with AP5 (10 pmol/100 nL), CNQX (250 pmol/100 nL) or Vehicle (0.9% saline, 100 nL), 10 minutes prior to a unilateral injection of ORX-A (300 pmol/100 nL) through the same guide cannula. Anxiety-like behavior was observed through the SI times within the SI test administered 30 minutes after the ORX-A injection (Figure 2.6A). This process was repeated every 48 hours until all rats received all three pre-ORX treatment conditions; the order of the pre-ORX treatments was counterbalanced. Pre-injections of the vehicle prior to ORX-A injections led to a significant increase in anxiety-like behavior, as seen by a significant decrease in SI time compared to baseline (RM ANOVA $F_{3,30}$=3.05, p=0.044; Dunnett's multiple comparisons, p<0.05). The pre-injections of the NMDA receptor antagonist AP5 blocked the anxiety-like behavior that was typically induced by ORX-A injections within the SI test, meaning that the AP5-ORX group SI time (mean ±SEM) was significantly higher compared to the Veh-ORX group SI times (Tukey's, p<0.05). However, the AMPA receptor antagonist CNQX did not fully block ORX-induced anxiety-like behavior, but instead attenuated the ORX-A effect on SI time. The SI times of the group

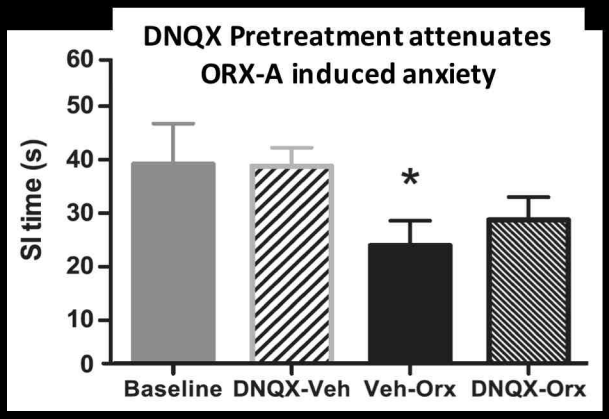
following the CNQX-ORX injections were not significantly different from the SI times of the group following Veh-ORX injections, nor were they significantly different from baseline SI times.

To demonstrate that CNQX and AP5 do not have intrinsic anxiogenic or anxiolytic effects or affect SI times in some other way, the AP5 and CNQX were injected followed by injections of Vehicle (0.9% saline, 100 nL) instead of ORX-A. In two subsets of the previous rats, injections of either CNQX (n=5) or AP5 (n=5) 10 minutes prior to Vehicle injections were given followed by SI testing (Figure 2.6B). Neither of the SI times following injections were different from baseline SI times ($p \geq 0.294$). These glutamate antagonists do not affect social interaction, demonstrating that the AP5 anxiolytic effect (seen in Figure 2.6A) is truly blocking the Orexin anxiogenic effect.

To further clarify if the attenuation of ORX-A injections on anxiety-like behavior by CNQX injections was due to its action on the AMPA receptor and not on non-selective NMDA receptor action, an additional AMPA receptor antagonist DNQX, with less affinity than CNQX for the NMDAr, was injected into the BNST prior to either Vehicle or ORX-A. In a new set of rats (n=9), DNQX (250 pmol/100 nL) was injected into the BNST 10 minutes prior to Vehicle or ORX-A (Figure 2.6C). The Vehicle-ORX-A injection served as the positive control, and we saw a significant increase in anxiety-like behavior by the reduction in SI time compared to Baseline (RM ANOVA $F_{3,18}=4.05$, $p=0.033$; Dunnett's $p<0.05$). Pre-injections of DNQX, followed 10 minutes later by ORX-A or Vehicle injection into the BNST, attenuated the anxiogenic effect seen in the Vehicle-ORX-A injections but did not fully block the anxiogenic effect. Compared to Vehicle-ORX injections, the DNQX-ORX injections only attenuated the decrease in SI time caused by the ORX-A injections. The SI times were not significantly different from either the baseline or the DNQX-Vehicle injections. Additionally, we see that the pre-injection of DNQX followed by Vehicle did not lead to a change in SI time, demonstrating that DNQX alone is not leading to alterations in anxiety-like behavior.


A.
Glutamates role in BNST
ORX-induced anxiety
SI time (s)
60
50
40
30
20
10
0
*
†
Baseline
Veh-ORX
CNQX-ORX
AP5-ORX


B.
Glutamate antagonists
in BNST do not affect SI
SI time (s)
60
50
40
30
20
10
0
Baseline
CNQX-Veh
Baseline
AP5-Veh


DNQX Pretreatment attenuates
ORX-A induced anxiety
SI time (s)
60
50
40
30
20
10
0
*
Baseline
DNQX-Veh
Veh-Orx
DNQX-Orx

Discussion

The key features of the animal model of SoFiA

This chapter began by first identifying the key features of a testing paradigm that would allow for studying social familiarity effects on anxiety. Through an examination of previous work, the SI-hab testing paradigm was identified as useful for observing this effect. The SI test used in this paradigm consists of a social component, and so the presence of a social partner does not confound the parameters of the test itself. The test additionally involves a repeated exposure paradigm, which allows several opportunities for the development of social familiarity memory.

I first asked whether the SI-hab testing would lead to any intrinsic pro-social effects. A key aspect to the SI-hab paradigm is repeatability, and the previous use of this paradigm was primarily done with a familiar partner in each testing session with UCN primed rats. Here it was found that under baseline (control) conditions the SI-hab training resulted in consistent SI behavior. I tested rats in the SI-hab paradigm with a novel partner under dim red light (non-anxiogenic conditions) for 5 consecutive days (Figure 2.1). Within this testing period, there was no generic increase or decrease in sociability of the rats under these baseline conditions. With a novel partner, there was no possibility for social familiarity development, but stability of the behavioral response within this testing paradigm allows us to observe measureable differences in anxiety behavior in response to changes in other factors. Such factors that can be altered within this testing paradigm are the presence of the anxiogenic stimulus or social familiarity.

To decide on an anxiogenic stimulus to use in conjunction with the SI-hab testing paradigm, I first identified the key features that would characterize my desired anxiogenic stimulus. The essential criteria are that the stimulus reliably induces anxiety-like behavior, and can be given repeatedly without habituation to the stimulus itself. The best features would be that the stimulus is easy to administer, not require extensive training, and ideally not require surgery or other procedures that may lead to unnecessary pain. Within this chapter I explored the anxiogenic stimuli of a Bright Light Challenge, Restraint and Orexin-A injections into the BNST. While each stimulus has unique benefits, it was determined that the BLC was most useful for our purposes based on the findings that point to the reliability and repeatability of the stimulus as an anxiogenic stimulus.

The Bright Light Challenge

The use of this BLC proved to be a useful tool to reliably induce anxiety-like behavior in a reproducible and non-habituating way. Previous uses of a brightly lit arena by File and colleagues for the SI test found that the highest social interaction times were when the lights were low and the testing arena was familiar, while the lowest social interaction times were when the lights were high and the testing arena was unfamiliar (File, 1984; File, Lippa, Beer, & Lippa, 2004). They found that a bright light is anxiogenic within the SI test, which led us to consider a bright light as a possible anxiogenic stimulus for testing during the SI-hab paradigm. However, to optimize the anxiogenic effect of the bright light, we modified the presentation of the stimulus and termed it the Bright Light Challenge (BLC). This stimulus is performed in a slightly different way than previously done in other labs, as we prevented the rats from habituating to the high illumination prior to behavior testing. Instead, the rats were habituated to a dim red light prior to testing. The BLC consists of quickly transitioning from the previously habituated dim red light to a bright white light after immediately placing the rats in the SI chamber. The initiation of the behavior test was paired with an abrupt onset of the BLC to enhance the anxiogenic effect of the BLC in the SI test. Optimizing the SI-hab paradigm with the use of the BLC is one step further in developing a rodent model to study the underlying mechanisms regulating the social enhancement of anxiolysis.

The BLC induced anxiety-like behavior in multiple tests

The BLC induced anxiety-like behavior in multiple behavioral tests. Firstly, within the Open Field (OF) test, the BLC induced changes in the exploratory behavior (Figure 2.2B-D). The OF test takes advantage of the exploratory behavior of rats, specifically, the natural tendency of rats to explore their environment as well as the natural aversion rats have towards open, exposed spaces. When rats spend more time in the middle and center zones, away from the protective walls of the outer zone, we interpret that as less anxious behavior. Within the OF test under the different lighting conditions, we observed reductions in the time spent in the center zone, with subsequent increased time spent in the middle and outer zones under the BLC (Figure 2.2C). These alterations in the times spent in the different zones represents an increase in anxiety-like behavior under this BLC. Additionally, the rats spent more time freezing under the BLC (Figure 2.2D), which also demonstrates that when the rats are tested alone the under the BLC, they demonstrate elevated anxiety-like behavior.

Our observations of increased anxiety-like behavior within the OF test under the BLC are supported by other studies. One study looking at OF behavior for 15 minutes in low-light (8-13 lux) and high-light (400-500 lux) conditions found that rats had increased anxiety-like behavior in the high-light versus the low-light conditions as measured through a reduction in locomotor activity and a reduction in the amount of time spent in the center of the OF arena coupled with an increase in the amount of time spent facing the corner of the arena (Bouwknecht et al., 2007). The high light conditions used in the study were equivalent to the light intensity in lux that was used in the current BLC exploration.

Results in the Elevated Plus Maze (EPM) demonstrated that under the BLC, rats had a significant decrease in the number of open arm entries, which is one measure of anxiety-like behavior within the EPM (Figure 2.2E). However, there was not a significant BLC effect in the amount of time in seconds the rats spent in the open arms (Figure 2.2F), which is an additional measure of anxiety-like behavior within this test. The BLC led to greater variability in the amount of time spent in the open arms compared to the rats tested under the dim red light. Therefore, we see that the EPM under the BLC had mixed results, which is a limitation to this stimulus. To further parse out the anxiety behavior elicited by the BLC, this test may need to be repeated, as there were a small number of animals tested in this initial preliminary trial.

Here the BLC resulted in consistent and reliable reductions in SI times, and across multiple days of testing, the rats did not habituate to this anxiogenic stimulus (Figure 2.3). We do not use an unfamiliar testing arena, and the rats are not habituated to the bright light before testing, unlike the tests done by the File lab. These elevations in anxiety-like behavior under the BLC are therefore more likely to be a result of the BLC itself and not from the context. This increase in anxiety-like behavior to the BLC has been replicated several times within this lab. Additionally, the SI times observed in these experiments were within the range of SI scores from similar experiments using the same SI scoring methodology (T. J. Sajdyk & Gehlert, 2000; A Shekhar, Sajdyk, Rainnie, & Gehlert, 2002; Truitt et al., 2007).

The BLC is effective on two strains of rats and within three facilities

We see that the BLC is a reliable anxiogenic stimulus that is not limited to one behavior test or strain of rats. This lab traditionally uses the Wistar strain of rats for all behavior testing. However, some labs utilize the Sprague Dawley strain, and so we tested a cohort of these rats to determine if the BLC would have the same effects on inducing anxiety-like behavior. The

intention here was to demonstrate the versatility of the BLC on various strains of rats to utilize this stimulus amongst various laboratories. The BLC was effective in inducing anxiety-like behavior in the SI test in the Sprague Dawley strain of rats (Figure 2.2G).

The BLC consistently induced anxiety behavior over multiple sessions

In order to observe measurable changes in the behavior as a consequence of the development of social familiarity, it is essential that the rats do not habituate to the anxiogenic stimulus itself if repeated. I show here that the BLC is anxiogenic in the SI test (Figure 2.2A) and with repeated testing the BLC induces increased anxiety-like behavior compared to baseline for all the testing sessions (Figure 2.3). The testing partner was novel in each repeated test and therefore social familiarity did not develop. Without social familiarity, the consistent testing conditions of the OF arena and the BLC did not lead to changes in behavior, with no main effect of time. A 24-hour inter-test interval was chosen because this is the interval used in the UCN priming study in which they repeated the SI test (Truitt et al., 2007).

The BLC provides advantages to use, such as that it is easy to administer, as it is an external stimulus and does not require any surgeries or invasive procedures. This is an advantage over the UCN priming procedure, which requires surgery as well as several injections to achieve the behavioral effect. The BLC is a more ecological stimulus, as it is naturally aversive for rats, requiring no training or other undesirable procedures such as food deprivation, which has been found to negatively affect SI times in rats (Genn, Tucci, Thomas, Edwards, & File, 2003). The BLC is aversive because it may represent the presence of a predator, which the animal is not protected against within the open space of the OF box. An actual predator presence may be the most ecologically significant stimulus; however, it would also initiate a fear response of fight or flight. For our purposes, the actual presence of a threat is not the goal, but rather the implied presence by the BLC serves the purpose of initiating the anxiety-like behavior that I aim to study. Additionally, the BLC does not require any pharmacological intervention to administer in order to get the desired behavioral effects. This prevents any possible confounding interactions between any onboard pharmacological compound used to initiate the anxiety and any future therapeutics that might be explored. These considerations make the BLC the anxiogenic stimulus of choice to pursue further.

Restraint is not an appropriate anxiogenic stimulus for our uses

Restraint stimulus as an anxiogenic stimulus

The restraint stimulus had a mixed ability to initiate anxiety-like behavior in the acute phase. The SI times following restraint exposure in a previous study had about a 50% reduction in the SI time compared to pre-restraint SI times, which also matches the reduction in SI that we saw to the BLC (T. J. Sajdyk et al., 2008). The time course used to achieve this was a 30-minute restraint with a 30-minute rest prior to SI testing. Therefore, I wanted to test the same time course with the restraint, hypothesizing that the restraint stimulus would significantly reduce the SI time. I additionally wanted to test the repeatability of a restraint/SI testing paradigm. Within this study, the use of restraint as an anxiogenic stimulus had inconsistent results. Compared to baseline SI times, the first day of SI testing following a pre-exposure to the restraint led to two distinct responses within the SI test. Three of the six animals had an increase in the anxiety-like behavior (responders), while the other three did not respond with increased anxiety-like behavior in the following SI test (non-responders, Figure 2.4). This was unexpected, as restraint has been used extensively to induce increases in anxiety-like behavior and reduced social investigation, and so my results do not fit with the literature (Doremus-Fitzwater et al., 2009; Padovan & Guimaraes, 2000; T. J. Sajdyk et al., 2008). Reasons for this not being an effective anxiogenic stimulus in my hands are unclear, as I attempted to replicate fully the procedure for implementing the restraint in the previous experiments. However, it could be that I needed to implement the restraint for a longer period of time or with a shorter rest period between the restraint and the SI test. It is possible that the amount of time of the restraint stimulus (30 minutes) was at a threshold of anxiety induction, which could explain the lack of effect in half of the animals.

Repeated Restraint led to habituation

Repeating the restraint exposure led to habituation within the rats that responded to the stimulus. The SI-hab paradigm necessitates repeated exposures to the anxiogenic stimulus without lessening in the anxiogenic effect or developing habituation. In my hands, the rats had different responses to repeated exposures to the restraint stimulus. Among the non-responders, there were no significant changes in the SI times across the repeated days. Among the responders, we see that with repeated testing, the initial increase in anxiety-like behavior was reduced, and so by the 6^{th} day of testing, the SI times were significantly greater than the 1^{st} day

(Figure 2.4). This increase in SI time for the responders across the repeated exposures demonstrates that these rats habituated to the stimulus itself, as the partners in each of the tests were novel, preventing social familiarity from developing. This habituation to the anxiogenic stimulus will interfere with our ability to identify measurable changes in the SI test as a result of the development of social familiarity, and therefore this stimulus cannot be used. This result is again not consistent with the literature, as chronic restraint has been used extensively to induce anxiety-like behavior (Chung, Martinez, & Herbert, 2000; Conrad, LeDoux, Magarinos, & McEwen, 1999; Padovan & Guimaraes, 2000). One limitation to this conclusion is that since half of the rats did not respond to the restraint, more rats may be needed in order to observe a significant effect on the subsequent SI test(s). However, the inconsistent effectiveness and habituation to the restraint seen in the rats is concerning and it is for these reasons that the restraint stimulus was ruled out as the possible anxiogenic stimulus.

Exploration of ORX-A as an anxiogenic stimulus

Orexin injections into the BNST is anxiogenic

Within this investigation, ORX-A injections into the BNST were a reliable anxiogenic stimulus. Following injections, rats demonstrated elevated anxiety-like behavior within multiple tests, the EPM and SI tests (Figure 2.5). My findings here show that the ORX-A has anxiogenic action within the BNST, and that it is also specific to the BNST, as injections into the medial septum were not anxiogenic (Figure 2.5D). The data presented also supports the role of the BNST in inducing anxiety-like behavior in rats. A previous report found that anxiety-like responses to a systemically delivered panicogenic stimulus (sodium lactate injections into rats with L-allylglycine infused into the hypothalamus) was blocked by placing an ORX1r antagonist directly into the BNST (Johnson et al., 2010). The anxiety-modulating effects of benzodiazepines and selective serotonin reuptake inhibitors have been suggested to have influence over the Orexin system that correlates with anxiety-like behaviors as well (Nollet et al., 2011; Panhelainen & Korpi, 2012). This study supports the role of the BNST as one of the neuronal sites responsible for regulating anxiety-like behavior, and that Orexin is a key neural substrate in this process (Johnson et al., 2010).

The anxiogenic specificity may not be limited to only the BNST. Some reports have shown that ORX-A injections into the paraventricular nucleus of the thalamus in rats and the central amygdala of hamsters induced anxiety-like behavior in the EPM (Avolio et al., 2011; Li et

al., 2010). Both of these loci are related via either proximity (paraventricular nucleus of the thalamus, caudal/medial border of caudal BNST) or function (central amygdala and BNST collectively form the extended amygdala) to the BNST. So, there could well be a network of sites modulated by Orexin input that are capable of enhancing anxiety-like responses. This method of inducing anxiety has some advantages as the method was not only shown to be reliable, but also the mechanism of action to induce the anxiety-like behavior is known, and the role of the BNST as a key site of anxiety regulation can help us to better understand anxiety regulation in general.

Blocking the anxiogenic effects of Orexin in the BNST

We performed additional experiments with ORX-A to explore the regulatory mechanisms underlying the anxiogenic effect of Orexin within the BNST. Pretreatment with the NMDA antagonists AP5 and CNQX were able to reduce the ORX-A mediated increases in anxiety behavior within the SI test (Figure 2.6A). This suggests a role for the NMDA receptor in the regulation of anxiety within the BNST. Additionally, the AMPA receptor antagonist DNQX did not fully block the ORX-A mediated anxiogenic effect but attenuated the SI time reduction (Figure 2.6C). This attenuation prevented the assertion that the AMPA receptor is definitively involved in anxiety regulation.

The action of ORX-A as an anxiogenic stimulus, as well as blocking this effect by NMDA receptor antagonists, suggests that the anxiogenic mechanism within the BNST may be mediated by Orexin interactions with glutamate. Further evidence of this is that ORX-A in the ventral tegmental area (VTA) has been demonstrated to potentiate neuronal responses to endogenous glutamate release, and ORX1r antagonism reduced VTA neuronal activity (Borgland et al., 2006; Moorman & Aston-Jones, 2010b). Low levels of Glutamate may be responsible for increased vigilance, and even higher vigilance may induce Orexin and glutamate to be released, inducing anxiety. The source of the glutamate in the BNST is not yet known, but Orexin and Glutamate could be co-released onto BNST neurons, mediating the anxiety behavior.

Rationale for not using Orexin

Although I have demonstrated that Orexin can act as a reliable anxiogenic source to induce anxiety-like behavior within the SI test, we made the decision not to pursue the Orexin injections further. The rationale for this is the procedure involves surgery, which requires recovery time. The surgery itself involves cannula implantation to be done within many animals

to acquire the number needed for behavior testing. As Figure 2.5D demonstrated, cannula implantation often involves misses, with several of the injection sites going into the septum instead of the BNST, which can lead to larger numbers of animals used. Also, this procedure involves a pharmacological manipulation. Through this I gained insight into the role of the BNST and anxiety regulation, and part of my goal is to find out how anxiety is regulated. However, the induction of anxiety through this circuit manipulation could possibly obscure understanding of the regulation of anxiety through processes that occur in response to a social presence specifically. Orexin is again an interoceptive stimulus, and the anxiety produced through these injections are likely a consequence of this neural manipulation. This means that social familiarity-induced anxiolysis to ORX-A injections may not give us insights into the role of other neuronal structures that are involved. Therefore, identifying an anxiogenic stimulus that does not involve circuit manipulation can help me gain insights into the endogenous circuit that regulates anxiety in a wild type animal. With the decision not to pursue ORX-A injections further, the repeatability of ORX-A injections into the BNST was not explored.

Conclusions

Anxiety-like behavior can be induced through various stimuli. I explored the Bright Light Challenge, restraint stimulus as well as ORX-A injections into the BNST. It was determined that restraint is not an ideal stimulus as anxiety-like behavior following restraint exposure was inconsistent and the rats habituated to the stimulus with repeated exposures. Additionally, the ORX-A injections did not fully fit our criteria for an anxiogenic stimulus, as it can be difficult to administer.

The best stimulus for our purposes has proven to be the Bright Light Challenge. The BLC fits my necessary criteria for an anxiogenic stimulus. It reliably induces anxiety-like behavior within the behavior tests that I aim to utilize. It also does not lead to alterations in behavior with repeated testing. It is easy to administer and requires no surgery or training, making it useful for utilization within the SI-hab testing paradigm. I next implement the BLC during the SI-hab testing paradigm to investigate the ability of social familiarity to induce anxiolysis.

Chapter 3: The validation and characterization of the model of Social Familiarity-induced Anxiolysis (SoFiA)

Introduction

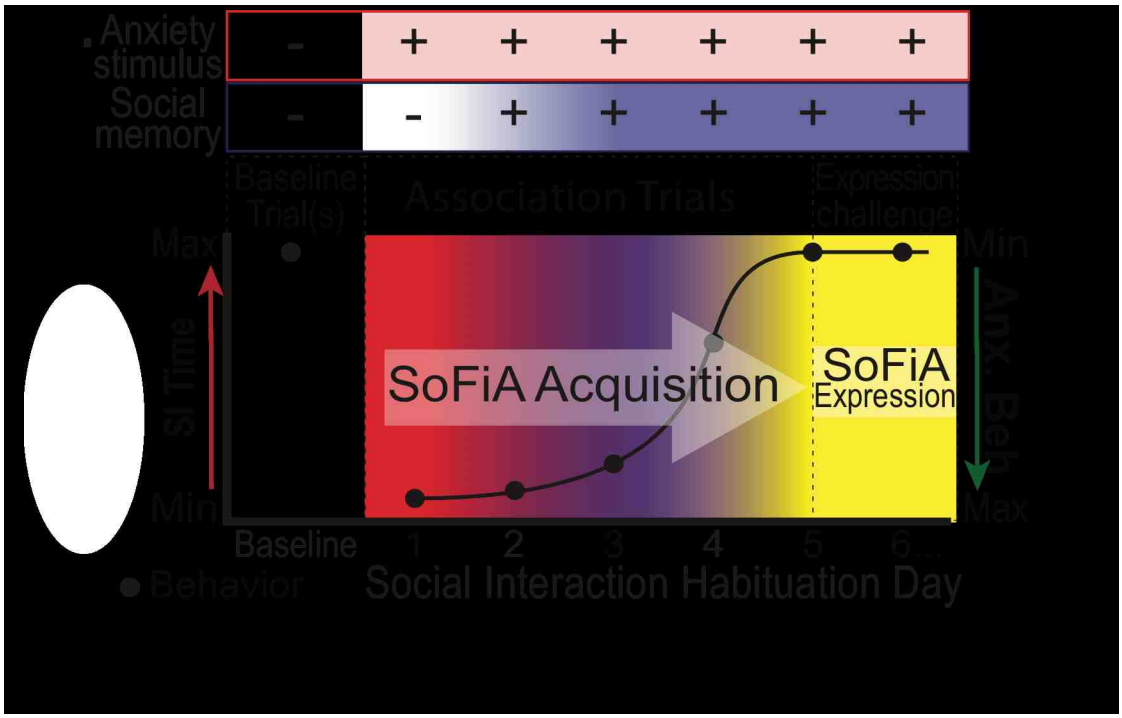
Goal of this Chapter

The goal for this chapter is to characterize a preclinical model of social familiarity-induced anxiolysis (SoFiA). The model of SoFiA demonstrates socially enhanced anxiolysis, specifically, the ability of social familiarity to alter behavioral responses to an anxiogenic stimulus. Within the previous chapter I identified the Bright Light Challenge (BLC) as a useful anxiogenic stimulus to initiate anxiety-like behavior. The testing paradigm in which the variables of social familiarity and anxiety are tested is in the SI-hab testing paradigm. The previous use of the SI-hab testing paradigm by Truitt and colleagues (2007) demonstrated that social familiarity obtained within this testing paradigm was sufficient to overcome the persistent anxiogenic state of the rats (Truitt et al., 2007). However, there remains unanswered questions and control studies to do in order to fully characterize and validate the SI-hab procedure as a model of social familiarity-induced anxiolysis. To characterize the model, I determine the conditions in which socially enhanced safety learning is achieved, and the relationship between social familiarity development and the presence of anxiety during training. Furthermore, validation of an animal model is an essential process to determine if the model is a true representation of the human processes it attempts to represent (Belzung & Lemoine, 2011). In order to ensure that the SI-hab testing under the BLC is a valid animal model of SoFiA, the types of validity key to animal models are tested. The three key types of validity that I will focus on in this chapter are face, construct, and predictive.

The main objectives within this chapter are to demonstrate that the SI-hab testing paradigm is a useful way to model social familiarity-induced anxiolysis, as well as establish three types of validity of the SoFiA model. To demonstrate validity, first I will determine that social familiarity is an effective way to overcome the BLC anxiogenic stimulus. I further characterize the SoFiA model by demonstrating the importance of the presence of the anxiogenic stimulus during SI-hab training. I identify the IL as a neural structure that is putatively involved in the regulation of the behavioral outcome following SI-hab training, demonstrating construct validity. Lastly, I demonstrate predictive validity by testing the enhancement of SoFiA acquisition with D-cycloserine.

Characterization of the SoFiA model

In this model, acquisition and expression of SoFiA is operationally defined according to the behavior observed. Anxiety is initially expressed during the first exposure to the BLC, seen as a statistically significant drop in SI time (in seconds) compared to baseline. This initial drop in SI time is then followed by a significant increase in SI time after repeated exposures to the SI-hab with a familiar partner. The acquisition of SoFiA is attained once the SI time is statistically significantly greater than the first exposure to the testing partner under the anxiogenic stimulus. Additionally, the expression of SoFiA is defined as the subsequent SI test times under the BLC with the familiar partner that remain significantly greater than Day 1. A schematic of the typical behavioral response is depicted in Figure 3.A.



Face validity of the model of SoFiA

In this chapter, I hypothesized that social familiarity attained through SI-hab testing will lead to anxiolysis to the BLC, similar to the SI-hab induced anxiolysis to UCN priming. A key behavioral outcome from SI-hab in UCN-primed rats is the development of anxiolysis in the presence of a socially familiar partner. A remaining question from the UCN study is if the familiar partner is an essential element to the development of anxiolysis and if a novel partner presented each day in the SI-hab testing can become anxiolytic. I addressed this by determining

the effect of SI-hab testing with a novel partner in each test. Face validity is established with the demonstration that the anxiolysis is partner specific, and not merely a pro-social effect. Additionally, I explore whether the initiation of anxiolysis can occur in the absence of the anxiogenic stimulus, as well as the specificity of the induced anxiolytic behavior to the SI test.

Exploring construct validity

Construct validity is an important element of an animal model. To this end, I explored the role of the medial prefrontal cortex (mPFC) in the acquisition and expression of SoFiA. The mPFC has been implicated in the regulation of both social behavior and top-down regulation of anxiety, making it a possible neural substrate pivotal to the regulation of SoFiA (Adolphs, 2010; Fossati, 2012; Hartley & Phelps, 2010; M. J. Kim, Gee, Loucks, Davis, & Whalen, 2011; Meyer-Lindenberg & Tost, 2012). Evidence for PFC involvement in anxiety regulation was found in humans with damage to their ventral medial prefrontal cortex (vmPFC). These patients had difficulty using emotional and social cue information to guide decision-making, implicating the vmPFC as the area of social-emotion regulation (Sotres-Bayon, Cain, & LeDoux, 2006). There is a functional relationship between the vmPFC and the BLA, and anxiety regulation by the vmPC may be from these connections (Hartley & Phelps, 2010; Milad & Quirk, 2002). Evidence for a functional connection of the vmPFC and BLA was seen in rats following unilateral disinhibition of the vmPFC by Bicuculline Methodide (BMI), which led to cFos expression increases within the ispilateral BLA compared to control injections (Truitt et al., 2007). The vmPFC and its connectivity to the amygdala are also tightly linked to emotion regulation, including anxiety, and the strength of this connection can predict positive outcome for cognitive behavioral therapies (Bishop, Duncan, Brett, & Lawrence, 2004; M. J. Kim, Gee, et al., 2011; M. J. Kim, Loucks, et al., 2011; Pezawas et al., 2005). This funcitonal relationship may point to a possible a top-down regulation of the mPFC on the BLA, making the mPFC an area of interest for exploring a possible role in anxiety behavior.

Additionally, sub-structures within the mPFC, including the infralimbic cortex (IL) of the rat, are critical for expression of key social behaviors during development (J. M. Spikman, M. E. Timmerman, M. V. Milders, W. S. Veenstra, & J. van der Naalt, 2012; van Kerkhof, Damsteegt, Trezza, Voorn, & Vanderschuren, 2013a; van Kerkhof, Trezza, et al., 2013). The IL and the similar human structure, the subgenual vmPFC, are well known sites for cortically driven reductions in anxiety through safety learning. The IL/vmPFC is activated in response to stimuli that signal

safety (Gupta et al., 2013b; Herry & Mons, 2004; Knapska & Maren, 2009; Phelps, Delgado, Nearing, & LeDoux, 2004; Schiller, Levy, Niv, LeDoux, & Phelps, 2008). Activating the IL can either enhance or emulate extinction of fear conditioning, likely via connections with the amygdala (Knapska et al., 2012; Milad & Quirk, 2002; Thompson et al., 2010). Likewise, activation of the IL facilitates the fear extinction retrieval (Do-Monte, Manzano-Nieves, Quinones-Laracuente, Ramos-Medina, & Quirk, 2015). Moreover, inhibition of the IL during the extinction phase, but not following the memory formation, prevents extinction memory formation, which could implicate the IL in a role of safety learning formation (Do-Monte et al., 2015). Based on these observations, I hypothesized that the PFC is likely one of the neural substrates involved in the regulation of SoFiA. I test for construct validity of the SoFiA model by exploring the role of the vmPFC in the expression of SoFiA to begin to understand the underlying circuitry involved.

The use of D-cycloserine to demonstrate predictive validity

Predictive validity is accomplished through the use of clinically effective pharmacological agents tested in animal models. In this chapter, this validity is supported by the use of the pharmacological agent D-cycloserine (DCS), an allosteric NMDA receptor partial agonist that binds at the glycine site, inducing glutamatergic activity (Gomperts, Rao, Craig, Malenka, & Nicoll, 1998). DCS action as a partial agonist means that its activity can both enhance NMDA receptor activation or, at sufficient doses, can act to reduce the efficiency of the receptor by blocking it from endogenous glycine (Emmett et al., 1991; Hood, Compton, & Monahan, 1989). Both processes are proposed possible mechanisms of action for DCS to enhance fear extinction (Watson, Bolanowski, Baganoff, Deppeler, & Lanthorn, 1990). DCS has been studied in both the clinical and preclinical spheres (Davis, Ressler, Rothbaum, & Richardson, 2006; Myers & Carlezon, 2012). Cognitive behavioral therapies (CBT), such as exposure therapy, are a form of safety learning that can be enhanced (reduction in the number of exposures or pairings) in numerous human studies by DCS (Ganasen, Ipser, & Stein, 2010; Hofmann, Meuret, et al., 2006; Hofmann, Pollack, & Otto, 2006; Myers & Carlezon, 2012). The use of DCS in conjunction with CBT can enhance the effectiveness of the therapy and reduce specific fears or phobias. For example, in humans, DCS enhanced exposure therapy in patients with generalized social anxiety disorder (Hofmann, Meuret, et al., 2006; Hofmann et al., 2013a).

In animals, DCS has been shown to enhance fear extinction training in rats (Davis, 2011; Davis et al., 2006; Gupta et al., 2013a; Ledgerwood, Richardson, & Cranney, 2003; Walker, Ressler, Lu, & Davis, 2002). DCS's effects on safety learning appear to occur by augmenting the IL–amygdala circuitry, making it a candidate to a possibly affect SoFiA (Chang & Maren, 2011; Gupta et al., 2013b; Ledgerwood et al., 2003; Walker et al., 2002). In this chapter, I test whether DCS could augment the anti-anxiety effect of the SI-hab training by reducing the number of partner pairings that are necessary to acquire anxiolysis (SoFiA).

Methods

Animals

Adult male Wistar rats (Harlan Laboratories, Indianapolis, IN) between 300-350g were used for all behavioral experiments. The care of the animals was the same as described in Chapter 2.

Behavioral testing methodology

Elevated Plus Maze

The Elevated Plus Maze (EPM) testing paradigm was implanted in the way as described in Chapter 2. Additionally, for these tests, the EPM was scored using ANY-maze video tracking software (Stoelting Co. Wood Dale, IL, version 4.8).

Specific Experimental Protocols

Experiment 1. SI-hab in BLC or DL

Figure 3.1A. All rats were given a baseline SI test in dim red light with a novel partner rat. Forty-eight hours later, rats were divided into 2 groups based on lighting conditions during SI-hab testing: Dim red light (n=8) and BLC (n=7). On the first SI-hab day, rats were paired with a novel partner for the SI test. On SI-hab Days 2-5, rats were then re-exposed to the same partner (familiar) used in SI-hab Day 1.

Experiment 2. SI-hab with a Novel/Familiar Partner

Figure 3.1B. The SI-hab paradigm was performed for 6 consecutive days. Rats were divided into two groups based on partner condition; Novel Partner group (n=8) were paired with a novel (unfamiliar) partner, or the Familiar Partner group (n=8) were paired with the same partner rat in each SI test. All SI testing sessions were performed under the BLC conditions.

Experiment 3. Novel Partner Challenge, Novel Environment Challenge

Figure 3.2. The SI-hab paradigm was done with rats (n=8) paired with a familiar partner in the SI test for 6 consecutive days under the BLC. On the seventh day, under the BLC, rats were paired with a novel partner rat in the SI test. After 5 days of no behavior testing, on the 13th day, the rats were paired with the previously familiar partner, but in a novel environment, which was a novel social interaction box placed in a different part of the testing room. The novel

environment had black colored walls instead of the light blue colored walls of the SI apparatus used for the previous SI sessions.

Experiment 4. SI-hab in dim light followed by Bright Light Challenge

Figure 3.3A. The SI-hab testing was given a familiar partner, but instead of testing in the BLC, the testing was done in the Dim red lighting for 5 consecutive days. On day 6, rats were divided into two groups; the novel partner (NP) group (n=6) or the familiar partner (FP) group (n=6), which was the same partner they had been paired with for the first 5 days. The SI session on day 6 was performed under BLC conditions for all rats.

Experiment 5. Extended SI-hab in dim light

Figure 3.3B. The SI-hab protocol from the previous experiment was extended from 5 SI tests to 10. Rats (n=10) were given a familiar partner in each of these 10 days and tested under dim red lighting. On Day 13, the animals were given the familiar partner and tested in the SI test under the BLC.

Experiment 6. SI-hab followed by Elevated Plus Maze

Figure 3.4. SI-hab training preceded the EPM. This SI-hab training was done for 5 consecutive days under either the BLC (n=4) or dim red lighting conditions (n=7). The SI testing was done with a familiar partner in each testing session for both lighting groups. The rats tested in the SI-hab in dim red lighting were split into two groups to be tested in the EPM under either dim red lighting or the BLC. This split created three groups based on the SI-hab lighting/EPM lighting; Dim/Dim, Dim/BLC, and BLC/BLC. Twenty-four hours following the last day of the SI-hab test the three groups were tested in the EPM under the designated lighting conditions.

Experiment 7. Acute Restraint testing following SI-hab

Figure 3.5. The restraint stimulus was given 24 hours following the completion of the SI-hab testing paradigm. Initially, the SI-hab testing was done with a familiar partner for 5 consecutive days under the BLC. The SI times were determined by manual scoring with ODlog for Mac OS X version 2.6.1 by Macropod Software. On Day 6, each rat was given 30 minutes of restraint followed by 30 minutes of rest within the home cage. SI testing then followed this rest with a novel (n=6) or familiar (n=6) partner in dim red lighting. Restraint was accomplished by placing the animals in a decapicone with the end securely closed and the tips cut slightly larger

for the animal to have space to breath. The animal remained in the decapicone for the duration of the restraint stress after which he was carefully removed and returned to his home cage. SI testing with either a novel or familiar partner was then given 30 minutes later under dim red lighting.

Experiment 8. Muscimol injections

Surgical Techniques

Surgical procedures were performed similar to the surgeries described in Chapter 2, but here the rats were anesthetized by placing them in a Plexiglas box connected to an Isoflurane system (MGX Research Machine, Vetamac, Rossville, IN). The animals were then placed on a stereotaxic instrument (Kopf Instruments, Tujunga, CA) with the incisor bar set at -4.5 mm and kept under a constant flow of isoflurane through a Plexiglas nose cone. Rats were implanted bilaterally with a 26-gage microinjection guide cannula (Plastics One, Roanoke, VA) directed towards the Infralimbic region of the Prefrontal Cortex (AP +3.2mm, ML ±0.7, DV -5mm) according to the Paxinos and Watson Atlas (2005) of the rat brain. All rats were given a minimum of 4 days recovery prior to any behavioral testing. During recovery, rats were gently handled each day for a minimum of 2 min.

Histology

Figure 3.6A. Rats with guide cannula were sacrificed following the conclusion of experiments and brains were removed, frozen and stored at -80^0 C until processed. Frozen brains were sliced coronally at 30µm and every 3rd section (separated by 90µm) was placed on a microscope slide. The sections were stained on the slides with cresyl violet. The location of bilateral injection sites was determined by damage left by cannula and injectors from these 30µm coronal Nissl-stained sections through the frontal cortex at 5x magnification, and confirmation at 40x (when needed), using rat brain atlas for guidance (Paxinos & Watson, 2005). For inclusion in analysis, both injection sites were located within the area designated IL.

Acute Muscimol injections and SI-hab testing

Figure 3.6B. SI-hab testing was performed for eight consecutive days under BLC conditions with the same partner rat. On Days 1-5 and again on Day 8, rats were given a sham (mock) intracranial (IC) injection 10 minutes prior to SI testing. Ten minutes prior to SI testing on Day 6 and 7, rats were given bilateral IC injections into the IL of either 90pmol muscimol (Musc,

Sigma-Aldrich, St. Louis, MO) dissolved in 0.9% saline, or 0.9% saline vehicle (Veh) at an injection volume of 100µl. The experiment was done in a balanced, cross-over design (n=11). An injection of one treatment (either Veh or Musc) was given on day 6 and the opposite treatment on day 7. This dose of Musc was similar to what has been used to suppress mPFC nuclei specifically in relation to social or fear/anxiety studies (Sierra-Mercado, Padilla-Coreano, & Quirk, 2011; van Kerkhof, Damsteegt, Trezza, Voorn, & Vanderschuren, 2013b). Infusions were done at a rate of 100 ml/min and injectors were allowed to remain in for an additional minute before removal.

Figure 3.6C. In an additional test, rats (n=11) were given bilateral IC injections into the IL of either 90pmol muscimol dissolved in 0.9% saline, or 0.9% saline vehicle at an injection volume of 100µl. Injections were given 10 minutes prior to SI testing with a novel partner rat in BLC or dim red lighting in a balanced cross-over design.

Repeated Muscimol injections and SI-hab testing

Figure 3.6D. In an additional muscimol experiment, rats (n=4) were given a Baseline SI test in dim red light followed by three days of muscimol injections (90pmol muscimol dissolved in 0.9% saline) into the IL 10 minutes prior to SI testing under the BLC with the same partner rat. For the remaining 2 days, rats received 0.9% saline vehicle injections at a volume of 100µl prior to SI testing with the same partner rat.

Experiment 9. SI-hab and D-cycloserine systemic injections

Figure 3.7. To habituate the rats to systemic subcutaneous (SC) injections, rats (n=10 total) were given a saline vehicle injection (0.9% saline (1.0 ml/kg)) once per day for two days 30 minutes prior to a habituation to the SI box. This procedure was repeated for two days. 24 hours following the second habituation session, rats were given a saline injection followed 30 minutes later by Baseline SI testing with a novel partner rat in dim light. 72 hours following baseline, Day 1 began for the SI-hab paradigm testing, rats were given a saline injection 30 minutes prior to SI testing in BLC with a novel partner rat. On Days 2-5, rats were split into two groups, receiving either a saline vehicle (n=5) or 10mg/kg D-cycloserine (Sigma-Aldrich, St. Louis, MO, Cat#C6880) (n=5) injection 30 minutes prior to SI in BLC with the familiar partner rat. On Day 6, rats were given either saline or D-cycloserine injection followed 30 minutes later by SI in BLC with an unfamiliar partner rat.

Statistics

All data were analyzed using the same techniques described in the Chapter 2 Statistics section, using Prism 6.0 Software (La Jolla, CA) and data are presented as mean ±SEM where appropriate with the confidence level for significance set at $p \leq 0.05$. With consecutive day training, a Repeated Measures one-way or two-way ANOVA was performed when appropriate. In the presence of significant main effects, post-hoc pairwise comparisons were conducted using Dunnett's to compare back to baseline values and Tukey's HSD tests between groups.

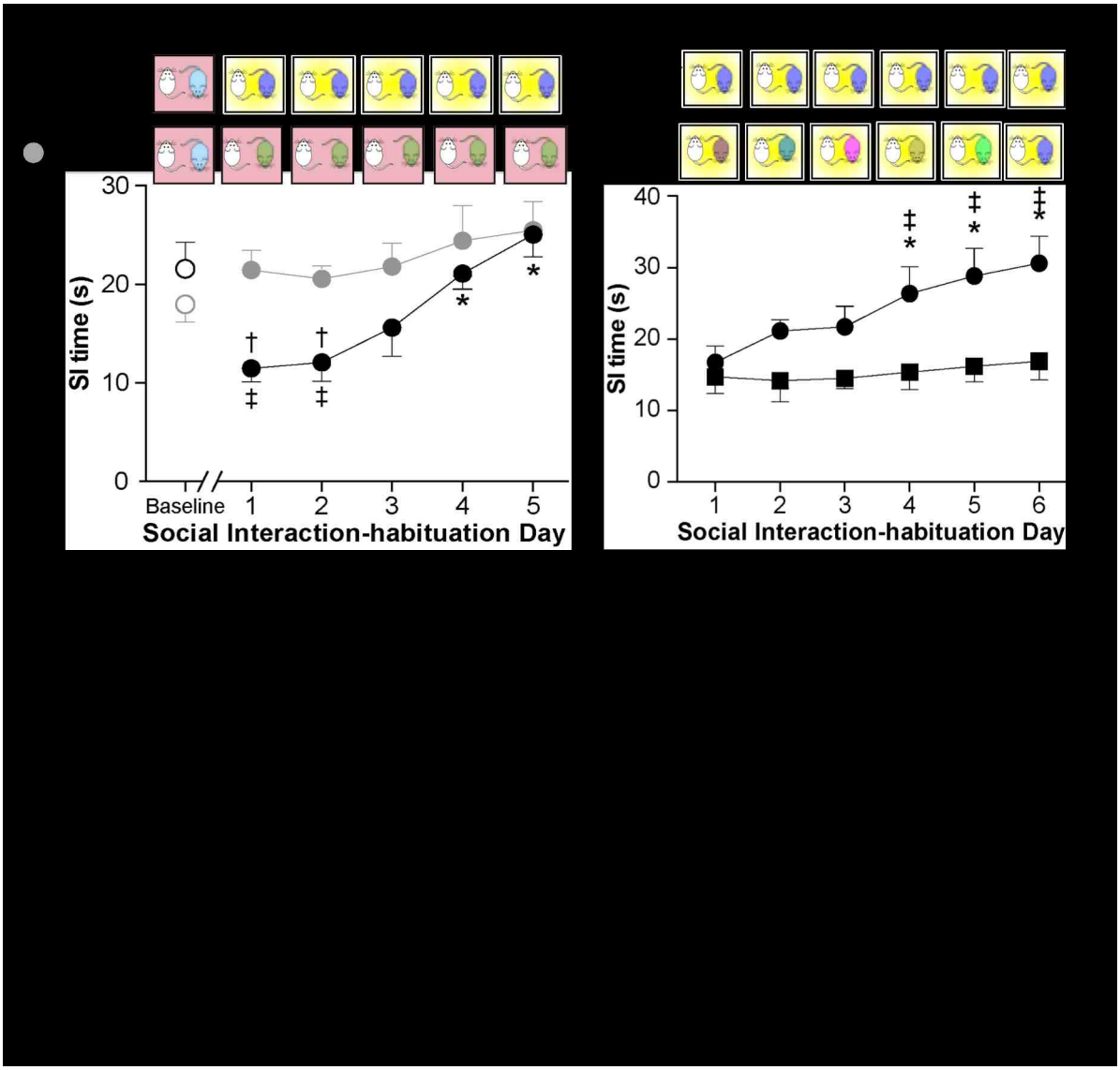
Results

Social Familiarity leads to anxiolysis in the presence of the BLC.

To determine if social familiarity acquired through repeated exposures in the SI test could reduce the anxiogenic effect of the Bright Light Challenge (BLC), rats were tested in the SI-hab testing paradigm. Rats were divided into two groups based on lighting conditions during testing, dim red light (n=8) and BLC (n=7). All rats received an initial 5-minute baseline SI testing session (with a novel partner under dim red lighting). Forty-eight hours after baseline testing the SI-hab protocol was initiated, and all rats underwent daily SI testing sessions for 5 consecutive days (SI-hab days 1-5). On the first SI-hab day, rats were paired with a novel SI partner and then re-exposed to that same partner (familiar) for the remainder of the experiment (SI-hab days 2-5) under the designated lighting condition (see top of Figure 3.1A for a procedural schematic). This paradigm produced a main effect of Day and Day x lighting condition interaction on SI times (RM two-way ANOVA, Day $F_{5,65}$=7.56, p<0.0001; Day x lighting condition $F_{5,65}$=3.95, p=0.0034, Figure 3.1A). The BLC significantly reduced SI times for the BLC group on the first two days of SI-hab testing (Days 1 and 2) compared to baseline (Tukey's p=0.0043 and p=0.0088, respectively) and compared to control rats (Bonferroni's p=0.013 and p=0.047, respectively). Compared to the first exposure to the partner rat (Day 1), repeated exposures to the same partner in the BLC group significantly increased SI times on the fourth and fifth days (Day 4 and Day 5, Dunnett's p=0.0028 and p<0.0001, respectively). This increase in SI time observed with the SI-hab paradigm under the BLC was not observed in the rats tested under the dim red light (control) conditions. Thus, the anxiety-like response to the BLC was diminished with multiple exposures to the familiar partner, but not in the absence of the BLC, which demonstrates that repeated testing with a familiar partner does not produce a generic increase in social interaction but rather a reduction in the anxiety-like behavior initiated by the BLC.

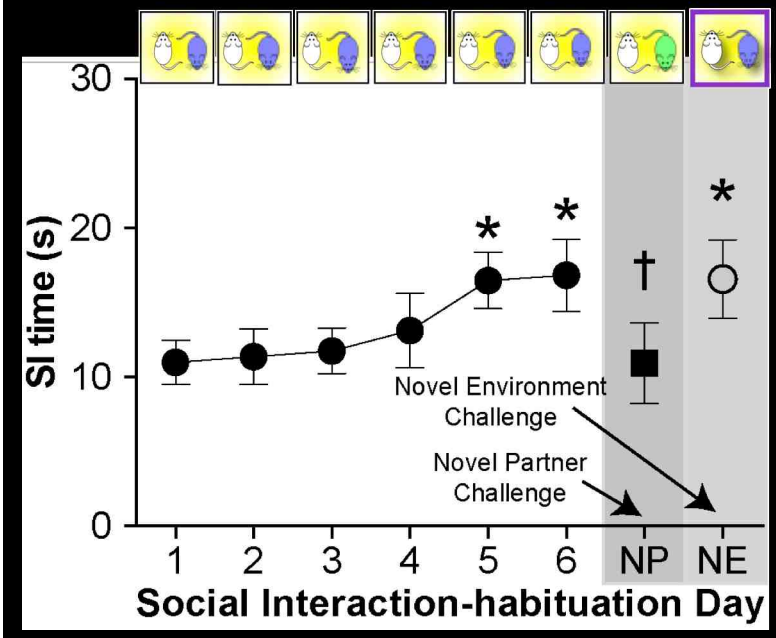
In the previous chapter I demonstrated that rats tested under the BLC with a novel partner did not habituate to this anxiogenic stimulus (Chapter 2, Figure 3). The next experiment was designed to investigate the specific role of the familiar partner in the reduction of anxiety-like behavior initiated by the BLC. This was to determine if the anxiolytic behavior observed was a consequence of specifically social familiarity and not merely contextual familiarity. Rats were paired with either a familiar (n=8) or novel (n=8) partner each day for 6 consecutive SI-hab days under BLC conditions (Figure 3.1B schematic on top). Here, main effects of Day, partner

condition, and a Day x partner condition interaction were observed (RM two-way ANOVA, day $F_{5,70}$=7.53, p<0.0001; partner condition $F_{1,14}$=7.13, p=0.018; Day x partner condition $F_{5,70}$=3.72, p=0.0048 Figure 3.1B graph). SI times increased over the SI-hab days in the familiar partner group but not in the novel partner group, with SI times in the fourth through sixth sessions being significantly increased compared with SI times of the first exposure to the partner on Day 1 (Dunnett's p≤0.0003), and significantly higher than the SI times of the novel partner group (Bonferroni's p≤0.038). The significant increase in SI times from Day 1 on Days 4-6 demonstrate an acquisition of the social familiarity-induced anxiolysis (SoFiA) effect, and we see therefore that social familiarity is an essential requirement for the for the acquisition of anxiolytic behavior.



SoFiA expression is specific to the Familiar Partner.

The next experiment was designed to determine the role of social and environmental context specificity in the expression of anxiolytic-like behaviors. This was accomplished by repeated pairing of a partner rat during the SI-hab testing paradigm under the BLC on testing Days 1–6. Similar to observed behavior in previous experiments, repeated SI testing with the same partner under BLC conditions led to a main effect of time (RM one-way ANOVA $F_{5,30}$=4.88, p=0.015; Figure 3.2). The mean ±SEM SI times on Days 5 and 6 were significantly increased compared with the first exposure to the partner on Day 1 (Dunnett's p≤0.014), and thus SoFiA was acquired. Rats were then exposed to a novel partner challenge on SI-hab Day 7. Exposure to the novel partner, under BLC conditions, resulted in SI times similar to Day 1 and significantly reduced from Day 6 (Tukey's p=0.043), suggesting that the presence of the familiar partner is required for the expression of the anxiolytic-like behavior (SoFiA). Rats were then exposed to a novel environment challenge on Day 13 where they were once again paired with the Familiar Partner that was previously used on Days 1–6, and under BLC conditions but tested in an alternative novel SI box as described in the methods. The mean ±SEM SI times in this novel environment were once again significantly greater than Day 1 SI times (Dunnett's p=0.025) and SI times during the previous Novel Partner challenge (Tukey's p=0.0012). Therefore, we see that the expression of SoFiA is partner specific but not environmentally specific (when paired with the familiar partner).


30
20
10
0
SI time (s)
*
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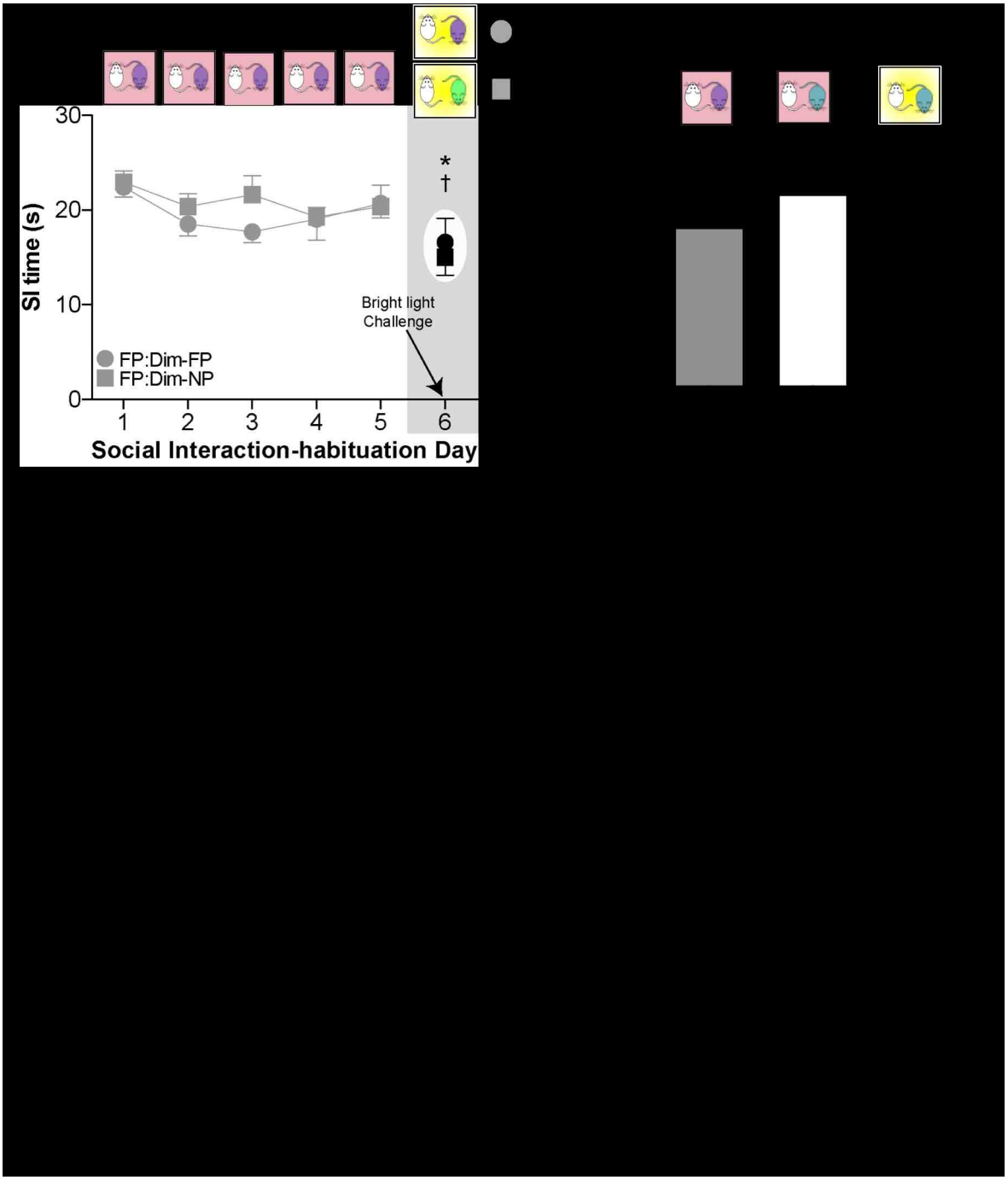
†
*
Novel Environment
Challenge
Novel Partner
Challenge
1
2
3
4
5
6
NP
NE
Social Interaction-habituation Day

SoFiA acquisition requires the presence of the anxiogenic stimulus and the familiar partner.

The next experiment was designed to determine if social familiarity could reduce anxiety-like responses to the BLC when social interaction pairings occurred under dim red light in the absence of the anxiogenic stimulus. To accomplish this, all rats were paired with the same partner rat (familiar partner, FP) for 5 consecutive days of SI-hab sessions (Day 1-5) under dim red light conditions (FP:Dim). For the sixth SI-hab session (Day 6), the effect of social familiarity under a BLC was then tested (See Figure. 3.3A top for procedural schematic). Here rats were either paired with the same partner they had the previous five sessions (FP) or they were paired with a novel partner (NP), resulting in two groups of rats trained with a FP in dim red light conditions and exposed to a NP on the challenge Day 6 (FP:dim-NP, n=7), and rats trained with a FP in dim red light conditions and exposed to the same partner on the challenge Day 6 (FP:dim-FP, n=7). This procedure resulted in a significant main effect of Day (Two-way RM ANOVA, day $F_{5,55}$ =4.36, p=0.0021), but neither partner condition main effect nor the interaction reached significance. The mean ±SEM SI times on Day 6 (under the BLC) were significantly lower than SI times on Day 1 or Day 5, regardless of the familiarity of the partner rat (Tukey's p=0.0005 and p=0.035 respectively; Figure 3.3A). These results suggest that social familiarity alone is not sufficient to reduce anxiety-like responses to the BLC when the social familiarity pairings are done under non-anxiogenic conditions.

I next asked the question of whether an extended amount of exposure time under non-anxiogenic conditions would induce social familiarity sufficient to overcome an anxiogenic stimulus. This was done by increasing the number of SI test sessions in the absence of the anxiogenic stimulus to provide additional opportunity for social familiarity to develop. The number of SI-hab sessions in the dim red light was increased from the normal 5-6 sessions to 10. Following the conclusion of the 10 Day SI-hab pre-testing in dim red light, the rats were challenged in the SI test with the familiar partner but under the BLC (Figure 3.3B). Comparing the mean ±SEM SI times in the BLC on Day 13 to the baseline and 10^{th} day of SI-hab testing in dim red light, there was a significant effect of treatment day (RM one-way ANOVA $F_{2,18}$=7.374, p=0.0046). Comparing Day 13 under the BLC to the baseline and Day 10 under the dim red light, we see that the BLC reduced the mean SI time compared to the previous SI tests (Tukey's multiple comparisons, Baseline vs Day 13 and Day 10 vs Day 13, $p<0.05$). The social familiarity developed within the 10 SI sessions between the test rat and the partner rat was not sufficient

to overcome the BLC. Therefore, the SI-hab testing must involve pairing the social familiar partner and the anxiogenic stimulus to initiate the acquisition of anxiolysis.

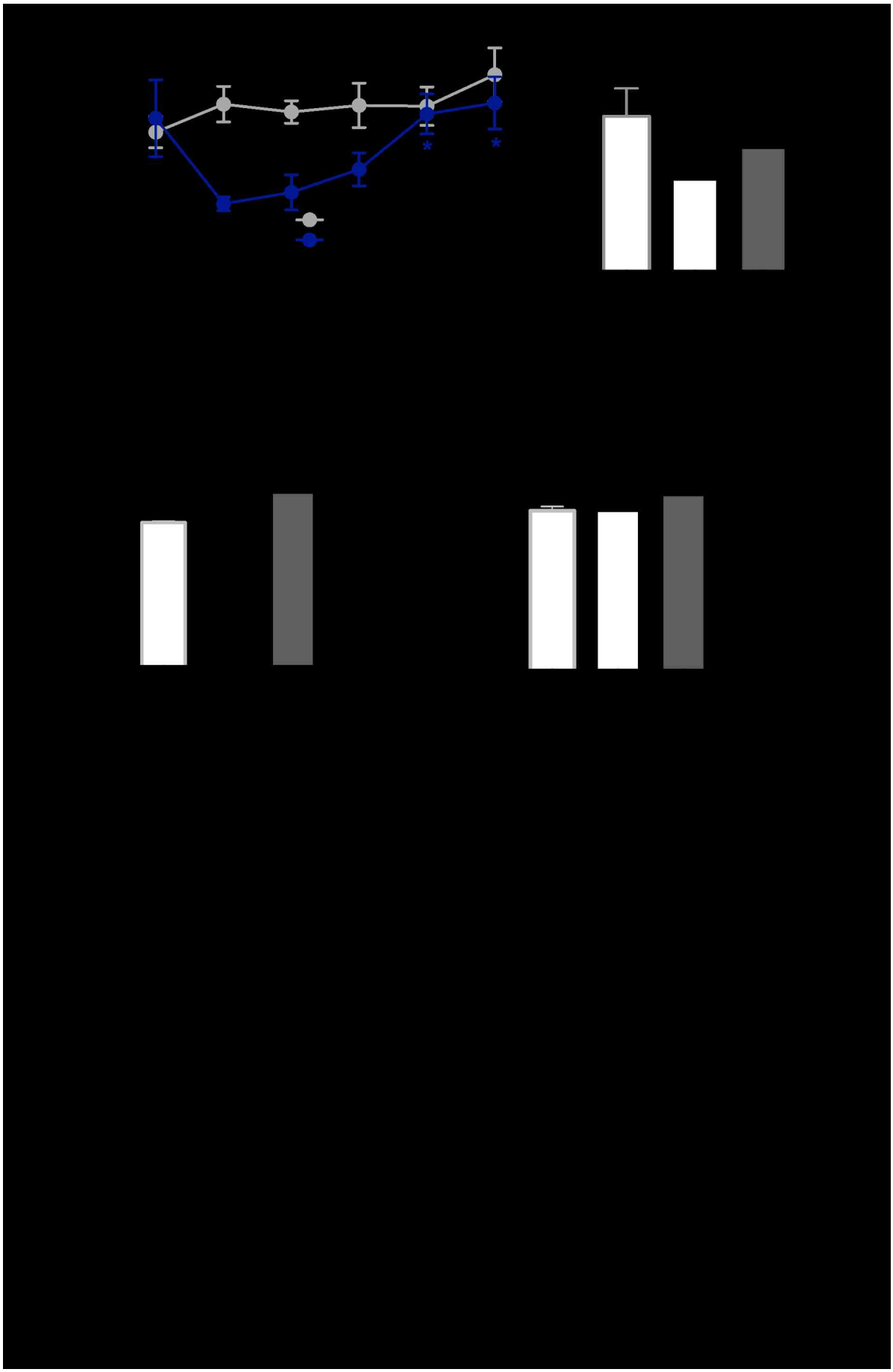


Effect of BLC in the Elevated Plus Maze following SoFiA acquisition.

The next experiment was designed to determine if the anxiolysis to the BLC following SoFiA acquisition would translate to anxiolysis to the BLC within a different test of anxiety-like behavior. Therefore, I tested rats in the Elevated Plus Maze (EPM) test following either the acquisition of SoFiA under the BLC, or no acquisition of SoFiA through the SI-hab training done under dim red lighting. The hypothesis here was that if the rats had overcome the anxiogenic stimulus of the BLC following the acquisition of SoFiA, then the BLC would not be anxiogenic during the EPM as well. To test this hypothesis, the rats were tested in the SI-hab testing paradigm, with one group of rats tested with a familiar partner in dim red lighting (n=7) and one group tested with the familiar partner under the BLC (n=4) (Figure 3.4A). SI-hab testing went for 5 consecutive days, producing the SoFiA effect within the group that was tested in the BLC each day, while as expected, the rats tested in the dim red light did not acquire the SoFiA effect. There was a main effect of Day (RM two-way ANOVA $F_{4,36}$=6.023, p=0.0008), main effect of light condition ($F_{1,9}$=6.297, p=0.0333) and an interaction ($F_{4,36}$=2.662, p=0.0482). The SoFiA effect is seen in the rats tested under the BLC, as evidenced by the SI times becoming significantly greater than Day 1 times on Day 4 and 5 (Dunnett's multiple comparisons test p<0.05). The rats also demonstrated an elevated anxiety-like behavior on Day 1 in the BLC, as the SI times were significantly lower in the BLC group compared to the dim red light group (Sidak's multiple comparisons test p<0.05). While the rats tested in the dim red lighting did not have significant alterations in the SI times across the SI-hab days, there was a significant increase from Baseline on Day 5 (Dunnett's, p<0.05).

On Day 6, rats were tested on the EPM apparatus under either the BLC or dim red lighting conditions. The group of rats tested in the SI-hab in dim red light (Control) was separated into two groups for the EPM, either dim red light or BLC. While the rats tested in the BLC for the SI-hab training were all tested in the EPM under the BLC. This created 3 groups based on SI-hab lighting/EPM lighting, Control/Dim (n=3), Control/BLC (n=4), and SoFiA/BLC (n=4). There were differences in the number of open arm entries, with a main effect of treatment (SI-hab/EPM lighting) (One-way ANOVA $F_{2,8}$=4.979, p=0.0394) (Figure 3.4B). BLC resulted in a reduction in number of open arm entries for the rats trained in dim red light, compared to the Dim light control group (Control/Dim vs Control/BLC, Tukey's multiple comparisons test, p=0.0330). SI-hab training under BLC appears to have attenuated the BLC effect, as no reduction in number of open arm entries was observed between Control/Dim vs.

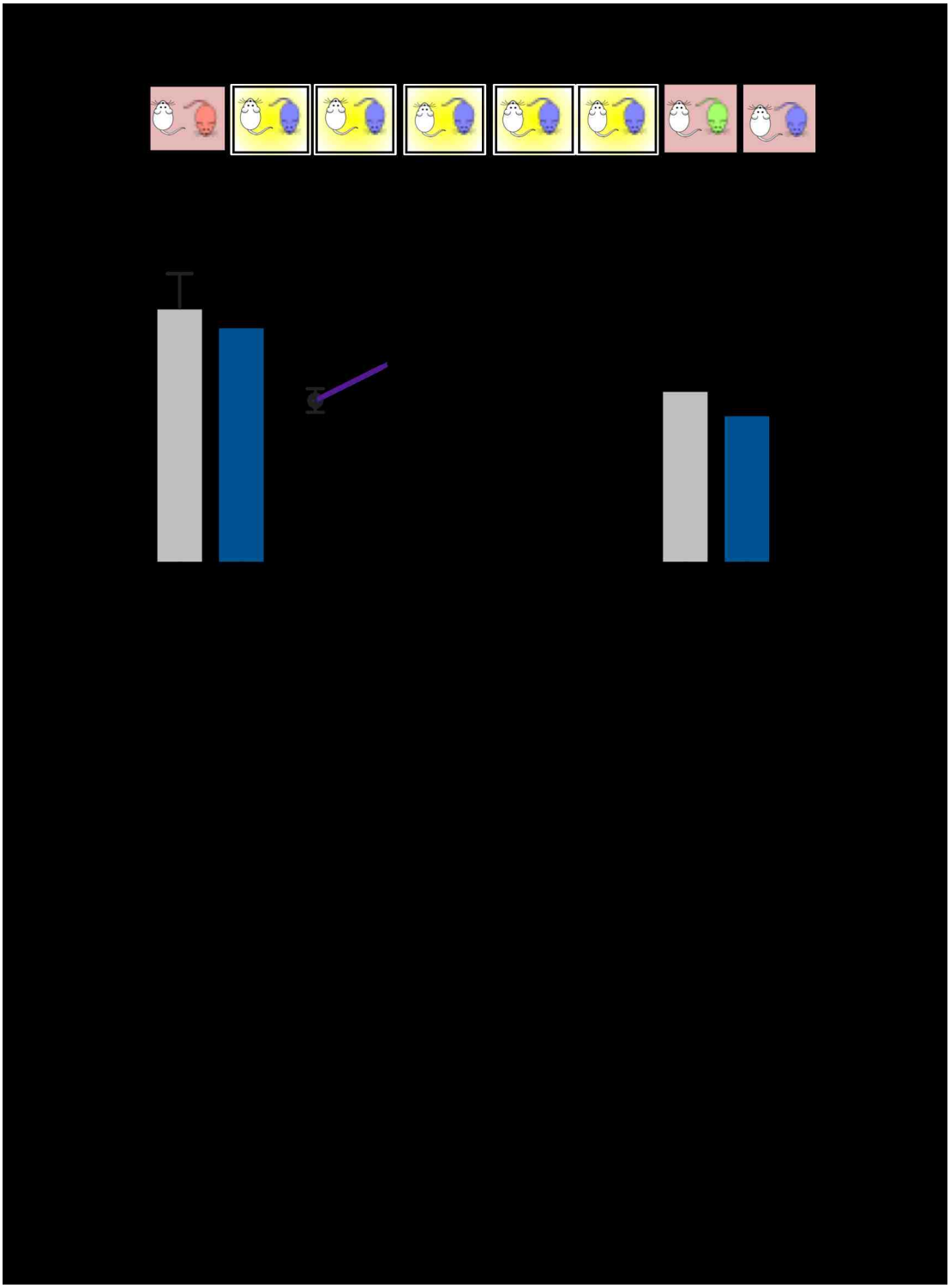
SoFiA/BLC groups. The prior treatment of the SI-hab training under the BLC influenced the anxiety-like behavior in the EPM, and a t-test comparing the two groups tested in the EPM under the BLC is significant (Control/BLC vs. SoFiA/BLC, unpaired t-test p=0.0074). This comparison shows that looking at the prior SI-hab training conditions of either BLC or dim red lighting, the EPM open arm entries under the BLC was altered. This differential effect of the SI-hab training conditions on the EPM behavior was not seen in the open arm time (Figure 3.4C). There is not a difference between the three groups tested, and so the pre-training of the SI-hab in the dim versus BLC groups did not lead to changes in the open arm time in the EPM under BLC. Additionally, the total distance traveled within the EPM was not significantly different between the three groups during testing (Figure 3.4D).



B. On Day 6, the rats were split into three groups depending on the SI-hab testing conditions/EPM lighting conditions, creating; Control/Dim (open gray outlined bar), Control/BLC (open black outlined bar), and SoFiA/BLC (gray black outlined bar). Presented here are mean ±SEM for number of open arm entries. The number of Open Arm entries is significantly different between the Control/Dim group and the Control/BLC group (* Tukey's multiple comparisons test p=0.0330). Comparing the two groups tested in the EPM under BLC conditions but having different SI-hab pre-training conditions, there was a significant difference in the number of open arm entries, with the SoFiA acquired group from pre-training in the BLC having more open arm entries than the non-SoFiA acquisition group trained in dim red light during the pre-training (unpaired t-test, p=0.0074). **C.** Presented are the mean ±SEM times of the amount of time the rats spent in the open arms during the EPM testing. Looking at the open arm time between the three groups produced no significant differences between the times spent in the open arms, regardless of SI-hab pre-training conditions, or the EPM lighting conditions. **D.** Presented are the mean ±SEM total distance (m) traveled within the EPM during testing. There is no difference between the different treatment groups in the total distance traveled.

Following SoFiA acquisition, the presence of a familiar partner did not alter the anxiety-like response to an acute restraint.

Restraint exposure reliably induces anxiety like behavior in rats in the SI test when given as an anxiogenic stimulus (T. J. Sajdyk et al., 2008). Therefore, following what we see in the EPM, I asked whether following the acquisition of SoFiA, would a pre-treatment of restraint followed by SI testing with the familiar partner be anxiolytic compared to SI testing with a novel partner? Although I previously saw a habituation to the restraint stimulus when given repeatedly (Chapter 2, Figure 4), I instead used it as a single acute anxiogenic stimulus following SoFiA acquisition. Rats were given the SI-hab testing paradigm under the BLC with a familiar partner to initiate the acquisition of SoFiA, which was seen in all rats (n=12), with a significant effect of Day (RM One-Way ANOVA, $F_{2.789,30.68}$=9.594, p=0.0002) (Figure 3.5). There was a light effect seen on Day 1 with a significant decrease in SI time from Baseline (Tukey's multiple comparisons test p<0.01), while across the repeated exposures, a significant increase in SI time from the Day 1 occurred on Days 3, 4 and 5 (Tukey's multiple comparisons test p<0.05). On Day 6, the rats were split into two groups and given the Restraint stimulus similar to previously reported methods (T. J. Sajdyk et al., 2008); 30 minutes of restraint and 30 minutes rest before SI testing in dim red lighting with either the Familiar Partner (n=6) from the previous SI-hab testing, or a Novel Partner (n=6). This 30 min of restraint significantly altered the SI times for both groups compared to baseline, with a main effect of restraint (2-way RM ANOVA $F_{1,10}$=11.15, p=0.0075) but no familiarity x restraint effect. Regardless of the presence of social familiarity, the familiar partner rat did not protect against the anxiety induced by the restraint pre-treatment. The SI on the Restraint day was lower for both groups from baseline (Uncorrected Fisher's LSD, baseline vs Restraint/SI with NP, p=0.0453, baseline vs Restraint with FP, p=0.0350).



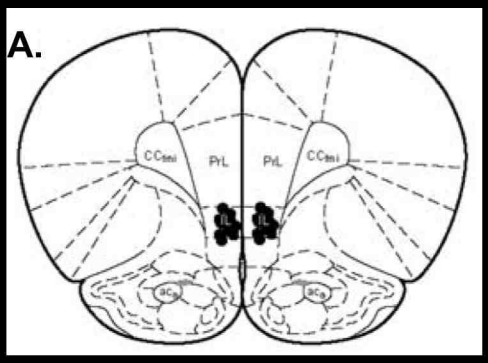
Inhibition of the mPFC disrupts SoFiA expression.

To determine if an active medial prefrontal cortex (mPFC) was necessary for expression of SoFiA, rats (n=11) implanted with bilateral guide cannulae directed towards the IL of the mPFC (Figure 3.6A). These rats were tested in the SI-hab paradigm under the BLC conditions with the same partner rat for eight consecutive SI-hab sessions (see top of Figure 3.6B procedural schematic). Prior to sessions 1-5 and 8, rats were given a sham injection 10 min before SI testing in the BLC. On test Days 6 and 7 rats received intracranial injections of either muscimol (Musc, 90 pmol/100 nl) or saline as the vehicle control (Veh, 100 nl) 10 minutes prior to testing. The injections were done in a counterbalanced cross-over design where six rats received Veh injections on day 6 and Musc injections on day 7, and the other five rats received Musc injections on day 6 and Veh on day 7 (Figure 3.6B). Repeated exposure to the same partner rat led to significant increases in SI time across the days tested (main effect of day, RM one way ANOVA $F_{10, 90}$=4.60, p<0.0001, Figure 3.6B), with SI times significantly increased from D1 on Days 4, 5 and 8, and following the IL vehicle injection (Dunnett's $p \leq 0.037$). Interestingly, muscimol injections into the IL blocked the expression of SoFiA (increase in SI time from D1) (Dunnett's p=0.77) and significantly reduced SI times compared to vehicle injections into the IL (Tukey's p=0.042).

The reduction in SI time following muscimol injections into the IL appears specific to SoFiA expression. Additional controls were done to determine if muscimol itself would lead to a decrease in SI time without the prior acquisition of social familiarity. In this separate experiment done with the same cannulated rats starting 3 days after the completion of the initial experiment, rats were again given muscimol or vehicle injections into the IL followed by SI testing in either BLC or dim red light. The treatments were counterbalanced so all the rats were given vehicle and muscimol across 2 days followed by SI testing with a novel partner in dim red light. This was then repeated across two more days with rats receiving vehicle and muscimol injections followed by SI testing with a novel partner in the BLC (Figure 3.6C). Taken together, there were 4 treatment days of drug infusion/SI lighting (n=11 in each group), Vehicle/Dim Light, Muscimol/Dim, Vehicle/BLC, and Muscimol/BLC. Among these groups, there was a main effect of light (2-way ANOVA $F_{1,40}$=6.563, p=0.0143), but not an effect of treatment or an interaction. There was no significant difference between the two treatment groups in each lighting condition, but there was a significant difference between the light conditions for the Muscimol,

but not the Vehicle treated rats (Sidak's multiple comparisons test, $p<0.05$). This demonstrates that muscimol into the mvPFC itself did not affect anxiety-like behavior with a novel conspecific.

To test the role of the IL in the acquisition of SoFiA, I injected muscimol into the IL prior to SI testing on the days during which the acquisition of SoFiA normally occurs (Days 1-3) (Figure 3.6D). A new cohort of rats (n=4) were given a Baseline test consisting of SI testing in dim red lighting with a novel partner. The SI-hab training then began with SI tests in the BLC with a familiar partner. On days 1-3, the rats received Muscimol injections into the IL 10 minutes prior to SI testing with a familiar partner, while on days 4-5, rats received vehicle injections into the IL 10 minutes prior to SI-hab testing. Here there was a significant effect of Day (RM one-way ANOVA $F_{5,15}=9.078$, $p=0.0004$), which was a result of the BLC effect seen on the first day of testing in which the SI times were significantly reduced compared to Baseline SI times (Tukey's multiple comparisons test, Baseline vs Day 1, $p<0.05$). The remaining days of testing Days 2-5 were not significantly different from Day 1 (Tukey's multiple comparisons test, $p>0.05$). There was not an increase in SI time from Day 1 across the days tested, demonstrating a lack of the acquisition of SoFiA according to our a priori operational definition of SoFiA being a significant increase in SI time from Day 1 SI times.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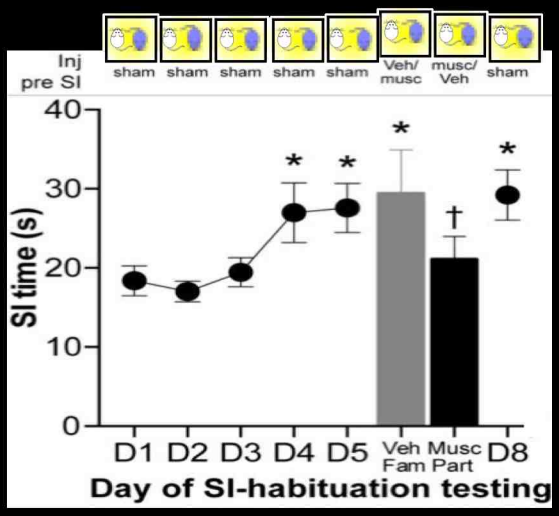
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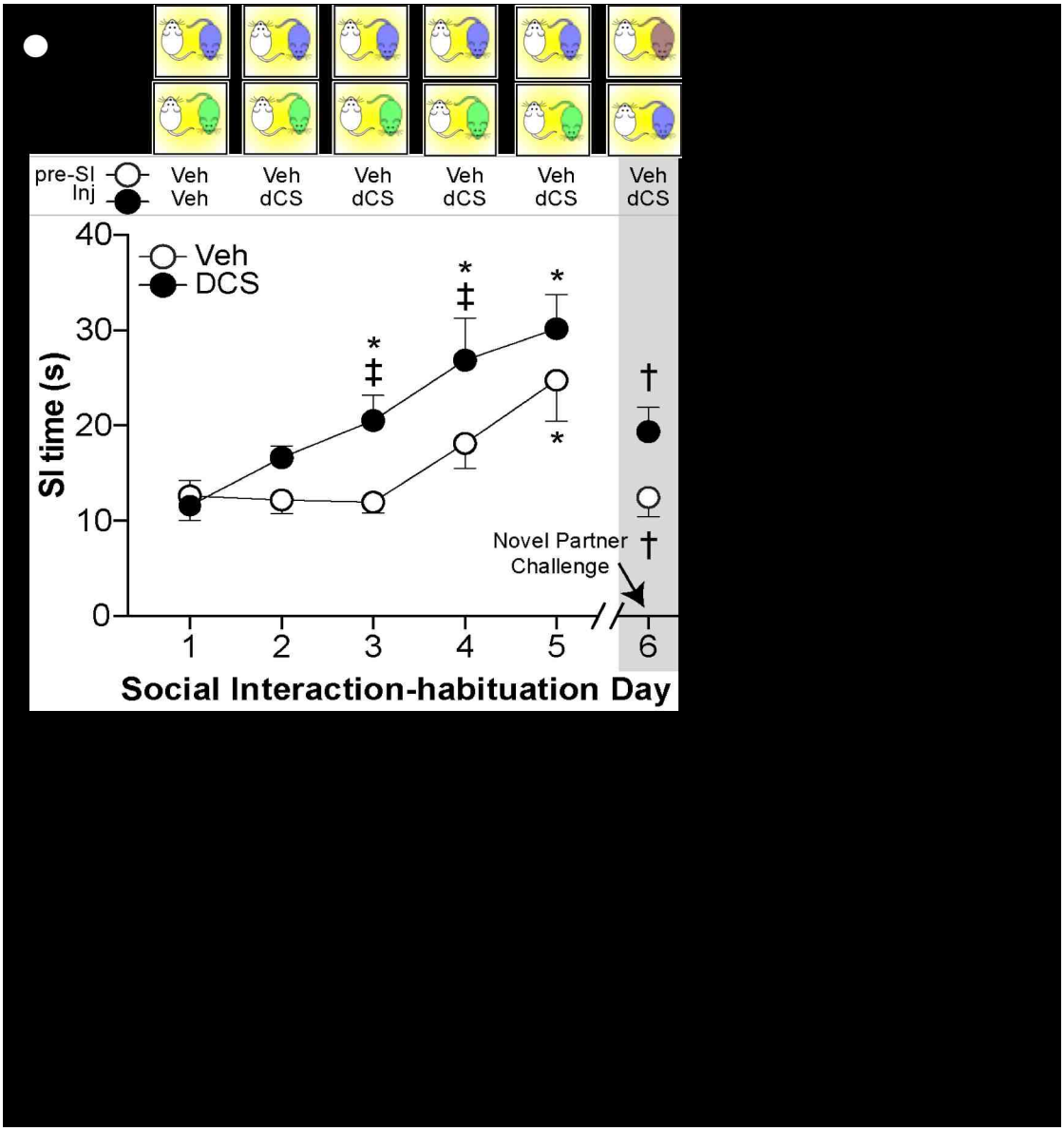
C. As a control to establish that the Muscimol was not inherently anxiogenic, rats were given Muscimol (black bars) or Vehicle (gray bars) injections followed by SI testing in either Dim light or BLC with a Novel Partner for the SI test. Presented here are the mean ±SEM SI times for the rats given these treatments (n=11, cross-over, counterbalanced design). Within the Muscimol group, there was a significant change in SI time between the lighting conditions (* Sidak's multiple comparisons test, $p<0.05$). **D.** Presented here are the mean ±SEM SI times of a separate groups of rats (n= 4) treated with injections of Muscimol into the IL just prior to SI testing on Days 1-3 during the acquisition of SoFiA. Following Baseline exposure in the absence of the anxiogenic stimulus and the muscimol injection, Days 1-3 involved injections of Muscimol prior to SI testing. The remaining two days involved vehicle injections prior to Social Interaction testing. † Tukey's multiple comparisons test, Baseline vs Day 1, 4 and 5, $p<0.05$.

D-cycloserine enhances the acquisition of SoFiA.

With the use of the cognitive enhancer D-cycloserine (DCS) to enhance safety learning in other studies, I next asked whether pairing social familiarity exposure with systemic injections of DCS could enhance the acquisition of SoFiA. SoFiA acquisition was a priori defined as a significant increase in SI time compared with the first exposure to the partner (SI-hab Day 1), and the rate of acquisition as the number of SI-hab pairings required to achieve this significant increase in SI time. To habituate rats to being injected, rats were brought into the behavior staging room and were subcutaneously (s.c.) injected with 0.9% saline (1.0 ml/kg) once per day for 2 days prior to the SI-hab paradigm. Rats were injected 30 min before SI-hab testing (Figure 3.7 top); each SI session was performed under BLC conditions and with the same partner rat for SI-hab days 1–5. All rats were injected with saline (1.0 ml/kg s.c.) on SI-hab day 1, the first exposure to the partner rat. Rats were then divided into two groups based on injection type on SI-hab days 2–6. On these days, rats were either injected with saline (Veh group, n=5) or DCS (10 mg/kg in a volume of 1.0 ml/kg; DCS group, n=5). The dose of 10mg/kg for DCS was chosen because it was in the low-dose range that was still effective in enhancing safety learning in rats (Ledgerwood et al., 2003; Walker et al., 2002). As previously observed, social familiarity produced an increase in SI time across days (RM two-way ANOVA main day effect $F_{5, 40}$=13.16, p<0.0001 Figure 3.7). However, DCS treatment affected the rate at which this increase in SI time occurred between the two treatment groups over the first 3 days of repeated exposure to the partner rat (day x treatment interaction $F_{2,16}$=7.84, p=0.0042). Rats treated with DCS had significantly increased SI times on the third SI session (SI-hab day 3) and lasting through session 5, compared with the first day of exposure to the partner (Dunnett's p≤0.031), whereas SI times of Vehicle-treated rats were not significantly increased, compared with Day 1, until the fifth exposure to the partner rat (Dunnett's p=0.002). In addition, the SI times of the DCS group were significantly increased compared with Vehicle group SI times on SI-hab Days 3 and 4 (Fisher's LSD p≤0.027). As DCS has previously been reported to have pro-social effects in mice, both Vehicle- and DCS-injected rats were exposed to a Novel Partner challenge for the sixth SI session under the BLC (Jacome, Burket, Herndon, & Deutsch, 2011). If the effect of the DCS injections was to simply produce pro-social effects, then we would expect that the SI times during the Novel Partner challenge would remain elevated compared with the SI times of the first SI-hab session (Day 1) and with SI times of the Vehicle group. Pairing with a novel partner resulted in a significant reduction in SI time compared with SI times of the rat's previous SI session (Day 5) for

both DCS and Vehicle-treated rats (Tukey's p=0.018 and p=0.005, respectively). Furthermore, the SI times for each group were not significantly different compared with Day 1 SI times (Dunnett's; Vehicle, p=0.999 and DCS, p=0.073) or between groups (Day 6, Fisher's LSD p.0.072). Here I demonstrated an enhanced rate of SoFiA acquisition following injections of DCS. However, comparing D-cycloserine effects on SI time only during the novel partner conditions (day 1 and day 6) resulted in a main effect of day (RM two-way ANOVA main day effect $F_{1,8}$=5.89, p=0.041) and a day X treatment interaction ($F_{1,8}$ = 6.40 p = 0.035). In this, less stringent analysis (as a result of reducing the multiple comparisons) SI times of DCS rats are significantly greater on day 6 compared to day 1 (within) and compared to SI time of Veh rats on day 6 (Fisher's LSD p=0.008 and p=0.024, respectively). Thus, interpretations of these data are limited.



Discussion

Social Familiarity-induced anxiolysis

The concept that overcoming fear and anxiety is easier in the presence of a familiar person (e.g. friend or therapist) is commonly accepted and social support enhances the efficacy of cognitive behavioral therapy (Baldwin, Wampold, & Imel, 2007; Martin et al., 2000; McHugh, Whitton, Peckham, Welge, & Otto, 2013; Roshanaei-Moghaddam et al., 2011). Yet, little is known of the neural mechanisms that regulate this social familiarity-induced anxiolysis (SoFiA). The current chapter is the first systematic investigation of SoFiA as a preclinical animal model. Here social familiarity selectively reduces anxiety-like responses to a naturally anxiogenic stimulus, the Bright Light Challenge (BLC), but does not alter baseline anxiety behaviors as measured by the social interaction (SI) test (Crawley & Goodwin, 1980; de Jongh, Groenink, van Der Gugten, & Olivier, 2002; DeFries, Hegmann, & Weir, 1966; Walker & Davis, 1997). The behavioral testing paradigm of Social Interaction-habituation, or SI-hab, in the presence of the BLC is shown here as a valid test for measuring positive effects of social familiarity on changes in anxiety-like behavior.

Social Familiarity induces safety learning

Here we observed that social familiarity led to anxiolysis, produced through the SI-hab training sessions. The reduction in SI times induced by the BLC was overridden by the fourth or fifth pairing with a familiar partner rat. As seen in Figure 3.1A and B, social familiarity is obtained through repeated exposures to the partner while in the presence of an anxiogenic stimulus reducing the anxiety-like behavior. The acquisition of this anxiolytic-like behavior appears to be specifically linked to the familiar partner as rats failed to acquire an anxiolytic-like response to the BLC when a novel partner was used for each of the repeated SI sessions. The familiarity of the partner is thus essential to anxiolytic responses to the BLC in the SI test. These increases in SI times with repeated exposures to a familiar partner rat appear to be an anxiolytic-like response rather than a general increase in pro-social behavior, as demonstrated by the lack of behavioral changes in the absence of the BLC (Figure 3.1A).

The familiar partner is a vital aspect to the acquisition of SoFiA, as novel partner exposures did not lead to SoFiA acquisition (Figure 3.1B). The rats did not habituate to the BLC with novel partner exposures, and so the change that we see in the SI times across days is likely due to the social familiarity to the partner instead. After SoFiA is acquired, the presence of the

familiar rat remains pivotal for the expression of the anxiolysis because anxiety-like behavior returns when the familiar rat is replaced with a novel rat [Figure 3.2 and (Truitt et al., 2007)]. These results are similar to past findings, where social familiarity had no effect on control rats, but reduced anxiety-like behaviors in rats made persistently anxious by the UCN priming, a procedure that, as described previously, led to lasting increases in anxiety-like behaviors and increased excitability of the Basolateral amygdala (BLA) (Rainnie et al., 2004; Truitt et al., 2007).

Furthermore, the anxiolytic-like response in the presence of a socially familiar partner remained even when the SI test was done in a different testing environment. When the test rat was challenged with a novel environment, the anxiolysis to the familiar partner was retained, and SI times in the novel environment were not different from the times post acquisition of SoFiA in the previous environment (Figure 3.2). These results support the idea that the acquisition of anxiolytic-like behavior in this paradigm is unlikely a result of habituation to the testing environment or the BLC stimulus. This also demonstrates that the behavioral response was not contextually driven, and the rat was cueing off of the partner rat specifically. We did not extend testing a further day, but a novel partner challenge in this new environment would be expected to lead to increased anxiety-like behavior again.

Collectively these data could be interpreted that SoFiA is a conditioned response, and the familiar rat acts as a cue. The behavior shift occurring during SoFiA acquisition is similar to extinction training, as both paradigms involve behavior shifts across time in response to repeated exposures. However, typically when cues are repeatedly paired with unconditioned aversive stimuli, they are avoided or the cue itself starts to induce fear or anxiety responses (Maren & Quirk, 2004; Thielen & Shekhar, 2002). Since the presence of the familiar partner reduces anxiety-like behavior, the partner rat may be acting as a safety cue, in which case SoFiA could be considered a form of safety learning (Christianson et al., 2012).

The SI-hab testing under the BLC is different from Fear conditioning

The current understanding of the prefrontal cortex often emerges from studies that focus on fear conditioning. Fear conditioning creates a memory connection between a neutral, conditioned stimulus (CS) and an aversive unconditioned stimulus (US), creating fear behavior in response to the CS. This is often followed by extinction training, which consists of repeated exposures of the conditioned stimulus (CS) without the noxious unconditioned stimulus (US) paired with it. In the absence of the aversive stimulus new memories form that override the fear

response and leads to a dissociation of the CS and US being paired, or that the CS indicates the impending presentation of the US, leading to a reduction in the fear response to the CS (Sotres-Bayon, Bush, & LeDoux, 2004). Fear extinction means that new learning or the stimulus meanings has occurred, and it does not involve an override of the emotional responses to the US itself, which can still elicit responses when presented again in later trials. This stands as an additional way in which safety learning is not the same as fear extinction. Safety learning involves a change in the response to the US itself, while in the presence of the safety cue.

We have shown that the BLC itself is a naturally aversive US that remained anxiogenic even after several exposures. The BLC cannot be escaped from, but the presence of the partner rat can act as a source of safety from the BLC. Safety learning is a conditioned inhibition of the fear. In this case, the CS (the familiar partner) becomes associated to indicate positive instead of negative. As the SoFiA behavior develops we argue that this is learned safety, which is the opposite of learned fear. The approach taken with the SI-hab testing is to not eliminate the aversive stimulus, but instead create a memory of safety in relation to the presence of the familiar partner in the face of the aversive stimulus. Therefore, with the SI-hab training with a familiar partner we still see the reduction of the anxiety-like behavior even in the actual presence of the BLC. The partner, instead of becoming a stimulus to elicit fear or anxiety, becomes a safety cue as the test rat learns to utilize the presence of the partner to overcome the anxiety produced by the BLC.

Social familiarity and the anxiogenic cue need to be paired concurrently

We see that the familiar partner is necessary for the acquisition and expression of SoFiA, and so we further asked; is it the anxiogenic stimulus that is triggering the formation of the social memory or is it the social memory that leads to the formation of the partner rat becoming an anxiolytic (safety) cue? In Figure 3.3, I tested the rats in the SI-hab paradigm with a familiar partner but in the absence of the BLC anxiogenic stimulus during the testing sessions. We observed that even after repeated exposures to a familiar partner in dim red light (low anxiety-like) conditions, subsequent exposure to the BLC on Day 6 with the familiar partner still produced anxiety-like behavior. This social familiarity acquired in the absence of the BLC was not sufficient to overcome the anxiogenic stimulus, and we saw a light effect in rats challenged in the BLC regardless of partner type. In the dim red lighting conditions, the familiarity to the partner rat is not established as a safety cue and so testing in the BLC yields anxiety-like

behavior that was not alleviated by the presence of the familiar partner rat. Acquisition of the anxiolytic-like behavior appears to be linked to the anxiogenic conditions under which the familiarity was formed. At this point, it is unclear if the presence of the anxiogenic stimulus during the social familiarity training session enhances the social memory of the partner rat or is required to activate an anxiolytic pathway specific to the type of anxiogenic cue.

Next the question was asked; if the opportunity for social familiarity was augmented through expanding the number of SI tests in dim red lighting, would the anxiety response to the BLC change? Under dim red lighting the SI behavior does not significantly change over time, making the formation of social familiarity impossible to definitively observe within these test sessions; however, the absence of a significant behavior change over many days without the anxiogenic stimulus does not signify a lack of social familiarity being established. Increasing the number of SI tests did not increase the chances of overcoming the BLC, as we see reductions in SI time with the familiar partner in the BLC (Figure 3.3B). This demonstrates that the social familiarity gained in the absence of an anxiogenic stimulus does not lead to a protection against it when it is presented as a novel stimulus, supporting the idea that the training conditions need to be done by pairing the anxiogenic stimulus with the familiar partner. One possibility to explain this pairing necessity is that the training sessions did produce social familiarity, but in the absence of the anxiogenic stimulus, the familiarity was not associated with the anxiogenic stimulus. This substantiates the idea that during the acquisition of SoFiA, the test rat is cueing off of the partner, which becomes a safety cue when paired with the anxiogenic stimulus. A future step could be to isolate the anxiogenic stimulus, accomplished through exposing the test rat to the BLC in the absence of a partner for several sessions, and test the anxiety-like behavior to the BLC in the SI test. Preliminary testing of this (not shown) indicate that the rat displays elevated levels of anxiety-like behavior following these sessions, pointing to the necessity for the pairing of the familiar partner and the anxiogenic stimulus to initiate the formation of the safety learning.

SoFiA acquisition affects the Elevated Plus Maze

I next asked; following the acquisition of SoFiA, would the anxiolysis response to the BLC stimulus carry over to any other tests of anxiety behavior? The Elevated Plus Maze (EPM) was chosen, as it is a commonly used test for anxiety-like behavior in rats. The EPM also allowed for the use of the BLC to be present during the test, allowing for us to test the effect the BLC, and

the effect of SoFiA acquisition, in an alternate test. Therefore, following the acquisition of SoFiA through the SI-hab testing under the BLC, the rats were challenged in the EPM under the BLC as well (Figure 3.4). The control group was given the SI-hab testing paradigm under dim red lighting, leading to a lack of SoFiA. This control group was then challenged on the EPM under the BLC. The group that had acquired SoFiA behaved with less anxiety-like behavior in the EPM compared to the non-SoFiA acquired group, having significantly more Open Arm entries (Figure 3.4B). This suggests that the rats were able to transfer the anxiolysis acquired to the BLC during the SI-hab testing to the EPM. Another parameter of the EPM did not produce significant differences in the groups based on SoFiA acquisition, as the Open Arm time was not different between groups (Figure 3.4C). The distance traveled was also not different between the groups, but this is less indicative of the anxiety behavior level (Figure 3.4D). The lack of an anxiolytic response to all the parameters of the EPM are a limitation to the versatility of the SI-hab training under the BLC. Also, the specificity of the anxiolysis to the BLC is unknown; the use of a different anxiogenic stimulus instead of a BLC during the EPM testing may have produced different results. Alternatively, it could be speculated that the partner needs to be present only during the acquisition of the SoFiA, but the anxiogenic stimulus that was present during the training is the only stimulus that the rat now has a reduced anxiety towards. This speculation was addressed in the next experiment in which we test an alternative anxiogenic stimulus following the acquisition of SoFiA.

The BLC, but not an acute restraint, is overcome through social familiarity

In the previous chapter, we saw that restraint was not an anxiogenic stimulus to give rats repeatedly, as they habituated to the stimulus in my hands. A different question regarding the restraint stimulus as an anxiogenic stimulus was instead asked; will an acute restraint stimulus still lead to increased anxiety-like behavior in the SI test with the Familiar partner following SoFiA acquisition? We found that regardless of partner type, SI times were significantly lower following exposure to the restraint (Figure 3.5). The reduction in anxiety-like behavior in the SoFiA group did not transfer to reductions in anxiety-like behavior in response to restraint. There could be several reasons for this; the restraint could be producing a non-selective type of anxiety-like behavior effect that is not overcome through social familiarity. This restraint stimulus may be too different from the BLC as an anxiogenic stimulus, and the association of the Familiar partner is not connected to this stimulus as it may be with the BLC.

The restraint was given alone, and the association of the familiar partner as a safety cue to the restraint may not be formed, as it presumably does under the BLC since the two stimuli are presented together. So, the anxiety that was produced by the restraint carried over into the subsequent SI test under the dim red light, and regardless of the familiarity of the partner, the rats all demonstrated elevated anxiety-like behavior. Thus, this further supports the idea that the acquisition of SoFiA is specific to the anxiogenic stimulus that is presented during testing.

The medial prefrontal cortex and SoFiA

The rodent and human medial prefrontal cortex (mPFC) are implicated in both social processing and cortical regulation of anxiety/fear, making it a compelling target as the cortical site for regulation of SoFiA (Adolphs, 2010; Fossati, 2012; Hartley & Phelps, 2010; Meyer-Lindenberg & Tost, 2012; Milad & Quirk, 2002; van Kerkhof, Damsteegt, et al., 2013a; van Kerkhof, Trezza, et al., 2013). Particularly, the Infralimbic (IL) cortex of the prefrontal cortex, in rodents, and the analogous human structure, the ventral medial prefrontal cortex (vmPFC), is an area of interest because this area of the brain is associated with inhibition of fear, safety learning and social regulation (Quirk & Beer, 2006; Quirk, Russo, Barron, & Lebron, 2000; Uylings, Groenewegen, & Kolb, 2003). The IL/mvPFC is involved in the discrimination between stimuli that signal fear and safety, as well as the consolidation and recall of extinction of conditioned fear (Sangha, Robinson, Greba, Davies, & Howland, 2014). Extinction from conditioned fear is a form of safety learning that is depended on an active IL, particularly the consolidation and recall of extinction, as the recall of extinction learning is prevented by lesioning the IL, and stimulation of the IL exacerbates extinction recall (Sangha et al., 2014). Using muscimol as an inhibitor, Sierra-Mercado and colleagues found that the IL sub-region of the mPFC impairs fear extinction memory and extinction acquisition (Sierra-Mercado et al., 2011). Therefore, I targeted the IL with the inhibitory compound muscimol during SoFiA acquisition to test the safety signal memory formation of the test rat for the partner rat, and during SoFiA expression to determine if the expression of this safety signal memory relay could be disrupted.

Temporary inhibition of the IL after SoFiA acquisition, completely blocked the anxiolytic effect induced by social familiarity, reducing SI times to the level of the first exposure. Interestingly, inhibition of the IL had no effect on SI time during SI sessions with novel partners (in either BLC or control conditions). Collectively, these data are interpreted as the IL apparently

needs to be functioning in order for the SoFiA memory to be accessed. This effect was transient and the social memory was not lost, because testing the following day with the familiar partner rat in the absence of the IL inhibition rescued SI times to the level displayed during SoFiA expression. These data suggest the value of familiar rats as a safety signal is dependent on the mPFC and that SoFiA is a form of socially enhanced safety learning.

While inhibiting the mPFC appears to selectively suppress the anxiolysis induced by social familiarity, an alternate explanation may be that inhibition of the mPFC disrupts more basic form of social cognition, such as the ability to recall the partner rat as familiar. We see that the acquisition does not happen when the IL is inhibited prior to SI testing in the first three days of the SI-hab training. These first three days appear to be critical to the acquisition of SoFiA, as this is the period when the SI times often transition from being in the higher anxiety levels to the lower anxiety levels relative to Baseline. Suppressing the IL during the acquisition of SoFiA could be preventing the formation of the partner rat as familiar, more so than disrupting the association of the partner as a safety cue to the BLC, which we see remains anxiogenic throughout the SI-hab testing. This could be tested by determining if inhibition of the IL disrupts social memory using the Social Recognition test. An additional follow-up to this experiment would be to extend the days of the SI-hab testing in which the animals received vehicle injections. If the safety learning is delayed by the inhibition of the muscimol in the first three days of testing, an eventual SoFiA acquisition may occur after several more days of pairing the familiar partner in the SI-hab under the BLC.

Not only is a functional IL necessary for SoFiA expression, a functional IL, during social training sessions, also appears to be necessary for SoFiA acquisition. Temporary inhibition of the mPFC, by local injections of muscimol into the Infralimbic area (IL) of the mPFC during the initial 3 days of Si-hab, blocked the subsequent SoFiA acquisition through Day 5. It is important to note, that in both of these studies, all of the injections were localized within the IL, however it is possible that the effects of the muscimol injections were a result of diffusion beyond the IL and thus interpretation of these results are limited to the mPFC. Without anatomical controls into adjacent PFC regions, it cannot be claimed that these findings are specific to the IL but rather the mPFC more generally.

In terms of social cognition, the mPFC is sited as a locus for integration of social stimuli and emotional responses (Adolphs, 2009; Amodio & Frith, 2006). Thickness of the (v)mPFC is associated with social functioning and ability to correctly interpret emotion from social cues

(Holmes et al., 2012). In a recent animal study the importance of the mPFC in developmentally relevant social behavior, social play, was demonstrated through inactivation of the mPFC (van Kerkhof, Damsteegt, et al., 2013a). The authors also reported that inactivation of the mPFC (in a non-threatening environment) increased social investigation, which is a similar measure to the increase in SI time reported under similar control conditions in the current study. We did not find such general increases in SI time in our mature rats given muscimol injections and tested with novel partners in the dim red light in the SI test or during the BLC. Thus, the mPFC may be involved in the expression of anxiolytic social learning.

Evidence that points to the role of the mPFC as a regulator of anxiety-like behavior but in contrasting roles to the current study, was demonstrated in a study in which inducing activity in the mPFC through the $GABA_A$ antagonist bicuculline methiodide infusions into the IL of mice led to increased anxiety-like behavior in the OF and EPM tests, while inactivation of the IL led to anxiolytic effects (Bi et al., 2013). This was specific to the IL, as the effects were not seen when the PL was targeted (Bi et al., 2013). This study is not in line with previous findings regarding the IL and conditioned fear. With this study inactivation of the IL was anxiolytic, while inactivation of the IL in the current study with Muscimol, led to blocking of SoFiA expression and acquisition, but not increased anxiety itself in the SI test. The differences in observations could be explained by learned and innate fear having different regulatory mechanisms.

The role of the cognitive enhancer DCS in the acquisition of SoFiA

The current data support the idea that in the process of SoFiA acquisition, the familiar partner rat becomes a safety signal. This is based on the observations that acquisition of SoFiA appears to require repeated pairings of the socially familiar partner rat with the anxiogenic stimulus, the presence of the familiar conspecific is necessary for the expression of SoFiA, and SoFiA is dependent on an active mPFC which is a pivotal site for safety learning. Pairing the safety learning with the drug D-cycloserine (DCS) can enhance safety learning in humans and rodents (Davis et al., 2006; Gupta et al., 2013b; Hofmann, Pollack, et al., 2006). DCS augmentation of cognitive behavioral therapy for social anxiety in particular, was associated with a faster rate of improvement (Hofmann et al., 2013b). Again, the operational definition of SoFiA that we are aiming to affect is a significant increase in SI time from the first exposure to the partner and BLC following repeated exposures. The hypothesis here was that DCS would decrease the number of exposure sessions to the familiar partner in the presence of the anxiogenic stimulus that were required to acquire SoFiA.

Pretreatment with DCS was carried out as systemic injections prior to the SI pairings with the familiar conspecific. To initially get the rats habituated to being injected and to reduce the possibility of enhancing the initial high-anxiety state on the first day of exposure, the first day involved vehicle injections, with DCS injections beginning on Day 2. Waiting until Day 2 was intended to prevent enhancing the memory of the first encounter with the partner rat, which is initially potentially anxiogenic. The second encounter has a greater possibility of the partner rat being familiar, positive, and potentially starting to become a safety cue, and so that is the encounter we aimed to enhance.

In the current study, pretreatment with DCS prior to pairings with the familiar conspecific reduced the number of SI training sessions required to reduce the anxiety-like response to the anxiogenic challenge (Figure 3.8). A potential caveat of this observation is that DCS treatment increased pro-social behavior in other rodent models, which could confound the current interpretations of the SI behavior (Myers & Carlezon, 2012). To determine the extent to which pro-social effects of DCS were contributing to the enhanced SoFiA acquisition, rats were pretreated with DCS and tested with a novel partner after SoFiA was established (Day 6). Here, in the presence of a novel partner, SI times were significantly reduced compared to the last SoFiA session and no longer significantly higher than Day 1, regardless of receiving DCS or vehicle injection. From these data, it can be interpreted that the enhanced acquisition of SoFiA observed with DCS was at least in part due to enhanced social safety learning rather than enhanced pro-social behavior. Alternatively, the DCS treated rats displayed a strong trend towards increased SI times during the novel partner challenge, which reached significance when not controlling for multiple comparisons, implying the possibility of a slight pro-social or possibly a hedonistic effect induced by DCS, which appears to be additive with the SoFiA response. Further studies are needed to fully resolve the mechanism by which DCS enhances the acquisition of SoFiA.

SoFiA differs from Social Buffering

Contrary to the concept that in SoFiA the familiar partner becomes a safety signal is the idea of a social buffering response. Social buffering studies have demonstrated that the presence of a conspecific can reduce fear and stress responses in conditioned fear paradigms without any training (Davitz & Mason, 1955; Kiyokawa et al., 2009; Kiyokawa, Wakabayashi, Takeuchi, & Mori, 2012; Latane, 1969; Terranova et al., 1999), suggesting that a conspecific may

serve as an external inhibitor of fear or anxiety rather than a safety signal (Christianson et al., 2012). Social buffering effects were not directly investigated in the current study of SoFiA. However, SoFiA differs from social buffering in several key areas. First, SoFiA overrides an unconditioned anxiogenic stimulus while most social buffering experiments use a conditioned fear as the stimulus. Next, SoFiA was acquired only following "training", requiring between 4 – 5 pairings of the familiar conspecific with the anxiogenic stimuli, suggesting that the social familiarity is acting more like a safety signal than an external inhibitor of the behavior. Finally, expression of SoFiA appears to require an active mPFC while social buffering effects in response to the presence of a conspecific at the time of testing appear to be independent of mPFC activation, meaning the two effects are separate and likely involve different mechanisms and possibly different neural circuitry (Kiyokawa et al., 2007; Kiyokawa et al., 2009).

Partner effect on the anxiogenic state of the test rat

A consideration in regard to the anxiolytic outcomes is whether the anxiety state of the partner rat affects the state of the test rat. Looking at previous work with the UCN priming we know that the partners were not made anxious with any internal or external stimuli (Truitt et al., 2007). Scoring these partners did not result in any overt reductions in the SI times, unlike the UCN primed rats, demonstrating that within this testing paradigm, the anxiety level of the test rats did not affect the anxiety level of the partners (personal communication, S. Fitz). Alternatively, some studies have shown that an elevated stress state of the partner can increase the stress state of the conspecific exposed to it, possibly through olfactory cues (Davitz & Mason, 1955; Kiyokawa, Kikusui, Takeuchi, & Mori, 2004). The exchange of the emotional state between partners was also seen in a study looking at the exploration of a partner rat towards a rat that was either shocked or not shocked, the rat that was shocked elicited more exploration behavior of the partner rat, demonstrating a transfer of the emotional state of the rat to elicit behavioral alterations in the partner (Knapska et al., 2006). With the BLC, both of the animals in the dyad receive exposure to the BLC stimulus at the same time. This means that both of the rats are experiencing elevated levels of the anxiety-like behavior. When partner rats are scored for SI time during the first test sessions of the SI-hab in the BLC, the times are similar to the test rats, as the rats tested were usually untreated. This supports the idea that even when the rat is experiencing elevated levels of anxiety–like behavior, the state of the rat did not prevent the acquisition of SoFiA in these rats.

Conclusions

This chapter presents the SI-hab testing paradigm as a valid preclinical animal model of social familiarity-induced anxiolysis, or SoFiA. This model was developed through the concept of reverse translation of human exposure therapy. Here we took a complex human process and simplified it to a basic level in order to study and better understand the underlying processes that drive the changes in behavior that we observe. Once we understand these underlying processes, we can then utilize the model to develop novel treatments and therapies, and finally apply it back translationally to patients with anxiety disorders.

This model demonstrates that reductions in anxiety-like responses are selective to social familiarity. SoFiA appears to be a learned response, where the context in which social familiarity is established determines the extent to which familiarity will induce anxiolysis. This is based on the observations that acquisition of SoFiA appears to require repeated pairings (4-5) of the socially familiar partner rat with the concurrent presence of the anxiogenic stimulus. This reduction in anxiety behavior in the presence of a socially familiar conspecific represents face validity. This type of reduction in anxiety-like behavior is similar to exposure therapies that are a common form of cognitive behavioral therapy.

The rodent model of SoFiA is also characterized by having some specifications that were observed in this chapter. The acquisition of SoFiA requires the concurrent exposure of the socially familiar partner with the anxiogenic stimulus, and training in the absence of the anxiogenic stimulus prevents the possible association of the social partner being a safety cue in the presence of the anxiogenic stimulus. The acquisition of SoFiA while concurrently under the BLC however was shown to reduce anxiety-like behavior to the BLC in the EPM, but did not protect against anxiety-like behavior induced by exposure to Restraint exposure.

Additionally, the mPFC is critical to expression of SoFiA; similar to findings in other safety-learning paradigms, and demonstrating construct validity. While the neural circuitry is yet to be fully elucidated, we have determined that SoFiA is dependent on an active mPFC for the acquisition and expression, and that is a pivotal site for safety learning. Lastly, the cognitive enhancer D-cycloserine enhanced the acquisition of SoFiA, demonstrating postdictive validity. Additional forms of validity exist and in the next chapter I will explore a pathology acquired by humans, and modeled in rodents to demonstrate a pathogenic validity of the SI-hab paradigm.

Chapter 4: Utilizing the SoFiA model to detect psychosocial learning deficits in a pathological animal model

Introduction

Goal of this chapter

Traumatic brain injury (TBI) is a growing issue, and the consequences of it are still being realized. Research has shown TBI and mental health issues are strongly linked, and tools to study these links are of critical importance to furthering our understanding of the challenges people face following a TBI. The preclinical model of SoFiA is one such tool that can be used to systematically investigate the mechanisms contributing to the core issues, such as psychosocial deficits, that emerge following TBI. My aim is to use the rodent model of SoFiA to detect the psychosocial deficits that can emerge following a pathological model of rodents exposed to a blast-induced mild traumatic brain injury (bmTBI). Deficits in social functioning can be detected through alterations in the acquisition or expression of SoFiA within blast-exposed rats following the SI-hab training paradigm. We hypothesized that any social deficits that develop as a consequence of exposure to the bmTBI would be detectible through a lack of the acquisition or expression of SoFiA. Such findings would demonstrate additional validity of the SoFiA model and represent a first step into elucidating mTBI-induced psychosocial deficits.

Mild Traumatic Brain Injury

Mild traumatic brain injury (mTBI) is defined as the result of a sudden non-penetrating impact of the head that leads to acceleration, deceleration or rotation of the brain (Bigler, 2008). Mild TBI is the most common type of TBI, and is characterized by an initial set of symptoms that arise immediately following this kind of trauma (Bazarian, McClung, Cheng, Flesher, & Schneider, 2005). These symptoms include initial confusion or disorientation, possible loss of consciousness that lasts less than 30 minutes and post-traumatic amnesia that lasts less than 24 hours (Bazarian et al., 2005; Bigler, 2008). Most mild TBI patients make a full, uncomplicated recovery within a few months (Heather G Belanger, Curtiss, Demery, Lebowitz, & Vanderploeg, 2005; Carroll et al., 2014; Mooney & Speed, 2001; Schretlen & Shapiro, 2003). However, numerous unfavorable neuropsychiatric sequelae are associated with TBI, including psychosocial impairments such as social isolation, interpersonal problems, and unemployment (Jacoba M Spikman, Marieke E Timmerman, Maarten V Milders, Wencke S Veenstra, & Joukje

van der Naalt, 2012). These impairments are cited as the most detrimental factors impacting quality of life after TBI (Kristy Draper, Ponsford, & Schönberger, 2007a; Jacoba M Spikman et al., 2012). Mild TBIs often go unreported or disregarded as minor in the acute injury phase, but TBI-associated patterns of dysfunction can emerge days to years later. For a minority of patients (generally agreed 15-20%), mild TBI results in persistent neuropsychiatric sequelae that can significantly reduce quality of life (Stein & McAllister, 2009). The association of seemingly minor, subclinical TBIs with delayed emergence of life-long psychiatric consequences underscores a pressing need for greater understanding of the neural mechanisms contributing to the development of mental illness after TBI.

Psychosocial deficits and TBI

Emergence of psychosocial deficits after TBI has proven difficult to predict, as injuries often lack clear or consistent clinical presentations in the acute post-injury period (Konrad et al., 2011; Millis et al., 2001). A study looking at the occurrence and prevalence of psychological disorders that emerge following a mTBI event found that the percent of psychiatric disorders following an injury more than doubled and was nearly three-fold, compared to the prior rate before injury (Mooney & Speed, 2001). Anxiety, depression and PTSD were among the most common newly developed disorders (Mooney & Speed, 2001). They also found that among the patients who had prolonged recovery and worse outcomes than those who recovered, 60% had a comorbid psychiatric condition, which did not correlate with pre-existing psychiatric conditions (Mooney & Speed, 2001).

The most devastating neuropathological outcome of traumatic brain injury is cited as changes to personality or psychosocial impairments, which are at the core of almost every psychiatric disease and a main predictor of unfavorable reintegration into society and a lower overall quality of life (Bombardier et al., 2010; Kristy Draper, Ponsford, & Schönberger, 2007b; J. M. Spikman et al., 2012). Social support is a vital aspect to patients who have suffered a mTBI, as social isolation can be common amongst this cohort (Bryan, Clemans, Hernandez, & Rudd, 2013; Wright, Kelsall, Sim, Clarke, & Creamer, 2013). However, the ability to utilize social support may also be impaired, creating a Catch-22 for these patients, introducing new challenges for treatment and rehabilitation. Additionally, these psychological outcomes may be present at subclinical levels, delaying vital treatment that could potentially prevent the onset of

psychological pathologies (H. G. Belanger, Kretzmer, Yoash-Gantz, Pickett, & Tupler, 2009; Bryan et al., 2013).

Blast-induced mild Traumatic Brain Injury

Blast-induced mild Traumatic Brain Injury (bmTBI) is a specific type of mTBI injury of special interest because it is regarded as the signature injury of modern warfare (DePalma, Burris, Champion, & Hodgson, 2005; Terrio et al., 2009). Blast-related traumatic brain injuries caused by improvised explosive devices have become a common mode of TBI injury in warzones (Rosenfeld et al., 2013). The increased prevalence of this type of injury and the number of survivors living with the aftermath is contributing to the rising incidence of soldiers and veterans with the neuropathological sequelae of TBI, including anxiety, depression, PTSD, psychosocial deficits and suicidal ideation (Bombardier et al., 2010; K. Draper, Ponsford, & Schonberger, 2007; Rosenfeld et al., 2013; Tsaousides, Cantor, & Gordon, 2011; Wright et al., 2013). Veterans with mTBI experience a lower quality of life than their demographically matched non-injured counterparts (Schiehser et al., 2014). Among Operation Iraqi Freedom and Operation Enduring Freedom veterans surveyed, the presence of mTBI was associated with a prevalence ratio of 3.85 to also having PTSD (Schneiderman, Braver, & Kang, 2008). Additionally, within this survey, of those who sustained mTBI, 35% self-attributed three or more psychiatric symptoms to a possible head injury (Schneiderman et al., 2008). Mechanisms by which TBI leads to psychosocial deficits remain elusive, in part because of challenges with systematic investigation and a lack of a preclinical model capable of reliably assessing psychosocial deficits (Rosenfeld et al., 2013).

Blast-induced mild Traumatic Brain Injury modeling in rodents

The model of SoFiA is an excellent putative tool to identify the presence of deficits in the psychosocial learning ability of rodents following an exposure to a bmTBI. This provides us a way of looking at the resulting pathology in rodents following the same trigger factor as humans, the blast exposure (Belzung & Lemoine, 2011). Observing the resulting phenotypic outcome, I asked whether human social deficits that can occur following bmTBI will be reflected in the model of SoFiA in rats that have also been subjected to a bmTBI. Additionally, there are many benefits to specifically investigating the consequences of *mild* bTBIs, mainly because it represents the largest portion of bTBI survivors. Also, mTBI does not lead to gross motor deficits, which would confound the interpretability of the SI-hab testing. Furthermore, deficits seen in this behavioral model could have implications for future research into how social behavior is utilized and

regulated within the global mTBI patient population, as well as provide crucial insights into the neural mechanisms of SoFiA.

Within this chapter, I identify the extent to which an exposure to a blast-induced mild traumatic brain injury will lead to a pathology, specifically deficits in acquisition of social familiarity-induced anxiolysis. This is modeled in rats using techniques developed by the Shi lab at Purdue University. This model consists of rats receiving a closed-head exposure to an overpressure shockwave "blast" through an open-ended shock tube. The model system is of the primary injury only, which refers to the shockwave produced by the compressed gas. No penetrating injuries (secondary injury), no acceleration/blunt impact conventional TBI (tertiary injury), and no chemical exposure, extreme heat or radiation (quaternary injury) was present in this model system.

Previously, in the Shi lab, they have demonstrated that the oxidative stress resulting from the blast shockwave exposure is indicated by measurements of urine levels of 3-hydroxypropylmercapturic acid (3-HPMA) following Blast or Sham exposure (Shi, Rickett, & Sun, 2011). 3-HPMA is the stable metabolite of acrolein, an established neurotoxin that is both product and initiator of oxidative stress (Yan, Byrd, Brown, & Borgerding, 2010; Zheng et al., 2013). Acrolein is known to increase after CNS trauma, including blast (Shi et al., 2011; Uchida et al., 1998). Therefore, urine 3-HPMA levels are assessed to determine the presence of injury following the blast exposure in the rodent model.

Methods

Animals

Adult male Sprague Dawley rats (Harlan Laboratories, Indianapolis, IN) between 350-450g were used in all experiments. Rats were individually housed upon arrival to the Purdue facility and housed in a temperature and humidity-controlled room (21-22C, 40-45% humidity) with a light/dark cycle of 12 hours (lights on at 0600 hours). Rats had free access to food and water. Rats were each handled daily for a minimum of 3 days prior to behavioral testing. All animal procedures were conducted under approved animal use protocols overseen by the Purdue Animal Care and Use Committee (Protocol #1111000280).

Blast Exposure

For Blast exposure, animals were fully anesthetized with a ketamine/xylazine cocktail (80mg/kg and 10mg/kg, respectively) and secured in open-ended shock tube blast apparatus with body protection and head fixation using ear bars. The blast shockwave was generated by using compressed gas to burst a Mylar membrane resulting in a blast overpressure magnitude of 150kPa (side-on) with 1.5msec overpressure duration. The blast overpressure shockwave was directed to impact the rat's head in a top-down manner. This procedure has been demonstrated to result in a primary blast exposure that is considered mild (no acute observable neuromotor or neurocognitive deficits), without generating secondary (penetrating injury) or quaternary (heat, radiation, chemical exposures) blast-related effects (Walls et al., 2016). The body shielding and fixation of the head reduce confounding systemic injuries and eliminate tertiary (impact-acceleration) bTBI effects (Walls et al., 2016). Throughout the procedure, sham rats received identical treatment including anesthesia, head fixation, and exposure to loud blast sound by bringing them into the room with the blast apparatus, but not the injurious shock wave exposure.

Urine Collection and Analysis

The changes in Acrolein, a known post-trauma neurotoxin, were assessed in excreted urine via its stable glutathione-reduced metabolite, 3-HPMA (Carmella et al., 2007; Eckert, Drexler, & Goen, 2010; Parent et al., 1998; Schettgen, Musiol, & Kraus, 2008; Yan et al., 2010; Zheng et al., 2013). Noninvasive urine collection was conducted in blast and sham exposed rats on each of the 2 days prior to blast/sham exposure and daily for 4 days starting at 1-day post-

injury using standard metabolic collection cages. Rats spent 4 hours per collection session in a free-roaming wire cage with ready access to unlimited water supply. Urine 3-HPMA was collected and quantified as demonstrated in our prior publications (Walls et al., 2016; Zheng et al., 2013). Briefly, a solid phase extraction was used to prepare urine for elution and ensuing liquid chromatography with tandem mass spectrometry (LC/MS/MS) analysis. Assuming healthy kidney function, 3-HPMA levels were normalized to urine creatinine concentrations, which are relatively stable and commonly used to normalize and calibrate measurements across with variable water content due to differential hydration status of subjects (Carmella et al., 2007; Eckert et al., 2010; Parent et al., 1998; Schettgen et al., 2008; Yan et al., 2010; Zheng et al., 2013).

Behavioral experiments

Social Interaction testing

The Social Interaction (SI) testing was done similarly to the protocol as described previously Chapter 2. The post-exposure baseline SI test occurred at 9 days after blast or sham exposure and was performed under the low anxiogenic-like conditions of the dim red lighting. Scoring was done by an observer blinded to the treatment (Blast vs. Sham). Partner rats were used a maximum of 2 times per day and were paired with one Blast and one Sham rat each day, tests being separated by at least 30 min. Testing was done between 1100-1500 each day, during the rat's light cycle.

Social interaction-habituation Training

Twenty-four (cohort 1) or forty-eight (cohort 2) hours after baseline SI testing, SoFiA acquisition was measured through the SI-habituation testing paradigm as described previously in the previous Chapter 3.

Open Field Test

Seven days following either blast or sham exposure, the Open Field (OF) test was given to evaluate gross motor and anxiety-like behavior of the rat. OF testing was performed similarly as described in Chapter 2. The test was performed under dim red lighting conditions. The OF test served as the habituation to the Social Interaction testing arena.

Rotorod

The Rotorod test for locomotor coordination and maximal motor activity was conducted following the OF test on day 7 after sham or blast exposure in cohort 1 (Whishaw, Li, Whishaw, Gorny, & Metz, 2008). This test consists of placing a rat on a rod that rotates at steadily increasing speed from 3-30RPM over 5 minutes. The longer the rat stays on the rod, the faster it will rotate. After successful acclimation: 3 consecutive trials of 60 seconds or more, the test was performed three times per rat. Session end criteria were the rat falling off the rod or being spun in one complete revolution without locomotion by holding on tightly. If the rat initially jumped off the rod prior to test start or within the first 15 seconds, the trial was repeated.

Social Recognition test in Blast rats

The Social Recognition test was done in the same SI testing arena as the all the SI tests, with the exception of 2 sets of plastic bar inserts placed into two opposite corners. The inserts are placed to accommodate the full length of the partner rat's body from the corner to the bars. The bars are wide enough apart to allow physical contact between the test rat and partner rat through touching noses and front paws. Prior to the placement of the test rat into the center of the testing arena for testing, the partner rats were placed behind to plastic bars in the corners. One corner contained the familiar partner rat from the previous SI-hab testing days, and the opposite corner contained a novel partner rat. The familiar and novel corners were randomized to prevent any place preferences for either corner in the rats. To begin the test, the test rat was placed into the center of the testing arena and allowed to move freely between the two partner corners for 5 minutes. The lighting for the test was done under the BLC. For scoring, the testing arena was split diagonally across the center to designate the half of the arena nearest the Familiar partner corner, and the half of the arena nearest the Novel partner corner. Behavior scoring was automated by ANY-maze video tracking software (Stoelting Co. Wood Dale, IL, version 4.8) to calculate the amount of time the test rat spent in each partner zone.

Social Recognition testing in uninjured rats

For a set of uninjured rats, the Social Recognition testing was done following the SI-hab testing. The SI-hab testing paradigm was done in the typical procedure with baseline SI testing in dim red lighting followed by the SI-hab testing paradigm in either BLC (n=11) or dim red light (n=11) with a familiar partner each day in the SI test. The SI-hab testing with the Familiar partner

was repeated for 6 consecutive days. 24 hours after the completion of the SI-hab testing paradigm on Day 7, the Social Recognition test was executed. For the Social Recognition test, the two SI-hab groups were further split into two more groups, with each of those groups getting the Social Recognition test in either dim red lighting or under the BLC. This created 4 groups across the two SI-hab treatments (Control/SoFiA) and two Social Recognition testing conditions (Dim/BLC). The four groups were Control/Dim (n=5), Control/BLC (n=6), SoFiA/Dim (n=5), SoFiA/BLC (n=6).

Tail suspension

Twenty-four hours after the last day of Social Familiarity training (on day 17), Blast cohort 2 rats were tested for depression-like measures through the inescapable stress test, the Tail Suspension test (TS)(Chermat, Thierry, Mico, Steru, & Simon, 1986). The TS consists of wrapping the tail of the rat completely in cloth medical tape, followed by a layer of duct tape that never touches any part of the rat. The duct tape was extended from the end of the rat's tail and wrapped around a horizontal metal bar 4 feet above the ground. The rat was suspended from the metal bar for the duration of the 5-minute test, and video recorded from the side. The videos were scored by ANY-maze automated software that measured the amount of time the rat spent immobile as the measure for this test.

Novel Object Recognition

For the second cohort of rats, 7 days after exposure to either blast or sham, the rats were given the Novel Object Recognition (NOR) test. This test consists of placing a rat alone in an open field for 5 minutes under dim red lighting. In each of two opposite corners, are two objects secured to the floor with tape. In this case the two objects were either both a small car of the same kind or a small grouping of blocks about the same size as the car. After the 5-minute test in which the rat is free to explore each of the objects, the rat is taken out and placed inside his home cage for a 10-minute inter-test-interval, while the box and objects are wiped down. Following the 10-minute rest, the rat was replaced into the open field for another 5-minute test in dim red lighting. In this second test, one of the two objects was replaced with the kind of object that the rat was not exposed to previously. The type of object used in the first exposure was pseudo randomly assigned as well as which corner the new object was assigned to in the second exposure. This pseudo random assignment was to ensure that there was not an effect of the corner or object that would produce false preferences in the rats that were not true to the

rats recognizing the objects themselves. The behavior was videotaped from above and the amount of time the rat spent interacting with the two objects in the second test phase was measured with the ANY-maze automated software.

Immunohistochemistry

Tissue Preparation

Seventeen days after Blast or Sham exposure and 24 hours following the cessation of behavioral testing, rats were deeply anesthetized with a cocktail of Ketamine and Xylazine at 80 mg/(kg+50g) and 10 mg/(kg+50g) respectively. The rats were then perfused via a peristaltic pump with 300ml of saline followed by 100ml of 4% paraformaldehyde in phosphate buffered saline (PBS) through the ascending aorta. Following perfusion, the rats were decapitated and the brain removed and post-fixed in 4% paraformaldehyde in PBS for 1 hour followed by 30% sucrose in phosphate buffer (PB) and stored in 4°C until further use. Brains were frozen and mounted onto a specimen block with tissue mounting medium and sliced at 30µm thickness with a sliding microtome (Microm). Tissue slices were collected in serial collection, with every 6^{th} slice going into one of 6 wells. The sliced tissue was saved in cryoprotectant for storage.

NeuN Staining

Forebrain slices were separated out of a single well and washed in PBS 3 times for at least 5 minutes for each wash. The tissue was then blocked in 1% H_2O_2 for 10 minutes, followed by washing in PBS and blocked in PBS+ (PBS, 0.1% Bovine serum albumin, 0.4% Triton X-100) for a minimum of 1 hour and subsequently washed again in PBS. The tissue was then incubated overnight at room temperature in primary monoclonal antibody mouse anti NeuN (Millipore, Chemicon MAB377, lot #0604027006) at a concentration of 1: 10,000 in PBS+. Following incubation in primary antibody, the tissue was washed in PBS and incubated in biotinylated secondary antibody, goat anti mouse (Vector BA 9200, lot# X0623) at 1:500 for 1 hour at room temperature. Following that, the tissue was washed in PBS and incubated in the avidin-biotin complex (ABC Elite) at 1:1000 for 1 hour at room temperature. Next the tissue was washed in PBS and stained with the chromagen 3,3'-Diaminobenzidine (DAB) for 10 minutes. The tissue was then washed in phosphate buffer and mounted onto charged slides and cover slipped.

NeuN Tissue Imaging and Cell Counting

Cell counting of NeuN stained cells was performed under brightfield microscopy on an Axio Imager M2 microscope (Zeiss) using Stereo Investigator software version 11 (MBF Bioscience). This was to obtain an unbiased estimate of the population of neurons within the Infralimbic (IL) area of the Prefrontal Cortex. The area of the IL was determined using Paxinos and Watson's atlas of the rat brain and counts were performed on slices that fell between Bregma 3.18-3.7. IL location was identified under 2.5x magnification and contours of the IL were drawn using the software contours. Using the Optical Fractionator Probe within the software, grid size was made to 200 X 200µm with the counting frame of 50 X 50µm. The grid was randomly generated and placed over the contoured area by the software and counts where performed by counting cells that had NeuN staining. The estimated cell population was provided by the software based on contour volume and cell counts within the counting frames.

Statistics

All data were analyzed as described in the text with significance set at $p \leq 0.05$. The statistical software used was Prism 6.0 Software (La Jolla, CA).

Treatment Timelines for two cohorts in the Blast experiments

Presented in Figure 4.A is a schematic of the procedural time line used for rats in Cohort 1 (n=6 sham and n=7 blast) and Cohort 2 (n=9 sham and n=9 blast). The data presented from each cohort is specified prior to each result. The days relative to the blast or sham exposure are listed across the top, and the procedures performed on the days are listed. Abbreviations: OF = Open Field test; RR = RotoRod test; NOR = Novel Object Recognition; SI = Social Interaction test; SI-hab = Social Interaction-habituation procedure; Soc. Rec. = Social Recognition test; TS = Tail suspension test.

Results

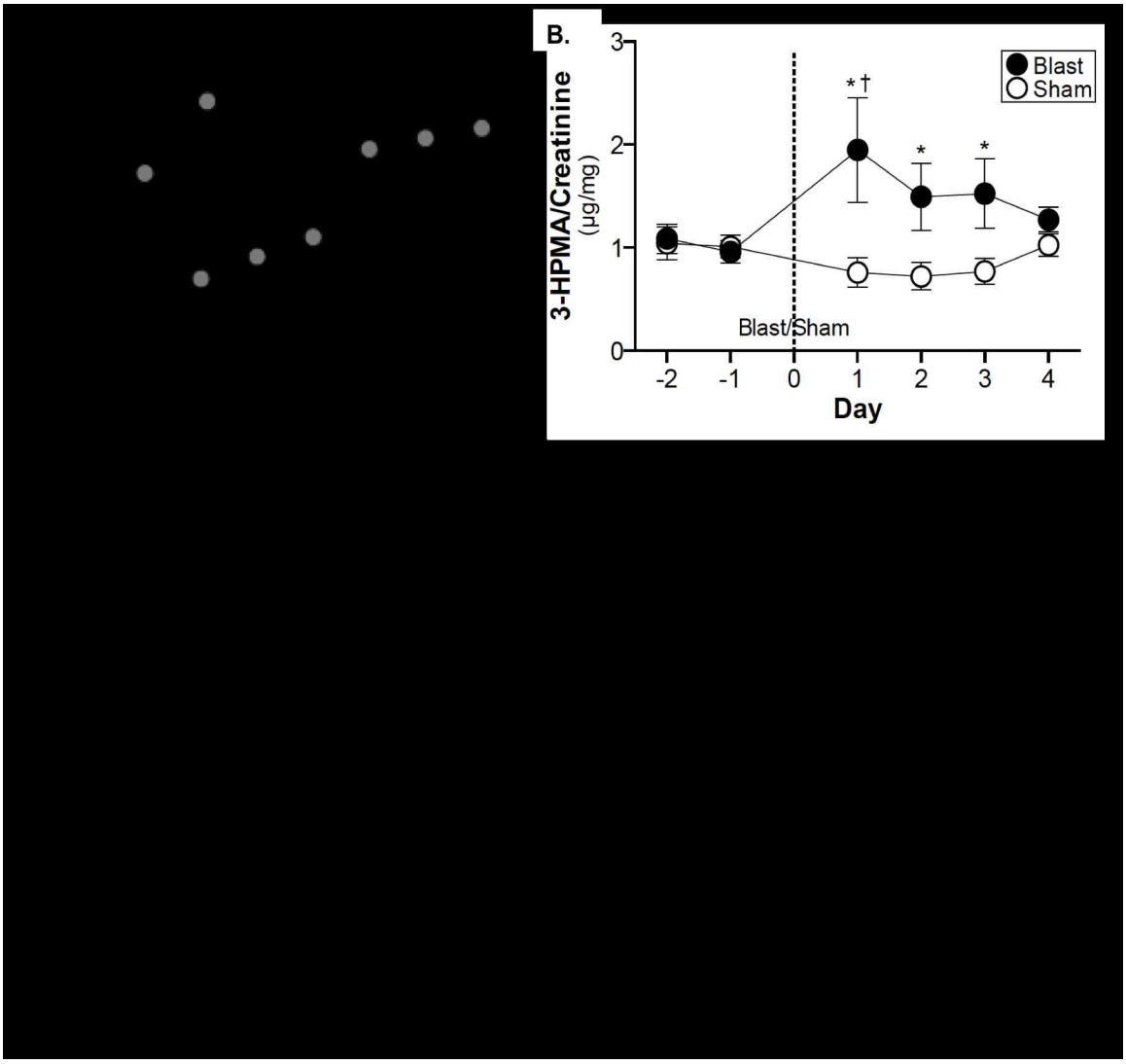
Blast exposure led to a deficit in SoFiA acquisition that correlated with an indicator of neural trauma

Rats were tested in the SI-hab training paradigm starting at 9 days post blast (n=7) or sham (n=6) exposure. Rats were given a Baseline SI test under dim red lighting conditions on Day 9. On day 10, the standard SI-hab training protocol began, in which rats underwent 6 consecutive days of SI testing with the same partner rat under BLC conditions (Figure 4.1A). Both exposure groups responded to the BLC initially with an increase in anxiety-like behavior, however, the two exposure groups responded differently to the SI-hab training. There was a main effect of blast exposure (RM 2-Way ANOVA, exposure main effect $F_{1,11}$=7.49, p=0.019), a main effect of day ($F_{6,66}$=11.01, p < 0.0001) and an exposure by day interaction ($F_{6,66}$=5.28, p=0.0002). Baseline SI times did not differ between treatment groups (Bonferroni's difference between groups, p>0.05). Both treatment groups also had an equivalent response to BLC where both groups had significantly reduced SI time on Day 1, the first exposure to the BLC and a novel partner, compared to their own baseline SI time (Tukey's p ≤ 0.028). Thus, Blast exposure did not alter the basal anxiety levels of the rats, or their ability to perceive the BLC as anxiogenic.

The divergence between the two exposure groups occurred when the SI-hab training was repeated across multiple days, the sham exposed rats acquired the SoFiA effect by Day 4, as indicated by a significant increase in SI time compared to Day 1, and this increased SI time remained significantly elevated through SI-hab Day 6 (difference from Day 1, Dunnett's p ≤ 0.0051). However, the blast-exposed rats' SI times remained significantly lower than their baseline across all SI-hab days (Tukey's p ≤ 0.0059) and compared to Sham rats on Days 4-6 (Bonferroni's p ≤ 0.0355). The lack of SoFiA acquisition in light of these normal baseline and BLC responses demonstrates a selective deficit in psychosocial safety learning.

Urine levels of the acrolein metabolite, 3-HPMA/Creatinine (µg/mg), have been used to determine neurotrauma (Shi et al., 2011), thus these metabolites were measured 2 days prior to and 4 days following the exposure to the blast (Figure 4.1B). Blast (n=7) exposure, compared to Sham (n=6), produced an increase in 3-HPMA/Creatinine (µg/mg) urine levels across days as evidenced by a main effect of blast exposure (RM two-way ANOVA, exposure main effect $F_{5,55}$=15.54, p=0.0023) and an exposure by day interaction ($F_{5,55}$=2.65, p=0.0322). The Blast rats had a significant increase in the urine 3-HPMA/Creatinine ratio levels on post Blast exposure Day

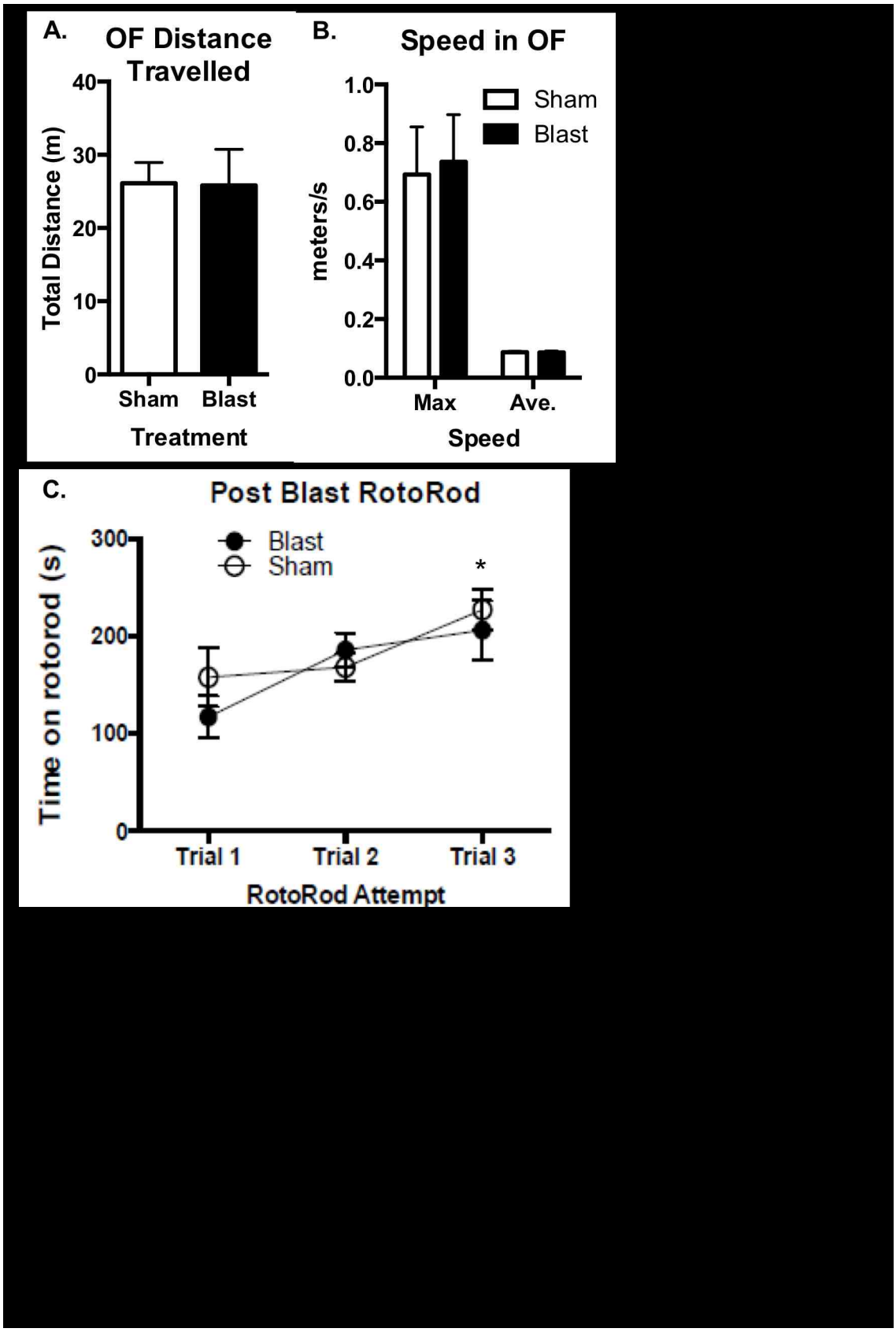
1 compared to the pre-Blast Day -1 (Dunnett's p=0.0094). Between the exposure groups, the 3-HPMA/Creatinine levels are significantly higher than the Sham rats on post Blast Days 1-3 (Fisher's LSD t ≥ 2.547 p ≤ 0.0132). Suggesting the presence of neural trauma in the Blast exposed rats.



Blast exposure did not affect motor ability

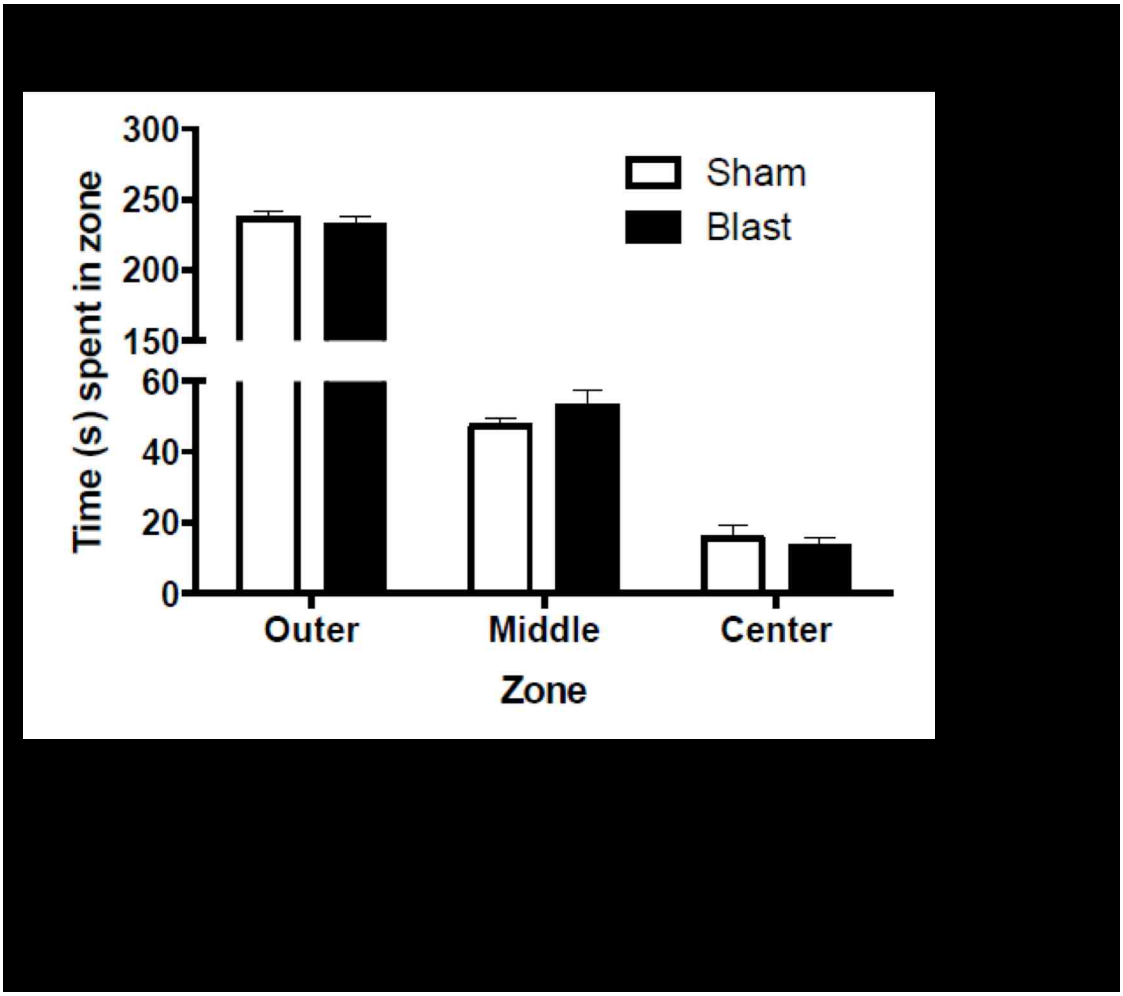
As the ability to socially interact in the SI test demands motor capability, and could be confounded by motor deficits, it was important to determine if Blast exposure produced gross motor deficits. Blast exposure effects on gross motor ability were assessed in a five-minute Open Field test under dim red lighting 7 days after Sham (n=6) or Blast (n=7) exposure. No differences were found between groups for any of the measures; the mean (± SEM) total distance traveled (Unpaired t-test, p= 0.475), maximum speed (p=0.932) and average speed (p=0.417) (Figure 4.2A and 4.2B).

Further, the motor ability of the rats on the Rotorod was also assessed to add another layer of motor ability assessment to make certain that the rats had no motor learning or balance deficits following blast exposure. The Rotorod test was given approximately 2 hours after the gross motor session in the Open Field test on Day 7 post Blast (n=7) or Sham (n=6) exposure (Figure 4.2C). Performance was assessed by the amount of time the rat spent balanced on the rotating rod across three consecutive test trials. There was no difference between the Blast and Sham groups across the 3 consecutive test trials, with both groups having a main effect of trial (RM two-way ANOVA p=0.0065). Both groups increased in their performance across the testing sessions and had a significant increase in time spent on the rod from test session 1 to test session 3 (Tukey's p=0.016). The time spent on the rotating rod was not significantly different between groups in each testing session, nor was there a significant group by trial interaction, suggesting that both groups display motor ability, balance ability and the ability of motor learning. Collectively, these results suggest that the motor ability was not affected by blast exposure and motor deficits are not a confounding factor for why the Blast rats failed to acquire SoFiA.


A.
OF Distance Travelled
Total Distance (m)
40
30
20
10
0
Sham
Blast
Treatment
B.
Speed in OF
meters/s
1.0
0.8
0.6
0.4
0.2
0.0
Sham
Blast
Max
Ave.
Speed
C.
Post Blast RotoRod
Time on rotorod (s)
300
200
100
0
Blast
Sham
*
Trial 1
Trial 2
Trial 3
RotoRod Attempt

Blast exposure did not affect anxiety-like behavior in the Open Field

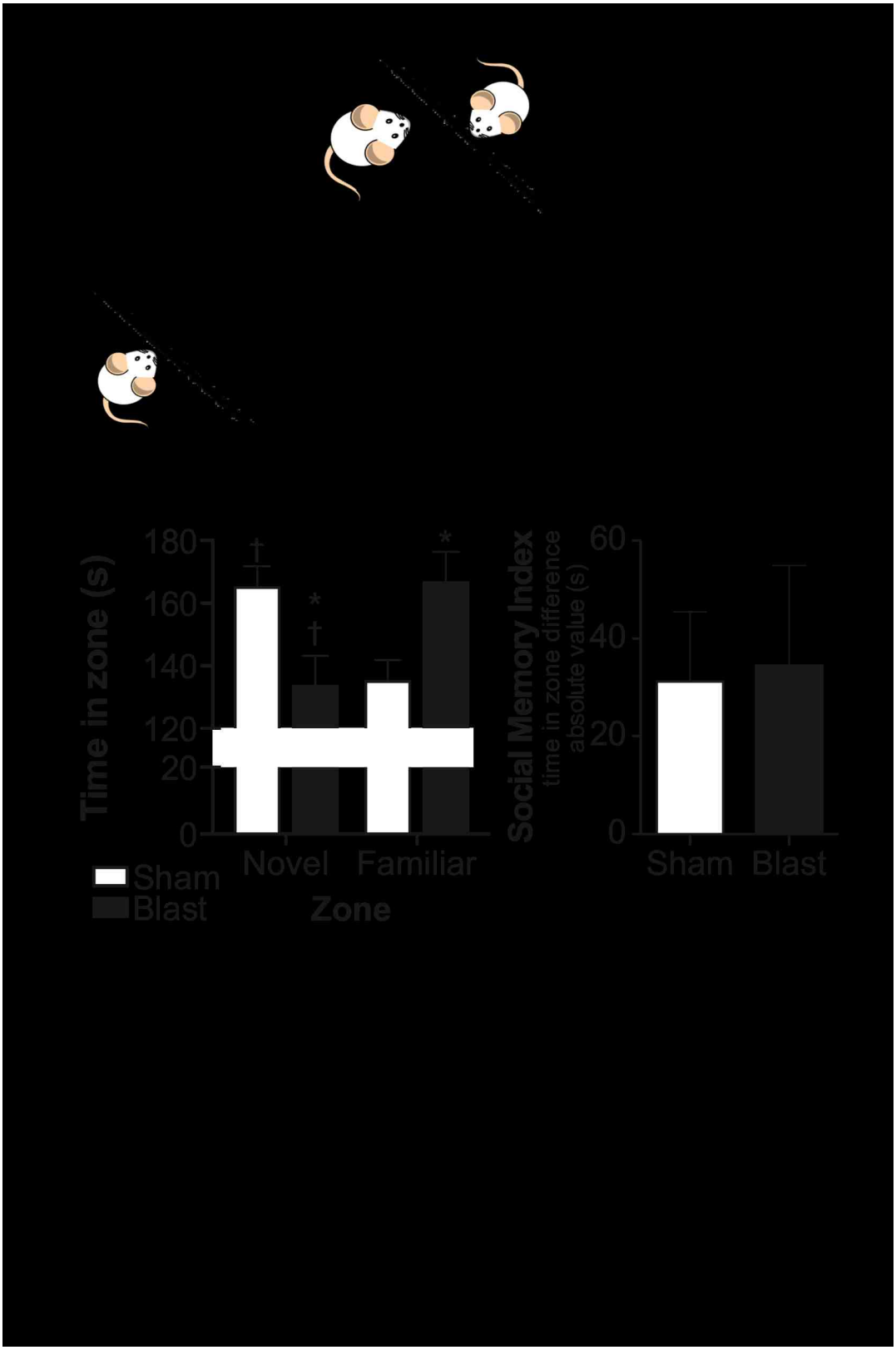
To ensure that the exposure to blast was not affecting the basal anxiety behavior of the rats, which could confound the ability of us to measure social familiarity effects on anxiety, open field behavior was assessed. The Blast (n=7) and Sham (n=6) rats underwent the Open Field test at 7 days post-exposure (48 hours prior to the Baseline SI test) to assess basal anxiety-like behavior within another behavioral test (Figure 4.3). The amount of time the test rats spent within each of the outer, middle and center zones of the Open Field was determined by the video tracking software ANY-maze. The amount of time the two groups spent in each of the Open Field zones was not significantly different between treatment groups (unpaired t-tests: outer; p=0.589, middle; p=0.298, center; p=0.583).



Blast exposure does not affect social recognition

To determine if social memory is still intact in the Blast exposed rats, the Social Recognition test was performed. This was to determine if a lack in the ability to recognize the conspecific partner as "familiar" was compromised by the exposure to Blast, and thus possibly contributing to the lack in the acquisition of SoFiA. Blast (n=7) and Sham (n=6) rats from cohort 1 were given this Social Recognition test post SI-hab training, and so the testing was done after the rats had had a chance to develop social familiarity with the partner rat, having been exposed to that partner in the SI test for 5 minutes a day for 6 consecutive days. On the day following the completion of the SI-hab testing (Day 16 post blast/sham exposure), the rats were given the Social Recognition test under the BLC and in the SI testing arena modified as described in the methods (Figure 4.4A), allowing the rats free choice between spending time in the familiar zone (partner rat from the SI-hab training) or the novel zone. Differences in the partner preference of which zone the rats spent the most time were seen in the behavior of each group. Here there was a main interaction effect (two-way ANOVA, Exposure x Zone interaction $F_{1,22}$=12.93, p=0.0016). Blast and Sham rats differentiated familiar and novel partners, as both groups of rats had significantly different times spent in the Novel versus Familiar partner zones (Fishers LSD, Sham Familiar vs Novel, p=0.0295; Blast Familiar vs. Novel, p=0.0109). Additionally, the different exposure groups had opposite behavior, preferring to spend more time in opposite partner zones both (Fishers LSD, Familiar zone Sham vs. Blast, p=0.0185, Novel zone Sham vs. Blast, p=0.0185). The graph in Figure 4.4B shows that the rats, when given a choice to move freely between the two zones in the partitioned SI testing box under the BLC, the Blast rats shifted the partner preference and spent more time in the Familiar partner zone, while the Sham rats spent more time in the Novel partner zone. This could be explained by learned and innate fear having different regulatory mechanisms.

To better visualize the social memory of the different partner types; a Social Memory Index is presented in Figure 4.4C. The absolute value of the total time spent interacting with a given partner is presented in the graph, showing that there was no difference between the Blast and Sham groups in their discrimination between the two partner types. Both Sham and Blast rats differentiated the Familiar rat from the Novel rat (spent significantly different amounts of time in the familiar versus novel zone) to an equivalent degree. This indicates that the rats equally discriminated between the partner types, demonstrating a social recall that was still intact in the blast rats.

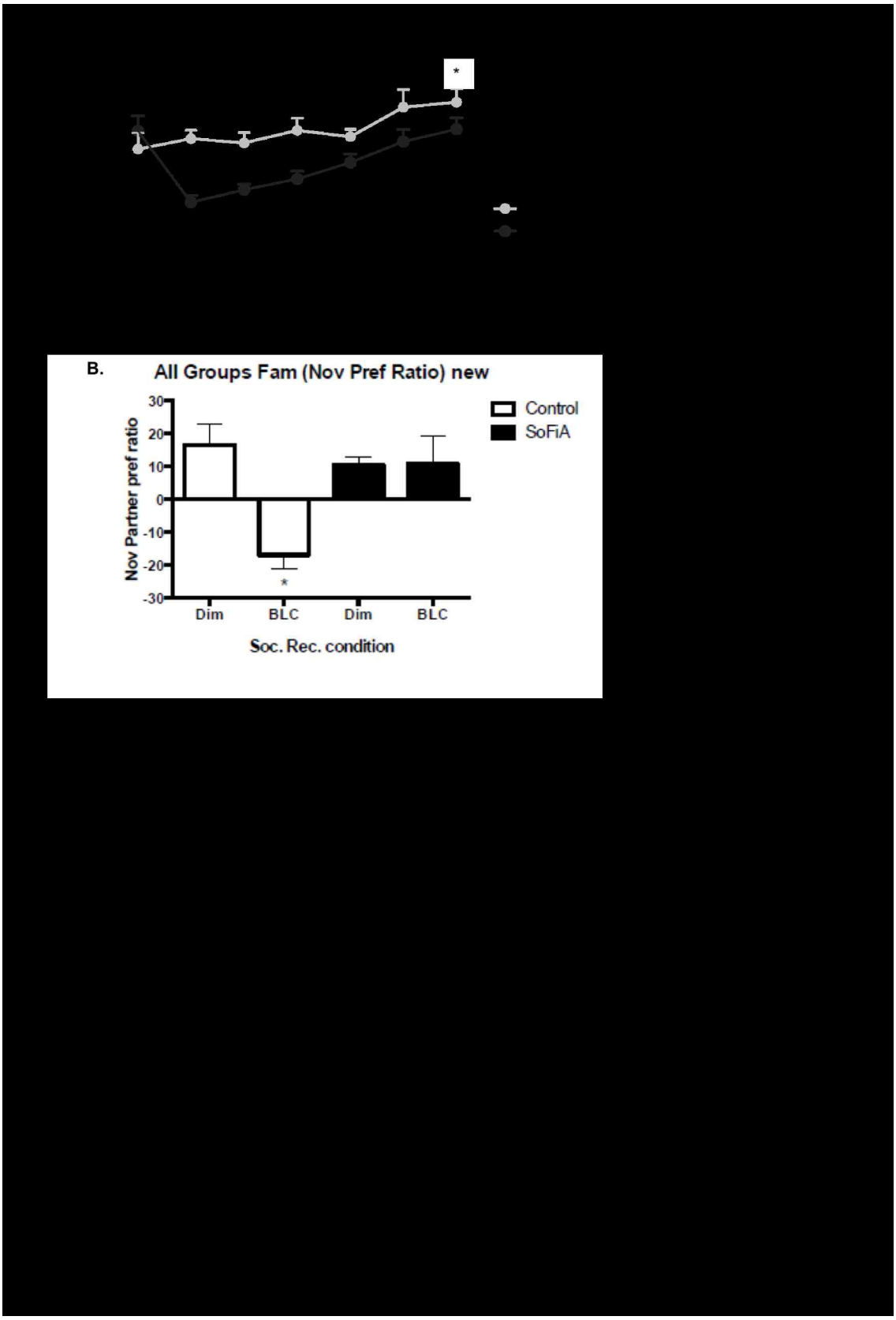

Time in zone (s)
180
160
140
120
20
0
†
*
†
*
Novel
Familiar
Zone
Sham
Blast
Social Memory Index
time in zone difference
absolute value (s)
60
40
20
0
Sham
Blast

Social Recognition testing in non-injured rats

An additional experiment was designed to better understand the role of social familiarity on anxiolysis by quantifying the social familiarity that develops following SI-hab training. The Social Recognition testing paradigm was used for these rats the same as the procedure for the Blast rats. The experiment was designed to answer the question of what type of social training conditions affect partner preference. Specifically, whether previous exposure to the anxiogenic stimulus during the SI-hab training would lead the test rat to behave differently in the Social Recognition test than if the exposure to the anxiogenic stimulus had not previously occurred. Here we gave rats the SI-hab training paradigm, testing in either dim red light (n=11) or BLC (n=11) each day for 6 consecutive days (Figure 4.5A). The SI-hab testing in the BLC vs Dim red light produced different SI times, with a main effect of time (RM two-way ANOVA, $F_{6,120}$=7.903, p<0.0001) and a main effect of lighting condition ($F_{1,20}$=12.90, p=0.0018), and an day by light interaction effect ($F_{6,120}$=3.193, p=0.0061). The BLC group had decreased SI times from Baseline on Days 1-3 (Tukey's multiple comparisons test, p<0.05), and a difference between groups was present for the first 3 days of SI testing (Sidak's multiple comparisons test p<0.05). By Day 5 and 6, the BLC group had developed the SoFiA effect and the SI times were no longer significantly lower than Baseline, and were significantly higher than Day 1 (Tukey's multiple comparisons, p<0.05). The Dim red light group had SI times that did not differ from Baseline significantly across Days 1-5, however SI time on Day 6 was significantly higher than baseline but not different from Days 1-5 (Sidak's multiple comparisons test p<0.05).

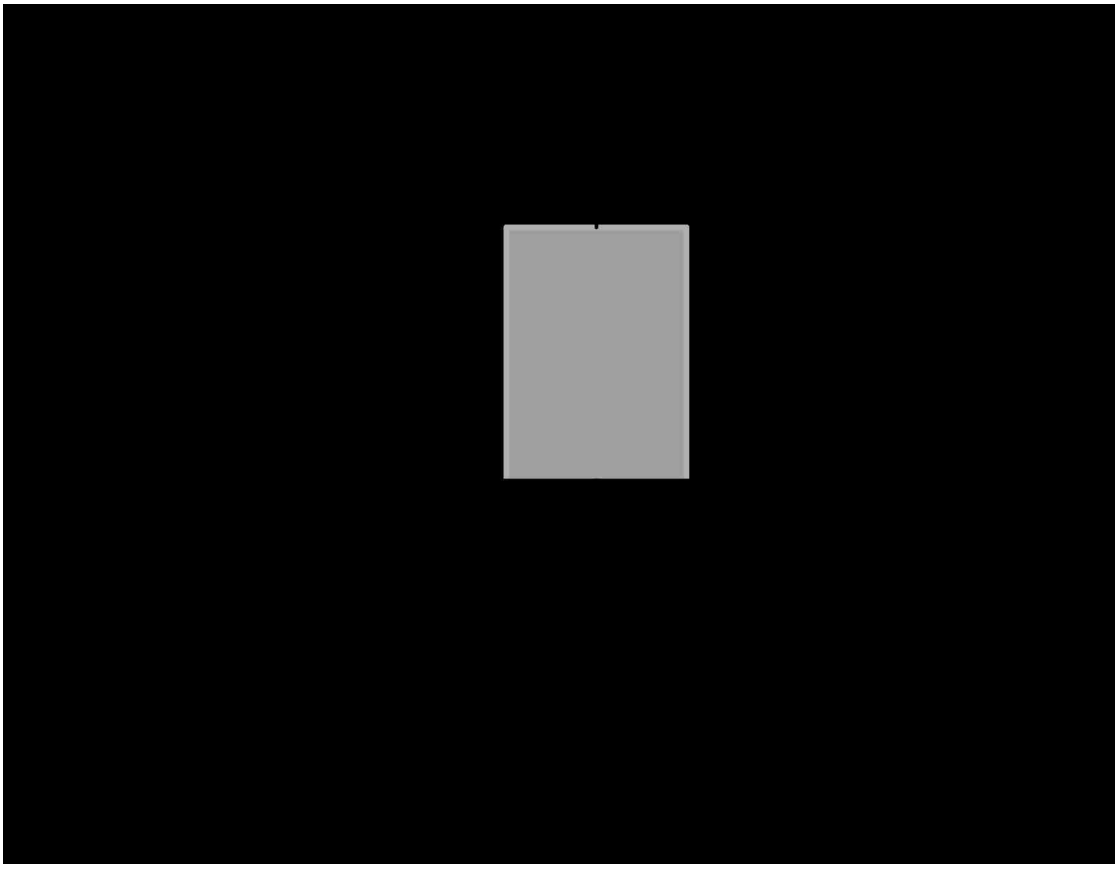
Twenty-four hours following the last day of SI-hab training, the two groups were then split into two additional groups, BLC (n=6 rats from each SI-hab treatment) or dim red light (n=5 rats from each SI-hab treatment) exposure for the Social Recognition test, creating 4 groups according to the training lighting conditions and Social Recognition lighting conditions, SoFiA/Dim, SoFiA/BLC, Control/BLC, Control/Dim (Figure 4.5B). Following the SI-hab protocol in either dim red light (Control) or BLC (SoFiA) and with a familiar social partner in each test session, the presence of the acquisition of the SoFiA effect determined the preference of the rat for the social partner in the Social Recognition test. The preference ratio is calculated as a novel preference coefficient ratio= (time in novel zone-time in familiar zone)/(time in familiar zone + time in novel zone) x100. The more positive the number, the more preference for the novel partner, the more negative the number, the less preference for the novel partner and more preference for the familiar partner. Here there was a main effect of treatment, or light

conditions among the groups (two-way ANOVA $F_{3,18}$=6.063, p=0.0049). The rats spent more time in the Novel partner zone than in the Familiar partner zone in all the groups except for the Control/BLC group. This Control/BLC group had a reversed preference for the partner rat as demonstrated by the negative value on the graph in Figure 4.5B for the Novel Partner Preference Ratio. This Control/BLC group was significantly different from the other test groups (Tukey's multiple comparisons test comparing the Control/SoFiA group to the other groups; p=0.0059 for Control/Dim, p=0.019 for SoFiA/Dim, and p=0.018 for SoFiA/BLC), while the other groups were not significantly different from each other. This indicates that there is an effect of the training conditions on the Social Preference of the rats when they are given the Social Recognition test under different lighting conditions. Another possible explanation is that the social preference shifts from novel to familiar in the presence of perceived anxiogenic stimuli.


*
B.
All Groups Fam (Nov Pref Ratio) new
Control
SoFiA
Nov Partner pref ratio
30
20
10
0
-10
-20
-30
*
Dim
BLC
Dim
BLC
Soc. Rec. condition

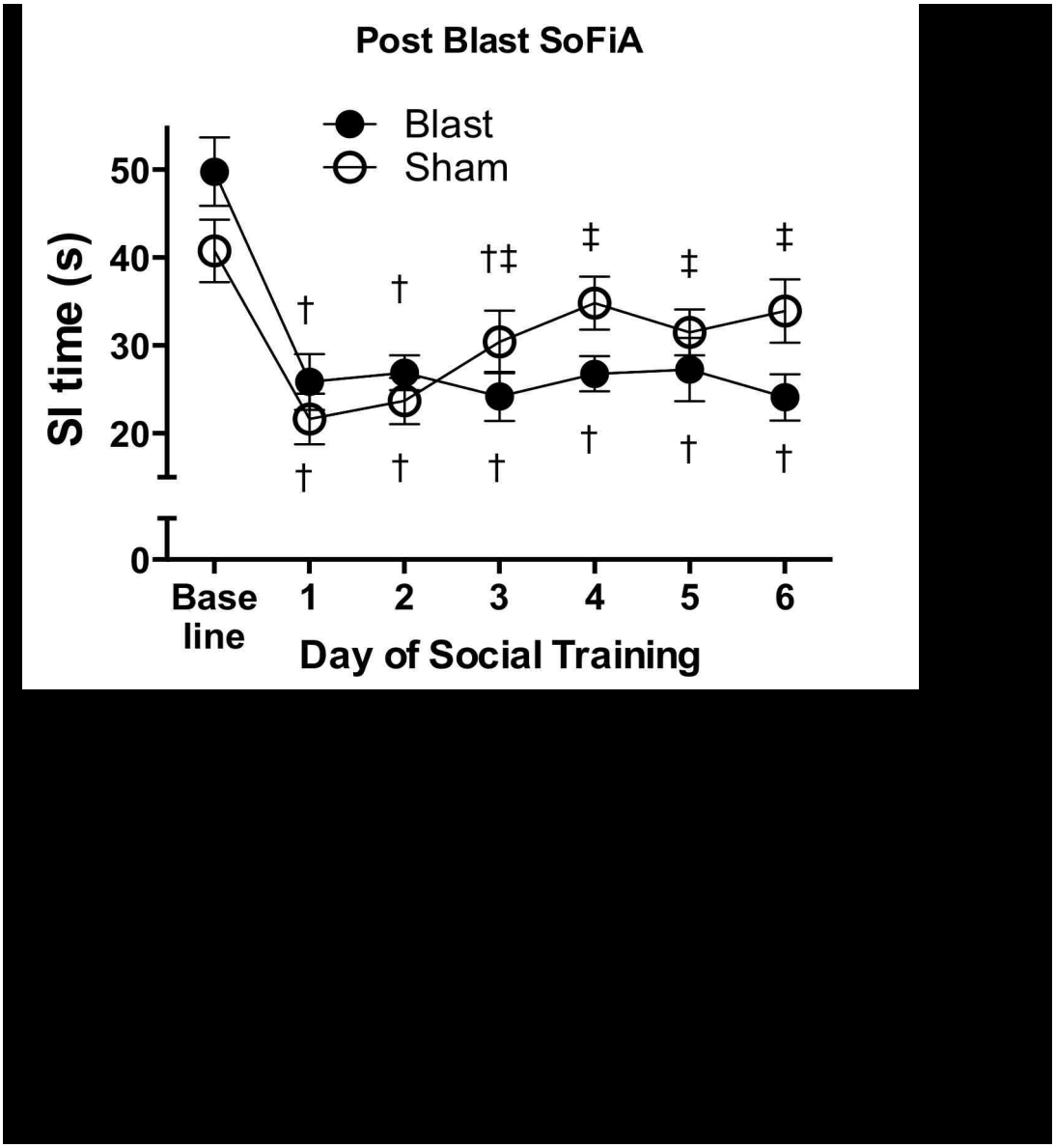
Blast exposure association with a neuronal marker

In an effort to begin the exploration into possible tissue damage effects from blast exposure, the total number of neurons present in the Infralimbic area of the prefrontal cortex was counted (Figure 4.6). The neurons were visualized through immune staining against the NeuN protein, a neuron-specific protein. The IL was the focus of the neuron immune staining counts because this area was determined through previous experiments in chapter 3 to be involved with the regulation of SoFiA. Through counting the number of NeuN positive neurons within the IL area of the PFC, we see that there are no differences in the number of cells/volume (μm^3) area counted in each Blast (n=7) and Sham (n=6) group (unpaired two-tailed t-test; t=0.7878, df=11, p=0.4475).



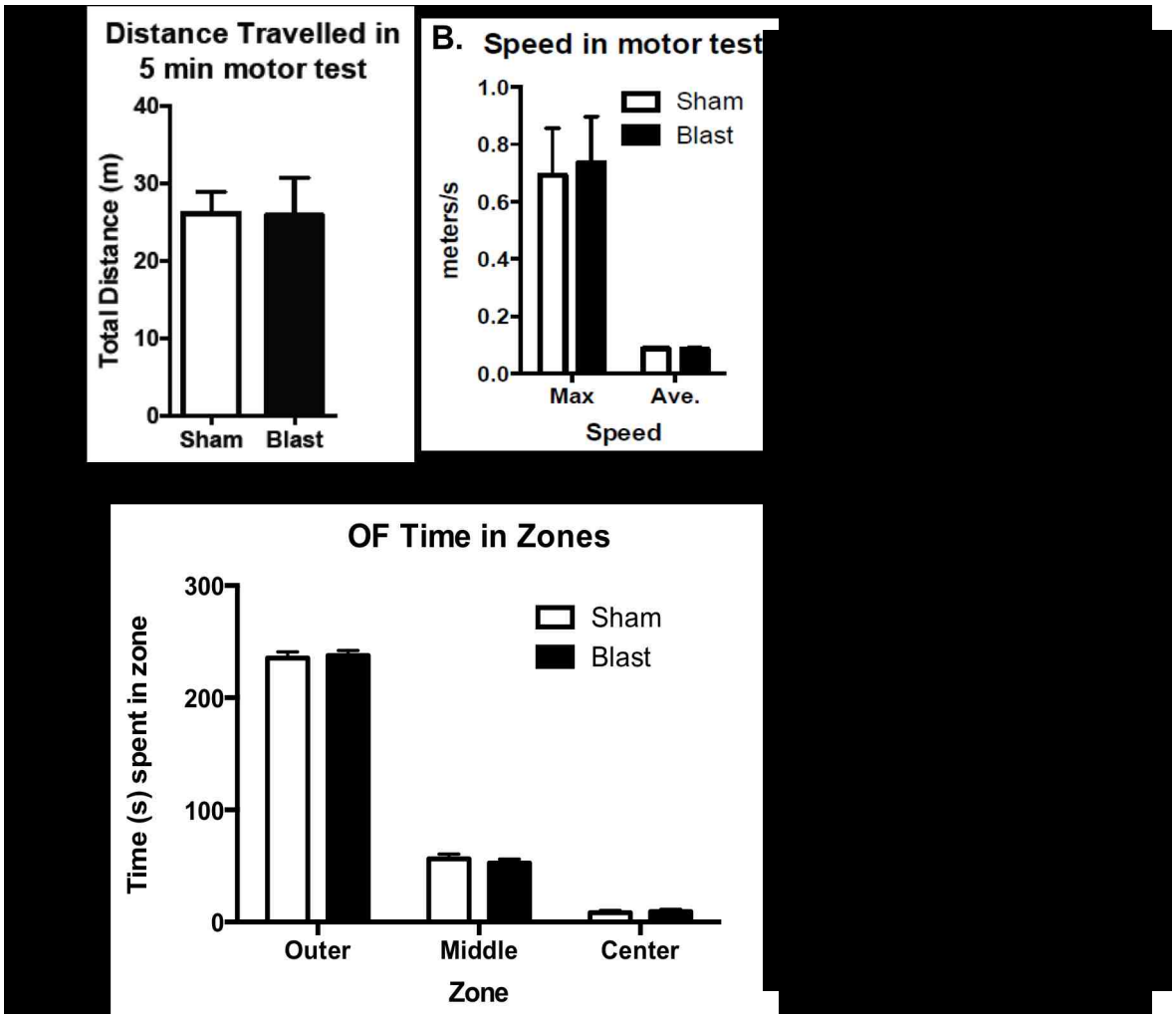
Replication of bmTBI-induced SoFiA deficits

SoFiA acquisition was similarly affected in a duplicate cohort of rats that were similarly Blast (n=9) and Sham (n=9) exposed. In Figure 4.7, the behavioral SI-hab procedure was the same as the first cohort and that the Blast and Sham groups had differential responses to the SI-hab training under the BLC, with a main effect of Day (RM two-way ANOVA main effect of Day $F_{6,96}$=18.30, p<0.0001) and a day by exposure interaction ($F_{6,96}$=4.625, p=0.0004). For the Sham rats, the BLC induced a significant reduction in SI time compared to Baseline SI times on social training Days 1-3 (Tukey's p≤0.0383). This group of Sham rats acquired the SoFiA effect and had significant increases in SI time from the initial Day 1 on Days 3-6 (Dunnett's multiple comparisons test p ≤0.0461). However, SI times for the Blast group remained significantly lower than Baseline SI times on all the days tested (Tukey's p<0.0001). The baseline Si scores where appear different but are not significantly different in this cohort, underlining the necessity for baseline testing for each test. This duplicate cohort demonstrates the robustness of the effect of the blast exposure on the deficits in SoFiA acquisition.


Post Blast SoFiA
Blast
Sham
SI time (s)
50
40
30
20
0
Base line
1
2
3
4
5
6
Day of Social Training

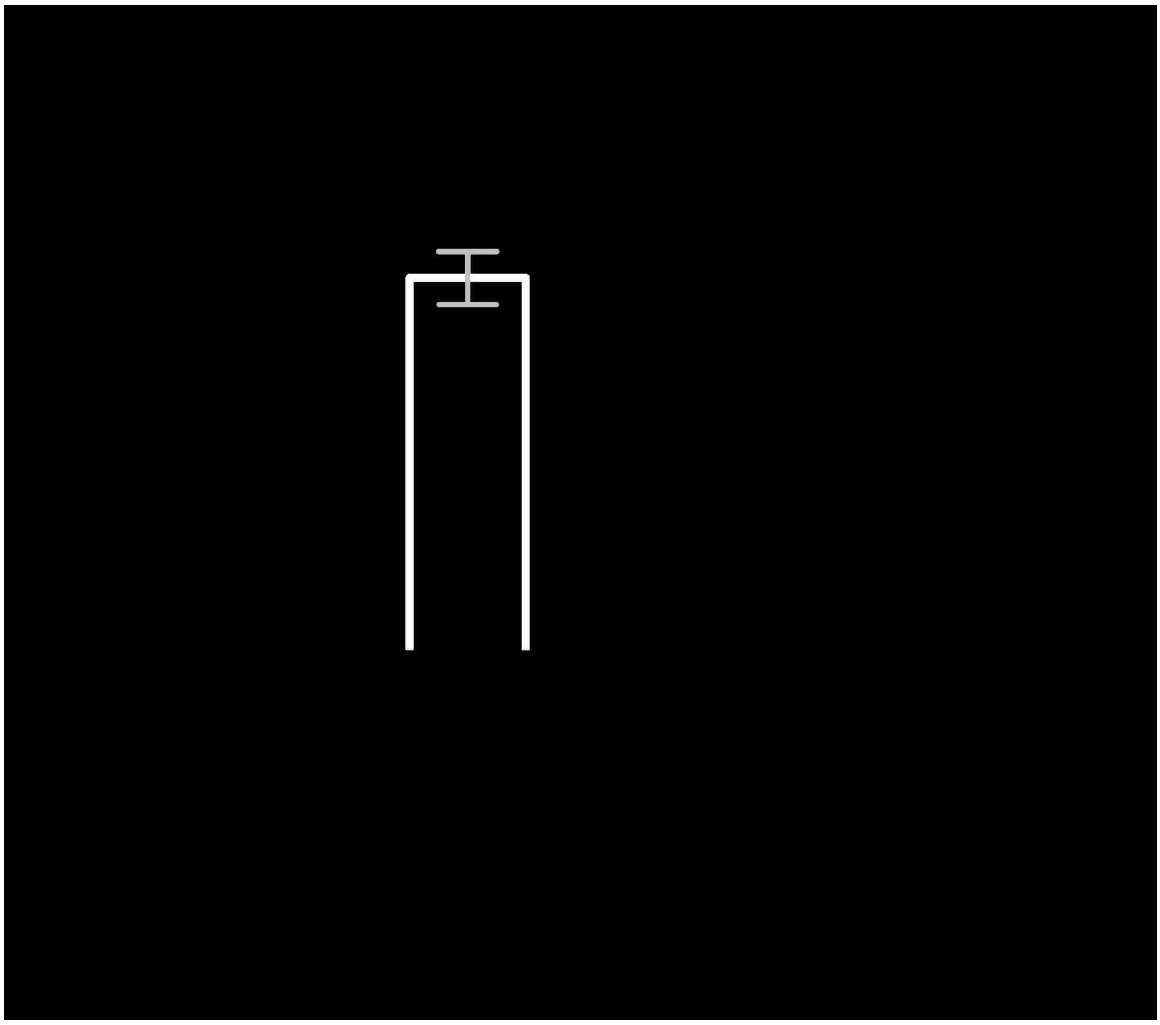
Open Field Motor/Anxiety assessment in Blast or Sham cohort 2

Gross motor ability of the second cohort of rats was measured on Day 7 post Blast (n=9) or Sham (n=9) exposure and 48 hours prior to SI-hab testing. Total distance traveled, maximum speed and average speed were calculated (Figure 4.8A and 4.8B), and there were no differences between the two exposure groups in these parameters (unpaired t-test, distance traveled; p=0.8717, maximum speed; p=0.853, average speed; p=0.890). Therefore, between both cohorts, we see that gross motor ability performance in the Open Field was not affected by the Blast exposure. Additional to the motor assessment in the Open Field, the anxiety measurement within the Open Field was assessed in this cohort. The time spent in each of the three zones was not different between treatment groups (unpaired t-tests: outer; p=0.740, middle; p=0.533, center; p=0.693) (Figure 4.8C).



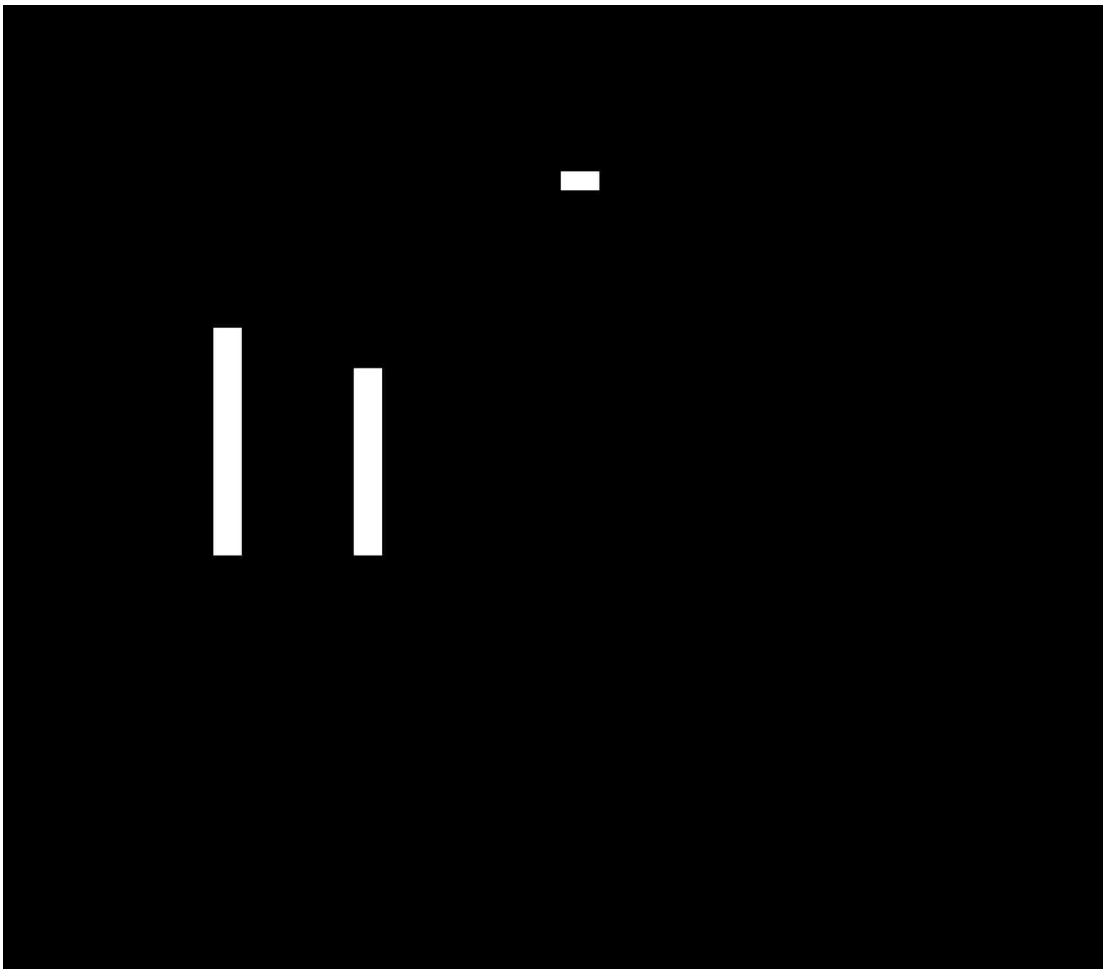
Depression-like behavior is not altered following blast exposure

To explore a different phenotypic outcome that could have arisen as a consequence of blast exposure, we tested the second cohort in the Tail Suspension test, a test for depression-like behavior. The Tail Suspension test was done on Day 17 post exposure to the Blast (n=9) or Sham (n=9) (Figure 4.9). Time spent immobile, the measure of behavioral despair indicative of depression-like behavior, was determined through Any-maze automated scoring software. There were no differences between Blast and Sham groups in the total time spent immobile during this 5-minute test (unpaired two-tailed t-test; t=0.9673, df=16, p=0.3478). This lack of a difference on time spent immobile between the two exposure groups demonstrates that a depression-like phenotype is not pervasively present following blast exposure. An acute depression-like phenotype (within the first 24-72 hours) was not determined.



Novel Object recognition is not affected by exposure to blast

To assess the memory formation ability of the rats exposed to the blast, we tested the Blast and Sham rats with a Novel Object Recognition test on Day 7 post exposure. This test exposes rats to two similar objects for a 5-minute pre-test and after a 10-minute inter-test-interval, are re-exposed to the open field with one object the same one as the previous exposure and one object replaced with a novel object. Looking at the time spent investigating the different objects for the two groups, there was a main effect of object (two-way ANOVA $F_{1,16}$=11.35, p=0.0039), but no effect of blast treatment (two-way ANOVA $F_{1,16}$=1.304, p=0.2703), or an interaction (two-way ANOVA $F_{1,16}$=3.249, p=0.0903). The Blast rats spent significantly more time interacting with the novel object than the familiar object (Sidak's multiple comparisons test, novel vs familiar, p=0.0042), indicating an ability to discriminate the object type. However, the Sham rats did not demonstrate a discrimination between the novel and familiar objects (Sidak's multiple comparisons test, p=0.4877).



Discussion

Pathogenic Validity

The rodent model of SoFiA demonstrates the main types of validity critical to establishing a rodent model of behavior. These are Face, Predictive and Construct. The next logical course for this rodent model is to explore another type of validity that we can use to further explore the uses and implications of the model. A common type of validity that many animal models attempt to emulate is translational validity in order to have clinical relevance. Translational validity of a model promotes a better understanding of human disease states. One kind of translational validity that we wanted to test for was to identify a pathological condition in humans that leads to social deficits and determine if our model of SoFiA could identify social deficits within that pathological state in rodents, thus establishing pathogenic validity (Belzung & Lemoine, 2011).

With our rodent model of SoFiA in mind, we aimed to identify a patient population in which psychosocial deficits are often present. One particular growing patient population that we were able to target through preclinical modeling was people who have had an exposure to a blast-induced mild traumatic brain injury (bmTBI). This population has been shown to have a common prevalence of psychosocial deficits, and modeling this patient population will provide useful information when developing treatment strategies. In this chapter, exposure to a bmTBI transformed rats to display a phenotype of psychosocial deficits, seen in the form of a failure to acquire SoFiA, which demonstrates a pathogenic validity to the SI-hab model of psychosocial behavior. Through this testing paradigm, we have discovered a way in which to study the presence of a pathology that is not well understood yet. Modeling bmTBI in rodents through the blast exposure procedure and further testing them for the presence of psychosocial deficits is now one way of preclinical modeling for patients within this population.

Blast exposure leads to psychosocial deficits

Improving our understanding of the mechanisms through which the social deficits are induced by a blast exposure will help future patients with targeted therapies and possibly better intervention strategies. Through the work in this chapter, we observed that the psychosocial deficits were selective. Observations of these kind and further examinations into the behavioral and physiological outcomes of blast exposure can help future researchers to begin looking into different ways to help treat those that are affected by bmTBIs.

Within this chapter, I observed that the Blast rats failed to acquire SoFiA with a familiar partner, unlike the Sham rats (Figure 4.1A). This lack of SoFiA acquisition can be present for several reasons, and we explored some possible reasons that could contribute to the behavioral change we observed. Initially the Blast rats had the exact same behavior in the SI test under non-anxiogenic (dim red lighting) conditions as the Sham rats did, demonstrating that the blast exposure did not lead to exaggerated levels of anxiety-like behavior or a lack thereof. Also, the Blast rats had the same increase in anxiety-like behavior to the BLC that the Sham rats did, demonstrating a normal response to anxiogenic stimuli (Seen in Figure 4.1A Day 1 SI times). This also demonstrates that the Blast rats have retained their ability to perceive the anxiogenic stimulus in this testing condition, ruling out a sensory deficit acquired from exposure to blast. We have ruled out that the Blast rats were not capable of physically moving towards the partner or able to recognize the Familiar partner as familiar. Instead, the rats are displaying an elevated level of anxiety-like behavior that is not overcome, regardless of the repeated opportunities to socially interact with a familiar partner that would normally lead to anxiolysis.

The key finding that a lack of the ability of the Blast rats to acquire SoFiA could be explained by the blast exposure leading to an inability of the rats to utilize the Familiar partner to overcome the BLC anxiogenic stimulus. The Blast rats may lack the ability to make the connection of the partner rat as a safety cue. Translationally, in humans, social support is a vital aspect of therapy, and if the patients are not able to perceive the presence of the social support through the relationship with those helping such as the therapist, the therapy is futile. A study looking a mTBI in Chinese patients as a result of traffic accidents found that the perception of the presence of social support was associated with less mental fatigue (Zeng et al., 2016). Improving the social support of the mTBI patients with mental fatigue led to a decrease in the reported fatigue, pointing to a need for mTBI patients to engage in rehabilitation that involves the integration of social support (Zeng et al., 2016).

We see that the lack of SoFiA acquisition behavior following blast exposure was persistent to the number of days tested for the Blast exposed rats. The last day of SI-hab testing was 15 and 16 days post exposure for cohort 1 and 2 respectively. Here we observed that the Blast rats still were not able to acquire the SoFiA effect (Figure 4.1A and Figure 4.7). This SoFiA acquisition deficit was not an anomaly of just one experiment, as this deficit effect was seen in 2 duplicate cohorts of rats tested in two completely different times. The fact that the Blast rat SoFiA deficits are replicated in the two cohorts of rats demonstrates that this effect is strong

and highly specific. As I also demonstrate in this chapter, this selective psychosocial-like learning deficit is present in the absence of other obvious motor, affective or learning abilities.
A loss of mental flexibility may help to explain why the rats have not been able to acquire SoFiA, they have social recognition as we have shown, but the mental flexibility to adjust emotional and behavioral responses to changing social environmental cues is not seen in the Blast rats. A future study looking at the mental flexibility of the rats may help to determine if the rats have lost this ability (Brady & Floresco, 2015).

Acrolein

Acrolein has numerous sources of endogenous production including lipid peroxidation, arachidonic acid catabolism, and polyamine catabolism, all of which increase in the acute term following CNS trauma (Moghe et al., 2015; Shi et al., 2011). Following blast exposure, we saw that the urine levels of an acrolein metabolite were elevated in the Blast rats (Figure 4.1B). This 3-HPMA increase is of significant importance because acrolein elevations point to the presence of oxidative stress and inflammation following the blast exposure, which demonstrates the presence of neurotrauma (Luo & Shi, 2005). It additionally suggests a putative biomarker to help predict post-bmTBI psychosocial learning deficits, especially within the SI-hab model. Taking this into consideration, future explorations into the usefulness of acrolein as a potential diagnostic or prognostic target may hold promise. Additionally, acrolein may also prove to be a target for treatment intervention strategies for blast exposure patients, due to the direct neuro-damaging properties (Leung et al., 2011; Park et al., 2014). Ongoing processes from the oxidative damage from acrolein induction could lead to future damage, leading to newly developed pathologies. More work is needed to better understand the role that this compound is playing following blast exposure.

SoFiA deficits are selective following Blast exposure

The effect of blast exposure on the Blast rats has, in the set of experiments presented here, proven to be highly specific. Here we see a specific deficit in the acquisition of SoFiA, and this was replicated over two separate cohorts of rats. A comprehensive behavioral assessment has not yet been possible to accomplish with these cohorts of rats. There is a chance that a less salient effect could still exist that we have not yet discovered, which could help to explain the SoFiA deficit. However, we attempted to observe tests that covered as many of the possible outcomes that could be a result of blast exposure or confound the SI-hab testing.

The blast exposure led to a lack in SoFiA acquisition, and this deficit was seen while in the absence of other physical and psychological changes. The type of Blast exposure that was used in this chapter is considered mild based on previous data and a lack of observed motor deficits seen in the Open Field test and Rotorod test, producing no outward physical incapability (Walls et al., 2016). In this chapter, we see that the Blast rats moved throughout the Open Field and balanced on the Rotorod at similar rates as the Sham rats (Figure 4.2). This eliminates the possibility of the Blast rats having artificially decreased SI times due to an inability to move about the Open Field. Therefore, the ability to move about the SI testing arena plus vestibular information combined with motor coordination (on the Rotorod) was not a confounding factor to the Blast rats not acquiring the SoFiA effect during the SI-hab training.

The Blast rats demonstrated normal levels of anxiety-like behavior as seen within the SI test under non-anxiogenic Baseline (dim red light) conditions. We saw that the basal anxiety levels of the blast-exposed rats were typical to the levels seen in the sham-exposed rats in the SI test. This was additionally true for the Open Field test. The anxiety-like behavior was similar in both groups during the Open Field test under baseline conditions, meaning that the blast exposure does not lead to basal anxiety-like behavior in non-anxiogenic conditions (Figure 4.3). The lack of abnormally increased anxiety-like behavior in the Open Field test demonstrates that the basal non-social anxiety levels were not increased as a consequence of Blast exposure.

Additionally, the blast exposure did not lead to aberrant anxiety-like behavioral responses to anxiogenic stimuli (BLC). The BLC increased anxiety-like behavior similarly in the Blast rats as it did in the Sham rats, demonstrating the normal response of the Blast rats to an acute anxiogenic stimulus. This normal response to the BLC means that the blast exposure did not affect the ability of the rodents to perceive, process and respond normally to anxiogenic stimuli. Lastly, blast exposure did not lead to another affective phenotype such as depression-like behavior as seen in the Tail Suspension test, which was not different between the treatment groups.

Social Recognition testing

In order to acquire SoFiA, we see that the test requires multiple test sessions, with each session increasing the available amount of time the test rat has to become familiar with the partner rat. In response to the Chapter 3 data in which we see that in order to acquire SoFiA, pairing of the familiar partner and the anxiogenic stimulus during SI-hab training is required, I

wanted to demonstrate social familiarity through quantifiable means. Social memory is demonstrated through the test rats' discrimination of different partner types, familiar or novel. The Social recognition is quantified through the amount of time the test rats spends interacting with the different partner rats (Familiar or Novel), in which the test rat chooses between two partner types simultaneously presented.

Some research has pointed to rats lacking social recognition for juveniles lasting beyond 30-45 minutes (Noack et al., 2010; Sekiguchi, Wolterink, & van Ree, 1991). However, our research differs in that we use two adult rats, and they are not partitioned away from each, but instead are allowed to have full physical contact during the SI test. Therefore, we believe that through our testing, in 24 hours, it is still possible for the rats to have quantifiable social recognition. I gave a cohort of blast-exposed rats as well as a group of non-injured rats the Social Recognition test to not only demonstrate the presence of social recognition, but also to better understand the partner preference we saw following blast exposure.

Blast exposure affects partner preference in the Social Recognition test

A possible confounding factor preventing the acquisition of SoFiA in the blast-exposed rats could be that the blast exposure led to a lack of social memory formation. To test for and demonstrate social memory in the Blast rats, the Social Recognition test was done following the completion of the SI-hab training. The testing conditions were chosen to mimic the conditions under which the rats where given the SI-hab testing, and where the Sham rats had acquired SoFiA. We hypothesized that the Sham rats, having acquired SoFiA, would prefer the Novel partner, while the Blast rats, lacking SoFiA acquisition, would prefer the Familiar partner.

Within the Social Recognition test, both exposure groups demonstrated the ability to differentiate between the two partner types, Familiar versus Novel (Figure 4.4B). The differentiation is evident because each group of rats spent more time near one type of partner rat over the other. However, the Blast rats showed a different response to the two partner types than the Sham rats. This differentiation between partner types in both groups demonstrates the ability to distinguish them, and indeed the absolute value of the difference between the times in each of the two zones was similar in both the Blast and Sham groups (Figure 4.4C). Therefore, social recognition deficits were ruled out as confounding the ability of the Blast rats to develop SoFiA.

The Sham rats spent more time in the Novel Zone, which is what we expected as the typical response if these control animals are behaving similar to the typical response of rodents in the Social Recognition test, to prefer novelty. However, the Blast rats showed an opposite response, spending more time with the Familiar partner over the Novel one. This data means that the Blast rats are still capable of forming a social memory, but we see that they are also demonstrating an aberrant social preference. If the Blast rats were displaying typical responses, we would expect them to have the Novel partner preference similar to the Sham rats.

Since the Blast rats had not yet developed anxiolysis to the BLC in the SI test, they may have been experiencing elevated levels of anxiety in the presence of the BLC during the Social Recognition test as well. This could help explain the partner preference switch that we see. Although the Blast rats did not show increased SI times with the Familiar partner, that partner may be less aversive than the Novel partner to interact with or be nearer to in the Social Recognition test. Additionally, even with the familiarity of the partner intact, the Blast rats could have had the inability to perceive the partner rat as a safety cue, preventing the acquisition of SoFiA.

SoFiA acquisition affects partner preference in the Social Recognition test

To demonstrate that social familiarity is developing during the SI-hab testing under the different anxiogenic conditions, the Social Recognition test was given under different lighting conditions following the SI-hab training under different lighting conditions, either in dim red light or BLC. Therefore, the social memory was tested while in different anxiogenic conditions as well. The expectation was that the presence of the anxiogenic stimulus during the Social Recognition test would have differential effects on the test rat depending on whether the rat had undergone SI-hab testing and developed SoFiA. Specifically, the hypothesis was that the rats that had developed SoFiA would have less anxiety-like behavior and so would likely prefer the Novel partner even under the anxiogenic stimulus (BLC). Additionally, the rats that were trained in the SI-hab test in the dim red light, having not developed SoFiA, would likely have elevated levels of anxiety-like behavior when tested in the Social Recognition test under the BLC, and so would prefer the Familiar partner. These testing conditions were also done to better understand the partner preferences of the Blast and Sham rats. The Blast rats had not acquired SoFiA, and

so I expected the rats given the SI-hab testing in the dim red lighting to behave similarly to the Blast rats in having a Familiar partner preference.

The results indicate that the SI-hab training conditions are important to the development of anxiolysis, and that this affects the partner preference within the Social Recognition test. Here we see specifically post SoFiA acquisition leads to novel partner preference in the Social Recognition test, regardless of lighting conditions during the Social Recognition test. For the rats that were tested in the SI-hab training in dim red light, and did not acquire SoFiA, the presence of the anxiogenic stimulus during the Social Recognition test had an alternate effect on the partner preference, switching from Novel to Familiar (Figure 4.5B). Possible explanations for this could be that the rat was experiencing elevated levels of anxiety to the anxiogenic stimulus during the Social Recognition test, leading the test rat to spend more time with the Familiar partner. While testing the Social Recognition test in dim red light does not introduce any additional anxiogenic stimulus, leading the rat to interact preferentially with the Novel partner, which is the typical response in a Social Recognition test (Engelmann, Wotjak, & Landgraf, 1995; van der Kooij & Sandi, 2012). When the rats had developed SoFiA, the rats had possibly decreased anxiety in the presence of the anxiogenic stimulus. The BLC in the Social Recognition test, therefore, was not novel, and having already overcome this stimulus, the rats acted in the way that the rats tested in the dim red light did.

The Social Recognition test is not a test for anxiety-like behavior, but is a test for the presence of social memory and learning. All of the rats in this experiment are demonstrating social discrimination, spending significantly more time in one partner zone in lieu of the opposite partner zone. If the rats were not able to discriminate between the two partner types, the test rats would spend equal amounts of time in each partner zone, demonstrating no preference. This experiment may support a certain state dependent learning, as the animals all displayed social learning, even those who were faced with different lighting conditions in the two different tests, but the behavior does appear to be related to the anxiety state. When the rats have overcome the anxiety stimulus through SoFiA acquisition or if no anxiety stimulus is present, the Novel partner is preferred, while the rats that have not overcome the anxiogenic stimulus will prefer the Familiar partner when presented with the anxiogenic stimulus.

Other types of memory in the Blast rats

Although we have shown the social memory is still intact, spatial memory was also addressed in the second cohort of Blast rats. A study found that spatial and object recognition memory formation is linked to the prefrontal cortex, and if damage is occurring in this area from blast exposure, it could impair the ability of the rat to form these types of memory (Barker, Bird, Alexander, & Warburton, 2007). However, we saw that the Blast rats did not have deficits in the ability to discriminate between a novel and familiar object when presented them during the NOR test (Figure 4.10). Interestingly, we saw that the Sham rats did not demonstrate a significant discrimination between the two objects, and it is unclear as to why the rats failed to recognize the familiar object.

SoFiA expression and acquisition was linked to the activity of the prefrontal cortex in Chapter 3, and if this area is damaged as a result of blast exposure, it could mean that spatial memory is disrupted. It could still be argued that a lack of spatial memory in the Blast rats exists, which could mean that the environment is novel in each day of the SI-hab testing, which could possibly be a source of anxiety to the rats, leading to increased anxiety-like behavior and contributing to the SoFiA acquisition deficits we see. Also evidence has shown that these two types of memory are modulated within two different areas of the Hippocampus, suggesting different neuronal circuitry underlying the two types of memory (Hitti & Siegelbaum, 2014). Social memory is specifically integrated in the CA2 region of the dorsal hippocampus (Hitti & Siegelbaum, 2014). The pathways from the CA2 region do not directly project to the IL of the Infralimbic cortex, but the pathways that go to it can be through the social memories processed by these neurons may reach the IL via one of two disynaptic pathways, CA2–CA1–IL or CA2–supramammilliary nucleus (SUM)–IL (Hitti & Siegelbaum, 2014; Jay & Witter, 1991).

However, this does not fully explain the lack of the ability of the Blast rats to utilize the Familiar partner to help in overcoming the BLC. We would expect that if the Blast rats were still capable of acquiring SoFiA but are just under higher anxiety in each session, that the presence of the Familiar partner would still lead to increases in SI time across the repeated days, even if it is less than Sham rats, but the SI times remain flat for the Blast rats across all the days tested. Additionally, we have shown in previous data that a novel environment challenge with a familiar partner did not significantly increase the anxiety-like behavior, further nullifying the argument that the environment is the cause of the lack of SoFiA acquisition. Extending the number of SI-

hab days in the Blast exposed rats would help to clarify this point and determine if the rats need additional time to acquire SoFiA.

Neuronal effect from Blast

This model of bmTBI can be used for the basis of beginning to study the neural impact of a bmTBI. Blast exposure injury to the brain is still being assessed and no definitive conclusions can yet be made on the specific neuronal mechanisms affected by a bmTBI. However, at a first round of looking at possible neuronal effects following blast, we looked at the Infralimbic cortex of the Prefrontal Cortex. This area is of interest based on the previous experiment presented in Chapter 3 that identified the IL of the PFC as involved in SoFiA. When we targeted the IL, and inhibited the PFC with Muscimol, we saw that the expression and also the acquisition of SoFiA were inhibited. A gambling task challenge given to patients with vmPFC lesions demonstrated that the patients were unable to adjust cognitive decision making to reflect new information about the reward/punishment of the game, and instead always opted for instant gratification, regardless of the long-term disadvantage (Bechara, Tranel, & Damasio, 2000). These studies support the idea that the vmPFC is needed for mental flexibility in adapting to changing situations, which is vital for appropriate social behavior, and is a reason for our exploration of this area in the Blast rats. Additionally, the Blast rats did not demonstrate any social memory deficits, and though the hippocampus is located towards the dorsal part of the brain and close to the top where the blast wave encountered the head of the rat, we did not anticipate finding any neuronal death within this area. Therefore, based on the only overt behavioral deficit that we detected, we decided to look within the IL.

Here we asked whether the IL had any detectible physical damage as a consequence of blast exposure, and to identify obvious damage, we did immunohistochemistry to detect NeuN (*Neu*ronal *N*uclei) immunopositive cells within the IL of a brain slice from each Blast and Sham rat. NeuN is a nuclear marker for neuronal cells that appears at the final stage of neuron differentiation (Mullen, Buck, & Smith, 1992). NeuN was chosen as a putative marker for this first round of immunohistochemistry staining because it is capable of detecting most neuronal cell types within vertebrates, and so would be a good first round of detection if there are any major cell death that occurred following blast exposure (Mullen et al., 1992). However, cell counts indicated that there were no differences in the number of NeuN stained cells between the two treatment groups (Figure 4.6). The lack of differences in this specific type of neuronal

marker only means that the physical damage to the brain did not lead to a loss of or increase in neuron death within the IL. This does not rule out other types of damage that could have occurred that could explain the SoFiA deficits.

Although we did not find differences in the amount of NeuN immunostaining, this does not mean that there are other markers that have yet not been explored that are different between the two exposure groups. It is important to note that the rats did not lapse into a coma following blast exposure. Coma would be indicative of severe tissue damage that would likely have led to more obvious physical and behavioral outcomes. Other markers may need to be considered, and there are numerous cell activity and protein level markers that can still be explored. Also, NeuN does not stain glial cells and so any damage to those cell types were not observed (Mullen et al., 1992). Additional other biochemical disruptions could have occurred as a result of the blast exposure. For example, micro-axonal damage could have occurred that causes neurofilament structural damage preventing axonal transport along microtubules. There is also the chance that epigenetic alterations are occurring as a result of blast exposure that could be the reason for the differences in SoFiA acquisition.

Another way to explore the physical effect on the brain from blast exposure could be to detect gene expression differences that result from blast versus sham exposure. This extensive project would require an initial whole genome scan that would then need further parsing out to find candidate genes to explore further. This type of project is beyond the scope of the current chapter and the focus of the presented work as a whole. But further explorations are needed to better understand the physical damage that can help to explain the specific behavioral deficit that we see following this blast exposure.

Conclusions

The blast exposure that we used in this chapter was found to cause a highly specific deficit in the ability of rats to acquire social familiarity-induced anxiolysis, SoFiA. This was in spite of a lack of other obvious motor, memory, affective deficits or neuronal cell death within the IL. This specific SoFiA deficit may represent an intermediate phenotype, preceding other pathologies or psychological phenotypes that could emerge later in time. This makes it a possible strategy to be used for first screening of the presence of a bmTBI. Additionally, Blast exposure led to an increase in the Acrolein metabolite, which holds promise as a future biomarker indicating the presence of blast exposure.

This chapter demonstrates the utility of the SI-hab rodent model of SoFiA as a valid preclinical model for observing psychosocial outcomes from a pathology-inducing condition. The rodent model of SoFiA, in general, will allow us to systematically study psychosocial deficits that can occur from blast injury or even other diseases. We could possibly expand to use this model for investigation into other potential known conditions in humans that lead to psychosocial deficits. This knowledge will also help us to begin to better understand the way in which anxiety is regulated and overcome.

The underlying causes for the observed SoFiA acquisition deficits remain elusive and may require extensive further research into the neural mechanisms that may be disrupted. This does however provide us with vital information into the utility of the SI-hab model to be used to identify social deficits following the induction of a bmTBI injury. This ability of the SI-hab model is here demonstrated as sensitive enough to tease out a pathology that has occurred following this blast exposure, seen here as a lack of SoFiA acquisition. Interestingly, this closely models pathology in humans, in which we see psychosocial deficits with a lack of other motor or cognitive deficits following exposure to a blast mTBI. This correlation indicates an additional validity of the SI-hab model in modeling pathology with a similar inductor as we see in humans. This makes the SI-hab model a useful tool for studying psychosocial outcomes of blast exposure.

The work in this last chapter was done in an effort to begin to understand bmTBI to help those whom have suffered. This devastating occurrence has continued to rise and it is through research efforts that a better understanding of the effects that the bmTBI is having on the brain, the better we can assess the cause for the psychosocial deficits that result. The selective SoFiA deficit observed in these current experiments suggests bmTBI may lead to inability to learn via social support or it may lead to a diminished perception of social support and its emotional benefits. Such outcomes could manifest in a number of mental illness symptoms. This work allows us to begin the investigation into the future development of therapies for combat troops exposed to bmTBI. Considering the frontline treatment for many mental illnesses are based on the formation of social support such as interpersonal therapy and group therapies, specifically bmTBI patients may require more unique targeted treatment strategies for mental illness that can aid in the perception of social support.

Chapter 5: Significance and future directions

The work presented is the initial identification and validation of this preclinical animal model, in which the use of social familiarity is used to reduce anxiety-like behavior in rodents. The behavioral testing paradigm that I used was based on previous work in this lab, the Social Interaction-habituation test (SI-hab). With this testing paradigm, we were able to observe a behavioral phenomenon of social familiarity-induced anxiolysis (SoFiA). This model of SoFiA is a valid model of socially enhanced safety learning demonstrating face, predictive, and construct validity.

The model of SoFiA was validated through observing face, construct and predictive validity, as well as a pathogenic validity. Face validity is observed in this model through the observations that the presence of a familiar partner is anxiolytic in the face of a feared or anxiogenic stimulus. The human parallel to this behavior is seen in the social enhanced safety learning that accompanies training for an aversive job (e.g. soldiers and firefighters) as well as the social enhanced safety learning that occurs with therapist lead exposure therapies for phobias. Predictive validity is observed when effective treatments in humans also produce the predicted results in the animal model. We saw predictive validity with the cognitive enhancer D-cycloserine improved the acquisition rate of SoFiA, similar to its effects on humans in reducing the time needed for overcoming an aversive stimulus in exposure therapies. Construct validity is when the mechanism of action of the disease is similar in the model as it is in the human condition. Construct validity is demonstrated through the identification of the mPFC as a necessary neural structure for the expression of socially enhanced safety learning in the SoFiA model. I then found that we can use this model to identify social deficits resulting from a pathology inducing exposure to a blast mild traumatic brain injury (bmTBI).

Socially-enhanced safety learning in people is a commonly utilized strategy, but our understanding of the underlying mechanisms regulating this are limited. It is vital that we understand the ways in which behavior is normally regulated in order to understand aberrant behavior processing and regulation. The elucidation of the neural mechanisms underlying the regulation of behavior responses to social cues such as anxiety was previously prevented by the lack of a valid preclinical animal model. This model does not represent a panacea of all instances of socially enhanced safety learning, however, it is a representation of one type of safety learning, that we can now systematically observe and study. Now we can start to take the next

steps in our understanding of the neural circuitry regulating such an important element in human social interactions.

Translational significance

The social environment can have major impacts on the mental health of an individual, on the emotional regulation and response to the environment and ultimately the development of psychopathologies as well (Meyer-Lindenberg & Tost, 2012). It is for this reason that we continue to seek a better understanding of how behavior is regulated through social interactions and support. The rodent model of SoFiA is preclinical, and therefore direct translational applications of the discoveries here are limited and premature. However, the model of SoFiA was created to begin laying the groundwork on which we can build an understanding of the underlying neural circuitry that regulates this behavior. The implications of the knowledge gained can potentially be to understand environmental, genetic, physiological and developmental factors that influence socially enhanced safety learning.

More targeted therapies could be developed with a better knowledge of the circuitry involved. Patient populations with psychosocial deficits stemming from various psychological illnesses could potentially benefit through the knowledge we will gain about how anxiety is regulated through top-down cortical regulation of emotions. For example, the patient population that we modeled with the Blast exposure demonstrates that this preclinical model can potentially lead to future therapeutic applications of targeted therapies for patients that have unique psychological sequelae. Furthering this research, the group at Purdue is exploring the therapeutic applications of Acrolein as a biomarker for TBI as well as developing scavengers to reduce the initial increase in the Acrolein levels that we saw in the initial days following the exposure to Blast. Another patient population that we could target in the future is patients with attachment disorders. People with attachment disorders are unable to utilize social support to overcome anxiety in some instances, and an understanding of aberrations in the underlying circuitry will help in the development of therapeutics for these patients (Ditzen et al., 2008).

Limitations to the SoFiA Model

The model of SoFiA contains some limitations that must be noted. First, it is important to note that both the strains of Wistar and Sprague Dawley rats are albino, with pigment free red eyes, which leads to these two strains having lower visual acuity than pigmented or wildtype strains (Prusky, Harker, Douglas, & Whishaw, 2002). The bright light of the BLC could potentially

be painful to these albino rats, much more than colored rats and it is a limitation that we do not know whether the light is leading to physical discomfort for the animals. High intensity light can be potentially harmful and degenerating to the vision of albino rats (De Vera Mudry, Kronenberg, Komatsu, & Aguirre, 2013). However, the light intensity is high for our studies, but lasts only a relatively short duration during each session. If the BLC was prohibitively painful to the rats during this time, we would see that the SI times are not consistent, and later, that under the BLC, an anxiolytic effect would not be attainable. If the rats where in too much pain to socially interact, there would be no increases in SI times at any point and therefore no acquisition of the social familiarity-induced anxiolysis.

The specific sensory inputs that are necessary for the acquisition and expression of SoFiA are still unknown. Other sensory modalities have not been explored within this model. It is unknown the specificity of the partner's presence for the acquisition of SoFiA, or whether other visual or olfactory stimuli would be sufficient for this effect. Additional experiments are needed to elucidate the specific sensory inputs that are necessary and sufficient for this behavior.

Additionally, we saw that the anxiolytic effect of SoFiA was sensitive to the anxiogenic conditions, and that the anxiolysis did not cross over to other anxiogenic stimuli such as the restraint stimulus. It may be that there were errors in the application of the restraint stimulus, or that SoFiA expression is highly specific to the acquisition conditions. I have not been able to reproduce the SoFiA effect with other anxiogenic stimuli as of yet, and so future work is needed to elucidate the power of a social presence in reducing anxiety and possibly fear.

Neural Circuitry elucidation

We have remaining gaps in knowing the full neural circuitry of the SoFiA acquisition and expression construct. Further testing needs to be performed to fully understand the neural mechanisms that control each of the constructs of SoFiA, and how these areas work together. The constructs of SoFiA that will be isolated and explored are the circuits involved in the social memory formation, anxiety behavior induction, safety learning and anxiolytic behavioral output.

We see that the IL is involved in the expression and acquisition of the SoFiA effect, as inhibition of this area prevents the behavioral response. The IL as a critical hub for the integration of social memory and anxiety behavior make it a possible node of regulation and the purpose for the targeted injections within this thesis. Social memory is specifically integrated in the CA2 region of the dorsal hippocampus (Hitti & Siegelbaum, 2014). Possible connections

between the Hippocampus and the IL could be to integrate the social memory and the association of the social memory with safety. To explore any IL connections with social memory information, an approach could be to inhibit the IL during social recognition testing. Targeting the IL during the Acute Social Recognition test will indicate if IL inhibition will block access to social familiarity knowledge. This will provide insight into how the IL inhibition is affecting the lack of the acquisition and expression of SoFiA. Additionally, the IL could be targeted with Muscimol in the 24-hour Social Recognition test, which would involve further inhibition of the IL after the social familiarity test and prior to re-testing 24 hours later. Any social recognition deficits would demonstrate if we could block consolidation of the social memory, if the social memory draws upon the IL in any way during the Social Recognition test.

Numerous connections exist between the amygdala and other areas of the brain associated with social processing that have been identified through human imaging studies (Bickart, Dickerson, & Barrett, 2014). Additionally, Truitt and colleagues previously found that ablation of the NK1 receptor-containing interneurons within the amygdala led to total impairment of SoFiA acquisition, demonstrating an important role of these interneurons in learning SoFiA (Truitt et al., 2007). Further studies into the role of the amygdala, in which cellular activation visualization through cFos expression is being done to identify the possible role of the amygdala during the acquisition and expression of SoFiA. Additionally, other structures can be further explored for a role in modulating anxiety behavior within the SoFiA model. The mesolimbic dopamine system is usually associated with reward, but is also involved in the response to stress stimuli, and increases in dopamine neuronal firing occurs in the projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) in response to stress stimuli (Trainor, 2011). The VTA projects to the limbic system, including the NAc, amygdala, hippocampus and frontal cortex, and activation in response to stressful stimuli indicates that this system is also involved in the response and possibly the regulation of behavior, although the functioning is still not fully understood (Trainor, 2011).

The acquisition and expression of SoFiA likely involves an integration of structures that have roles that evolve across time as the behavior shifts from anxiety to anxiolysis. Therefore, identifying the changing roles of the various structures across time is needed to better understand the regulation and change in behavior output. A time course study, in which the structures are examined for neural activity throughout the process of the SI-hab training protocol will be done.

Work with Females

By default, we worked with only male rats in all of the studies within this thesis, making this a significant limitation to our understanding of the model of SoFiA. However, being male is not the default, and it is important to note that sex differences exist in response to stress. For example, the menstrual cycle of humans causes variations in the HPA axis response to stress (Kajantie & Phillips, 2006). For example, estrogen has effects on anxiety-like behavior in rats, being anxiolytic in high doses (Pandaranandaka, Poonyachoti, & Kalandakanond-Thongsong, 2009). Additionally, sex differences have been found in the central nervous system. The BNST is sexually dimorphic, which given its role in the response to stress and anxiety, could help to explain the any differences that emerge between sexes (Allen & Gorski, 1990). Females have been shown to have different levels of brain-derived neurotrophic factor protein expression within the BNST in response to social defeat stress compared to males, demonstrating a very specific sex difference in the response to different social stimuli (Greenberg et al., 2014). Sex differences also exist in the expression of dopamine in the nucleus accumbens following social defeat, with males having higher increases in dopamine protein expression following the defeat in both mice and rats (Campi, Greenberg, Kapoor, Ziegler, & Trainor, 2014). Sex differences have been found in the presence of antecedent adolescent social isolation and the vulnerability to ethanol consumption (Butler, Carter, & Weiner, 2014). They found that male long evans rats were vulnerable to this type of social isolation while female long evans rats were not.

This evidence points to the fact that major sex differences exist in the response to both stress and social stimuli and it would behoove me to in the future develop a way to work around, or with, the Estrus cycle to incorporate females into my research. The sex differences in social behavior could have unique outcomes to the SoFiA effect, but I expect that females also would be capable of gaining social support through social familiarity in order to overcome an anxiogenic stimulus as well.

Age and SoFiA

Modeling human behavior and anxiety can be sensitive to the developmental stage in which we are modeling. For example, early life stress in the form of emotional abuse measured in human males reduced the functional connectivity of the prefrontal anterior cingulate cortex with the amygdala as measured with fMRI (Fan et al., 2014). This loss of functional connectivity points to a breakdown in the regulatory ability of the prefrontal cortex over the emotional

responses elicited by amygdala activation, and that this loss of regulation can begin with early life stress. This also means that when working with rodent models, the age of the rodent is important to consider. Stressors can have different impacts on animals depending on the time and stage of development of the animal (Sandi & Haller, 2015).

Studies have found Social Isolation in post weaned rodent pups leads to reductions in volume of medial amygdala and right medial PFC in rats (Cooke, Chowanadisai, & Breedlove, 2000; Schubert, Porkess, Dashdorj, Fone, & Auer, 2009). The area of the hypothalamus has been shown to increase in volume following maternal separation stressor in male rats. This stressor associated increase in hypothalamus volume can possible confer differential behavioral responses and possibly play a role in the regulation of the increased anxiety-like behavior seen in these rats (Irles, Nava-Kopp, Moran, & Zhang, 2014). Differences between early life and later life social isolation exist in rodents. Juvenile (4-5 weeks) and adult (6 weeks+) rats have been shown to have differential responses to social isolation, decreasing motivation for social contact and increasing aggressive behavior in juveniles, while increasing social behavior in adults (M. Toth, Halasz, Mikics, Barsy, & Haller, 2008; Van den Berg et al., 1999).

The current work uses animals that are adult age (3-4 months), which undergo a short bout of social isolation upon arrival at the facility prior to testing. A future direction in my work in SoFiA with rats could be to include rats that had an early life stressor. Early life stress would involve maternal separation, a possible diathetic model used to identify any SoFiA deficits that could occur later in life as a consequence of the early stress exposure. Exposure to anxiogenic-inducing stimuli during Post-weaning time points but prior to adult stage (postnatal day ~75), could have impacts on the ability of the rat to acquire SoFiA. A social isolation model in which Dr. Weiner and colleagues have involves socially isolating rats on the day of weaning on postnatal day 21, until postnatal day 70 (Skelly, Chappell, Carter, & Weiner, 2015). They found that this type of social isolation led to increases in anxiety in the Elevated Plus Maze, disruptions in fear extinction leading to increased fear potentiated startle (Skelly et al., 2015).

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Curriculum Vitae

Elizabeth Ann Lungwitz

Education

2003-2005 University of Iowa Iowa City, IA
Biology Major

- Made Deans List Fall 2003 and Spring 2004
- Inducted into the National Society of Collegiate Scholars in 2004
- Maintained a cumulative GPA of 3.4

2006-2008 Valdosta State University Valdosta, GA
Biology Major

- Dean's List Spring/Summer/Fall 2006, Spring/Fall 2007, Spring 2008
- Graduated Cum Laude May 2008 with cumulative GPA of 3.58

2009-2017 Indiana University Indianapolis, IN
Medical Neuroscience Ph.D. Program

- Graduate research assistant, Laboratory of William Truitt
- Medical Neuroscience Major, Life Sciences Minor
- PhD. Candidacy Fall 2011
- Cumulative GPA 3.81

Research and Training Experience

June 2008-July 2009 Lab of Dr. Gannon, Circadian Neurobiology
Valdosta State University Valdosta, GA
Laboratory Technician

- Perform experimental procedures with hamsters
- Maintain standards of care and husbandry for hamsters
- Coordinate lab activities with student assistants
- Train student assistants in lab techniques

September 2006-May 2008 Lab of Dr. Gannon, Circadian Neurobiology
Valdosta State University Valdosta, GA
Student Laboratory Assistant

- Perform immunohistochemistry procedure on hamster brain tissue
- Maintained standards of care and husbandry for hamsters
- Assisted in experimental procedures with hamsters

E. Castillo: Alone in the Dark: Drugs, Rhythms and Hamsters. Invited talk at the Council for Undergraduate Research Faculty/Student Colloquia. November 19, 2008.

** E. Castillo; former name.

Professional Experience

University of Iowa Hospitals and Clinics Fall 2003-Spring 2004

Beta Beta Beta member Fall 2006-May 2008
- Treasurer Fall 2007-May 2008
- Volunteered Habitat for Humanity September 30th 2006 and September 15th 2007
- Volunteered at the Math and Science Extravaganza November 3rd, 2006
- Organized car wash fundraiser October 20, 2007 for Halloween gifts for children at The Haven Women's Shelter

Started Students for Stem Cells Club at VSU
- Vice-President Jan 2007-May 2008
- Petitioned and received full club status fall 2007
- Volunteered at America's Second Harvest for Make a Difference Day
- Organized clothing drive for The Haven November-December 2007
- Organized Relay for Life team April 2008

IBMG Recruitment Weekend Jan/ Feb 2010, Feb 2011
- Student Ambassador

Summer Mentorship
- Mentored and taught summer students in techniques and procedures within the laboratory
- Project SEED, 2011, 2012, 2013, 2015, 2017
- Independent student volunteer, 2012, 2013
- Undergraduate Neuroscience Research Program, 2014

Awards

Best Poster Award- Science Division
- Participated in Council for Undergraduate Research Symposium April 2007
- Presented poster entitled *PER1* Gene Expression in the Hamster Suprachiasmatic Nucleus.

Travel Award from Center for Applied Research
- Participated in Society for Neuroscience Convention November 5-8, 2007 in San Diego
  - Presented poster entitled Post-light Temporal Efficacy of 5-HT1A-mediated Potentiation of Circadian Phase Shifts in the Syrian Hamster.

- Finalist for Student Employee of the Year 2007-2008

- Nominated Outstanding Student Award Spring 2008

Publications

Peer reviewed articles

Sanford, A. E., Castillo, E., Gannon, R.L.: Cannabinoids and hamster circadian activity rhythms, *Brain Res.* (2008); 1222: 141-148.
** E. Castillo; former name.

Lungwitz, E. Gannon, R.L., Serotonin$_{1A}$-mediated amplification of light-induced phase advances of circadian rhythms in the Syrian hamster: Post-light effects, *Brain Res*. (2008), doi:10.1016/j.brainres.2008.11.010.

R. Gannon; E. Lungwitz; N. Batista; I. Hester; C. Huntley; A. Peacock; P. Delagrange; M. Millan. The Benzodiazepine Diazepam Demonstrates the Usefulness of Syrian Hamsters as a Model for Anxiety Testing: Evaluation of Other Classes of Anxiolytics in Comparison to Diazepam. *Behavioural Brain Research*. (2011) 218: 8-14.

E.A. Lungwitz*, A. Molosh*, P.L. Johnson, B.P. Harvey, R.C. Dirks, A. Dietrich, P. Minick, A. Shekhar, W.A. Truitt. Orexin-A induces anxiety-like behavior through interactions with glutamatatergic receptors in the bed nucleus of the stria terminalis of rats. *Physiol Behav*. (2012) 107(5): 726-32. doi: 10.1016/j.physbeh.2012.05.019. Epub 2012 May 28

Lungwitz EA, Stuber GD, Johnson PL, Dietrich AD, Schartz N, Hanrahan B, Shekhar A, Truitt WA. The role of the medial prefrontal cortex in regulating social familiarity-induced anxiolysis. *Neuropsychopharmacology*. (2014) 39(4):1009-19. doi: 10.1038/npp.2013.302. Epub 2013 Oct 25.

Lungwitz E.A.*; Race N.S*; Vega Alvarez S.M.*; Warner T.R.; Andrews K.; Shekhar A.; Shi R.; Truitt W.A. Selective psychosocial-like impairment after blast traumatic brain injury correlates with urine biomarker in rats. *(In progress).*

Book Chapter

- Sreeparna Majumdar*, Elizabeth A. Lungwitz*, Katharine D. Andrews*, Joanna E. Chambers and William A. Truitt. (2018). Animal models to investigate social support induced anxiety reductions. In Sangha S. & Foti D. (Eds.), *Neurobiology of Abnormal Emotion and Motivated Behaviors.* Cambridge, MA: Academic Press. ISBN: 9780128136935

Published Abstracts

E. Castillo, R. L. Gannon; *PER1* Gene Expression in the Hamster Suprachiasmatic Nucleus. Abstract. Council for Undergraduate Research Symposium. 2007.

Castillo E., Gannon, RL; Post-light Temporal Efficacy of 5-HT1A-mediated Potentiation of Circadian Phase Shifts in the Syrian Hamster. Society for Neuroscience Annual Meeting. 2007(on CD).

E. Castillo, R. L. Gannon; ERK Gene Expression in the Hamster Suprachiasmatic Nucleus. Abstract. Council for Undergraduate Research Symposium. 2008.

Gannon, R. L., Sanford, A. M., Castillo, E., Cannabinoid Modulation of Hamster Circadian Activity Rhythms. Abstract. International Behavioral Neuroscience Society Annual Meeting. 2008.

Lungwitz, E.; Johnson, P.; Harvey, B.; Deal, R.; Dietrich, A.; Minick, P.; Shekhar, A.; Truitt, W. Orexin-A injections into the BNST induced Anxiety-Like Behavior via Interactions with Glutamatergic Receptors in the rat. Abstract. International Behavioral Neuroscience Society Annual Meeting. 2011.

E.A. Lungwitz; S. Janasik; L. Federici; S. Fitz; W.A. Truitt; P.L. Johnson; A. Shekhar. Behavioral and Molecular Characterization of Serotonin Transporter Deficient Rats Across Development Stages. Abstract. Society for Neuroscience Annual Meeting. 2011.

Lungwitz, E.A.; Sanghani, S.; Harvey, B.; Bah, A.; Dietrich, A.; Truitt, W.A. The Cognitive override of anxiety is accomplished by social familiarity and is mediated by the medial prefrontal cortex. Indianapolis Chapter of the Society for Neuroscience Annual Meeting. 2012

Truitt W.A.; Lungwitz E.A.; Minick P.; Dietrich A.; Shekhar S. Social Familiarity, in rats, reduces anxiety through a medial ventral prefrontal cortex dependent pathway. Society for Neuroscience Annual Meeting. 2012.

Truitt, W.A.; Lungwitz E.A.; Dietrich A.; Minick P.; Shekhar A. Social interaction-familiarization, a valid preclinical model of social processing and behavioral therapy for anxiety. American College of Neuropsychopharmacology Annual Meeting. 2012.

Lungwitz, E.A.; Sanghani, S.; Harvey, B.; Bah, A.; Dietrich, A.; Truitt, W.A. The cognitive override of anxiety is accomplished by social familiarity and is mediated by the medial prefrontal cortex. International Behavioral Neuroscience Society Annual Meeting. 2012.

Truitt W.A.; Lungwitz E.A.; Hanrahhan B.; Walls M.K.; Shi R. Mild blast-induced brain injury produce social deficits, depression-like phenotype and exaggerated responses to anxiogenic cues in rats. Society for Neuroscience Annual Meeting. 2014.

Lungwitz, E.; Race, N.; Vega Alvarez, S.; Dietrich, A.; Shi, R.; Truitt, W. Blast exposure selectively induces psychosocial deficits in rats. Indianapolis Chapter of the Society for Neuroscience Annual Meeting. 2014.

Knight C.P.; Toalston J.E.; Lungwitz E.A.; Deehan Jr. G.A.; Hauser S.R.; Waeiss R.A.;McBride W.J.; Rodd Z.A. Is anxiolysis reinforcing? Ethanol has both rewarding and anxiolytic properties within the central nucleus of the amygdala. Research Society on Alcoholism Annual Meeting. 2015.

S. Majumdar, E.A. Lungwitz, R. Du, K.D. Andrews, A.D. Dietrich, W.A. Truitt. Elucidating the neural circuitry of social familiarity induced

anxiolysis. Society for Neuroscience Annual Meeting. 2016.

S. Majumdar, E.A. Lungwitz, N. Bharadwaj, K.D. Andrews, A.D. Dietrich, W.A. Truitt. Elucidating the neural circuitry of social familiarity induced anxiolysis. International Behavioral Neuroscience Society Annual Meeting. 2017.