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Targeted Helping in Rodents: Sex Differences and the Role of the Insula in Empathic Behavior

Stewart Shelby Cox
Medical University of South Carolina

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By
Stewart S. Cox

Department of Neurosciences

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Chairman, Carmela M. Reichel

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Sudie E. Back

_____
Heather A. Boger

_____
Brett E. Froeliger

_____
Thomas C. Jhou

_____
M. Foster Olive

ABSTRACT

STEWART SHELBY COX**.** Targeted Helping in Rodents: Sex Differences and the Role of the Insula in Empathic Behavior. (Under the direction of CARMELA REICHEL).

Empathy, the capacity for shared emotional valence with others, allows for cooperativity and social bonding between individuals. The evolutionary basis of the empathic behaviors observed across numerous species can be described using the Perception Action Model (PAM), in which shared affect can promote an action that eliminates the distress of both the "Target" and, by extension, the "Observer". However, clinical studies indicate empathy is dysregulated in neuropsychiatric disorders like autism and addiction, which makes the elucidation of underlying behavioral, affective, and neurobiological variables of empathy paramount. We first introduce and validate a novel model of targeted helping, in which rats learn to aid a distressed conspecific in the absence of social reward. Next, using this model, we identify sex differences in sensory and affective signaling, including the impact of direct visualization of a distressed conspecific, and the type of ultrasonic vocalizations (USV) made between animal pairs, during the task. Further, neuronal activity in cortical and subcortical regions of interest showed distinct sex-specific patterning across time during targeted helping. Finally, we directly examined the effect of the anterior insula, a region active during perspective taking and emotional regulation hypothesized to be a critical node in the empathic brain, during empathic behavior. We not only confirmed that the anterior insula was activated during the task, but inhibition of the insula, both pharmacologically and chemogenetically, significantly attenuated helping behavior. Further, tracer studies were performed in order to elucidate critical insula-specific circuits that may modulate targeted helping. These studies, using a newly validated model of targeted helping, work to inform the underlying affective and biological variables that modulate empathic behavior in the hopes of improving the treatment outcomes and quality of life of those diagnosed with neuropsychiatric disorders.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

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CHAPTER 1: Background and Significance

INTRODUCTION

Empathy is broadly understood as the capacity to share the feelings of another and generate an appropriate response to those shared feelings (Hoffman, 1975; de Waal, 2009; Lahvis, 2017; Meyza et al., 2017). It is a multidimensional concept that lays the groundwork for cooperation and prosocial altruistic behaviors, both of which are critical for the formation and maintenance of interpersonal relationships and societal cohesion (Decety, 2011; de Waal and Preston 2017). Empathic processes that help shape social behaviors and ultimately guide complex societal norms are therefore critical for reproduction and individual and group survival (Decety et al. 2015; Sivaselvachandran et al., 2016; Adriaense et al., 2020). Further, it is becoming more apparent that empathy is dysregulated in numerous DSM-V-defined psychiatric disorders. An increasing number of studies have concluded patients with diagnoses of Substance Use Disorder (McCown, 1989; McCown, 1990; Massey et al. 2018), Major Depressive Disorder (Cusi et al. 2011; Ekinci and Ekinci, 2016; Kupferberg et al., 2016), Autism Spectrum Disorder and alexithymia (Bird and Cook, 2013; Cook et al., 2013; Harmsen, 2019), and Antisocial Personality Disorder (Mealy, 1995; Blair and Cipolotti, 2000; Decety and Moriguchi, 2007), to name a few, have blunted empathic behaviors that are correlated with the severity of the disease (Cusi et al., 2011) and a reduced quality of life (Kronmuller et al., 2011). The role empathy plays in social behaviors and the prevalence of its dysregulation in psychiatric disorders make the behavioral and neurobiological understanding of empathy paramount. Furthermore, the development and use of a translationally relevant rodent model allows for significant advancements in our current understanding of empathic behaviors. Because of the recent explosion of empathy-related research, this introduction seeks to outline the prevailing theories of empathy, the psychiatric disorders in which empathy is affected, the rat and mouse models of empathy currently used, and the important social, environmental and neurobiological factors that affect empathic behavior discovered using these models. There are

numerous recent reviews focused on the evolution (Decety, 2011; Decety et al., 2012) and comparison of empathy across species (Panksepp and Panksepp, 2013; Perez-Manrique and Gomila, 2017), as well as neural correlates of empathy based on fMRI studies (Bernhardt and Singer, 2012; Decety, 2015; Keysers and Gazzola, 2018; Marsh, 2018). There is currently no single agreed-upon rodent model of empathic behavior (Keum and Shin 2016; Uysal et al. 2020), in part because of its complexity. Moreover, specific models evaluate different behavioral outputs of components of what we broadly call empathy. Overall, however, rodent models could advance the treatment of psychiatric disorders through the elucidation of the underlying motivation and neurobiology of empathic behaviors.

THEORIES OF EMPATHY

Despite its ubiquity and importance, empathy has many definitions due to its multidimensional and complex nature (Preston and de Waal, 2002; Sivaselvachandran et al., 2016; Yamamoto, 2017; Uysal et al, 2020). Often, researchers will define the parameters of empathy to best fit their experimental question, and this often leads to discrepancies amongst researchers. Some early definitions of empathy were cognitively focused, putting emphasis on the ability to simulate how others are feeling, like Theory of Mind (Goldman, 2008). Empathy had been described as a "leap of imagination into someone else's headspace" (Baron-Cohen, 2005) or putting oneself in the place of another (Allport, 1937). These definitions, however, do not allow for a full affective description of what occurs during empathically-driven behaviors and was more exclusive to adult humans. In the 20th century, a new theory of empathy grew from the notion that empathy was an involuntary affective response in which individuals were able to "feel into," (Einfülung) without extensive cognition or reasoning (Lipps, 1903; Zahavi, 2008). Although most researchers accept empathy involves both aspects, much of the recent debate surrounding empathy has focused on this distinction between cognitive and emotional empathy (discussed in Preston and de Waal, 2002). Emotional empathy, often used interchangeably with emotional contagion, is a subcortical and evolutionarily conserved process by which one is able to state-match with a conspecific (Panksepp

and Panksepp, 2013; Keysers and Gazzola, 2018). Because of its relative simplicity, forms of emotional empathy have been observed in numerous animal species across the tree of life (Mogil 2012; Sivaselvachandran et al., 2016; Lahvis, 2017; Meyza et al., 2017). Cognitive empathy, on the other hand, is a top-down, cortically-driven process that allows for perspective-taking and targeted helping towards a distressed conspecific (Lamm, et al., 2007; Shamay-Tsoory et al., 2009; Lamm and Majdandžić, 2015; Barrett et al., 2016). Historically, cognitive empathy was thought to be exclusive to species with developed forebrains, such as non-human primates (de Waal, 2009; Yamamoto and Takimoto, 2012; Perez-Manrique and Gomila, 2017), and humans (Singer et al., 2004; Shamay-Tsoory, 2011; Bernhardt and Singer, 2012; Zaki and Ochsner, 2012). Although this distinction may be useful for studying similar phenomena across species (Panksepp and Lahvis, 2011) or distinguishing high-order, cognitively-driven empathy tasks in humans versus animals, it may hinder the advancement of translational rodent research of empathic processes. Further, growing evidence suggests behaviors canonically within the spectrum of cognitive empathy, such as targeted helping, are observed in rodents (Bartal et al., 2011; Sato et al., 2015; Bartal et al., 2016; Karakilic et al., 2018; Cox and Reichel, 2019). Therefore, it is likely these forms of empathy are highly interconnected in evolution and across species (de Waal and Preston, 2017). However, theoretical models of empathy continue to be numerous and differ across the research fields. In the next few paragraphs, we will briefly outline some of the current and more prominent theoretical frameworks for understanding empathic behaviors.

Perception -Action Model of Empathy

A major milestone came with Preston and de Waal's Perception Action Model of empathy (Preston and de Waal, 2002; de Waal, 2012; de Waal and Preston, 2017; Preston and de Waal, 2017). The model asserts that attended perception of an individual's affective state automatically activates the observer's representations of the state, situation, and target. Additionally, activation of these representations automatically primes or generates the associated autonomic and somatic responses unless inhibited (Preston and de Waal, 2002). When an observer attends to another's

affective state, they are able to access information about them through an associative process evolved from the nervous system's ability to map others' states onto our own (Preston and de Waal, 2017). The PAM lies at the core of empathy and the myriad component parts are built from it, similar to a Russian nesting doll (de Waal, 2012; de Waal and Preston, 2017). Simple empathic behaviors, like emotional contagion and motor mimicry, are nearest the PAM core of the nesting doll, while more complex behaviors, like perspective-taking and targeted helping, are the outermost layers of the doll and require additional cognitive capabilities and emotional self-regulation. However, all of these abilities are reliant and are built upon the PAM (Preston and de Waal, 2002; de Waal, 2012; de Waal and Preston, 2017; Preston and de Waal, 2017). This model allows for an understanding of how affective transfer may then promote helping behavior (Bartal et al., 2011; Meyza et al., 2017). Emotional transfer between a distressed target and an observer occurs, causing shared affect. The now distressed observer must self-regulate to perform an action (e.g. consolation or aid) to reduce the distress of the target and, by a second emotional transfer, themselves (de Waal and Preston, 2017). This model links cognitive and emotional empathy by their underlying process and links behaviors ranging from emotional contagion to altruistic behavior, as they all require or involve some level of affective transfer. It also sheds light on the fact that, although there is a top-down/bottom-up circuitry distinction, it has been overestimated or, at the very least, perseverated upon. There is empirical evidence to support this theory in numerous species (Perez-Manrique and Gomila, 2007) including rats and mice (described in the Models and Affective Transfer and Models of Targeted Helping sections). The PAM's simplicity and usefulness in the evolutionary development and translational understanding of empathic behaviors makes it one of the most widely cited theories of empathy today.

Three Factor Model of Empathy

While the PAM is a widely accepted model of empathy, there are others that have differing theoretical frameworks. Some argue that the "elegant simplicity" of the PAM may not sufficiently describe the underlying complexity of empathy (Hollis and Nowbahari, 2013; Yamamoto, 2017;

Adriaense et al., 2020). Yamamoto suggests that, while many animals do exhibit empathic behaviors, the species differences and the cognitive requirements of the observed behaviors may be interpreted in a way other than a simple, linearly developing model (Yamamoto, 2017; Adriaense et al., 2020). In the PAM, it is assumed that more complex empathic behaviors are built linearly from more evolutionarily-conserved ones (Preston and de Waal, 2002). However, according to Yamamoto, it is not necessarily the case that helping behaviors are present if more simplistic state matching is observed. For example, chimpanzees are highly capable of perspective-taking, but they often do not engage in helping behavior proactively and instead react to begging (Yamamoto and Tanaka, 2009; Yamamoto et al., 2009). For this reason, Yamamoto proposes a re-organization into a combinatorial model. It is therefore suggested that three main component factors of empathy, matching with others, understanding of others, and prosociality, can be present individually or in combination with one another. This theory focuses on the complexities of species-specific differences and cognitive sophistication in empathic behaviors. Yamamoto's theory allows for independent evaluation of, as well as the interaction between, the separate components of empathy, especially through the lens of comparative evolutionary biology.

Dualistic Theories of Empathy

There are other theoretical models of empathy that, based on the research question or model used, focus more specifically on the elucidation of human empathy. As such, a more strict distinction between cognitive and emotional empathy is maintained within each theory. For example, the Dual Route Model of Empathy (Yu and Chou, 2018) was developed to understand the neural mechanisms of affective and cognitive empathy. The authors proposed that empathy, like emotion generally, has a "low road" and a "high road" (LeDoux, 1998). A fast, subcortical, short-latency pathway drives the affective "low road" of empathy that is processed automatically and subconsciously. In contrast, the slower, more complex and conscious process of cognitive empathy is moderated by attention and involves cortical structures more exclusively (Bernhardt and Singer, 2012; Decety 2015; Marsh, 2018). These routes, although separated by their neural

substrates, are hypothesized to be interconnected in several ways, such as familiarity with others (Levine et al., 2005; Han and Northoff, 2008; Serino et al., 2009; Liew et al., 2011) and prior experience (Lamm et al., 2011; Meyer et al., 2012,). Further, prosocial behavior, they suggest, flows from both routes because, "people who share and understand others' mind will finally care about others and generate desires to help others" (Yu and Chou, 2018). Additionally, activity in brain regions that are associated with both affective (e.g. amygdala, anterior insula) and cognitive empathy (e.g. dorsolateral prefrontal cortex, anterior cingulate cortex) in fMRI studies can positively predict the willingness to perform prosocial behaviors (Singer et al., 2008; Rameson et al., 2012; Waytz et al., 2012; Keysers and Gazzola, 2018).

Another framework known as the Self-Other Model of Empathy (SOME), addresses the complexity of "how empathy is shared" or how affective and cognitive systems allow for empathy to be experienced (Bird and Viding, 2014). The SOME is comprised of several subsystems, all of which are required to meet the criteria for an empathic process. These systems are separated into the following: the situation understanding system (which provides information on emotional state of another), the affective representation system (which represents the currents affective state of the self), the affective cue classification system (which allows for emotional pattern matching to signal the affect state of another), and Theory of Mind system (which represents the mental states of self and other) (the mirror neuron system component of the theory is outside the scope of this review but Bird and Viding, 2014 provides an excellent discussion). According to the authors, emotional contagion can occur using these systems once the affective state of one is matched in another and the perceiver understands that state is being experienced by the other. However, empathy only occurs following the self-other switch. In brief, this switch is the process by which the empathizer pays more attention to the other compared to self and the resulting affective state is more appropriate to the conspecific compared to one's own. The self-other switch indicates the empathizer's emotional state matches the other through Theory of Mind. This model puts forth a framework of a more 'advanced' form of empathy directed more towards specifically

understanding empathy in human research of psychiatric disorders. In fact, in a study of individuals with alexithymia, researchers concluded enhancing self-other distinction potentiates alexithymics' ability to use advanced empathy, but not primitive empathy (Saito et al, 2016).

Empathy is a highly complex emotional state that can be manifested and measured in many ways. Developing an underlying theoretical framework that is able to incorporate every aspect and nuance of empathy may not be feasible, or warranted, across all fields of study. Some theories, like the SOME, seem to have an anthropocentric understanding of empathy framed to best answer questions derived from human experimentation. On the other hand, the PAM of empathy is more focused on the evolutionary development and comparison of empathic processes across species. In this dissertation, I do not wish to take a stance on the validity of any one theory, as I acknowledge the scientific question may inform the theory used. However, because this article describes the importance of evaluating empathic behaviors translationally through rodent models to improve our understanding of empathy and our treatment of psychiatric disorders, the discussions within this article will be framed through the lens of the PAM of empathy.

PSYCHIATRIC DISORDERS WITH ALTERED EMPATHIC PROCESSES

The translational study of empathy has garnered increasing attention due to the growing research indicating empathic processes are affected by, or contribute to, a majority of Diagnostic and Statistical Manual (DSM)-V-defined psychiatric disorders (2013). In the following section, we will discuss some of these disorders and how empathy is dysregulated in each.

Substance Use Disorders

To date, only a handful of clinical studies have considered the relationship between empathy and Substance Use Disorders (SUD), yet the consistency of the results is striking. Stimulant users, poly drug users, and those with Alcohol Use Disorder all show impairments in empathy (McCown 1989; McCown, 1990; Massey et al., 2018; Robinson et al., 2018). Further, targeting empathic processes may be a treatment-modifiable risk factor for maintaining drug-free abstinence. For example, higher levels of empathy, as evaluated by questionnaire, in patients

participating in twelve-step addiction programs was correlated with enhanced involvement in such programs and prolonged abstinence periods (McCown, 1989; McCown, 1990). These findings, alongside evidence that social support and maintenance of personal relationships via empathy improves the overall success of treatment outcomes in SUD, suggest restoration of empathic behaviors in individuals suffering from SUD may improve treatment outcomes and reduce the chance of relapse (Massey et al., 2018; Robinson et al., 2018).

Major Depressive Disorder

Along these same lines, patients suffering from Major Depressive Disorder (MDD) display overall lower empathy, emotional accuracy, and affective response compared to control patients (Cusi et al., 2011; Ekinci and Ekinci, 2016; Kupferberg et al., 2016). One conceptual framework posits that empathic dysregulation as a consequence of major depression causes social impairments (Kupferberg et al., 2016), strains interpersonal relationships (Kronmuller et al., 2011), and may be positively correlated to the severity of the disorder (Cusi et al., 2011).

Autism Spectrum Disorder

Many researchers agree that empathy, broadly defined, is altered in those diagnosed with Autism Spectrum Disorder (ASD). The underlying cause of the deficits in empathy is poorly understood, although some posit that children with ASD have difficulties expressing or feeling emotion, which prevents them from engaging empathically with others (Decety and Moriguchi, 2007). Others believe that empathic deficits can be more specifically tied to alexithymia, the understanding and processing of one's own emotions, as opposed to the more heterogeneous spectrum of symptoms that underlie autism. Cook et al. (2013) demonstrated that, in those patients with ASD, the severity of co-occurring alexithymia, not the severity of the ASD, directly correlated to reduced emotional recognition. Focusing on understanding and evaluating changes in empathy could have both diagnostic and therapeutic implications for ASD (Harmsen, 2019) by maintaining a distinction between social and emotional impairments, with ASD being associated with the former, not the latter (Bird and Cook, 2013).

Personality Disorders

There are numerous personality disorders described in the DSM V that exhibit dysregulated empathic processes. Antisocial Personality Disorder is, by its very nature, defined by a paucity of empathy towards others. This deficit has been postulated to originate from a reduced ability to feel and respond to changes in others' emotional states (Blair, 1995). However, it has been suggested the reduced empathic ability could be related to a disruption in the affective processing of emotion rather than the inability to adopt the emotions of others (Decety and Moriguchi, 2007). Higher-order emotional abilities are also disrupted in Bipolar Disorder. Bipolar individuals have augmented emotional recognition and affective responsiveness but maintain perspective-taking similar to that of healthy controls. This may suggest they have difficulty identifying emotion on other's faces and identifying emotions they would experience in the same situation, but they may still be able to take the perspective of another, depending on the severity of their symptoms (Seidel et al., 2012). Interestingly, empathic dysregulation in patients with Bipolar Disorder may depend on whether they are experiencing an acute manic or depressive episode. For example, cognitive empathy was attenuated during both manic and depressive episodes, but affective empathy was potentiated during a manic episode while remaining unchanged in a depressive one (Bodnar and Rybakowski, 2017).

Schizophrenia

Finally, studies have consistently demonstrated patients with schizophrenia have blunted cognitive empathy, including Theory of Mind, emotional processing, and affective face processing (Savla et al., 2013; Green et al., 2015; Varcin et al., 2019), the degree of which was correlated to the severity of schizophrenic symptoms (Shamay-Tsoory et al., 2007). However, the changes may be more nuanced as they related to emotional empathy. For example, one meta-analysis concluded affective empathy is disrupted in patients with schizophrenia (Bonfils et al., 2016), and another study demonstrated schizophrenics have blunted emotional expressivity to faces compared to controls (Varcin et al., 2019). In contrast, affective sharing, as defined by the activation of the

emotion-related neural substrates in a person observing the emotional expression of another (Green et al., 2015), were comparable between healthy controls and patients with schizophrenia. In some cases, schizophrenics reported significantly higher reactivity to the feelings of others (Achim et al., 2011; Michaels et al., 2014). The subtleties regarding empathic dysregulation in schizophrenia likely means subcomponents of empathy, like emotional processing, should be treated and trained individually (Green et al., 2015). Overall, continual improvement in the understanding of the association between schizophrenia and empathy will improve the social disability and overall quality of life in schizophrenic individuals.

This list, far from extensive, is expanded upon in review articles focused on empathic dysfunction in psychiatric disorders (Decety and Moriguchi, 2007; Farrow and Woodruff, 2007). However, the multitude of disorders with dysregulated empathy demonstrates translational models of empathic behavior are imperative to elucidate its underlying neurobiology, as well as directly discern the relationship between psychiatric disorders and empathic dysregulation as a potential diagnostic and therapeutic tool to clinical populations.

MODELS OF AFFECTIVE TRANSFER

The study of empathy in rodents began in earnest in the 1950-1960s with two seminal studies. In the first, by Russel Church (1959), rats learned to stop pressing a lever that had previously been associated with an appetitive food reward when it became associated with an aversive shock stimulus to a rat in an adjacent cage. This was the first evidence to intimate rodents were capable of recognizing and sharing the affective state of a conspecific. Soon thereafter, Rice and Gainer (1962) utilized an apparatus in which a rat was hoisted off of the ground to generate distress. A second animal was able to quickly, and without prior training, learn to press a lever to lower the conspecific back to the ground, thereby alleviating its distress. These initial studies spurred a great debate over animal empathy that continues to this day. Because of their seminal work, an exponential number of experiments have been performed to better explain the phenomena observed in their studies. Importantly, they also inspired a myriad of research and novel behavioral

models. One such line of research is the evaluation of consolation behavior in prairie voles (*Microtus ochrogaster*) as a proxy for empathy. Prairie voles are uniquely useful for the understanding of social affiliation because they form and maintain monogamous pair bonds (Bosch and Young, 2018; Lee et al., 2019). Recently, research has also determined they are capable of emotional contagion, state-matching, and self-other discrimination (Burkett et al., 2016; Meyza et al., 2018; Stetzik et al., 2018). Moreover, these prosocial behaviors have been shown to be modulated by the neuropeptides oxytocin and vasopressin in a sex-dependent manner (Winslow et al., 1993; Wand and Aragona, 2004; Burkett et al., 2016; Tickerhoof and Smith, 2017). This research has been widely reviewed (Demas and Jansow, 2016; Tabbaa et al., 2016; Bosch and Young, 2018; Meyza et al., 2018; Pohl et al., 2019), and therefore the following sections focus on models that use rats and mice to evaluate empathic behaviors of varying cognitive degrees. The major findings using each of the models discussed below are summarized in **Table 1**.

Table 1. Rodent models of empathy, along with species tested, main behavioral findings, as well as any other manipulations performed, and neurobiology discovered with each model. Table is continued through page 14.

Model of Empathic Behavior	Species Studied	Outcome
Emotional Contagion - Behaviors		
Langford et al., 2006	mice	Potentiation of pain-related behaviors tested in the presence of a familiar conspecific that also received painful stimulus
Smith et al., 2016 Carnevali et al., 2017	mice rats	Hyperalgesia observed in a "bystander" animal tested in the same environment as a conspecific that received painful stimulus, even without the shared experience of the stimulus
Li et al., 2018 Du et al., 2019	rats rats	Prosocial behaviors (allo-licking and -grooming) made by "bystander" directed at conspecific that received noxious stimulus
Li et al., 2014 Martin et al., 2015	rats mice	Expression of emotional contagion may be modulated by circulating glucocorticoid levels, depending on the type/severity of noxious stimuli used
Laviola et al., 2017	mice	Low levels of emotional contagion (paw licking) correlated with potentiated oxytocin and vasopressin levels in behaviorally-relevant brain areas absent any changes in receptor density. Low emotional contagion also associated with impaired reactivity to external stressors
Emotional Contagion – Brain Areas Associated		
Li et al., 2014	rats	Prefrontal cortex (PFC)
Knapska et al., 2006	rats	PFC and amygdala
Zaniboni et al., 2018	mice	Insula
Observational Fear Learning - Behaviors		
Bredy and Barad, 2009	mice	Context-dependent modulation of the social transmission of fear learning to an observer from a conspecific
Guzman et al., 2009	mice	
Bruchy et al., 2010	rats	
Knapska et al., 2010	rats	
Atsak et al., 2011	rats	
Jeon and Shin, 2011	mice	
Sanders et al., 2013	mice	
Jeon et al., 2010	mice	

Jeon and Shin, 2011	mice	Vicarious freezing behavior due to affective transfer to observer from conspecific receiving footshocks
Hernandez-Lallement et al., 2020	rats	Instrumental harm aversion tested when observers became directly responsible for the footshock of the conspecific. This behavior was shown to be subject to effort
Guzman et al., 2009 Watanabe 2011; 2015 Kiyokawa et al., 2014	mice mice rats	Social buffering, both passive and active, modulated observational fear learning in observer. This phenomenon is dependent on the level of familiarity with the fearful conspecific
Pisansky et al., 2017	mice	Oxytocin administration, or chemogenetic activation of oxytocin neurons in the paraventricular nucleus of the hypothalamus (PVN), caused enhanced sensitivity to distress of the conspecific
Guzman et al., 2014	mice	Oxytocin infusion into Lateral septum (LS) accelerated social buffering of observational fear
Observational Fear Learning - Brain Areas Associated		
Pisansky et al., 2017	mice	PVN
Guzman et al., 2014	mice	LS
Jeon et al., 2010 Carrillo et al., 2019	mice rats	Anterior cingulate cortex (ACC)
Restraint Stress Model - Behaviors		
Bartal et al., 2011 Ueno et al., 2019a	rats mice	Animals learned to release a conspecific from a restraint tube
Bartal et al., 2014	rats	Release of a conspecific is modulated by social experience and familiarity
Ueno et al., 2019b	mice	Underlying motivation for release behavior in mice is less clear and may be driven by social interest and curiosity with the apparatus
Blystad et al., 2019	rats	Release behavior may be modulated or dependent on contents of the container
Silberberg et al., 2014	rats	Release behavior may be modulated or dependent on social contact
Carvalheiro et al., 2019 Bartal et al., 2011	rats rats	Release behavior may be modulated or dependent on alternative choices afforded to the observer
Bartal et al., 2016	rats	Midazolam administered to observers before the session reduced door openings
Restraint Stress Model – Brain Areas Associated		
Tomek et al., 2020	rats	Insula
Soaked Conspecific Model - Behaviors		
Sato et al., 2015	rats	

Cox and Reichel, 2019	rats	Animals learned to release a "soaked conspecific" into a dry chamber. Release behavior specific to presence of conspecific, and previous experience in the distressing condition potentiates the helping behavior
Karakilic et al., 2018	rats	Mild stress (footshock) reduced latency to release distressed conspecific
Yuksel et al., 2019	mice	8 weeks of voluntary wheel running improved door opening latency in females
Yamagishi et al., 2019	rats	Oxytocin injections enhanced early acquisition of helping behavior in single-housed, but not pair-housed rats
Ueno et al., 2019b	mice	Oxytocin had no effect on door opening
Kandis et al., 2018	rats	Dose-response relationship observed between acetaminophen concentration administered and reduction in door opening latency

Targeted Helping - Behaviors		
Cox and Reichel, 2019	rats	Rats will release distressed conspecific from a pool of water independent of social interaction. Release behavior is specific to a distressed conspecific, modulated by previous experience, and is dependent on effort

Emotional Contagion

The ability to share an affective state of pain with another has been widely explored in human fMRI studies (Keysers and Gazzola, 2007; Bernhardt and Singer, 2012; Keum and Shin, 2016). In fact, Church's rodent model took advantage of this phenomenon, suggesting for the first time rats are able to recognize and share the affective state of a conspecific (Church, 1959). In a typical model of emotional contagion of pain, animals are subjected to a painful stimulus and placed with an animal that undergoes the same, a different, or no pain treatment, and pain related behaviors like writhing and paw licking are assessed. Mice tested with another animal that also received a painful stimulus (intraperitoneal acetic acid injection or subcutaneous formalin injection) displayed potentiated pain related behaviors as compared to mice that were tested alone or with a non-treated mouse (Langford et al., 2006). A bidirectional modulation of pain behavior occurred in familiar mice; pain behavior was potentiated when the partner received a more noxious stimulus and decreased when the partner received a less noxious stimulus (Langford et al., 2006). However, these effects were not observed when stranger mice were used. Instead, pain related behaviors were attenuated if the conspecific was unfamiliar (Langford et al., 2006). The apparent analgesic effect of an untreated stranger mouse suggested the social threat of an unfamiliar conspecific may play a role in the reduction of the pain behaviors. These results indicate mice are capable of emotional contagion, a more primitive form of empathy, and the social threat of an unfamiliar animal may attenuate it.

Stress also modulates observed emotional contagion in mice (Martin et al., 2015). Mice subjected to the social stress of an unfamiliar animal significantly reduced emotional contagion to a noxious stimulus. The observer mice paired with a stranger had higher levels of circulating glucocorticoids compared to mice paired with a familiar mouse or tested alone. Further, pharmacological inhibition of stress hormone synthesis enabled the expression of emotional contagion in the presence of an unfamiliar animal (Martin et al., 2015). However, other studies that demonstrate changes in emotional contagion in relation to familiarity do not show the same

glucocorticoid-mediated regulation of pain-related behaviors (Li et al., 2014), possibly due to a difference in the type and/or severity of the noxious stimuli used.

More directly related to empathic processes are accounts of emotional contagion of pain even when the experience is not shared. For example, mice will develop corresponding hyperalgesia by observing a conspecific experience pain (Smith et al., 2016). When a "bystander" mouse is housed and tested in the same environment as mice that were subjected to inflammatory (CFA injections) or alcohol or morphine withdrawal-induced pain, the bystander rats will develop corresponding hyperalgesia as measured through mechanical, thermal, and chemical sensitivity tests (Smith et al., 2016). Interestingly, observer rats will exhibit not only emotional contagion, but also prosocial consolation behaviors, like allo-licking and allo-grooming, towards the conspecific that received the painful stimulus (Li et al., 2018; Du et al., 2019).

Social defeat stress also taps into the emotional contagion of fear (Carnevali et al., 2017). In this case, a rat is exposed to a cage mate that was either previously exposed to an aggressive male or simply to a novel cage. Following exposure to a rat that had previously undergone social defeat, observer rats show state matching behaviorally (social avoidance) and physiologically (elevated heart rate and circulating corticosterone). In this version of the model, the social communication of interaction-mediated stress and fear between animals can be easily evaluated (Carnevali et al., 2017; Carnevali et al., 2020).

Emotional contagion is a highly conserved process that provides a rapid assessment and adaptation to social and environmental challenges (Hatfield et al., 1993). This behavior can be easily observed across species, including rats and mice (Meyza et al., 2018). All of the models currently utilized take advantage of the transfer of negatively valent emotions, such as fear or pain, purportedly due to a difficulty in standardization of models associated with positive affect transfer (Meyza et al., 2018). However, emotional contagion, due to its biological prevalence and importance, makes it a simple model ideal for the exploration of well-defined neuronal pathways

of empathy (Meyza et al., 2017; Meyza and Knapska, 2018; Zaniboni et al., 2018), as well as neurotransmitters involved in normal and dysregulated emotional sharing (Zoratto et al., 2018).

Observational Fear Learning

Fear is an appropriate response to many environmental stimuli, and the acquisition of fear through indirect measures (social observation) is critical for the survival within social groups (Olsson and Phelps, 2007; Keum and Shin 2016; Keum and Shin, 2019). The process of evaluating observational fear learning involves Pavlovian conditioning, in which a conditioned stimulus is paired with an aversive unconditioned stimulus. However, in this case, the fear behaviors, such as freezing, are developed vicariously when an animal observes a conspecific undergoing an aversive stimulus (i.e., foot shock) (Keum and Shin 2016; Kim et al., 2018). Models of observational fear learning have generated several interesting findings. For example, in several rodent studies (Bredy and Barad, 2009; Guzman et al., 2009; Knapska et al., 2010; Jeon and Shin, 2011), social interaction with a familiar conspecific that had previously been exposed to the fear condition modulated the acquisition, retention, and/or subsequent extinction of cue-fear associations of observers. Conditioned fear was significantly increased when observers were pre-exposed to a conditioned animal when testing occurred in a novel context (mice: Guzman et al., 2009; rats: Knapska et al., 2010; Atsak et al., 2011). In contrast, when social interaction between familiar animals occurs in a home cage, observer mice's fear acquisition is impaired when exposed to a recently conditioned familiar mouse (Bredy and Barad, 2009). Together, these findings propose an interesting caveat to this model and suggest a possible context-specific modulation of the social transmission of fear (Bruchy et al., 2010).

In the aforementioned studies of conditioned fear, animals are subjected to conditioning and transferred to safe environment where social interaction occurs with the observer (remote danger) (Jones et al., 2014). In contrast, a shift in the paradigm exposes the rat to an aversive stimulus in an environment without the possibility of escape (imminent danger) while a conspecific views the distressing event (Meyza and Knapska, 2018). This allows for an elucidation of vicarious

freezing behavior specifically due to affective transfer. In one example, observer animals viewed demonstrators receiving foot shocks without ever receiving the shocks themselves. Both animals showed simultaneous freezing behavior during the task. Further, when the observers were placed back in the same chamber the next day absent the demonstrators, the observer still exhibited freezing behavior, indicating a contextual fear memory even though they were never shocked (Jeon et al., 2010; Jeon and Shin, 2011). The observers made a connection between the stressed state of the conspecific and the environment in which they were placed. Therefore, this variation of the model seems to test both initial emotional contagion and prolonged affective transfer (Kim et al., 2018). However, there is some discrepancy as to whether observing a conspecific alone is sufficient to induce vicarious freezing behaviors. In one study, a similar protocol showed that observing a conspecific was not enough to generate vicarious freezing (Sanders et al., 2013). Instead, animals had to be subjected to the same foot shock as the demonstrators 24 hour before the test to exhibit a shared affective response. The authors suggest that in naïve observers, a high level of aversive stimuli occurring to demonstrators is required to obtain freezing, but if observers have had a prior experience with the aversive stimulus, they respond to lower levels of the stimulus (Sanders et al., 2013).

Advancing the field further, observational fear behaviors were evaluated when the observer was directly responsible for the delivery of the harm to their conspecific (Hernandez-Lallement et al., 2020). Here, a preferred lever (developed from sucrose training) became paired with foot shock for a conspecific in an adjacent compartment and rats had to learn to stop pressing the lever to avoid inflicting harm on the target (i.e., instrumental harm aversion). Both males and females learned to switch levers over time, but pre-exposure to the shock makes the harm aversion effect stronger. Interestingly, this effect was limited by effort. For example, rats would readily avoid pressing the lever if that meant not receiving a single pellet of sucrose, but they would not stop if it meant reducing the number of sucrose pellets from three to one (Hernandez-Lallement et al., 2020). Overall, observational fear models of emotional empathy have been correlated with trait measures

of empathy (Olsson et al., 2007; Kleberg et al, 2015), which suggests this model evaluates a fundamental feature of empathic behavior (Panksepp and Panksepp, 2013) and can help offer insights into more basic and conserved forms of human empathic processes (Kim et al., 2018).

MODELS OF TARGETED HELPING

Because empathy allows for the sharing of affective states between individuals, it can also motivate prosocial behaviors like targeted helping. This phenomenon has historically been considered specific to humans and non-human primates. However, in recent years, several groups have demonstrated that rodents will release a distressed conspecific from an aversive situation such as being trapped in a restraint tube (Bartal et al., 2011; 2014; 2016) or a pool of water (Sato et al. 2015; Kandis et al., 2018; Karakilic et al., 2018; Cox and Reichel, 2019; Yamagishi et al., 2019). The following section will describe these models of targeted helping behavior, along with the proposed advantages and limitations of each. We also discuss the influence of social contact on these tasks and how this potential issue can be overcome. In accordance with the PAM model of empathy, the rat charged with rescue or helping is termed as the "observer" throughout and the distressed conspecific is termed the "target". This nomenclature removes any preconceived biases connoted by the other terms.

Restraint Stress Model

In a restraint stress model, rats (Bartal et al., 2011) and mice (Ueno et al., 2019a) exhibited targeted helping toward a conspecific trapped inside a Plexiglas tube. Specifically, an observer learned to release the target from the restraint tube, and this release behavior is maintained even if direct physical contact is blocked following a training period. Further, when given a choice of opening a restrainer with chocolate chips inside or releasing a distressed cage mate, the observers' latency to open either restrainer was not significantly different from one another. The authors conclude from these data that the value of the helping behavior is roughly equivalent to that of palatable food reward (Bartal et al., 2011).

In accordance with primate studies showing that neither likeness nor kinship was required for prosocial behavior (Horner et al., 2011, Baden et al., 2013), social experience modulates targeted helping. For example, rats pair-housed with an animal of their strain or cross-fostered with a different strain helped the trapped conspecific with which the observer was familiar. In fact, cross fostered rats helped unfamiliar animals from the strain with which they were raised but not an unfamiliar rat of their own strain, suggesting targeted helping, as with other empathic behaviors like fear contagion (Jeon and Shin, 2011), is modulated by social experience and familiarity rather than genetic relatedness (Bartal et al., 2014). Reliance on social experience as opposed to kinship alone can allow for more behavioral flexibility in adapting to various social circumstances (Dugatkin et al., 2002).

Helping behavior can also be modulated pharmacologically. Midazolam (a benzodiazepine) administered to observer rats before the session in which the target was restrained reduced door openings (Bartal et al., 2016). Further, this effect was not due to gross sedative or cognitive effects of the drug, because rats retained the same door opening latency when the restrainer was instead filled with chocolate chips. These findings suggest that affective processing and transfer are required for targeted helping behavior (Bartal et al., 2016).

Whether the restraint stress model extends to empathic process in mice is less clear. For example, mice will open a clear restrainer to release a cagemate (Ueno et al., 2019a) and not a ball of yarn resembling a mouse (Ueno et al., 2019b). However, mice will also open an opaque tube with no animal in it when given the opportunity. In fact, mice show so much interest in the tube after releasing a cagemate that they prioritize its investigation over releasing another cagemate placed in a second restrainer (Ueno et al., 2019b). As such, lid-opening behavior in mice may be more driven by social interest and curiosity with the lid apparatus itself (Ueno et al., 2019b) rather than empathy as previously hypothesized (Ueno et al., 2019a).

Helping behavior in rats is also shaped by other variables. For example, release behavior is subject to the contents of the container (Blystad et al., 2019). Rats pre-trained to open a tube for

food and subsequently tested with an empty restrainer had shorter release latencies in response to the trapped conspecific compared to an empty restrainer, but longer compared to a restrainer filled with food (Blystad et al., 2019). Therefore, helping behavior may be reinforcing, but less so than appetitive rewards. Another example intimates selfless behavior may not take precedence over behaviors that directly improve the outcome for the observer. Providing the rats an opportunity to escape from a well-lit arena (which is inherently distressing) into an adjacent dark compartment instead of releasing a distressed conspecific from a restraint tube placed in the lit arena significantly delays the latency of release behavior (Carvalheiro et al., 2019). This alteration elucidates the motivation of rescue behavior if an escape alternative was present for the observer rat. One can conclude from these data that self-benefit may supersede empathically-driven behaviors, or perhaps helping behavior itself may be directed toward increasing self-welfare compared to another (Batson et al., 1983).

Social contact may also be a driving force behind the observed rescue behavior because, in a modified version of the restraint stress paradigm, rats did not learn to release a conspecific into a separate compartment if they were not first trained on the task in which social contact was permitted (Silberberg et al., 2014). Additionally, rats show equal preference for conspecifics in a closed restraint tube (distressed) or in an open one that allows for social contact (Hachiga et al., 2018). Overall, while the motivation and nuances behind the behavior need to continue to be explicated and replicated, the restraint stress targeted helping task is a very promising model of a higher-order helping behavior that can help elucidate more cognitively driven empathic processes.

Soaked Conspecific Model

In another model of targeted helping, a rat is placed in a pool of water to incite distress. An observer rat is placed in an adjacent dry chamber of the apparatus separated by Plexiglas and given access to a movable door. The observer must open the door to release the wet target into a dry chamber of the apparatus (Sato et al., 2015; Cox and Reichel, 2019). Observer rats quickly learn to release the target over subsequent trials. Latency to door opening increased or was eliminated in

response to an empty pool of water, when the target was replaced by an inanimate object, and the water was removed entirely following acquisition of the task (Sato et al., 2015; Cox and Reichel, 2019). Further, observers did not open the door for access to the pool of water alone or when a rat was placed on the opposite side of the Plexiglas partition absent any water (i.e. absent any distress). However, when water was omitted from the task and targets were placed in a dry compartment, observers still executed the chain pull response, indicating that social interaction is a factor in the soaked conspecific model (Cox and Reichel, 2019). In a choice task between releasing the soaked target and receiving a palatable food reward (chocolate cereal), releasing the cage mate had a higher value than the food reward (Sato et al., 2015). Finally, acute and repeated acetaminophen given to observers reduced door openings to release the target (Kandis et al., 2018). Interestingly, a reduction in empathic behaviors due to acetaminophen is also observed in clinical research (Mischkowski et al., 2016).

Reversing the role of the observers and targets demonstrated that previous experience with the distressing condition enhanced learning of the release behavior compared to the distress-naïve rats (Sato et al., 2015; Cox and Reichel et al., 2019). While it is true rats learn by observing the behavior of others (Heyes and Dawson, 1990), the modulation of learning through experience also suggests the behavior observed is empathically driven. According to the PAM, prior exposure to the same stressor could more readily enable the reversed observer to state match with its target (Preston and de Waal, 2002; Mogil, 2012; Sanders et al., 2013; de Waal and Preston, 2017), perhaps by activating salient emotional memories that drive empathic behavior. Overall, these data suggest that first-hand experience enhanced acquisition of the helping behavior and supports that the measured behavior is based on empathic processes (Sato et al., 2015; Cox and Reichel, 2019).

The debate as to whether rescue behavior is driven primarily by empathic processes or other confounding variables continues for this targeted helping model. Based on a series of control experiments, it has been postulated that the behavior may be driven by social contact or interest in the presence of a water-filled compartment (Schwartz et al., 2017; Cox and Reichel, 2019).

Therefore, it is necessary to consider these alternative motivators when interpreting the results from this model. Although empathy as driver of the outcome cannot be ruled out, using Lloyd Morgan's canon, according to the authors, the simpler behavior should be the preferred explanation over the complexity introduced in empathy (Schwartz et al., 2017).

It is likely the restraint and soaked conspecific models of helping behavior discussed above are driven, in part, by empathy; however, data from our lab and others (Silberberg, et al., 2014; Schwatrz et al., 2017; Hachiga et al., 2018; Hiura et al, 2018; Cox and Reichel, 2019), suggest that these models are also driven by the opportunity for social reward. Social contact, whether through play fighting (Vanderschuren et al., 2016), maternal care of pups (Lee et al., 2000), or sexual behavior (Trezza et al., 2011) is reinforcing and activates circuits associated with reward (Preston, 2017). In addition, animals readily exhibit preference to social interaction in conditioned place preference (CPP) paradigms (Calcagnetti et al, 1992; Thiel et al., 2008; Fritz et al 2011; Peartree et al., 2012), and they have been shown to value social contact above both natural rewards (palatable foods) (Ikemoto and Panksepp, 1992) and drugs of abuse (Normansell and Panksepp, 1990; Yates et al., 2013). In fact, volitional social reward reliably attenuated both methamphetamine and heroin self-administration in rats in an operant choice model (Venniro et al, 2018a, b; 2019; 2020b). Some effort has been made to parse apart the effects of social reward and direct empathic behavior. For example, Bartal and colleagues (2011) demonstrated rats will release a target from a restraint tube into a separate compartment, but only after they had been trained to release into the same environment. It is important to note that this methodological consideration does not render the current models inadequate in the evaluation of targeted helping behavior. On the contrary, they have strong ethological validity, as empathically-driven behavior can certainly be driven by or include social contact (Decety et al., 2016; Lahvis, 2017). Further, these models could lend great insight into more complex empathic behaviors directed at aiding a distressed conspecific. However, our lab has worked to address the impact of social interaction on targeted helping, as is discussed in detail in **Chapter 3**.

SOCIAL & ENVIRONMENTAL FACTORS THAT CONTRIBUTE TO EMPATHIC BEHAVIORS

In both humans (Bowen et al., 2014; Gunnar and Hostinar, 2015) and non-human primates (Sanchez et al., 2015) social interaction with a familiar conspecific helps to ameliorate the effects of aversive or stressful stimuli. This phenomenon, known as social buffering (DeVries et al., 2003; Kikusi et al., 2006), also occurs in rodents, in which social contact modulates stress-related behaviors (Davitz and Mason, 1955; Kiyokawa et al., 2004; Watanabe, 2011, 2015). Social buffering of a conditioned fear response is potentiated when the conspecific is familiar as opposed to unfamiliar (Kiyokawa et al., 2014). Interestingly, the presence of fear-expressing partners (those exposed to the same fearful stimulus) also reduce fear-induced hyperthermia and freezing behavior, just less so compared to non-fearful partners (Kiyokawa et al., 2004). A slight variant is pro-active social buffering (Meyza et al., 2018), in which interaction with a conspecific occurs prior to an aversive event compared to following it. In this case, pre-exposure to a non-fearful conspecific reduces long term (but not short term) contextual fear memories in mice (Guzman et al., 2009). Social buffering is therefore common in situations where social interaction takes place before and after an aversive stimulus (Morozov, 2019). Its effects must be noted during the use of some of the models described above, and additional research on its effects would aid in our understanding of emotional transfer.

Stress and voluntary exercise can also influence empathic behavior. In a recent study with male mice, low levels of observed emotional contagion was correlated to reduced circulating corticosterone following restraint stress, suggesting adequate HPA reactivity is required for empathic behaviors (Laviola et al., 2017). Additionally, a mild stressor (low intensity foot shock) increased rats' latency to release a conspecific in the soaked conspecific model (Karakilic et al., 2018). Finally, in male and female mice, 8 weeks (6 alone and 2 with a cage mate) of voluntary wheel running improved empathy responses (Yuksel et al., 2019). Given these studies, it is clear many social and environmental factors are involved in empathic processing and subsequent

prosocial behaviors, and with the help of animal research, we are beginning to explore an ever-increasing number of variables, from genes (reviewed in Kim et al., 2018), sex (Bartal et al., 2011), strain (Keum et al., 2016), and even the intestinal microbiome (Sgritta et al., 2019).

NEUROBIOLOGY & PHARMACOLOGY OF EMPATHIC BEHAVIORS

Neurobiology of Empathic Behaviors in Rats and Mice

A large library of fMRI studies exists that have evaluated the neurobiology of multiple components and cognitive levels of empathy. Brain regions that have been correlated with aspects of empathy include those involved in emotional salience and interoceptive valence, specifically the amygdala and insula (Adolphs et al., 2002; Singer et al., 2004; Fusar-Poli et al., 2009; Keysers and Gazzola, 2018; Marsh, 2018), as well as substrates necessary for perspective-taking, motivation, and cognition, like the prefrontal (PFC), anterior cingulate (ACC), and orbitofrontal (OFC) cortices (Jackson et al., 2005; Singer and Lamm, 2009; de Waal and Preston, 2017; Decety, 2015; Cerniglia et al., 2019; Uysal et al., 2019). More causational and region-specific research using rats and mice are beginning to corroborate some of these imaging studies, and these findings are discussed in detail in other review articles (Panksepp and Panksepp, 2013; Sivaselvachandran et al., 2016; Meyza and Knapska, 2018; Meyza et al., 2018). Below, we will briefly discuss some neural underpinnings of rodent empathy that have translational importance.

In an emotional contagion paradigm, amygdala and PFC activity, as indicated by *c-fos* staining, in non-stressed rats generally mirrored that of stressed conspecifics (Knapska et al., 2006). Interestingly, the central amygdaloid nucleus was activated to a higher level in unstressed observer rats, suggesting it is likely specific subnuclei of the amygdala are sensitive to the distress of others (Meyza et al., 2018). Additionally, bilateral lesions of the prefrontal cortex, but not the amygdala, were sufficient to inhibit empathy for pain in rats (Li et al., 2014). Overall, these results point towards the amygdala and PFC contributing to emotional contagion, but likely the amygdala playing a more direct role in the salience of another's pain.

The ACC, a region known for its role in pain, affect, and emotional motivation (Keum et al., 2016), is necessary for the acquisition of observational fear learning. More specifically, the deletion of the Cav1.2 Ca^{2+} calcium channel within the ACC was adequate to impair social fear learning in mice (Jeon et al, 2010). Further, ACC dopamine D_2, but not D_1 receptors are required for vicarious fear (Kim et al., 2012). And while inactivation of the ACC led to disrupted fear learning, lateral amygdala inactivation led to impairments in both fear learning and fear expression (Jeon et al, 2010). Pharmacologic deactivation of the ACC abolished harm aversion in a model in which rats were directly responsible for a conspecific's harm, which also helps corroborate the ACC's role in affective transfer (Hernandez-Lallement et al., 2019). One of the more intriguing findings comes from an elegant study of neuronal populations within the ACC by Carrillo and colleagues (2019), in which they were able to identify neurons that respond to pain of self and pain in others, but not from another emotionally-salient emotion (fear from a conditioned stimulus). Deactivating this region of the ACC reduces freezing in rats observing a conspecific receiving footshocks, but not while hearing a conditioned stimulus. The authors concluded mirror-like neurons are present in the rat ACC that encode the pain of others into a similar or overlapping ensemble for personal experiences of pain (Carrillo et al., 2019)

Lastly, increasing research points to the insular cortex, a region in humans known for its role in predicting the valence of both self and others (Gogolla, 2017). Pharmacological inactivation of the insula was shown to cause an alteration in emotional contagion of pain (Zaniboni et al., 2018). Also, chemogenetic activation of the anterior insula restored a heroin-induced decrease in targeted helping (Tomek et al., 2019; Tomek et al., 2020), indicating this brain area may be a potential translationally-relevant therapeutic target for individuals suffering from SUD. The insula in its role in empathixc behaviors is expanded upon in detail throughout **Chapter 4**. Overall, the volume of research is expanding in regard to the neurobiology of empathy using rodent models. There is still much to understand, including the interaction and circuitry between these regions of interest (Jeon et al., 2010), as well as the role of the emotional processing neural networks in

different empathic behaviors and how they communicate with one another (Panksepp and Panksepp, 2013).

Oxytocin's Role in Empathy

There is overwhelming evidence in human studies linking oxytocin (Oxy) to several components of empathy (Domes et al., 2007; Hurlemann et al., 2010; Decety, 2011; Theodoridou et al., 2013; Christov-Moore et al., 2014; Rilling et al., 2014; Jones et al., 2017; Ebert and Brüne, 2018; Uysal et al., 2020). While rodent studies have focused on the importance of Oxy in areas such as maternal care (Arletti and Bertolini, 1985; Borrow and Cameron, 2012) and mating (Pederson et al., 1982; Champagne et al., 2001), they have only recently begun to explore its importance in prosocial behaviors (Burkett et al., 2016). For example, mice that were identified as having low levels of empathic behaviors, as measured by emotional contagion of fear, showed potentiated neural Oxy levels absent any changes in receptor density (Laviola et al., 2017).

Additionally, the Oxy system plays a role in observational fear learning, as well as the social buffering of observational fear. Intranasal Oxy, both acute and chronic, enhanced vicarious freezing in mice in response to an unfamiliar conspecific that had undergone a foot shock test. What's more, chemogenetic stimulation of hypothalamic Oxy neurons caused mice to become sensitive to distress of unfamiliar demonstrators (Pisansky et al., 2017). Systemic Oxy injection bolstered socially transmitted fear from a familiar animal (Pisansky et al., 2017), while direct infusion of Oxy into the lateral septum (important in fear responses) accelerates the social buffering effect on observational fear learning, as measured by a reduction in the exposures with the conspecific needed to decrease the contextual freezing (Guzman et al., 2014).

Oxytocin has also been shown to modulate targeted helping in a socially-dependent manner. Single-housed rats that received Oxy injections (1 mg/kg, 5 days) had faster door opening latencies at the beginning of task acquisition compared to animals that were pair-housed and received Oxy injections (Yamagishi et al., 2019). This may suggest Oxy reduces social anxiety or improves prosocial behavior in certain social contexts. In contrast, Oxy administration had no effect

on targeted helping behaviors in mice, which was another indicator to the authors that rescue behavior in mice may not be mediated directly by empathic processes (Ueno et al., 2019b). With the field's continued advancement of translationally-relevant models, there is little doubt other neurotransmitters will receive growing attention. There is already bourgeoning research to discern the role of serotonin (Kim et al., 2014) and dopamine (Lichtenberg et al., 2018) as other critical neurotransmitters involved in empathic behavior.

CONCLUSIONS AND FUTURE DIRECTIONS

Empathy is a process critical for group cohesion and social development. Further, empathic dysregulation is extremely prevalent in psychiatric disorders, ranging from personality disorders, substance use disorders, and schizophrenia. And although the study of the underlying neurobiology of empathy has gained increasing attention in humans, there are inherent limitations to human research. The current neuroimaging techniques do not provide high temporal or spatial resolution that would allow for the study of specific circuits underlying the observed behavior. That is why rodent models of empathic processes have become increasingly important; with these paradigms, we are able to utilize technologies such as chemo- and optogenetic tools that offer precision in deciphering specific neural substrates and mechanisms underlying various empathic processes (Meyza and Knapska, 2018). With the increasingly prodigious library of genetic tools to study neuropsychiatric diseases in rodents, these models can assess the impact specific genetic modifications have on empathic behaviors directly. This field is advancing rapidly and there has been promising research using rodent models. It is important we do not anthropomorphize the findings or over-extend a particular theory of empathy. However, multiple theories describing empathy can be parsimonious, granted the research questions being studied have an appropriate scope. Further, as it has been pointed out previously (Mogil, 2012), the accumulating evidence of rodents sharing or responding to the emotional state of a conspecific makes repudiating their empathic abilities a form of anthropodenial. It is imperative that models like these are replicated, validated, and focused on translating their findings to understand the underlying neurobiology of

empathy to improve the outcomes for those with neuropsychiatric disorders. Therefore, the goal for the following chapters is to expand upon the current literature by developing and validating a rodent model of targeted helping that removes the confounding variable of social contact in order to specifically evaluate empathic behavior within the task. The goal of this PhD dissertation is to build upon the current literature and develop a rodent model of empathic behavior that eliminates the rewarding effects of social interaction as a confounding variable and use it to examine sex differences in the neuronal, affective, and sensory correlates of targeted helping. Further, we also examine the potential neural mechanisms of empathic behavior through direct manipulation of the insular cortex.

CHAPTER 2: General Methods:

In this brief chapter, we will discuss methodological points that are used throughout the remainder of this thesis. Most importantly, the 2 chamber and novel 3 chamber helping tasks used to explore different aspects of empathic behavior will be described in full in the following sections. Any modifications made to these general methods are detailed in further chapters.

Animals

Male and female Sprague Dawley rats weighing 250-275g were pair-housed with the same sex on a 12-hour reversed light cycle (lights on at 1800). Animals were given food and water *ad libitum* until behavioral testing, when they were then switched to a daily stable intake (20g) of rat chow (Harlan). Rats were given at least 5 days to acclimate to their cage mate. Following acclimation, one rat was randomly selected to be the "Observer" and the other the "Target". Animals were handled and weighed for 2 days, 5 min/day before the behavioral assessment. For all behavioral evaluations, rats were transported to the experiment room and left undisturbed for 5 minutes. The tasks were performed in a sound-attenuated room with the lights off except for a single lamp used for the experimenter to view the test. All experimental procedures were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources on Life Sciences, National Research Council) and approved by the IACUC of the Medical University of South Carolina.

2 Chamber Prosocial Task

Evaluation of targeted helping with the opportunity for social reward was performed in a custom-made operant box (34.2x33.9x30.5 cm) by Med Associates (Fairfax, VT, USA; **Figure 2.1A**). Targets were placed in 100 mm of water in the wet compartment (19.5x34.2 cm), while the Observer was placed on a dry platform (19.5x34.2 cm) with access to a chain that, when pulled, opened an automatic guillotine door. Once the door was opened, it allowed Targets to be released into the same dry compartment as the Observer.

3 Chamber Empathy Task

Social interaction-independent helping behavior was evaluated using a custom (Med Associates; Fairfax, VT, USA) operant box (34.2x33.9x30.5 cm) developed with three chambers. In this apparatus, Targets were again placed in 100 mm of water in the wet compartment (19.5x19.5 cm) and Observers on a dry platform (19.5x34.2 cm) with access to a chain that would open an automated door. In this case, however, the Target was released into a dry compartment (17x19.5 cm) separate from the Observer (3 chamber, **Figure 2.1B**). Additionally, in the 3 chamber apparatus, small holes (0.25 cm radius) were drilled exclusively between the wet compartment and the Observer's dry compartment, which allowed for improved scent detection, but were small enough to prevent any physical contact between the two rats. Importantly, no holes existed between the separated dry chambers to prevent any opportunity for social interaction after the chain pull.

In both the 2 chamber and 3 chamber tasks, latency to chain pull was taken as an index of helping behavior. Trials (20 total across 10 days, labelled "Acquisition") lasted a total of 300 s (5 min) regardless of the chain pull latency in order to reduce the likelihood that removal from the apparatus was a motivating factor for the behavior. If the Observer did not pull the chain within the allotted time, the experimenter ended the trial and released the Target. In some of the following experiments, the role of each rat in a pair was subsequently reversed ("Reversal" phase), such that a Target became the Observer (labelled "R-observer"), and the Observer becomes the Target ("R-target"). This reversal phase was carried out for 5 days (10 trials). Two trials were conducted daily during the rats' dark cycle.

Immunohistochemistry (IHC)

Subjects were sacrificed and perfused approximately 90 minutes following behavior and brains were collected. Rats were anesthetized with Equithesin and then transcardially perfused with 150-200 mL cold 0.9% saline followed by 400-500 mL of 10% buffered formalin. Brains were removed and postfixed in 10% formalin for 24 hours, submerged in 20% sucrose/0.1%sodium azide solution for 48 hours, and then sectioned into 50-µm tissue sections.

For Fos visualization using a 3,3' diaminobenzidine (DAB) stain, tissue sections were incubated in a rabbit anti-Fos primary antibody (Millipore; 1:1000) overnight, followed by a 2-hour incubation in donkey anti-rabbit secondary antibody (Jackson ImmunoResearch; 1:500) amplified with an avidin biotin complex method (Thermo Scientific). The sections were then visualized with DAB (Sigma) + nickel ammonium sulfate to produce a blue-black nuclear reaction product. Slices were coverslipped using Permount and regions of interest were photographed at 10x magnification using a Leica microscope and VideoToolbox software.

For fluorescent IHC, 50-µm tissue slices were permeabilized and blocked in 2% normal goat serum (NGS) and 2% Triton X-100 in PBS and were incubated in a rabbit anti-Fos primary (Millipore; 1:1000) overnight at 4°C, followed by a 5-hour incubation in donkey anti-rabbit (Millipore; 1:1000) at room temperature while protected from light. Slices were mounted and coverslipped with Prolong Gold, and representative images were taken using a Nikon fluorescent microscope. Total count analysis was performed using ImageJ (NIH). Additional staining performed in experiments throughout the dissertation are discussed specifically in their respective chapters.

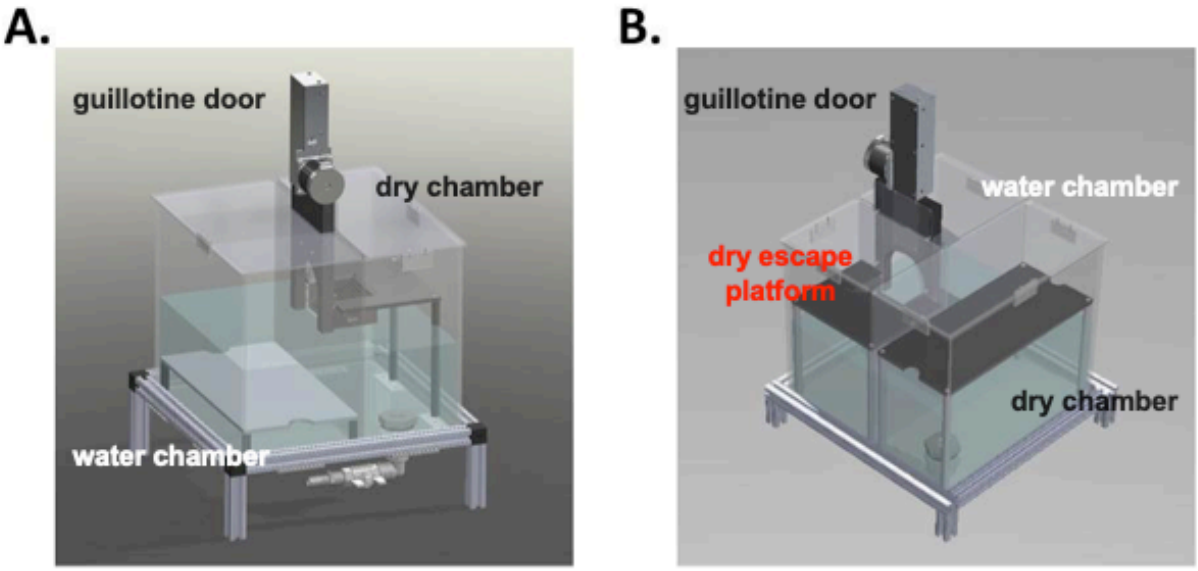


Figure 2.1. **Illustrations of the 2 and 3 Chamber Behavioral Apparatuses**. **A)** This illustration depicts the 2 chamber apparatus that allowed for social contact. Observer rats placed in the dry chamber have access to a chain that, when pulled, releases the target rat from the wet chamber onto the same side as the Observer through an automated guillotine door. **B)** An illustration of the 3 chamber empathy apparatus created to better model empathic behavior via the elimination of social reward as a confounding variable. Observer rats learn to pull a chain to release target rats from the water chamber into a separate dry chamber.

CHAPTER 3: Male Rats Display Empathic Behavior Independent of the Opportunity for Social Interaction

INTRODUCTION

As discussed in **Chapter 1**, empathy can be defined as the capacity to share the emotional valence of others, which generates shared affective states and therefore drives behaviors most appropriate to the emotional condition of others (Meyza et al., 2017). Overall, this ability is essential for cooperation towards common goals and the formation of social bonds (Preston and de Waal, 2002; de Waal and Preston, 2017). Moreover, empathic behavior is dysregulated in numerous disorders defined in the DSM-V, including, but not limited to, SUD (Maurage et al., 2011), ASD (Bird and Cook, 2013; Harmsen, 2019), and MDD (Cusi et al., 2011; Ekinci and Ekinci, 2016). These findings suggest restoration of empathic behaviors may improve treatment outcomes and reduce the chance of relapse (Lamm et al., 2011; Robinson et al., 2018). It is therefore imperative that a translational model for the study of empathy is available in order to discern the relationship between psychiatric disorders and empathic deficits as a potential diagnostic and therapeutic tool for clinical populations.

Empathy is a continuum of phenomena that, at its core, lies the perception-action model (PAM). As described in **Chapter 1**, the PAM posits that an "Observer" must attend to another's distress, thereby generating a shared affective state (Preston and de Waal, 2002; de Waal and Preston, 2017). The Observer must regulate their emotional responsivity to effectively perform a behavior that will eliminate the distress of the "Target" and, by extension, their own. While it has long been assumed that empathy was exclusive to humans and non-human primates, a growing body of evidence suggests that many mammals, including rodents, express these more complex empathic behaviors (Panksepp and Panksepp, 2013; Preston and de Waal, 2017; Meyza and Knapska, 2018).

Early studies (Church, 1959; Rice and Gainer, 1962), as well as more recent experiments (Panksepp and Panksepp, 2013; Sivaselvachandran et al., 2016; Meyza et al., 2017; Meyza and

Knapska, 2018), have demonstrated that rats will actively work to reduce the distress of a conspecific, such as releasing them from restraint (Bartal et al., 2011; Bartal et al., 2014) or a water-filled compartment (Sato et al., 2015). Helping behavior is potentiated when the level of distress of the conspecific is elevated (Bartal et al., 2011), and animals will forgo or delay personal reward (e.g., chocolate) to aid the conspecific (Bartal et al., 2011; Sato et al., 2015). However, in these aforementioned tasks, distressed rats were released into the same environment as their rescuer, allowing for direct social interaction. This methodological consideration obscures whether release behavior was driven by empathic processes or the reward of social contact (Silberberg et al., 2014; Hachiga et al., 2018).

For these reasons, we have developed a novel rodent model for the study of empathic behavior independent of social interaction. In our task, a three-chamber apparatus was used in which a distressed "Target" rat was placed in a pool of water. An "Observer" rat was placed in a dry chamber with access to a chain that, when pulled, opened an automatic guillotine door and released the target into a separate dry chamber, thereby eliminating its distress (**Figure 2.1B**). In the current report, we show that rats learned to aid a distressed conspecific in the absence of social reward, and previous experience of the distressing event significantly potentiates the rate rats learn the task. We denote tasks that include social contact after being released as "social" and the task that prevents social contact as "empathic". We validated the specificity of this new model to empathic processes and characterized the importance of the presence of a distressed Target on the Observer's chain pull latency. We also demonstrated that the Observer's level of familiarity with the target modulates helping behavior. This model may be a valuable tool to directly evaluate empathic processes in rodents and to understand the interplay between blunted empathic behavior and psychiatric disorders.

MATERIALS AND METHODS

Animals: Male Sprague Dawley rats (n=102, 51 pairs) weighing 250-275g were utilized for these experiments, as described in **General Methods.** Rats were handled and weighed for 2 days, 5

min/day before the behavioral assessment. All experimental procedures were conducted in accordance with the "Guide for the Care and Use of Laboratory Rats" (Institute of Laboratory Animal Resources on Life Sciences, National Research Council) and approved by the IACUC of the Medical University of South Carolina. The distribution of rats across experimental cohorts is detailed in **Table 2**.

Apparatus and Behavioral Testing: Evaluation of social and empathic behaviors occurred in a 2 or 3 chamber apparatus, respectively, as described in **General Methods**. Briefly, Observers were placed on a dry platform with access to a chain that, when pulled, opened an automatic guillotine door and allowed the Target to be released into either the same dry compartment as the Observer (2 chamber, **Figure 2.1A**) or to a dry compartment (17x19.5 cm) separate from the Observer (3 chamber, **Figure 2.1B**). Latency to chain pull was taken as an index of helping behavior. Trials (10 days, total, "Acquisition") lasted a total of 300 s (5 min) regardless of the chain pull latency to reduce the likelihood that removal from the apparatus was a motivating factor for the behavior. If the Observer did not pull the chain within the allotted time, the experimenter ended the trial and released the Target. The role of each rat in a pair was subsequently reversed ("Reversal") such that a Target became the Observer ("R-observer"), and the Observer becomes the target rat ("R-target"). This reversal phase was carried out for 5 days (10 trials). Two trials were conducted daily during the rats' dark cycle. In order to evaluate the importance of the Target's level of distress for the Observer's chain pull behavior in both tasks, we conducted separate experiments in which Targets were placed in the chamber without any water (i.e., a dry chamber with no distressor present) and chain pull latency was recorded. We also tested motivation to release the target by increasing the response requirement to an FR5 and then an FR10 schedule.

Characterization of Empathic Behavior in Rats: We conducted a series of tests on the Observers and R-observers to characterize the helping behavior. First, the specificity of the empathic response was tested by replacing the live Target/R-target with a 'fake' rat (white Styrofoam of approximately equal length and width as a rat; 3 consecutive days) and an empty pool of water (3 consecutive

days). We also evaluated the time course of empathic responding by allowing rats to remain in their home cage and reintroduced them to the empathy chamber 5, 10, and 15 days after acquisition ended. We determined if helping behavior was specific to the cage mate of R-observers by replacing the R-target rat with an unfamiliar rat. Three 'stranger' rats were used twice in random order for a total of 6 trials. Latencies in these experiments were compared to a baseline (BL) calculated by the average of the final 3 days of the empathic responding.

Data Analysis: Chain pull latency was the primary dependent variable and a daily mean for each rat was calculated by averaging the two trials performed each day. No animals were excluded from the analysis in order to establish patterns of social and empathic responding. One-way repeated measures (RM) analyses of variance (ANOVAs), with day being the repeated measure, were performed to evaluate the change of chain pull latency over time for Observers and R-observers. Baseline (BL) latencies used for comparisons were calculated by finding the mean latency from the equivalent number of final days of the previous phase. One-way RM ANOVAs were then performed to evaluate changes in latency from BL. In addition, two-way RM ANOVAs were performed to directly compare Observers and R-observers. All post hoc comparisons were conducted using a Holm-Sidak's correction for family wise error when appropriate, with the alpha set at 0.05. All analyses were conducted with Prism Software version 8.4.3. All data are expressed as the mean ± SEM.

Table 2. Description of animal use throughout Chapter 2.

Experiment/Cohort	***# of rats = 102 Observers/Targets***	***Behaviors***			
		Acquisition	**Reversal**	**Tests**	**Group tested**
2-chamber social	7/7 = 14	✓ Increased FR	✓	Increased FR	none
2-chamber social	8/8 = 16	✓ Dry Chamber	✓ Dry Chamber	none	none
3-chamber empathy					
Cohort 1	8/8 = 16	✓	✓	Fake rat, empty chamber	R-Observers
Cohort 2	8/8 = 16	✓ Dry Chamber	✓ Dry Chamber	none	none
Cohort 3	8/8 = 16	✓ Increased FR	none	Fake rat, empty chamber, extended time course	Observers
Cohort 4	8/8 = 16	✓	✓	Strangers	R-Observers
Cohort 5	4/4 = 8	✓ Water pre-exposure	none	none	none

RESULTS

Rats Readily Release a Conspecific from a Pool of Water: Rats performed a helping task similar to one previously described (Sato et al., 2015) (n=7 pairs) in which release of the Target resulted in social interaction. Observers' (**Figure 3.1A**) chain pull latency decreased throughout the acquisition phase [$F(7, 42) = 18.64$, $p<0.0001$], with significantly shorter latencies on days 2-8 compared to day 1 ($p<0.05$). R-observers (**Figure 3.1B**) also had decreased latencies [$F(4, 24) = 36.6$, $p<0.0001$], with a significant reduction observed on days 2-5 compared to day 1 ($p<0.0001$). A direct comparison of Observers and R-observers (**Figure 3.1C**) over the first 5 days of the acquisition and reversal phases revealed a significant group by day interaction [$F(4, 48) = 3.133$, $p<0.023$] in which Observers had longer latencies than R-observers on days 2-4 ($p<0.01$). Consistent with Sato et al. (Sato et al., 2015), R-observers had significantly decreased latencies to release a distressed conspecific compared to Observers.

Rats will Perform an Operant Task for Social Interaction: Target rats (n=8 pairs) were placed in the 'wet' compartment without water (i.e. no distress was present). Latency decreased over days [**Figure 3.1D**, $F(7, 49) = 10.14$, $p<0.0001$] such that days 4, 5, 7, and 8 were significantly less than day 1 ($p<0.05$) demonstrating acquisition of the social task in the absence of a distressed target. The R-observers' latencies also decreased over days [**Figure 3.1E**, $F(4, 28) = 11.47$, $p<0.0001$] with days 2-5 significantly lower than day 1. In direct comparisons, Observers had higher latencies than R-observers [group by day interaction, [$F(4, 56) = 3.602$, $p<0.0111$] on days 2-4 ($p<0.0001$, **Figure 3.1F**). These data suggest previous experience still plays a role in acquiring this social task in spite of the conspecific's lack of distress.

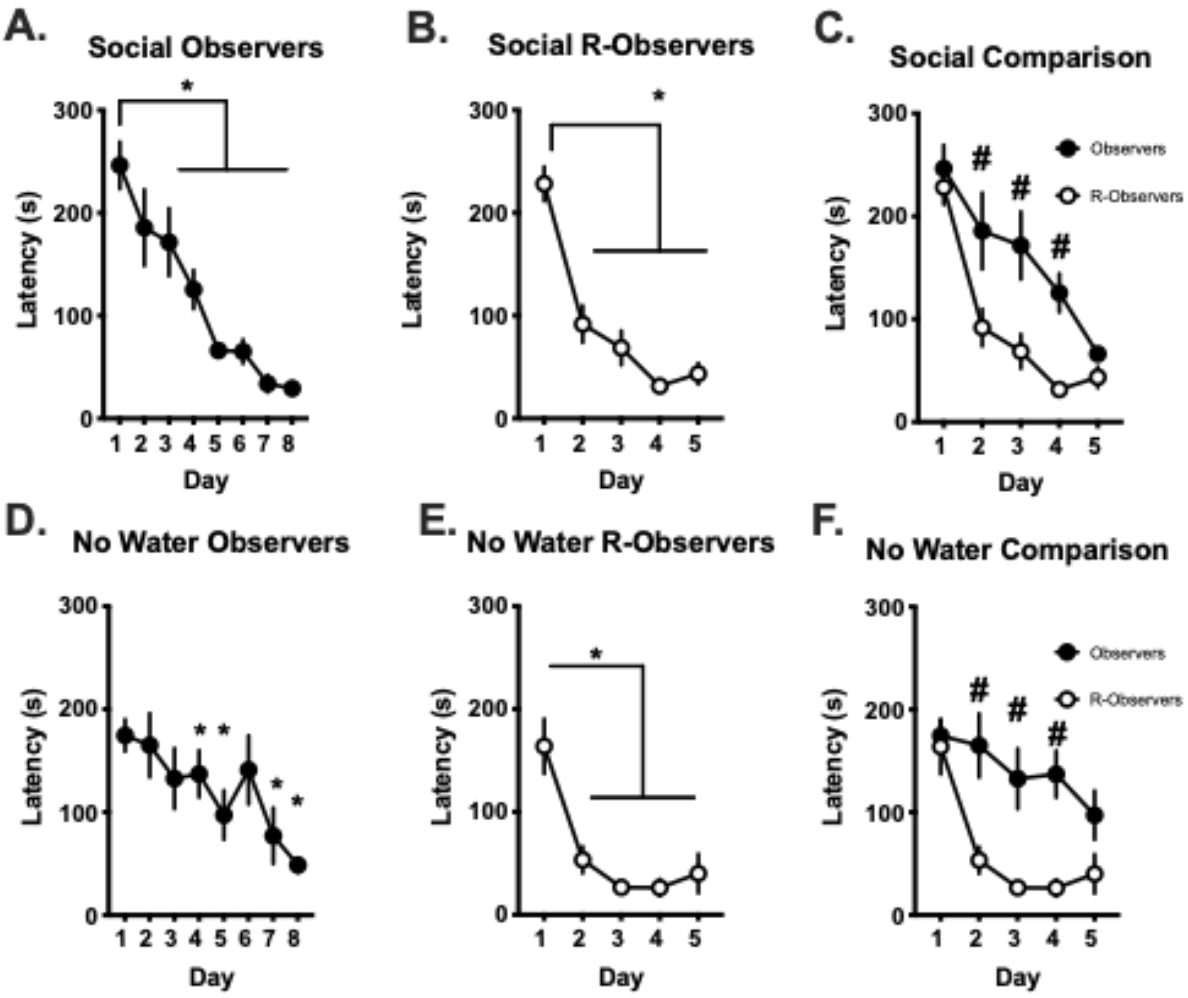


Figure 3.1. **A-C) Rats readily release a conspecific from a pool of water.** Performance of 7 pairs of male rats during the social 2 chamber task. **A)** Latency for observer rats to chain pull to release a distressed partner decreased over 8 days. Significantly shorter latencies occurred on days 2-8. **B)** R-observer rats showed a decrease in response latency by day 2 with significantly shorter latencies occurring on days 2-5. **C)** When the first 5 days of the acquisition and reversal phases were directly compared, R-observer rats performed the task significantly faster on days 2-4. **D-F) Rats will perform an operant task for social interaction.** Performance of 8 pairs of male rats during the social 2 chamber task with the target placed in a dry compartment (i.e. no distress present). **D)** Observer rats' latencies to release a non-distressed target decreased over the course of 8 days with significantly shorter latencies on days 4-5 and 7-8. **E)** R-observer rats had a decrease in the latency to release the R-target over 5 days with significantly shorter latencies on days 2-5. **F)** When the first 5 days of the acquisition and reversal phases were directly compared, R-observer rats performed the task significantly faster on days 2-4.

*Significant difference from day 1 or baseline, ($p<0.05$).
#Significant difference from R-Observers, ($p<0.05$).

Rats Readily Engage in Empathic Behavior Independent of Social Interaction: Behavior was conducted in three different cohorts of rats to provide replications of the task. Rats (n = 24 pairs) were placed in the 3 chamber apparatus and latency was recorded. Observer rats (**Figure 3.2A**) had decreased latencies over days [$F(9, 207) = 14.33$, $p<0.0001$] with days 4-10 significantly faster compared to day 1 ($p<0.05$). When the conditions were reversed, R-observers' (**Figure 3.2B**) latencies also decreased over days [$F(4, 60) = 6.976$, $p=0.0001$], with days 2-5 being significantly lower than day 1 ($p<0.05$). In a direct comparison, Observers had longer latencies than R-observers across all 5 days [**Figure 3.2C**; main effect of group $F(1,38) = 28.99$, $p<0.0001$], suggesting that previous experience to a distressing situation modulates task learning in this empathic model. In a separate cohort, target rats (n=8 pairs) were placed in a dry compartment rather than one filled with water, thereby removing the distress of the Target. There were no differences in latency across days in Observers (**Figure 3.2D**) or R-Observers (**Figure 3.2E**). In the absence of a distressed conspecific and social interaction, chain pull behavior was not readily acquired and prior experience did not modulate acquisition of the task.

Empathic Responding is Subject to Effort: We tested motivation to release a conspecific in both assays by increasing the FR requirement to 5 and then 10 for 3 consecutive days each. Social Observers (**Figure 3.3A**) had faster latencies under the FR1 (i.e. baseline) than under an FR10 schedule but still executed the chain pull response under higher ratio schedules [main effect FR, $F(2, 18) = 4.96$, $p=0.019$]. In contrast, Empathy Observers took significantly longer to release the Target under an FR5 and an FR10 schedule [Figure 4B, ($F(2, 21) = 25.27$, $p<0.0001$].

Specificity of the Chain Pull Response Depends on the Targets' Distress: To characterize the specificity of the release behavior to the distress of the conspecific, the Target was replaced by a "fake" rat or removed, leaving an empty pool of water. For both Observers (n=8, **Figure 3.4A**) and R-observers (n=8, **Figure 3.4B**) latencies were significantly increased in both conditions relative to BL [Observers: ($F(2, 14) = 6.27$), $p=0.02$ and Holm Sidak's $p<0.05$; R-observers: ($F(2, 14) = 7.079$), $p=0.0075$, and Holm Sidak's $p<0.05$]. These data suggest that chain pull behavior in both

Observer and R-observers is dependent on the presence of a distressed conspecific. We also determined that empathic behavior persisted over time because Observers (n=8) maintained release behavior 5, 10, and 15 days following the final day of acquisition, indicated by a lack of change from BL (**Figure 3.4C**). Additionally, we found that release behavior was sensitive to familiarity of the conspecific. Specifically, in a cohort of R-observers (n=8 pairs), a familiar conspecific was replaced with an unfamiliar rat in the wet chamber over 6 trials and empathic responding was evaluated (**Figure 3.4D**). There was a significant change in latency between trials [$F(6, 42) = 8.581$, $p<0.0001$]. Specifically, the time to release the 'stranger' was significantly slowed during the first 2 trials ($p<0.0005$), but subsequently returned to BL levels, indicating rats were capable of learning to release an unfamiliar conspecific.

As a pilot study to discern if the differences in latency between Observers and R-observers resulted from a drive to reduce the distress of a conspecific or a result of experience with the associative structure of the task, a cohort of Observers (n=4 pairs) were pre-exposed before beginning the empathy task to the pool of water without being rescued. The time spent in the pool each day was yoked to the baseline acquisition curve depicted in **Figure 3.2A** to emulate the distressing condition experienced by a cohort of Targets. Following 10 days (20 trials) of yoked pre-exposure to the water, Observers performed 10 days (20 trials) of the empathy task. A RM one-way ANOVA was significant across days [$F(9, 27) = 2.5$, $p=0.03$], however there were no differences between days 1-10 on post hoc analysis (**Figure 3.4E**).

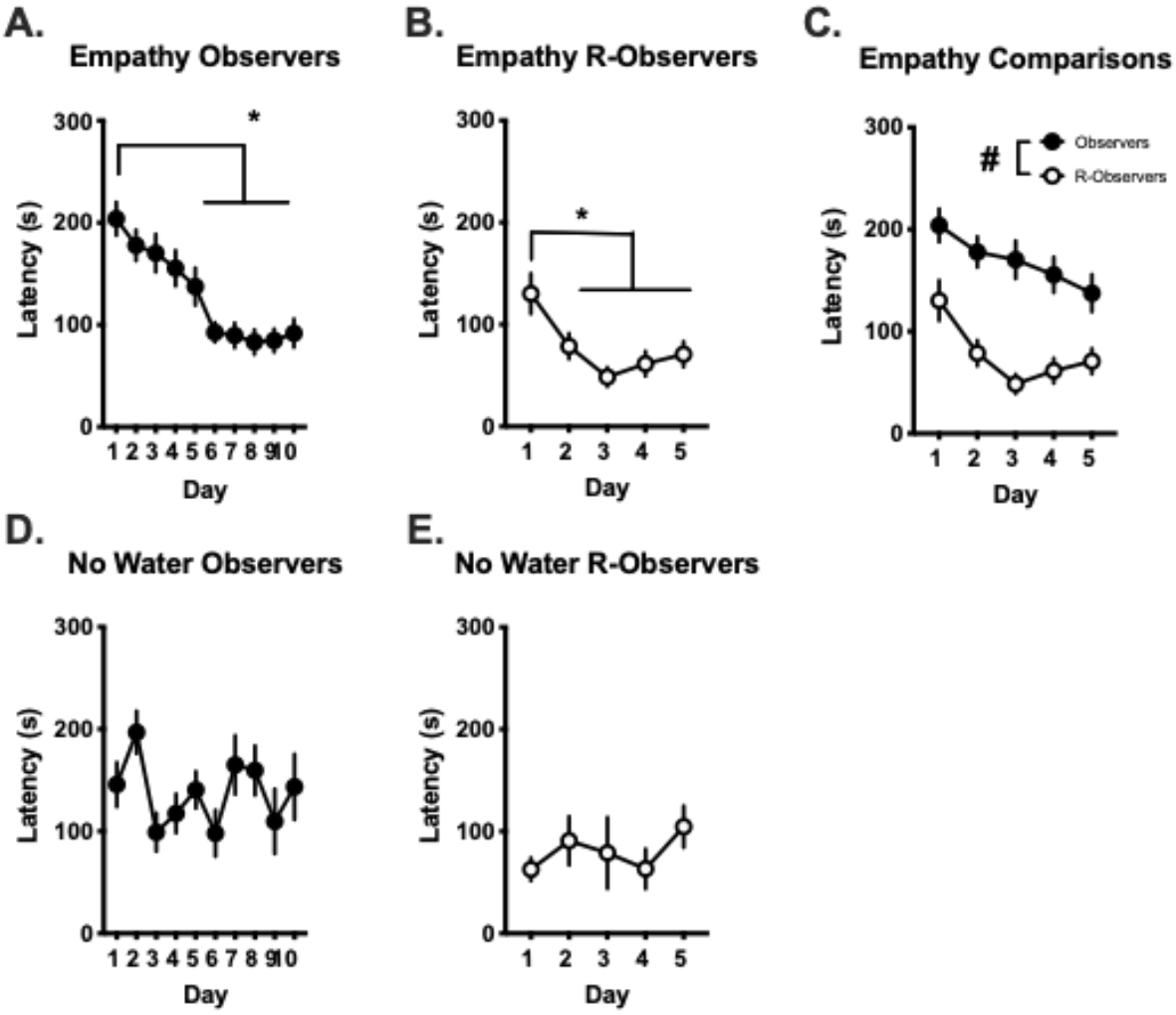


Figure 3.2. **A-C) Rats engage in empathic behavior in the absence of social interaction.** Performance of 24 (acquisition: 8 pairs/cohort over 3 cohorts) or 16 (reversal: 8 pairs/cohort over 2 cohorts) of male rats during our novel empathy task. **A)** Latency for observer rats to chain pull to release a distressed partner decreased over 10 days. Significantly shorter latencies occurred on days 4-10. **B)** R-observer rats that previously experienced the water showed a decrease in response latency by day 2. Specifically, significantly shorter latencies occurred on days 2-5. **C)** When the first 5 days of acquisition and reversal were compared to each other, significantly shorter latencies were found across all 5 days in the reversal compared to the acquisition phase. **D-E) Rats do not respond in the absence of distress or social reward.** Performance of male rats (n=8 pairs) during the novel empathy task, when the target was placed in a dry compartment (i.e. no distress present). **D)** No clear response pattern emerged when observer rats opened the guillotine door for target rats placed in a dry compartment rather than in water. **E)** R-observer rats also showed no difference across days.

*Significant difference from day 1 or baseline, ($p<0.05$).
#Significant difference from R-Observers, ($p<0.05$).

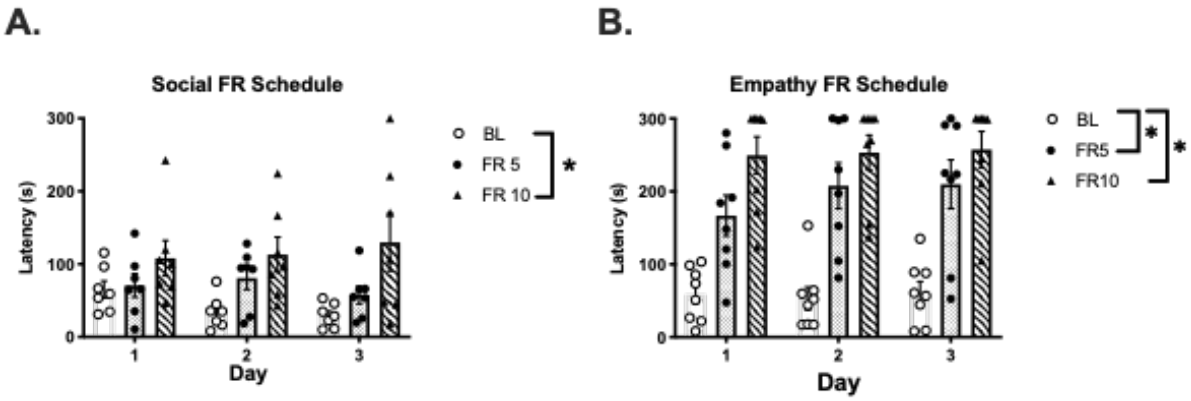


Figure 3.3. Prosocial behaviors are subject to effort. Observer rats underwent an increasing FR schedule moving from FR5 to FR10 (3 days each) to release the distressed target following acquisition of either the 2 or 3 chamber task. **A)** In the social task, latency significantly increased on an FR10 relative to an FR1. **B)** In the empathy task, latency increased significantly on both an FR 5 and FR 10 relative to BL. In fact, in the case of Removal of social interaction as a reward, there was diminished motivation for maintaining the chain pull behavior in the empathy task, or eliminated it altogether, as compared to the social task.
*Significant difference from baseline, ($p<0.05$).

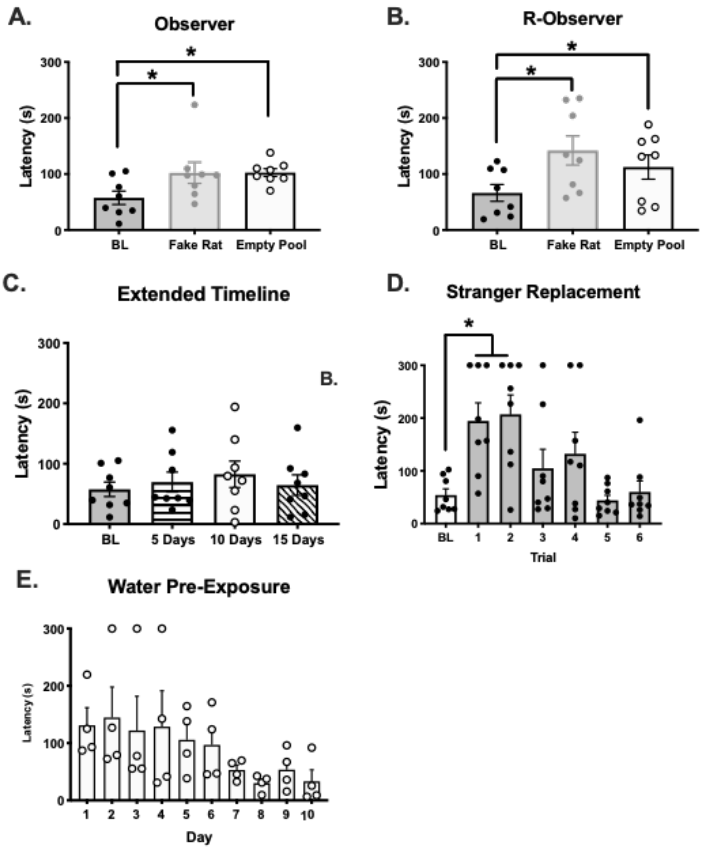


Figure 3.4. **Elucidating the specificity of the chain pull response on the targets' distress.** Performance of male rats across three cohorts (8 pairs/cohort) in separate control experiments using the three-chamber empathy task. **A)** Observer rats significantly increased their chain pull latency when the distressed conspecific was removed and replaced by either a fake rat or an empty pool of water (3 consecutive days of each condition) when compared to baseline (BL; baseline (BL, average of final 3 days of acquisition). **B)** In a separate cohort, R-Observer rats also demonstrated an increased latency to chain pull in response to a fake rat and an empty pool relative to BL (average of final 3 days of reversal). **C**) Following 10 days (20 trials) of acquisition, Observer rats (n=8) maintained release behavior for 15 days. Specifically, observers were reintroduced to the 3 chamber empathy task and latencies to release the distressed targets were recorded 5, 10, and 15 days following their last acquisition session. Latencies did not change over time compared to BL. **D)** In another study, a rat that was completely unfamiliar to the R-Observer rat was introduced into the wet side of the empathy chamber ('stranger'). Latency to release the 'stranger' was significantly potentiated compared to baseline during the first 2 trials, but subsequently returned to levels not significantly different from BL. **E)** A cohort of observer rats (n=4) were pre-exposed to the pool of water (yoked to time spent by targets in Experiment 3) without being rescued prior to the start of acquisition of the empathy task. There was a significant effect across days, however there were no differences between days 1-10 on post hoc analysis.
*Significant difference from baseline, ($p<0.05$).

DISCUSSION

Our 3 chamber task conforms to the perception action model (PAM) of empathy and may be a translational tool to understand empathy in neuropsychiatric disorders. We have expanded current models by removing social interaction as an underlying interpretation for the observed behavior and showed that rats will still learn to release a distressed cage mate (**Figure 3.2A**) and retain the task for an extended time period (**Figure 3.4C**). Further, previous experience with the same distressing environment attenuates the time it takes for rats to release a distressed conspecific (**Figure 3.2B**). Taken together, we believe that these data support and adhere to the PAM (described in de Waal and Preston, 2017) as a translational evaluation of empathy.

According to the PAM, the presence of a distressed animal initially causes emotional transfer of that distress, thereby promoting a shared affect. Observer rats must regulate their own emotional state in order to respond appropriately to lower the Target's distress. The relief of the Target will then allow for another emotional transfer between animals, and therefore reinforce the Observer's actions. Prior exposure to the same stressor more readily enables the R-observer to state match with the R-target perhaps by activating salient emotional memories that drive empathic behavior. When the distressed Target or R-target was either replaced or removed in the 3 chamber task, the chain pull latency of the Observers and R-observers significantly increased (**Figures 3.4A & 3.4B**). We can conclude that the rapid release latency seen in both Observers and R-observers is dependent on the presence of a distressed cage mate.

Social Observers did not maintain the chain pull response under an FR10 but did continue under an FR5 when the outcome involved direct contact with the conspecific (**Figure 3.3A**). However, Empathy Observers did not maintain responding past an FR1 (**Figure 3.3B**). Combined, these findings suggest that social interaction aids in maintaining this helping behavior. Along this notion, prosocial behaviors are sensitive to changes in motivation and when the effort to respond is increased. Although there is a dearth of research in rodents evaluating the motivational aspects of helping behavior, a recent study gave rats the opportunity to release a distressed conspecific or

escape into a darkened cage to alleviate their own distress. Rats learned to release their cage mate, but they did so slower and less often compared to rats that did not have the choice to escape (Carvalheiro et al., 2019). Further, clinical studies found that empathy in human subjects decreased when the effort to help increased (Lockwood et al., 2017) and engaging in empathy was avoided because it was considered more effortful compared to an alternative strategy (Cameron et al., 2019). These studies suggest that empathic behaviors, especially those requiring more than minimal effort, are significantly less motivating when compared to a behavior that ultimately leads to a personal reward like social interaction. Future studies could directly evaluate the motivation to perform a social task compared to an empathic task.

Familiarity between conspecifics has a role in helping behavior. For example, rats will aid a distressed stranger only if it is a strain with which the observer rat is familiar. Further, pair-housing rats with a particular strain prompt them to aid strangers of that strain, indicating prior social experience may drive helping behavior (Bartal et al., 2011). Thus, we tested the importance of familiarity for R-observers to perform the empathy task in the presence of rats with which they were completely unfamiliar. Interestingly, chain pull latencies in the first two (of 6) trials were significantly increased compared to baseline while the last four were not (**Figure 3.4D**). This finding was not due to habitation to an individual rat because three different stranger rats were used randomly over the course of the six total trials. This response pattern indicates rats quickly acclimated to the concept of aiding an unfamiliar conspecific. Prosocial behaviors in humans are modulated by the degree of affiliation, with in-group members preferentially helped as compared to unaffiliated individuals (Cialdini et al., 1997; Levine et al., 2005; Echols and Correll, 2012). However, group affiliation can be overcome by changes in social experience (Batson et al., 2005), even in rats (Bartal et al., 2011). The stranger rats used in our experiment were of the same strain as the R-observers, corroborating the conclusion that the motivation to perform helping behaviors in rats is extended to strangers of familiar strains (Bartal et al., 2011). This evidence may indicate that rats form groups based on social experience, similar to humans (Mathur et al., 2010). As it

pertains to the PAM, rats are less likely to generate shared affect with an out-group animal, reducing the motivation to act empathically. However, this can be more readily overcome with an unfamiliar rat of a familiar strain. Future experiments using this novel model will work to evaluate the importance of group membership via cross-fostering rats of different strains to determine if they demonstrate selectivity in empathic behavior towards strangers of the strain with which they are familiar.

It is likely that the behavior seen in our lab and other researchers' (Bartal et al., 2011, 2014; Sato et al., 2015; Karakilic et al., 2018) social helping model, in which rats will aid a distressed conspecific (**Figure 3.1A**), and previous experience with the same distressing environment directly modulates helping behavior (**Figure 3.1B**), is driven in part by empathy. However, data from our lab and elsewhere (Silberberg et al., 2014; Hachiga et al., 2018) suggest that these models are also driven by the opportunity for social reward. There is a wealth of evidence that demonstrates rats find various forms of social interaction, such as play fighting (Vanderschuren et al., 2016), maternal care of pups (Lee et al., 2000), and sexual behavior (Trezza et al., 2011) reinforcing. Further, animals readily exhibit preference to social interaction in conditioned place preference (CPP) paradigms (Calcagnetti and Schechter, 1992; Douglas et al., 2004; Fritz et al., 2011), and they have also been shown to value social reward above both natural rewards (palatable foods) (Ikemoto and Panksepp, 1992) and drugs of abuse (Normansell and Panksepp, 1990; Fritz et al., 2013; Yates et al., 2013). Venniro et al. (2018a) demonstrated volitional social reward reliably attenuated both methamphetamine and heroin self-administration in rats in an operant choice model. Like an operant model of social reward, Targets in the 2 chamber social task were released into the same environment as the Observer. When the water was removed as a distressor, there was a delay in the acquisition of the chain pull (**Figure 3.1D**) when compared to the task in which water is present (**Figure 3.1A**). We posit that this delay is because a driving force of the behavior, reducing the distress of the cage mate, was removed. However, social reward was still present and thus helped to eventually promote a decrease in release latency. Moreover, it is likely that R-observers more

readily acquired the task in the absence of a distressed cage mate (**Figure 3.1E**) because they were still able to associate the chain pull with social reward from being given access to the dry chamber during the acquisition phase.

In contrast, when water was removed from the 3 chamber empathy task, meaning there was neither a distressed conspecific nor the opportunity for social interaction, there was no change in chain pull response across days, indicating that Observers/R-observers did not systematically acquire a directed chain pull task (**Figures 3.2D & 3.2E**). The absence of a distressed conspecific, as described by the PAM, prevents the motivating force of state matching in order to drive the helping behavior. Moreover, there is no prior affective experience that allows for enhanced state matching in the absence of the distressing event, which, we suggest, is why there is no difference over days in the chain pull responses in Observer or R-observer rats when water was removed during the empathy task.

In conclusion, we have expanded on the current literature to develop a method to study empathic behavior in a translationally relevant rodent paradigm based on Preston and de Waal's perception action model (PAM) (Preston and de Waal, 2002; de Waal and Preston, 2017). Rats display empathic behaviors indexed as a progressive decrease in latency to release a distressed conspecific. Moreover, prior experience with the event allows for enhanced state matching and improved targeted helping. The observed empathic behavior, although subject to low effort as previously described (Lockwood et al., 2017; Cameron et al., 2019; Carvalheiro et al., 2019), is specific to the presence of a distressed conspecific, and rats will also learn to aid an unfamiliar animal. Overall, we believe this model could be an excellent translational tool to better understand the underlying neurobiology of empathy, as well as understanding how empathy is altered in psychiatric disorders (Heilig et al., 2016; Venniro and Golden, 2020b).

CHAPTER 4: Neuronal, Affective, and Sensory Correlates of Targeted Helping Behavior in Male and Female Rats

INTRODUCTION

As noted previously in this dissertation, empathy is a complex suite of behaviors that works to convey an understanding of the affective state of others. The ability to generate a shared affective state can have myriad benefits, including group cooperation, reproduction, and survival (Decety, 2011; de Waal and Preston 2017). Empathic processes therefore help to inform interpersonal relationships, as well as guide complex social norms (de Waal and Preston 2017; Adriaense et al., 2020).

Sex has long been considered a critical variable in empathy. Indeed, clinical research has long concluded women have consistently shown higher levels of empathic compared to males (Hall, 1978; O'Brien, 2013). However, these results have increasingly been called into question, as evidence has accumulated to suggest that the observed differences are a direct result of the methodology of the study (Eisenberg and Lennon, 1983; Baez et al., 2017). Unfortunately, the vast majority of rodent studies investigating empathic processes are performed in males, but the studies that do test sex differences are conflicting. For example, one study of targeted helping found female rats released a distressed conspecific from a restraint tube faster than males (Ben-Ami Bartal et al., 2011). In another, males and females were used but no sex difference was reported, and data were collapsed across sex (Sato et al., 2015). Additionally, male and female rats demonstrated indistinguishable levels of emotional contagion (Han et al., 2020), and either minimal or no differences in a battery of consolation behaviors in an empathy of pain model (Du et al., 2020). It was therefore imperative that sex was evaluated as a variable in our 3 chamber model of targeted helping.

According to the Perception Action model (PAM) of empathy as described in **Chapter 1**, empathic behaviors like targeted helping stem from an affective transfer between conspecifics. Sensory cues are critical for understanding the state of a conspecific and can modulate prosocial behaviors, but the mechanism behind this affective transfer is poorly understood and likely multimodal. Research suggests that rodent prosocial behaviors, such as social learning, may require the availability of visual cues for the best facilitation of learning (Paraouty et al., 2020). As it more precisely relates to empathy, rodents have the ability to recognize the distress of conspecifics, at least in part, though visual cues (Fanselow, 1985), as vicarious freezing was attenuated if an Observer animal's view of the Target was obstructed by an opaque partition (Jeon et al., 2010). More recently, it was demonstrated that emotional contagion, specifically empathic observational contagious pain in mice, requires the image-forming visual system (Geng et al., 2020). We therefore examined the role direct visualization of the distressed conspecific during the targeted helping task has on Observers' chain pull latency in both males and females.

It is unlikely that the visual system is the only sensory modality that is important in the transfer of affective information between Observer and Target (Knapska et al., 2006; Kim et al., 2018). As highly social creatures, rats use a rich repertoire of sensory cues to communicate and increasing attention has been given to ultrasonic vocalizations (USV) as an effective proxy for understanding the affective state of rodents. Although there is still a dearth of evidence regarding the behavioral consequence or specificity of USV, they can broadly be categorized in rats into two groups classified by their frequencies. Low frequency USV, often called 22 kilohertz (kHz) USV, with a range of approximately 18-35 kHz, are emitted in the presence of aversive stimuli, such as the presence of a predator or predator odor (Blanchard et al., 1991) and other stressors, like inescapable foot shock (Borta et al., 2006). It is therefore hypothesized that low frequency USV serve as alarm calls or indicate an aversive affective state. High frequency USV, also known as 50-kHz USV, fall within the range of >35 kHz and are emitted in prosocial situations like social exploratory activity, mating behavior, and other positive affective states (Panksepp and Burgdorf,

2003; Simola and Brudzynski, 2018; Wöhr, 2018). Research is still needed to explore affective states of rats during empathic behavior (Chen et al., 2009; Atsak et al., 2011), but some evidence points to USV as critical for evoking an empathy-like state and promoting helping behavior (Ben-Ami Bartal et al., 2011). Therefore, USV of both the Target and Observer were recorded during multiple timepoints throughout our targeted helping task in males and females to understand their respective affective states.

As mentioned in **Chapter 1**, a large library of fMRI studies exists that have evaluated the neurobiology of multiple components and cognitive levels of empathy. Brain regions that have been correlated with aspects of empathy include those involved in emotional salience and interoceptive valence, specifically the amygdala and insula (Adolphs et al., 2002; Singer et al., 2004; Fusar-Poli et al., 2009; Keysers and Gazzola, 2018; Marsh, 2018), as well as substrates necessary for perspective-taking, motivation, and cognition, like the prefrontal (PFC), anterior cingulate (ACC), and orbitofrontal (OFC) cortices (Jackson et al., 2005; Singer and Lamm, 2009; de Waal and Preston, 2017; Decety, 2015; Cerniglia et al., 2019; Uysal et al., 2019). More causational and region-specific research using rats and mice are beginning to corroborate some of these imaging studies (Panksepp and Panksepp, 2013; Sivaselvachandran et al., 2016; Meyza and Knapska, 2018; Meyza et al., 2018). However, no rodent research to date has done a large, brain-wide exploration of neural activity during empathic behavior, nor has there been a sex comparison of activity in these regions. For these reasons, neural activity of cortical and subcortical regions of interest were evaluated through immunohistochemical analysis of the immediate early gene *c-fos* across time and between sex.

Finally, **Chapter 1** also discusses the critical and complex role oxytocin (Oxy) plays in empathic behavior. The primary region of Oxy release, the paraventricular nucleus of the thalamus (PVN) can be divided into two nuclei based on the cellular morphology and electrophysiology. Canonically, the magnocellular nucleus mainly project to the posterior pituitary gland and release Oxy in the bloodstream to influence peripheral functions as a neuronal hormone, while the

parvocellular nuclei controls autonomic function via projections to the spinal cord and midbrain (Liao et al., 2020). However, recent evidence suggests neurons from the PVN project to cortical regions and regulate complex behaviors, including empathy (Donaldson and Young, 2008; Liao et al., 2020). Oxytocin neural levels have been associated with differences in emotional contagion (Laviola et al., 2017), observational fear learning, and socially transmitted fear (Guzman et al., 2014; Pisansky et al., 2017). Oxy has also been shown to modulate targeted helping in a socially dependent manner (Yamagishi et al., 2019), suggesting its role may be more nuanced and context-dependent than previously thought. To begin to understand the relevance of Oxy within our model of empathic behavior, Oxy neuronal activity within the PVN was analyzed through *c-fos* activity.

In the following experiments, we utilized our lab's 3 chamber model of targeted helping to investigate the behavioral, affective, sensory, and neural differences in empathic behavior between males and females across three timepoints; early acquisition (EA), as an indication of an initial helping response; late acquisition (LA) to examine if habituation to aiding a familiar conspecific modulates helping behavior as it does social interaction (Choleris et al., 2006); and reversal (Rev) to see if previous experience plays an equal role in helping behavior in males and females (Sato et al., 2015; Cox and Reichel, 2019). We conclude that sex differences are not a ubiquitous presence in empathic behavior; it is likely that sex differences may instead be a convergent phenomenon, in which the behavior of males and females are similar, but the underlying biological mechanisms are distinct (Becker et al., 2017). Having a sex-specific picture of the distinct sensory, affective, and neural components of empathy can help us better understand the underlying process of a complex prosocial behavior in males and females, but also act as a steppingstone towards improving treatment outcomes and quality of life for individuals suffering from psychiatric disorders with dysregulated empathy.

MATERIALS AND METHODS

Animals: Size-matched male and female Sprague Dawley rats weighing 250-275g were pair-housed with the same sex as described in **General Methods**. All experimental procedures were conducted in accordance with the "Guide for the Care and Use of Laboratory Rats" (Institute of Laboratory Animal Resources on Life Sciences, National Research Council) and approved by the IACUC of the Medical University of South Carolina.

Behavioral Testing: There is conflicting evidence regarding the presence of sex differences during empathic tasks in rats (Han et al., 2020; Bartal et al., 2011) and mice (Du et al., 2019). For this reason, we also compared targeted helping between males and females (n=21 pairs/sex) using our 3 chamber model of targeted helping (described in **General Methods**). Male and female rats (n=4/sex) were also evaluated in the 2 chamber task (described in **General Methods**), where contact between conspecifics is permitted, in order to also determine if social interaction may drive sex differences in prosocial behaviors (Borland et al., 2018). Finally, in order to determine the importance of the Observer visualizing the Target to learn to release the distressed conspecific, a separate cohort of male and female rats (n=8/sex) were tested in the 3 chamber operant box where the Plexiglas divider present between Observer and Target was painted black to prevent either animal from seeing through it. In all tasks, trials (20 total across 10 days, labelled "Acquisition") lasted a total of 300 s (5 min) regardless of the chain pull latency to reduce the likelihood that removal from the apparatus was a motivating factor for the behavior. If the Observer did not pull the chain within the allotted time, the experimenter ended the trial and released the Target. The role of each rat in a pair was subsequently reversed ("Reversal" phase) such that a Target became the Observer (labelled "R-observer"), and the Observer becomes the target rat ("R-target"). This reversal phase was carried out for 5 days (10 trials).

Immunohistochemistry (IHC): In order to elucidate the neural substrates important during empathic behavior, subjects were sacrificed and perfused approximately 90 minutes following the task at three different time points (EA, LA, and Rev, n=4-6/group) and brains were collected. Briefly, rats

were anesthetized and brains were removed and postfixed as described in **General Methods (Chapter 2)**. In ofrder to analyze Fos expression in regions of interest, tissue was stained with 3,3' diaminobenzidine (DAB) + nickel ammonium sulfate to produce a blue-black nuclear reaction product as previously described (**Chapter 2**). Slices were coverslipped using Permount and regions of interest were photographed at 10x magnification using a Leica microscope and VideoToolbox software.

The same brains were utilized to determine the activity of oxytocin neurons within the paraventricular nucleus of the thalamus (PVN) during empathic behavior. Different 50-µm tissue slices containing the PVN were permeabilized and blocked in 2% normal goat serum (NGS) and 2% Triton X-100 in PBS and were incubated in a rabbit anti-Fos primary (Millipore; 1:1000) and mouse anti-oxytocin (Millipore; 1:2000) overnight at 4°C, followed by a 5-hour incubation in donkey anti-rabbit 488 (Millipore; 1:1000) and donkey anti-mouse 594 (Millipore; 1:1000) at room temperature while protected from light. Slices were mounted and coverslipped with Prolong Gold, and representative images of the magnocellular and parvocellular regions of the PVN were taken at 20x magnification using a Nikon fluorescent microscope and averaged together to create an average PVN activity estimate.

IHC Quantification and Analysis: At the three timepoints (EA, LA, and Rev), rats either performed the targeted helping task (Empathy) or remained in their homecages (homecage control, HCC) as controls. For the DAB stain, blue-black nuclear immunoprecipitate from Fos-positive cells in regions of interest were quantified using a brain atlas for comparison (Paxinos and Watson, 2007). Fos-positive (Fos+) cells that fell within each region of interest were automatically counted using a macro and averaged across sections for each rat. On average, 3 bilateral sequential sections for each region were used for analysis. Anterior-posterior coordinates for each analyzed region are as follows: PL: 3.7 to 2.7; IL: 3.2 to 2.2; ACC: 3.2 to 2.2; OFC: 4.2 to 2.7; LHb: -2.5 to -3.3; PVT: -2.5 to -3.6; BLA: -2.5 to -3.3; CeA: -2.5 to -2.8. In order to compare the change in Fos+ cells

between sex and across time, each group was compared to their own homecage control (HCC) by calculating a percent change from HCC in the analysis.

In the Fos/oxytocin double label experiment, fluorescently labelled Fos+ and oxytocin+ (Oxy+) cells were identified within the PVN and overlap of the two labels were counted under experimenter-blind conditions. Data were expressed as the percentage of total overlapped Fos+/Oxy+ cell counts per total Oxy+ cells. All images were quantified using ImageJ software (NIH).

Ultrasonic Vocalization Detection and Analysis: Ultrasonic vocalizations (USVs) were recorded in a subset of male and female (n=4-8) rats to understand their affective states and level of communication during empathic behavior. Two high-quality condenser microphones (Avisoft Bioacoustics) were fastened to the lids of the operant box, one on the Observer's dry side and one on the Target's wet side. The microphones were connected to Avisoft UltraSoundGate 416Hb multichannel recording system and processed using Avisoft-SASLab Pro software (Avisoft Bioacoustics, Glienicke, Germany). USVs were recorded for one complete trial (300s) at three different time points (EA, LA, and Rev) with a sampling rate of 250kHz, and analyzed with DeepSqueak version 2.6.0 (Coffey et al., 2019) in MATLAB. Due to background noise during the task, post-hoc denoising was carried out and subsequently rechecked for errors by an experimenter. Calls with tonality of <0.35 were considered to be background and were rejected manually from analysis. Remaining USVs were reviewed by an experimenter blind to the conditions and calls that were picked up in both microphones were assigned to a particular rat by comparing power of the USV across both channels.

Data Analysis: Two-way mixed analysis of variance (ANOVA) was used to compare the latency of chain pulls latency during both the 2 chamber and 3 chamber tasks across sex, with the between-subject variable being sex (males vs. females) and the repeated measure being days (1-10 Acquisition and 1-5 Reversal). Unpaired t-tests were used to compare latencies at EA and LA timepoints between sex. Comparisons in total Fos+ counts across time and sex were performed

using a 3-way mixed ANOVA with sex (male vs. female), group (HCC vs. Empathy), and time (EA vs. LA vs. Rev) as the independent variables (see **Table 3**). Comparisons of total count between HCC groups, as well as percent change from HCC comparisons in Fos+ and Fos+/Oxy+ analyses were performed using 2-way mixed ANOVAs with sex (male vs. female) and time (EA vs. LA vs. Rev) as the independent variables. One-sample t-tests were also performed to determine whether empathy groups were significantly different than HCC. Finally, 2-way mixed ANOVAs were performed to compare USV total counts, while ordinary 1-way ANOVAs were used to compare the distress and social frequencies. All post hoc comparisons were conducted using a Holm-Sidak's correction for family wise error when appropriate, with the alpha set at 0.05. Mixed effect models were used when necessary to account for any missing data points. All analyses were conducted with Prism Software version 8.4.3. Unless noted, all data are expressed as the mean ± SEM.

RESULTS

Sex Differences during Empathic Behavior: Groups of male and female Observers (n=21 pairs/sex) performed the 3 chamber empathy task in order to discern if any sex differences in chain pull latency were present during Acquisition or Reversal. For the acquisition phase, a mixed effects 2-way ANOVA was used to account for missing trials in a small subset of animals. The analysis showed a main effect of time [$F(9,344) = 13.00$, $p<0.0001$] and sex [$F(1,40) = 7.272$), $p = 0.0102$], with males having faster chain pull latencies compared to females. However, the time x sex interaction was not significant ($p = 0.1780$). Post hoc analysis on the main effect of time revealed the latencies on days 2-10 were significantly faster ($p<0.0005$) compared to day 1 (**Figure 4.1A**). In order to determine if the sex differences were present during early acquisition (EA) or late acquisition (LA), averages were taken for latencies on days 1-2 (EA) and 9-10 (LA) for each sex and subsequently compared (**Figures 4.1B and 4.1C**). Unpaired t-tests showed no significant difference between males and females at the EA or LA timepoints. In the Reversal

phase, there was again a main effect of time [F (4,88) = 13.72, $p<0.0001$], with days 2-5 latencies significantly faster than day 1 ($p<0.05$) shown in post hoc analysis of the main effect of time (**Figure 4.1D**). However, no effect of sex was seen between male and female R-Observers.

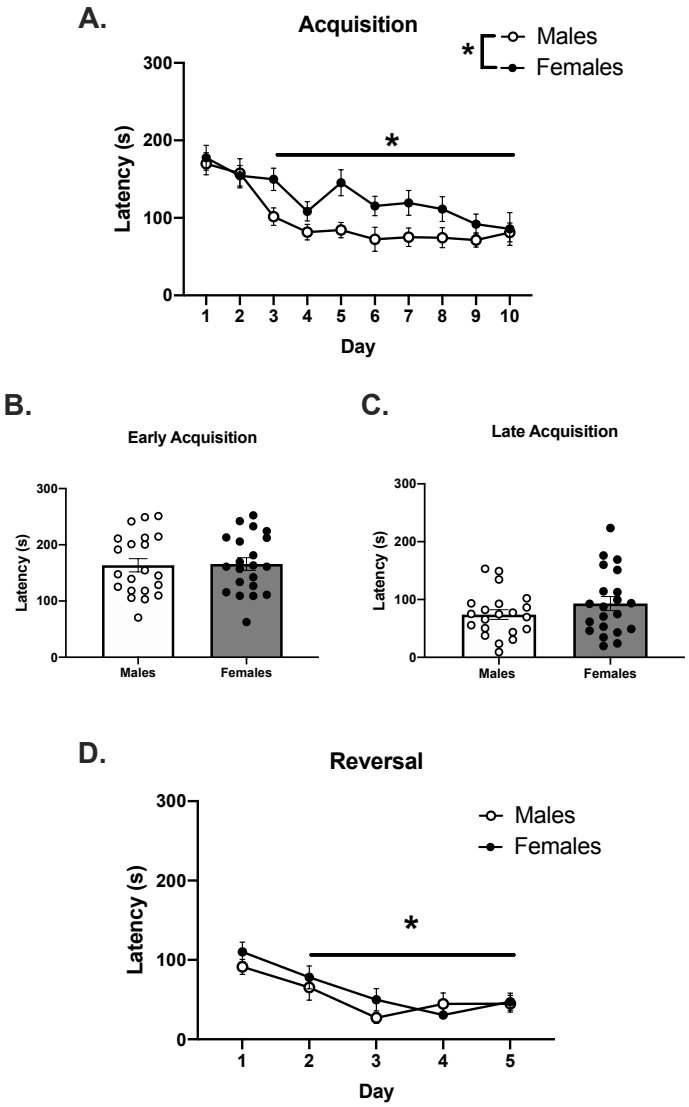


Figure 4.1. **Elucidation of sex differences during the 3 chamber empathy task. A).** Performance of male and female (n=21 pairs/sex) rats during the 3 chamber empathy task revealed latency for Observers to release a distressed partner decreased over 10 days. Significantly shorter latencies occurred on days 3-10 compared to day 1. A main effect of sex was also seen in acquisition, with male latencies being faster compared to females. **B-C)** Unpaired t-tests comparing chain pull latencies during early (**B**) and late (**C**) acquisition did not show a difference between males and females. **D**) Sex differences were also not present during task Reversal. While chain pull latency decreased, with days 2-5 significantly shorter than day 1, no main effect of sex was seen in Reversal. *p<0.05.

Sex Difference is Driven by 'Low Acquisition Index' Rats: In order to better understand individual and sex differences in chain pull latency, an Acquisition Index was calculated: $AI = \frac{Acq_{BL} - Acq_F}{Acq_{BL} + Acq_F}$, where Acq_{BL} is an average of the first 2 days of acquisition used as baseline chain pull latency, and Acq_F is an average of the final 2 days of acquisition. Positive AI reflect a reduction in latency over the course of acquisition, with AI=1 the maximum possible given each animal's baseline. An unpaired t-test comparing the AI of all males compared to all females revealed no significant difference (**Figure 4.2A**). The median of each sex's AI was then used to divide each sex into two groups, "Low AI" and "High AI" (Hernandez-Lallement, et al., 2020). A mixed effects 3-way ANOVA comparing all of these groups indicated main effects of time [F (9,197) = 9.069, $p<0.0001$], sex [F (1,197) = 24.61, $p<0.0001$], and index group [F (1,167) = 5.091, $p = 0.0253$]. Further, significant time x index group [F (9,167) = 3.935, $p = 0.0001$] was seen. In order to discern the sex differences within the AI groups, low and high AI groups of each sex were compared. A mixed effects 2-way ANOVA comparing high AI male and female rats only showed a main effect of time [**Figure 4.2B**, F (4.252,81.74) = 16.89, $p<0.0001$]. In contrast, the same analysis showed a main effect of sex when comparing males and females with low AI [F (1,18) = 5.23, $p = 0.0345$], with only a trend for a main effect of time ($p = 0.0586$) (**Figure 4.2C**). Taken together, these data indicate no sex effect is present in any of the timepoints analyzed or in high AI animals, but the main effect of sex is driven almost exclusively by sex differences with animals with low AI.

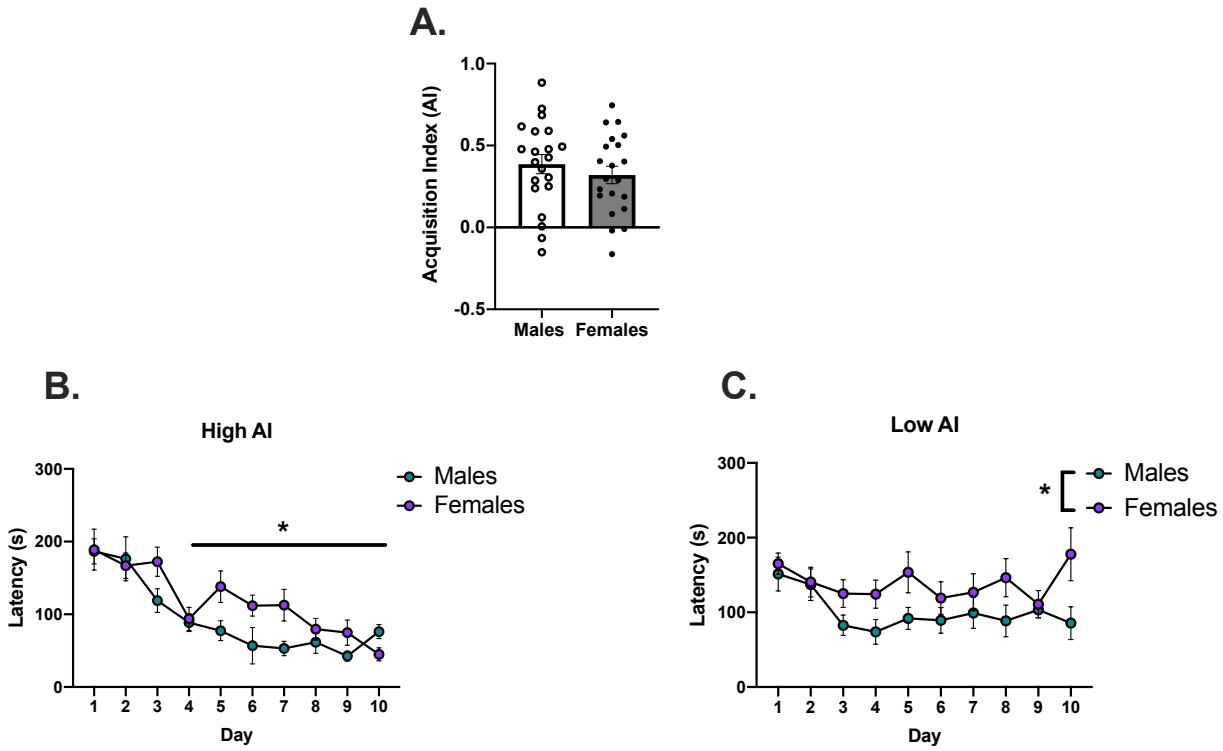


Figure 4.2. Comparison of Acquisition Index Calculation Between Sex. **A**) The calculated $AI = \frac{Acq_{BL} - Acq_F}{Acq_{BL} + Acq_F}$, did not differ between males and females in the empathy task. **B-C**) Males and females were divided into 'high' and 'low' AI groups based on the median of each sex's AI (n=10-11/group). **B**) Chain pull latency decreased overall in the 'high AI' groups, with days 4-10 significantly faster than day 1. However, no effect of sex was observed. **C**) In contrast, 'low AI' females had significantly slower latencies when compared to 'low AI' males. *p<0.05.

Males and Females Readily Release a Conspecific if Social Interaction is Possible: **Figure 4.3A** demonstrates that, during acquisition, males and females (n=4/sex) release a distressed conspecific in the 2 chamber helping task that allows for social contact, at similar rates (main effect of time [$F(9,54) = 10.03$, $p<0.0001$]). Post hoc analysis of the main effect showed chain pull latency on days 4-10 were significantly faster compared to day 1 ($p<0.05$). The 2-way ANOVA did not reveal a main effect of sex during acquisition. Indeed, no differences were seen in the unpaired t-tests comparing male and female latencies during EA (**Figure 4.3B**) or LA (**Figure 4.3C**). Similarly, during Reversal, only a main effect of time [$F(4,24) = 29.34$, $p<0.0001$] was found. Specifically, Reversal; days 2-5 were significantly faster compared to day 1 ($p<0.0001$) (**Figure 4.3D**).

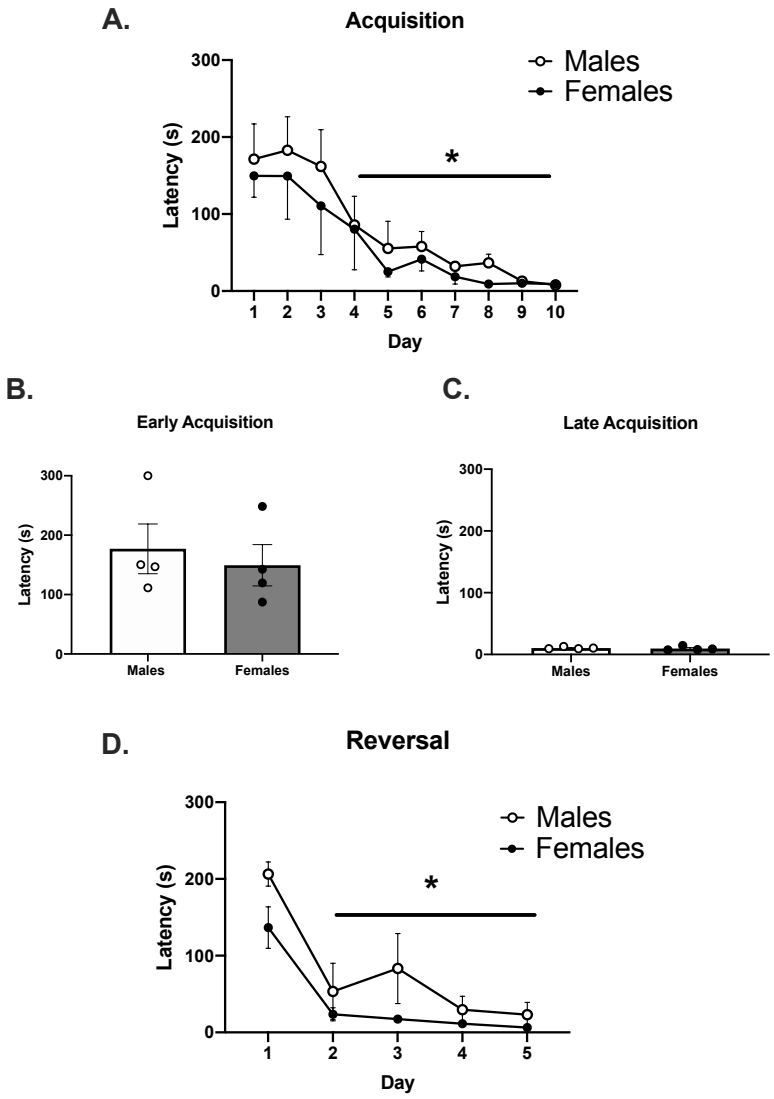


Figure 4.3. **Males and Females Readily Release a Conspecific if Social Interaction is Possible.** Performance of male and females (n=4 pairs/sex) rats during the 2 chamber helping task where social contact is possible **A**) Chain pull latencies for males and females during acquisition did not differ; latencies decreased over time, with days 4-10 significantly faster compared to day 1. **B-C**) Unpaired t-tests comparing chain pull latencies during early (**B**) and late (**C**) acquisition did not show a difference between males and females. **D**) Latencies again decreased in reversal, specifically on days 2-5 compared to day 1. However, no main effect of sex was identified. *Significant difference from day 1, ($p<0.05$).

Sex Differences in Empathic Behavior when Visualization of the Conspecific is Prevented: **Figure 4.4** shows the chain pull latency of males and females when the Plexiglas divide between the Observer and Target was blacked out. Only a main effect of time [F (4.829, 67.61) = 9.733, $p<0.0001$] was revealed by the 2-way ANOVA during acquisition, with days 4-10 significantly different from day 1. However, there was a strong trend for an effect of sex ($p = 0.0589$). Unpaired t-tests comparing males and females during EA and LA revealed females were significantly faster during EA compared to males (**Figure 4.4B**, $p = 0.043$), but not during LA (**Figure 4.4C**). Finally, a main effect of time [F (4,56) = 4.764, $p = 0.0022$] was observed during reversal when visualization of the conspecific was prevented (**Figure 4.4D**), but neither a main effect of sex, nor a time x sex interaction was shown to be significant.

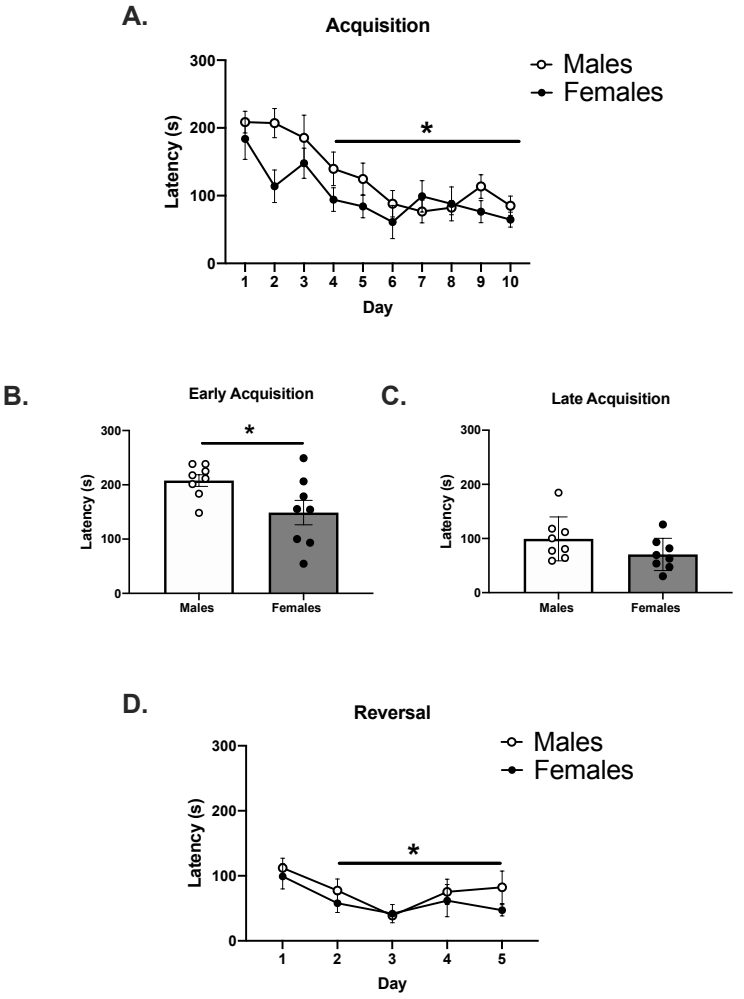


Figure 4.4. **Sex Differences in Empathic Behavior when Visualization of Conspecific is Prevented. A)** Male and female chain pull latencies were similar across acquisition; days 4-10 were significantly faster than day 1. **B-C**) In order to specifically evaluate sex differences during early and late acquisition, unpaired t-tests were performed comparing the average latencies for each time frame. **B**) Female latencies were significantly faster compared to males at the early time point. **C**) During late acquisition, however, there was no sex difference in chain pull latency. **D**) While the overall latency decreased in reversal, specifically on days 2-5 compared to day 1, no main effect of sex was identified. *p<0.05.

C-Fos *Total Count Varies Across Substrate, Time, and Sex:* In order to discern the neural substrates critical during empathic behavior, as well as understand the temporal changes and sex effects during the task, groups of male and female rats (n=4-6) either performed the 3 chamber task (Empathy), or were left in their home cages as controls (HCC), and were sacrificed for Fos expression at three different timepoints; the third day of acquisition (EA), the final day of acquisition (LA), or on the final day of reversal (Rev), and the total number of Fos+ cells within each region of interest was calculated. For each region, a 3-way ANOVA was performed, with the variables being time (EA vs. LA vs. Rev), sex (males vs. females) and group (HCC vs. Empathy). **Table 3** shows the average total Fos+ count within each region of interest, as well as the pertinent findings of the 3-way ANOVAs.

Table 3. Average Fos+ cells at EA, LA, and Rev in male and female rats. Values expressed as means ± SEM.

	Group				
Region	***Male HCC***	***Female HCC***	***Male Empathy***	***Female Empathy***	***Effects (p value)***
AI	**EA**: 102.91± 18.59	**EA**: 120.51 ± 23.23	**EA**: 232.52 ± 29.07	**EA**: 260.36 ± 24.08	Time (0.0002) Group (<0.0001) Sex (<0.0001) Time x Sex (0.0003)
	LA: 42.71 ± 4.18	**LA**: 123.58 ± 22.81	**LA**: 114.32 ± 10.05	**LA**: 293.14 ± 17.61	
	Rev: 65.04 ± 12.26	**Rev**: 80.92 ± 12.50	**Rev**: 141.74 ± 28.14	**Rev**: 164.55 ± 22.60	
PL	**EA**: 189.0 ± 12.42	**EA**: 226.48 ± 54.76	**EA**: 419.96 ± 49.66	**EA**: 450.62 ± 39.95	Time (0.0001) Group (<0.0001) Sex (0.0012) Time x Sex (<0.0001) Time x Group (0.0173) Group x Sex (0.0165)
	LA: 209.29 ± 35.57	**LA**: 362.70 ± 18.42	**LA**: 366.49 ± 33.36	**LA**: 699.21 ± 43.89	
	Rev: 302.48 ± 25.16	**Rev**: 175.12 ± 11.06	**Rev**: 332.39 ± 35.80	**Rev**: 342.81 ± 11.02	
IL	**EA**: 171.90 ± 35.91	**EA**: 226.95 ± 12.27	**EA**: 351.76 ± 27.75	**EA**: 381.98 ± 24.05	Group (<0.0001) Sex (0.0007) Time x Sex (0.0023)
	LA: 191.31 ± 19.31	**LA**: 291.10 ± 47.52	**LA**: 263.48 ± 18.99	**LA**: 479.94 ± 28.27	
	Rev: 249.96 ± 32.36	**Rev**: 194.25 ± 17.98	**Rev**: 266.69 ± 36.10	**Rev**: 322.77 ± 46.94	
ACC	**EA**: 82.02 ± 15.69	**EA**: 85.60 ± 24.30	**EA**: 142.01 ± 15.00	**EA**: 137.65 ± 13.12	Time (<0.0001) Group (<0.0001) Sex (<0.0001) Time x Sex (<0.0001) Group x Sex (0.0172)
	LA: 72.29 ± 24.51	**LA**: 492.46 ± 28.44	**LA**: 129.35 ± 28.67	**LA**: 652.07 ± 59.83	
	Rev: 151.75 ± 19.91	**Rev**: 152.37 ± 7.41	**Rev**: 149.56 ± 17.43	**Rev**: 277.22 ± 34.35	
OFC	**EA**: 201.84 ± 19.57	**EA**: 270.58 ± 32.23	**EA**: 452.81 ± 22.03	**EA**: 454.31 ± 28.22	Group (<0.0001) Sex (<0.0001) Time (0.0412) Time x Sex (<0.0001) Group x Sex (0.0365) Time x Group x Sex (0.03)
	LA: 98.78 ± 39.37	**LA**: 379.17 ± 50.84	**LA**: 253.96 ± 22.17	**LA**: 652.9 ± 53.09	
	Rev: 222.88 ± 33.83	**Rev**: 168.09 ± 23.27	**Rev**: 272.49 ± 33.82	**Rev**: 463.59 ± 61.41	
LHb	**EA**: 13.44 ± 1.56	**EA**: 23.76 ± 4.39	**EA**: 22.64 ± 3.47	**EA**: 35.39 ± 3.45	Group (0.0001) Sex (0.0002)
	LA: 13.88 ± 1.81	**LA**: 28.63 ± 5.58	**LA**: 31.63 ± 5.02	**LA**: 41.07 ± 3.38	
	Rev: 20.98 ± 3.41	**Rev**: 18.47 ± 3.78	**Rev**: 18.55 ± 2.84	**Rev**: 33.63 ± 6.39	
PVT	**EA**: 98.5 ± 14.79	**EA**: 89.45 ± 11.31	**EA**: 151.62 ± 20.20	**EA**: 139.98 ± 27.39	Group (0.0001) Sex (0.0001) Time (0.0247) Time x Sex (0.0048) Group x Sex (0.0499)
	LA: 89.88 ± 41.64	**LA**: 148.67 ± 12.56	**LA**: 128.08 ± 16.33	**LA**: 251.83 ± 34.33	
	Rev: 116.31 ± 5.09	**Rev**: 162.54 ± 13.26	**Rev**: 121.08 ± 3.10	**Rev**: 275.83 ± 30.57	
BLA	**EA**: 34.86 ± 5.76	**EA**: 43.42 ± 3.55	**EA**: 63.87 ± 1.30	**EA**: 78.4 ± 7.36	Time (<0.0001) Group (<0.0001) Sex (0.0002) Time x Group (0.0001)
	LA: 47.08 ± 7.17	**LA**: 50.23 ± 5.98	**LA**: 88.46 ± 8.68	**LA**: 107.88 ± 4.64	
	Rev: 29.8 ± 1.96	**Rev**: 39.97 ± 2.96	**Rev**: 37.52 ± 3.04	**Rev**: 58.69 ± 4.84	
CeA	**EA**: 57.33 ± 5.03	**EA**: 57.75 ± 7.91	**EA**: 61.25 ± 6.16	**EA**: 74.58 ± 7.13	Time (<0.0001) Group (<0.0001) Sex (0.0149) Time x Group (0.0042) Group x Sex (0.0402)
	LA: 49.17 ± 5.70	**LA**: 54.13 ± 4.19	**LA**: 76.63 ± 7.52	**LA**: 105.88 ± 15.34	
	Rev: 33.9 ± 3.34	**Rev**: 33.44 ± 2.73	**Rev**: 38.96 ± 2.86	**Rev**: 50.32 ± 2.96	

A specific comparison of the HCC groups within each region was also performed. For each cortical (**Figure 4.5**) and subcortical (**Figure 4.6**) region of interest, a 2-way ANOVA was calculated for HCC, with the variables being sex (males vs. females) and time (EA vs. LA. vs. Rev). Notably, HCC controls sacrificed at each time point (EA, vs LA vs Rev) experienced all training phases prior to the sacrifice time, but on the day of sacrifice were left in the home cage in lieu of that particular training phase. Post hocs that were reported specifically focused on comparing across sex at the same timepoint and within sex across all timepoints. Within the prelimbic cortex (PL), the analysis revealed a significant sex x time interaction [**Figure 4.5A**, $F(2,18) = 10.83$, $p = 0.0008$]. Post hoc comparisons of note showed significant differences in total Fos+ cells between LA males and females ($p = 0.0275$) and LA and Rev females ($p = 0.0055$). The was a very strong trend towards a sex x time interaction in the infralimbic cortex (IL), although it did not reach significance ($p = 0.0508$, **Figure 4.5B**). The 2-way ANOVA in the anterior insular cortex (AI) revealed a significant effect of sex [$F(1,18) = 5.53$, $p = 0.0303$], but neither a main effect of time, nor an interaction were found (**Figure 4.5C**). **Figure 4.5D** depicts the total count of Fos+ cells within the anterior cingulate cortex (ACC). Within the ACC, main effects of sex [$F(1,18) = 66.75$, $p<0.0001$] and time [$F(2,18) = 45.27$, $p<0.0001$], along with a significant group x time interaction [$F(2,18) =64.79$, $p<0.0001$] were seen. Pertinent post hoc analyses showed that total counts in LA females were significantly higher than those of LA males ($p<0.0001$), LA females ($p<0.0001$), and Rev females ($p<0.0001$). The 2-way ANOVA of the orbitofrontal cortex (OFC) gave a main effect of sex [$F(1,18) = 11.96$, $p = 0.0028$] and significant sex x time interaction [$F(2,18) = 11.90$, $p = 0.0005$]. Specifically, as is seen in **Figure 4.5E**, LA females had a higher Fos+ count than LA males ($p = 0.0003$) and Rev females ($p = 0.0061$).

Similar analysis was performed on subcortical regions of interest (**Figure 4.6**). While there was only a significant effect of sex in the lateral habenula (LHb) [**Figure 4.6A**, $F(1,18) = 6.215$, $p = 0.0226$], no effects were observed within the paraventricular nucleus of the thalamus (PVT, **Figure 4.6B**). In both the basolateral (BLA) and central (CeA) nuclei of the amygdala, only main effects

of time were significant. **Figure 4.6C** demonstrates that post hoc analysis of the main effect in the BLA [F (2,18) = 4.10, p = 0.0341] revealed the Rev timepoint has significantly attenuated Fos+ cells compared to LA (p = 0.0354). Similarly, the post hoc on the effect of time within the CeA [F (2,19) = 11.05, p = 0.0007] indicated Rev rats had significantly fewer Fos+ cells compared to both EA (p = 0.0007) and LA (p = 0.005, **Figure 4.6D**).

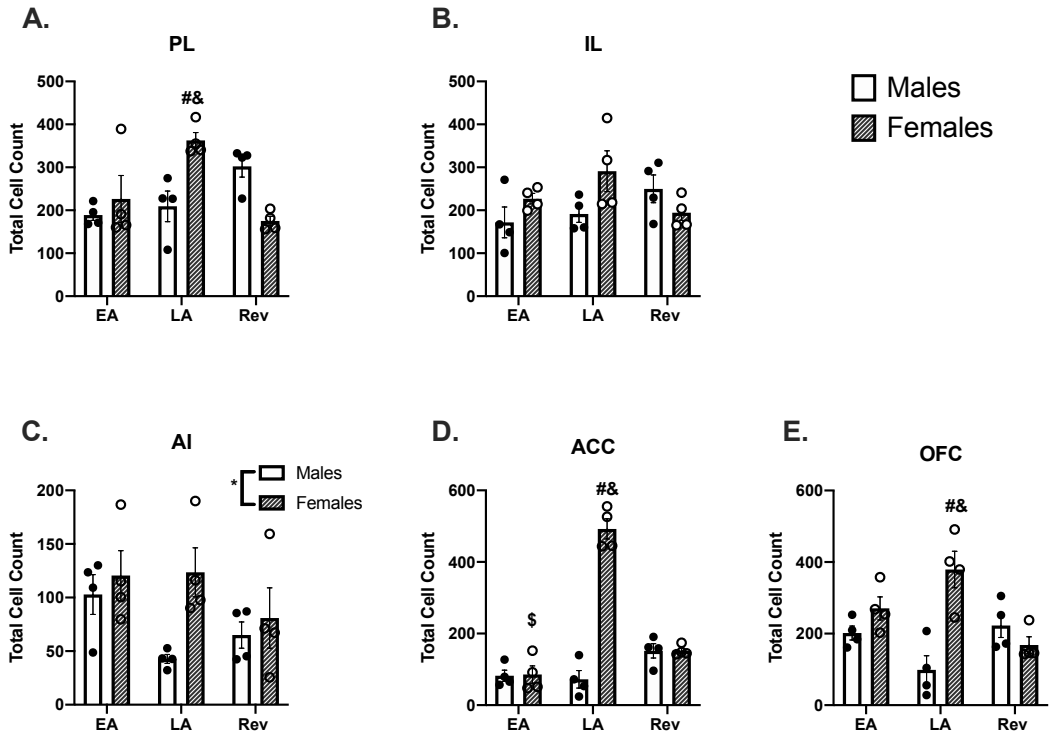


Figure 4.5. **Sex Differences in Controls within Cortical Regions of Interest.** Male and female home cage control (HCC) rats were sacrificed at one of three time points; EA, LA, or Rev. Total Fos+ cells for HCC rats were counted in each region of interest and compared at each time point. **A**) Post hoc analysis of the prelimbic cortex (PL) showed females at LA were significantly different than males at LA, as well as females during Rev. **B**) Although there was a strong trend ($p = 0.0508$) for a sex x time interaction in the infralimbic cortex (IL), nothing significant was seen in the analysis. **C**) A main effect of sex was present in the anterior insula (AI), but an effect of time was not seen. **D**) Within the anterior cingulate cortex (ACC), relevant post hocs concluded Fos+ cells for females at the LA time point were significantly higher than males at LA, as well as females at both EA and Rev. **E**) Analysis in the orbitofrontal cortex (OFC) revealed females at LA had significantly higher Fos+ cell counts compared to LA males, as well as Rev females.
*$p<0.05$
#Significant difference from same timepoint of other sex, $p<0.05$
&Significant difference from reversal timepoint of own sex, $p<0.05$
$Significant different from late acquisition of own sex, $p<0.05$

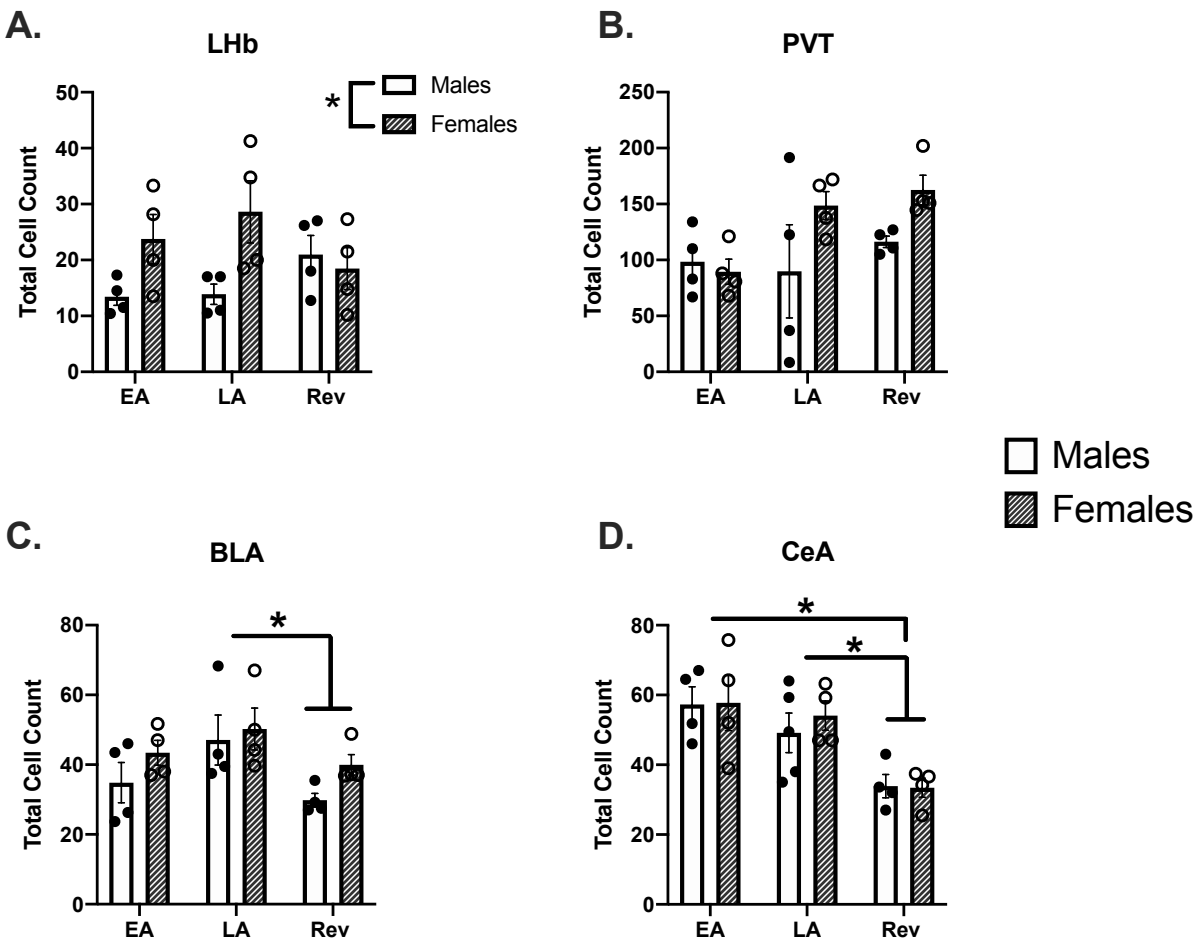


Figure 4.6. **Sex Differences in Controls within Subcortical Regions of Interest.** Male and female (HCC) rats were sacrificed following the 3 chamber empathy task at one of three time points; EA, LA, or Rev. Total Fos+ cells for HCC rats were counted in each region of interest and compared at each time point. **A**) Only a main effect of sex was seen in the lateral habenula (LHb). **B**) Neither a main effect of sex, nor a main effect of time was observed in the analysis for the paraventricular nucleus of the thalamus (PVT). **C**) Within the basolateral amygdala (BLA), post hoc analysis on the main effect of time reveled Fos+ cell counts in Rev to be significantly lower compared to LA. **D**) Similarly, Fos+ cell counts in the central amygdala (CeA) at the EA time point were significantly higher than both LA and Rev.
*$p<0.05$

Male and female rats seem to exhibit a similar pattern of behavior during the 3 chamber empathy task during EA (**Figure 4.1B**), LA (**Figure 4.1C**), and Rev (**Figure 4.1D**), but there are numerous sex effects in the neural substrates that are activated during this task. The sex effects seen in the total Fos+ cells during HCC may represent a quantitative sex effect, in which females exhibit a potentiated response in the same condition (Becker et al., 2017). For this reason, we converted the total Fos+ cell counts into a percent of HCC for each group by dividing the total Fos+ cell count in the Empathy group by its own HCC (**Figures 4.7-4.8**). That way, the overall sex differences in neural activity can be accounted for and the sexes can be directly compared between the three timepoints. For each neural substrate of interest, a 2-way ANOVA was performed and, where appropriate, post hocs comparing each time point (EA vs. LA vs. Rev) were compared within sex to understand if the trend in activity across time in each region was different in males compared to females. In the PL, a main effect of time [$F(2,20) = 3.839$, $p = 0.039$], as well as a significant interaction [$F(2,20) = 4.605$, $p = 0.023$] was found. Post hoc analysis revealed male Rev rats had a significantly attenuated change of Fos+ cells from HCC as compared to EA ($p = 0.0024$) or LA ($p = 0.0483$) males. In contrast, female timepoints were not significantly different from one another (**Figure 4.7A**). The IL analysis showed main effects of both time [$F(2,23) = 9.526$, $p = 0.001$] and sex [$F(1,23) = 5.127$, $p = 0.0333$]. Specifically, EA males had a potentiated % change in Fos+ cells compared to both LA males ($p = 0.0108$) and Rev males ($p = 0.0013$), with no changes being seen within females (**Figure 4.7B**). An approximate two-fold increase in Fos+ cells compared to HCC were seen within the anterior insula across all groups. Therefore, nothing of significance emerged from the analysis (**Figure 4.7C**). Next, in the ACC, a 2-way ANOVA revealed a significant sex x time interaction [$F(2,19) = 5.741$, $p = 0.0112$], with post hocs showing strong trends for EA and LA males to be significantly higher than Rev males ($p = 0.064$), but neither reached significance (**Figure 4.7D**). The final cortical region examined, the OFC (**Figure 4.7E**), also showed a significant sex x time interaction [$F(2,21) = 17.45$, $p<0.0001$]. Post hoc analysis indicated opposite trends for males and females. While the Rev males had the lowest percent change from HCC

compared to male EA ($p = 0.0114$) and LA ($p = 0.001$) time points, female Rev was significantly higher than both female EA and LA ($p = 0.0065$).

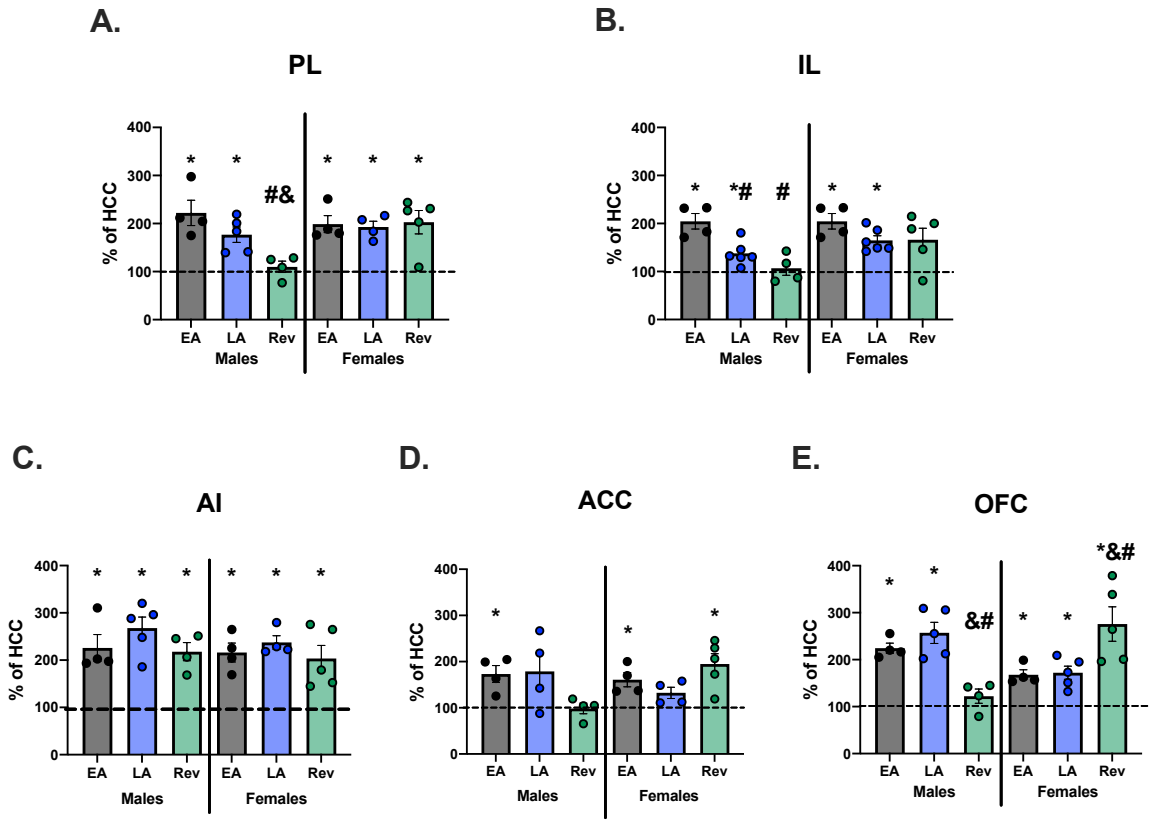


Figure 4.7. Percent Change Differences in Fos+ cells across Cortical Regions of Interest. Total Fos+ cells for male and female rats that underwent the 3 chamber empathy task were directly compared to HCC rats from the same time point to create a percent of home cage control (%HCC) value for each rat. **A**) Within the PL, the % change of males during Rev were significantly lower compared to males at both EA and LA time points. **B**) In the IL, male LA and Rev had a significantly attenuated % change compared to males at EA, but no differences were seen in the females across time. **C**) No significant effects were seen within the AI, although there was a significant increase from HCC in all groups. **D**) While a sex x time interaction was observed in the ACC, post hoc analysis did not reveal any significant differences. **E**) Males and females showed opposite trends in the OFC, specifically at the Rev timepoint. Male Rev % change was significantly attenuated, while female Rev was potentiated, compared to their respective EA and LA time points.
*Significant change from own HCC, $p<0.05$
- Significant difference from EA, $p<0.05$
&- significant difference from LA, $p<0.05$

Similar analyses were performed in subcortical regions of interest. Within the PVT, there was a trend towards a main effect of sex (p = 0.083), but nothing significant was revealed in the analysis (**Figure 4.8A**). The LHb, however, had both a significant main effect of time [F (2,23) = 3.776, p = 0.0382], and time x sex interaction [F (2,23) = 7.585, p = 0.003]. The post hocs revealed that EA and LA males (p = 0.0417), as well as LA and Rev males p = 0.0004) were significantly different from one another, but no females timepoints differed (**Figure 4.8B**). The 2-way ANOVA of changes in Fos+ within the BLA (**Figure 4.8C**) only revealed a main effect of time [F (2,18) = 13.65, p = 0.0002], and post hocs on the main effect shows differences in EA (p = 0.0044) and LA from Rev (p = 0.0002). Finally, main effects of both time [F (2,19) = 7.609, p = 0.0037], and sex [F (1,19) = 6.909, p = 0.0165] were found in the CeA (**Figure 4.8D**). Post hocs on the main effect of time showed the LA time point was significantly higher as compared to the EA (p = 0.0041) and Rev (p = 0.0199) timepoints.

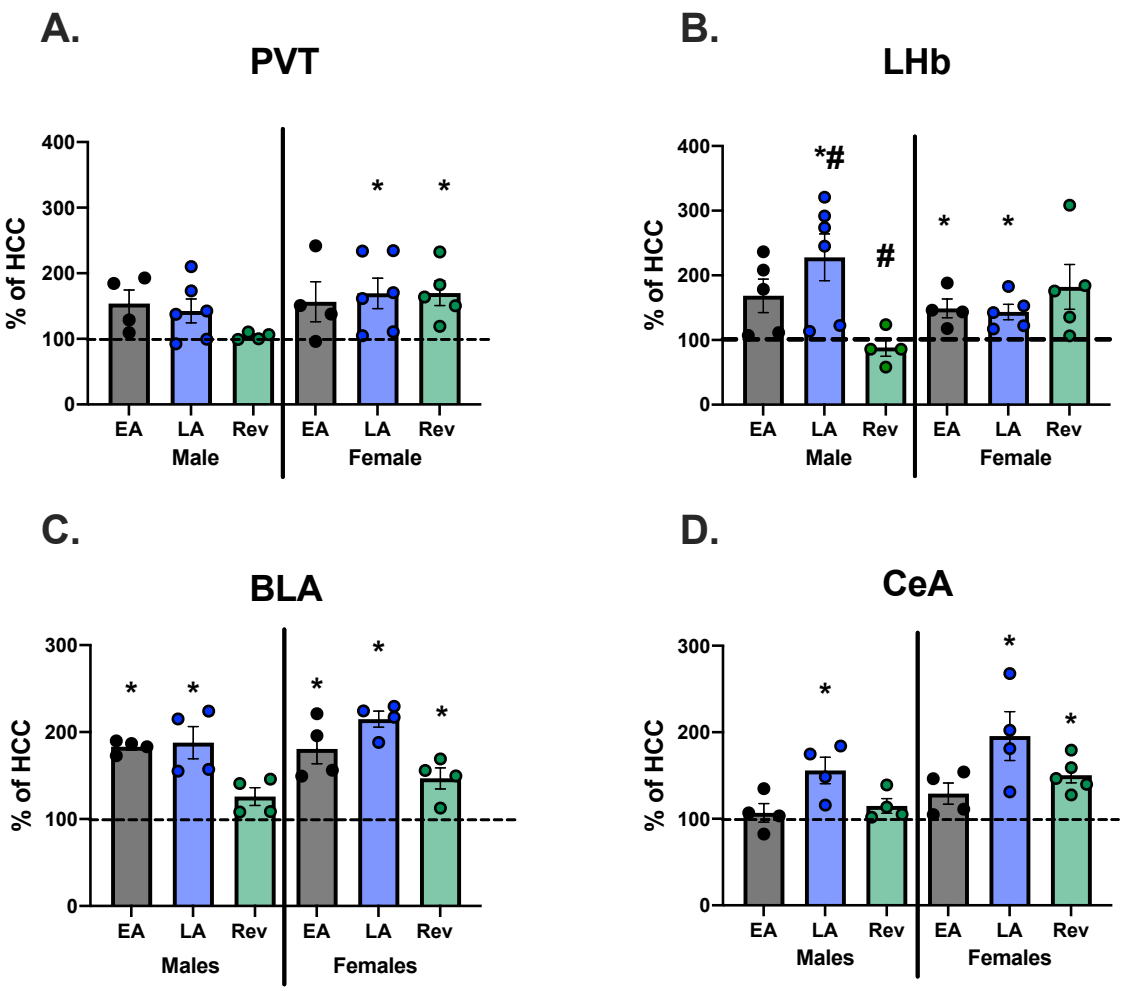


Figure 4.8 Percent Change Differences in Fos+ Cells across Subcortical Regions of Interest. Total Fos+ cells for male and female rats that underwent the 3 chamber empathy task were directly compared to HCC rats from the same time point to create a percent of home cage control (%HCC) value for each rat. **A**) No effects were observed in the PVT, although female LA and Rev were significantly potentiated compared to HCC. **B**) Differences across time were seen in the LHb in males; particularly, male LA was potentiated, while male Rev was attenuated, compared to males at the EA time point. No differences were seen across time for females. **C**) Only a main effect of time was significant in the BLA; further, all groups but male Rev were significantly potentiated compared to their HCC. **D**) Main effects of both time and sex were observed in the CeA. LA of both sexes and Rev females were significantly potentiated compared to their HCC.
*Significant change from HCC, $p<0.05$
- Significant difference from EA, $p<0.05$
&- significant difference from LA, $p<0.05$

Ultrasonic Vocalizations: In adult rats, two main USVs have been categorized; aversive 22 kHz (18-35 kHz) calls during stressful events, and prosocial/appetitive 50 kHz (>35 kHz) calls (Wöhr et al., 2005; Takahashi et al., 2010; Wöhr, 2018). In order to determine the range and proportion of communicative frequencies during the task in males and females, calls for each sex in EA, LA, and Rev were used to generate a frequency of distribution graph of the call frequencies (kHz). Call frequencies were binned in 5 kHz bins and relative frequencies (percent of total) was calculated for each time point. In order to determine if there was a difference in the affect, as evaluated by call frequency, of rats during the 3 chamber task, USVs of each rat were categorized 'distress' (18-35 kHz) or 'prosocial' (>35 kHz), and each category was analyzed as a percent of total calls using 2-way ANOVAs, with sex (male vs. female) and group ((R-) Targets vs. (R-) Observers) as the variables. During EA, the frequency of distribution analysis indicated a bimodal distribution of call frequencies (**Figure 4.9A**). When the calls were split into 'distress' or 'prosocial', main effects of sex [$F(1,20) = 7.33$, $p = 0.0136$], group [$F(1,20) = 5.278$, $p = 0.0325$], and a sex x group interaction [$F(1,20) = 7.655$, $p = 0.0119$] was seen. Post hoc analysis indicated female Targets had a significantly larger proportion of their total calls within the distress range, and therefore significantly fewer within the prosocial range, compared all other groups (**Figures 4.9B and 4.9C**). The same analysis was performed for males and females at the LA and Rev timepoints. While a bimodal distribution does still seem present at LA (**Figure 4.10A**), there is no difference in the percent of distress (**Figure 4.10B**) or prosocial (**Figure 4.10C**) calls. Even fewer calls in the distress range were recorded during Rev (**Figure 4.11A**), and again no differences in the percent of distress (**Figure 4.11B**) or prosocial (**Figure 4.11C**) calls were observed.

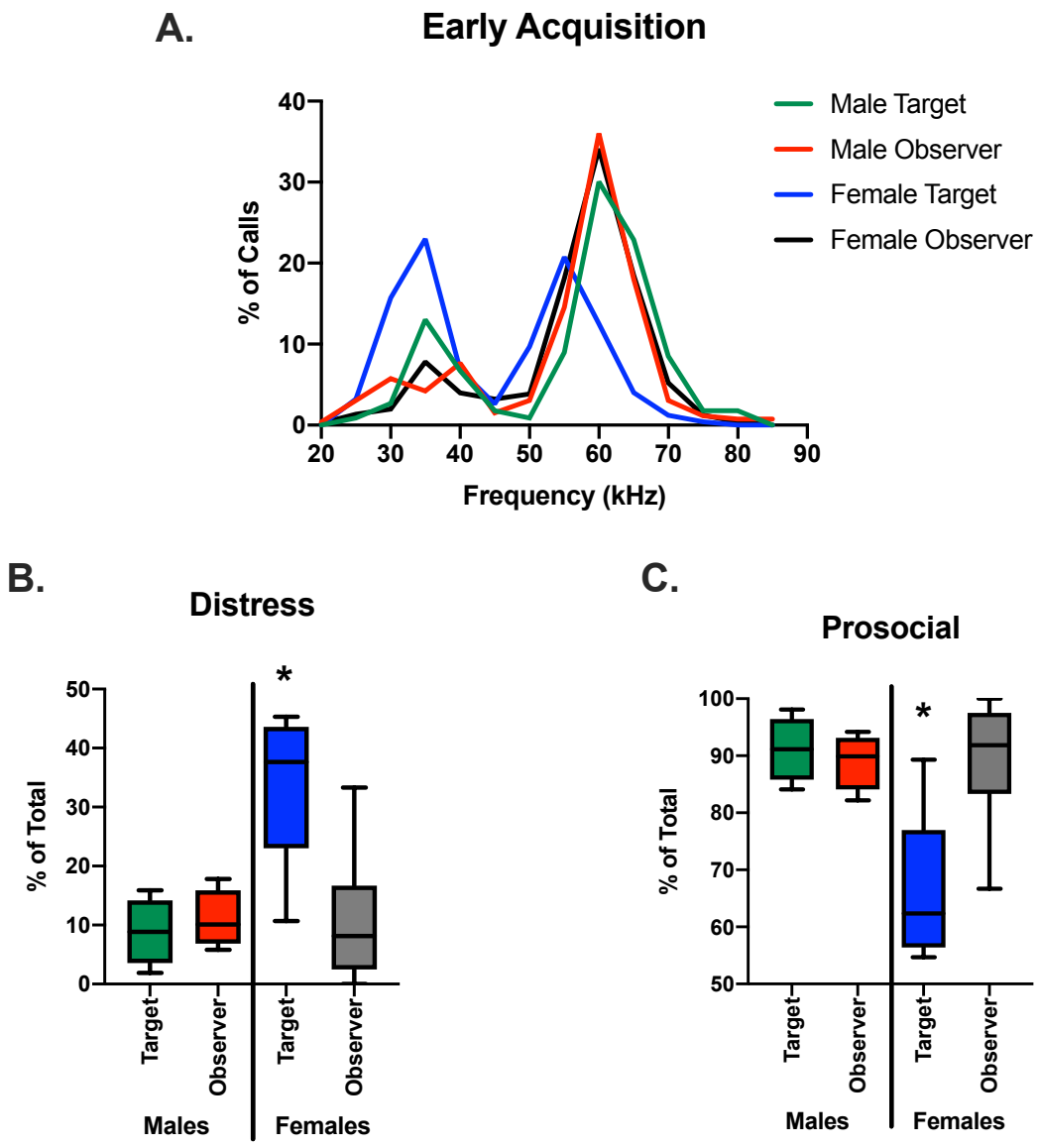


Figure 4.9. **Comparison of USVs Frequencies during Early Acquisition between Males and Females. A**). Frequency of distribution graph indicates a bimodal distribution of call frequencies, roughly corresponding to the 'distress' and 'prosocial' ranges. **B-C**) An analysis of the calls in both call types for each group as measured by a percent of total calls made, expressed as mean ± min to max. **B**) Female Targets make a significantly greater percentage of distress calls in EA compared to all other groups. **C**) Correspondingly, calls in the prosocial range make up a significantly smaller percentage of female Targets' total calls compared to the other groups.
*Significant difference from all other groups, $p<0.05$

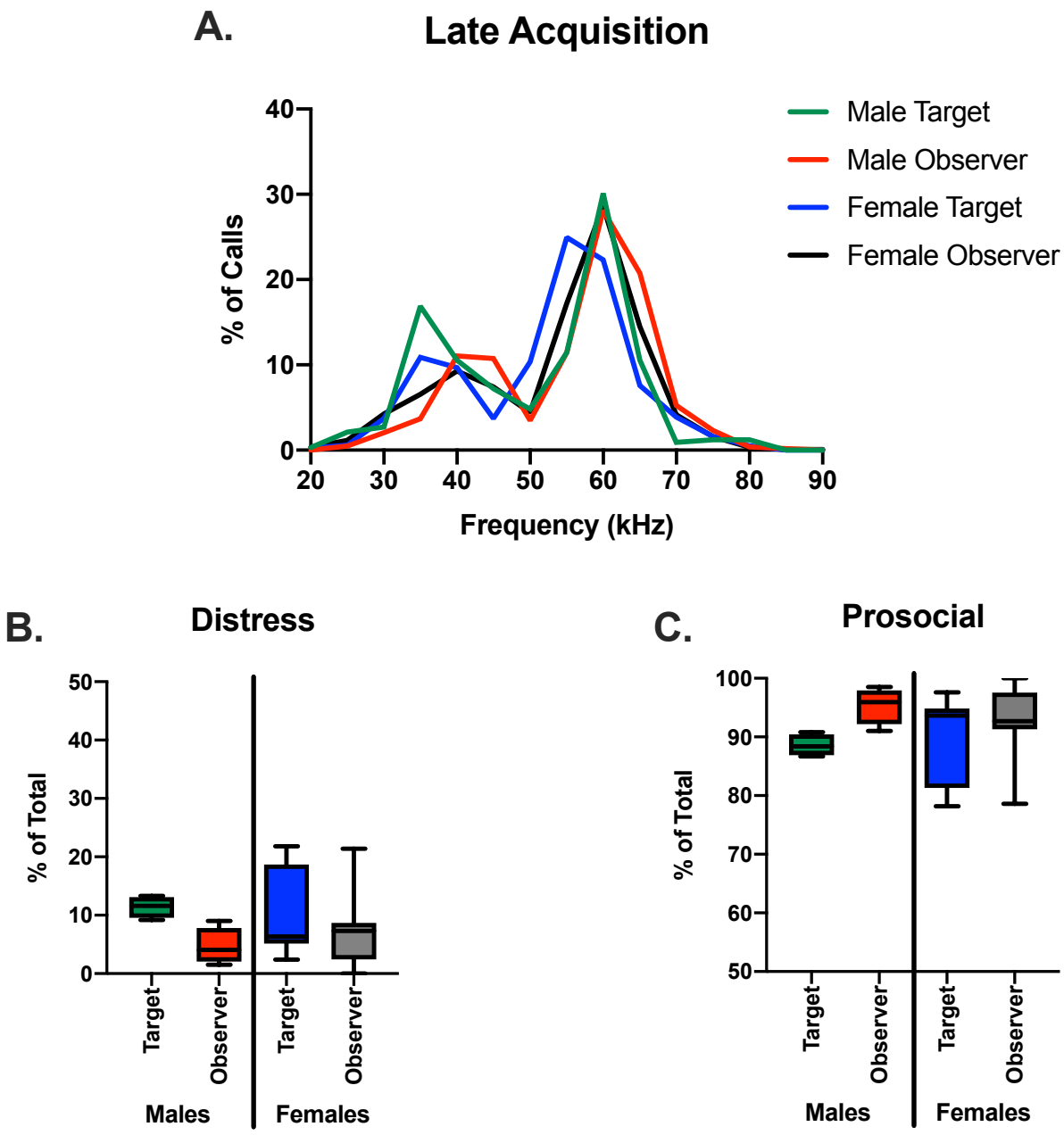


Figure 4.10. **Comparison of USVs Frequencies during Late Acquisition between Males and Females. A**). Frequency of distribution graph showing the distribution of call frequencies during LA. **B-C**) An analysis of the calls in both call types for each group as measured by a percent of total calls made, expressed as mean ± min to max. **B**) No differences were seen in distress calls as a percent of total calls made. **C**) Similarly, no difference was seen in the prosocial call range across the four groups.

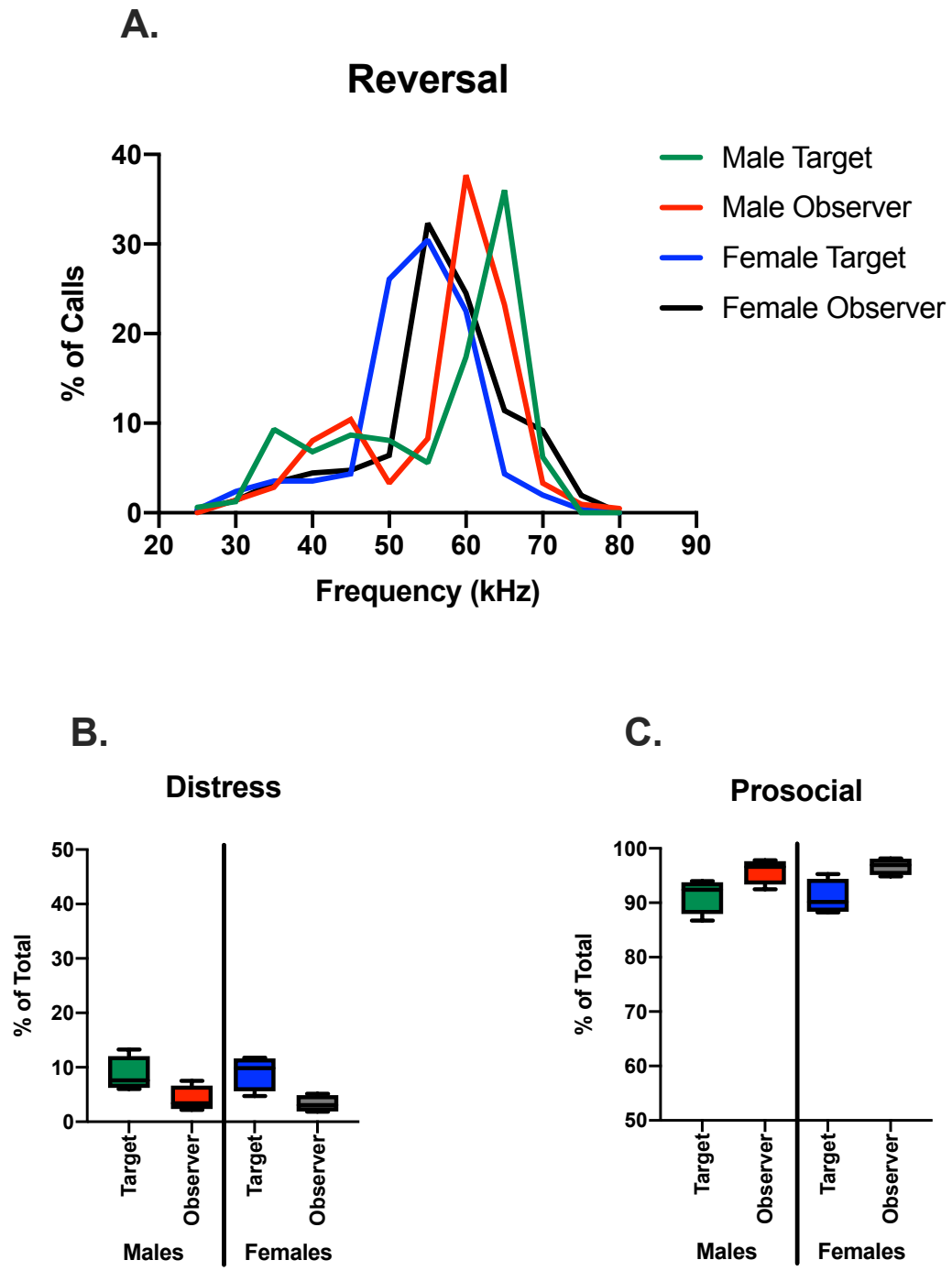


Figure 4.11. **Comparison of USVs Frequencies during Reversal between Males and Females.** **A**). Frequency of distribution graph indicate the distribution of call frequencies is almost entirely within the prosocial call range. **B-C**) An analysis of the calls in both call types for each group as measured by a percent of total calls made, expressed as mean ± min to max. **B**) No differences were seen in the number of distress calls as a percent of total calls. **C**) No difference was seen in the prosocial call range across the four groups.

Sex Differences in Oxytocin Neuron Activity in the PVN following Empathic Behavior: Two representative images of the PVN were counted and combined for each animal to generate an estimate of overall PVN activity. Observers either underwent the empathy task or remained in their home cages and were sacrificed to evaluate Fos/oxytocin overlap at EA, LA, and Rev. Cells that were Fos+ and Oxytocin+(Oxy+) were quantified and expressed as a percent of total Oxy+ cells. The data were then converted into a percentage of home cage control (HCC) for each group, with HCC being set to 100%. A 2-way ANOVA with timepoint (EA vs. LA vs. Rev) and sex (male vs. female) as the two independent variables was performed. When appropriate, post hoc analyses were performed comparing males and females at each timepoint and differences within sex across time. Additionally, one-sample t-tests were performed for every sex at every timepoint to determine if Fos+/Oxy+ overlap changed following the completion of the empathy task compared to HCC. Results from the 2-way ANOVA for revealed a significant timepoint x sex interaction [$F(2,20) = 13.89$, $p = 0.0002$]. Post hoc analysis further demonstrated that, during EA, the Fos+/Oxy+ in males was significantly higher than females, while the opposite was true during Rev. The percent change for males at LA and Rev were significantly lower than EA, but LA and Rev did not differ from one another. In contrast, female Rev had the highest percent change compared to female EA and LA, while EA was also significantly lower compared to LA (**Figure 4.12B**). One-sample t-tests indicated that Fos+/Oxy+ overlap was decreased compared to HCC in females during EA ($p<0.05$). While there were strong trends suggesting that the percent overlap was increased in males during EA and females during Rev, neither t-test reached significance.

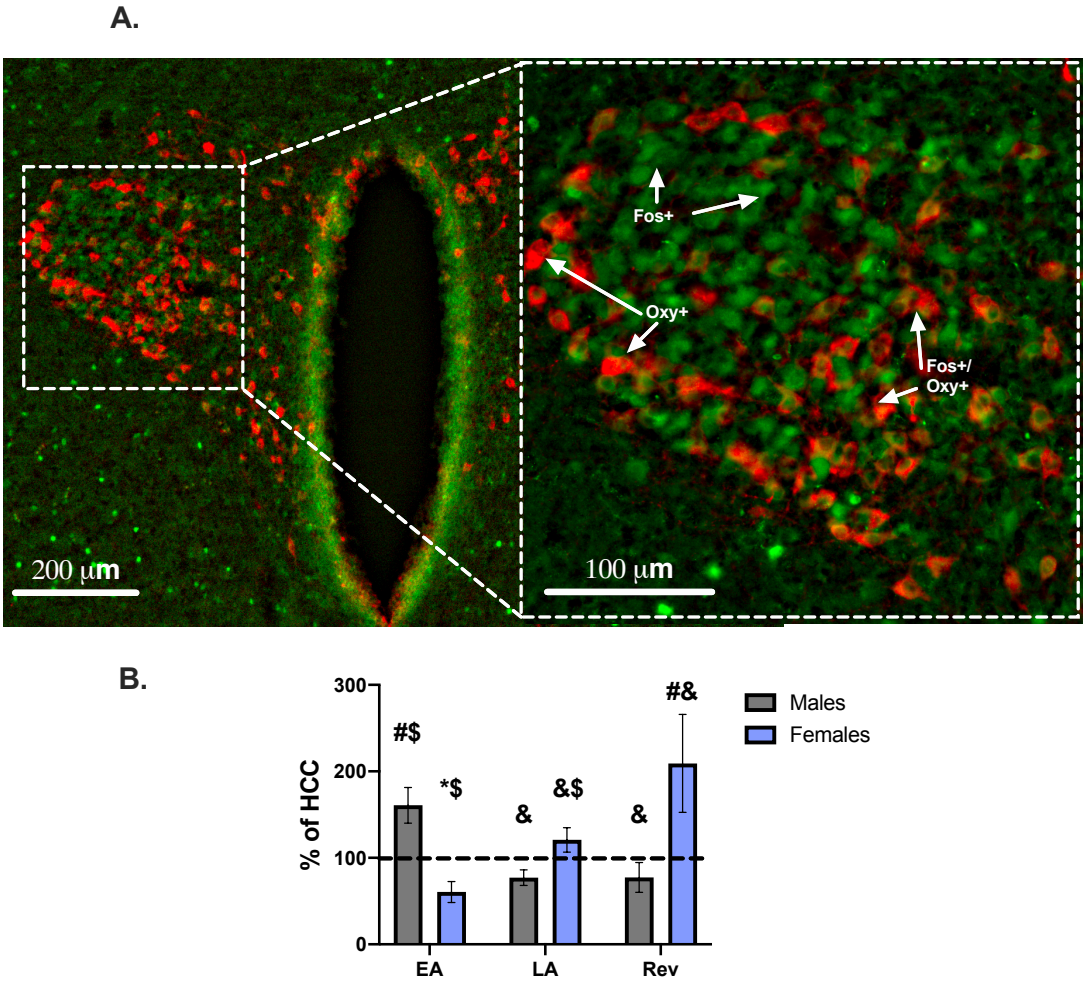


Figure 4.12. Sex Differences in Oxytocin Neuron Activity in the PVN following Empathic Behavior. A) A 10x representative image of the PVN and a 20x inset example of the magnocellular nucleus of the PVN with Fos+, Oxy+, and Fos+/Oxy+ overlap cells identified. Total Fos+/Oxy+ cells for male and female rats were counted and quantified as a ratio of total Oxy+ cells. Representative images of the PVN from rats performing the 3 chamber task were counted then directly compared to HCC rats from the same time point to create a percent of home cage control (%HCC) value for each rat. **B)** During EA, the percent change in Fos+/Oxy+ levels were significantly higher compared to females. Female levels were significantly higher than males during Rev. There were also differences seen across time within both sexes. Male EA activity was significantly higher than LA and Rev, but no difference was seen between LA and Rev. In contrast, female percent change in activity exhibited a stepwise increase over time; EA was the lowest, with LA significantly higher than EA, and Rev significantly higher than LA. Finally, while there was a strong trend for the percent change in males during EA and females during Rev to be significantly higher compared to their respective HCC, it did not reach significance. Only females during EA differed significantly from their HCC.

#-Significant difference from other sex at the same timepoint, $p<0.05$
&-Significant difference from EA of own sex, $p<0.05$
$-Significant difference from Rev of own sex, $p<0.05$
*-Significant change from HCC, $p<0.05$

DISCUSSION

In Chapter 3, we sought to elucidate any behavioral, as well as neural, sensory, and affective differences between males and females over time during our 3 chamber model of targeted helping. Overall, our results indicate four main findings. First, while an effect of sex was seen in the latency to release a distressed conspecific during acquisition, it was not present in the three timepoints evaluated (EA, LA, or Rev; **Figure 4.1**), nor was there a difference in a helping task that afforded the animals social contact (**Figure 4.3**). Instead, this sex effect was driven primarily by a subset of 'low acquisition index (AI)' females (**Figure 4.2C**), suggesting that any differences in overall acquisition between males and females may be due to female rats that did not acquire the task, as defined by a decrease in latency over time. Second, males and females exhibit differing neuronal activity, especially within the cortical substrates studied. While males exhibited a decrease in relative cortical activity compared to HCC across time, activity was maintained, or increased, in females (**Figure 4.7**). Third, it appears there are sex differences in visual (**Figure 4.4**) and auditory cues (**Figures 4.9**) during the task that differentially drives behavior in males and females, particularly in early acquisition. Finally, oxytocin (Oxy) neuron activity within the PVN exhibits a sex-specific pattern of activity across time during targeted helping (**Figure 4.12**). Taken together, while males and females may be similar behaviorally, different mechanisms or socio-emotional cues may be used to respond to their distressed conspecific.

Canonically, it has been understood in clinical research that females exhibit higher levels of empathic concern compared to males (Hall, 1978; O'Brien, 2013), but this broadly held assumption is now in question (Eisenberg and Lennon, 1983; Baez et al., 2017). Further, the admittedly few experiments in rodents exploring the effect of sex on empathic behavior have conflicting results; while some also show females more empathic than males (Ben-Ami Bartal et al., 2011), others show no difference (Du et al., 2020; Han et al., 2020). In our experiment, males exhibited faster chain pull latencies across acquisition (**Figure 4.1A**), no such effect was seen in reversal (**Figure 4.1D**), suggesting no sex-specific difference in previous experience on empathic

behavior. Further, no differences were seen at the two timepoints in acquisition (EA and LA) that were analyzed (**Figures 4.1B-4.1C**). Nor was there a difference between male and female latency to release a distressed conspecific in the 2 chamber model of helping behavior (**Figure 4.3**) that affords social interaction (see **Chapter 3**), suggesting social contact does not drive the observed sex difference in the 3 chamber task (Cox and Reichel, 2019; Silva et al., 2020). In fact, the sex difference was only present when rats were split into low and high groups based on their 'acquisition index'(AI), a calculation examining the change in chain pull latency over the course of acquisition. Low AI females showed a significantly slower chain pull latency over acquisition compared to low AI males.

Our lab and others (Silva et al., 2020) utilize change in latency over time as a proxy for learning and mastery of a behavioral task. While it cannot be ruled out that this subset of females exhibits lower levels of empathy compared to males, it seems less likely since no differences were seen in any of the specific timepoints evaluated. Instead, we suggest males and females demonstrate similar levels of helping behavior, but low AI females may have a diminished ability to understand the association between chain pull and target's contingencies, possibly due to differences in anxiety-like behaviors (Scholl et al., 2019), which may play a role in the overarching task acquisition. Evidence from other targeted helping models confirm stress' role in empathic behavior. Specifically, male Observers subjected to a low amplitude foot shock prior to the task improved their targeted helping (Karakilic et al., 2018), and, in a separate study, prior administration of the anxiolytic Midazolam potentiated conspecific release latencies (Ben-Ami Bartal et al., 2016). In contrast, high amplitude foot shock did not alter release latency, indicating there is a 'Goldilocks effect' of stress on empathy. As females are generally more stress responsive than males (Rincón-Cortés et al., 2019), it is possible the stress response in female Observers as a consequence of their distressed conspecific reaches levels detrimental to memory formation and task acquisition. An interesting follow-up study would be to explore whether or not low AI females exhibit higher physiological stress responsivity compared to high AI females and males, and whether that directly

correlates to attenuated chain pull latency. We would hypothesize that this potentiated stress responsivity would limit low AI females' ability to regulate their emotional state in order to perform the task and reduce the distress of their conspecific, as is described in the PAM of empathy. Another variable that needs additional exploration as it relates to stress responsivity and empathy is the impact of the estrous cycle in female rats. There is limited evidence on the subject, but one study of emotional contagion showed females in the diastral cycle behaved similarly to males, but females in their estrus phase were especially unresponsive to fear contagion (Mikosz et al., 2016). These results support the notion circulating estrogens are correlated with reduced anxiety (Mora et al., 1996), and warrant further study on the impact of female estrous cycle on targeted helping.

As mentioned previously, evidence points to complex, situational, and multimodal sensory communication to allow for affective transfer and empathic behaviors (Knapska et al., 2006; Kim et al., 2018). Our data suggest that visualization of the distressed Target may in fact hinder release behavior in females during the initial trials of acquisition (**Figure 4.4A**). Further, during EA, female Targets exhibit a significantly higher proportion of distress USV compared to male Targets (**Figure 4.9**). These USV data expand on the findings of Ben-Ami Bartal et al. (2011) that also demonstrated in a model of targeted helping that Targets made more stress calls in EA, when release was rare. We can intimate from our USV data that females find the water more distressing initially compared to males. Overall, these data may imply that the female Targets' enhanced stress cause Observers to spend more time at the clear Plexiglas in an effort to console or interact with them, as is hypothesized in the "tend and befriend" theory of empathy (Christov-Moore et al., 2014). When direct visualization is no longer possible, it is likely the distress calls become an aversive stimulus and females work to eliminate them, as indicated by a reduced chain pull latency in EA when a black Plexiglas divider is used (**Figure 4.4A**). In order to answer this question, future studies could use a Black Plexiglas divider without a Target, and instead play back distress calls to determine if it is sufficient to produce release behavior. Finally, these data also suggest distress calls alone may not be necessary to drive empathic behavior (Ben-Ami Bartal et al., 2011). The high frequency

calls shown to communicate prosocial information broadly (Panksepp and Burgdorf, 2003; Simola and Brudzynski, 2018; Wöhr, 2018) may also drive targeted helping, even in the absence of direct social contact. Overall, our studies indicate sex differences in sensory and affective communication between males and females, especially early in acquisition when release is less frequent.

Our analysis of neural activity also yielded interesting results. We first noted a sex difference inherent in the baseline activity of home cage control (HCC) animals between sex in many of the regions examined (**Figures 4.5-4.6**), suggesting a quantitative sex effect. Neural activity of each region was analyzed set to the HCC of each sex. From there, distinctive sex-specific activity patterns began to emerge. For example, the percent change in cortical activity in males was generally highest in EA and decreased to its lowest in Rev. In contrast, female activity remained stable across all timepoints or was highest in Rev (**Figure 4.7**). This pattern was particularly clear in the regions comprising the medial prefrontal cortex (PL and IL), and the orbitofrontal cortex (OFC). These substrates are necessary for cognition, decision-making, and perspective-taking (Jackson et al., 2005; Singer and Lamm, 2009; de Waal and Preston, 2017). The maintenance of their activity over time may indicate the task retains salience or continues to require higher-order regulation across time in females, while becoming more habitual in males. Although research in other operant training tasks have concluded females express habitual behavior earlier than males (Schoenberg et al., 2019), these tasks are often for a personal reward and do not include the presence of a distressed conspecific. Future studies into the neurobiology of targeted helping will examine the sex differences between regions that are necessary for habitual behaviors, such as the basal ganglia (Yin and Knowlton, 2006), to discern whether the presence of strong emotional salience like a distressed conspecific attenuates the transition to habitual behaviors in females but not males.

Another sex effect of note was present in both the central (CeA) and basolateral (BLA) nuclei of the amygdala. Within the BLA, percent change in EA and LA in males and females was approximately double HCC, but only females exhibited a difference from HCC during Rev (**Figure 4.8C**). Similarly, males and females had the same pattern of activity in the CeA during EA and LA,

but again, only females showed a significant increase from HCC in Rev (**Figure 4.8D**). In a separate empathy model, amygdala activity in non-stressed rats generally mirrored that of stressed conspecifics (Knapska et al., 2006), with the CeA activated to a higher level in unstressed observer rats. The authors concluded the CeA therefore is highly sensitive to the distress of others (Meyza et al., 2018). We could then surmise from our data that previous experience with the distressing condition in females may drive increased CeA activity compared to males. In addition, the BLA has long been considered a substrate that encodes emotionally salient memories, particularly of fear (Sun et al., 2020). The fact that females during Rev show higher activity in the BLA compared to males also suggests that the water is more distressing for females, and that memory may drive enhanced activity when the roles of the task are reversed. It is of note that an apparent convergent sex effect of neural activity during targeted helping may be informed by a quantitative sex effect between male and female homecage rats (Becker et al, 2017). One limitation to this evaluation of sex differences in neural activity during targeted helping is the relatively low number of animals per group. For every timepoint for each sex, n=4-6 for all groups. Increasing the power of these studies to confirm the sex-specific changes observed across numerous regions of interest will be important moving forward. Overall, the patterns observed in neural activity change within regions critical for emotional regulation, salience, and perspective-taking between males and females may help shed light on sex-specific neuronal drivers of empathic behaviors.

Finally, we examined the activity of oxytocin (Oxy) neurons within the paraventricular nucleus of the thalamus (PVN) during targeted helping. A distinct pattern in the percent change of Fos+/Oxy+ compared to HCC was seen across time between males and females. Males had the highest activity change during EA; it was significantly higher compared to EA females, as well as all other male timepoints. In contrast, female EA had the lowest percent change in activity, it increased in a stepwise fashion over time, with Rev significantly higher than males at that time and all other females timepoints (**Figure 4.12**). These data suggest, like an increasing number of studies, as it pertains to complex prosocial behaviors, Oxy's role is heavily dependent on social

context and the type of empathy under analysis. Administration of Oxy improved targeted helping in males, but only early in acquisition and only when the Observer was single-housed and the Target was unfamiliar; pair-housed animals, on the other hand, showed no difference in time to release the distressed conspecific (Yamagishi et al., 2019). While this finding has not been explored in females, it, along with the research that demonstrated social isolation activates PVN neurons (Grippo et al., 2007), suggests the Oxy levels and their impact on empathic behavior is not ubiquitous, but is dependent on social context. Further, Oxy activity and impact may vary depending on the type of empathic behavior under investigation. In a model of emotional contagion, mice shown to have the lowest levels of empathic responding had the highest levels of Oxy in the brain (Laviola et al., 2017). It is possible our task combines components top-down cognitive and bottom-up emotional components of empathy (Panksepp and Panksepp, 2013). A follow up experiment to examine Oxy's direct effect on targeted helping would be to chemogenetically inhibit the activity of the PVN during the 3 chamber task in males and females. Additionally, like the previous studies evaluating sex differences in neural activity in this chapter, groups are underpowered, with n=4-6/group. Replication of these findings to confirm the trends seen within the PVN is also critical. These results point towards Oxy as another sex-dependent variable, whose activity across time between sex could indicate different drivers of empathy, cognitive or emotional, that modulates helping behavior.

In conclusion, this chapter sought to elucidate the role of sex on targeted helping, along with sensory, affective, and neural components that may contribute to any behavioral differences. We believe that males and females exhibit similar targeted helping, but multiple variables work together to generate a convergent sex effect of empathic behavior. Additionally, empathic sex differences previously understood to be canon are likely dependent on contextual cues and task type (Langford et al., 2006). Subtle nuances, like housing (Yamagishi et al., 2019) estrous cycle (Meyza et al., 2017), and chronicity of the task must be considered when any affective or physiological variable is being studied as it pertains to empathy. Overall, we believe this lays a

groundwork for future studies to explore variables that drive empathic behavior in males and females, even if the overarching behavior between the sexes is similar.

CHAPTER 5: The Role of the Insular Cortex during Empathic Behavior in Male Rats

INTRODUCTION

Throughout this dissertation, empathy has been described as a multidimensional concept that allows for an understanding of the affective states of others. Affect sharing afforded by empathy helps to lay the groundwork for cooperation and prosocial behaviors, both of which are critical for the formation and maintenance of interpersonal relationships and societal cohesion (Decety, 2011; Sivaselvachandran et al., 2016; de Waal and Preston 2017; Adriaense et al., 2020).

Although there is still a dearth of understanding in the underlying neurobiology of empathic behaviors, one region that has gained significant interest is the anterior insular cortex (see **Chapter 1**). The insula is an anatomically and functionally heterogeneous region that consists of three main subdivisions: the agranular (anterior), a middle dysgranular, and the posterior granular insular cortex (Butti and Hof, 2010; Gu et al., 2013; Uddin et al., 2017). The insula is uniquely positioned to be a primary node in multiple complex functions including sensory processing, risk prediction and decision-making, and emotional representation (Damasio and Carvalho, 2013; Uddin, 2015; Gogolla, 2017). Moreover, the insula, particularly the anterior insula (AI), has been implicated in integrating interoceptive and affective information (Craig, 2009; Bernhardt and Singer, 2012). For this reason, the AI has garnered increased attention as it relates to empathic behavior (Fan et al., 2011; Decety, 2015). Functional neuroimaging studies demonstrate an overlapping activity pattern in the AI when personally receiving a painful stimulus and the vicarious pain experienced by watching another receive a painful stimulus (Singer et al., 2004; Saarela et al., 2007; Singer and Leiborg, 2009). Human lesion studies corroborate the role of the AI in prosociality; patients with epilepsy having undergone insular resection had impairments in the ability to recognize facial expressions on other's faces (Boucher et al., 2015; Uddin et al., 2017). In addition, aberrant insular activity plays a role in the dysregulation of empathic behaviors seen in patients with ASD (Gu et al., 2015). In animal studies, the insula has also been implicated in prosocial behaviors, including social approach and avoidance (Rogers-Carter et al., 2018). More specifically to empathy,

inactivation of the insula reverses hyperalgesia induced by emotional contagion of pain (Zaniboni et al., 2018), while chemogenetic activation of the AI restores a heroin-induced decrease in targeted helping (Tomek et al., 2020). Therefore, our lab evaluated the role of the AI during a social contact-independent targeted helping model of empathy (Cox and Reichel, 2019).

We first confirmed the activity of the AI following the completion of the helping task in Experiment 1 by staining for the immediate early gene *c-fos*. In Experiment 2, the critical afferent and efferent circuitry to and from the insula were explored using retro- and antero-grade viral tracers. Finally, in order to determine the causal impact of the AI during empathic behavior, the AI was inhibited pharmacologically (Experiment 3) using a baclofen/muscimol (B/M) cocktail. Chain pull latency was the dependent measure for targeted helping. Ultrasonic vocalizations (USVs) were measured during the task and on test days, as described thoroughly in **Chapter 4,** to understand the resulting change in affect from an alteration in helping behavior. Finally, a chemogenetic approach (Experiment 4) was also used to selectively inhibit the AI during the 3 chamber task. The chemogenetic experiment utilized Designer Receptors Exclusively Activated by Designer Drugs (DREADDs), engineered muscarinic acetylcholine receptors (hM4Di) that are activated exclusively in the presence of an exogenous ligand clozapine-N-oxide (CNO). An additional social task was performed during this experiment to elucidate the specificity of the behavioral changes observed following AI inhibition. In the following experiments, we demonstrate the insula is activated during empathic behavior, there are multiple afferents and efferents of the AI the also are strongly activated during the task, and that inhibition of the AI selectively blunts empathy, but not prosocial behaviors per se.

MATERIALS AND METHODS

Animals: Male Sprague Dawley rats weighing 250-275g were pair-housed as described in **General Methods (Chapter 2)**. All experimental procedures were conducted in accordance with the "Guide for the Care and Use of Laboratory Rats" (Institute of Laboratory Animal Resources on Life

Sciences, National Research Council) and approved by the IACUC of the Medical University of South Carolina.

General Surgical Procedures: Observer rats were anesthetized using isoflurane vaporized for inhalation (4-5% for induction in a chamber, 2%–3% through a nosecone for preparation and 1%-3% for surgical anesthesia maintenance). In all experiments, the anterior insular cortex was targeted using the following coordinates relative to the skull and bregma: +3 mm anteroposterior, ±4 mm mediolateral, and −4 mm (guide cannula) or -5mm (glass micropipette) dorsoventral according to a stereotaxic atlas (Paxinos and Watson, 2007). Following surgeries, ketorolac (2.0 mg/kg, IP; Sigma Chemical, St. Louis, MO, USA) and cefazolin (0.2 g/kg, Patterson Veterinary, Saint Paul, MN, USA) were given as an analgesic and antibiotic, respectively, and rats were given at least 5 days to recover before any behavior assessment began.

Drugs: *Experiment 3*: baclofen/muscimol (B/M) cocktail was obtained from Tocris (Bristol, UK), dissolved in PBS, and combined in a 1.0mM/0.1 mM solution as previously described (Arguello et al., 2017). Rats were bilaterally infused with the B/M solution at a volume of 0.5 µL/side.

Experiment 4: Clozapine-N-oxide (CNO) hydrochloride was purchased from Hello Bio (Princeton, NJ), dissolved in water and administered at a dose of 10 mg/kg i.p. Although this dose is higher than others have suggested, our lab has seen behavioral changes with it in the past independent of the potential sedative effects from the back-metabolism of CNO to clozapine (Peters et al., 2018; Siemsen et al., 2019). Further, proper control groups have been included to define the effects of CNO during behavior. Experimenters were blind to injection condition.

3 Chamber Apparatus and Behavioral Testing: As discussed in previous chapters, rats underwent empathy testing using the 3 chamber operant box (**General Methods**), where no opportunity for social contact existed during or after the chain pull.

Social Interaction and Social Reward Place Preference: The insular cortex has numerous other behavioral effects, including the modulation of social approach (Rogers-Carter et al., 2018), as well predicting circumstances of reward and guiding behavior dependent on expected outcomes

(Gogolla, 2017). In order to assess whether the changes seen during empathic behavior impacted other prosocial components, rats in Experiment 4 underwent a social interaction and social reward place conditioning task (see **Figure 5.5**). This task occurred in a round open field (125-cm diameter), and behavior was recoded with EthoVision tracking software in 10-min sessions. On day 1, as a pre-interaction evaluation, rats were placed in the open field and given 10 min to explore the environment. White tape was used to mark two 16 × 18 in. squares on the floor on opposite ends of the apparatus in order to indicate two zones; the social zone (SZ, the area with the unfamiliar rat) and the object zone (OZ, with a novel object) would go on subsequent sessions. The amount of time spent in each of these areas was recorded. On days 2, 3, and 4, an unfamiliar rat was placed in a wire cage (11 × 7 × 7 in) in the SZ, and a novel object (battery, pencil holder, or a soda can) was placed in the center of the OZ on the opposite end of the arena. Rats were injected with either CNO or water 30 minutes prior to these sessions. Finally, on day 5, rats were returned to the empty open field for a post-interaction assessment. Each day, we evaluated the amount of time rats spent in the SZ and OZ throughout the 10-min session.

IHC/Placement and Quantification: 90 minutes following behavior, rats were anesthetized, transcardially perfused, and brains were removed and sliced into 50 µm tissue sections as previously described (**Chapter 2**).

In Experiment 1, blue-black nuclear immunoprecipitate from DAB-stained Fos-positive (Fos+) cells in the AI were quantified using a brain atlas for comparison (Paxinos and Watson, 2007). Fos+ cells that fell within the AI were automatically counted using a macro and averaged across sections for each rat. On average, 3 bilateral sequential sections were used for analysis. Anterior-posterior coordinates for the insula ranged from 3.7 to 2.7. Slices were coverslipped and photographed at 10x magnification using a Leica microscope and VideoToolbox software. Total count analysis was performed using ImageJ (NIH).

In Experiments 2 and 4, tissue was blocked and incubated over-night at 4°C in rabbit anti-Fos (1:1000, Synaptic Systems) primary with 2% normal goat serum (**Chapter 2**). Tissue was then incubated at room temperature for 5 hours while protected from light in: Experiment 2; goat anti-rabbit Alexa 594 (1:1000); Experiment 4; goat anti-rabbit Alexa 594 (1:1000, Abcam) in the rats infused with the control viral construct, or and goat anti-rabbit Alexa 488 (1:1000, Invitrogen) in those infused with the inhibitory DREADD. Slices were mounted and coverslipped, and representative images of the insula were taken at 10x magnification using a Nikon fluorescent microscope. Surgical placement was determined by visualizing the spread of the viral fluorophore and comparing it to the corresponding coronal section of the insula from the atlas of Paxinos and Watson (2007). Subjects were eliminated from the final dataset if no expression was visible in the cell body region or if there was substantial spread into adjacent regions. In Experiment 2, regions of interest were determined by examining the overlay of native EGFP with Fos+ cells, and the percent of overlap compared to the total number of EGFP+ cells ((Fos+/GFP+)/GFP+) in a region was calculated. CNO-mediated insular activity change in Experiment 4 was determined by counting the overlap of Fos+ cells and either mCherry (hM4Di) or EGFP (control virus) under experimenter-blind conditions. All images were quantified using ImageJ software (NIH).

Ultrasonic Vocalizations: During Experiment 3, USVs were recorded during test days, as described previously (**Chapter 4 Methods**). Two high-quality condenser microphones (Avisoft Bioacoustics) were fastened to the lids of the operant box, one on the Observer's dry side and one on the Target's wet side. USVs were recorded during test days, and analyzed with DeepSqueak version 2.6.0 (Coffey et al., 2019) in MATLAB. USVs were reviewed by an experimenter blind to the conditions. The proportion of calls that fell within the distress range (18-35 kHz) compared to the rats' total calls made per trial were calculated and compared on test days. Additionally, in order to discern the impact the change in latency between baseline test day latencies (as calculated by the formula $Release\ Ratio = \frac{Acq_{BL} - Acq_{Test}}{Acq_{BL} + Acq_{Test}}$; an increase in latency from BL to test days is reflected

by a positive ratio) had on Targets' affect, a correlation analysis was performed between the release ratio and the proportion of distress calls made by Targets on those respective days.

Baclofen/Muscimol Infusions: In Experiment 3, the AI of Observers (n=8) was bilaterally cannulated with two 26-gauge single barrel steel guide cannulae (Plastics One), which were anchored to the skull with cranioplastic cement and steel screws (Plastics One). Rats were given one week to recover before starting the 3 chamber empathy task. Rats underwent the empathy task as previously described for 8 days of acquisition. On days 9 and 10, awake Observers had either B/M or PBS (counter balanced) bilaterally, microinfused at 0.5 μL/side into the AI (Arguello et al., 2017) using a microinjector (Plastics One) that extended 1 mm beyond the implanted cannula and a gas-tight Hamilton syringe mounted on a microinfusion pump (Harvard Apparatus). The injectors were left in place for 5 min to allow for diffusion of the virus 30 minutes prior to behavior. Each animal served as their own controls in a within-subjects model. Following the empathy task, animals were sacrificed and placement of cannulae were grossly observed with the aid of ABC quickstain.

Viral Infusions into the Anterior Insula: In the tracer studies (Experiment 2) and the chemogenetic inhibition of the insula (Experiemnt 4), viral vectors were directly infused into the AI using a glass micropipette via Auto-Nanoliter Injector (Nanoject II, Drummond Scientific) at the time of surgery, directly targeting the AI. During intracranial surgery, the virus was infused at a volume of 50.6 nL per injection every 30 seconds at a rate of 23 nL/second followed by an additional 5 minutes to allow the injected virus to diffuse prior to removal of the pipette. Cranial holes were covered with bone wax dental cement and the wound was sutured closed with 4–0 Perma-Hand silk sutures. DREADDs were allowed 3 wk to reach maximal expression levels before behavioral testing began.

Experiment 2 worked to elucidate the role of circuits that include the anterior insula (AI), by infusing either a retrograde-traveling AAVrg (AAVrg-hSyn1-eGFP-Cre, Addgene) or anterograde-traveling AAV (AAV1-hSyn1-eGFP-Cre, Addgene) (n=4/cohort) into the AI.

Following the final day of acquisition, animals were sacrificed, and brains were stained for Fos+/GFP+ cell overlay in the afferent or efferent terminals of the insula to determine which circuits were active during the task.

Prior to Experiment 4, rats were randomly assigned to receive microinjections of either the control virus ("EGFP", AAV8-CaMKIIα-EGFP, Addgene; n=8) or the inhibitory DREADD virus ("hM4Di," AAV8-CaMKIIα-hM4D(Gi)-mCherry, Addgene; n=10). Following infusion and incubation (described above), rats underwent Acquisition for 8 days. On days 9-10, 30 minutes prior to behavioral testing, Observers received i.p. injections of either CNO or water (counter balanced). Following the conclusion of the empathy task, rats underwent the social interaction task (see above), in which animals also received CNO or water injections 30 minutes prior to days 2-4 of testing (see **Figure 5.5**). Placement and DREADD efficacy were confirmed by double-labeling for the viral fluorophore and Fos+ cell overlap within the AI.

Data Analysis: An unpaired t-test was performed to compare the total Fos+ cells in the anterior insula (AI) in rats that performed the task (behaving, BEH) compared to homecage controls (HCC) in Experiment 1. Paired t-tests were performed to determine whether the latency to release the distressed conspecific decreased from early acquisition (EA, days 1-2) to late acquisition (LA, days 9-10) in Experiments 2-4. The number of Fos+/GFP+ overlap cells were quantified as a percentage of total GFP+ cells in each region of interest in the tracer studies of Experiment 2. In the baclofen/muscimol inhibition study (Experiment 3), a one-way RM ANOVA was used to compare the change in chain pull latency in the empathy task between groups (BL vs. PBS vs. B/M). Additionally, a mixed effects 2-way ANOVA was used to compare changes in distress vocalizations as a percent of total calls on test days, while a Pearson correlation was performed between Observers' BL/Test latency ratio and their respective Targets' proportion of distress calls. Finally, for the study examining the chemogenetic inhibition of the AI (Experiment 4), a RM 2-way ANOVA was used, with group (EGFP vs. hM4Di) and injection (water vs. CNO) as the two

variables. One-way RM ANOVAs were also performed to elucidate the differences in time spent in the zones between groups during the social interaction task. Any post hoc comparisons were conducted using a Holm-Sidak's correction for family wise error when appropriate, with the alpha set at 0.05. All analyses were conducted with Prism Software version 8.4.3, and data are expressed as the mean ± SEM.

RESULTS

Experiment 1; AI is activity is potentiated during empathy task: The immediate early gene *c-fos* was stained for in the AI as a measurement of activity during empathic behavior. Rats either performed the 3 chamber task (Behaving, BEH) or were left in their home cages as controls (homecage control, HCC) (see **Figure 5.1A**). Rats readily acquired the targeted helping task, as seen by the significant decrease in chain pull latency from early (EA) to late acquisition (LA) [$t(10) = 4.831$, $p = 0.0007$, **Figure 5.1B**]. The total number of Fos+ cells within the AI were compared between both groups using an unpaired two-tailed t-test. The analysis indicated the Empathy group had significantly more Fos+ cells in the AI compared to the animals that had stayed in their home cages [$F(4,3) = 7.221$, $p = 0.0006$; **Figure 5.1B**].

Experiment 2, Elucidation of AI efferents and afferents: In order to discern potentially critical insula-specific neural circuits modulating empathic behavior, anterograde and retrograde viral tracers were bilaterally infused into the AI in two separate cohorts (n=4/cohort). Regions of interest were identified by calculating the number of Fos+ cells that overlapped with cells expressing viral fluorophore (GFP), as a percentage of the total number of GFP+ cells within the region of interest. Representative images of each region evaluated, as well as the percent overlap comparisons are in presented in **Figures 5.2 and 5.3**.

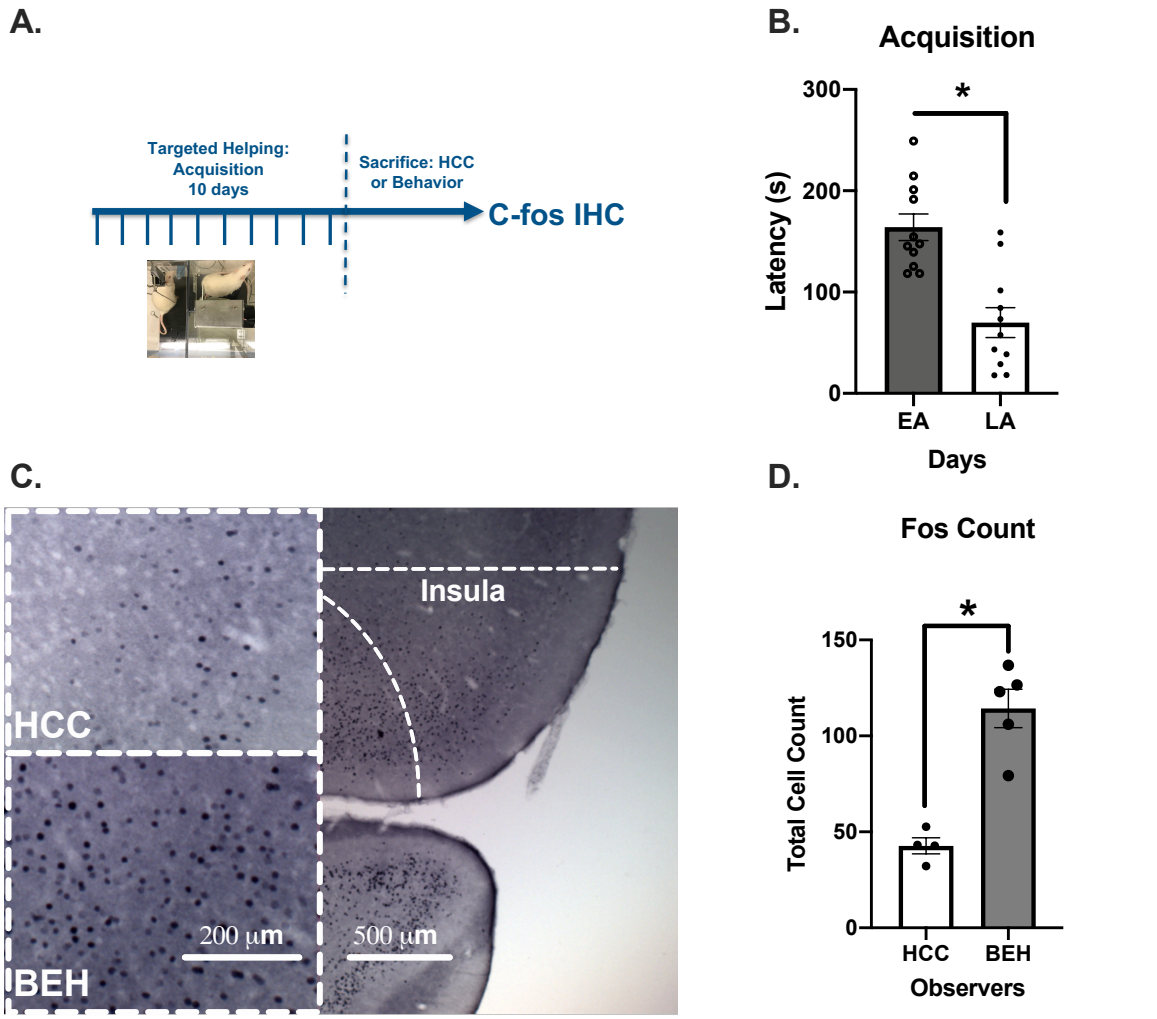
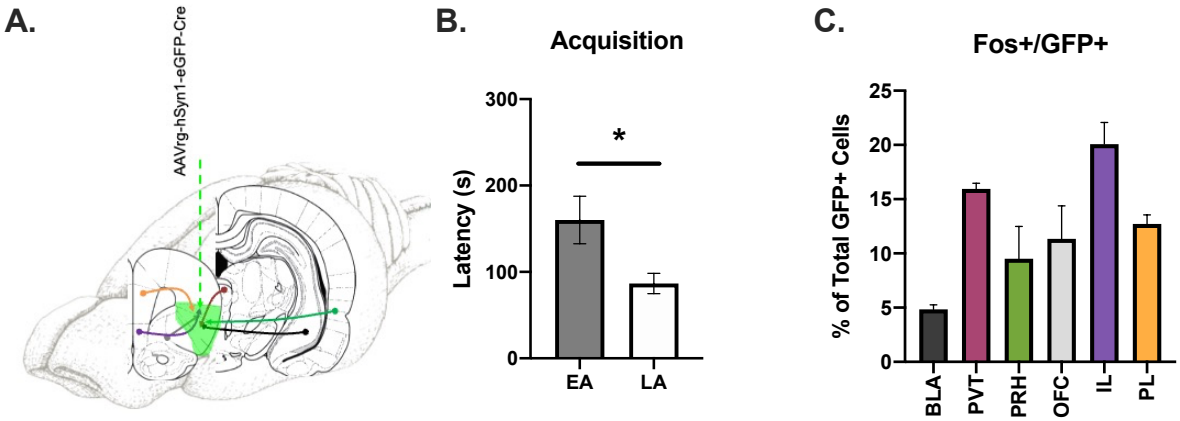


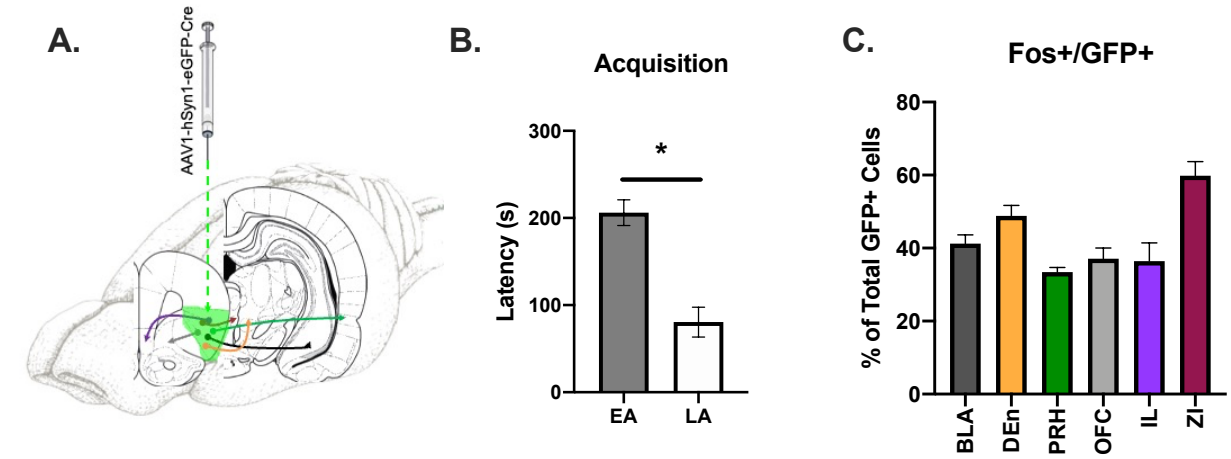
Figure 5.1. The Insula is Active during Empathic Behavior. A) A timeline of the experimental procedures. Male Sprague Dawley rats underwent the empathic behavioral paradigm for 10 days. On test day, rats either performed the task (Behaving, BEH) or remained in their home cages (home cage control, HCC), and were sacrificed 90 minutes following behavior in order to analyze *c-fos* activity in the insula. **B)** Rats' chain pull latency significantly decreased from early (EA) to late (LA) acquisition, indicating task acquisition. **C)** A representative image of the insula (4x) DAB stain used to quantify Fos+ cells in the insula, with 10x insets of a representation of HCC and BEH rats. **D)** Total Fos+ cell counts within the insula. On average, significantly higher total counts were seen in the insula of rats that had performed the task compared to HCC.
*Significant difference between HCC and Empathy groups, $p<0.05$.



D.

Region	BLA	PVT	PRH	OFC	IL	PL
Fos						
Gfp						
Overlay						

Figure 5.2. Elucidation of Insular Afferents Active during Empathic Behavior. **A**) Male Sprague Dawley rats were infused bilaterally with the retrograde AAVrg-hSyn1-eGFP-Cre into the anterior insula (AI) in order to determine important afferents into the insula. **B**) Rats underwent the empathy task for 10 days, and the latency to release a distressed conspecific decreased over time. Specifically, chain pull latency in late acquisition (LA) was significantly shorter compared to early acquisition (EA). **C**) Rats were sacrificed 90 minutes following the final trial in order to stain for *c-fos*. Fos+/GFP+ overlay cells were counted in regions with strong viral staining and presented as a percent of total GFP+ cells in the region. **D**) Representative images for each region of interest evaluated.
*Significant difference between EA and LA, $p<0.05$.



D.

Region	BLA	DEn	PRH	OFC	IL	ZI
Fos						
Gfp						
Overlay						

Figure 5.3. Evaluation of Empathy-Related Insular Efferents. **A**) Male Sprague Dawley rats were infused bilaterally with a anterograde AAV1-hSyn1-eGFP-Cre into the AI. in order to determine important efferents from the insula. **B**) Rats underwent the empathy task for 10 days, and the latency to release a distressed conspecific decreased over time. Specifically, chain pull latency in late acquisition (LA) was significantly shorter compared to early acquisition (EA). **C**) Rats were sacrificed 90 minutes following the final trial in order to stain for *c-fos*. Fos+/GFP+ overlay cells were counted in regions with strong viral staining and presented as a percent of total GFP+ cells in the region. **D**) Representative images of labeled soma for each region of interest evaluated. Labeling of these regions is transsynaptic.
*Significant difference between EA and LA, $p<0.05$.

Experiment 3, Pharmacological inhibition of the AI: A total of 8 pairs of animals were used to elucidate the impact of direct pharmacological inhibition of the AI bilaterally using a baclofen/muscimol (B/M) cocktail. The Observers underwent stereotax surgery in which indwelling cannulae were implanted bilaterally into the AI (**Figure 5.4A**). After surgical recovery, pairs were tested in the behavioral apparatus, in which the Observers progressively learned to release the distressed conspecific over the course of acquisition. Specifically, the latency to release the Target decreased from early acquisition (EA, days 1-2) to late acquisition (LA, days 7-8) [$F(7,7) = 4.435$, $p<0.001$); **Figure 5.4B**]. LA latency averages were used as a baseline (BL) to that test days were compared to (set as % of BL). A paired t-test comparing the change in latency between days in which rats received PBS or B/M infusions indicated a significant effect of treatment [$t(7) = 4.422$, $p = 0.0031$)], and a one-sample t-test also showed B/M was significantly higher compared to BL ($p = 0.0088$), while PBS was not (**Figure 5.4C**). Finally, a one-way mixed effects analysis of USVs within the distress frequency range (18-35 kHz) compared as a percent of total calls found a main effect of test [$F(1, 9) = 22.26$, $p = 0.0011$)], as well as a test x group interaction [$F(1,9) = 7.724$, $p = 0.0214$)]. Post hoc analysis showed Target rats with Observer partners that received B/M infusions had a significantly larger proportion of their total calls ($p = 0.0012$) fall within the distress range compared to Targets whose Observers received PBS control infusions, as shown in **Figure 5.4D**. In order to determine the relationship between the attenuated helping behavior and increased proportion of distress calls, a correlation was performed between the Observers' ratio of the change in chain pull latency from BL to either PBS or B/M test days ($Release\ Ratio = \frac{Acq_{Test} - Acq_{BL}}{Acq_{Test} + Acq_{BL}}$) and the respective proportion of distress calls for Targets. While there was no significant correlation in the PBS trials, a positive correlation was found between the change in Observers' latency and the proportion of the Targets' calls in the distress range ($r = 0.8618$, $p = 0.0126$, **Figure 5.4E**).

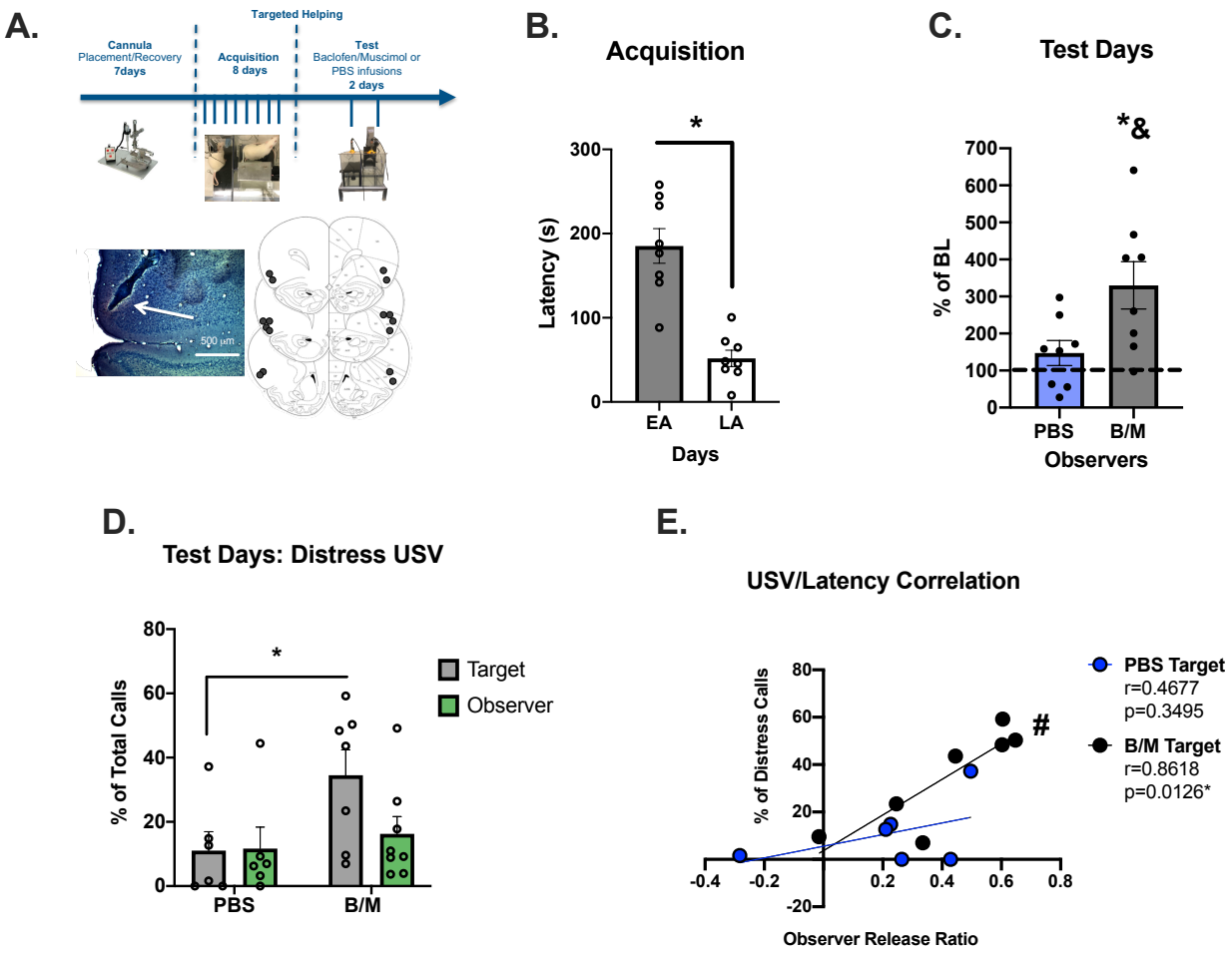


Figure 5.4. Pharmacological Inhibition of the AI Attenuates Empathic Behavior. Direct pharmacological inhibition of the AI via bilateral indwelling cannulae was performed to determine if insula activity is necessary for the observed rescue behavior in male Sprague Dawley rats. **A**) A timeline of the experiment and representative images of the cannulation of the AI and the location of the cannulae of each rat bilaterally. Rats performed 8 days of acquisition in the empathy task. **B**) The latency to release the Target significantly decreased from EA to late acquisition (days 7-8). **C**) On test days, rats that received bilateral infusions of the baclofen/muscimol cocktail (B/M) demonstrated significantly potentiated latency to release the distressed conspecific compared to baseline (BL) or animals that received the control PBS infusions. **D**) Target rats that made significantly larger proportion of their ultrasonic vocalization (USV) calls within the distress frequency range (18-35 kHz) on tests days when their conspecific received B/M infusions compared to PBS infusions. **E**) To understand the relationship between the blunted helping behavior and enhanced distress calls, a Pearson correlation was performed between Observers' BL/Test latency ratio and the percent of distress calls made by their respective Targets. A significant correlation was found on days when Observers received B/M infusion, but not PBS.
*p<0.05

Experiment 4, Chemogenetic inhibition of the AI: To corroborate our findings in Experiment 3, Observer rats had either a viral vector control (EGFP) or an inhibitory DREADD (hm4Di) bilaterally infused into the AI, as depicted in **Figure 5.6A**. Viral placement and efficacy was confirmed following behavior by staining for *c-fos* and quantifying Fos+/virus+ cells within the AI as a percentage of total virus+ cells. A 2-way ANOVA demonstrated main effects of viral group [$F(1, 12) = 29.95$, $p = 0.0001$)] and injection [$F(1, 12) = 20.50$, $p = 0.0007$)], and a significant viral group x injection interaction [$F(1, 12) = 33.99$, $p < 0.0001$)]. Post hoc analysis showed that rats microinfused with the Gi DREADD and that received an i.p. injection 30 minutes prior to the empathy task had significantly less Fos+/virus+ overlap cells compared to any other group, confirming the efficacy and selectivity of the inhibitory DREADD (**Figure 5.6B**). Observer rats first performed the empathy task. **Figure 5.7A** shows that rats acquired the task, with chain pull latency attenuated on in LA compared to EA ($p < 0.0001$). LA were again used as a BL for comparison. For test days, latencies were converted to a percent of BL and compared between groups. A RM 2-way ANOVA showed main effects of both group [$F(1,16 = 7.168$, $p = 0.0165$)] and injection [$F(1,16) = 5.386$, $p = 0.0338$)]. Post hoc analysis confirmed hm4Di rats that received CNO injections had percent changes in latency significantly higher than hM4Di x H2O and EGFP x CNO, meaning the. Additionally, unpaired t-tests indicated the hM4Di x CNO group latency was significantly higher than BL ($p<0.05$), while all other groups did not differ (**Figure 5.7B**). Rats were also tested in a modified social interaction task in order to elucidate the specificity of the AI in modulating empathic behaviors compared to prosociality per se. Because no behavioral differences were seen in the EGFP group between water and CNO injections, these data were combined for analysis. The cumulative duration of EGFP and hM4Di groups did not differ during pre-interaction in either the SZ or OZ (**Figure 5.8A**). On Interaction days, a main effect of zone was seen [$F(1,13) = 16.94$, $p<0.0001$)], with all groups spending significantly more time in the SZ compared to the OZ (**Figure 5.8B**). However, no group differed from one another in their time spent within the SZ. In addition, other indicators of social interaction, like nose-nose contact

between animals and time spent cage climbing, as well as motor activity, were measured on interactions days. No difference was seen in any of these additional variables, further confirming inhibition of the AI had no effect during interaction (data not shown). Finally, on post-interaction day 5, a main effect of zone was again found [**Figure 5.8C inset**; $F(1,13) = 5.028$, $p = 0.043$)], however, there was no difference in cumulative time spent in the SZ compared to the OZ in any specific group (**Figure 5.8C**).

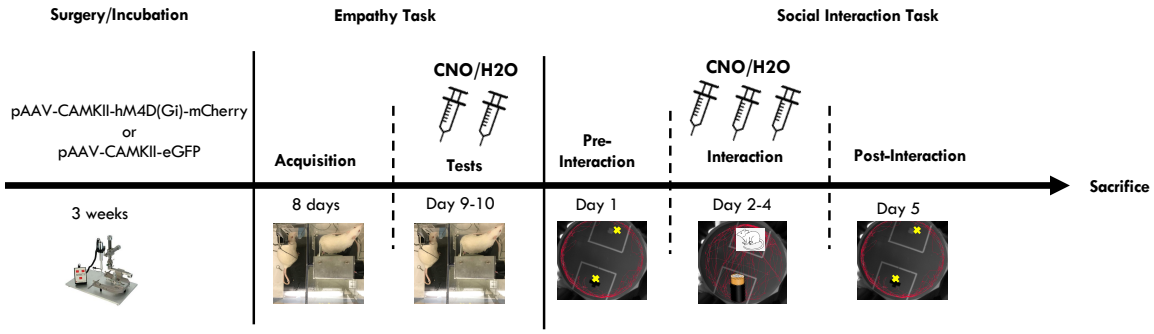


Figure 5.5. Timeline for Experiment 4. Male Sprague Dawley Observer rats underwent stereotax surgeries and received microinfusions of either the control virus (AAV8-CaMKIIα-EGFP, n=8) or the inhibitory DREADD virus (AAV8-CaMKIIα-hM4D(Gi)-mCherry, n=10). After 3 weeks of recovery and viral incubation, rats went through the empathy task for 8 days. On the final 2 days of the empathy task, Observers received either CNO or water injections (i.p.) in a within-subjects experimental paradigm. Next, rats underwent a social interaction and social reward place conditioning task as described in **Chapter 5 Methods.** Specifically, animals were tested on days 2-4 and received either CNO or water injections (i.p.) 30 minutes before placement in the arena in a between subjects design. On the day animals were sacrificed, rats were injected with either CNO or water and again performed the empathy task. 90 minutes following behavior, the animals were sacrificed in order to stain for *c-fos* in the AI to confirm both placement and activity of the inhibitory DREADDs.

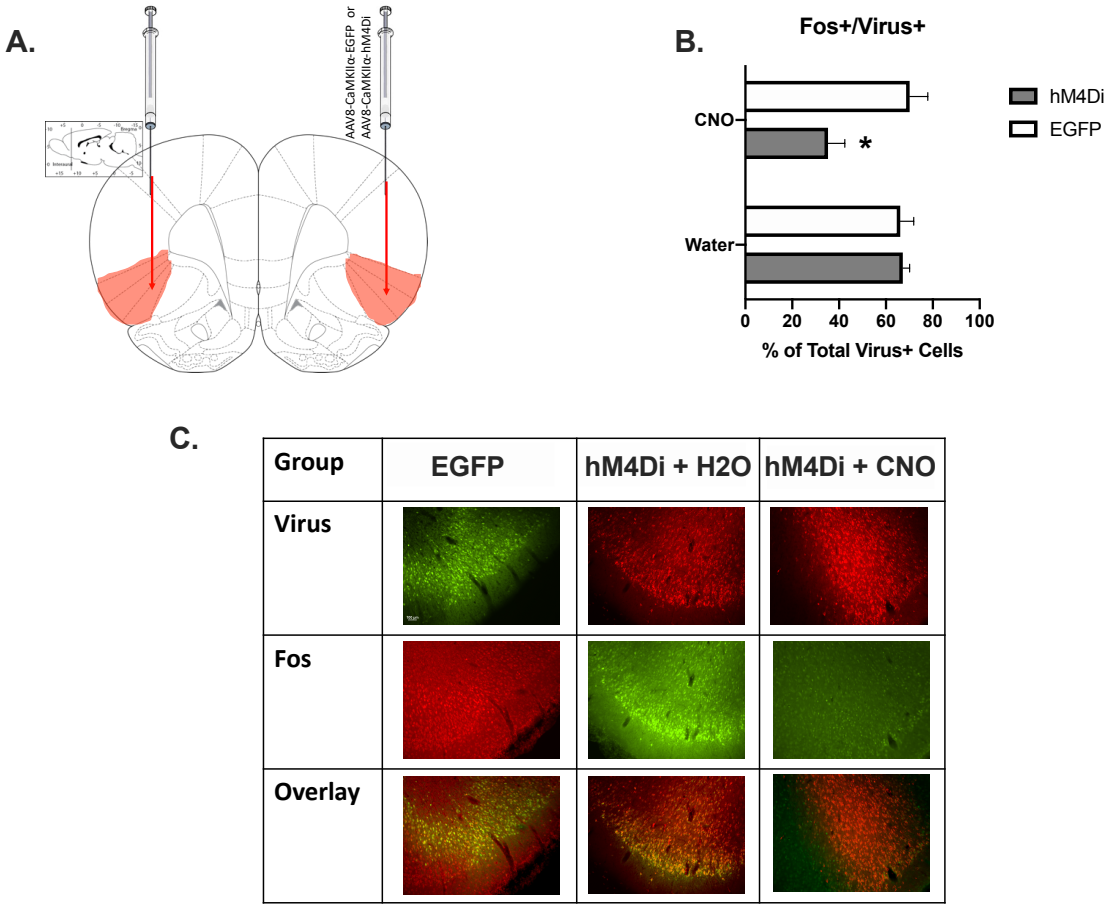


Figure 5.6. **Inhibitory DREADDs are Activated Specifically in the Presence of CNO. A**) Rats were bilaterally infused into the AI with either the control virus (AAV8-CaMKIIα-EGFP, n=8) or the inhibitory DREADD virus (AAV8-CaMKIIα-hM4D(Gi)-mCherry, n=10). **B**) Following behavior, rats were sacrificed and Fos+/Virus+ cells were quantified in the AI as a percentage of total Virus+ cells. Rats having received the Gi DREADD infusion into the AI and CNO injections 30 minutes prior to behavior had significantly fewer Fos+/Virus+ cells compared to all other animals. **C**) Representative images of the AI in the EGFP control group, the hM4Di + H2O injection, and the hM4Di + CNO injection.
*Significant difference in Fos+/virus+ cell overlap, $p<0.05$.

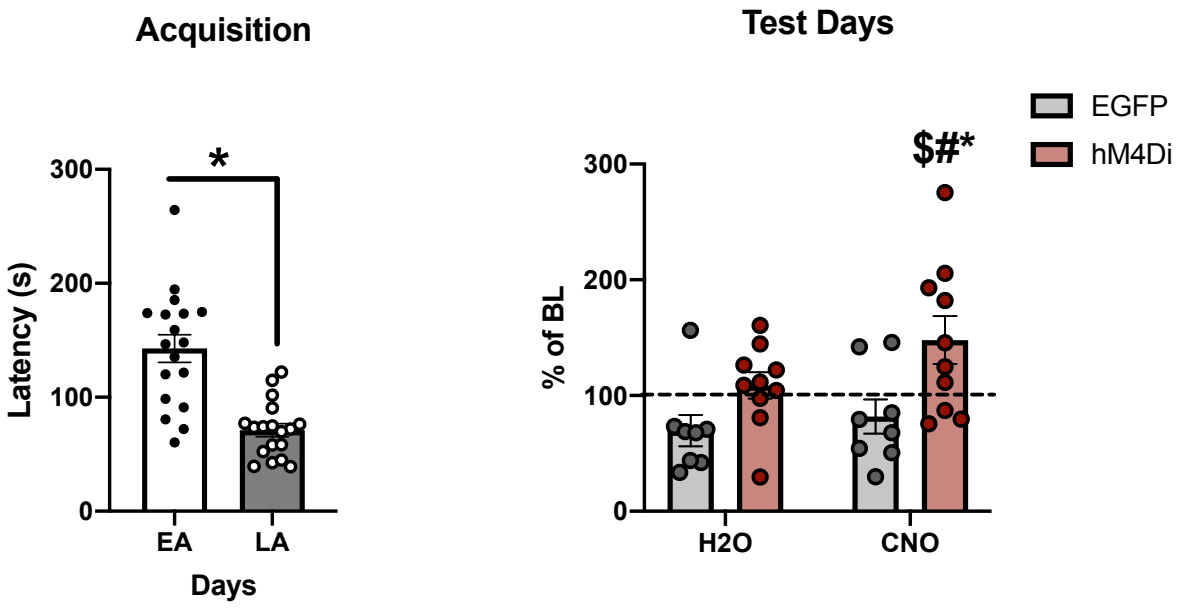


Figure 5.7. CNO Activation of hM4Di DREADD Attenuates Empathic Behavior. Rats performed 8 days of acquisition in the empathy task prior to testing **A**) Observers learned to release a distressed conspecific onto the dry side. Specifically, late acquisition (LA, days 7-8) chain pull latency was significantly attenuated compared to early acquisition (EA, days 1-2). *p<0.05. **B**) On test days, Observers were injected with either water or CNO (i.p.) 30 minutes prior to the first behavioral trial. Rats infused with the Gi DREADD injected with CNO had latencies significantly higher than their baseline (days 7-8) latencies. Further, the percent change of baseline in the hM4Di +CNO group was significantly higher compared to the hM4Di +H2O and the EGFP+CNO groups.
*Significantly different than BL, p<0.05
$Significantly different from hM4Di x H2O, p<0.05
#Significantly different from EGFP x CNO, p<0.05

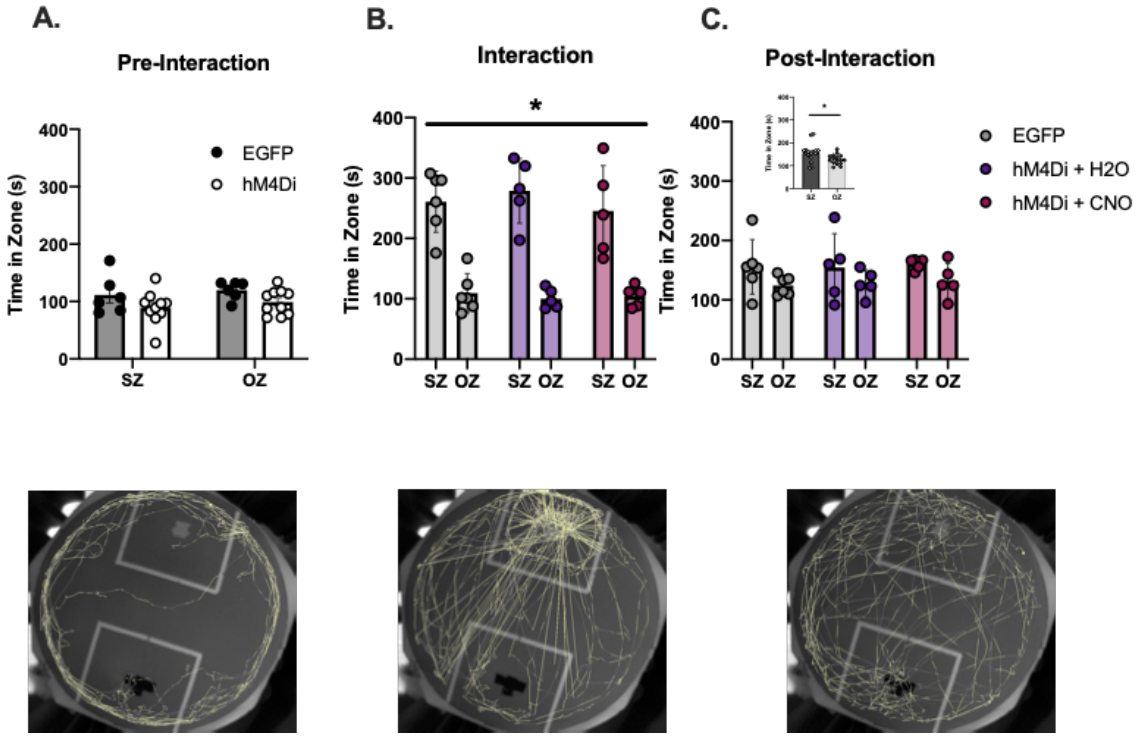


Figure 5.8. Social Interaction Does Not Differ Following Insula Inhibition. Rats underwent a social interaction and social reward place conditioning task. On day 1, as a pre-interaction evaluation, rats were placed in the open field and given 10 min to explore the environment. On days 2, 3, and 4, rats were injected with water or CNO 30 minutes prior to the task and given the opportunity to explore an unfamiliar rat and an unfamiliar object simultaneously. Finally, on day 5, rats were returned to the empty open field for a post-interaction assessment. Time spent in social zone (SZ) and object zone (OZ) were recorded throughout the task. **A**) Rats did not exhibit a side bias within the open field, equally preferring the SZ and OZ on pre-interaction day. **B**) On interaction days, a main effect of zone was observed in which all groups preferred the SZ over the OZ. **C**) On post-interaction day, a main effect of side was again observed, in which the time spent in the SZ was greater than the OZ (inset). However, the post hoc of the main effect did not reveal any significant differences in time spent when the groups were compared.
A representative locomotion tracking image for each day is placed below their respective figures.
*Significant effect of zone, $p<0.05$

DISCUSSION

In the previous series of studies, we evaluated the role of the anterior insula (AI) during a social contact-independent helping task used as an evaluation of empathic behavior. Here, we report three main findings. First, the insular cortex is significantly more active in Observers compared to homecage controls during empathic behavior late in acquisition (**Figure 5.1B**). Second, there are multiple insular efferents and afferents that may act as critical circuits to modulate the behavior. Finally, inhibition of the AI in Observers, both pharmacologically (**Figure 5.4**) and chemogenetically (**Figure 5.7**), is enough to significantly blunt the release of a distressed conspecific. This insula-dependent modulation seems to be specific to targeted helping, as AI inhibition did not affect social exploration in a social interaction or novel object exploration in a social preference task (**Figure 5.8**). These findings provide further insight into the neural basis of empathic behaviors, and consistent with both preclinical (Zaniboni et al., 2018) and clinical research (Singer et al., 2004; Saarela et al., 2007; Singer and Leiborg, 2009; Boucher et al., 2015; Uddin et al., 2017) point to the insula as a primary node in the empathic brain.

The AI acts as an informational integration hub, receiving external sensory and interoceptive inputs from the thalamus, sensory cortex, and olfactory bulb, while also having strong connectivity with the limbic system and cortical regions (Gogolla, 2017; Gehrlach et al., 2020). This unique connectivity permits the AI to detect salience and assess valence of both self and other, and therefore generate an appropriate prosocial behavior (Gogolla, 2017; Rogers-Carter and Christensen, 2019). Because many of these insular pathways are bidirectional (Gehrlach et al., 2020), it was unsurprising to see that both afferent (**Figure 5.2**) and efferent (**Figure 5.3**) circuits of the insula were active during the 3 chamber helping task. However, it was surprising to note the disparity of activity (as measured by Fos+/GFP+ overlap) in afferents as compared to efferents. One methodological consideration for this differene is the transsynaptic path the anterograde tracer must take make alter expression levels compared to the retrograde tracer. It is also possible that the cortical, limbic, and thalamic regions evaluated in this study did not fully incorporate the strongest

inputs into the AI. Connectivity studies have concluded the AI receives strong intra-insular and sensory cortex inputs, which may make up over half of the cortical inputs into the insula (Gehrlach et al., 2020). Due to the path of the microinjector and the strength of the viral fluorescence within the insula, we were unable to quantify overlay of Fos within these regions in Experiment 2. Insular outputs may also show comparatively higher activity following the integration of multimodal inputs to the AI to generate an appropriate behavioral output.

A region of interest that emerged in our analysis was the infralimbic region (IL) of the medial prefrontal cortex (mPFC). The mPFC has been implicated in many goal-oriented tasks, including reward valuation, working memory, flexible behavior, and social cognition (Bicks et al., 2015; Ito et al., 2015; Suto et al., 2016). In fact, while activity of the prelimbic region (PL) of the PFC may be associated with goal valuation (Ito et al., 2015), recent evidence suggests the IL is more responsive to alternative choices (Suto et al., 2016), with distinct neuronal engrams within the IL playing a role in both promotion and suppression of rewarding, goal-directed behavior (Mukerjee and Caroni, 2018). This dualistic role of the IL may have a temporal component, in which the IL may suppress behavior during consolidation of a behavioral task, but is required for the acquisition of ongoing, habitual behaviors (Barker et al., 2014). As it related to the Perception-Action Model (PAM) of empathy, it is possible the IL's role is two-fold. It may initially play a role in affect regulation in order to formulate an appropriate response in the presence of a distressed conspecific. However, once the chain pull task is acquired, its activity may be necessary for the habitual release behavior seen in the latter parts of acquisition. Future studies in our lab will utilize a dual viral approach to directly manipulate the AI → IL pathway to determine if its function in behavioral flexibility is necessary for the observed release of a distressed conspecific.

Our results indicted the inhibition of the AI attenuated empathic behavior, as measured by a potentiation of chain pull latency in our 3 chamber task. This finding was confirmed using two separate methods of insular inhibition; pharmacologic, using a baclofen/muscimol (B/M) cocktail directly infused into the AI, as well as chemogenetic, utilizing an inhibitory DREADD construct.

An interesting finding was that when chain pull latency was potentiated as a result of B/M infusion, the proportion of Targets' USV that were within the distress frequency range increased significantly (**Figure 5.4D**) compared to trials in which Observers received PBS. Further, there is a significant correlation between the Observers' B/M/BL release ratio and their Targets' proportion of distress USV (**Figure 5.4E**). These data suggest that Targets recall their quick release in the latter parts of acquisition by the Observer. When there is a mismatch of outcome with previous experience of release (i.e., a significantly longer time spent in the water), the Targets' level of distress increases, and therefore their proportion of distress calls rises. These results strengthen the concept that, per the perception action model (PAM) of empathy, the actions of the Observer, or lack thereof during the trials in which their insula were inhibited, have a direct impact on the affective state of the Target.

Although we observed an attenuation of helping behavior following AI inhibition, it did not affect exploration of a novel object or social interaction with an unfamiliar conspecific. Not only did the time spent in the respective social and object zones not differ between hM4Di and EGFP groups, other variables like locomotor activity, time spent climbing the object or the cage, and nose-to-nose contact with the unfamiliar animal, did not vary across groups (data not shown). These data help corroborate AI inhibition did not alter social or novel object exploration. It has been demonstrated previously that there is a large dopamine increase in the insular cortex during the exploration of a novel object (Guzman-Ramos et al., 2012). Further, there is a direct involvement of the insular cortex in object recognition memory (Bermudez-Rattoni et al., 2005). However, it is not surprising that the rats did not spend significant time in the OZ, as animals consistently spend more time exploring an unfamiliar conspecific compared to a novel object (Miura et al., 2020). Therefore, while it is possible the AI does modulate novel object exploration, its relatively low salience compared to a conspecific may have drowned out the effect during insular inhibition. Our results also showed no change to social interaction with a conspecific, which is antithetical to other research that has shown inhibition of the insula disrupts a social affective

preference test (Rogers-Carter et al., 2018), while its activation promotes social exploration (Rogers-Carteret al., 2019). Further, chemogenetic activation of the AI subsequently restored heroin-induced deficits in prosocial behaviors (Tomek et al., 2020). One methodological reason for our findings could be due to the fact that inhibitory DREADDs have been found to only suppress approximately 50-60% of the neural activity of affected cells following CNO injections (Smith et al., 2016, Tomek et al., 2020), as was corroborated in our analysis (**Figure 5.6A)**. The DREADD-induced insular inhibition may not have been robust enough to attenuate social interaction to a significant degree, but adequate to see changes in empathic behavior.

There are several methodological considerations that also need to be addressed in this series of experiments. In the Experiment 4, a dose of 10 mg/kg CNO was used on test days. Although this is higher than some suggest as a recommended range (Smith et al, 2016), our lab has consistently utilized 10 mg/kg based on reports that this may be the minimally effective dose for some cortical systems (Chiarlone et al., 2014; Augur et al., 2016). Additionally, the use of the EGFP control virus lacking the DREADD coding sequence, ensured that CNO or the reverse metabolite clozapine alone did not produce any observable behavioral effects (Chen et al., 2015), and using hM4Di animals injected with water provided evidence the inhibitory DREADD does not innately alter neural activity.

It is also likely the perception action model (PAM) of empathy, as measured by our 3 chamber task, incorporates other behavioral components, such as learning and memory and emotional contagion. Although we did replicate our findings showing targeted helping is attenuated following AI inhibition, additional behavioral control experiments should be performed to better elucidate the specific component of empathy that is dysregulated when the AI is inhibited. Another variable that needs further study is the effect of sex on the impact of AI inhibition on targeted helping. In **Chapter 4**, we demonstrated similar changes in insular activity compared to homecage control, between male and females Observers across all timepoints (**Figure 4.7**). However, there was a sex effect in total number of Fos+ cells between male and female HCC (**Figure 4.5**), so it is

unclear if equivalent change or a difference in absolute activity may more strongly modulate facets of the empathic process in males and females.

In conclusion, our results have established the importance of the anterior in targeted helping by showing, in two separate studies, its activity is necessary for release of a distressed conspecific. Further, the behavioral change was specific to empathic dysregulation, as social and novel object exploration was unchanged by insular inhibition. Finally, the tracer experiments performed revealed potential insula-specific circuits that may mediate empathic processes that can be explored in future experiments. Overall, elucidating both critical nodes and circuits involved in empathic processes can not only better help us understand a complex, requisite social behavior, but it can help to improve the treatment outcomes and quality of life for those diagnosed with myriad psychiatric disorders.

CHAPTER 6: Conclusions and Future Directions

Rats Display Helping Behavior Independent of the Opportunity for Social Contact

There are multiple animal models that strive to elucidate specific components of empathic behaviors. One such model, targeted helping, evaluates the ability for a rat to release a distressed conspecific. However, social interaction has remained a confounding variable within this task (Bartal et al., 2011; Sato et al, 2015). We first confirmed that rats indeed learned to release another rat in a conventional helping task, and that the release behavior was driven by the presence of a conspecific in distress. In agreement with some behavioral studies, we identified the opportunity for social contact as at least part of the reduced chain pull latency over time. We therefore utilized our lab's novel 3 chamber targeted helping task, in which social contact was not possible, to show that indeed rats release a distressed conspecific. Like the 2 chamber task, latency decreases with subsequent trials, and previous experience significantly attenuates chain pull latencies. We validated our paradigm by showing the chain pull latencies increase in the absence of a distressed conspecific. We further demonstrated rats are able to learn to release an unfamiliar rat, and that empathic behavior is dependent on effort. Taken together, the results from our experiments in **Chapter 3** work to expand upon the current rodent models of empathic behaviors to include one that removes the confounding effects of direct social interaction during the task, while still adhering to the perception action model (PAM) of empathy.

A goal for our lab is to utilize this targeted helping task in order to explore translationally relevant disorders that exhibit empathic dysregulation, such as substance use disorder (SUD) and Autism Spectrum Disorder (ASD). As mentioned in **Chapter 1**, individuals with a mono- or poly-substance abuse show impairments in empathy and restoring empathic behaviors may improve treatment outcomes like relapse (Massey et al., 2018; Robinson et al., 2018). Other groups have begun to explore the impact of prosocial behaviors, specifically "social self-administration" on drug taking in rodents (Venniro et al., 2020b). Our lab hopes to utilize our targeted helping task in

conjunction with an operant drug self-administration model to examine the effects of prior heroin self-administration on empathic behaviors. Additionally, patients with ASD have difficulty recognizing the emotions of others (Harmsen, 2019). Recent studies have shown aberrant prosocial behavior in a chemical model (valproic acid) of ASD (Fontes-Dutra et al., 2019). Future studies in our lab could utilize improved genetic knockout rat models of ASD in our apparatus to evaluate whether changes in social interaction seen in autism models are driven by dysregulated empathic processes. Overall, we believe this model will be translationally useful to explore critical variables and mechanisms underlying changes in empathic behavior in neuropsychiatric disorders.

Sex Differences in Targeted Helping

One such variable that has garnered great attention in the field of empathy is that of sex. There is little consensus regarding the presence of a sex difference in empathic behavior (Ben-Ami Bartal et al., 2011; Du et al., 2020; Han et al., 2020). Results from our studies (**Chapter 4**) are in congruence with research that suggests there is little or no inherent behavioral differences in animal models of empathy. Any sex difference observed was due to an attenuation in helping behavior within the "low AI" females, which showed no significant reduction in their chain pull latency over time. As was hypothesized in **Chapter 4**, stress may be the driver of this behavioral difference. Female Targets may find the water more distressing, as suggested by the potentiated proportion of distress calls, early in acquisition. This may lead observers (which in this study were also female) to have more difficulty regulating their own affect, altering the rate at which the behavior is acquired (de Waal and Preston, 2017). One way of comparing the stress response during the task between sexes is an assessment of circulating corticosterone (CORT) during targeted helping. We would hypothesize serum CORT levels in female Observers would be higher than males and may match the levels of Targets. Research also indicates stress directly alters rats' licking and grooming time and patterning (Kalueff et al., 2015). Adding these assays to directly assess stress would help to correlate physiological and behavioral stress responses to helping behavior.

A major limitation of this work mirrors that of the field at large, which is the significance and nuance of rat USV have yet to be fully elucidated. While there is strong evidence confirming rats emit 22 kHz calls during a stressful event (Borta et al., 2006), it is more unclear what the importance of prosocial calls on a complex behavior like empathy. Some work has tried to decipher the importance of subgroups of prosocial calls by further dividing them into two subcategories, flat and frequency modulated (Wohr, 2017). While some may signal affiliative or appetitive function, others may work as social coordination (Simola et al., 2018), but research is still needed to elucidate the importance of these calls in prosocial and empathic settings. Specific distress or prosocial-specific frequency playback studies using the blacked-out Plexiglas in the absence of a Target could help determine if these calls are necessary for helping behavior. However, we believe USV are a useful tool in behavioral studies to assess the underlying affective state of the animals.

Oxytocin (Oxy) continues to garner significant attention in the field of empathy research. It has been suggested that Oxy may modulate empathic processes spanning from emotional contagion to socially transmitted fear (Guzman et al., 2014; Laviola et al., 2017; Pisansky et al., 2017). However, more evidence is growing to point towards social-context and sexually dimorphic effects of Oxy release and receptor binding (Bredewold and Vennema, 2018). Therefore, while we found interesting sex-specific Oxy neuron activity within the paraventricular nucleus if the hypothalamus (PVN) over the course of the targeted helping task (**see Chapter 4**), the overall behavioral effect of these differences have yet to be confirmed. Our lab is currently working to directly inhibit Oxy neuron activity within the PVN of males and females during targeted helping to elucidate the importance of Oxy release in empathic behaviors. In addition, there is significant binding overlap between receptors for Oxy and vasopressin, another pituitary neuropeptide, due to their molecular similarity (Dumais and Veenema, 2016). The vasopressin system has received more attention for its role in prosocial behaviors, particularly in males. While Oxy may drive certain prosocial

behaviors, like pair-bonding, the same behaviors seem preferentially driven by vasopressin in males (Lu et al., 2019).

The Role of the Anterior Insula on Targeted Helping

In agreement with extensive clinical and preliminary preclinical literature, the data presented in **Chapter 5** of this dissertation demonstrated the anterior insula (AI) is necessary for targeted helping. Inhibition of the AI using two different methods, both pharmacological and chemogenetic, significantly attenuated the release of a distressed conspecific. Further, in trials when the AI was inhibited using and the latency to release the Target was increased, the proportion of distress calls made by the Target rats also increased. More importantly, the Observers' change in the ratio of chain pull latency from B/M test days compared to BL latency significantly correlated to the proportion of distressed calls made by the Targets, which points toward a direct association between the change in behavior of Observers to a change in affect of the Targets. Finally, the behavioral effects were specific to targeted helping, as there was no difference in social interaction with AI inhibition.

The absence of an effect AI inhibition had on social interaction was somewhat surprising, as several studies have indicated the insula directly modulates social approach and avoidance, or social interaction (Rogers-Carter et al., 2018; 2019; Tomek et al., 2020). As mentioned in **Chapter 5**, a methodological variable to consider regarding inhibitory DREADDs is they may not fully suppress neuronal activity (Smith et al., 2016, Tomek et al., 2020). Therefore, it is possible the level of inhibition afforded by Gi DREADDs is insufficient to blunt the drive for social interaction. Other labs have used muscimol or optogenetic methods (Rogers-Carter et al., 2018; 2019), which have limitations of their own, but may suppress neuronal excitability at a higher proportion. Therefore, future studies will explore the impact of AI inhibition on social interaction using a baclofen/muscimol cocktail as in **Chapter 5, Experiment 3**.

Another limitation of these studies is they were done exclusively in males. Our lab did explore insular activity in males and females in **Chapter 4** and saw the change in activity compared to home cage controls were relatively unchanged between sex and across time. However, there was a significant sex effect in the HCC rats, suggesting the total Fos+ cells between males and females differ. No studies to date have compared the impact of the insula between sexes, and it is unclear if the absolute or relative change in activity within the insula has more of an effect on targeted helping behavior. Therefore, future studies should also examine the role of the AI in female rats.

Finally, our research has identified potential afferents and efferents of the AI that may comprise a critical targeted helping circuit. A promising finding was there are several regions implicated in emotional recognition and perspective-taking that receive strong efferents from the AI. It must be noted that there is a large discrepancy between the relative activity in the efferent projections compared to the afferents (see **Chapter 5**). While several tracing studies have shown many insular circuits to be bidirectional (Gehrlach et al., 2020), the relative activity of these circuits, especially during empathic behavior, is not known. It is possible the discrepancy is due to a difference in the transfection rate of the retrograde AAV compared to the anterograde AAV1. While some have thought AAV1 may transduce collateral or second-order neuron, recent evidence suggests that AAV1 only transduces first-order postsynaptic neurons similar to rAAV (Zingg et al., 2017; Zingg et al., 2020). Overall, set of experiments in **Chapter 5** not only point to the AI as a critical neural node in the empathic brain, but it also lays the groundwork for future studies to identify specific circuits that mediate empathic behaviors.

Final Thoughts

Empathy is a complex, multivariate social behavior that is observed throughout the animal kingdom. A growing body of literature indicates empathic behavior is not only critical for social cohesion and individual relationships, but it is strongly dysregulated in myriad neuropsychiatric disorders, including SUD, ASD, and others. A translationally

relevant model that has minimal confounding variables is thus critical for expanding our understanding of empathy and striving to improve treatment outcomes and quality of life in patients with psychiatric illness. The current dissertation introduced and validated a novel rodent model of targeted helping that eliminates social contact as an underlying motivator for release of a distressed conspecific. We further identified sex differences contributing to empathic behaviors, and proved the insula is a critical node in the empathic brain. A number of questions remain regarding the convergent sex effect of neural activity and affect transfer, as well as the role of the AI within a circuit that governs empathic behavior. Further, more work is necessary to combine our novel model with other operational tasks to explore the relationship between empathy and other behaviors like drug self-administration. However, these experiments help to advance the field and lay a solid groundwork for future studies to examine these interdependent variables on empathic behavior.

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