Social Fear Learning in Oxytocin Receptor Knockout Mice and People with Autism Spectrum Conditions

Neural, Genetic, and Psychophysiological Translational Investigations

Submitted for the degree of Doctor of Philosophy by

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Declaration

This dissertation is the result of my own work and includes nothing that is the outcome of work done in collaboration except as specified in the text.

It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University of similar institution except as specified in the text

This dissertation does not exceed the 60,000 word limit as prescribed by the Degree Committee.

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Abbreviations

ACUC: Animal care and use committee
ADHD: Attention deficit hyperactivity disorder
AQ: Autism quotient
ANOVA: Analysis of variance
ARC: Autism Research Centre
ASC: Autism spectrum condition
BLA: Basolateral amygdala
BNST: Bed nucleus of the stria terminialis
BOLD: Blood oxygenation level-dependent
CeA: Central amygdala
CRH: Cortico-tropin releasing hormone
CS: Conditioned stimulus
DHHS: Department of Health and Human Services (USA)
DSM-5: Diagnostic and statistical manual of mental disorders, Fifth Edition
EDA: Electrodermal activity
EQ: Empathy quotient
fMRI: Functional magnetic resonance imaging
GWAS: Genome-wide association studies
HPA: Hypothalamic-pituitary-adrenal
IADS: International affective digital sounds
IAPS: International affective picture system
KO: Knockout
MeA: Medial amygdala
MEMRI: Manganese-enhanced magnetic resonance imaging
MR: Magnetic resonance
NIH: National Institutes of Health (USA)
NIMH: National Institute of Mental Health (USA)
NPY: Neuropeptide Y
OT: Oxytocin peptide
Oxtr: Oxytocin receptor gene
PTSD: Post-traumatic stress disorder
PVN: Paraventricular nucleus of the hypothalamus
RDoC: Research domain criteria
ROI: Region of Interest
SCR: Skin conductance response
SON: Supraoptic nucleus of the hypothalamus
UCS: Unconditioned stimulus
VPA: Valproic acid
WT: Wildtype

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Abstract

The processing of fear-related emotions is a crucial component of typical social behavior as well as mood disorders (e.g., post-traumatic stress disorder, social anxiety, bipolar disorder, and depression) and developmental disorders (e.g., autism spectrum disorder and social communication disorder). The research described in this thesis explores the genetic, neural, physiological, and behavioral factors involved in the processing of social fear in animal models and human participants. Objectives of this thesis are: 1) To examine the role of oxytocin in the expression of fearful emotions in a social context. How does oxytocin mediate fear responses in social contexts in mice at the neural, behavioral, and physiological levels? [Chapter 2 (Behavioral), Chapter 3 (Neural), Chapter 4 (Physiological)]; 2) to assess the psychophysiological effects of fear when presented in social contexts in high-functioning adults with an autism spectrum condition (ASC). [Chapter 5]; 3) to evaluate the translational aspects of research examining behavioral and mood disorders in animal models. How do physiological results from the oxytocin receptor gene (*Oxtr*) knockout (KO) mouse model compare to those drawn from human ASC cases? [Chapter 6].

Experiments conducted to evaluate Objective 1 included behavioral observation of *Oxtr* KO and WT male mice during social defeat interactions (examined via the Resident-Intruder Paradigm). Results indicated that *Oxtr* KO male mice act more submissively (as evaluated via measurement of fleeing and freezing postures in this study) than WT animals when socially interacting with a more aggressive conspecific despite exposure to similar levels of social aggression. This elicitation of a more submissive phenotype potentially indicates a difference in emotional fear processing that may be related to the absence of oxytocin receptors in the brain. To further probe Objective 1, this research piloted a method of neuroimaging in mice known as manganese enhanced magnetic resonance imaging (MEMRI), where we demonstrated that Mn^{2+} accumulates throughout the whole brain of the mouse, and also concentrates preferentially in the more active regions of the brain (i.e., olfactory bulbs, hippocampus, amygdala). Qualitative evidence indicated that signal enhancement was greater when manganese was infused over a shorter period of time (higher effective rate of infusion). Subsequent studies employing MEMRI

to functionally image socially defeated *Oxtr* KO and WT adult male mice indicated that there are differences in amygdalar activation (localized to the medial and basolateral nuclei) in socially defeated WT mice that was not observed in socially defeated *Oxtr* KO (or sham handled WT mice). Specifically, this research links oxytocin receptor activity in the mouse brain to basolateral and medical amygdala-specific response to social defeat. This research also evaluated a secondary measure of social fear conditioning via physiological response. Results indicated that core body temperatures are differentially regulated in *Oxtr* KO and WT adult male mice when subjected to a 3-day social defeat paradigm. It was observed that social stress (i.e., exposure to aggressive and dominant behaviors exhibited by a conspecific) induced acute hyperthermia in *Oxtr* KO mice *during* social defeat encounters. Intriguingly, evidence of early chronic hyperthermia was also observed over the 3-day period—as indicated by the increasing body temperature measurements collected from *Oxtr* KO animals prior to social defeat exposures that exceeded increases in body temperature exhibited by WT animals.

In the human studies portion of this thesis research we employed classical fear conditioning in a social context as a paradigm for social anxiety in humans. Utilizing a psychophysiological approach to assess sympathetic nervous response to social stimuli paired with threatening cues, investigators in this research evaluated skin conductance response during the Habituation, Acquisition, and Extinction phases in control subjects and ASC cases. Control participants demonstrated increased skin conductance in response to CS+ during the Acquisition Phase of the fear conditioning paradigm, while ASC cases indicated attenuated SCR to CS+ that was not significantly distinguishable from SCR to CS-, indicating a potential elevated fear response. Similarly, Control subjects displayed expected decreases in SCR during the Extinction Phase of the fear conditioning paradigm while ASC cases indicated residual elevation of SCR (to both CS+ and CS-). Such results suggest that individuals with ASC may have difficulty forming associations to specific cues during associative fear learning (perhaps due to the inability to discriminate between social cues as suggested by the data). Results from the acquisition and extinction phases of the fear conditioning paradigm in this study thus support previous findings of attenuated SCR due to an increased fear response and impaired differentiation between conditioned and non-conditioned stimuli in individuals with ASC.

This research demonstrates in mice that oxytocin receptors play an active role in the amygdala during the processing of social stimuli in a fear-related context. This research also indicates that experiences of social fear may elicit physiological responses in humans that may be related to oxytocin function in the brain. As the precise mechanism through which oxytocin receptors interact with other hormones and neurotransmitters in the mediation of social behaviors is not wholly characterized, it is important to employ comprehensive methods that examine the receptor's functions at the systems level. Our analysis of oxytocin's response to social stimulation through a multimodal approach measuring the neuronal, behavioral, and physiological outcomes provide broader insight into the psychosocial system of fear cognition. The results in this study linking abnormal responses to fearful contexts of a social nature to sympathetic arousal in ASC patients draw the amygdala theory of autism to consideration. The theory that the amygdala is a crucial player in the neural processing of stimuli essential for the expression of typical social behaviors also raises the question of how it may affect fear processing. As the amygdala is a key area of the brain involved in the relay of threatening cues and initiation of behaviors critical to survival, how the processing of fearful and social stimuli intersect in the amygdala to potentially manifest in the form of anxiety or phobias in individuals with ASC is a key area of concern that requires further examination.

Table of Contents

List of Figures

List of Tables

Chapter 1: Introduction

1.1 SOCIAL BEHAVIORS AND FEAR-RELATED EMOTION PROCESSING

The processing of fear-related emotions is a key area of cognitive function and a topic of interest in the understanding of social behaviors related to mood disorders (e.g., post-traumatic stress disorder, anxiety, bipolar disorder, and depression) and developmental disorders (e.g., autism spectrum conditions and social communication disorders). The research described in this thesis report explores the genetic, neurological, physiological, and behavioral factors involved in the emotional processing of social fear via a series of translational studies conducted in both animal models and human participants.

1.1.1 Implications of fear in social behaviors

Fear, or the emotional processing of threatening stimuli, is a basic emotion experienced by humans and animals, evolutionarily rooted in the instincts for survival. Feelings of fear are triggered by specific events and often induce such instinctive behaviors as freezing, fleeing, or confrontation of the perceived threat (Öhman, 1986). The process of fear learning, however, involves a form of conditioning in the brain where the association of fear to neutral stimuli allows for the signaling of aversive events. It is often though fear learning in the context of social situations that humans and animals develop social anxiety, aggression, and distrust (Mineka & Zinbarg, 1996). The occurrence of traumatic or emotional experiences, may, as a result, elicit such enduring behaviors as social avoidance, aberrant social interactions, and phobias (Rogan, Staubli, & LeDoux, 1997). Though fear is an innate, basic emotion common among all humans and many animals, subgroups of individuals who exhibit extreme levels of fear-related social anxiety or phobias are often diagnosed for mood, developmental, and social communication disorders. Such individuals exhibiting these behaviors at a level that interferes with their quality of life are commonly diagnosed for clinical anxiety disorders (e.g., social anxiety disorder, panic disorder, post-traumatic stress disorder, social phobia), mood disorders (e.g., depression, bipolar disorder), developmental disorders (e.g., autism spectrum conditions), and social communication disorder (*Diagnostic and statistical manual of mental disorders: DSM-5*, 2013). The importance of understanding how the brain processes fear learning is thus apparent considering the clinical

significance of fear-related social behaviors in the manifestation of mental health and neuropsychiatric conditions.

1.1.2 Fear learning and conditioning

The quintessential paradigm to explore the processes underlying learning in both animals and humans is the Pavlovian model of classical conditioning. The creator of the most well-known paradigm of classical conditioning, Ivan P. Pavlov, first communicated his findings in 1927 through a series of lectures titled: "Conditioned Reflexes: an investigation of the physiological activity of the cerebral cortex" (Pavlov, 1960). In these discussions, Pavlov described a model of conditioning in which he observes the physiological response of dogs to food; Pavlov termed this physiological response to food the "salivary reflex." Today, a cue such as food that naturally produces a response is known as the unconditioned stimulus (UCS). A conditioned stimulus (CS) is one which does not elicit an inborn reflex, and Pavlov observed that, after several presentations of the UCS (e.g., food) paired with the sound of a beating metronome (the CS), salivation would occur upon presentation of this CS alone. Pavlov described this phenomenon as a form of signalization of food via sound, which today is called classical conditioning.

The integration of fearful emotions with classical conditioning, pioneered by Pavlov, is considered a type of associative learning, also known as fear learning, or fear conditioning. In this paradigm, entities formulate an aversive associated response to specific cues or contexts. In such experimental designs, animals are introduced to a novel environment or cue (conditioned stimulus, CS) and presented with an aversive unconditioned stimulus (UCS)—commonly a shock or unpleasant auditory tone. After several trials in which repeated pairings of the CS to UCS facilitate associative learning between the neutral cue/context, the animal will begin to elicit an unconditioned response to the CS in the absence of the UCS. One standard model of classical fear conditioning consists of a paradigm composed of three phases: Habituation, Acquisition, and Extinction. In this protocol, the CS is presented without the UCS in the Habituation Phase, the CS is paired to the UCS in the Acquisition Phase, and the CS presented

without the UCS again in the Extinction Phase (Curzon, Rustay, & Browman, 2009). Through this three-phase paradigm, investigators can subsequently observe for changes in neural, behavioral, or physiological activity during each stage of classical conditioning—from the associative learning of the aversive stimulus to the extinguishing of this learned association.

Traditional models of contextual and cued fear conditioning are commonly practiced on animals to observe for behavioral, neural, and physiological changes during fear learning. These studies are often conducted for translational purposes in order to better understand the neurological, cognitive, and autonomic mechanisms underlying fear processing in humans. Rodent studies employing fear conditioning have helped to elucidate the neuronal circuits and brain regions of greatest influence in fear learning. Such animal models of fear conditioning have also shed light on the molecular processes contributing to learned memory consolidation and retrieval. Human studies that employ classical conditioning tackle the exploration of fear learning from the opposite end of the translational spectrum. Through functional magnetic resonance imaging (fMRI) and psychophysiological studies, researchers examine how individual differences in genetics, gender, and contextual experiences impact the processing of fear in humans. Such studies on fear conditioning in both animal models and humans are increasing in prevalence as public and scientific awareness broadens concerning fear-related social behaviors manifested in many mental health conditions (Delgado, Olsson, & Phelps, 2006).

1.1.3 Neurobiology of fear learning

As described in Section 1.1.1 and 1.1.2, the process through which associative learning formulates a trained response to fearful contexts or stimuli may be a key factor in the development of certain social behaviors. With increased motivation among the scientific community to identify the neural, molecular, and morphological causes of social deficits observed in depression, post-traumatic stress disorder, autism spectrum conditions, and anxiety disorders—an understanding of the neurobiological mechanisms underlying associative fear learning thus emerges as a key topic of interest. Through comprehensive knowledge of the neurocircuitry and molecular factors influencing neural development and plasticity, it will be

possible to approach the identification of molecular targets and therapeutic interventions associated with fear learning more strategically.

1.2 NEUROCIRCUITRY OF FEAR PROCESSING

The neural pathways and mechanisms involved in the processing of fear-related emotions and learning comprises a complex system that draws from multiple regions of the brain and converges at the amygdala—a key area that filters and relays cues important to the learning of fearful contexts and external threats (Hariri & Whalen, 2011; Kim et al., 2011). The amygdala is a key structure within the limbic system of the brain that also includes the hippocampus and hypothalamus, thus influencing the endocrine and autonomic pathways of the nervous system. Composed of several nuclei, the amygdala can be partitioned into the basolateral, medial, and central nuclei, which collaboratively process incoming sensory information for the downstream exhibition of behavioral and physiological responses (Kim et al., 2011). The amygdala can also be considered a core interface for which olfactory, visual, spatial, and cognitive cues are translated into freezing, fleeing, affiliative, and avoidance behaviors that are necessary for survival.

As depicted in **Figure 1.1**, both rodent and human studies have found that the processing of fearful stimuli begins with sensory signaling within areas of the cortex, thalamus, and hippocampus. Regions of the cortex and thalamus relay auditory cues, while the hippocampus transmits visuospatial information related to contextual stimuli and olfactory bulbs transmit scent cues. Somatosensory signals are collected from areas of the insular cortex known as the primary and secondary somatosensory—historically found to be involved in touch-related sensory learning (Friedman, Murray, O'Neill, & Mishkin, 1986; Treede, Kenshalo, Gracely, & Jones, 1999). All sensory information is then transmitted to the basolateral nucleus of the amygdala, which can be further defined into lateral, basal, and accessory basal subnuclei. From the basolateral nucleus, signals are subsequently conveyed via a series of intra-amygdala projections to other regions within the amygdala for further processing and conditioning of the fear-related stimuli. These intra-amygdala projections include pathways between the basolateral to the

central nucleus, lateral to medial nucleus, and lateral to central nucleus. Direct projections from the central nucleus extend to the brainstem and hypothalamus to initiate autonomic and endocrine responses related to cortisol release and the sympathetic nervous system. Pathways from both the prefrontal cortex and central nucleus also direct learned fear behavioral responses such as freezing and fleeing. Neural activities within the basolateral nucleus itself are also theorized to be involved in the learning of avoidance behaviors toward aversive stimuli, as well as fundamental memory consolidation of memories related to fearful stimuli. A structure outside the amygdala known as the bed nucleus of the stria terminalis (BNST) is responsible for unconditioned fear processing and behaviors, and is also considered to be an extension of the amygdala (Kim et al., 2011; Pitkanen, Savander, & LeDoux, 1997; Turner & Zimmer, 1984).

Responses activated via fear-related neural signaling from the amygdala consequently induce endocrine activity consisting of cortisol and corticotropin-releasing hormone (CRH) release into the body—a standard stress response regulated by the hypothalamic-pituitary-adrenal (HPA) axis. Alternate pathways from the amygdala activate the autonomic system, which initiates sympathetic responses via elevated blood pressure, increased heart rate, perspiration, and dilated pupils. Such physiological mechanisms also facilitate the exhibition of fear-related behaviors consisting of freezing, fleeing, and confrontation (Kim et al., 2011; LeDoux, 2003; Maren, 2001; Phelps & LeDoux, 2005; Rosen, 2004).

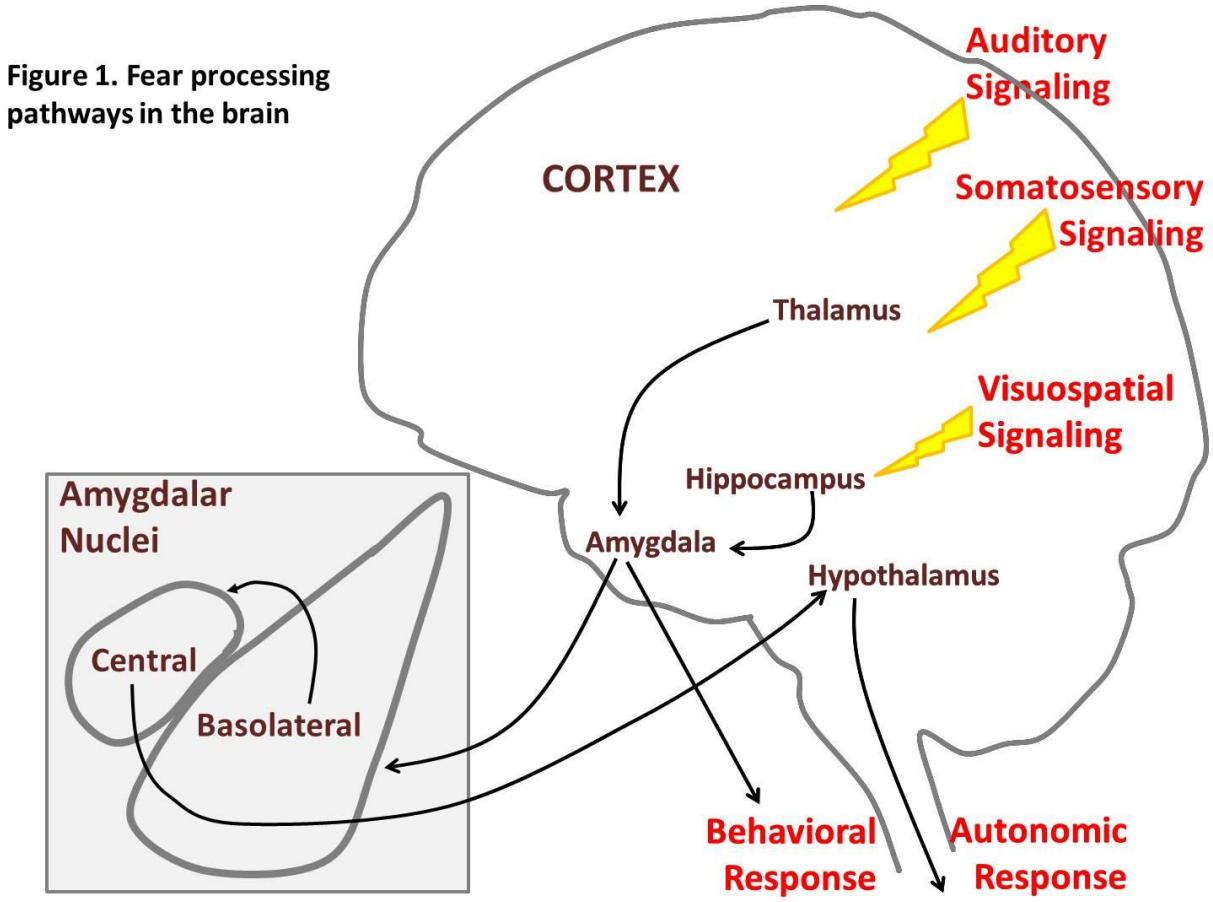

Figure 1.1 Fear processing pathways in the brain. Projections in the brain relay important signaling information from the environment to be processed in the amygdala, which initiates behavioral and neuroendocrine responses.

1.2.1 Role of neuropeptides in the amygdala

It is true that understanding the neurocircuitry involved in fear conditioning helps elucidate the general pathways through which the brain processes fearful stimuli. However, it is the molecular mechanisms underlying the neural activities of these pathways that are important in the identification of biological signatures and markers for the diagnosis and treatment of mood and developmental disorders.

Neuropeptides acting in the amygdala have been increasingly examined in the exploration of neuropsychiatric disorders as research has demonstrated their prominent role in

the development of social behaviors and interactions. Two such peptides are oxytocin and vasopressin. These nine amino acid molecules, also known as nonapeptides, exhibit structurally similar properties and often complementary functions in the brain.

While vasopressin has been tied to memory consolidation, anxiety, and aggression, oxytocin has been strongly linked to social affiliation, pair bonding, anxiolytic effects, and maternal behaviors. Both oxytocin and vasopressin influence the emotional pathways processed in the brain by way of the amygdala. However, oxytocin and vasopressin each have distinct receptor populations in both the hippocampus and amygdala (Huber, Veinante, & Stoop, 2005). It is theorized that with their reciprocal interactions and distinct neuronal populations, oxytocin performs inhibitory actions on vasopressin by way of activation of receptors that turn off vasopressin activity (Huber et al., 2005). Oxytocin (OT) and its receptor (OXTR) have risen as a top neuromodulator of etiological interest as evidence drawn from animal models, genome-wide association studies, and clinical trials have indicated oxytocin's potential role in the regulation of social behaviors related to ASCs. Chapter 2, Section 2.1 will delve into the biological properties and role of oxytocin from its synthesis, release, and reception throughout the body. Other molecular factors active in the amygdala include corticotropin-releasing hormone (CRH) and neuropeptide Y (NPY), which function in the central amygdala to regulate anxiogenic and anxiolytic activities. Through signaling mechanisms initiated by these molecules within the amygdala, it is believed that such activities affect plasticity in synapses, subsequently formulating the networks necessary to consolidate and express fear-related memories and responses. Furthermore, it is hypothesized that variations in oxytocin and vasopressin peptide and receptor expression patterns are fundamentally involved in the development of phenotypic differences exhibited by individuals concerning behavioral responses to social fear, anxiety, and avoidance (Carter, Grippo, Pournajafi-Nazarloo, Ruscio, & Porges, 2008; Donaldson & Young, 2008; Veinante & Freund-Mercier, 1997).

1.2.2 Fear and anxiety and relevant neurobiologial substrates

While fear and anxiety are similar in that both are considered negative sensations induced

by perceptions of threat, there are diagnostic differences between fear and anxiety in the context of emotion processing and elicited behaviors. Fear is defined as a phasic response that is triggered by specific stimuli and lends to stereotypic "fight-or-flight" behaviors upon sensory observation of harmful cues and subsequent neural processing via the hypothalamic-pituitary-adrenal axis. In contrast, anxiety is a sustained response that may not necessarily be initiated as a result of a specific cue. Oftentimes, anxiety is exhibited by feelings of extreme apprehension that may occur pre- or post-stimulus and continue for indefinite lengths of time (regardless of the continued presence of the threatening cue) (Barlow et al., 1996; Quinn and Fanselow 2006; Handbook of emotions).

Neurobiological substrates involved in the processing of fear and anxiety are overlapping, yet distinguished in that both fear and anxiety involve neural pathways intersecting at the amygdala. However, while behaviors induced by fear are triggered by way of signaling from the amygdala to the hypothalamus and brainstem, exhibition of anxiety occurs through release of CRH that has receptors in both within the amygdala as well as in the BNST (which also has projections to the hypothalamus and brainstem) (Davis et al., 2010). Neuroimaging studies of anticipatory anxiety in non-social contexts conducted in humans have demonstrated activation patterns in regions throughout the paralimbic system (Chua et al., 1998) and rodent studies have further distinguished the BNST as a key area distinguishing transient from sustained fear (which may be interpreted to be related to anxiety-like emotions in humans) (Davis et al., 2010). While the prefrontal cortex of the brain is strongly associated to elicitation of fear, the ventromedial PFC (vmPFC) appears to have a specific role in regulating anxiety.

These studies demonstrate variations that exist between the neural responses that underlie fear and anxiety. Still, both states are induced by amygdalar response. This thesis will not dissect the nuances of acute social fear in contrast to social anxiety, but will consider the elevated amygdalar response involved in both.

1.3 CLINICAL RELEVANCE OF RESEARCH ON SOCIAL FEAR PROCESSING

As discussed in section "1.1.1: Implications of Fear in Social Behaviors," the process through which fear is contextually associated with social stimuli and manifested through extreme emotional states can have potentially life-changing consequences to individuals' mental health and wellbeing. Such anxiety disorders as panic disorder, post-traumatic stress disorder (PTSD), and social phobia are commonly characterized by long-term bouts of excessive feelings of fear when exposed to specific social environments or more general social situations (*Diagnostic and statistical manual of mental disorders: DSM-5*, 2013). Depression, estimated to affect approximately 8% of the adult population in the United States ((CDC), 2015), and other mood disorders commonly co-occur with anxiety disorders. In addition to mood and social anxiety disorders, autism spectrum conditions (ASC) and social communication disorders are two developmental conditions broadly characterized by their deficits in social communication and interactions (*Diagnostic and statistical manual of mental disorders: DSM-5*, 2013). Multiple studies have cited varying reports of the prevalence of anxiety disorders among adolescents and young adults with autism, with some observing rates of 30% (Simonoff et al., 2008) to over 80% (Muris, Steerneman, Merckelbach, Holdrinet, & Meesters, 1998). With growing evidence of the prevalence of anxiety, social phobia, and aggression in individuals with ASC, it is increasingly important to families and to the scientific community to determine the cause of comorbid conditions of ASC and learn how to treat associated symptoms.

Observations of these social phenotypes in ASC, mood disorders, and social anxiety disorders have prompted investigators to study the underlying cognitive processes that may be responsible for the development of these deficits in emotional behaviors. Scientists and clinicians alike hope that a greater fundamental knowledge of the biological, genetic, and neural mechanisms will identify biological markers that will improve the future of diagnostics and facilitate the design of therapeutic interventions or treatments. This public health motivation thus reinforces the importance of exploring fear-related emotion processing and learning as a first step towards pursuing clinical targets for social symptoms of ASC, mood disorders, and social anxiety disorders.

1.3.1 Autism spectrum conditions (ASC) and fear processing

As headlines of increasing reports of ASC prompts greater public awareness and concern over rising autism incidence ((CDC), 2014), scientific discrimination of the heterogeneity of social symptoms observed in individuals with ASC has further defined its clinical profile . Within the core trio of behavioral pathologies employed in the DSM-IV to diagnose ASC (i.e., deficits in social communication, social interactions, and repetitive behaviors)—child psychiatrists are also noting differences in social anxiety and phobias among adolescents and adults with ASC. In a study conducted by Kuusiko *et al*., investigators observed increased social anxiety and avoidance behaviors with age in high-functioning adults with ASC and Asperger syndrome (Kuusikko et al., 2008). In another study examining comorbid psychiatric disorders in adolescents with ASC aged 10-14 years, it was discovered that the most common co-occurring conditions are social anxiety disorder, attention-deficit hyperactivity disorder, and oppositional defiant disorder (Simonoff et al., 2008). Intriguingly, first-degree relatives of individuals with ASC have also been found to demonstrate elevated levels of depression and social phobias (Smalley, McCracken, & Tanguay, 1995), pointing to a link between emotional behaviors and heritable genetic factors. These clinical observations relating ASC to social anxiety, phobia, and depression have thus prompted basic science researchers to explore the molecular mechanisms influencing manifestation of these conditions. In a valproic acid (VPA) rat model of autism, rodents treated with the anti-convulsant (suspected to be a potential cause of ASC in humans) demonstrated abnormal fear conditioning and differential processing in the amygdala (Markram, Rinaldi, La Mendola, Sandi, & Markram, 2008). In a study translating such findings in animal models of ASC to humans, classical fear conditioning conducted in a non-social context indicated differences in individuals with Asperger syndrome relative to age-matched neurotypical participants (Gaigg & Bowler, 2007). [Note: The term "neurotypical" is a broadly accepted term that describes individuals not on the autism spectrum]

As indicated by the aforementioned studies, the clinical observation of comorbid conditions like social anxiety, depression, and phobia among individuals with ASC has prompted scientific interest in determining how fundamental processes in fear learning and conditioning

may lead to the development of such psychiatric conditions in patients with autism. One such approach into exploring the realm of fear processing in the context of social anxiety involves the amygdala theory of autism, first proposed by Simon Baron-Cohen in 2000 (S. Baron-Cohen et al., 2000). The amygdala theory of autism suggests that the amygdala may be one of several key areas of abnormality and a possible underlying source of social deficits exhibited in ASC. This theory was developed from a literature review of the existing evidence denoting amygdala deficits in individuals with ASC. This evidence, in conjunction with an fMRI study employing the Reading of the Mind in the Eyes Test (which found that individuals with ASC exhibited decreased activation in the amygdala in comparison to that of control groups), all point to a central theme concerning abnormal neural processing within the amygdala among individuals with ASC.

While the role of the amygdala in fear learning has been well-established in scientific literature since the 1930s (Kluver & Bucy, 1939), precisely how the amygdala is involved in the development of fear-related social deficits is not well understood.

1.3.2 Role of oxytocin in ASC: genetic variation as a contributor to individual differences in fear-related behavioral phenotypes

As discussed in section "1.2.1: The role of neuropeptides in the amygdala," such neuropeptides as oxytocin and vasopressin activate key receptors in the amygdala to regulate behavioral responses associated with anxiety, social interactions, and aggression. The amygdala theory of autism ties amygdala morphology, activity, and development to the occurrence of social deficits associated with ASC. Therefore, understanding the role of signaling molecules within the amygdala is important to the future identification of biological signatures and design of therapeutic targets for ASC subgroups. Oxytocin is a key molecule of interest in ASC research as genetic studies and clinical observations demonstrate its involvement in processes concerning social cognition and behavioral responses in humans (Kirsch et al., 2005). Moreover, nonclinical evidence of oxytocin's specific role in regulating social affiliation and memory, as well as fear processing, in the amygdala in animals (Calcagnoli et al., 2015; Ferguson, Aldag, Insel, & Young, 2001) point to a molecular basis of neuromodulation that may have parallel ties

to expression of social anxiety in humans with ASC.

Oxytocin and its receptor genes have also been linked to ASC in several candidate gene association studies in adult populations (Jacob et al., 2007; Wu et al., 2005). Animal models of autism employing gene knockout mice lacking oxytocin in the brain have been observed to exhibit aberrant social behaviors including deficits in social recognition (Ferguson et al., 2001). The social deficits observed in mice are also specifically localized to activity of oxytocin within the amygdala (Ferguson et al., 2001; Michael Lukas, Toth, Veenema, & Neumann, 2013). Functional activity observed in humans during emotional social processing has demonstrated oxytocin-regulated activity within regions of the amygdala (Domes, Heinrichs, Glascher, et al., 2007; Petrovic, Kalisch, Singer, & Dolan, 2008). In adults with Asperger Syndrome, it was observed that intranasal administration of oxytocin improved social cognitive performance (Hollander et al., 2007). Moreover, in high-functioning individuals with ASC, oxytocin administration also increased social interactions (Andari et al., 2010).

The relationship between oxytocin and social behaviors in the amygdala has been extended to include other aspects of social cognition, such as social processing and fear in neurotypical adults (Kirsch et al., 2005). Oxytocin has also been observed to enhance such social skills as "mind-reading", or feelings of empathy, in neurotypical adults (Domes, Heinrichs, Michel, Berger, & Herpertz, 2007). Furthermore, researchers have noted that oxytocin may increase one's tendency to gaze into the eyes of others, as demonstrated by one study conducted in neurotypical adults (Guastella, Mitchell, & Dadds, 2008). As for mood disorders, it has been determined that in generalized anxiety disorder, individuals demonstrate increased functional activity within the amygdala when presented with fear-inducing social images (Labuschagne et al., 2010). This activity was subsequently found to decrease upon intranasal administration of oxytocin. Social memory has also been shown to be improved via administration of oxytocin in humans (Savaskan, Ehrhardt, Schulz, Walter, & Schachinger, 2008) and animal models (Ferguson et al., 2001).

1.3.3 Applications of translational research in animal models

Animal models are an important means through which scientists probe neuropsychiatric disorders in research. Numerous rodent models have been developed via genetic manipulation of target genes that have been identified in genetic disorders, or manifest similar phenotypes to those observed in human patients suffering from mental health issues. Just as many neuropsychiatric disorders such as fragile X syndrome have specific genetic causes, ASC has demonstrated a strong hereditary influence (Sandin et al., 2014) that may be drawn from numerous inherited and *de novo* (spontaneously arising) mutations from multiple genes that collaboratively confer risk (Li, Zou, & Brown, 2012; Sanders et al., 2012). While animal models typically cannot exhibit the comprehensive array of symptoms manifested in human patients of neuropsychiatric disorders, animal models are an invaluable resource for exploring molecular, cellular, neuroanatomical, and functional mechanisms underlying the development of the behavioral, physical, and social deficits observed in many patients.

With familial genetic factors estimated in latest findings to contribute approximately half the risk of developing autism (Sandin et al., 2014), animals naturally emerge as candidate models for the further exploration of how genetic risk factors may lead to the development of symptoms in autism. While the majority of autism cases are considered non-syndromic forms of autism (not attributed to any single gene), more gene variants have emerged in genetics studies linked to non-syndromic autism. How these gene variants actually perturb developmental networks to produce autism phenotypes, however, is still a largely undefined phenomenon.

With genetic manipulation in mice, scientists can explore the behavior of these linked gene variants in their integrated networks and attempt to make sense of how such pathways behave. While human brain imaging studies have hinted at potential physiological evidence of differences in cortical organization and morphology in the brains of autism patients (Stoner et al., 2014), it is only in animal studies that highly invasive analytical techniques may be employed to longitudinally examine the specific mechanisms of precise interactions in neural development. While the range of autism phenotypes is a broad and diverse spectrum, the composite base of knowledge from the many "mouse models of autism," each aiming to manifest some aspect of the morphological, behavioral, or neural traits associated with autism, is necessary in the

discovery of diagnostic markers and therapeutic targets.

With the latest edition of the DSM-5 seeking to better define autism spectrum conditions despite the ambiguities that the diverse phenotypes present in patients, there arises a growing need in the scientific community for better biological markers to be identified—as demonstrated by the latest movement by the National Institute of Mental Health (Dept. of Health and Human Services, USA) to promote a better system of mental health disorders classification based on biological markers and established behavioral measures (Research Domain Criteria, RDoC). Finally, the animal model in mental health research is an invaluable means of developing and testing therapeutic strategies. With increasing acknowledgement of identified comorbidities associated with autism (e.g., sleep disorders, epilepsy, anxiety, depression) there has been much public interest in better understanding the connection of these comorbidities to autism and directly treating these symptoms.

There are acknowledged drawbacks to animal models in mental health research—namely the inability to find any single animal model that fully recapitulates the full range of behavioral and physiological phenotypes associated with autism. Moreover, attempting to translate animal behavior to humans, particularly in the cognitive and social contexts, is a controversial and subjective issue that is still the center of much scientific debate. The lack of higher-level "human" social behaviors and cognitive processes in animals makes such models prone to misinterpretation and mistranslation of results to humans—presenting a challenge in leaping the pre-clinical divide into the realm of early-stage human studies. While these caveats in employing animal models in researching mental health disorders presents significant challenges to the translation of results from animal studies to humans, with proper understanding of their limitations, animal models remain crucial to better understanding the precise neural mechanisms and developmental processes underlying such complex conditions as ASC.

1.4 THESIS EXPERIMENTAL DESIGN AND OBJECTIVES

Goals of this thesis include the following objectives:

1) Examine the role of oxytocin in the expression of fearful emotions in a social context. How does oxytocin mediate fear responses in social contexts in mice at the neural, behavioral, physiological levels? [Chapter 2 (Behavioral), Chapter 3 (Neural), Chapter 4 (Physiological)]
2) Assess the psychophysiological effects of fear when presented in social contexts in high-functioning adults with autism spectrum conditions (ASCs). [Chapter 5]
3) Evaluate the translational aspects of research examining behavioral and mood disorders in animal models. How do physiological results from the oxytocin receptor gene (*Oxtr*) knockout (KO) mouse model compare to that drawn from human ASC cases? [Chapter 6]

To explore these objectives, parallel studies conducted in both animals and humans were performed in collaborating laboratories at the National Institutes of Mental Health (NIH, DHHS, USA), and the Autism Research Centre (Department of Psychiatry, University of Cambridge, UK). Each of the studies conducted in mouse models and human subjects were designed with a translational approach in mind so that findings would not only answer questions pertinent to better understanding social emotion processing from both animal model and clinical viewpoints, but to also observe how generalizable basic research is to human patients when considering behavioral and emotional processes.

1.4.1 Research aims, methodological approach, and outcomes

The techniques employed in this thesis to explore the objectives (1) and (3) in an *Oxtr* KO mouse model consist of manganese-enhanced magnetic resonance imaging (MEMRI), behavioral observation, and telemetry acquisition. The technique utilized to assess objectives (2) and (3) in ASC cases and control participants consisted of skin conductance response (SCR) measurement. The thesis concludes with a discussion of the results in relation to the objectives posed, and identifies areas of future interest in the continued exploration of emotional processing in humans and animals.

1.5 SUMMARY

While far-reaching in its impact on human behavior and cognition, social emotion processing can be tied to several specific structures within the brain and therefore specific physiological pathways that combine to determine the complex cognitive and autonomic interactions that direct how individuals behave in social contexts. With its serious implications for the ability to socially communicate effectively, and the prevalence of social anxiety disorders affecting autism patients with known social deficits, it is crucial that a better understanding of social emotion processing be pursued to determine what biological markers and therapeutic targets may one day improve the lives of individuals suffering from debilitating social phobias or with difficulty communicating socially. This thesis presents a dual-method approach conducted in both a genetic knockout mouse model and human participants with autism to explore the behavioral and physiological effects of social fear processing. With the data collected in this dissertation research, the findings will provide much needed insight into the molecular underpinnings of social fear processing in relation to specific behavioral phenotypes, while demonstrating the translational applications of basic research through comparison to a clinical psychology study designed in parallel. The following three sections, Chapters 2-4, of this dissertation delineate the tri-part study conducted in a mouse *Oxtr* knockout model to explore the behavioral, neural, and physiological effects of social defeat. Chapter 5 describes data collected from a human sample of neurotypical individuals and participants diagnosed with ASC where physiological data was gathered during a social fear conditioning paradigm.

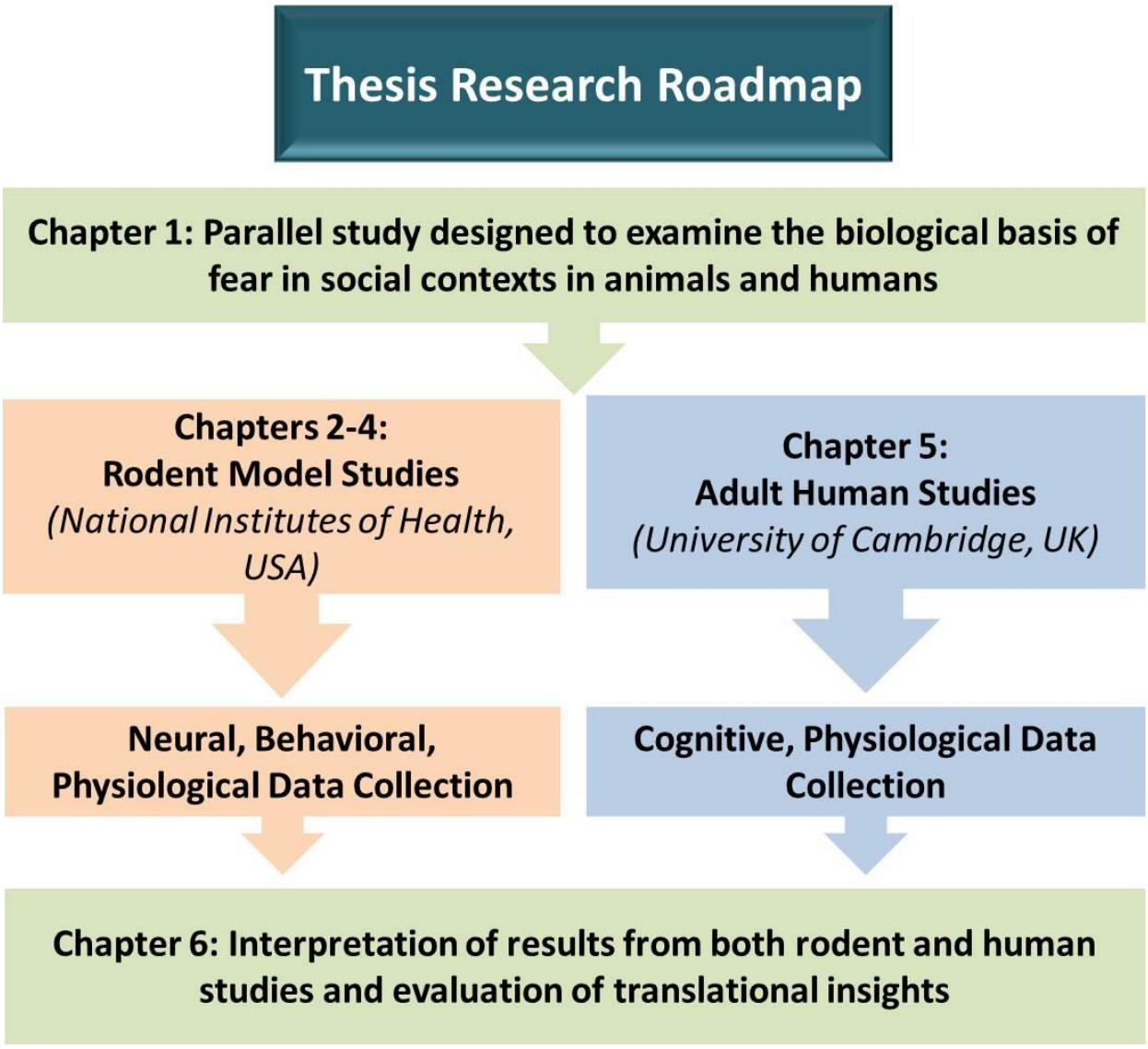

Figure 1.2 Thesis research roadmap. Flowchart depicting the structure of this dissertation by chapter and experimental topic. Research was designed to incorporate analogous data collection methods in both rodent models and human participants.

Chapter 2: Behavioral response to social stress in an oxytocin receptor gene knockout mouse model

2.1 *OXTR* KNOCKOUT MOUSE AS A MODEL TO ASSESS THE ROLE OF GENETIC VARIATION IN THE DEVELOPMENT OF BEHAVIORAL PHENOTYPES ASSOCIATED WITH SOCIAL FEAR PROCESSING

Defined by the American Psychiatric Association DSM-5, autism spectrum condition (ASC) is diagnosed early in development upon observation of a series of impairments that consist of repetitive behaviors and deficits in verbal communication and social interactions (*Diagnostic and statistical manual of mental disorders: DSM-5*, 2013). As scientific exploration into the etiological and pathophysiological factors associated with ASC advances, animal models, genome-wide association studies, and clinical trials have promoted much interest in the nonapeptide oxytocin (OT) and its receptor (OXTR). Oxytocin is a nine amino acid peptide produced in the paraventricular (PVN) and supraoptic nuclei (SON) of the hypothalamus. Peripheral release of oxytocin from the posterior pituitary plays a role in parturition via the regulation of uterine contractions and lactation. More recently, it is hypothesized that central release of oxytocin in the amygdala may contribute to distinct social and aggressive behaviors (Knobloch et al., 2012; M. Lukas et al., 2011; Veinante & Freund-Mercier, 1997; Viviani et al., 2011). Such studies describe oxytocin to be a neuromodulator involved in the mediation of aggression, anxiolytic stress response, and maternal behaviors (Consiglio, Borsoi, Pereira, & Lucion, 2005; Labuschagne et al., 2010; Pagani, Lee, & Young, 2011). Rodent studies have demonstrated oxytocin, broadly referred to as the "prosocial hormone," to be influential in pair bonding and social recognition (Insel & Hulihan, 1995; Macbeth, Lee, Edds, & Young, 2009). Furthermore, translational research in humans conducted via genome-wide association studies (GWAS) and clinical intranasal hormone administration have revealed that oxytocin and its receptor are involved with performance in social memory, trust, social cognition, and autism diagnostic measures (Anagnostou et al., 2012; Chakrabarti et al., 2009).

Oxytocin receptors are expressed prominently in the amygdala (Gould & Zingg, 2003; Veinante & Freund-Mercier, 1997), a key activator of the hypothalamic-pituitary-adrenal (HPA) axis, and research has indicated that oxytocin transmission imparts an anxiolytic effect during stress response (Knobloch et al., 2012). Evidence in humans of fear-induced amygdala

activation and aggressive behaviors in rodent oxytocin models delineate an emotional pathway composed of oxytocin signaling, amygdala activity, and social processing (Gamer & Buchel, 2009; Kleinhans et al., 2010; Mak, Broussard, Vacy, & Broadbear, 2012; Pagani et al., 2011). Comprehensive functional observation, however, of oxytocin receptor-dependent signaling in the amygdala and its impact on the expression of mammalian social behaviors is still a vastly undefined mechanism. With recent advancements in imaging technologies, the evaluation of mammalian functional activity in the brain during the processing of social behaviors is now possible.

2.2 SOCIAL DEFEAT AS A PARADIGM OF SOCIAL FEAR BEHAVIOR IN MICE

Research has created an expansive base of scientific literature describing the established phenotypes observed in animals after exposure to social stress. Such phenotypes include signs of depression, anxiety, decreased appetite, lower activity levels, and social avoidance. The expression of such symptoms by rodents within the laboratory is achieved by traditional methods of social defeat that induce physiological and behavioral responses, which elicit analogous symptoms to those observed in human mood disorders (Meerlo, Overkamp, & Koolhaas, 1997; Ruis et al., 1999). The standardized resident-intruder paradigm test for aggression is the most widely used method conducted on rodents that is utilized to not only evaluate aggressive behaviors, but also submission, social anxiety, depression, social affiliation, and social recognition (Koolhaas et al., 2013). As depicted in **Figure 2.1**, the Resident-Intruder paradigm consists of dual housing strategies that assist in establishing the resident and intruder roles of the involved rodent subjects. Phase 1 depicts a resident male housed with an ovariectomized female (female mice housed with intact males are required to first undergo removal of ovaries to prevent any influence of the female's estrous cycle on male behavior) for at least 2 weeks prior to behavioral testing. Alternatively, resident males may also be singly housed for at least two weeks if female companions are not available, which is the housing strategy employed in this thesis experimental protocol. Phase 2 displays the timed exposure of the resident male to the intruder male (who has been group housed with same sex littermates prior to testing) during the

dark phase of the light cycle. Behaviors are scored during the timed exposure in Phase 3 of the paradigm and animals are returned to their respective housing environments post-testing.

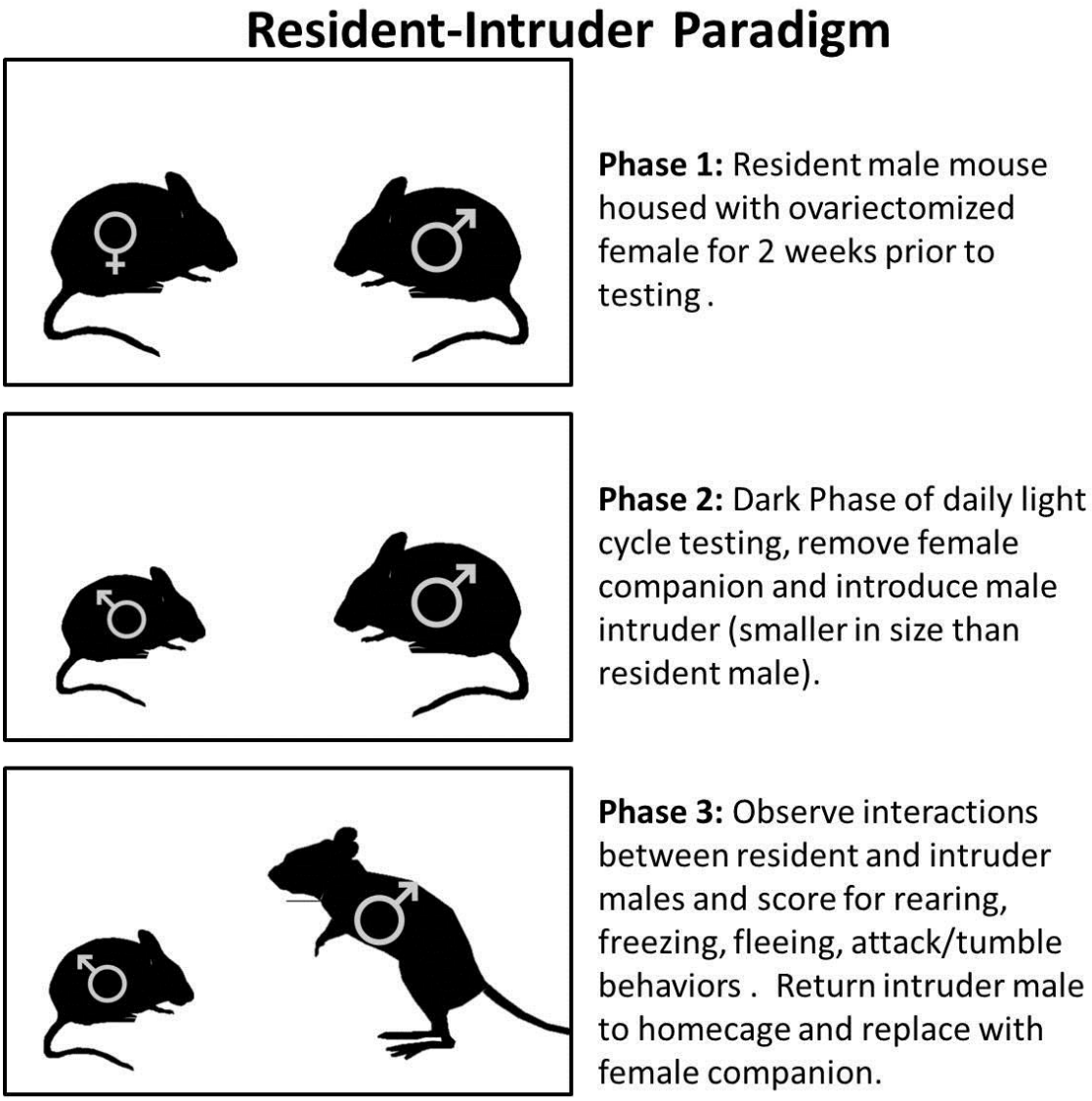

Figure 2.1 Resident-intruder paradigm

Evidence from literature examining the neurobiological mechanisms underlying social behaviors have linked oxytocin directly to pro-social behaviors and consequently social

avoidance and phobias. Using oxytocin antagonists in rats and mice, researchers have been able to observe the effects of depressed oxytocin activity in the brain on attenuation of social approach and social exploration behaviors, which could be rescued with direct injection of synthetic oxytocin in the brain. Furthermore, researchers of this study distinguished the diminished social approach effect, a potential indicator of social anxiety, to not translate over to non-social contexts as demonstrated by normal behaviors during the elevated plus maze test (Lukas et al.. 2011). Due to oxytocin's implications on the exhibition of anxiety-like behaviors in social contexts during social approach tasks, it is thus an objective of this thesis to examine if absence of oxytocin similarly results in enhanced social anxiety in situations of social aggression.

2.3 BEHAVIORAL DIFFERENCES DURING REPEATED SOCIAL DEFEAT IN *OXTR* KO AND WT MICE

In the translational approach presented in this thesis, repeated social defeat is employed as a paradigm to explore the effects of social stress on a transgenic mouse model with known social deficits that is genetically modified to lack oxytocin receptor proteins. Oxytocin has been associated directly with behaviors in affiliation and pair bonding across different species of animal models (i.e., finches, voles, sheep, rats, mice) (Young & Wang, 2004).

Scientific discovery within animal models of the regulatory effects of oxytocin in both endocrine and neural systems has prompted much interest in further understanding oxytocin's role within the human brain. Such research has led to clinical application of oxytocin in human subjects to observe for its effects on human cognition, emotion, and affiliative behaviors with conspecifics (Hollander et al., 2007; Kirsch et al., 2005; Kosfeld, Heinrichs, Zak, Fischbacher, & Fehr, 2005).

2.3.1 Objectives

This experiment sought to determine the behavioral outcomes following repeated exposure to social defeat as a paradigm for simulating social anxiety in a mouse model with a global knockout of the oxytocin receptor gene. With oxytocin implicated in regulating a series of social behaviors including trust, affiliation, and aggression, this research was designed to examine how oxytocin is related to social stress and anxiety from behavioral, neural, and physiological perspectives within an animal model. This chapter describes the behavioral outcomes of repeated social defeat stress on *Oxtr* KO and wildtype (WT) mice.

2.3.2 Hypothesis

In the behavioral study conducted on *Oxtr* KO and WT animals, it is hypothesized that oxytocin receptor knockout mice will demonstrate different behavioral outcomes compared to those of wild type mice when exposed to repeated incidents of social defeat stress. The *Oxtr* KO mice used in this study show elimination of oxytocin binding in the Oxtr-rich brain areas of amygdala, anterior olfactory nucleus, cerebral cortex, hippocampal formation, lateral septum, medial amygdala, olfactory bulb, piriform cortex, and ventral pallidum (Lee, HJ et al., 2008). Not only are oxytocin receptors expressed in regions of the brain linked to aggression and social behaviors, but their presence in amygdala is linked to social recognition and social avoidance—of which the latter behavior may be related to exhibition of submission during social defeat. (Ferguson et al., 2001). It is therefore hypothesized that the reduced Oxtr expression in limbic circuitry of *Oxtr* KO mice willlead to higher levels of social anxiety-like behaviors during repeated social defeat exposures, manifested as increased exhibition of submissive behaviors compared to that of WT mice during social defeat trials with more aggressive conspecific animals.

2.3.3 Methods

Generation of the Oxtr knockout: *Oxtr* KO (C57BL/6J background) mice were generated by first developing a line of mice floxed for the Oxtr allele (Otr $^{+/flox}$), A male Oxtr+/flox mouse containing germ cell expression of Cre recominbinase (Oxtr$^{+/flox,cre}$) was crossed with homozygous floxed females (Oxtr$^{flox/flox}$), which produced heterozygous offspring containing one inactive Oxtr allele (Oxtr$^{+/-}$). These mice were crossed with each other to produce homozygous total Oxtr knockout mice (Oxtr$^{-/-}$). Oxtr-/- animals utilized in this study were genotyped via PCR prior to behavioral testing and imaging. For a more detailed discussion of the methods utilized in the laboratory to generate and genotype Oxtr KO mice, see Lee *et al*. (H.-J. Lee, Caldwell, Macbeth, Tolu, & Young, 2008).

Animal care and handling: Mice were individually housed and provided food and water ad libitum throughout the duration of the infusion treatments. The physical health and activity of all treated animals were assessed daily by animal care staff and veterinarians of the National Institute of Mental Health (NIMH) small animal facility. The NIMH Animal Care and Use Committee (ACUC) approved all animals and methods utilized in this study. Resident mice consisted of male, age-matched Swiss Webster (SW) mice [purchased from Jackson Laboratories©] singly housed for 2 weeks and screened for aggressive behaviors (screening was conducted by exposing singly housed SW mice to group housed WT animals not used in the study cohorts and observing for social defeat of the WT animals within 5 minutes of exposure). All subject *Oxtr* KO and WT mice were group housed with same sex littermates throughout the duration of the study. All social defeat exposures were conducted during dark cycle hours and video recorded for post-testing blinded scoring of behaviors via Noldus Observer XT.

Social Defeat Testing*: Oxtr* KO and WT adult males (C57BL/6J background, ~22–30 g, ~90-100 days old, n = 10 per cohort) experienced daily social defeat exposures for three consecutive days [n=10 per genotype per cohort; 2 cohorts of animals tested—the experimental cohort was socially defeated while the control cohort was sham handled]. The behavioral paradigm was based on a resident-intruder protocol in which *Oxtr* KO and WT animals were introduced for 30

minutes into a resident animal's homecage. The social defeat protocol consisted of a 10 minute habituation period where *Oxtr* KO and WT animals roamed freely in their homecages. Habituation was followed by a 30 minute resident-intruder exposure where *Oxtr* KO or WT subjects were introduced into the homecage of a resident Swiss Webster male. Both body temperature and mobility measurements were collected during the habituation and social defeat phases of the procedure. Following the conclusion of the 30 minute social defeat exposure, subject mice were examined for any physical injuries and returned to their homecages. Subject mice were introduced to a different aggressive resident animal on each day of their 3-day social defeat time course. An investigator monitored all behavioral procedures in real-time and intervened immediately upon any observation of physical harm between the subject animal and the aggressor. Control groups were not socially defeated during the 3-day paradigm, but sham handled similarly to the socially defeated groups during the dark cycle hours of each day. See **Figure 2.2** for a general timeline demonstrating the process of behavioral testing that was utilized in this study. As shown in **Figure 2.2**, a 3-day social defeat paradigm was flanked by surgical implantation of the telemetry monitoring device and the mini-osmotic infusion pump (filled with a manganese chloride solution). Behavioral data was recorded, blinded, and scored post-social defeat sessions using Noldus Observer XT. After behavioral testing was concluded, a final MRI scan was conducted for each animal.

Figure 2.2 Implantation and behavioral testing timeline: Following implantation of the telemetry E-mitter and the mini-osmotic manganese infusion pump, a 3-day Social Defeat conditioning paradigm was conducted and followed by a final MRI scan.

2.3.4 Results

Oxtr KO mice are more susceptible to social stress: Evaluated behaviors during social defeat included submissive postures (scored as fleeing and freezing behaviors), defensive postures (scored as rearing and tumbling behaviors), and neutral behaviors (scored explorative and grooming behaviors), as previously characterized for this paradigm. (Miczek, 1979; Kabbaj *et al*, 2001). Behavioral data was scored from blinded pre-recorded samples using Noldus Observer XT where time spent performing each of the aforementioned behavioral tasks was quantified retrospectively from videotaped social defeat sessions (a subset of recorded observations were blinded and scored by two investigators. Quantified behaviors observed between the two evaluators were not statistically different [data not shown]; the subsequent recorded observations were scored by a single investigator due to resource and time constraints]. Results indicated that *Oxtr* KO animals exhibited a significantly greater fraction of time performing fleeing and freezing behaviors (interpreted in this study as potential measures of "submissive postures") over the composite 3 days of social defeat compared to that observed from socially defeated *Oxtr* WT animals [independent samples t-test indicated significant difference in the time spent exhibiting submissive postures between *Oxtr* KO (M=0.58, SD=0.07) and WT mice (M=0.37, SD=0.08); t(18)=3.80, p=0.004; **Figure 2.3**]. Defensive postures (i.e., rearing/tumbling) between the *Oxtr* KO and WT animals were not statistically different [independent samples t-test did not indicate significant differences in the time spent exhibiting defensive postures between Oxtr KO (M=0.21, SD=0.09) and WT mice (M=0.22, SD=0.08); t(18)=-0.21, p=0.83; **Figure 2.3**] indicating that both groups encountered similar levels of aggression during their social defeat encounters [**Figure 2.3**].

Aside from submissive and defensive postures, the remaining behaviors exhibited by animals consisted of exploratory and grooming behaviors, which were considered "neutral-like behaviors" for the purposes of this social defeat study. These behaviors were exhibited for the remaining fraction of time that each animal was not directly interacting with the aggressor (i.e., tumbling and defensive posturing) or exhibiting submissive behaviors (i.e., fleeing and freezing)

and thus Oxtr KO (M=0.21, SD=0.08) significantly differed in the exhibition of neutral behaviors from WT mice (M=041, SD=0.07), t(18)=3.80, p=0.004).

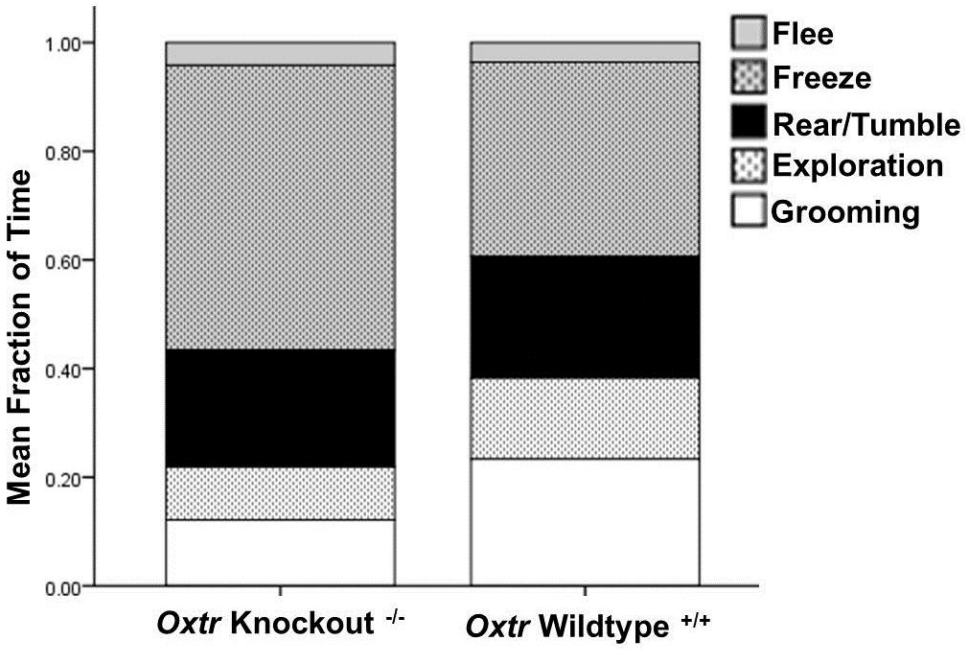

Figure 2.3 Behaviors Exhibited During Social Defeat Testing: *Oxtr* KO mice expressed significantly more time exhibiting submissive behaviors compared to that of WT mice.

2.4 SUMMARY

Behavioral observation of *Oxtr* KO and WT male mice indicated intriguing differences when test subjects were evaluated for exhibition of submissive behaviors during daily social defeat via the Resident-Intruder Paradigm. Results indicated that *Oxtr* KO male mice act more submissively than WT animals when socially interacting with a more aggressive conspecific despite exposure to similar levels of social aggression. This elicitation of a more submissive phenotype potentially indicates a difference in emotional fear processing that may be related to the absence of oxytocin receptors in the brain.

Existing scientific literature on the relationship between oxytocin and fear-related social behaviors in mice have produced an array of findings ranging from evidence that *Oxtr* KO mice demonstrate *increased* aggression (Dhakar, Rich, Reno, Lee, & Caldwell, 2012)—to observations that direct injections of oxytocin into the central nucleus of the amygdala *decreases* aggression in rats (Calcagnoli et al., 2015). Other research exploring social behaviors and oxytocin in rodent models have found that *Oxtr* KO mice demonstrate impaired social recognition or other social deficits, but *not* increased aggression (M. Sala et al., 2013).

When this dissertation's findings are considered in relation to such existing research, the elicitation of increased submissive behaviors by *Oxtr* KO mice may be interpreted as a type of anxiety-induced behavioral response to social stress. Rather than behaving more aggressive to the combative conspecific (as would be expected from the literature), *Oxtr* KO mice instead acted more submissively than their WT counterparts due to their heightened anxiety when placed at a physical and territorial disadvantage. It is possible that in an alternate behavioral context, these *Oxtr* KO mice may have acted more aggressively if placed in a different paradigm that minimized anxiogenic factors. It should also be considered what role oxytocin plays in the distinct behaviors elicited during the social defeat paradigm. While absence of oxytocin in the Oxtr KO mice resulted in both fleeing and freezing behaviors in socially defeated mice, it is worth considering whether the diminished exploratory and grooming behaviors are directly suppressed by absence of oxytocin or whether such behaviors were not as frequently exhibited due to the overwhelming motivation to spend time fleeing and freezing in such animals. Thus, further experiments specifically examining the effects of oxytocin on individual behaviors observed in this behavioral paradigm are necessary.

With sources citing the potential anxiolytic effects of oxytocin in mammals (Knobloch et al., 2012), this study's findings that *Oxtr* KO mice demonstrate increased submissive postures may be an indicator of increased social anxiety due to the absence of oxytocin signaling in the brain. However, this hypothesis connecting oxytocin activity to social stress regulation is limited by the assumption that submission is a phenotypic marker of socio-emotional anxiety in the mouse. Further exploration is needed to better understand the limbic activation experienced in

mice when exhibiting submissive behaviors in social situations. It would also be important to verify whether submissive behaviors during social defeat is a marker of anxiety through observations of socially defeated Oxtr KO mice (who exhibited increased submission) via commonly used behavioral tests of anxiety (e.g., elevated plus maze, light/dark exploration, and social interaction test). The social defeat experiments presented in this chapter do not rule out the possibility that amygdalar oxytocin signaling modulates anxiety independently of social variables. However, the well-supported role of oxytocin in bonding and affiliative behaviors, considered in conjunction with its anxiolytic profile, supports a unique role for oxytocin in socially-induced anxiety, perhaps through modulation of the salience of social cues (Shamay-Tsoory et al). While such behavioral motivations of Oxtr KO mice remain to be individually characterized, these results underline the importance of OXTR function to social interaction.

CHAPTER 3: Functional imaging of neural activity during social defeat via manganese-enhanced MRI in mice

3.1 PRINCIPLES OF MEMRI TECHNOLOGY AND APPLICATIONS

Magnetic resonance imaging (MRI) has emerged as a major non-invasive technique to visualize the brain in both animal and clinical studies. Harnessing the physical properties and interactions of some atomic nuclei via the application of an external magnetic field, MRI provides structural and functional data that can be examined in vivo longitudinally (Budinger, Benaron, & Koretsky, 1999; Farrall, 2006). Such uses have promoted MRI as a popular method to observe anatomical and functional characteristics of the brain; particularly, regarding transgenic animal studies of disease.

Despite MRI's ability to provide images with spatial resolutions of <0.2 mm, there has been much recent interest in contrast agents to enhance the observation of morphological structures and functional activity in the brain (Budinger et al., 1999). These agents include paramagnetic metals such as gadolinium and manganese (Caravan, Ellison, McMurry, & Lauffer, 1999) (Schwert, Davies, & Richardson, 2002). Manganese-enhanced MRI (MEMRI) is becoming an increasingly popular means of imaging animals due to the ability of Mn^{2+} to enter neurons via voltage-gated calcium ion channels. Upon depolarization, these voltage-dependent calcium channels permit the entry of Mn^{2+} due to the ion's similar ionic radius to that of Ca^{2+} (Kuo, Herlihy, So, & Bell, 2006; Lin & Koretsky, 1997; Yu, Wadghiri, Sanes, & Turnbull, 2005). Thus, Mn^{+2} uptake indicates direct activity in the brain, which is observed in MRI as enhanced signal intensity proportionate to the frequency of neuron depolarization. This method of activity-dependent imaging differs from traditional methods of functional MRI that interpret activation as a function of hemodynamic fluctuations in oxygen concentration. The limitations of blood oxygenation level-dependent (BOLD) fMRI are apparent in its requirement that animals be immobilized completely during stimulation and functional activity acquisition. Subsequently, extended periods of physical restraint in a low-temperature, loud magnet bore preclude the expression of many natural behaviors exhibited typically in the animal's normal environment (Ferris et al., 2008).

The combination of manganese's ionic properties in biological systems, its physical ability to shorten T_1-relaxation times (thus enhancing signal), and its slow rate of clearance from

rodent brains (half-life>1.5 months) has proven MEMRI to be an invaluable technique to delineate brain structure, function, and neuronal connectivity (Aoki, Wu, Silva, Lynch, & Koretsky, 2004; Pautler, Mongeau, & Jacobs, 2003; Takeda, Sawashita, & Okada, 1995). Manganese's chief disadvantage, however, is its cellular toxicity, which can lead to neurological deficits and organ failure when administered at high doses (Aschner & Aschner, 1991; Crossgrove & Zheng, 2004; Malecki, 2001). As a result, attempts to increase Mn^{2+}concentration per dose to further enhance MR signal have been inhibited. Such an obstacle is critical to overcome in functional studies that require physiologically healthy and normally behaving animals.

The use of several fractionated dose administrations of manganese to improve image contrast has been demonstrated to minimize toxicity and produce greater signal enhancement at higher total doses (Bock, Paiva, & Silva, 2008; Grunecker et al., 2010). However, multiple injections conducted intraperitoneally in awake animals or intravenously in anesthetized animals are too intrusive for some behavioral studies, such as those focused on anxiety-like behaviors. A recent study examined the use of an osmotic pump to achieve continuous slow, systemic release of Mn^{2+} in rats (Eschenko et al., 2010). These authors used intra-abdominal osmotic pumps to deliver a total of 80 mg/kg of $MnCl_2$ over 7 days and found no decreases in motor performance. In contrast, an equivalent single bolus dose administered systemically was found to reduce wheel running, as well as food intake and body weight. The method of manganese infusion via osmotic pump implantation is thus less disruptive to the animal's mobility and optimal in studies on animal behavior. Nevertheless, systematic analysis of the relationship between the rate of continuous systemic manganese infusion and signal intensity in the brain has not been explored thoroughly in research. This relationship is important considering the diversity of behavioral tasks employed commonly for rodent models that range in frequency and duration.

3.2 OSMOTIC INFUSION-BASED MEMRI: METHODOLOGY DEVELOPMENT AND OPTIMIZATION FOR APPLICATION AS A FUNCTIONAL IMAGING TOOL

Manganese-enhanced magnetic resonance imaging is a technique that employs the divalent ion of the paramagnetic metal manganese (Mn^{2+}) as an effective contrast agent to visualize the mammalian brain in vivo. As total achievable contrast is directly proportional to the net amount of Mn^{2+} accumulated in the brain, there is a great interest in optimizing administration protocols to increase the effective delivery of Mn^{2+} to the brain while avoiding the toxic effects of Mn^{2+} overexposure. This is particularly important if the research objective is to conduct MEMRI as a functional imaging tool to visualize neural activity in animals that are awake and behaving normally.

3.2.1 Objectives

Due to the toxicity of Mn^{2+} accumulation and low tolerance to large bolus administrations of Mn^{2+} in small rodents, it was necessary to first optimize the methodology of systemic Mn^{2+} administration. After optimization of an effective and safe method of utilizing MEMRI in mice, MEMRI could then be employed as a useful tool for visualizing brain activity in *Oxtr* KO and WT animals following social defeat.

3.2.2 Hypothesis

It is hypothesized that by utilizing a novel method of continuous, slow systemic infusion of manganese chloride ($MnCl_2$) into the mouse via mini-osmotic pump administration, visualization of features within the brain can be achieved while maintaining normal levels of physical activity.

3.2.3 Methods

Generation of the Oxtr knockout: *Oxtr* KO (C57BL/6J background) mice were generated by first developing a line of mice floxed for the Oxtr allele (Otr $^{+/flox}$), A male Oxtr+/flox mouse containing germ cell expression of Cre recominbinase (Oxtr$^{+/flox,cre}$) was crossed with homozygous floxed females (Oxtr$^{flox/flox}$), which produced heterozygous offspring containing one inactive Oxtr allele (Oxtr$^{+/-}$). These mice were crossed with each other to produce homozygous total Oxtr knockout mice (Oxtr$^{-/-}$). Oxtr-/- animals utilized in this study were genotyped via PCR prior to behavioral testing and imaging. For a more detailed discussion of the methods utilized in the laboratory to generate and genotype Oxtr KO mice, see Lee *et al*. (H.-J. Lee et al., 2008).

Animal care and handling: Mice were individually housed and provided food and water ad libitum throughout the duration of the infusion treatments. The physical health and activity of all treated animals were assessed daily by animal care staff and veterinarians of the National Institute of Mental Health (NIMH) small animal facility. The NIMH Animal Care and Use Committee (ACUC) approved all animals and methods utilized in this study. All subject *Oxtr* KO and WT mice were group housed with same sex littermates throughout the duration of the study. The cohort of mice in the socially defeated 3-day MEMRI experiment were weighed daily 30 minutes before the investigator prior to social defeat exposures to note any substantial physiological effects of Mn^{2+} infusion on animal weight. The animal core facility staff and resident veterinarian also monitored the subject mice's outward health and movement via daily checks (mice were generally monitored to observe for natural exploratory behaviors in the home cage, expected levels of movement during the light cycle, and for interaction with group housed littermates). Health monitoring consisted of qualitative checks of animal behavior and physiology—no quantitative measurements were collected. No other procedures were employed to measure the specific changes in health in the mice due to the resource and time limitations of the investigator.

Manganese enhanced MRI: Three cohorts of male *Oxtr* KO mice (C57BL/6J background) and control male C57BL/6J age-matched adult mice (~22–30 grams) were evaluated (n = 4 per cohort) in this study. As shown in **Figure 3.1**, each cohort was assigned to one of three Mn^{2+} infusion treatments: 3-day (1.0 μL/hour), 7-day (0.5 μL/hour), or 14-day (0.25 μL/hour). Each treatment provided a cumulative total dose of 180 mg/kg of $MnCl_2$ (~3.96–5.4 mg/mouse) ($MnCl_2 \cdot 4H_2O$, Sigma–Aldrich, St. Louis, MO, USA). While alternate studies have been conducted administering Mn^{2+} via slow osmotic infusion utilizing lower cumulative doses (80 mg/kg $MnCl_2$) with no evidence of motor-impairing deficits (Eschenko et al., 2010), the total cumulative dose of 180 mg/kg of $MnCl_2$ utilized in this research was based on previously examined MEMRI outcomes utilizing fractionated dose Mn^{2+} administration (Bock et al., 2008). It was shown in Bock et al. that Mn^{2+} could be administered in more fractionated doses to achieve a greater cumulative dose, resulting in improved MRI signal enhancement. The greater cumulative dose was thus piloted in this research to observe for health impairments in mice and signal contrast in MR images.

An additional cohort of *Oxtr* KO and wildtype age-matched adult male mice treated with the 3-day Mn^{2+}infusion (total dose of 180 mg/kg) was also examined in this study (n = 7 WT, n = 5 KO). In these animals, we conducted daily measurements of weight (n = 6). All $MnCl_2$ solutions were prepared in 400 mM bicine buffer, pH of 7.4 (adjusted with 1 Molar NaOH). Continuous slow systemic infusion of $MnCl_2$ was achieved through subcutaneous implantation of mini-osmotic pumps (ALZET, DURECT Corporation, Cupertino, CA, USA) on the midline of the mid-lower back of the mice. Osmotic pumps utilized in this study included the 1003D (3-day), 1007D (7-day), and 1002 (14-day) models, all of uniform mass and size (0.4 gram, 1.5 cm in length, 0.6 cm in diameter). The pumps contained stainless steel flow moderators and were thus removed prior to MR scanning. All surgical operations were performed on mice anesthetized with 2% isoflurane. Mice were individually housed and provided food and water ad libitum throughout the duration of the infusion treatments. The physical health and activity of all treated animals were assessed daily by animal care staff and veterinarians of the National Institute of Mental Health (NIMH) small animal facility. The NIMH Animal Care and Use Committee (ACUC) approved all animals and methods utilized in this study.

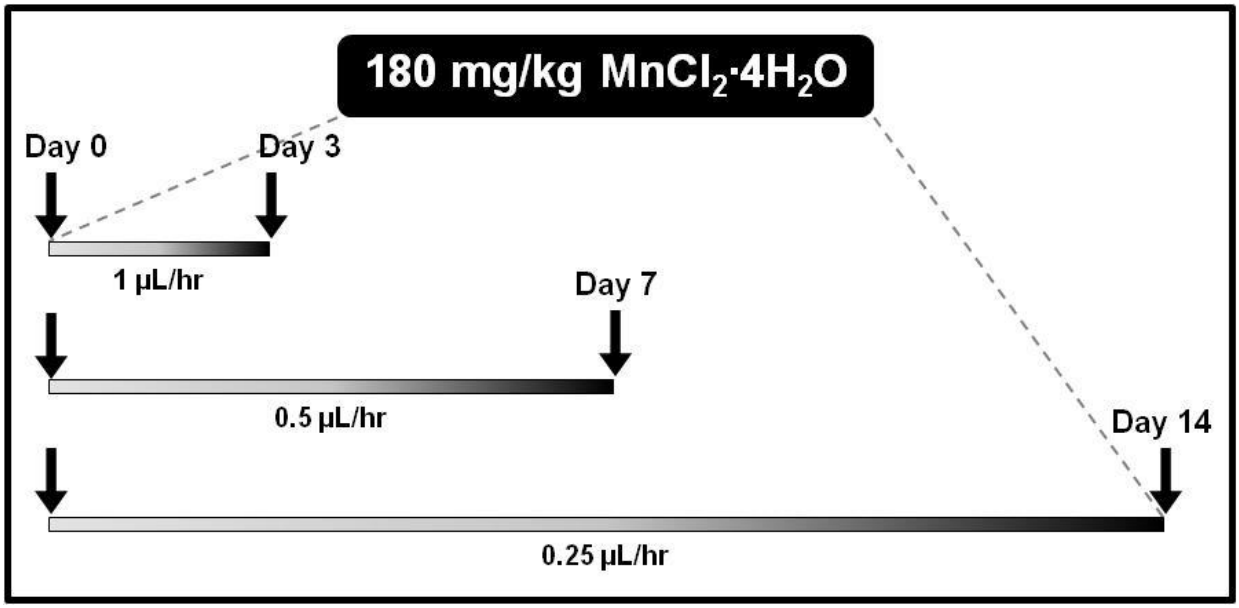

Figure 3.1 $MnCl_2$ treatment timeline for the three infusion periods (3-, 7-, and 14-day): An initial pre-contrast scan preceded each infusion treatment, and a final post-contrast scan concluded each treatment period. *Oxtr* knockout and control C57BL/6J adult male mice were tested in each treatment group (n = 4 per genotype). Total cumulative doses of 180 mg/kg of $MnCl_2$ were administered in each group via mini-osmotic pump infusion

T_1-weighted MR acquisition: For each of the Mn^{2+} infusion treatments, we performed T_1-weighted MRI at two time points: pre-infusion and post-infusion. Whole-brain 3-D images were acquired using a T_1-weighted gradient echo sequence in a 7 T 21-cm horizontal scanner (Bruker BioSpin, Billerica, MA). The MR acquisition protocol consisted of 1.5-hour scans collected over eight averages (echo time = 3.5 ms, repetition time = 30 ms, isotropic spatial resolution = 100 µm, field of view = 1.56 × 1.5 × 1.2 cm, image matrix = 256 × 150 × 120). In the additional cohort of *Oxtr* KO and WT mice (n = 7 WT, n = 5 KO) treated with a 3-day Mn^{2+} infusion, MR acquisition protocol consisted of 2-h scans collected over 40 averages (echo time = 3.5 ms, repetition time = 30 ms, spatial resolution = 75 × 75 × 150 µm, field of view = 1.4 × 1.4 × 0.48 cm, image matrix = 192 × 186 × 32).

All animals were secured in a stereotaxic holder and mounted in a 72-mm volume (transmit)/25-mm surface (receive) radio frequency coil ensemble. Mice were maintained under

anesthesia (2% isoflurane in a mixture of oxygen, medical air, and nitrogen) and core body temperature was regulated at 37°C using a circulating water pad and monitored via a rectal temperature probe. A pressure transducer (SA Instruments, Inc., NY, USA) was placed on the dorsal side of the mice to monitor breathing rate, which was maintained at 60 ± 10 breaths/minute. A copper sulfate fiduciary marker was placed adjacent to the mouse head in each scan.

Region of Interest (ROI) analysis: We conducted region of interest (ROI) analysis on all acquired pre- and post-infusion images to assess for differences in Mn^{2+} accumulation by treatment group (3-, 7-, and 14-day) and genotype (*Oxtr* KO vs. controls). In the methodological pilot experiments, regions of Mn^{2+} accumulation examined included the bilateral olfactory bulbs, hippocampal formation, and amygdalae. These areas were chosen for Mn^{2+} systemic infusion ROI analysis due to the high density of Mn^{2+} accumulation in these areas of the brain, and thus facilitated the discrimination of signal enhancement differences observed from each of the infusion treatment groups. T_1-weighted MR data were analyzed with the ImageJ software (Rasband). ROI's in coronal brain slices were identified and delineated using the Allen Institute Mouse Brain Reference Atlas (Seattle, WA, USA). Raw signal measurements analyzed in each ROI included signal intensity and area in arbitrary units. Signal activity in a ROI was interpreted as a function of Mn^{2+} accumulation—calculated as the product of mean signal intensity and area. All ROI mean signal intensities were thresholded at a constant level and normalized to that of the fiduciary marker included in each scan. We employed the non-parametric (tests for the distribution of signal intensity indicated that such outcomes were not normally distributed) independent samples *t*-test (Wilcoxon–Mann–Whitney test) and repeated measures analysis of variance (Kruskal–Wallis test) in SPSS Statistics 17.0 (IBM, Somers, NY, USA) to assess the significance between normalized mean signals in each experimental group. Significance was set at $P < 0.05$ for each statistical test.

3.2.4 Results

Mn^{2+} accumulates throughout the whole-brain and concentrates preferentially in active regions: The signal enhancement examined in three mutually perpendicular planes in T_1-weighted images of *Oxtr* KO and control mice demonstrate the extent to which Mn^{2+}distributes throughout the mouse brain and the anatomical structures in which Mn^{2+}accumulates. **Figure 3.2** displays representative slice images drawn from a control C57BL/6J adult male mouse after a treatment of 180 mg/kg $MnCl_2$ infused systemically at a rate of 1 μL/hour over 3 days. Signal enhancement is observed in the posterior to anterior areas of the brain as indicated in coronal slice images (**Figure 3.2a**). Alternate axial views of the brain also indicate manganese distribution in slice images left to right and ventral to dorsal in the sagittal (**Figure 3.2b**) and horizontal (**Figure 3.2c**) planes, respectively.

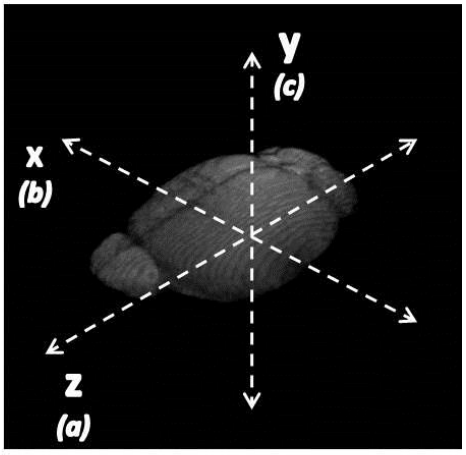

Figure 3.2 T_1-weighted whole-brain. 3D MRI (100 µm isotropic resolution) of a control C57BL/6J adult male mouse after 3-day $MnCl_2 \cdot 4H_20$ infusion (180 mg/kg). Representative coronal (a), sagittal (b), and horizontal (c) slice images demonstrate Mn^{2+} accumulation as enhanced signal throughout the entire brain

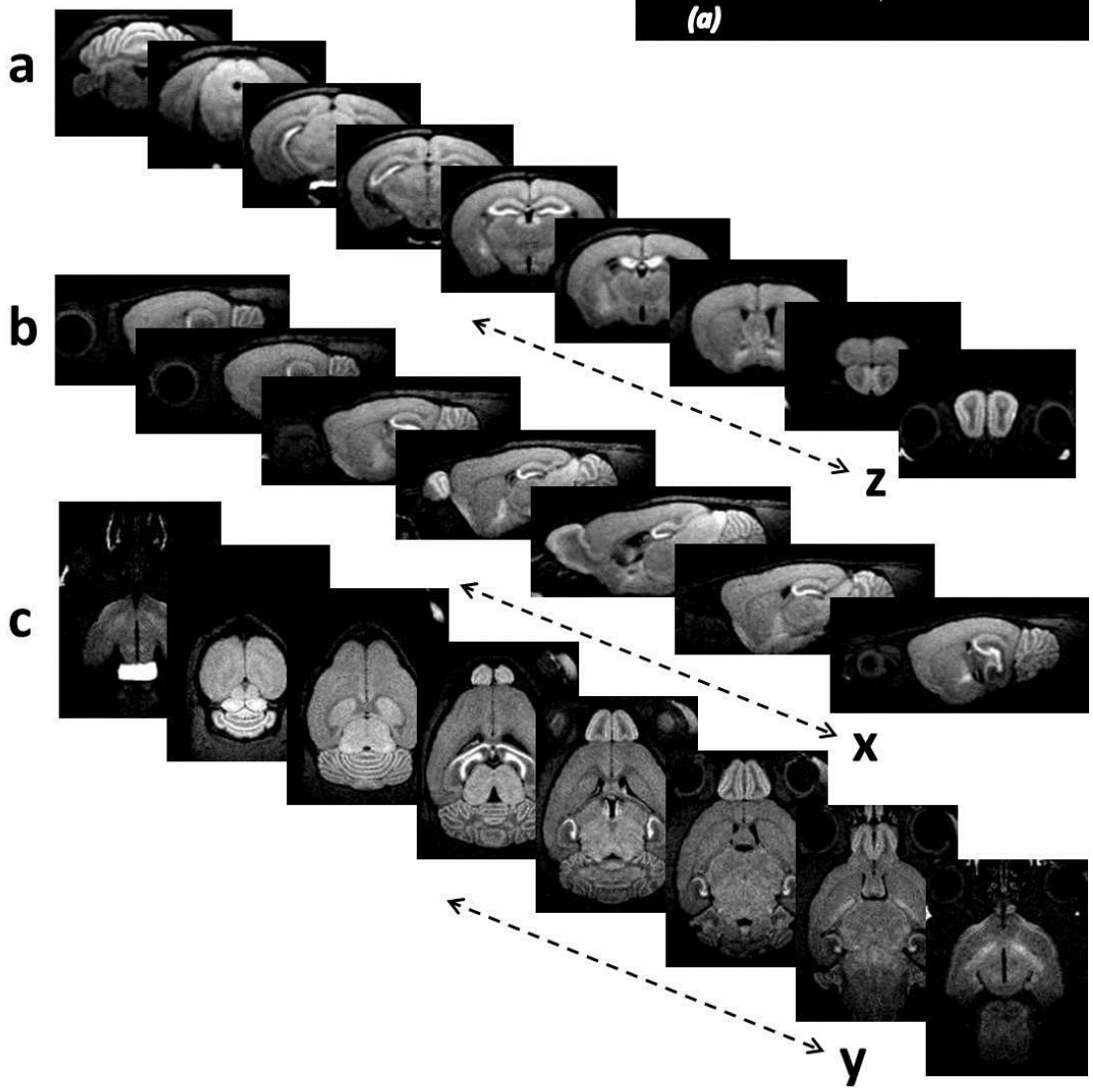

Figure 3.3 presents enlarged slice images from a control adult male mouse after a treatment of 180 mg/kg $MnCl_2$ infused systemically at a rate of 1 µL/hour over 3 days. Representative horizontal (**Figure 3.3a**), sagittal (**Figure 3.3b**), and coronal (**Figure 3.3c**) views demonstrate abundant accumulation of Mn^{2+} in various structures of the brain. Such regions include the olfactory bulbs, areas of the cortex (i.e., infralimbic cortex, motor cortex, and visual cortex), habenula, hippocampal formation (CA1, CA2, CA3, and dentate gyrus), cerebellum, inferior colliculus, amygdala, pituitary, thalamus, and hypothalamus. It should be noted that many of these cited regions of high Mn^{2+}accumulation are key components in sensory and limbic systems regulating olfaction, emotion, learning, and memory (Isaacson, 2003; Mega, Cummings, Salloway, & Malloy, 1997).

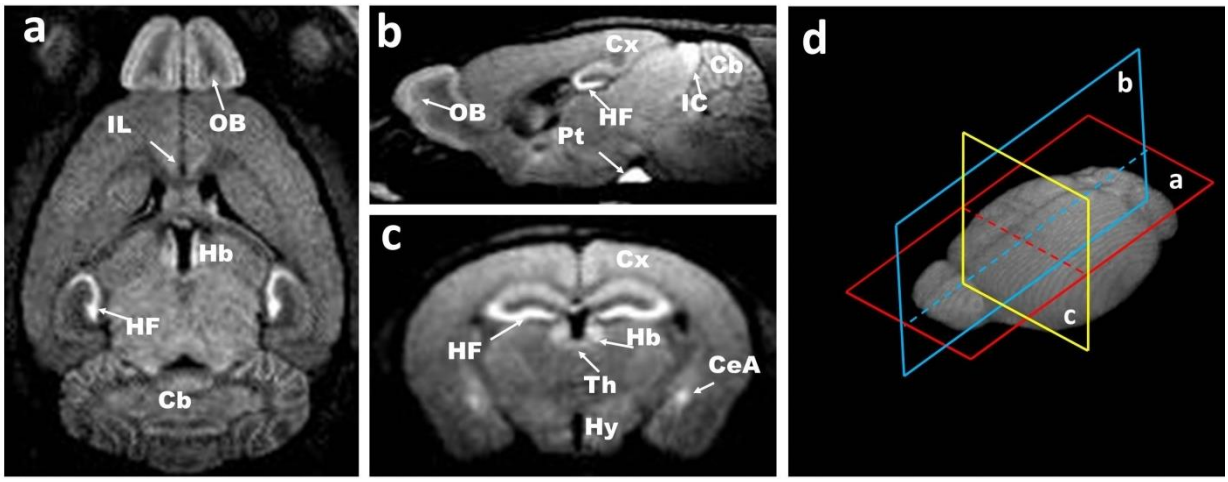

Figure 3.3 Selected horizontal (a), sagittal (b), and coronal (c) T_1-weighted slice images (100 µm isotropic resolution) from a control C57BL/6J adult male mouse brain following a 3-day $MnCl_2{\cdot}4H_20$ infusion (180 mg/kg cumulative dose). Structures of significant Mn^{2+} accumulation include the olfactory bulbs (OB), areas of the cortex (Cx) (i.e. infralimbic cortex (IL), motor cortex, visual cortex), habenula (Hb), hippocampal formation (HF) (i.e. CA1, CA2, CA3, dentate gyrus), cerebellum (Cb), inferior colliculus (IC), central amygdala (CeA), pituitary (Pt), thalamus (Th), and hypothalamus (Hy). 3D whole-brain composite model demonstrates location from which representative slice images were selected (d).

Equal doses of Mn^{2+} infused at different rates produce comparable signals*:* To test differences in image quality pre- and post-infusion of the contrast agent, we analyzed mean signals measured bilaterally from the hippocampal formation, olfactory bulbs, and amygdalae (**Figure 3.4**). We found normalized mean signals in post-infusion images to be significantly greater than that of pre-infusion images (P <0.001) (**Figure 3.5**) in both *Oxtr* KO and control animals. Despite an apparent trend of greater signal corresponding to higher rates of infusion, non-parametric statistical analysis of signal means did not reveal significant differences in the bilateral olfactory bulbs, hippocampal formations, or amygdalae (or the whole-brain, data not shown) in the 3-, 7-, 14-day infusion treatments (equal cumulative doses of 180 mg/kg, significance levels set at P < 0.05; **Figure 3.5**).

Qualitative observations of signal intensity are presented from sagittal (**Figure 3.6a**), coronal (**Figure 3.6b**), and horizontal (**Figure 3.6c**) perspectives in representative slice images from each infusion treatment. No significant differences were observed between the *Oxtr* KO and control mice in mean signals measured bilaterally from the olfactory bulbs, hippocampal formation, and amygdalae (data not shown, significance levels set at P <0.05). Repeated analysis conducted on an additional cohort of 3-day Mn^{2+} infused animals also found no significant differences in normalized mean signal measured from the hippocampal formation and whole amygdalae between ***Oxtr*** KO (n = 5) and WT (n = 7) adult male mice (data not shown, significance level set at P <0.05). The MR acquisition protocol performed for this cohort of animals allowed finer anatomical mapping of structural subregions of the amygdala (see **Figure 3.7a**, **3.7b**). As a result, signals in ***Oxtr*** KO and WT animals (n = 11) were determined among the basal, central, medial, and lateral nuclei of the amygdala in which the central nucleus of the amygdala exhibits the greatest signal intensity (P = 0.025). Furthermore, none of the Mn^{2+} infusion treatments was observed to produce any ostensible negative effects on animal physiology or behavior throughout the duration of manganese infusion. The additional cohort of *Oxtr* KO and WT mice infused with a 3-day Mn^{2+} treatment (total dose of 180 mg/kg, n = 6) showed no significant differences in daily body weights (*Oxtr* KO M=26.4 g, WT M=27.1 g, P=0.18). Other behavioral and physiological measures aside from weight were not collected due to the time and resource limitations of the investigator. For a complete description on how

physiology and animal behavior was monitored during the daily checks on general animal health, see the Animal Care and Handling section in the methods portion of this chapter. Future studies examining the behavioral and physiological effects of MEMRI in mice would ideally include a comprehensive battery of mobility and sensory assays in addition to evaluation on animal weight and appetite over a longer observation period (e.g. several weeks versus the 3-day observation in this study).

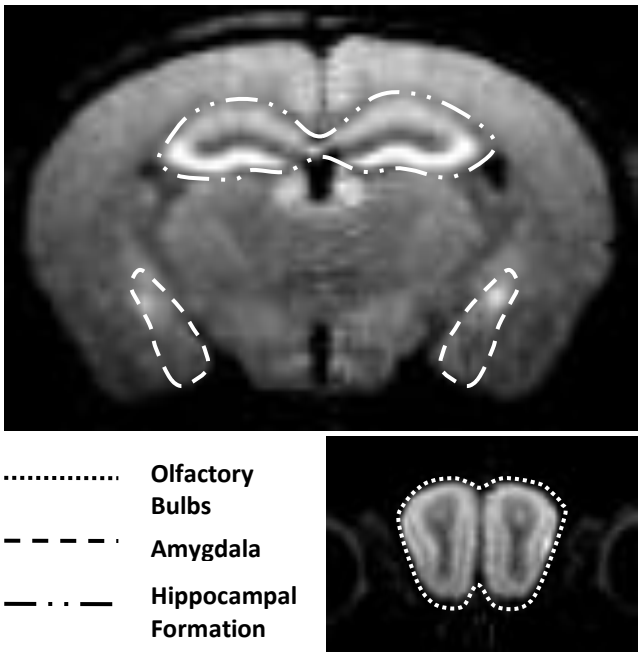

Figure 3.4 ROI demarcations of the hippocampal formation, amygdala, and olfactory bulbs: T_1-weighted coronal slice images

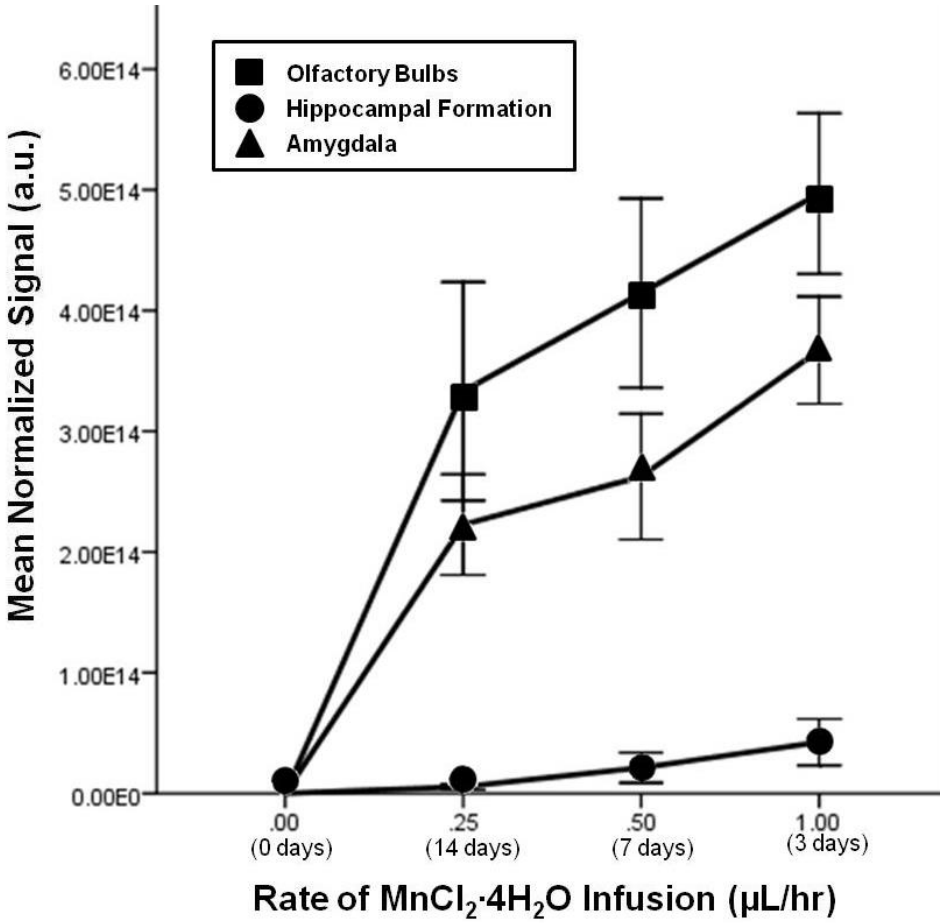

Figure 3.5 Normalized mean signal comparisons of olfactory bulb, hippocampal formation, and amygdala ROIs by Mn^{2+} treatment group (equal cumulative 180 mg/kg dose). Error bars indicate ±1 standard error. Mean signal measured from T_1-weighted coronal slice images. Data includes *Oxtr* knockout and control C57BL/6J mice (*n*=10 per treatment group)

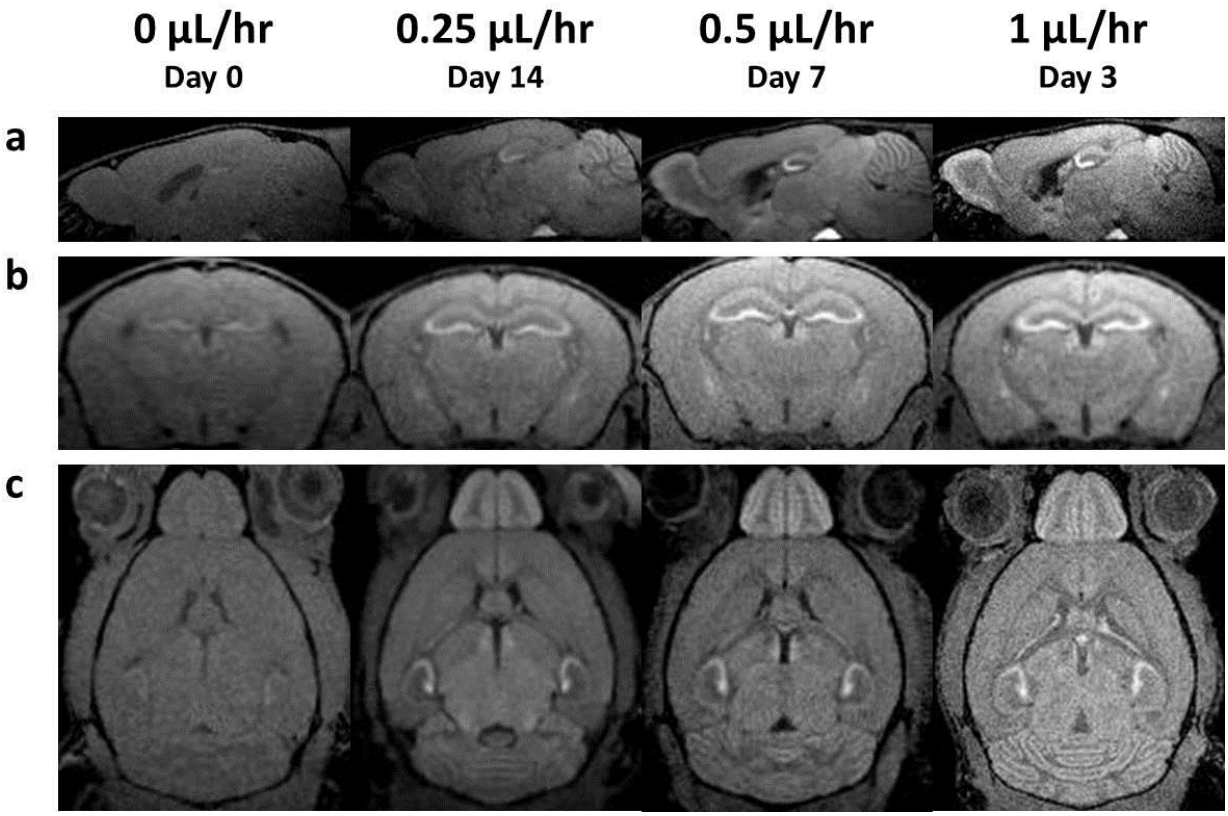

Figure 3.6 Sagittal (a), coronal (b), and horizontal (c) T_1-weighted slice images (100 µm isotropic resolution) from control C57BL/6J adult male mouse brains prior to and following 14-day, 7-day, and 3-day $MnCl_2 \cdot 4H_20$ infusion treatments (180 mg/kg cumulative dose). Progressively greater signal enhancement observed at higher rates of Mn^{2+} infusion over shorter infusion periods

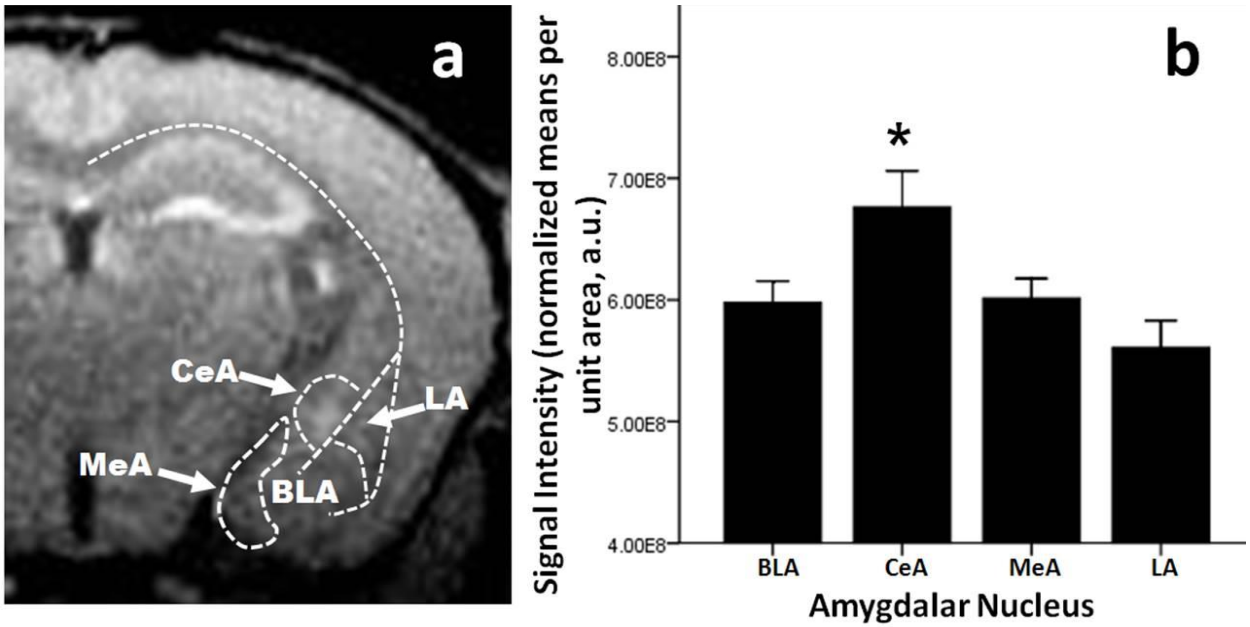

Figure 3.7 (a) Demarcations of the central (CeA), lateral (LA), basal (BLA), and medial (MeA) amygdala on a T_1-weighted coronal slice image. (b) Signal intensity (normalized signal means per unit area) comparisons by amygdalar nucleus. Error bars are s.e.m. Mean signals measured from T_1-weighted coronal slice images following 3-day Mn^{2+} infusion treatments (180 mg/kg cumulative dose). Data includes *Oxtr* knockout and wildtype mice (n=12). Signal intensity in the central amygdala is significantly greater than that of other nuclei (* P<0.05)

3.3 MEMRI IN *OXTR* KO MICE DURING SOCIAL DEFEAT: FUNCTIONAL ANALYSIS

3.3.1 Objectives

In this study, we applied the optimized MEMRI functional imaging technique employing slow systemic infusion in a rodent model to investigate oxytocin-related signaling patterns in the amygdala in the context of social fear processing. To better understand how oxytocin receptors regulate social fear behaviors, the investigator employed a neuroimaging approach consisting of manganese-enhanced magnetic resonance imaging (MEMRI) and related the functional activity results to behavioral response findings as described previously in Chapter 2.

3.3.2 Hypothesis

It was hypothesized that functional activity patterns in the brains of *Oxtr* KO and WT mice will correspond to differences in behavioral outcomes to social defeat. It is specifically hypothesized that such differences in neural activity during social defeat will be localized in areas within the amygdala of the mouse brain. This hypothesis is based on previous findings linking amygdalar activity to social fear processing in rodents (Bourne et al., 2013), and specifically activity within particular nuclei of the amygdala to non-social fear learning and long term anxiety in animals (Arendt et al., 2014; Clinard, Bader, Sullivan, & Cooper, 2015; Sears, Schiff, & LeDoux, 2014).

3.3.3 Methods

Generation of the Oxtr knockout: See Chapter 3.2

Animal care and handling: See Chapter 3.2

Repeated social defeat paradigm: See Chapter 2, section 2.3.3

***Manganese-enhanced magnetic resonance imaging*:** Adult male oxytocin receptor gene (*Oxtr)* knockout (KO) and wildtype (WT) mice were evaluated in this study (C57BL/6J background, ~22–30 g, ~90-100 days old, n = 10 per cohort). All animals were generated and genotyped as previously described (H. J. Lee et al., 2008). Cohorts were administered a 3-day 1.0 µL/h infusion of $MnCl_2{\cdot}4H_2O$, cumulative total dose of 180 mg/kg (~3.96–5.4 mg/mouse, Sigma–Aldrich, St. Louis, MO, USA) according to previous methodology [see Chapter 3.2 for MEMRI methodology design and pilot experiments] (Mok, Munasinghe, & Young, 2012). All $MnCl_2$ solutions were prepared in 400 mM bicine buffer, pH of 7.4 (adjusted with 1 M NaOH). Continuous slow systemic infusion of $MnCl_2$ was achieved through subcutaneous implantation of mini-osmotic pumps (ALZET, DURECT Corporation, Cupertino, CA, USA) on the midline of the mid-lower back of the mice. Osmotic pumps utilized in this study included the 1003D (3-

day) model (0.4 g, 1.5 cm in length, 0.6 cm in diameter). The pumps contained stainless steel flow moderators and were thus removed prior to MR scanning.

T_1-weighted MR acquisition: For each of the Mn^{2+} infusion treatments, we performed T_1-weighted MRI at two time points: pre-infusion and post-infusion. 3-D images were acquired using a T_1-weighted gradient echo sequence in a 7T 21-cm horizontal scanner (Bruker BioSpin, Billerica, MA). The MR acquisition protocol consisted of 2 hour scans collected over 40 averages (echo time = 3.5 ms, repetition time = 30 ms, spatial resolution = 75.9 x 75.9 x. 150 µm, field of view = 1.49 x 1.49 x 0.48 cm, image matrix =192.9 x 186.9 x 32). All animals were secured, maintained under anesthesia, monitored for breathing, and core body temperature regulated as described in the methods section of Chapter 3.2. A copper sulfate fiduciary marker was placed adjacent to the mouse head in each scan.

MEMRI region of interest analysis: We conducted region of interest (ROI) analysis on all acquired pre- and post-infusion images to assess for differences in Mn^{2+} accumulation by genotype (*Oxtr* KO vs. WT). Areas of ROI evaluation consisted of the central, medial, and basolateral nuclei of the amygdala. The amygdala was targeted as the main region of interest in this experiment of socially defeated *Oxtr* KO animals due to region's many links to regulation of social defeat behaviors and involvement in the processing of fear [see Chapter 1]. While other areas of the brain, such as the medial frontal and temporal cortex are also known to be involved in social cognition, due to limitations in time and scanner availability (experiments were all conducted and analyzed by a single investigator and the scanner was part of a core imaging facility shared by multiple research groups), the amygdala was selectively imaged and analyzed T_1-weighted MR data were analyzed with the ImageJ software (Rasband). ROI's in coronal brain slices were identified and delineated using the Allen Institute Mouse Brain Reference Atlas (Seattle, WA, USA). Raw signal measurements analyzed in each ROI included signal intensity and area in arbitrary units. Signal activity in a ROI was interpreted as a function of Mn^{2+} accumulation—calculated as the product of mean signal intensity and area. This calculation was employed as a method to roughly account for both Mn^{2+} signal intensity and neuronal density, two factors that are hypothesized in this research to be important factors in indicating neural

activity. Other factors, such as the particular region of the brain (i.e., the hippocampus and olfactory bulbs are examples of areas of the brain that display constant levels of high signal intensity), also affect the observation of Mn^{2+} accumulation in the brain. Since this research limited comparison of only amgydalar nuclei between Oxtr KO and WT animals, with control cohorts indicating similar levels of amygdalar Mn^{2+} accumulation, a study assumption was that any changes in Mn^{2+} accumulation within the amygdala was a potential outcome of variation in neural activity. All ROI mean signal intensities were normalized to that of a copper sulfate fiduciary marker included in each scan. We employed the non-parametric independent samples t test (Wilcoxon– Mann–Whitney test) and repeated measures analysis of variance (Kruskal–Wallis test) in SPSS Statistics 17.0 (IBM, Somers, NY, USA) to assess the significance between normalized mean signals in each experimental group. Non-parametric tests were utilized in this analysis due to the non-normal distribution of the ROI data. Significance was set at $P<0.05$ for each statistical test.

3.3.4 Results

***Differences in amygdalar activation during fearful social processing in Oxtr KO mice*:** To assess functional activation in the amygdala during social fear processing, we employed MEMRI acquisition following the performance of social defeat behaviors in *Oxtr* WT and KO mice. Behaviorally tested and control animals were administered manganese via a mini-osmotic pump implant that systemically infused a continuous volume of $MnCl_2 \cdot 4H_2O$ over a 3-day social defeat period (see **Figure 2.2**). Behaviorally tested animals were subjected to daily 30-minute resident-intruder tests of aggression, during which they interacted freely with a more aggressive resident conspecific. To distinguish functional activity in the amygdala of socially defeated groups, all animals were magnetic resonance imaged prior to manganese administration and again after completion of infusion. Region of interest analysis on normalized signal measurements of amygdala nuclei collected via MEMRI reveal significant signal enhancement in the basolateral (Mann-Whitney U non-parametric two-tailed hypothesis, $\alpha=0.05$; U-value=2, Z-score=3.5907, p=0.0034) and medial amygdalae (Mann-Whitney U non-parametric two-tailed hypothesis, $\alpha=0.05$; U-value=22, Z-score=2.0788, p=0.0375) in socially defeated *Oxtr* WT mice (**Figures**

3.8b and **3.8c)** compared to that of socially defeated and sham handled *Oxtr* WT controls. In contrast, no significant signal enhancement was observed in control (sham handled) or socially defeated *Oxtr* KO mice in the basolateral or medial nuclei of the amygdala as that exhibited by socially defeated WT animals (Mann-Whitney U non-parametric two-tailed hypothesis, α=0.05; U-value=30, Z-score=-1.474, p=0.145; **Figures 3.8d and 3.8e**). The central nucleus of the amygdala was uniformly enhanced in all control and socially defeated subjects (**Figure 3.8b-e**). Control *Oxtr* KO and WT animals were sham handled over a 3-day period, but not socially defeated. For quantified ROI data, see **Figure 3.9** and **Table 3.1**.

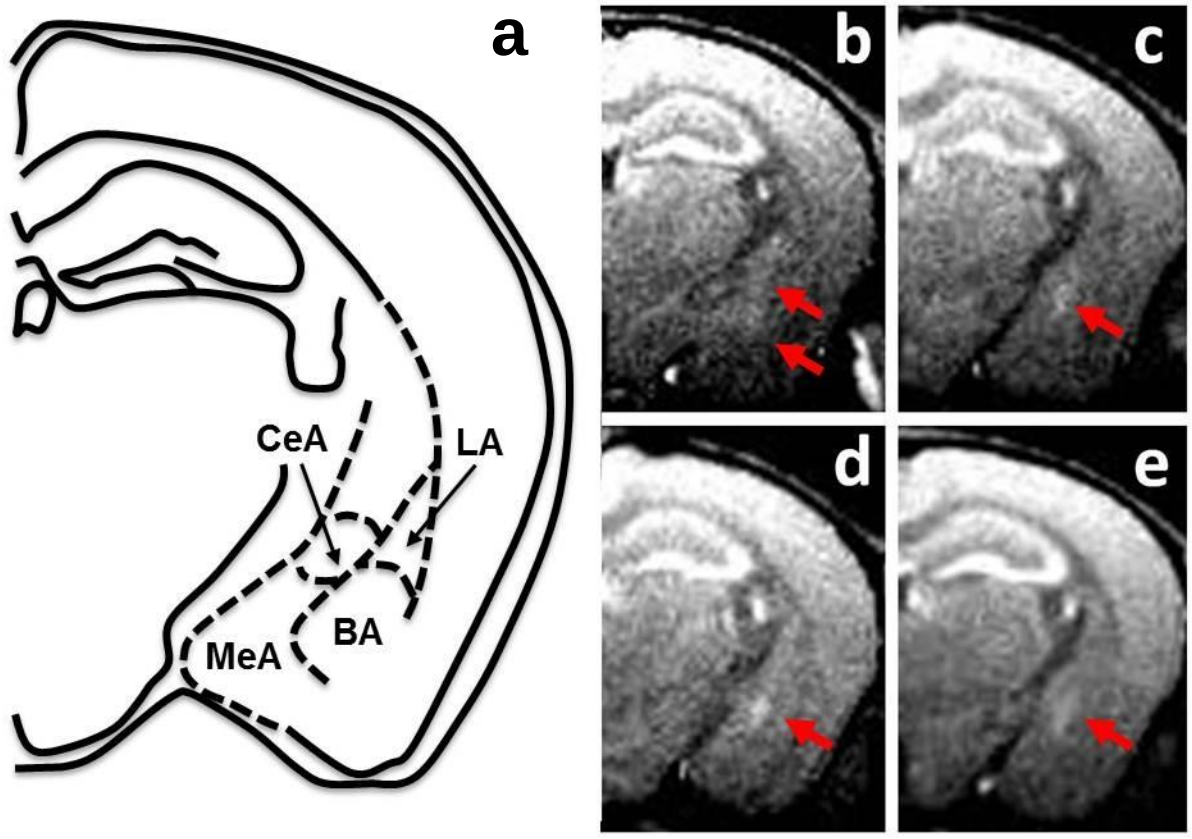

Figure 3.8 MEMRI of amygdalar nuclei in *Oxtr* KO and WT during social defeat. a) Schematic of an adult mouse coronal brain section with amygdala nuclei delineated; Representative coronal slice images from b) socially defeated WT, c) control WT, d) socially defeated *Oxtr* KO, e) control *Oxtr* KO with red arrows demarcating significant areas of signal enhancement in the Basolateral (WT only) and Central amygdala nuclei (WT and *Oxtr* KO mice).

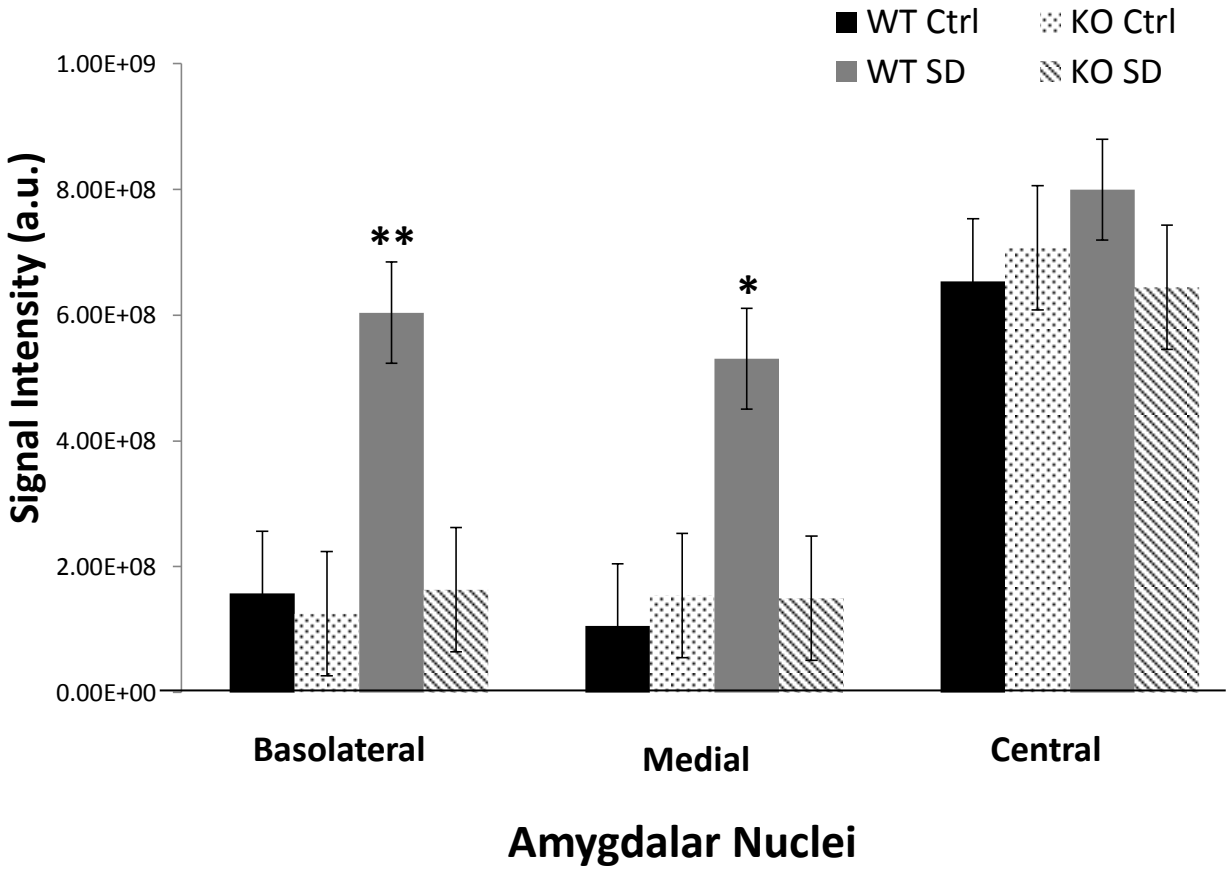

Figure 3.9 Region of Interest (ROI) analysis of amygdalar nuclei in *Oxtr* KO and WT mice. Signal intensity (normalized signal means per unit area) comparisons by amygdalar nucleus. Mean signals measured from T_1-weighted coronal slice images following 3-day Mn^{2+} infusion treatments (180 mg/kg cumulative dose) and repeated social defeat (or sham handling for control animals). Data includes *Oxtr* knockout and wildtype mice (n=10). Signal intensity in the basolateral and medial amygdalae of the WT socially defeated mice are significantly greater than that of respective nuclei in Oxtr KO socially defeated mice (also significnatly greater than that of all control subjects) (* P<0.05; * P<0.01). Error bars are one standard error of the mean (S.E.M.).

Experimental Treatment	Group	Mean Signal Intensity a.u. (S.E.) CeA	MeA	BLA
Social Defeat	*Oxtr* KO	6.447×10^8 (5.896×10^5)	1.496×10^8 (5.231×10^5)	1.632×10^8 (4.276×10^6)
	WT	7.996×10^8 (8.287×10^5)	5.305×10^8 (2.819×10^5)	6.063×10^8 (1.628×10^5)
Control	*Oxtr* KO	7.072×10^8 (4.658×10^5)	1.538×10^8 (4.161×10^5)	1.252×10^8 (6.896×10^5)
	WT	6.541×10^8 (1.958×10^6)	1.0568×10^8 (2.887×10^5)	1.577×10^8 (0.984×10^5)

Table 3.1 Region of Interest (ROI) data of amygdalar nuclei in *Oxtr* KO and WT mice. Signal intensity (normalized signal means per unit area) comparisons by amygdalar nucleus (CeA=Central, MeA= Medial, BLA=Basolateral). Mean signals measured from T_1-weighted coronal slice images following 3-day Mn^{2+} infusion treatments (180 mg/kg cumulative dose) and repeated social defeat (or sham handling for control animals). Data includes *Oxtr* knockout and wildtype mice (*n*=10). Errors are s.e.m.

3.4 SUMMARY

Experiments conducted with *Oxtr* KO and WT adult male mice demonstrated the utilization of mini-osmotic systemic infusion as an effective means of slowly administering manganese chloride in a small animal model as a functional imaging tool. The method piloted in this thesis research not only indicated that Mn^{2+} accumulates throughout the whole brain of the mouse, but that it also concentrates preferentially in the more active regions of the brain (i.e., olfactory bulbs, hippocampus, amygdala). Furthermore, qualitative evidence indicated that signal enhancement was greater when manganese was infused over a shorter period of time (higher effective rate of infusion). Subsequent studies employing MEMRI to functionally image socially defeated *Oxtr* KO and WT adult male mice indicated that there are differences in amygdalar activation (localized to the medial and basolateral nuclei) in socially defeated WT mice that was

not observed in socially defeated *Oxtr* KO (or sham handled WT mice). Specifically, this research links oxytocin receptor activity in the mouse brain to basolateral and medical amygdala-specific response to social defeat.

Previous research has similarly linked activity within the amygdala (as well as the hippocampus and prefrontal cortex) to social fear processing in rodents during fear conditioning (Bourne et al., 2013). Moreover, there is evidence in the scientific literature that the lateral nucleus in the amygdala is a key center in the processing of fear learning, and indications that receptors aside from oxytocin (e.g., serotonin, orexin 2/hypocretin) in the basolateral amygdala are important in both the production and reduction of anxiety in animals (Arendt et al., 2014; Clinard, Bader, Sullivan, & Cooper, 2015; Sears, Schiff, & LeDoux, 2014). While, the medial amygdala has been linked to regulation of social recognition abilities in rodents (Ferguson et al., 2001), and bilateral medial amygdala-lesioned mice have been observed to exhibit impairments in learning/memory as well as indications of depression (Wang et al., 2015)--- the relationship between the medial amygdala activity and the processing of social threats in mice is still not clear.

The current MEMRI findings reported here demonstrate that *Oxtr* KO mice may be experiencing abnormal neural activity in the medial and basolateral regions of the amygdala during social defeat, which the literature suggests may be linked to oxytocin-dependent signaling (due to the density of oxytocin receptors in these regions and oxytocin's strong association to anxiolytic properties). These findings both support and supplement the existing scientific knowledge on oxytocin's role in amygdalar function. These findings also provide further evidence of a potential role for the intra-amygdalar pathway between the basolateral and medial nuclei of the amygdala (Savander et al., 1998)) in oxytocin-related signaling during social fear processing. As demonstrated by this research, MEMRI's ability to "record" functional imaging signaling in normally behaving animals is an invaluable tool. However, it is recognized that it lacks real-time imaging capability and is therefore limited in its measurement of signaling nuances that accompany more transient and 4esubtle behaviors. Indeed, while this method identifies changes in amygdalar activity as a result of social defeat exposure, its temporal

limitations prevent the live visualization of amygdalar activity *during* the social defeat procedure (as opposed to post-social defeat, which is utilized in this MEMRI-based methodology). Behavioral results from Chapter 2 would appear to suggest *greater* activation of the amygdala in *Oxtr* KO mice would occur during elicitation of behaviors that suggest anxiogenic activity in the body, i.e., elevated freezing/fleeing (Fekete et al. 2009; Dabrowska et al. 2011). It is possible that the compensatory activity of inhibitory neurons in the amygdala may be functioning in the absence of oxytocin during fear conditioning. However, previous research has indicated that presentation of a fearful stimulus during conditioning results in the extracellular *reduction* of GABA in the mouse amygdala, which is shown to be sustained for several hours after the presentation of the stimulus (Stork et al., 2002). Thus the amygdalar hypoactivity captured by MEMRI in this research could indicate that extracellular GABA is modulated by oxytocin receptor activity, where GABA is elevated in the absence of such Oxtr activity following social defeat, resulting in increased inhibitory effects in the amygdala. A future direction of this research is thus to apply higher-resolution functional MRI techniques that will provide more accurate representations of real-time neural activity in behaving animals.

Chapter 4: Physiological effects of social defeat in mice

4.1 PRINCIPLES AND APPLICATIONS OF TELEMETRIC RECORDINGS IN ANIMALS

Telemetry, or the remote monitoring of measurable physiological factors, has been broadly utilized for decades in scientific research. In mouse models, telemetry has proven to be an invaluable tool in the wireless monitoring of neurological and physiological activities including vigilance states, core body temperatures, heart rate, respiration, and activity levels (Neuhaus and Borbely,1978; Drifhout et al., 1995; Kramer and Kinter, 2003). Telemetry is commonly performed in rodent models as a flexible means to monitor animal physiology and health during administration of therapeutic drugs or behavioral tasks (Dielenberg and McGregor, 2001; Edgar and Seidel, 1997). With telemetric technology, researchers are able to measure in real-time animal physiology in response to environmental conditions for the quantification of autonomic processes and behavioral outcomes. Particularly with regard to the translation of pre-clinical data drawn from animal studies to human trials, telemetry is a crucial means of acquiring important physiological evidence of the potential impact that a treatment may have on human participants.

4.2 ANALYSIS OF BODY TEMPERATURE IN *OXTR* KNOCKOUT MICE DURING REPEATED SOCIAL DEFEAT EXPOSURES

The relationship between emotional cognition and the autonomic nervous system in mammals has established such phrases as “fight-or-flight” due to the activation of the sympathetic nervous system in response to harmful or threatening situations. The increase in heart rate, breathing, and core temperature within the body in response to threatening stimuli are thus common physiological factors observed when inferring how a non-human animal subject may be responding to environmental stimuli.

4.2.1 Objectives

This research employs telemetric data acquisition to gather additional information on how *Oxtr* KO mice respond to social threat from a physiological viewpoint. The relationship between the sympathetic nervous system and the regulation of core body temperature has long been established in scientific research. Early studies have cited the importance of the sympathetic nervous system in the regulation of homeostatic processes in rats, as exemplified by modulation of core body temperatures (Maickel, Stern, Takabatake, & Brodie, 1967). The thermoregulation of body temperature has been linked directly to the hypothalamus as the regulatory center, which is also a key component in the hypothalamic-pituitary-adrenal (HPA) axis that plays a functional role in regulating the sympathetic response during threatening situations (Hamilton et al., 1964; Fusco et al, 1961). As a result of this relationship, analysis of core body temperature has been found to be a useful method of measuring conditioned behavioral response to fearful stimuli. This has been demonstrated by studies indicating elevations in body temperature in paired environments following pavlovian fear conditioning (Godsil, Quinn, & Fanselow, 2000), as well as in conditioning experiments involving social stress where studies have observed anticipatory rises in core body temperatures after repeated sensory contact with aggressive conspecifics (Pardon, Kendall, Pérez-Diaz, Duxon, & Marsden, 2004).

Research exploring the relationship between plasma oxytocin and sympathetic response (and subsequently thermoregulation as discussed previously) have found increased plasma concentrations of oxytocin to be correlated with decreased activity in sympathetic response in postpartum women—further promoting theories that oxytocin modulate anxiolytic activity (Grewen and Light, 2011). Moreover, an oxytocin receptor small nucleotide polymorphism (SNP) in a population based study of male humans found that the Oxtr SNP was associated with enhanced sympathetic activity both at baseline levels and during stressful conditions (Norman et al, 2012). The established evidence base of research linking the sympathetic control of temperature regulation to fear learning in mammals, combined with the human studies research examining Oxt and Oxtr's relationship to regulation of sympathetic response all contribute to the hypothesis that Oxtr is an important neuromodulator in sympathetic activity, and is likely

directly involved in elicitation of downstream sympathetic response (e.g., body temperature, galvanic skin response, etc.) during the processing of fear-inducing stimuli.

Using implantable wireless radiotransmitter telemetric technology, this thesis research monitored core body temperature in real-time as a secondary response to conditioned fear learning and also as a measure of the sympathetic nervous system as a function of social defeat. The physiological measurements collected were subsequently evaluated in relation to functional brain imaging and behavioral data gathered in parallel from the same *Oxtr* KO and WT mice.

4.2.2 Hypothesis

Previous work has linked the absence of oxytocin receptors in the brain to increased exhibition of aggressive behaviors and deficits in social behaviors (Mariaelvina Sala et al., 2011; Takayanagi et al., 2005). However, it is not known how oxytocin receptors in the mammalian brain may influence cognitive response to social aggression and the exhibition of anxiety-related behaviors. It was determined from the social defeat-induced behavioral response portion (Chapter 2.3) of this research that *Oxtr* KO male mice exhibit increased levels of submissive behaviors in response to more aggressive conspecifics during a resident-intruder test of aggression. It is therefore anticipated that physiological response in *Oxtr* KO mice to social defeat will include elevated core body temperatures compared to that of WT mice. This hypothesis is formulated upon the basis of existing research citing the relationship between increases in core body temperature to conditioned fear learning and social stress in rats (Bhatnagar, Vining, Iyer, & Kinni, 2006; Vianna & Carrive, 2005).

4.2.3 Methods

Generation of the Oxtr knockout: *Oxtr* KO (C57BL/6J background) mice were generated by first developing a line of mice floxed for the Oxtr allele (Otr $^{+/flox}$), A male Oxtr+/flox mouse containing germ cell expression of Cre recominbinase (Oxtr$^{+/flox,cre}$) was crossed with homozygous floxed females (Oxtr$^{flox/flox}$), which produced heterozygous offspring containing one

inactive Oxtr allele ($Oxtr^{+/-}$). These mice were crossed with each other to produce homozygous total Oxtr knockout mice ($Oxtr^{-/-}$). Oxtr-/- animals utilized in this study were genotyped via PCR prior to behavioral testing and imaging. For a more detailed discussion of the methods utilized in the laboratory to generate and genotype Oxtr KO mice, see Lee *et al*. (H.-J. Lee et al., 2008).

Animal care and handling: Mice were individually housed and provided food and water ad libitum throughout the duration of the infusion treatments. The physical health and activity of all treated animals were assessed daily by animal care staff and veterinarians of the National Institute of Mental Health (NIMH) small animal facility. The NIMH Animal Care and Use Committee (ACUC) approved all animals and methods utilized in this study. Resident mice consisted of male, age-matched Swiss Webster (SW) mice [purchased from Jackson Laboratories©] singly housed for 2 weeks and screened for aggressive behaviors (screening was conducted by exposing singly housed SW mice to group housed WT animals not used in the study cohorts and observing for social defeat of the WT animals within 5 minutes of exposure). All subject *Oxtr* KO and WT mice were group housed with same sex littermates throughout the duration of the study. All social defeat exposures were conducted during dark cycle hours and video recorded for post-testing blinded scoring of behaviors via Noldus Observer XT.

Social Defeat Testing*: Oxtr* KO and WT adult males (C57BL/6J background, ~22–30 g, ~90-100 days old, n = 10 per cohort) experienced daily social defeat exposures for three consecutive days [n=10 per genotype per cohort; 2 cohorts of animals tested—the experimental cohort was socially defeated while the control cohort was sham handled]. The behavioral paradigm was based on a resident-intruder protocol in which *Oxtr* KO and WT animals were introduced for 30 minutes into a resident animal's homecage.

***Physiological temperature acquisition*.** All animals were measured for changes in body temperature via implanted telemetry probes (Mini Mitter, Philips Respironics, Andover, MA, USA). G2 E-mitter (15.5 mm x 6.5 mm / 1.1 g) telemetry probes were utilized in this study and removed surgically prior to magnetic resonance scanning. The wireless radio transmitters were implanted through subcutaneous insertions at the midline of the lower backs of the mice. During

resident-intruder exposures, cages were placed atop a receiver platform (model ER-4000, Mini Mitter) that relayed all body temperature and mobility measurements to a PC workstation. The E-mitter implantable probes also measured activity counts of mobility, but that data was not analyzed in this study due to time limitations of the investigator and the absence of a standardized method to interpret the activity count values.

4.2.4 Results

***Social stress induces acute and chronic hyperthermia in Oxtr KO mice*:** To examine oxytocin receptor-dependent HPA activity, sympathetic physiological response in the form of body temperature recordings was monitored. Utilizing wireless radio transmitter telemetry implants, body temperature was measured at time points prior to and during each 30 minute daily social defeat encounter over the 3-day time course. Socially defeated *Oxtr* KO animals demonstrated higher baseline body temperatures during the 10 minute temperature acquisition period collected prior to Day 2 and Day 3 social defeat encounters ($P<0.05$, $n=10$, repeated measures ANOVA, **Figure 3.10a**) compared to that of socially defeated WT mice (and all non-socially defeated animals). Moreover, acute normalized body temperatures of socially defeated *Oxtr* KO mice grew increasingly elevated with successive social defeat encounters ($P<0.05$, $n=10$, repeated measures ANOVA, **Figure 3.10b**) compared to that of socially defeated WT mice (and all non-socially defeated animals). Thus, while socially defeated WT groups also exhibited similar trends in both acute and chronic hyperthermia, *Oxtr* KO animals demonstrated consistently more elevated body temperatures compared to that of WT mice during both baseline and social defeat time points. *Oxtr* KO and WT control groups not subject to social defeat did not indicate any signs of increased body temperatures over the 3-day time course (**Figures 3.10a and 3.10b**).

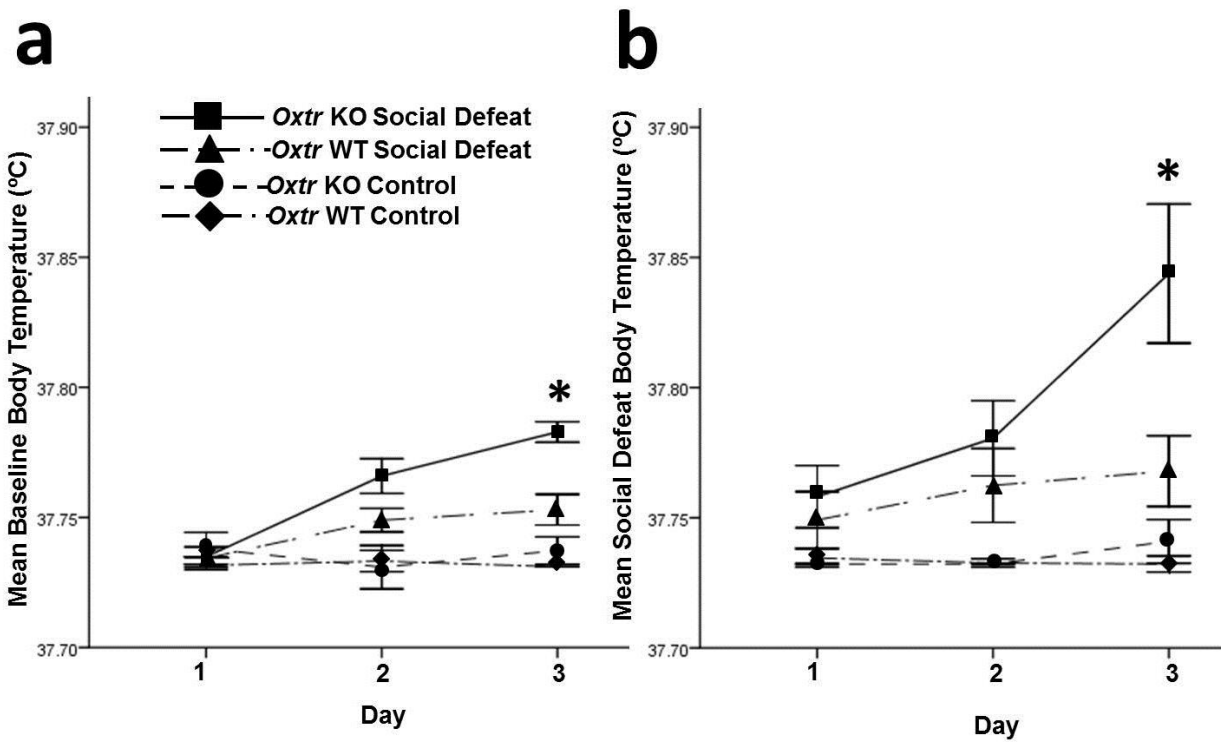

Figure 4.1 Mean body temperature measurements for *Oxtr* KO and WT mice before (a) and during (b) social defeat. a) Baseline body temperatures measured prior to social defeat for *Oxtr* KO and WT mice over a 3-day social defeat period. b) Mean body temperatures measured during social defeat for *Oxtr* KO and WT mice over a 3-day social defeat period (* $P<0.05$).

4.3 SUMMARY

Through evaluating a secondary measure of social fear conditioning via physiological response, this research determined that core body temperatures are differentially regulated in *Oxtr* KO and WT adult male mice when subjected to a 3-day social defeat paradigm. It was observed that social stress (i.e., exposure to aggressive and dominant behaviors exhibited by a conspecific) induced acute hyperthermia in *Oxtr* KO mice *during* social defeat encounters. Intriguingly, evidence of early chronic hyperthermia was also observed over the 3-day period—as indicated by the increasing body temperature measurements collected from *Oxtr* KO animals

prior to social defeat exposures that exceeded increases in body temperature exhibited by WT animals.

Results from this study are intriguing considering the many scientific findings linking oxytocin to both social behaviors as well as neuroendocrine control of the autonomic system in both animals and humans. In a study conducted with voles, it was observed that administration of an oxytocin solution decreased isolation-induced social stress—as measured by decreases in autonomic cardiovascular function (Grippo, Trahanas, Zimmerman, Porges, & Carter, 2009). Other rodent studies have noted specific sympathetic modulation as measured by changes in body temperature due to acute and repeated social defeat (Bhatnagar et al., 2006), as well as systemic and central injection of oxytocin (Ring et al., 2006). In comparison to animal models, human studies have demonstrated analogous results; research has shown that increased plasma OXT levels in female participants are correlated with decreases in sympathetic response (Grewen & Light, 2011) as measured by lower levels of cardiovascular and sympathetic activity after exposure to a stressor. Another study administering intranasal OXT was found to diminish electrodermal activity in neurotypical participants during the processing of emotion classification tasks (Gamer & Buchel, 2012).

In the context of existing research in both animal models and humans that demonstrate the anxiolytic relationship between oxytocin and stress reduction as measured by decreases in sympathetic activity, results from this study indicating increased sympathetic activity (as measured by body temperature) in *Oxtr* KO mice during social defeat supplements existing scientific literature by exhibiting how a model system lacking oxytocin receptors may respond when subject to a social stressor. These responses were measured behaviorally, via neuroimaging, and physiologically to indicate that the absence of oxytocin receptors in the brain and body may facilitate heightened sympathetic arousal and manifest behaviorally as a submissive-like fear response when the animal is presented with a threat. Moreover, the measurement of submissive-like behaviors and elevated sympathetic response to social stress is linked to observations of dampened amygdalar activation in the medial and basolateral nuclei.

Based on previous literature indicating oxytocin's strong role as an anxiolytic modulator as measured by sympathetic activity in humans and animals, the acute and chronic hyperthermia observed in this study may indicate *both* increased fear and anxiety in the *Oxtr* KO mice. In addition, the indication of more freezing and fleeing behaviors demonstrated by Oxtr KO mice (interpreted in this study as a submissive-like behavioral response), may be further explored as a potential marker of a behavioral deficit in response to social threat.

Since response to non-social threats were not compared in these studies, it is not possible to determine if the neural, physiological, and behavioral outcomes in this *Oxtr* KO mouse model is specific to social threat. However, the data in these experiments nonetheless aid in connecting the behavioral implications of oxytocin in social situations to its physiological impact on autonomic control in mammals. These findings further support the established evidence base mentioned in Section 4.2.1, which discusses the research linking the sympathetic control of temperature regulation to fear learning in mammals, and the human studies research examining Oxt and Oxtr's relationship to regulation of sympathetic response. Previous scientific research in conjunction with this study on thermoregulation in socially defeated Oxtr KO mice all contribute to the hypothesis that Oxtr is an important neuromodulator in sympathetic activity, and is likely directly involved in elicitation of downstream sympathetic response (e.g., body temperature, galvanic skin response, etc.) during the processing of fear-inducing stimuli.

Body temperature, however, is but one measure of autonomic regulation and cannot comprehensively characterize the full impact of oxytocin on sympathetic activity. It is therefore a key future direction of this research that additional measures of autonomic response (e.g., heart rate, respiratory rate) also be evaluated. Moreover, the role of oxytocin receptors in the brain versus peripheral populations of OXTR in the anxiolytic control of the sympathetic response cannot be determined in this research. Thus, it is important that conditional *Oxtr* KO models also be explored to identify the precise pathways through which oxytocin influences the processing of social information to elicit certain physiological responses.

Chapter 5: Examination of social fear processing in people with ASC and controls

The need for scientific understanding of the relationship between anxiety disorders and autism spectrum conditions grows as documentation of autism comorbidities rises among communities. Various studies have indicated high incidence of comorbid psychiatric disorders, which range from 70 to 80% among individuals with ASC (Lai, Lombardo, & Baron-Cohen, 2014; Simonoff et al., 2008). However, little is understood concerning the shared neurobiological pathways and mechanisms that may influence autistic symptoms and psychiatric health in patients with ASC.

As discussed in Chapter 1, Section 1.3.1, many researchers are considering whether aberrant fear processing may be responsible for increased comorbidity for anxiety disorders and social phobias among individuals with ASC due to the central processing of fear and anxiety behaviors in the amygdala. While multiple researchers have brought forth data citing elevated risk of social anxiety and social avoidance among adults and adolescents with ASC (Kuusikko et al., 2008; Simonoff et al., 2008), general anxiety was also found to be increased in high-functioning children with ASC in a UK study (Gillott, Furniss, & Walter, 2001). A cohort of children in Canada with ASC and Asperger syndrome have also been found to exhibit higher rates of both anxiety and mood disorders (such as depression) (J. A. Kim, Szatmari, Bryson, Streiner, & Wilson, 2000). Not only are adolescents with ASC found to display high levels of anxiety relative to neurotypical individuals, the increased anxiety has also been found to be correlated with deficits in social skills (Bellini, 2004). The comorbidity of anxiety and social deficits among individuals across the autism spectrum is a strong indicator that emotional processes underlying anxiety may be involved in the modulation of social behaviors in children and adults with ASC. It is thus important that the neurobiological and physiological mechanisms affiliated with both anxiety and ASC be thoroughly explored and characterized with the goal of understanding the development of social deficits in ASC. While it is acknowledged that variations that exist between the neural responses that underlie fear and anxiety, both states are regulated by amygdalar response. This thesis will not dissect the nuances of acute social fear in contrast to social anxiety, but will consider the role of amygdalar activity involved in both.

5.1 CLASSICAL FEAR CONDITIONING IN A SOCIAL CONTEXT AS A PARADIGM OF SOCIAL ANXIETY IN HUMANS

A target of interest in the examination of biological processes underlying the social phenotypes and anxiety-related comorbidities exhibited by individuals with ASC is the amygdala. One key reason is the central role of the amygdala in fear acquisition and memory, which is also a region of the brain highly implicated in abnormal activity and morphology in ASC functional imaging and post-mortem studies. Traditionally, the amygdala is believed to be key in the associative learning of fearful behaviors in relation to specific contextual factors due to its general role in discerning and selectively relaying cues from the environment to other regions of the brain (Adolphs, Tranel, Damasio, & Damasio, 1995; Hariri & Whalen, 2011; Kim et al., 2011; Kliemann, Dziobek, Hatri, Baudewig, & Heekeren, 2012). The "amygdala theory of autism" (S. Baron-Cohen et al., 2000) emphasized the importance of the amygdala as a chief regulator of the network of neurons that comprised what is termed to be the "social brain". While research has demonstrated links between amygdalar responsivity to visual processing tasks of social stimuli in humans (Whalen et al., 2004), it has also been found that administration of oxytocin can modulate the level of responsivity within the amygdala during the processing of social cues (Kirsch et al., 2005). The combined evidence of the amygdala's functions as a salience detector during associative fear learning and as a key modulator of the processing of social stimuli collectively serves to promote the hypothesis that the amygdala is an important region of the brain that may influence downstream manifestation of social deficits observed in individuals with ASC.

The utilization of classical fear conditioning as a model of associative fear learning is thus an essential tool in the examination of the amygdala's involvement in the development of social deficits observed among individuals with ASC. This research thus employs a classical fear conditioning paradigm as a model for anxiety processing in a social context.

5.2 PSYCHOPHYSIOLOGICAL ASSESSMENT OF RESPONSE TO SOCIAL FEAR IN ASC CASES

Non-invasive physiological recording are commonly utilized techniques with widespread appeal in psychological studies due to their ability to provide insight into neural processes regulating autonomic pathways. A prevalent method of gauging psychophysiological response is the measurement of skin conductance response (SCR). Favored as a robust and feasible technique to evaluate the sympathetic nervous system as a proxy for physiological and psychological response, SCR is a method that effectively measures the level of electrical resistance in the skin as a function of sweat gland activity (an indicator of sympathetic arousal) (Vetrugno, Liguori, Cortelli, & Montagna, 2003).

Due to its function as an index of sympathetic arousal and its advantages as a non-invasive technology, SCR measurement has been broadly utilized in ASC research. SCR has been employed to investigate sympathetic nervous system response in children with ASC while viewing face images, where researchers observed that arousal levels correlated with the degree of social impairments (i.e., social communication skills) (Kaartinen et al., 2012). In another study evaluating social processing employing SCR, investigators distinguished phenotypic subgroups among high-functioning adults with ASC—which was found to correspond to resting-state SCR levels (Mathersul, McDonald, & Rushby, 2013). In an emotional processing task that involved evaluation of facial expressions, it was observed that individuals with ASC exhibited decreased SCR levels compared to that of control subjects (Hubert, Wicker, Monfardini, & Deruelle, 2009). Researchers have also sought to elucidate the amygdala theory of autism through a series of classical fear conditioning experiments conducted on high-functioning individuals with ASC. Utilizing non-social stimuli, researchers in one study paired solid color tiles with unconditioned stimuli (i.e., foghorn recording) and measured SCR in test subjects with Asperger syndrome. Interestingly, investigators found that individuals with Asperger syndrome demonstrated attenuated physiological fear responses to conditioned, non-social stimuli (as measured by SCR) (Gaigg & Bowler, 2007).

5.2.1 Objectives

As established in the introduction (Chapter 1), associative learning of threatening stimuli is a key process in the exhibition of typical behaviors and has been implicated in the development of social behavioral phenotypes for mood disorders and autism spectrum conditions. To assess the psychophysiological correlates of social fear response in individuals with ASC, this research extended its scope to measure autonomic outcomes in humans experiencing feelings of fear in a social context. Through the translation of methodologies conducted in the *Oxtr* KO mouse models assessing fear-related social behaviors, this research employed analogous technologies in a human cohort of ASC cases and neurotypical controls.

This chapter describes the human study conducted in this research to evaluate the psychophysiological measures of social fear conditioning in high-functioning adults with ASC and neurotypical age-matched controls. Research aims and methods utilized to examine the psychophysiological responses to social fear processing in individuals with ASC (and control participants) are delineated in this chapter. Through the results gathered in this human study, this research seeks to understand the biological pathways modulated during social fear processing and to observe for any phenotypic variations in physiological response in individuals with ASC. Results from this study will be important in the exploration of underlying mechanisms implicated in cognitive activities involved in social fear processing that may provide insight into general fear-related social behaviors associated with developmental and mood disorders.

Furthermore, the results from the mouse model experiments in this research present a unique opportunity to evaluate the translational value drawn from the psychophysiological experiments conducted on the *Oxtr* KO mouse model. The findings in this research concerning increased submissive behaviors in *Oxtr* KO mice and the physiological evidence of both acute and chronic hyperthermia during the processing of fearful stimuli in social contexts all point to the involvement of the amygdala in the downstream regulation of social behavior and autonomic responses in the sympathetic nervous system. Therefore, the basis of the human studies portion

of this dissertation is established upon the relationship between amygdalar activity and modulation of the sympathetic nervous system as measured by changes in skin conductance response—a relationship that has been previously established in both primate models and humans (Laine, Spitler, Mosher, & Gothard, 2009; Williams et al., 2001).

5.2.2 Hypothesis

In this study examining psychophysiological response to social fear conditioning, it was hypothesized that participants with ASC will demonstrate elevated skin conductance levels while undergoing a classical fear conditioning paradigm within a social context. Existing research examining skin conductance arousal during non-social fear conditioning in high-functioning children with ASC have demonstrated evidence that SCR is positively correlated to social anxiety (South et al., 2007). Furthermore, previous studies have found that the intensity of eye-gaze (direct vs. indirect) in individuals with ASC to also be positively correlated to symptom severity---specifically concerning social behaviors (Kaartinen et al., 2012; Kyllainen et al., 2012). However, there is also evidence of impaired fear learning as indicated by diminished skin conductance response in high-functioning individuals with ASC during fear conditioning in non-social contexts (Gaigg and Bowler, 2007).

Considering the existing evidence of SCR arousal in response to social stimuli and decrease SCR during fear conditioning in individuals with ASC, it is unclear how such individuals may autonomically respond to fear conditioning conducted in social contexts. Based on the data collected from the *Oxtr* KO animal model examined in this research, where Oxtr KO mice exhibited elevated body temperatures in response to social defeat, in conjunction with existing evidence in the literature that individuals with ASC display signs of increased autonomic response when presented with social stimuli, this research hypothesizes that high-functioning individuals with ASC will demonstrate elevated SCR during social fear conditioning.

Due to time and resource limitations, SCR was selectively chosen as the main physiological factor of interest for the observation of sympathetic response in this behavioral paradigm. In comparison to peripheral body temperature, skin conductance response is capable of producing discrete, event-related signals that directly indicate sympathetic activity. Despite being utilized in the *Oxtr* KO animal model (SCR is difficult to observe in mobile animals), body temperature is not as capable in producing discernible event-related responses in humans and is thus a less informative psychophysiological tool in this behavioral paradigm {Dawson, 2007 #187}.

5.2.3 Methods

Participant selection: Participants in this study consisted of adult subjects drawn from the student community and general population in the Cambridge area and surrounding regions. Two cohorts of subjects were tested: A) 23 adults (15 male, 8 female) with a diagnosed ASC between the ages of 19-45 years, B) 23 typically developed (neurotypical) adults (7 male, 16 female) between the ages of 19-45 years. Requirements for inclusion in the ASC case cohort consisted of diagnosis for any ASC. Individuals co-diagnosed with ASC and attention deficit hyperactivity disorder (ADHD) or attention deficit disorder (ADD) were also permitted to participate in this study. Subjects in the ASC-case cohort thus included those with high-functioning ASC, and Asperger Syndrome (as diagnosed in accordance to criteria in the DSM-IV). ASC cases were diagnosed by clinicians independent of this study. Participants were excluded from both the ASC-case and control cohorts for such conditions as: pregnancy, presence of specific comorbidities (e.g., multiple sclerosis, cancer, Parkinson's disease, cardiovascular diseases). Participants were also excluded if they suffered from any respiratory illnesses or had a history of epilepsy. For a full list of inclusion and exclusion criteria, please view **Appendix A**. All participants were matched in age, body mass index (BMI), and IQ. Participants were not matched in gender due to time constraints on recruitment and the decreased prevalence of women with a diagnosis for ASC in the Cambridge, UK region.

Participant testing: Prior to arriving for the psychophysiological testing, all participants completed a pre-appointment questionnaire, which asked individuals for information on age, sex,

ethnic background, height, weight, past and current medical conditions, and medications. In addition to the pre-appointment questionnaire, participants were invited to respond to a series of online tests offered by the Autism Research Centre as follows: the Autism Spectrum Quotient (AQ, adult) (S. Baron-Cohen, Wheelwright, Skinner, Martin, & Clubley, 2001), Empathy Quotient (EQ, adult) (S. Baron-Cohen & Wheelwright, 2004), Systemizing Quotient-Revised (SQ, adult) (Simon Baron-Cohen, Richler, Bisarya, Gurunathan, & Wheelwright, 2003), Eyes Test (adult) (S. Baron-Cohen, Jolliffe, Mortimore, & Robertson, 1997), Emotional Faces Task (KDEF) (Lundqvist, Flykt, & Ohman, 1998), Mental Rotation Task (Vandenberg & Kuse, 1978), Embedded Figures Test (Witkin, Oltman, Raskin, & Karp, 1971), and the Ravens Progressive Matrices Test (Raven, 1965). Only participant scores for the AQ and EQ tests were evaluated in this research as the SQ, Eyes Test, KDEF, Mental Rotation Task, Embedded Figures Test, and Ravens Progressive Matrices were collected for the purposes of parallel and independent research. For the full pre-appointment questionnaire, see **Appendix B**.

Recruitment network: Recruitment for participants in this study were conducted using several methods that included referral from affiliated National Health Services (NHS) hospital networks, recruitment from the Autism Research Centre (ARC) participant database, Cambridge University student communities, National Autistic Society: Cambridge Branch, and local advertisements. All participants were informed of the study protocols prior to participation and provided written consent in accordance with Cambridge University's Department of Psychiatry's Research Ethics committee. Participants also received monetary reimbursement for their time.

Psychophysiology protocol: Participants arrived at Cambridge University main campus for all testing procedures. 12 hours prior to testing, subjects were advised to refrain from eating any food and drinking any fluids (specifically those containing caffeine, as it is a known stimulant with direct impact on sympathetic nervous activity (Corti et al., 2002), and thus could present as a potentially confounding factor in this research), with the exception of water. Upon arrival at the testing facility, participants were briefed on the study objectives/procedures, and signed a written consent form.

***Hardware and participant setup*:** Participants were seated at a desk within a soundproof booth for the psychophysiology paradigm and prepped for skin conductance measurement. Regions of the participants' skin near the inner ankles and wrists were thoroughly cleaned prior to adhesion of the electrode pads (containing electrolyte (GEL101, 0.5% Saline; *Biopac Systems, Inc*.). Adhesive electrodes were placed on the inner surfaces of both wrists and the inner area of the left ankle. Two Ag-AgCl electrodes were also filled with electrolytic gel and secured to the distal regions of the first and second phalanges on the left hand (see **Figure 5.1**). Subjects were briefed on the protocol's instructions, informed to remain as still as possible throughout the duration of the test, and provided a headset that transmitted sounds during the protocol.

All data was collected using the Biopac Systems acquisition hardware. The Biopac system consisted of the base module (MP150) and skin conductance amplifier (GSR100C). All Biopac acquisition parameters were calibrated prior to subject setup. Gain was set to 5 microsiemens (μS)/volt for all data acquisition and sampling rate was set to 1000 Hz. Electrolyte gel was applied at the interface of the electrode pads and the subjects' ankles, fingertips, and wrists, and allowed to soak for at least 5 minutes prior to data acquisition. A 5-minute baseline acquisition period was first collected prior to the initiation of the social fear conditioning protocol. Channels in the Biopac-compatible Acqknowledge acquisition software collected GSR and stimuli (or "event") data, which were scored offline for the evaluation of event-related SCR magnitudes. Stimuli for the social fear conditioning protocol were visually presented using a computer interface programmed with the E-prime psychological testing software.

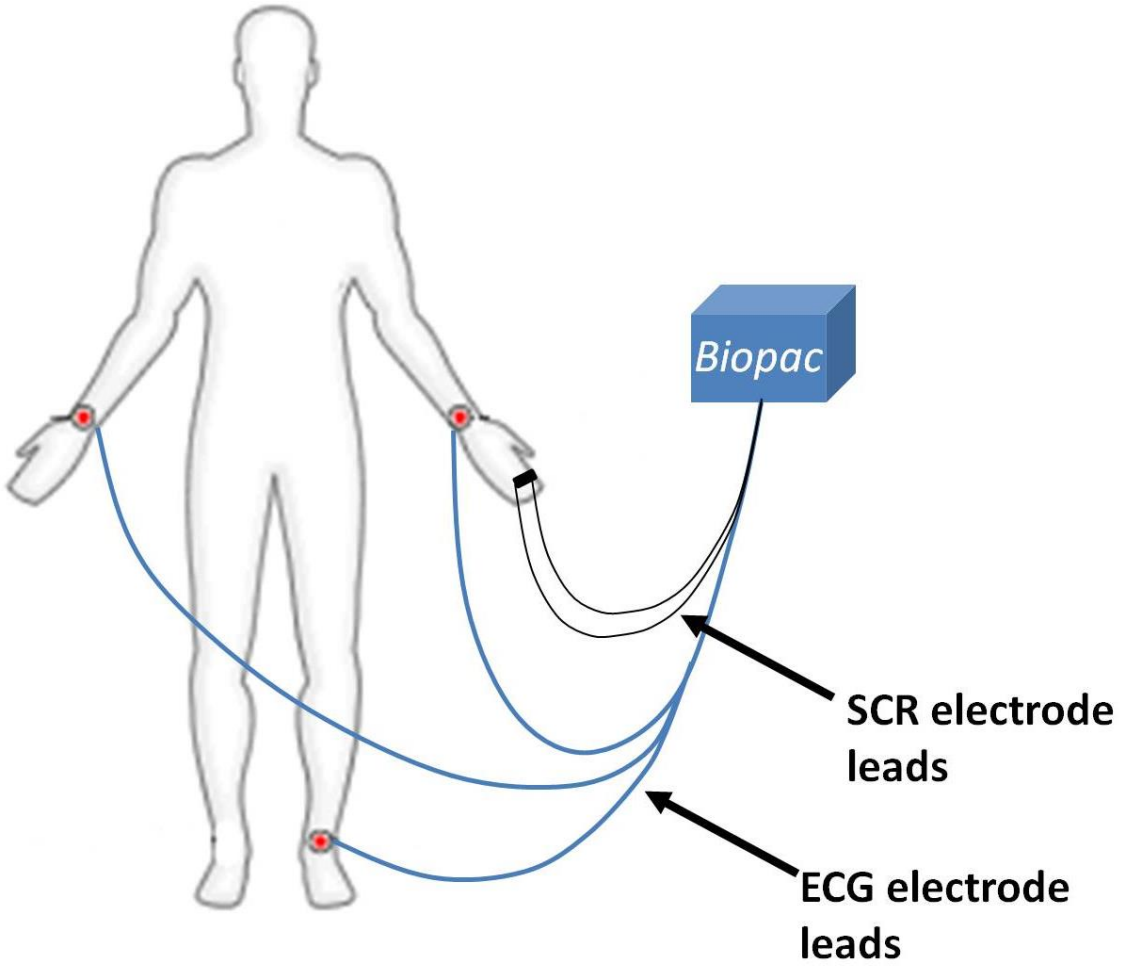

Figure 5.1 Physiological acquisition setup for skin conductance response collection

Social Fear Conditioning Protocol Design: E-prime is a programmable visual interface that allows for the design of computer tasks and presentation of stimuli. The E-prime social fear conditioning protocol employed in this psychophysiological study consisted of a 3-phase paradigm composed of habituation, acquisition, and extinction learning trials (see **Figure 5.2**). Containing a total of 42 trails, the conditioning protocol presented a mock computer task to the participant that involved the presentation of a series of visual images. Each image was depicted over a black background on a 15" computer monitor and consisted of either social or non-social representations. Social images consisted of photographs of female or male faces emoting neutral affect or object images of neutral emotional association (e.g., table, cup, spoon). All CS+ and

CS- stimuli were drawn from the International Affective Picture System (IAPS). All unconditioned auditory stimuli were drawn from the International Affective Digital Sounds (IADS) database (Bradley & Lang, 1999; Lang, Bradley, & Cuthbert, 2008).

After a 5-minute baseline acquisition period where no stimuli were presented to participants, participants were introduced to the protocol with a screen displaying instructions on how to complete the mock computer task. Participants were instructed to press the "space bar" on the computer workstation whenever an object image was presented on the screen. After this introductory screen, the Habituation Phase of the protocol was triggered and a series of 14 trials were presented in pseudorandom order.

The 14 trials presented in the Habituation Phase consisted of "pre-conditioned stimuli", which were presented to ascertain the baseline skin conductance response to CS+ and CS- before the introduction of the unconditioned stimulus and determine any differences between subjects and controls. These pre-conditioned stimuli included the 7 CS+ images (which were paired with an unconditioned stimulus during the Acquisition phase) and 7 CS- images (unpaired with an unconditioned stimulus during the Acquisition phase). Images were presented pseudorandomly, and each image was displayed for 5 seconds. CS+ and CS- social stimuli consisted of proportionally distributed male and female faces emoting neutral affect. CS- non-social stimuli consisted of object images of non-emotional association. Inter-trial intervals between each image presentation consisted of a black screen with a centered white cross as an attention fixation point. Inter-trial intervals (ITI) between successive stimulus presentations lasted 8-10 seconds, and were pseudorandomly "jittered" in duration between stimulus presentations. Inter-trial interval range was based on established SCR protocols accompanying classical fear conditioning paradigms (Gaigg & Bowler, 2007) and on piloted results collected during the design of this study [data not shown]. ITI programmed into the E-Prime conditioning paradigm was jittered in duration to reduce participant inattention and boredom—ideally facilitating participant conditioning to the visual stimuli. Participants were instructed to remain seated and still within the sound proof booth during the entire presentation (Habituation, Acquisition, and Extinction) of the social fear conditioning paradigm. To maintain participant visual attention

throughout the 15 minute conditioning paradigm, participants were given a mock task: to press the spacebar whenever an object image was presented on the computer screen. This mock task involved minimal movement in the free hand (not being measured for SCR), and ensured constant attention to the CS+ and CS- presented on the screen.

Following the 14 trials of the Habituation phase, the Acquisition phase was immediately initiated. Consisting of 14 trials, the Acquisition phase again displayed in pseudorandom order the pre-conditioned stimuli as that first presented during Habituation. During the Acquisition phase, the CS+ was paired to an aversive auditory stimulus (unconditioned stimulus, UCS), a shrill vocalization from a human female voice (Bradley & Lang, 1999). The UCS was transmitted at 100 decibels (dB) via headphones to the subject and lasted 2 seconds. The UCS was paired to either a male or female face for each protocol, and was presented to the participant 7 times during the Acquisition phase, a level of exposure sufficient to differentiate SCR between CS+ and CS- images. The pairing of the UCS to male or female faces was counterbalanced across participants.

In the final phase termed Extinction, 14 total trials were again presented where 7 CS- and CS+ (same as that displayed in the Habituation and Acquisition phases) were presented in the Extinction phase without the UCS in pseudorandom order to determine the persistence of conditioning through duration of elevated SCR to CS+ over CS= images After the end of the conditioning protocol, electrodes were removed from the subjects, participants were compensated for their time, and all individuals debriefed on the study prior to departure from the testing facility. The entire psychophysiological testing session lasted 30 minutes, which included participant preparation time and the 15-minute conditioning protocol.

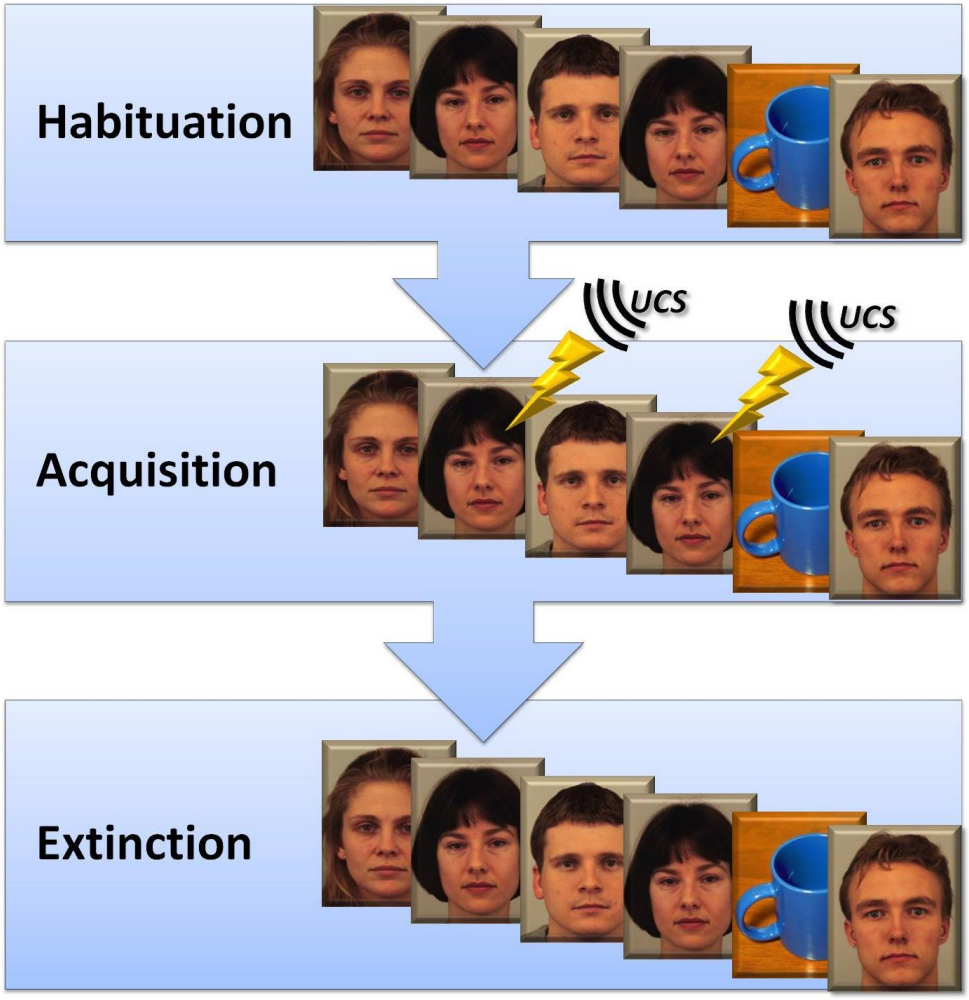

Figure 5.2 Human social fear conditioning paradigm. Three-part conditioning paradigm utilizing unconditioned and conditioned stimuli paired with human face or inanimate object images.

SCR Data Acquisition and Preparation: Stimulation presentation during the conditioning protocol was conducted using E-Prime, a psychology software tool. Skin conductance response data was acquired using Biopac Systems, Inc., which recorded stimulation events and physiological measurements. Acqknowledge, an acquisition software, was run on a desktop workstation outside a sound-proof testing booth, which allowed for the participant's SCR to be monitored continuously throughout the procedure. All SCR data were reviewed prior to analysis

for artifacts, recording errors, or hardware malfunctions. SCR data for one participant were excluded from the study due to physiological data acquisition difficulties. Stimulus events recorded in 3 different digital channels (marking CS+ and CS- stimuli in the Habituation, Acquisition, and Extinction phases) were transferred to the SCR channel for electrodermal event-related analysis on Acqknowledge. Offline, a 2 Hz low-pass filter was applied to all raw SCR data to filter noise recorded from electromagnetic disturbances. A 0.5 Hz filter was also applied to transform the phasic electrodermal activity (EDA, raw skin conductance signal) to tonic EDA.

Utilizing the transferred stimulus events from the digital channels, event-related SCR's (peak response signal associated with CS+ or CS-) were identified on the filtered signal and their magnitudes measured. Parameters restricting event-related SCR's consisted of specified time frames where onset of related events initiated between 1-3 seconds after each CS+ or CS- stimulus event. An EDA threshold was set at 0.02 microsiemens and all SCR's less than 10% of the maximum were rejected from the analysis. Peak amplitudes for event-related SCR's were calculated by subtracting baseline SCR measurements (measured immediately prior to the pairing of the UCS to the CS+) from the maximum SCR measurement within the defined latency window or 1-3 seconds after the CS+ presentation.

Through these pre-analysis processing methods, physiological responses to social fear conditioning were measured. Using the filtering techniques and identification of event-related SCR's, these methods effectively retrieved all SCR amplitudes corresponding to specific stimulus events for further statistical analysis.

Statistical analysis*:* Baseline or resting state physiological arousal was measured from a 5-minute acquisition period prior to the initiation of the Habituation phase of the conditioning protocol. One-way ANOVA statistical tests were applied to the baseline SCR recordings of ASC cases and control groups to detect any significant differences in resting state potentials between groups. One-way ANOVA statistical tests were applied to the measurements of combined CS+ and CS- responses in ASC cases and control groups during the Habituation phase to see any significant differences in CS response prior to UCS pairing.

Amplitudes of event-related SCR's corresponding to CS+ and CS- were assessed via repeated measures ANOVA using CS+ and CS- as the within-subjects factors. Normalized event-related SCR's were also evaluated using repeated measures ANOVA with ASC cases and control groups as the between-subjects factors. All statistical tests were conducted using SPSS 17.0. SCR data were evaluated for main effects between CS+ and CS- and between ASC cases and control groups *(P*<0.05). All 7 trials of the CS+ and CS- were included in the comparisons of the habituation response between diagnostic groups. The last four trials of the Acquisition Phase and the last four trials of the Extinction Phase were evaluated between groups. Repeated measures ANOVA were employed to examine Acquisition and Extinction event-related SCR over the last four trials of each phase (where mean event-related SCR for each phase were considered dependent variables of interest). Difference values between CS+ and CS- SCR readings were also examined in ASC cases and control groups.

Participant scores drawn from the autism and empathy quotient tests conducted online prior to each appointment were also analyzed in relation to diagnosis in both controls and participants with ASC. SCR data between subjects were examined according to phases of the conditioning protocol.

5.2.4 Results

Differences between baseline SCR measurements (collected during resting state) of autism cases and controls were first evaluated. No significant differences were observed between ASC cases and controls during this baseline measurement phase in which participants remained at rest without visual displays or auditory stimuli while seated in a soundproof booth prior to initiation of the social fear conditioning protocol (Independent Samples t-test, unequal variances; ASC Mean=0.096, S.E.=0.008; Control Mean=0.078, S.E.=0.012; t(39)=-0.79, p=0.434; **Figure 5.3**).

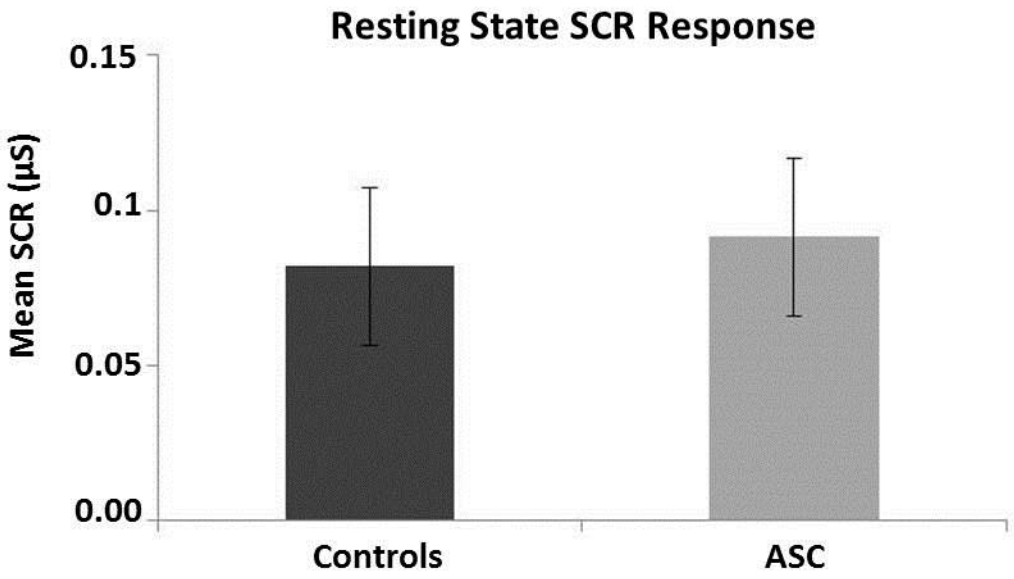

Figure 5.3 Baseline SCR responses in ASC cases and controls. Mean SCR responses collected during resting state in ASC subjects prior to social fear conditioning indicate no significant differences compared to that of controls [*P*>0.05; error bars indicate S.E.M].

Habituation Phase Results: One-way ANOVA was conducted between SCR measurements of ASC cases and controls during the Habituation Phase of the social fear conditioning protocol. Autism cases demonstrated significantly elevated SCR measurements compared to control subjects when presented visual stimuli consisting of neutral faces. Thus, there was a significant effect for autism diagnosis on SCR response during the Habituation phase of social fear conditioning ($F(1,1)=102.3$, $p=3.9E-06$) (see **Figure 5.4**). The null hypothesis that control subjects and autism cases have the same SCR measurement responses during Habituation was subsequently rejected.

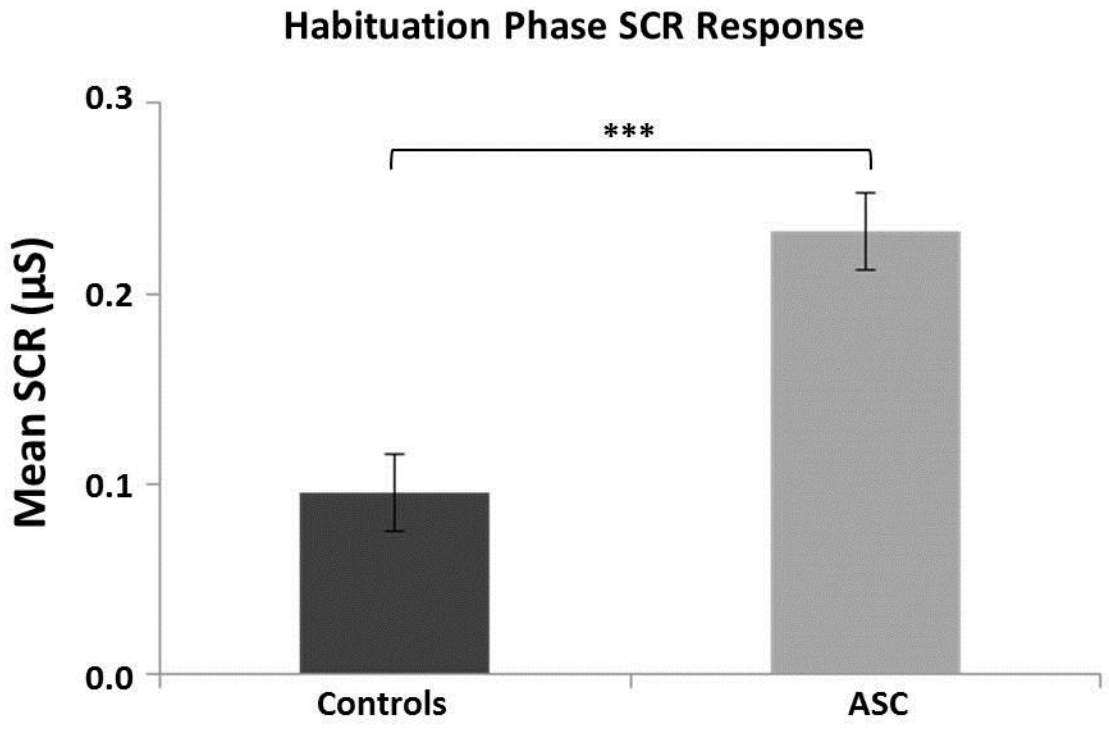

Figure 5.4 Habituation Phase SCR responses in ASC cases and controls: Mean SCR responses to conditioned stimuli (prior to pairing) in ASC subjects during the Habituation Phase of social fear conditioning indicate significant differences compared to that of controls [$p<0.001$; error bars indicate S.E.M].

***Acquisition Phase Results*:** A repeated measures ANOVA was utilized to evaluate the Acquisition Phase for main and interacting effects. Skin conductance response was assigned as the measurement variable and autism diagnosis (cases vs. controls) and stimulus type (conditioned stimuli (CS+) and non-conditioned stimuli (CS-)) as the nominal variables. Using SPSS, significance between subjects by ASC diagnosis was first evaluated. Results indicated statistically significant interaction effects between CS-type and ASC diagnosis [$F(1,1)=6.69$, $p= 0.0123$]. Thus it may be concluded that the main effect for ASC diagnosis on SCR response during the Acquisition Phase of fear learning is significantly influenced by the specific stimulus type being administered during the trials (CS+ vs. CS-) [Figure 5.5].

A repeated measures ANOVA results also confirmed the hypothesis that control subjects would demonstrate significantly different SCR responses to CS+ stimuli than that of ASC cases at the end of fear learning [F(1,1)=13.18, p=0.0012]. No main effects by CS-type was observed in ASC cases [F(1,1)=4.38, p=0.046]. Moreover, no significant main effects were observed by diagnostic group when examining the CS- stimulus type specifically [F(1,1)=2.31, p=0.14] (see **Figure 5.6**).

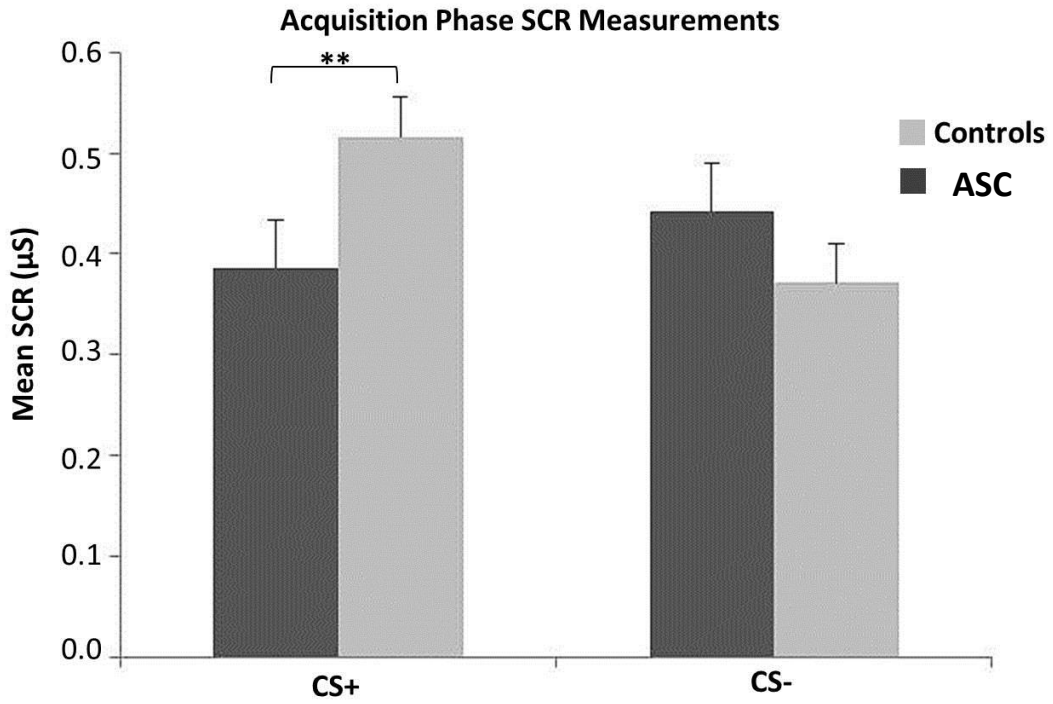

Figure 5.5 Acquisition Phase SCR Responses to CS+ and CS- in ASC cases and control subjects during fear learning. Control participants demonstrate significantly different SCR responses to CS+ stimuli than to CS- stimuli at the end of fear learning [p<0.01; F=13.18, p=0.0012; error bars indicate S.E.M].

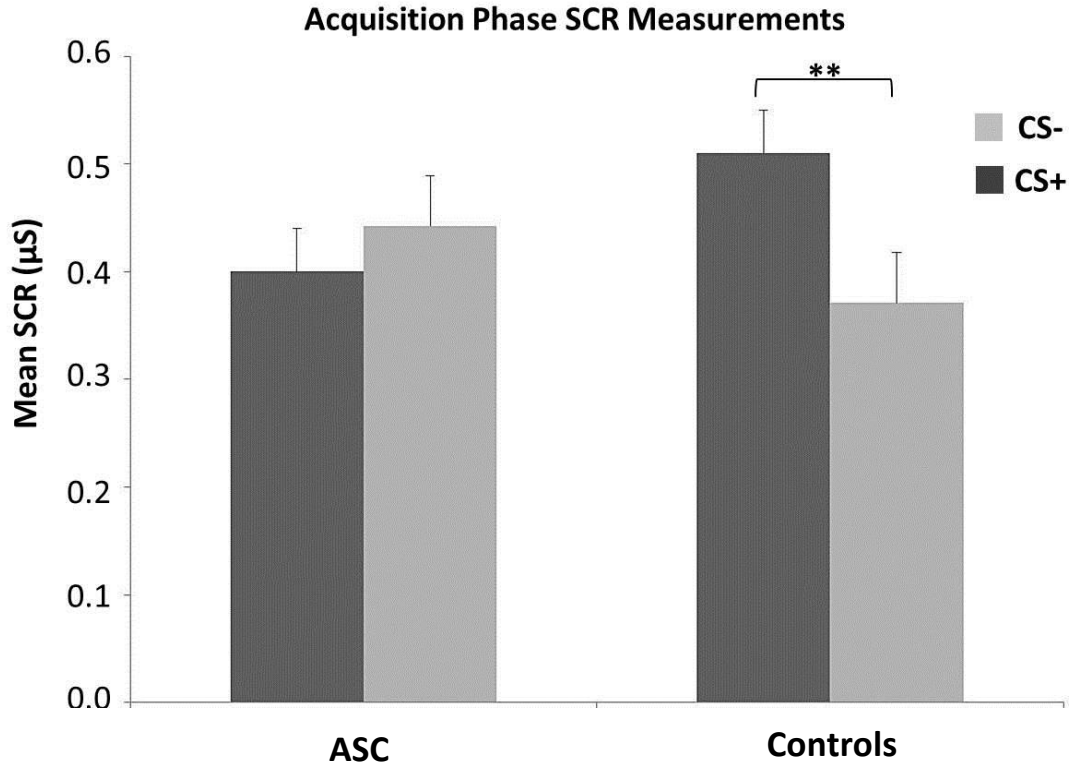

Figure 5.6 Acquisition Phase SCR responses to CS+ and CS- in ASC cases and control subjects during fear learning. Control participants demonstrated significantly different SCR responses to CS+ stimuli than to CS- stimuli at the end of fear learning [$P<0.01$; F=13.18, P=0.0012; error bars indicate S.E.M].

Extinction Phase Results:

A two-way ANOVA with replication was conducted in SPSS. Results indicated no significant interaction effects between CS-type and ASC diagnosis [F=0.161, p= 0.689]. Thus it may be concluded that the main effect for ASC diagnosis on SCR response during the end of the Extinction Phase of fear conditioning is not significantly influenced by the type of stimulus being administered during the trials (CS+ or CS-).

A repeated measures ANOVA was utilized to also evaluate the last four trials of the Extinction phase for main and interacting effects. With skin conductance response again as the

measurement variable and ASC diagnosis (cases vs. controls) and stimulus type (conditioned stimuli (CS+) and non-conditioned stimuli (CS-)) as the nominal variables, SPSS was used to assess for significance between subjects by stimulus response type (CS+ vs. CS-) in ASC cases and control participants. The repeated measures ANOVA results indicating elevated SCR to both CS+ and CS- after fear conditioning in individuals with ASC demonstrated that between subjects diagnosis classification is linked to significant main effects at the end of fear extinction [F(1,1)=10.065, p=0.002]. As seen in **Figure 5.7**, ASC cases exhibited elevated SCR levels to both CS+ and CS- at the end of fear extinction compared to that of control subjects. The repeated measures ANOVA results confirmed the null hypothesis that control participants and ASC cases would not demonstrate significant main effects within subjects by CS type at the end of Fear Extinction, as seen in **Figure 5.7** [F(1,1)=0.332, p=0.567].

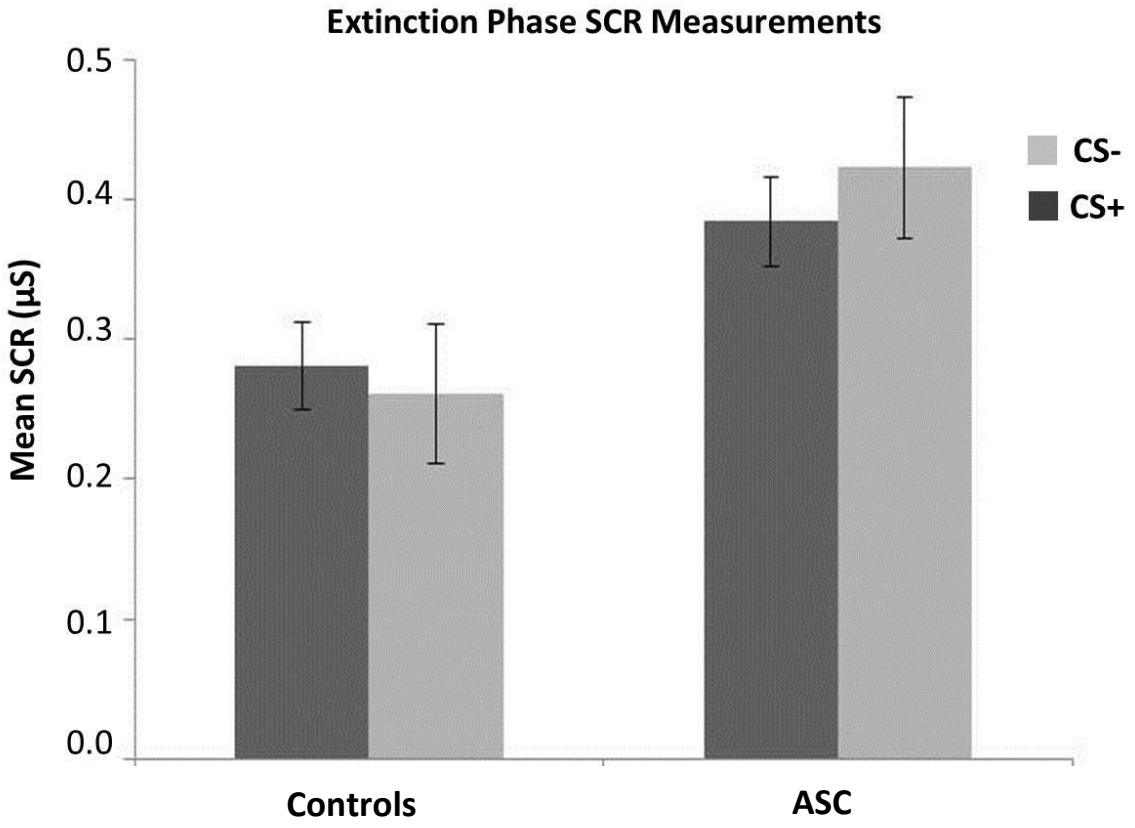

Figure 5.7 Extinction Phase SCR Responses to CS+ and CS- in ASC cases and control subjects after fear learning. The repeated measures ANOVA results confirmed the hypothesis that between subjects diagnosis classification would demonstrate significant main effects at the end of fear extinction [F(1,1)=10.065, p=0.002; error bars indicate S.E.M].

Qualitative Trends in SCR measurements: As exhibited in **Figure 5.8**, the conditioning process became more defined in the last four trials of the Acquisition Phase, which demonstrates upward trends of increasing SCR response to CS+ in both Autism cases and controls (though much more pronounced in control subjects). The SCR responses to CS- is less distinguishable with autism cases indicating a slight increase at the very end of Acquisition, and control subjects indicating a slight decline closer to the end of fear learning. See **Table 5.1** for a quantitative summary of mean SCR values for all ASC cases and control subjects.

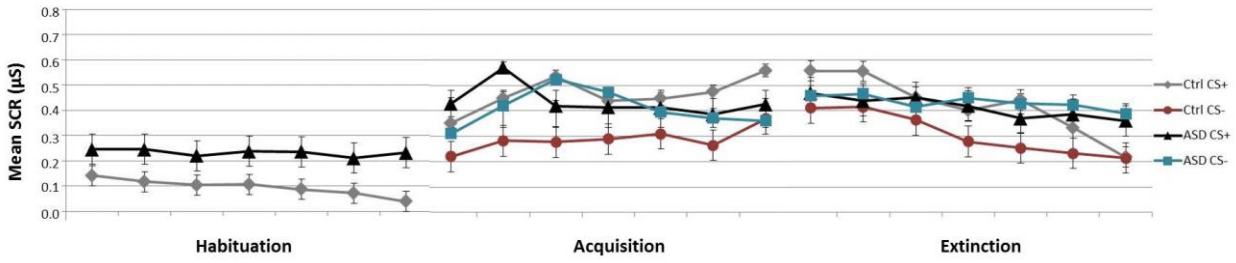

Figure 5.8 Time course of SCR magnitude in ASC cases and control participants before, during, and after fear learning. Error bars indicate S.E.M.

Conditioning Phase	Group		Mean µS (S.E.)	
Baseline	Controls		0.078	(0.012)
	ASC		0.096	(0.008)
Habituation	Controls		0.097	(0.006)
	ASC		0.23	(0.002)
Acquisition	Controls	(CS+)	0.521	(0.001)
		(CS-)	0.375	(0.012)
	ASC	(CS+)	0.401	(0.002)
		(CS-)	0.433	(0.004)
Extinction	Controls	(CS+)	0.281	(0.003)
		(CS-)	0.259	(0.067)
	ASC	(CS+)	0.388	(0.003)
		(CS-)	0.423	(0.042)

Table 5.1: Composite skin conductance response measurements from both ASC cases and control subjects during each phase of social fear conditioning. Mean SCR measurements indicated with S.E. displayed in parentheses. Event-related SCR to CS+ and CS- shown for each test group during the Acquisition and Extinction phases of the paradigm.

Results from Online Psychological Tests: Mean scores (**Table 5.2**) in control participants for the Autism Quotient (AQ) averaged at 16.6 (Standard Error=1.61), consistent with historical data on mean AQ values for individuals without ASC (Woodbury-Smith, Robinson, Wheelwright, & Baron-Cohen, 2005). In comparison, participants with a formal ASC diagnosis exhibited a mean AQ of 40.2 (Standard Error=1.74), also consistent with established findings on AQ scores among individuals with ASC (Woodbury-Smith et al., 2005). Regarding the empathy quotient (EQ), control participants scored well above the established threshold for ASC diagnosis (mean=40.9,

standard error=2.08) while participants with a formal ASC diagnosis scored on average 17.5 (standard error=3.24), which is below the established diagnostic threshold.

Diagnostic Group	Autism Spectrum Quotient (AQ) (S.E.) *max score=50*	Empathy Quotient (EQ) (S.E.) *max score=80*
ASC *(N=23)*	40.2 (1.74)	17.5 (3.24)
Controls *(N=23)*	16.6 (1.61)	40.9 (2.08)

Table 5.2: AQ and EQ results from ASC participants and control subjects. ASC means for both AQ and EQ fell within the established ranges commonly associated with clinical diagnosis for ASC (AQ range = 32-50; EQ range = 0-32). Standard errors indicated in parentheses below each corresponding mean value.

5.3 SUMMARY

This chapter describes the human studies portion of this thesis research, which employs classical fear conditioning in a social context as a paradigm for social anxiety in humans. Utilizing a psychophysiological approach to assess sympathetic nervous response to social stimuli paired with threatening cues, investigators in this research evaluated skin conductance response during the Habituation, Acquisition, and Extinction phases in control subjects and ASC cases. Comparison of results first established comparable baseline (resting state) SCR measurements in both controls and ASC cases. Habituation Phase results indicated that ASC cases exhibit elevated SCR levels compared to those of Controls during the basic viewing of

visual face images (unpaired with any other stimuli). However, Control participants supported hypothesized SCR patterns (increased skin conductance in response to CS+) during the Acquisition Phase of the fear conditioning paradigm, while ASC cases indicated attenuated SCR to CS+ that was not significantly distinguishable from SCR to CS-, indicating an elevated fear response. Similarly, Control subjects displayed expected decreases in SCR during the Extinction Phase of the fear conditioning paradigm while ASC cases indicated residual elevation of SCR (to both CS+ and CS-). Such results suggest that individuals with ASC may have difficulty forming associations to specific cues during associative fear learning (perhaps due to the inability to discriminate between social cues as suggested by the data). Results from the acquisition and extinction phases of the fear conditioning paradigm in this study thus support previous findings of attenuated SCR due to an increased fear response and impaired differentiation between conditioned and non-conditioned stimuli in individuals with ASC (Gaigg, 2007; South, 2011).

It has been widely shown in previous research that individuals with ASC may process fear learning differently from neurotypical individuals. One study employing fear conditioning in non-social contexts demonstrated that participants with Asperger syndrome exhibit diminished SCR during the acquisition of associative learning when compared to that of neurotypical individuals (Gaigg & Bowler, 2007). Research presented in this chapter supports previous work by also showing attenuated SCR during the Acquisition Phase of social fear conditioning in participants with ASC, and expands upon Gaigg's findings by conducting the paradigm in a simulated social context. Moreover, this research further explores differences in ASC cases and control participants during not only fear learning, but extinction—which found that individuals with ASCs may have residual sympathetic arousal *after* social fear learning (i.e., during the Extinction Phase). This difference in fear extinction may have implications on the chronic social anxiety and phobias experienced by some individuals with ASC and thus requires further scientific examination.

Other research has also found that not only do people with ASC differ in their response to fear conditioning, there is evidence of heterogeneity in SCR measurement that correlates with

social functioning—indicating that individuals with less severe symptoms perform more similarly to neurotypical participants during fear conditioning (South, Larson, White, Dana, & Crowley, 2011). While AQ and EQ data was collected from participants in this study, research limitations inhibited the participant recruitment of a large enough sample size that would permit a similar examination of how AQ and EQ scores are related to SCR outcomes during social fear conditioning. One of the original objectives of this research was to recruit participants with and without ASC, and to genotype all participants to observe for oxytocin receptor polymorphisms (all participants had their blood drawn at the end of data collection for a related biomarkers study, and the samples were also to be OXTR genotyped). It was thus intended in this study to observe for any associations between specific OXTR small nucleotide polymorphisms (SNPs) and SCR response, as well as AQ and EQ scores. Unfortunately, limitations in the recruitment of participants prohibited the compilation of sufficient sample size to conduct such an analysis.

Moreover, due to limitations in the recruitment of participants, while participants were matched for BMI and age, it was not possible to match according to gender between ASC cases and neurotypical controls. Future studies extending from this work would thus recruit a larger sample size of participants that are matched in both age and gender. Furthermore, as it is an aim of this research to better understand the implications of fear-related social processing on the development of comorbid anxiety and mood disorders, it is necessary that future studies specifically recruit participants with ASC who have also been diagnosed with such conditions as anxiety disorder, social phobia, or depression.

Chapter 6: General Discussion

6.1 ROLE OF OXYTOCIN IN THE REGULATION OF SOCIAL FEAR PROCESSING IN MICE

This research demonstrates that oxytocin receptors play an active role in the amygdala during the processing of social stimuli in a fear-related context. It was found that socially defeated transgenic mice with a global mutation in the oxytocin receptor gene experienced a distinct absence of functional activity in the basolateral and medial amygdalae compared to observed signal enhancement patterns in wildtype mice. In addition, it was determined that *Oxtr* KO mice exhibit greater submissive behaviors and elevated body temperatures when confronted by dominant aggressors in a stressful social context.

As the precise mechanism through which oxytocin receptors interact with other hormones and neurotransmitters in the mediation of social behaviors is not wholly characterized, it is important to employ comprehensive methods that examine the receptor's functions at the systems level. Our analysis of oxytocin's response to social stimulation through a multimodal approach measuring the neuronal, behavioral, and physiological outcomes provide broader insight into the psychosocial system of fear cognition.

6.1.1 Exhibition of fearful social behaviors

Current research into oxytocin's role in autism spectrum conditions are motivated largely by early findings in rodent models establishing oxytocin's involvement in pair-bonding and affiliative behaviors. More recent work exploring oxytocin's pervasive roles in sociability, social recognition, and aggression have expanded existing knowledge of the peptide's traditional functions in moderating reproductive behaviors and anxiolytic stress response. Such evidence of oxytocin's social implications in conjunction with findings from genome-wide association studies linking genetic variants in oxytocin and oxytocin receptor genes to ASC phenotypes emphasize the importance of defining oxytocin's neuromodulatory role in social behaviors.

Furthermore, the high expression of oxytocin receptors in areas of the limbic system, such as the amygdala, incite much interest in understanding how oxytocin regulates innate responses triggered by the hypothalamic-pituitary-adrenal axis in social circumstances.

6.1.2 Functional activity in the amygdala

Results from this study illuminate oxytocin's involvement in the processing of fearful stimuli of a social nature, particularly with regard to mediation of amygdala activation within the basolateral and medial areas. Prevalent findings throughout literature directly associate the basolateral amygdala to fear response and learning in both rodent models and human subjects (Knoll et al., 2011; Maiya, Kharazia, Lasek, & Heberlein, 2012; Terburg et al., 2012). However, the relationship between the medial amygdala, a region typically involved in chemosensory function, and the basolateral amygdala in the processing of social fear also indicates involvement in social recognition activities (Samuelsen & Meredith, 2011). Behavioral evidence gathered in this study further delineate the pathway through which oxytocin receptor-dependent targeting of neuronal populations within nuclei of the amygdala are translated into behaviors at both the behavioral and physiological levels. While the oxytocin peptide and its receptor's relationship to aggression is broadly described in research examining both social and maternal aggression in rodent models, how oxytocin is responsible for behaviors in alternate contexts involving social anxiety and fear is still unclear (Nephew, Bridges, Lovelock, & Byrnes, 2009; Takayanagi et al., 2005). Physiological measurements via assessment of body temperature as employed in this study demonstrate the strong affiliation between social anxiety and sympathetic response by way of the HPA axis. Results from this research portray the significant impact that a single social encounter imparts on both basal and acute body temperatures, and how such responses are moderated through the regulation of oxytocin receptors in the brain.

6.1.3 Regulation of body temperature

Theories of physiological activities involved in the psychological processing of fearful situations in social contexts (i.e., social phobias) have been demonstrated in animal models

(Grissom, Iyer, Vining, & Bhatnagar, 2007) and suggested in clinical psychology studies (Moscovitch, Suvak, & Hofmann, 2010). However, it is unknown how oxytocin receptors behave in the manifestation of psychophysiological outcomes in specific social situations. This research thus examined oxytocin receptor-dependent sympathetic activity via monitoring of body temperature recordings in mice. Observations collected before and during social defeat procedures utilizing *Oxtr* KO and wildtype mice determined that both chronic and acute hyperthermia can be induced through a social fear paradigm conducted with rodent models. This finding has meaningful implications as it elucidates the physiological impact that manifests during the emotional processing of social circumstances. As human studies on autism seek to better understand and link the behaviors exhibited in social situations to neural processes, these animal model-based examples are a fundamental platform from which researchers may expand with directed clinical studies.

6.1.4 General Research Findings

The differences in behavioral and physiological fear responses in *Oxtr* KO mice were observed in a paradigm dependent on social factors for fear induction. These experiments, however, do not specifically dissect out the social element of the fear response. In isolated circuits, oxytocin signaling has been demonstrated to directly impact general fear response. For example, optogenetic activation of oxytocin terminals in the central nucleus of the amygdala decreases the freezing response of contextually fear-conditioned rats (Knobloch et al., 2012). Additional studies will be required to determine the role of oxytocin signaling throughout the network circuitry responsible for social fear responses, and to determine any potential interaction of discrete fear modulation and affiliation- producing effects of oxytocin within this network.

Such dissection of the roles of oxytocin in social processing and fear processing would also serve to resolve the paradoxical findings that *Oxtr* KO mice demonstrate reduced amygdalar activation but enhanced submissive behaviors and physiological activation in a social fear paradigm. Amygdalar hypoactivation in *Oxtr* KO aligns well with previous human data that have demonstrated lower amygdalar activity in both resting-state and social contexts, as well as the

reduced functional connectivity of the amygdala that is central to the amygdala theory of autism (Von dem Hagen et al., 2012). Based on amygdalar hypoactivity alone, physiological and behavioral responses would be expected to decrease. In this case, however, the differential effects of depleted oxytocin on the social and fear-inducing features of the social defeat paradigm could produce elevated generalized fear responses, as observed by Knobloch et al., along with the impairment in social processing that results in reduced activation of the amygdala.

6.2 PHYSIOLOGICAL RESPONSES TO SOCIAL FEAR CONDITIONING IN INDIVIDUALS WITH ASC

In our human trials, a model analogous to the rodent model of psychophysiological response to social fear conditioning was used. With the observation of sympathetic responses in a mouse genetic knockout model of the oxytocin receptor, it was important to determine if such findings could be translated to a human context and interpreted with regard to behaviors relevant to ASC. This research thus examined the influence of social fear on skin conductance in human subjects with and without clinical ASC diagnoses. Utilizing a social fear conditioning protocol that employed the pairing of startling auditory stimuli to images of human faces, the experimental setup created a human simulation of "social defeat" using established conditioning methods in the psychological field. Psychophysiological results of human sympathetic response as measured by skin conductance recordings showed intriguing differences between individuals with autism and non-diagnosed control subjects. The observations that individuals on the autism spectrum demonstrate higher skin conductance levels prior to fear conditioning, as well as a decreased ability to distinguish between CS+ and CS- in both Acquisition and Extinction phases of conditioning indicates potential deficits in fear learning in individuals with autism.

The results in this study linking abnormal responses to fearful contexts of a social nature to sympathetic arousal in ASC patients draws the amygdala theory of autism to consideration. The theory that the amygdala is a crucial player in the neural processing of stimuli essential for

the expression of typical social behaviors also raises the question of how it may affect fear processing. As the amygdala is a key area of the brain involved in the relay of threatening cues and initiation of behaviors critical to survival, how the processing of fearful and social stimuli intersect in the amygdala to potentially manifest in the form of anxiety or phobias in individuals with ASC is a key area of concern that requires further examination.

6.3 TRANSLATION OF FINDINGS FROM *OXTR* KNOCKOUT MOUSE MODEL TO PEOPLE WITH ASC

With strong associations between genetic risk factors and molecular families in the development of autism, animal models are a critical piece of the neurobiological puzzle that leads toward understanding autism and its development in the human brain and body. While no single animal model of idiopathic or non-syndromic autism reliably exists, it is important that research explore the component pathways that are perturbed in exhibition of particular behaviors affected in ASC. While animal models have proven to be critical windows into syndromic autism where specific genes are found to be responsible for complete manifestation of autism traits, much more research is needed to understand how findings from animal models can be translated in valuable ways to the clinical setting.

Results from this study in both rodent models and human participants indicate distinct differences across both species in physiological response to social stimuli in fearful contexts. Despite the many species-specific methods utilized in basic and clinical research to observe biological phenomena in both animals and humans, the overarching observations gathered in this study will help explore the translational concepts that aid scientists in examining the molecular interactions regulating fearful emotion processing. Importantly, this thesis work's analogous design of protocols in both animals and humans enhances the ability to elucidate critical pathways that are perturbed in ASC. Such translational research will be important as therapeutics targeting ASC symptoms in humans are further explored in rodent and primate models. Moreover, insight into how physiological processes are affiliated with the emotional

and behavioral processes accompanying social deficits related to autism will enable scientists to approach therapeutics from a symptomatic-driven perspective and screen therapeutic candidates in animals.

6.3.1 Translational implications of physiological responses

The animal model used in this research to assess the effects of oxytocin receptor depletion on social fear consisted of *Oxtr* KO and WT adult males experiencing daily social defeat exposures for 30 minutes in a resident-intruder protocol for three consecutive days. The experimental animals were introduced into the homecage of a larger mouse; in this paradigm, the anxiogenic factors include both physical and territorial dominance of resident over intruder. The paradigm used in the human studies also relied on a pairing of social cues and anxiogenic stimuli. These factors, however, are less integrated in the human fear conditioning protocol; the social cues are entirely visual, in the form of 2D human face image presentation, while the anxiogenic stimulus is the application of an auditory shock that is temporally but not materially associated with the social cues.

While the behavioral and imaging data obtained from the mouse studies in this project can be compared to previous work in humans with ASC, the one aspect of the processing of fear in a social context that was measured here across species was physiological response. In *Oxtr* KO and WT mice, physiological response to social defeat was assessed using body temperature, while SCR was used to assess social fear conditioning in the human subjects. In both *Oxtr* KO mice and human subjects with ASC, these physiological measures were elevated compared to controls. In mice, this hyperactivation was pronounced in response to social defeat, while the SCR in ASC subjects was non-responsive to the social conditioning paradigm. The overlapping similarities between these approaches indicates that both removal of oxytocin signaling in the mouse and potential polymorphic expression in ASC result in augmented physiological activation in response to anxiogenic stimuli paired with social cues.

The human data presented here align well with the amygdala theory of autism discussed earlier; the lack of amygdalar integration into social networks posited by this theory suggests the sort of network disconnection that would result in a lack of physiological response flexibility that

tracks with social input. This is seen in the current dataset, in which ASC subjects do not show differential physiological response to conditioned and unconditioned stimuli. While the amygdalar hypoactivation seen in the mouse model also aligns with the amygdala theory of autism, further study is required to resolve the paradoxical behavioral and body temperature findings. As previously discussed, future directions should seek to dissociate the effects of oxytocin receptor depletion on social/affiliative behaviors and fear behaviors.

6.4 THE VALUE OF ANIMAL MODELS IN TRANSLATIONAL RESEARCH

The utilization of animal models in basic research is an incredible tool in the translation of scientific discoveries to human subjects. With the remarkable genetic similarity between rodents and humans, the value of the mouse in genetics research is an invaluable method of better understanding how particular genes function, respond to the environment, and are involved in disease pathways in humans. However, the role of animal models in psychological and behavioral studies is a challenging area of science that requires both further exploration and analytical consideration of its translational qualities. A common argument plaguing basic research that employs rodent models in the study of ASC is that data drawn from such analyses cannot be accurately translated to humans, as no rodent model is capable of successfully encompassing the myriad of complex traits that are observed in ASC. However, as scientists today emphasize, the concept of autism is not of a diverse developmental disorder lacking a single risk factor, but rather of hundreds of potential risk factors that perturb multiple pathways in both the brain and body of humans. It is therefore more important than ever that rodent models of key genetic pathways regulating social behavior, language skills, visual attention, (and more) be explored so that such complex developmental disorders (like ASC) and those affecting mental health can be further elucidated. Without a clear knowledge of the basic processes underlying the social behaviors and cognitive processes exhibited by humans with ASC, targeted therapeutics and treatments will be exceedingly difficult to develop.

6.4.1 Limitations of this research

It is recognized in this research that the translational depth spanning the divide between a genetic knockout model of oxytocin in mice and a human child on the autism spectrum is great. Therefore, this research is presented with the emphasis that animal studies modeling specific traits characteristic of autism can only provide a restricted glimpse of the larger picture that surrounds the entire disorder. High level cognitive processes that regulate social behaviors in humans are not easily represented by rodent models. The mouse social defeat paradigm utilized in this study to simulate human social behaviors as associated with emotions of fear is a very traditional and widely used model of an array of behaviors including, but not limited to: aggression, dominance, submission, social behavior, and emotional fear. Whether or not the social defeat paradigm is the most accurate and translational model of human social fear requires further exploration in itself. Moreover, the translational limitations of social fear conditioning conducted in human participants in this study are also notable. Is social fear conditioning an accurate model of social fear in the context of what is true in the real world? Is a subject seated in a dark, sound-proof booth while being exposed to unpleasant sensations during the presentation of social images realistic in comparison to what individuals encounter while at school or in the workplace around their peers and colleagues? These are all important questions that also require more scientific understanding and analysis.

In addition, it is acknowledged that while the social fear conditioning paradigms were modeled as analogously as possible in rodents and humans, due to limitations in the study and access to resources, different methods of data collection were employed to acquire physiological and behavioral data across the animal and human subjects. It is therefore a major goal in future projects to utilize more comparable data collection techniques (i.e., fMRI in humans) across animals and humans to further evaluate the translational benefits of rodent models of autism.

6.5 CONCLUSIONS AND FUTURE DIRECTIONS

Despite the aforementioned limitations on both the animal and human studies sides of this research, these experiments are important in providing fundamental information on the nature of oxytocin in mammals and its implications on the expression of social behaviors in fear-related contexts. With a multitude of genes linked to autism in humans, a systematic understanding of each gene's role in the processing of different stimuli, in the context of various emotions, is valuable information as increased recognition of the comorbidities accompanying ASC is established. With more clinical psychological studies demonstrating the prevalence of anxiety disorders and depression in patients with autism, it is critical that autism research expand from the traditional boundaries of evaluating deficits in social communication and cognition so that comorbid disorders may be addressed and treated.

Future research expanding upon the findings of this study aim to explore, with greater translational realism, the relationships between emotional fear and the development of social anxiety and phobias. An extended paradigm of the social defeat protocol in mice followed by repeated social approach is an example of one experimental approach toward understanding the chronic emotional and behavioral impact that repeated social defeat has on mammals. In parallel, human psychological trials expanding upon the social fear conditioning paradigm, but followed by an eye-gaze tracking task in which fear conditioned participants were presented images of faces previously paired with aversive sounds could serve as a simple test of social avoidance or phobia. In both animal and human experimental designs, it would be important to also monitor and evaluate physiological patterns during such social avoidance tasks as this data could also reveal how related such avoidance behaviors are tied to sympathetic responses typically elicited during the acquisition of social fear.

The findings of this research demonstrate the strong physiological and emotional links that are evident in both animals and humans when processing social situations. The work presented here demonstrates that global knockout of oxytocin receptors in mice elevates behavioral and physiological responses to social fear, and impairs amygdalar functioning after

social defeat. Methodologically, these experiments also revelated that Mn^{2+} accumulates throughout the whole mouse brain, concentrating preferentially in more active regions, and most effectively enhancing MRI with a higher rate of infusion. The human studies presented show a deficit in fear conditioning in ASC subjects due to globally elevated sympathetic response in social cues. While this research captures only one facet of the many neurobiological activities governing behavior and emotion in mammals, it provides, nonetheless, important knowledge and new avenues of exploration to further scientific understanding of how the brain processes emotions that lead to physiological and behavioral outcomes.

Appendix A:

Exclusion Criteria

- Participants who are pregnant
- Participants with a body mass index (BMI) less than 18.5 and greater than 35 kg/m^2
- Presence of the following co-morbidities: multiple sclerosis, all cancers, Parkinson's disease, rheumatoid arthritis, inflammatory bowel disorders, psoriasis, emphysema, chronic bronchitis, hay fever, diabetes and cardiovascular diseases.
- Participants taking any hormone-related or chronic medical condition therapies (such as diabetes, hypo/hyperthyroidism, hyperprolactinaemia, Cushing's syndrome, Congenital Adrenal Hyperplasia, severe asthma, hepatitis/liver deficiency, renal failure, congenital heart disease, auto-immune diseases such as psoriasis of arthritis, inflammatory bowel disease, cystic fibrosis, coeliac disease, thalassaemia, etc.), any female subjects on chronic or hormone therapies at the time of the study
- Participants on medications for depression
- Participants with respiratory diseases
- Participants with epilepsy

Inclusion Criteria:

- All participants with ASC's
- Participants age 19-45 years
- Participants co-diagnosed with ADHD/ADD may participate in the study
- Mild asthma is acceptable if patient is not taking any oral medication (intranasal sprays are acceptable)
- Participants on birth control *CAN* take part if they have the implant or depo-provera (injection birth control)

Appendix B: Pre-appointment Questionnaire

Participant ID:

Hormone & Biomarkers Study Questionnaire

Thank you for helping our research project concerning the relationship between different molecules (such as hormones, growth, inflammatory and immune factors) and Autism Spectrum Conditions (ASC). Please complete this questionnaire and then send it back to us.

ALL INFORMATION REMAINS STRICTLY CONFIDENTIAL

Personal Details

First name: Surname:

Date of Birth: Today's date:

Age in years: ***Gender:***

Weight in pounds: Height in feet and inches:

Highest qualification received:

Profession/ Job title:

Ethnicity: ☐ European ☐ Central Europe ☐ Southern Europe ☐ Non-Europe

MEDICAL BACKGROUND

Have you ever been referred (e.g. by your GP) AND/OR diagnosed with any of the following?

- Digestive disease (gastroesophageal reflux disease (GERD), gastritis, peptic ulcer, cholecystitis, gallstones, coeliac disease, malabsorption, irritable bowel syndrome (IBS, or spastic colon), inflammatory bowel disease (IBD, Crohn's disease or ulcerative colitis), constipation, etc.):

☐ No ☐ Yes

If yes, please specify:

- Hepatitis (viral hepatitis (A, B, C), autoimmune hepatitis, alcoholic hepatitis, fatty liver, cirrhosis, etc.):

If yes, please specify:

☐ No ☐ Yes

- **Heart disease (heart attack, heart failure, hypertensive heart disease, valvular heart disease, etc.)**

☐ No ☐ Yes

If yes, please specify:

- Autoimmune disease: ☐ No ☐ Yes

If yes, please specify:

- Thyroid disease (hypothyroidism (underactivity), hyperthyroidism (overactivity), benign thyroid nodules, etc)

☐ No ☐ Yes

If yes, please specify:

- Any hormone-related disease (Cushing's disease, hyperprolactinemia, hypogonadism, hypoandrogenism, hyperandrogenism, etc.)

☐ No ☐ Yes

- Cancer ☐ No ☐ Yes
- Diabetes ☐ No ☐ Yes
- Asthma ☐ No ☐ Yes
- Kidney disease ☐ No ☐ Yes
- HIV infection ☐ No ☐ Yes
- Epilepsy ☐ No ☐ Yes
- Physical disability ☐ No ☐ Yes
- Hearing or visual difficulties ☐ No ☐ Yes
- Genetic/chromosomal abnormality ☐ No ☐ Yes
- Tuberous Sclerosis ☐ No ☐ Yes
- Language delay/disorder ☐ No ☐ Yes
- Developmental delay ☐ No ☐ Yes
- Dyspraxia ☐ No ☐ Yes
- Dyslexia ☐ No ☐ Yes
- Autism Spectrum Condition (including Asperger Syndrome) ☐ No ☐ Yes

- Hyperactivity/Attention Deficit Disorder (ADHD, ADD) ☐ No ☐ Yes
- Tourette's Syndrome ☐ No ☐ Yes
- Multiple Sclerosis ☐ No ☐ Yes
- Parkinson's disease ☐ No ☐ Yes
- Rheumatoid arthritis ☐ No ☐ Yes
- Psoriasis ☐ No ☐ Yes
- Emphysema ☐ No ☐ Yes
- Chronic Bronchitis ☐ No ☐ Yes
- Hay fever ☐ No ☐ Yes

If you have Hay fever, how severe is it and do you take medication for it?

- Any other medical conditions not listed above? ☐ No ☐ Yes

If yes, please specify:

Are you undergoing any kind of pharmacological treatment? ☐ ***No*** ☐ ***Yes***

If yes, please specify how often:

Do you use cannabis? ☐ ***No*** ☐ ***Yes***

If yes, please specify:

Do you use any other recreational drugs? ☐ ***No*** ☐ ***Yes***

If yes, please specify how often:

Do you smoke? ☐ No ☐ Yes

If yes, please specify how often: (If you smoke daily, please specify how many you smoke per day)

If yes, please specify the intake of alcohol per week in units:

Do you drink alcohol? ☐ ***No*** ☐ ***Yes***

Do you exercise? ☐ ***No*** ☐ ***Yes***

If yes, please specify how often:

Do you have any particular dietary restrictions or preferences (vegetarian, gluten-free, lactose intolerance, etc.)?

☐ No ☐ Yes

If yes, please specify:

Have you lost or gained a lot of weight in the past month? ☐ No ☐ Yes

If yes, please specify:

IF YOU ARE FEMALE

Are you pregnant? ☐ No ☐ Yes

At what age did you have your first period? ☐ No ☐ Yes:

If you can recall, please specify also the month and year:

Have you ever been referred (e.g. by your GP) AND/OR diagnosed as having Polycystic ovary syndrome (PCOS) or any other androgen related conditions (such as acne, alopecia, excess of unwanted hair (hirsutism), etc.)?

☐ No ☐ Yes:

If yes, please specify:

Do you use any hormonal contraception? ☐ ***No*** ☐ ***Yes***

If YES, **which type and brand** of contraceptives are you taking:

Do you still have periods? ☐ ***No*** ☐ ***Yes***

When to your best recollection was the first day of your most recent period (DD/MM/YYYY)?

Do you have regular/predictable menstrual cycles? ☐ ***No*** ☐ ***Yes***

How often do you have your periods?

☐ Once a month

If NO, please specify:

☐ More than once a month

Please, provide any other information you think may be useful:

☐ Less than once a month
☐ Less than every two month

You are done! Please send this back to us. If you have questions or need clarification, please contact Dr Bonnie Auyeung (phone: 01223-746082) at the Autism Research Centre, University of Cambridge or email us at personperception@gmail.com.

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