EFFECTS OF OXYTOCIN ADMINISTRATION ON SOCIAL BEHAVIOR IN MALE AND FEMALE MICE

by

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ABSTRACT

Oxytocin has been well used in animal studies to determine its role in social behavior. However, the route of administration between studies often differs posing challenges in generalizing results. Additionally, oxytocin has been found to differentially affect male and female animals. The current study aims to compare routes of administration of the same dose of oxytocin and its effects on social and anxiety-like behaviors across male and female mice. Adult C57BL/6J mice were chronically pretreated with saline or oxytocin (12 μg) for 14 consecutive days, a dosage previously shown to alter prosocial and anxiety-like behavior in mice. Mice received either intranasal (i.n., 12 ml, 6 ml per nostril) or intraperitoneal (i.p., 120 ml) administration. On the fourteenth dosing day, mice completed the elevated plus maze (EPM) followed by the three-chamber sociability task (3C), and the free dyadic social interaction (FDSI) in a 24-hour completion cycle. General and social anxiety-like behaviors, social preference, and social novelty were coded utilizing Noldus EthoVision XT and human coders. Our results suggest that female and male mice have differing anxiety-like and social behavior after OT treatment, and that male mice are more susceptible to stress and behavioral changes depending on the route of peripheral administration route. These findings suggest that sex differences and route of oxytocin administration play an intricate role in anxiolytic and sociability behavior modulation.

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CHAPTER I: INTRODUCTION

Social Behavior

Throughout history, humans have relied on one another for survival, leading to intricate social systems arising as a byproduct (Alexander, 1974; Bergstrom, 2002).

Social behavior is a foundational skill in early development derived from learned responses of survival instincts passed generationally, leading to the formation of complex social expectations and hierarchies within human interaction (Geary & Flinn, 2001; Numan & Insel, 2006).

Abnormal social behaviors indicate potential symptoms related to mental disorders (Pelphrey & Carter, 2008). For example, affective disorders, such as anxiety and depression, and neurodevelopmental disorders, such as autism and fragile X syndrome, could all present social avoidance as a criterion for diagnosis (American Psychiatric Association, 2013). Specific genetic markers and abnormalities in neurocircuitry have been found in both clinical populations and animal models, emphasizing the importance of investigating the biological underpinnings of social behavior (Cacioppo et al., 2000; Johnson et al., 2005; Insel, 2010).

Neurocircuitry of Social Behavior

Neuroanatomy and Connectivity

Social behavior has been linked to activity in the cortico-limbic pathways (Peris et al., 2017). Several studies that examined the pathology of human brains demonstrating behavioral deficits have found damage to the medial pre-frontal cortex (mPFC; Ko et al,

2017). This area is associated with social salience and the regulation of anxiety levels in social situations (Ko et at., 2017; Son et al., 2018). Follow-up studies in mice found a correlation between the activity in mPFC and social approach behavior in a test mouse interacting with a conspecific mouse – where higher mPFC activity is associated with prosocial behaviors and reduced anxiety-like behaviors (Ko, 2017). These findings in human and animal studies appear to show there are neurocircuitry pathways from the mPFC that modulate anxiety into socially acceptable responses via communication with the ventral striatum, the brain area associated with decision making and reward-seeking behaviors (Pagnoni et al., 2002; Cox & Rissman, 2011).

Many human studies show an increase of activity in the ventral striatum when completing task for reward (Drevets et al., 2001; Pagnoni et al., 2002; Hung et al., 2017). Research has further demonstrated that the anticipation of receiving a reward causes increased activity in the ventral striatum, and the pleasure response plateaus with continuous reinforcement (Liu et al., 2020). Animal models of addiction primarily target the nucleus accumbens (NAc), a region within the ventral striatum, to explore the downstream effects of dopamine on other chemicals responsible for social behavior and social reward found within the limbic system (Yang et al, 2018).

Another primary brain area of interest, the amygdala, plays a role in modulating all components of social behavior, as its circuitry is connected to the pre-frontal cortex, the ventral striatum, and to all areas of the limbic system (Cardinal et al., 2002; Gouveia et al, 2019). The amygdala determines the affective perceptual response to external stimuli, such as signaling to hypothalamus to activate the autonomic nervous system,

leading to behavioral symptoms seen in anxiety or release of neurotransmitters to promote prosocial behaviors during social interactions (Liu et al., 2019; McDonald et al., 2020). Cellular activation in this area leads to release of several neurotransmitters related to affective behaviors including glutamate, serotonin, dopamine, and oxytocin (Cardinal et al, 2002; Gouveia et al., 2019).

Neurotransmitters

Many intricate neurotransmitter systems modulate behavior, and several work together to play a predominant role in social behavior (Liu et al., 2020; McDonald, 2020). Glutamate is an excitatory neurotransmitter responsible for social memory and emotional regulation when undergoing social stress as glutamatergic projections from the mPFC to the amygdala regulate the circuitry involved with social memory (Gunaydin et al., 2014; Son et al., 2018). Individuals diagnosed with attention-deficit hyperactivity disorder (ADHD) have less glutamatergic receptors in this pathway, suggesting that this neurotransmitter is responsible for regulating the attentional awareness needed to perform social behaviors normally (Naaijen et al., 2017). In mouse studies, glutamatergic activity in the mPFC plays a role in social approach and anxiety-like behavior. For example, mice given l mg/kg of a metabotropic glutamate receptor 1 (mGluR1) antagonist demonstrated less exploration in social chambers during the three-chamber sociability task and also demonstrated higher anxiety-like behavior such as grooming, rearing, or freezing (Zoicas & Kornhuber, 2019). Glutamate has also been linked to social reward behavior, as oxytocin receptors have been found on glutamatergic and dopaminergic neurons in the ventral tegmental area’s projections to the NAc (Peris et al., 2017).

Serotonin's role in social behavior partially overlaps with that of glutamate as it also contributes to social memory and emotional regulation (Canli & Lesch, 2007; Dölen et al., 2013). Lower serotonin levels are associated with affective mood disorders and atypical or antisocial behaviors (Canli & Lesch, 2007; Kane et al., 2012)s. Mice genetically modified to have reduced serotonin receptors in the amygdala have shown a variety of atypical behaviors, such as increased anxiety-like behaviors in social and non-social environments, decreased prosocial exploration of conspecific mice, and more aggressive behaviors during free dyadic exploration (Holmes, Murphy, & Crawley, 2002; Brenes, Rodriguez, & Fornaguera, 2008; Kane et al., 2012). Serotonin is also found in the ventral striatum and reduction of serotonin levels leads to lack of motivation seen during depressive episodes (Browne et al., 2019). Likewise, lower levels of serotonin receptors are found in individuals with depression, bipolar disorder, and schizophrenia (Brenes et al., 2008). This reduction of synaptic activity of serotonin plays a key role in describing the psychopathology of the emotional regulatory issues of affective disorders (Popa et al., 2008). The relationship between serotonin and social behavior has been widely explored and selective serotonin reuptake inhibitors are now used to treat a variety of affective mood disorders (Robbins & Everitt, 1992; Drevets et al., 2001; Canli & Lesch, 2007) and have been found to act with other neurotransmitters like oxytocin in the NAc acts with serotonin to mediate social reward behavior (Dölen et al., 2013).

Dopamine, the main neurotransmitter in reward-mediated behavior, is widely studied in addiction research since activity in the NAc mediates the reward system of the brain (Trainoe, 2011; Baik, 2013). When released in the NAc following exposure to a rewarding stimulus, dopamine elicits the downstream release of neurotransmitters that

lead to feelings of euphoria (Miczek et al., 2002). Receptors for dopamine and other neurotransmitters that drive social behaviors, particularly oxytocin, have been found at the same receptor sites, suggesting interactions between the neurotransmitters (Pearce et al., 2017; Kohli et al., 2019).

Oxytocin is a neuropeptide that has been demonstrated to increase sociability and approach in mice (Takayanagi et al., 2005, Yoshida et al., 2009; Teng et al., 2013; Sobota et al., 2015; Teng et al., 2016; Hung et al., 2017). Oxytocin has traditionally been known as the neurotransmitter involved during childbirth due to its role in parturition and lactation (Ring et al., 2006; Bartz & Hollander, 2008). Current research on oxytocin's influence on prosocial behaviors has attempted to utilize this neurotransmitter to alleviate the social stress and reduce the asocial behaviors in neurodevelopmental disorders, but the results have yielded mixed results in human studies (Tachibana et al., 2013; Dadds et al., 2014; Preti et al., 2014; Neumann & Slattery, 2016). When observing its pharmacodynamics, oxytocin receptor-null mice show increased asocial and anxiety-like behaviors. Oxytocin does appear to be linked to social adaptivity, and reversal of these asocial and anxiety-like behaviors has been demonstrated with oxytocin administration to autism and fragile X mouse models (Sala et al., 2011; Chadman et al., 2012; Yamasue et al., 2012; Peñagarikano et al., 2015; Teng et al., 2016).

Animal Models of Social Behavior

Examining neurochemical activity requires invasive methodology that could globally alter brain function and increase risk of harm to clinical populations. Therefore, examining psychopharmacological dynamics in basic science using animal models is

ideal. Many animals have complex social networks in all aspects of behavior from parenting to social hierarchy making them valid models for exploring social behavior (Ardesch et al., 2019; Dennis et al., 2020). For example, non-human primates demonstrate many social behavior parallels to human social behavior and studies often utilize fMRI and EEG measurements to evaluate the intersection of cognition and behavior as well as the neuropathology that occurs in brain injury and neurodegenerative disorders, as primates have very similar neuroanatomical mapping compared to humans (Ardesch et al., 2019). However, when looking at social development or neurochemical changes that require euthanasia for tissue harvest, the longer lifespan of primates makes this type of methodology unethical and uneconomical.

Using rodents as an alternative animal model bypasses this issue as rodents have comparable neuroanatomical structures to humans and high genetic concordance, while having significantly shorter lifespans of two to five years depending on the species. (Dennis et al., 2020). Social behavior remains complex in rodents and natural variation in behavior between strains and species allows for the specialization of models for specific research questions. For example, prairie voles exhibit monogamous mating which is linked to the expression of the *RS3* 334 gene, allowing the field to study unique parental and mating behaviors at the genetic level (Ophir et al., 2008).

Most commonly, rats and mice are the rodent models utilized in basic behavioral neuroscience and biomedical research due to their behavioral, genetic, and neuroanatomical similarities to humans (Dennis et al., 2020). Additionally, rodents are economical and allow tight environmental and genetic control. Mice tend to be a

commonly used model for behavior as the mice genome was sequenced before other rodent models and genetic modification of mouse models can be quickly created for exploration of target neurotransmitter receptors (Dennis et al., 2020). Given that mice actively seek out social and environmental novelty, researchers can use behavioral paradigms to quantitatively explore behavior patterns like sociability, social approach, and anxiety-like behaviors in social situations. The extent of these behaviors varies depending upon the genetic strain of mouse model (Peleh et al., 2019).

Genetic Variants of Mice Models

Genetic mouse models of human behaviors and conditions have contributed to significant strides in uncovering the neurocircuitry and neurotransmitters responsible in social behavior (Ricceri, Moles, & Crawley, 2007; Caruso et al., 2018). Researchers studying behavior with rodent models can choose to use either genetic models, environmental models, or wildtype mice. Knockout (KO) mice have had a single gene removed while knock-in mice have had a gene inserted or the function of a gene enhanced. Additionally, genetic mutant mouse models exist in which various genetic alterations may exist such as the deletion of a chromosomal region. These mouse models allow for the inspection of a single gene or multiple genes and the contribution to behaviors. Environmental models also exist to replicate environmental exposures, such as exposures to toxins or stress, to replicate environmental exposures in humans and their effects. Wildtype (WT) mice have had no genetic or environmental manipulation but instead descend from different background strains, which naturally vary in a range of behaviors. WT mouse strains are often used to represent natural behavioral variation and

individual differences in people. For example, the 129S1/SvlmJ WT and BTBRT^{+}*Itpr3*/J WT strains have low sociability/novel seeking behavior and high anxiety which is useful when research questions involving atypical social behavior. Conversely, the DBA/2J WT strain shows low sociability/novel-seeking behavior and low anxiety-like behavior. For more explorative questions, a commonly used model like the C57BL/6J WT strain would be a good option as it is middle-of-the-road in sociability/novel-seeking behavior and anxiety (Temme et al., 2014; Moy et al., 2007). C57BL/6J mice are also cost effective and fast breeders with observable hierarchies in housing allowing for researchers to easily observe the parenting behaviors, dyadic behaviors during development and adolescence, and dominance/submissive behavioral traits that contribute to their performance in behavioral tasks.

Purpose of the Current Study

As social behavior is naturally complex and variable, it is no surprise that the investigation of the neurocircuitry underlying these behaviors is currently incomplete. Ongoing research often seeks to elucidate oxytocin's role in social behavior. Oxytocin activity is central to many social behaviors, oxytocin interacts with many other neurotransmitters known to affect social behavior, and intranasal oxytocin is currently being investigated in clinical trials for the treatment of social symptoms in disorders such as autism spectrum disorder. However, the methodology of these studies in rodents often varies and poses challenges to generalization across experiments. Some rodent studies attempt to replicate the route of administration in humans by using intranasal (i.n.) administration in mice. Intranasal (i.n.) administration of oxytocin is the most common

route in clinical trials as it targets the mucosa layers of the nostrils to decrease absorption rate times (Yamasue et al., 2012, Gulliver et al., 2019, LeClerc & Easley, 2015). Other rodent studies use a more traditional method of pharmacological administration of intraperitoneal (i.p.) injection where the drug is delivered via injection into the adipose tissue of the lower abdominal region (Chui et al., 1995; Mierop et al., 2020). While Smith and colleagues (2019) have demonstrated that oxytocin successfully passes the blood-brain barrier with both peripheral administration methods, no studies to date directly examine the downstream behavioral differences between methods have been published to our knowledge. The current study aims to compare the behavioral effects of the two routes of administration of oxytocin.

Handling training and administration procedures also differ between i.n. and i.p. administration methods **(**Huang et al, 2014; Leng & Ludwig, 2016; Sakamoto, Sugimoto, & Uekita, 2019). Administration methods could lead to differences in behavior, as handling and invasiveness of each method could lead to habituation of different stressors. i.n. handling requires the mouse to be inverted in the palm of the researcher for a marginally longer time and could be more stressful than i.p., since it requires mice to habituate to pipettes coming into close contact with their noses. i.p. administration also presents different invasiveness that could lead to behavioral differences as injection requires habituation to a more painful method. Therefore, standardized handling training methods followed by saline-controlled drug delivery is necessary to parse apart potential differences between the methods.

Oxytocin has also been shown to differentially affect social behavior in male and female mice (Carter, 2007; Panksepp & Lahvis, 2016; Dumais & Veenema, 2016; Steinman et al., 2016). When interacting with the same sex, male mice typically demonstrate higher rates of negative social behavior, such as aggression, whereas female mice typically demonstrate more positive social approach, such as increased peaceful coinhabiting and playful activity (Amico et al., 2004; Ring et al., 2006; Choleris et al., 2007; Kercmar et al., 2011; Murakami et al., 2011). Differences in social behavior between male and female mice still exist when controlling for extraneous variables like diet and environment, and sex differences remain consistent in behavioral studies of oxytocin (Guo et al., 2004; Kercmar et al., 2014).

To determine the effect of the route of oxytocin on social behavior and how biological sex might play a role in behavioral differences, both chronic i.p. and i.n. administration of a standardized dose of 12 ug of oxytocin was delivered to male and female mice (see Smith, Korgan, & Young, 2019). Because of their moderate performance of anxiety-like and sociability in behavioral tasks, C57BL/6J mice were utilized. Anxiety-like behaviors and locomotor activity were explored in the elevated plus maze (EPM) as anxiety and hyper/hypo locomotion can confound the measurement of social behavior. Sociability and preference for social novelty following oxytocin administration was measured via the three-chamber sociability task (3C) and the free dyadic social interaction task (FDSI). Social behavior was examined by observing sociability and social novelty in the 3C task and by observing social approach, social avoidance, and social sniffing in the FDSI task. Given methodological differences and potentially differing psychopharmacological dynamics in administration routes, the

findings of this study sought to determine if i.n. or i.p. administration is more effective at altering social and anxiety-like behaviors. Potential findings could also determine any sex effects based on route of administration, suggesting necessary scrutiny when evaluating previous methodological generalizability in animal models and clinical studies.

CHAPTER II: METHODS

Animals

Sixty-eight C57BL/6J mice (#000664; M = 36, F = 32) were purchased from Jackson Laboratory at 9 weeks of age and allowed to habituate to housing one week before conducting experimental procedure. All mice ranged from 10 to 14 weeks old at start of the experiment. A priori power analyses estimated the number of mice to be used in this study is the minimum number necessary to achieve an appropriate projected effect size of 0.60 (see Erdfelder et al., 2005). All experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, approved by the Institutional Animal Care and Use Committee of Middle Tennessee State University, and ethical considerations regarding the minimization of pain and distress were used throughout the duration of the study.

Materials and Design

Housing

Mice were housed in standard caging with sex-matched littermates, ranging from 2-5 mice per cage. Food and water were provided ad libitum and the environment was controlled under a 12:12 hour light/dark cycle with environmental conditions constant at

approximately 22℃ with an average 55% humidity level. Cages were lined with 1 cm of wood shavings and cardboard enrichment squares given weekly throughout the duration of the experiment.

Animal Handling

A ten-day handling training (as outlined in Table 1) was completed to habituate subjects to the drug administration handling techniques necessary. Handling training was designed to gradually increase the intensity of the holding technique. Additionally, our study's animal handling design closely followed the training methods outlined by Bales and colleagues (2014). Upon completion of daily training, mice were rewarded with cheerios.

Oxytocin Administration

Mice were randomly assigned to receive either saline or oxytocin via i.n. or i.p. administration (see Figure 1 for experimental conditions). Twelve μg of oxytocin was delivered as 12 ml (3 ml in each nostril twice) for i.n. administration and 12 μg in 120 ml for a single i.p. injection. Both administration methods had around the same completion time of ~30 seconds. All mice underwent fourteen-days of daily oxytocin or saline administration. This fourteen-day chronic oxytocin administration schedule has been previously shown to reach oxytocin levels sufficient to influence behavior (Bales et al., 2014). On the fourteenth day of the oxytocin/saline administration, mice began a battery of behavioral tasks.

Behavioral Tests

The three behavioral tests were completed in 24-hour succession of each other, with the first beginning directly after the final drug administration. Anxiety and social behaviors were measured via the elevated plus maze (EPM), three chamber sociability task (3C), and the free dyadic social interaction test (FDSI). The measurement of anxiety (EPM) was conducted first to prevent reactivity from the social tests. FDSI was conducted last as it is expected to have the highest level of reactivity given direct interaction with an unfamiliar mouse. Previous studies support the uniform presentation of behavioral tests across all subjects to minimize reactivity such as anxiety for a more accurate measure of behaviors (McIlwain et al., 2001).

EPM is a well-used test of anxiety-like behaviors in mice. The test is conducted in an elevated cross-like apparatus that consists of four open arms (two closed in by walls and two open platforms, see Figure 2). Mice exhibit a behavioral preference to avoid open, elevated areas but also a propensity to explore novel spaces. Each test mouse was able to explore the maze's environment for 10 minutes and anxiety was determined as higher if the mouse shows greater preference for the walled arms (Yang et al, 2011).

Using 3C (Crawley, 2007; Yang et al., 2011), mice were placed in the center chamber of a custom plexiglass three-chambered apparatus, with inverted pencil cups in the left and right chambers to prevent free roaming of the conspecific mice. After habituating to the chamber, mice were tested for social approach and social novelty, each phase lasting 10 minutes (see Figure 3).

In the sociability stage of the 3C task, a sex-paired conspecific mouse was placed inside one of the inverted pencil cups. Prior to experimental testing, conspecific mice underwent a two-day habituation of remaining in the inverted pencil cups for the testing duration. The amount of time spent in the social chamber versus the empty chamber and time spent sniffing the conspecific mouse was measured for each test mouse. Directly after, a second conspecific mouse was placed in the previously empty cup in the opposite chamber.

In the social novelty stage of the 3C, each test mouse's preference of the novel conspecific mouse is compared to social preference of the previously exposed mouse from phase two by analyzing the time spent in each chamber and the time spent sniffing each mouse. In addition to analyzing time spent in each chamber, anxiety-like behaviors (including number of times rearing, number of times freezing, number of fecal pellets dropped, number and duration of time spent grooming) were collected and analyzed. Behavioral data were recorded by a *Hero Silver 7 GoPro* (30 and 60 fps, see Yang et al., 2011). Time spent within each chamber and time spent sniffing the conspecifics were analyzed via *Noldus Ethovision XT*.

FDSI task allows for observation of anxiety and social behavior within a freely explorable environment (see Figure 4). Each test mouse was placed in a 12"x12" white, acrylic, open field apparatus along with a novel, sex, age, and weight matched stimulus mouse. Behavioral interaction was recorded for 10 minutes. Social approach was defined as the frequency of times the experimental mouse oriented towards the stimulus mouse, while social avoidance was defined as frequency of the test mouse oriented away from

the stimulus mouse. Social anxiety-like behavior was analyzed by examining the frequency of the test mice's grooming behavior (Kraeuter, Guest, & Sarnvai, 2019).

CHAPTER III: RESULTS

Elevated Plus Maze

Descriptive statistics for locomotor activity and open arm exploration are shown in Table 2. A familywise alpha of 0.05 was used for all analyses. The GLM procedure in SAS Studio (version 3.80) was used to compare separate factorial ANOVAs to determine the effect of drug (saline and OT), route of administration (i.n. and i.p.), and sex (male and female on the following dependent variables: (1) total number of arm crossings and (2) time spent exploring the open arms.

Locomotor Activity

The main effect for drug indicated locomotor activity differed by drug type, F (1, 57) = 35.17, $p < 0.0001$, as saline-treated mice showed higher locomotor activity than OT-treated mice. The interaction between drug, administration, and sex was significant, F (1, 57) = 4.49, MSE = 77.17, p = 0.039, ω_p^2 = 0.05, 95% CI [0.00, 0.21]. Simple effect ANOVAs were conducted following the significant interaction. Pairwise comparisons were performed with the Tukey-Kramer procedure with an adjusted alpha of 0.025.

Total locomotor activity in males differed based on i.n. administration, F (3, 57) = 12.44, $p < 0.0001$. Male mice who were administered OT i.n. had significantly lower locomotor activity compared to males who were administered saline i.n. Total locomotor activity in males also differed based on i.p. administration, F (3, 57) = 8.05, p = 0.0001.

Male mice who were administered OT i.p. had significantly lower locomotor activity compared to males who were administered saline i.p. Finally total locomotor activity in saline-treated mice also differed based upon sex, F (3, 57) = 19.07, $p < 0.0001$. Male mice who were administered saline i.n. had significantly higher locomotor activity compared to male mice who were administered saline i.p. See Table 3 for pairwise comparison details.

Open Arm Exploration

The main effect for drug indicated open arm exploration differed by drug type, F (1, 57) = 27.87, $p < 0.0001$, where saline-treated mice explored open arms significantly longer than OT-treated mice. The interaction between drug, administration, and sex was significant, F (1, 57) = 14.42, MSE = 1026.00, $p = 0.0004$, $\omega_p^2 = 0.17$, 95% CI [0.04, 0.35]. Simple effect ANOVAs were conducted following the significant interaction. Pairwise comparisons were performed with the Tukey-Kramer procedure with an adjusted alpha of 0.025.

Open arm time exploration differed between mice who were saline-treated, F (3, 57) = 6.57, $p = 0.0007$. Female mice who were administered saline i.n. explored the open arms significantly less than male mice who were administered saline i.n. and female mice administered saline i.p. Male mice who were administered saline i.n. explored the open arms significantly longer than male mice who were administered saline i.p. Open arm exploration differed based on i.n. administration, F (3, 57) = 14.46, $p < 0.0001$. Male mice who were administered OT i.n. explored the open arms significantly less than male mice who were administered saline i.n. Open arm exploration also differed based on i.p.

administration, F (3, 57) = 6.19, p = 0.001. Female mice administered OT i.p. explored the open arms significantly less than female mice administered saline i.p. See Table 4 for pairwise comparison details.

Three-Chamber Sociability Test

Descriptive statistics for chamber exploration time and social sniffing time are shown in Table 5 and Table 6, respectively. A familywise alpha of 0.05 was used for all analyses. The GLM procedure in SAS Studio (version 3.80) was used to compare separate factorial ANOVAs to compare (1) time spent in the empty stimulus chamber during the sociability phase (2) time spent sniffing the empty stimulus cup during the sociability stage (3) time spent in the novel chamber during the social novelty stage and (4) time spent sniffing the novel mouse during the social novelty stage based on drug (saline, OT), administration (i.n., i.p.), and sex (female, male).

Sociability Phase

Time spent in the sociability chamber had a main effect of sex, F (1, 60) = 10.79, p = 0.0017. Female mice (N = 32, M = 241.75, SD = 68.04) explored the sociability chamber significantly less than male mice (N = 36, M = 287.72, SD = 60.32). Time spent sniffing the social stimulus mouse also had a main effect of sex, F (1, 60) = 11.93, p = 0.001. Female mice (N = 32, M = 127.09, SD = 46.93) explored the sociability chamber significantly less than male mice (N = 36, M = 171.82, SD = 66.49). Additionally, time spent in the open chamber differed by administration method, F (1, 60) = 7.93, p = 0.007. i.p. administered mice (N = 34, M = 130.04, SD = 53.14) explored the sociability chamber significantly less than i.n. administered mice (N = 34, M = 171.49, SD = 63.85).

Social Novelty Phase

There was a main effect of time spent exploring the social novelty chamber by sex, F (1, 59) = 10.27, p = 0.002. The interaction between drug and sex was significant, F (1, 59) = 4.14, MSE = 2517.76, p = 0.0464, ω_p^2 = 0.04, 95% CI [0.00, 0.19]. Simple effect ANOVAs were conducted following the significant interaction. Pairwise comparisons were performed with the Tukey-Kramer procedure with an adjusted alpha of 0.025. Time spent in the novel chamber differed between mice who were saline-treated, F (1, 59) = 11.55, p = 0.0012. Saline-treated, female mice spent significantly less time in the novel chamber compared to saline-treated, male mice. See Table 7 for pairwise comparison details.

Time spent sniffing the novel stimulus mouse differed by administration method, F (1, 59) = 5.06, p = 0.028. The interaction between drug, administration, and sex was significant, F (1, 59) = 4.40, MSE = 2069.96, p = 0.0, ω_p^2 = 0.05, 95% CI [0.00, 0.19]. Simple effect ANOVAs were conducted following the significant interaction. Pairwise comparisons were performed with the Tukey-Kramer procedure with an adjusted alpha of 0.025. Time spent sniffing the novel stimulus mouse differed between saline-treated mice, F (3, 59) = 6.89, p = 0.0005. Female mice who were administered saline i.n. spent significantly less time sniffing the novel mouse compared to male mice who were administered saline i.n. Additionally, male mice who were administered saline i.n. sniffed the social mouse significantly more than male mice who were administered saline i.p. See Table 8 for pairwise comparison details.

Free-Dyadic Social Interaction

The four stimulus mice, two female mice and two male mice, used throughout testing did not appear to show increased anxiety with repeated exposure to the test mice, $r(35) = 0.13$, $p = 0.47$ and $r(34) = 0.22$, $p = 0.22$, respectively. Descriptive statistics for social approach, social avoidance, and anxiety-like behaviors are shown in Table 9. A familywise alpha of 0.05 was used for all analyses. The GLM procedure in SAS Studio (version 3.80) was used to compare separate factorial ANOVAs to compare (1) orientation of test mouse towards stimulus mouse (2) orientation of test mouse away from stimulus mouse and (3) frequency of grooming behavior of test mouse during the social novelty stage based on drug (saline, OT), administration (i.n., i.p.), and sex (female, male).

Trained observers blind to experimental condition analyzed the frequencies of orientation of test mice towards and away from the stimulus mice and grooming for anxiety-like behaviors of the test mouse. Interrater reliability calculated between the two coders and discrepancies was determined by an expert coder. Intraclass correlation was calculated using the Irr package RStudio (version 4.0.2, model = one-way, type = consistency, unit = average) and test mouse grooming (ICC = 0.801 $F(6, 7) = 5.01$, $p = 0.026$) showed a significant correlation, while test mouse orientation towards conspecific (ICC = -0.971, $F(6, 7) = 0.51$, $p = 0.787$) and test mouse orientation away from conspecific (ICC = -1.760, $F(6, 7) = 0.36$, $p = 0.882$) did not show a significant correlation.

Social Approach

Frequency of orientation of the test mouse towards the stimulus mouse differed by both drug, F (1, 58) = 7.49, p = 0.008, and by administration method, F (1, 58) = 7.95, p = 0.007. The interaction between drug and administration method was significant, F (1, 59) = 8.30, MSE = 93.34, p = 0.006, ω_p^2 = 0.10, 95% CI [0.01, 0.27]. Simple effect ANOVAs were conducted following the significant interaction. Pairwise comparisons were performed with the Tukey-Kramer procedure with an adjusted alpha of 0.025. Frequency of orientation of the test mouse towards the stimulus mouse differed between mice treated with saline, F (1, 58) = 13.92, p = 0.0004. Mice administered saline i.n. had significantly less social approach compared to mice administered saline i.p. Frequency of orientation of the test mouse towards the stimulus mouse also differed between mice who received i.n. administration, F (1, 58) = 14.94, p = 0.0003. Mice administered OT i.n. had significantly more social approach compared to mice administered saline i.n.

There was also a significant interaction between administration method and sex, F (1, 58) = 24.82, p < 0.0001, ω_p^2 = 0.27, 95% CI [0.11, 0.44]. Orientation differed between males, F (1, 58) = 33.50, p < 0.0001. Males who were i.n. administered showed significantly less social approach compared to males who were i.p. administered. Orientation also differed between mice who were i.n. administered, F (1, 58) = 6.01, p = 0.017. I.n. administered female mice showed significantly more social approach compared to i.n. administered male mice. Additionally, orientation differed between mice who were i.p. administered, F (1, 58) = 21.70, p < 0.001. i.p. administered female mice

showed significantly less social approach compared to i.p. administered male mice. See Table 10 for pairwise comparison details.

Social Avoidance

Orientation of the test mouse away from the stimulus mouse differed by administration method, F (1, 58) = 25.44, $p < 0.0001$. There was a significant interaction between drug and administration method, F (1, 58) = 7.98, $p = 0.007$, $\omega_p^2 = 0.10$, 95% CI [0.01, 0.26]. Simple effect ANOVAs were conducted following the significant interaction. Pairwise comparisons were performed with the Tukey-Kramer procedure with an adjusted alpha of 0.025.

Orientation of the test mouse away from the stimulus mouse differed between saline-treated mice, F (1, 58) = 26.50, $p < 0.0001$. Mice administered saline i.n. showed significantly less social avoidance than mice who were administered saline i.p. Orientation of the test mouse away from the stimulus mouse differed between mice who were i.n. administered, F (1, 58) = 11.01, $p = 0.002$. Mice who were administered OT i.n. showed significantly more social avoidance compared to mice who were administered saline i.n.

There was also a significant interaction between administration method and sex, F (1, 58) = 9.45, $p = 0.003$, $\omega_p^2 = 0.11$, 95% CI [0.02, 0.28]. Orientation of the test mouse away from the stimulus mouse differed between males, F (1, 58) = 36.27, $p < 0.0001$. I.n. administered male mice showed significantly less social avoidance compared to i.p. administered male mice. Orientation of the test mouse away from the stimulus mouse also differed between mice who were i.p. administered, F (1, 58) = 13.38, $p = 0.0005$. I.p.

administered female mice showed significantly less social avoidance compared to i.p. administered male mice. See Table 11 for pairwise comparison details.

Anxiety-Like Behavior

Grooming behavior differed significantly by drug, F (1, 58) = 40.96, $p < 0.0001$, and by administration method, F (1, 58) = 11.97, $p = 0.001$. The interaction between drug, administration, and sex was significant, F (1, 57) = 20.35, MSE = 17.23, $p < 0.0001$, $\omega_p^2 = 0.23$, 95% CI [0.08, 0.40]. Simple effect ANOVAs were conducted following the significant interaction. Pairwise comparisons were performed with the Tukey-Kramer procedure with an adjusted alpha of 0.025.

Grooming frequency differed within OT-treated mice, F (3, 58) = 7.01, $p = 0.0004$. Female and male mice who were administered OT i.n. showed significantly more anxiety-like behaviors compared to female mice who were administered OT i.p. Additionally, male mice who were administered OT i.n. showed significantly more anxiety-like behaviors compared to male mice who were administered OT i.p.

Grooming frequency also differed within saline-treated mice, F (3, 58) = 11.63, $p < 0.0001$. Female mice who were administered saline i.n. showed significantly less anxiety-like behavior compared to male mice who were administered saline i.n. and female mice who were administered saline i.p. Male mice who were administered saline i.n. showed significantly more anxiety-like behavior compared to male mice who were administered saline i.p. Additionally, female mice who were administered saline i.p. showed significantly more anxiety-like behavior compared to male mice who were administered saline i.p.

Grooming frequency differed within i.n. administered mice, F (3, 58) = 8.47, p < 0.0001. Male mice who were administered OT i.n. showed significantly less anxiety-like behavior compared to male mice who were administered saline i.n. Additionally, grooming frequency differed within i.p. administered mice, F (3, 58) = 19.32, p < 0.0001. Female mice who were administered OT i.p. showed significantly less anxiety-like behavior compared to female mice who were administered saline i.p. See Table 12 for pairwise comparison details. This could suggest OT has sedative effects in higher doses, as mice treated with 0.1 mg/kg OT show higher exploration and lower anxiety-like behavior (Sakamoto et al., 2019).

CHAPTER IV: CONCLUSION

While open arm exploration during EPM can be an indicator of general anxiety-like behavior, our findings that 12 ug OT reduced open arm exploration time in conjunction with reduced locomotor activity might suggest that this dosing level could be sedative. When Bales and colleagues' (2014) demonstrated this dosing level passed the BBB, they did not utilize any behavioral assays, suggesting that lower dosing levels might need to be assessed for BBB permeability when OT is delivered via i.n. and i.p. injection within behavioral studies (Teng et al., 2013; Sobota et al., 2015, Sakamoto et al., 2019). Drug by administration by sex interactions suggest that OT effectiveness might depend on the route of PNS administration and sex, as male mice delivered OT intranasally and female mice delivered OT intraperitoneally both showed significantly less exploration time in EPM compared to control conditions. Social anxiety-like

behavior during FDSI also showed this trend, as i.n.-administered male mice and i.p.-administered female mice showed higher rates of grooming.

Time spent in the social chamber and time spent sniffing the social stimulus during the sociability stage of 3C and time spent in the social novelty chamber and time spent sniffing the novel stimulus during the novelty stage of 3C are indicators of social preference. Additionally, test mouse orientation towards and away from the stimulus mouse are indicators of social approach and avoidance, respectively. Mice who were i.p.-administered spent less time in the social chamber compared to i.n.-administered mice but not significantly less time sniffing the social stimulus. This might further support the anxiety-like behaviors in EPM that suggest administration methods have differing baseline anxiety-like affects. Additionally, male mice appear to vary in anxiety-like and social behaviors more intensely depending on route of peripheral OT administration compared to female mice. Prior studies have well documented that male mice, overall, have a larger number of OT receptors while female mice have higher endogenous OT levels (Carter, 2007; Panksepp & Lahvis, 2016; Dumais & Veenema, 2016; Steinman et al., 2016). These points support our finding, in that, male mice having significantly higher OT receptors throughout the brain increases the likelihood that differences in drug administration effectiveness will lead to more pronounced behavioral differences. Furthermore, female mice having higher endogenous levels of OT could help mitigate the variance in physiological impact the administration methods cause.

When comparing CNS vs PNS OT administration, Sakamoto and colleagues' (2019) found that 1.0 mg/kg OT delivered via i.p. injection reduced investigation time of

novel stimulus mice in the social preference task compared to saline and 0.1 mg/kg OT but did not see the same results in 0.05 µg/2 µL OT delivered via intracerebroventricular (i.c.v.) administration. This suggests that, when considering peripheral administration dosing, lower dosing of OT might generalize better to works using CNS administration methodology. Consideration of administration method based on sex was also a key finding when observing social approach and social avoidance in FDSI. Intranasally-administered male mice and i.p.-injected female showed more less prosocial behavior during FDSI, supporting that interactions between sex and route of administration play a key role in differing expression of anxiety-like and social behavior.

Overall, when utilizing peripheral delivery methods, OT appears to have varying affects on anxiety-like and social behaviors depending upon whether it is delivered via i.n.-administration or i.p.-injection, thereby requiring careful consideration when interpreting current OT behavior studies. Generalization of findings must be considered in conjunction with limitations to the design and data analysis. Additionally, while reliability checks were performed, lower inter-rated reliability for social approach and avoidance in FDSI added variance in determining behavior frequency. Additionally, group differences were only compared on performance within the behavioral assays. Future directions could add post-mortem histological assessment of oxytocin levels in the VTA, amygdala, and other target areas of the brain. This could better explain targeted functional changes of the neural correlates associated with behavior compared to observing behavior with a systemic change perspective. Exploration of targeted brain areas related to social behavior could also be explored through i.c.v. delivery of OT via cannular implementation.

Another potential addition to the design could be the incorporation of blood collection throughout the drug dosing schedule or following the behavioral assays to compare oxytocin levels between conditions. Because an important measure of the research design is perceived stress differences based upon the route of administration, additional procedures that could increase stress levels were excluded from the design. This also excluded collection of biomarkers of stress, such as cortisol, during behavioral assays as an additional measure of perceived stress. Given the limitations, a collection of general conclusions can be made regarding the importance of considering sex differences and delivery routes of OT in animal models and clinical patient populations.

Conclusively, our findings suggest that peripheral administration routes of OT do appear to modulate anxiety-like behaviors and social behaviors during various behavioral assays. Additionally, peripheral administration routes also target differing brain areas of mice depending on biological sex. These differences could explain, in part, conflicting findings of OT-dosing effectiveness in behavior modulation in pre-clinical drug trials in animal models and in clinical trials for patient populations living with affective mood and neurodevelopmental disorders.

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APPENDICIES

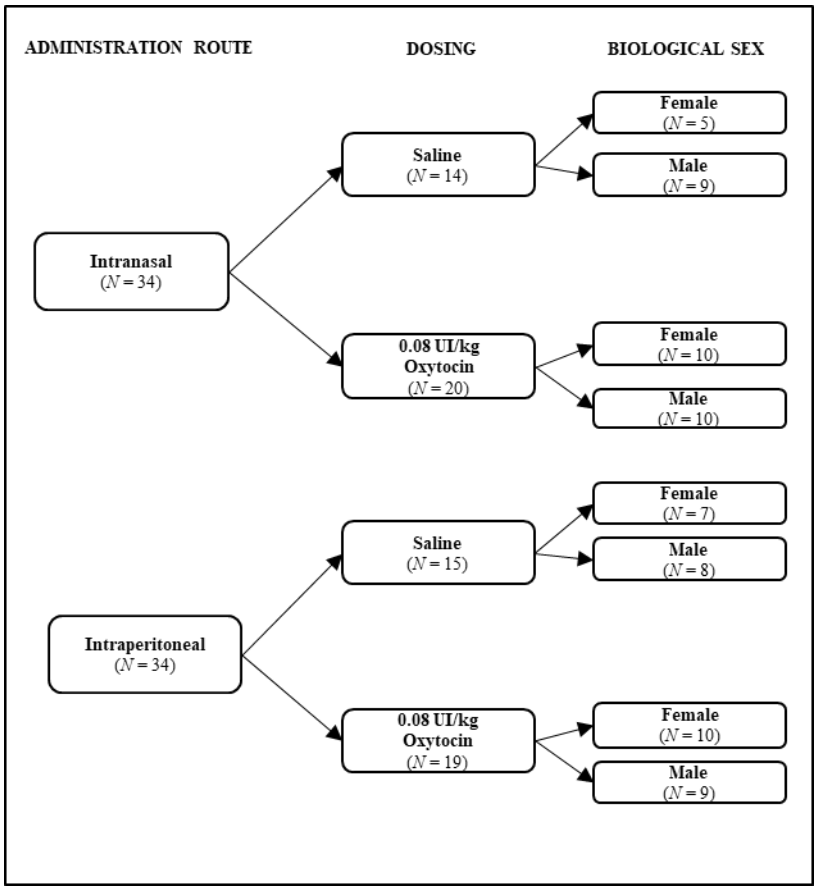
Appendix A: Animal handling procedure

Table 1.
Depicts ten-day animal handling training procedure.

Day	Description of Animal Handling Procedure
1	Pick up via tail; Hold in hand/on lab coat for 1 minute; Weigh
2	Pick up via tail; Hold in hand/on lab coat for 1 minute; Weigh
3	Pick up via tail; Hold in hand/on lab coat for 1 minute; Weigh
4	Pick up via tail; Hold in hand/on lab coat for 1 minute; Weigh
5	Lightly neck scruff while in hands; Weigh
6	Lightly neck scruff while in hands; Weigh
7	Neck scruff and invert mouse to expose stomach
8	Neck scruff and invert mouse to expose stomach
9	Neck scruff and i.p. or i.n. administration of saline; Neck scruff and intranasal administration of saline
10	Neck scruff and i.p. or i.n. administration of saline; Neck scruff and intranasal administration of saline

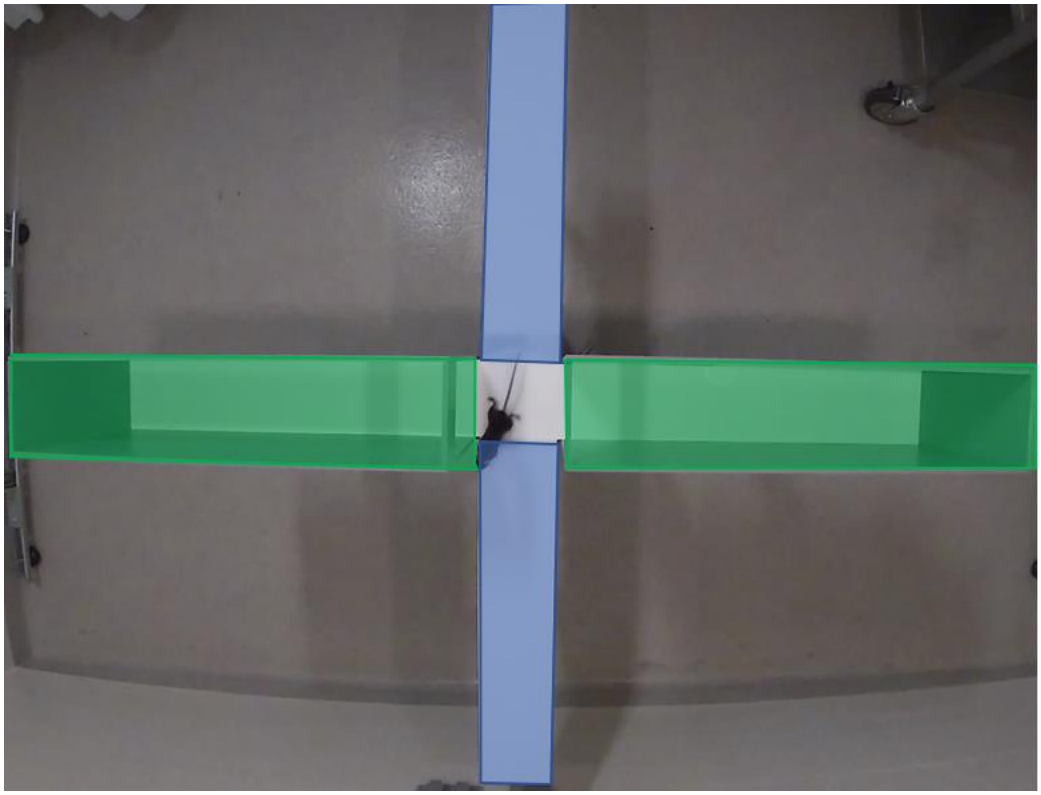
Appendix B: Experimental design

Figure 1.
Flowchart depicting study design and subject distribution.



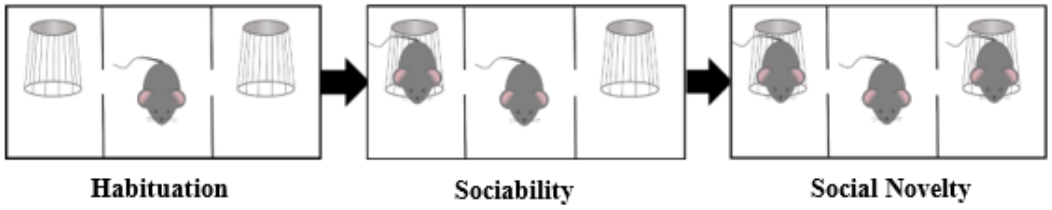
Appendix C: Elevated plus maze task

Figure 2.
Elevated plus maze task. Blue highlighted arms represent the open arms of the maze and green highlighted maze represent the closed arms of the maze.



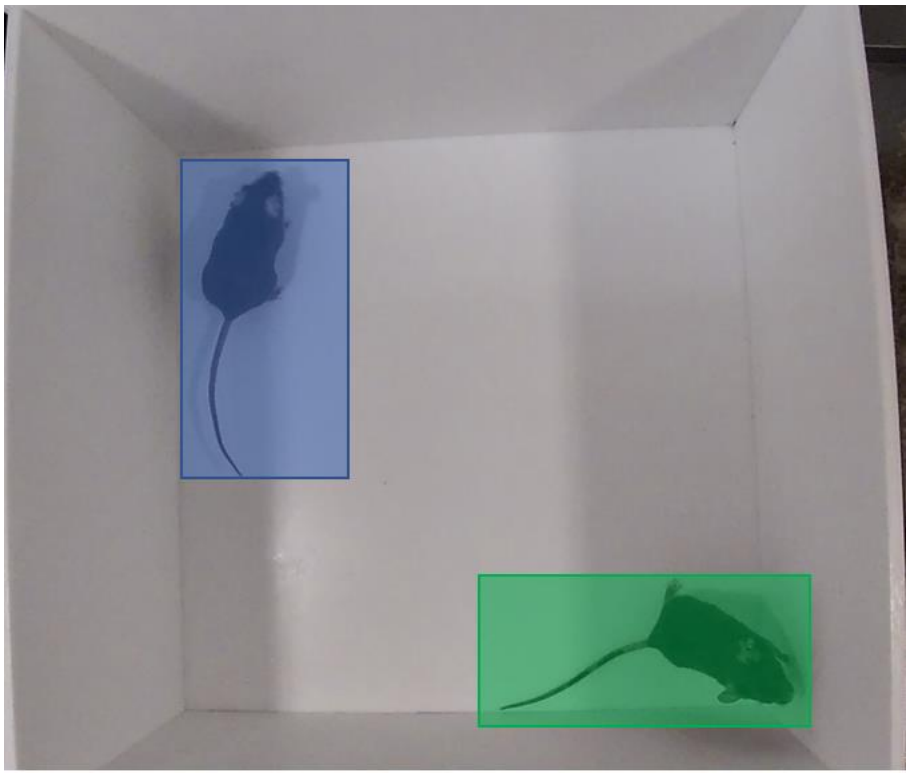
Appendix D: Three-chamber social task

Figure 3.
Three-chamber social task (reprinted from Meadows et al, in preparation). Illustration depicts the three phases of the task: Habituation – two empty chambers, Sociability – one empty chamber and one chamber with stimulus mouse, and Social Novelty – the familiar mouse chamber and a new stimulus mouse chamber.



Appendix E: Free dyadic social interaction task

Figure 4.
Open field test. At the beginning of the experiment, mice are placed on opposing chamber sides. Blue highlighted mouse represents the test mouse and green highlighted mouse represents the stimulus mouse.



Appendix F: Group means for elevated plus maze

Table 2.

Group Means for Total Crossings and Open Arm Time During the Elevated Plus Maze.

			Total Crossings		*Open Arm Time (s)*	
Drug	Administration	Sex	*M*	*SD*	*M*	*SD*
OT	i.p.	Female (*N* = 10)	34.00	7.86	60.58	22.79
		Male (*N* = 9)	26.33	5.57	52.93	32.13
	i.n.	Female (*N* = 9)	38.56	5.64	56.26	27.29
		Male (*N* = 10)	29.40	9.55	32.66	17.14
Saline	i.p.	Female (*N* = 6)	46.17	6.71	119.03	22.65
		Male (*N* = 8)	42.75	8.58	86.01	19.94
	i.n.	Female (*N* = 4)	39.50	6.95	47.80	31.53
		Male (*N* = 9)	53.89	14.30	124.93	59.81
Saline	i.p. (*N* = 14)		44.21	7.75	100.16	26.44
	i.n. (*N* = 13)		49.46	14.00	101.20	63.30
OT	i.p. (*N* = 19)		30.37	7.75	56.96	27.09
	i.n. (*N* = 19)		33.74	9.04	43.84	24.99
Saline		Female (*N* = 10)	43.50	7.28	90.54	44.38
		Male (*N* = 17)	48.65	12.93	106.61	48.62
OT		Female (*N* = 19)	36.16	7.10	58.54	24.41
		Male (*N* = 19)	27.95	7.86	42.26	26.72
	i.p.	Female (*N* = 16)	38.56	9.44	82.50	36.56
		Male (*N* = 17)	34.06	10.91	68.50	31.30
	i.n.	Female (*N* = 13)	38.85	5.79	53.66	27.60
		Male (*N* = 19)	41.00	17.15	76.36	63.07
OT (*N* = 38)			32.05	8.48	50.40	26.55
Saline (*N* = 27)			46.74	11.30	100.66	46.89
	i.p. (*N* = 33)		36.24	10.32	75.29	34.16
	i.n. (*N* = 32)		40.13	13.60	67.14	75.29
		Female (*N* = 29)	38.69	7.88	69.57	35.44
		Male (*N* = 36)	37.72	14.77	72.65	50.09

Appendix G: Tukey-Kramer pairwise comparisons for elevated plus maze

Table 3.

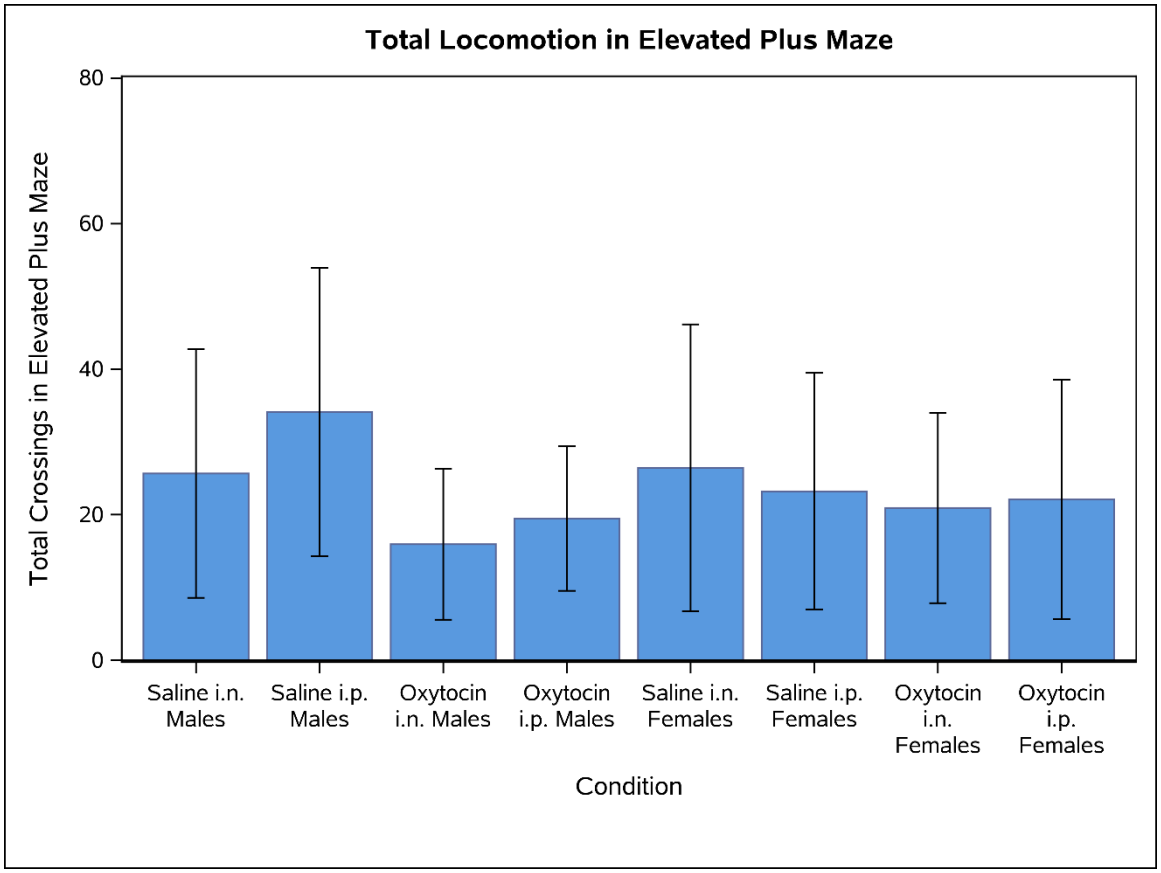
Tukey-Kramer Pairwise Comparisons for Locomotor Activity in the Elevated Plus Maze.

Comparison Split	Comparisons		Mean Difference	Std. Error	*p*-value
	Condition	Condition			
Oxytocin	i.n. Female	i.n. Male	9.16	4.04	0.118
	i.n. Female	i.p. Female	4.56	4.04	0.674
	i.n. Male	i.p. Male	3.07	4.04	0.872
	i.p. Female	i.p. Male	7.67	4.04	0.240
Saline	i.n. Female	i.n. Male	-14.39	5.28	0.041
	i.n. Female	i.p. Female	-6.67	5.67	0.645
	i.n. Male	i.p. Male	11.14	4.27	0.055
	i.p. Female	i.p. Male	3.42	4.74	0.474
i.n.	OT Female	OT Male	9.16	4.04	0.118
	OT Female	Saline Female	-0.94	5.28	0.998
	OT Male	Saline Male	**-24.49**	**4.04**	**< 0.001***
	Saline Female	Saline Male	-14.39	5.28	0.041
i.p.	OT Female	OT Male	7.67	4.04	0.240
	OT Female	Saline Female	-12.17	4.54	0.046
	OT Male	Saline Male	-16.42	4.27	0.002
	Saline Female	Saline Male	3.42	4.74	0.889
Female	OT i.n.	OT i.p.	4.56	4.04	0.674
	OT i.n.	Saline i.n.	-0.94	5.28	0.998
	OT i.p.	Saline i.p.	-12.17	4.54	0.046
	Saline i.n.	Saline i.p.	-6.67	5.67	0.645
Male	OT i.n.	OT i.p.	3.07	4.04	0.872
	OT i.n.	Saline i.n.	**-24.49**	**4.04**	**< 0.001***
	OT i.p.	Saline i.p.	**-16.42**	**4.27**	**0.002***
	Saline i.n.	Saline i.p.	**11.14**	**4.27**	**0.012***

Appendix H: Total locomotion during elevated plus maze graph

Figure 5.

Graph depicting frequency total of locomotor activity during the 10-minute elevation plus maze.



Appendix I: Tukey-Kramer pairwise comparisons for open arm exploration in elevated plus maze

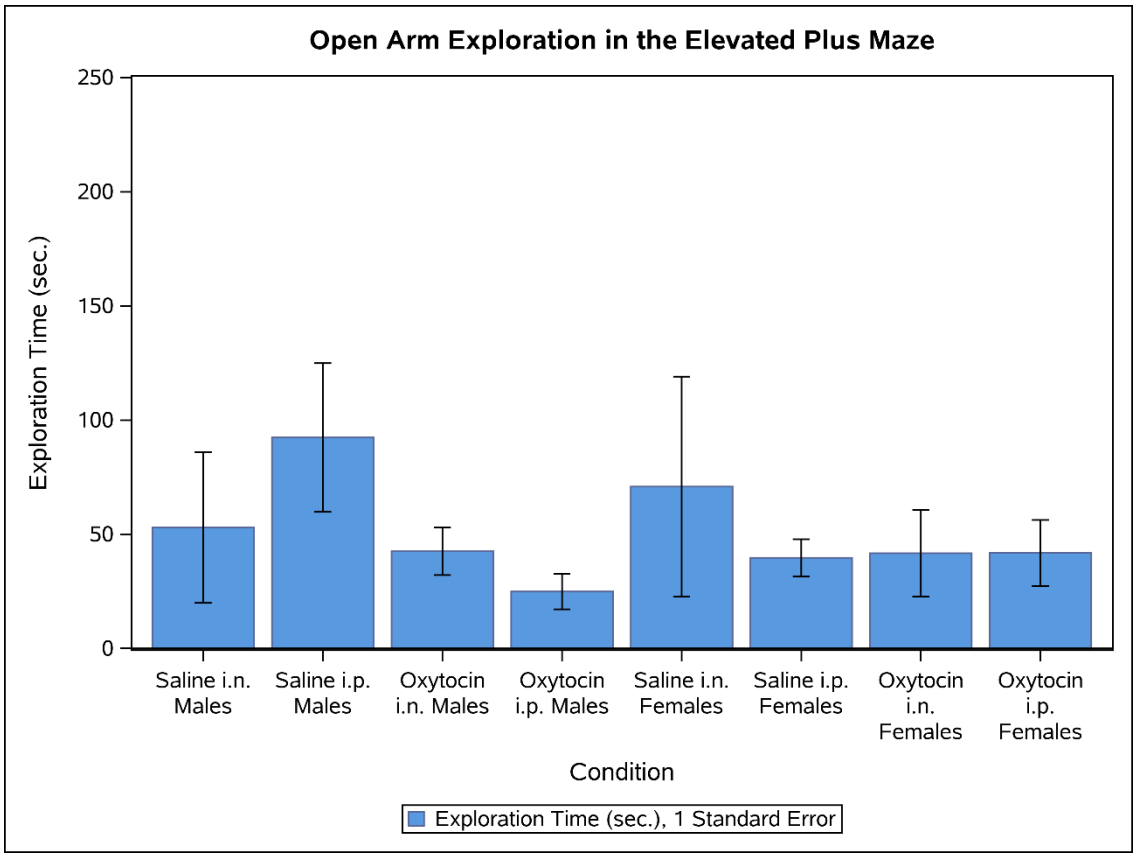
Table 4.
Tukey-Kramer Pairwise Comparisons for Open Arm Exploration in the Elevated Plus Maze.

Comparison Split	Comparisons		Mean Difference	Std. Error	*p*-value
	Condition	Condition			
Oxytocin	i.n. Female	i.n. Male	23.60	14.72	0.385
	i.n. Female	i.p. Female	-4.32	14.72	0.991
	i.n. Male	i.p. Male	-20.28	14.72	0.518
	i.p. Female	i.p. Male	7.65	14.72	0.954
Saline	i.n. Female	i.n. Male	**-77.13**	**19.25**	**0.001***
	i.n. Female	i.p. Female	**-71.23**	**20.68**	**0.006***
	i.n. Male	i.p. Male	38.92	15.56	0.071
	i.p. Female	i.p. Male	33.02	17.30	0.236
i.n.	OT Female	OT Male	23.60	14.72	0.385
	OT Female	Saline Female	8.46	19.25	0.971
	OT Male	Saline Male	**-92.27**	**14.72**	**< 0.001***
	Saline Female	Saline Male	**-77.13**	**19.25**	**< 0.001***
i.p.	OT Female	OT Male	7.65	14.72	0.954
	OT Female	Saline Female	**-58.45**	**16.54**	**0.004***
	OT Male	Saline Male	-33.08	15.56	0.158
	Saline Female	Saline Male	33.02	17.30	0.236
Female	OT i.n.	OT i.p.	-4.32	14.72	0.991
	OT i.n.	Saline i.n.	9.46	19.25	0.971
	OT i.p.	Saline i.p.	**-58.45**	**16.54**	**0.004***
	Saline i.n.	Saline i.p.	**-71.23**	**20.68**	**0.006***
Male	OT i.n.	OT i.p.	-20.28	14.72	0.518
	OT i.n.	Saline i.n.	**-92.27**	**14.72**	**< 0.001***
	OT i.p.	Saline i.p.	-33.08	15.56	0.158
	Saline i.n.	Saline i.p.	38.92	15.56	0.071

Appendix J: Open arm exploration during elevated plus maze graph

Figure 6.

Graph depicting time in seconds of open arm exploration during the 10-minute elevation plus maze.



Appendix K: Group means for sociability phase of three-chamber sociability task

Table 5.

Group Means for Sociability Chamber Time and Social Sniffing Time During the Sociability Stage of the Three-Chamber Sociability Task.

			Chamber Time (s)		*Social Sniff Time (s)*	
Drug	Administration	Sex	*M*	*SD*	*M*	*SD*
OT	i.p.	Female (*N* = 10)	227.23	67.60	122.65	42.54
		Male (*N* = 9)	287.72	42.26	160.51	38.29
	i.n.	Female (*N* = 10)	292.43	39.10	157.47	28.40
		Male (*N* = 10)	286.88	74.39	170.74	65.41
Saline	i.p.	Female (*N* = 7)	196.05	43.56	92.48	23.98
		Male (*N* = 8)	280.76	71.01	137.88	77.81
	i.n.	Female (*N* = 5)	233.38	92.85	123.64	76.52
		Male (*N* = 9)	294.85	58.22	214.51	66.43
Saline	i.p. (*N* = 15)		241.23	72.44	116.70	61.83
	i.n. (*N* = 14)		272.89	75.32	182.05	80.99
OT	i.p. (*N* = 19)		255.88	63.57	140.58	44.01
	i.n. (*N* = 20)		289.65	57.91	164.10	49.54
Saline		Female (*N* = 12)	211.60	67.37	105.46	51.97
		Male (*N* = 17)	288.22	62.88	178.45	80.06
OT		Female (*N* = 20)	259.83	63.30	140.06	39.47
		Male (*N* = 19)	287.28	59.67	165.89	53.12
	i.p.	Female (*N* = 17)	214.39	59.43	110.23	38.31
		Male (*N* = 17)	284.44	55.78	149.86	59.34
	i.n.	Female (*N* = 15)	272.74	65.40	146.19	49.64
		Male (*N* = 19)	290.66	65.50	191.47	67.85
OT (*N* = 39)			273.20	62.32	152.64	47.82
Saline (*N* = 29)			256.52	74.28	148.25	77.85
	i.p. (*N* = 34)		282.75	65.08	130.04	53.14
	i.n. (*N* = 34)		249.42	66.97	171.49	63.85
		Female (*N* = 32)	241.75	68.04	127.09	46.93
		Male (*N* = 36)	287.72	60.32	171.82	66.49

Appendix L: Group means for social novelty phase of three-chamber sociability task

Table 6.

Group Means for Novel Chamber Time and Novel Mouse Social Sniffing Time During the Social Novelty Stage of the Three-Chamber Sociability Task.

			Chamber Time (s)		*Social Sniff Time (s)*	
Drug	Administration	Sex	*M*	*SD*	*M*	*SD*
OT	i.p.	Female (*N* = 10)	237.61	57.67	131.58	48.39
		Male (*N* = 9)	277.41	69.08	141.96	42.27
	i.n.	Female (*N* = 10)	267.59	49.46	150.31	34.45
		Male (*N* = 10)	257.36	30.06	138.83	35.93
Saline	i.p.	Female (*N* = 7)	213.35	42.94	98.72	43.81
		Male (*N* = 8)	263.21	58.00	106.34	53.76
	i.n.	Female (*N* = 5)	219.14	36.94	105.30	28.49
		Male (*N* = 9)	301.67	38.84	187.16	62.63
Saline	i.p. (*N* = 15)		241.84	56.36	103.07	48.06
	i.n. (*N* = 14)		272.20	55.06	157.93	65.73
OT	i.p. (*N* = 19)		256.46	64.81	136.50	44.65
	i.n. (*N* = 20)		262.47	40.18	144.57	34.76
Saline		Female (*N* = 12)	215.99	38.43	101.71	36.01
		Male (*N* = 17)	283.57	51.16	149.13	70.39
OT		Female (*N* = 20)	252.60	54.50	140.94	42.00
		Male (*N* = 19)	266.86	51.75	140.32	37.98
	i.p.	Female (*N* = 17)	228.52	52.51	119.25	48.11
		Male (*N* = 17)	270.73	62.54	125.20	49.94
	i.n.	Female (*N* = 15)	251.44	50.21	135.31	38.44
		Male (*N* = 19)	278.35	40.48	161.73	54.80
OT (*N* = 39)			259.55	52.97	140.64	39.56
Saline (*N* = 29)			257.02	56.81	130.50	63.03
	i.p. (*N* = 34)		250.26	60.88	122.32	48.39
	i.n. (*N* = 34)		266.48	46.34	150.07	49.42
		Female (*N* = 32)	239.61	51.87	127.02	43.74
		Male (*N* = 36)	274.75	51.44	144.48	55.02

Appendix M: Tukey-Kramer pairwise comparison for social novelty phase of three-chamber sociability task

Table 7.

Tukey-Kramer Pairwise Comparisons for Time Spent in the Social Novelty Chamber in the Three-Chamber Sociability Task.

Comparisons				
Condition	Condition	Mean Difference	Std. Error	*p*-value
OT Female	OT Male	-14.78	16.09	0.362
Saline Female	Saline Male	**-66.19**	**19.48**	**0.001***
Female OT	Female Saline	36.35	18.89	0.059
Male OT	Male Saline	-15.06	16.78	0.373

*Significant based on a Tukey-Kramer adjusted alpha of 0.025.

Appendix N: Tukey-Kramer pairwise comparisons for social sniffing during three-chamber sociability task

Table 8.

Tukey-Kramer Pairwise Comparisons for Social Sniffing of the Novel Mouse in the Three-Chamber Sociability Task.

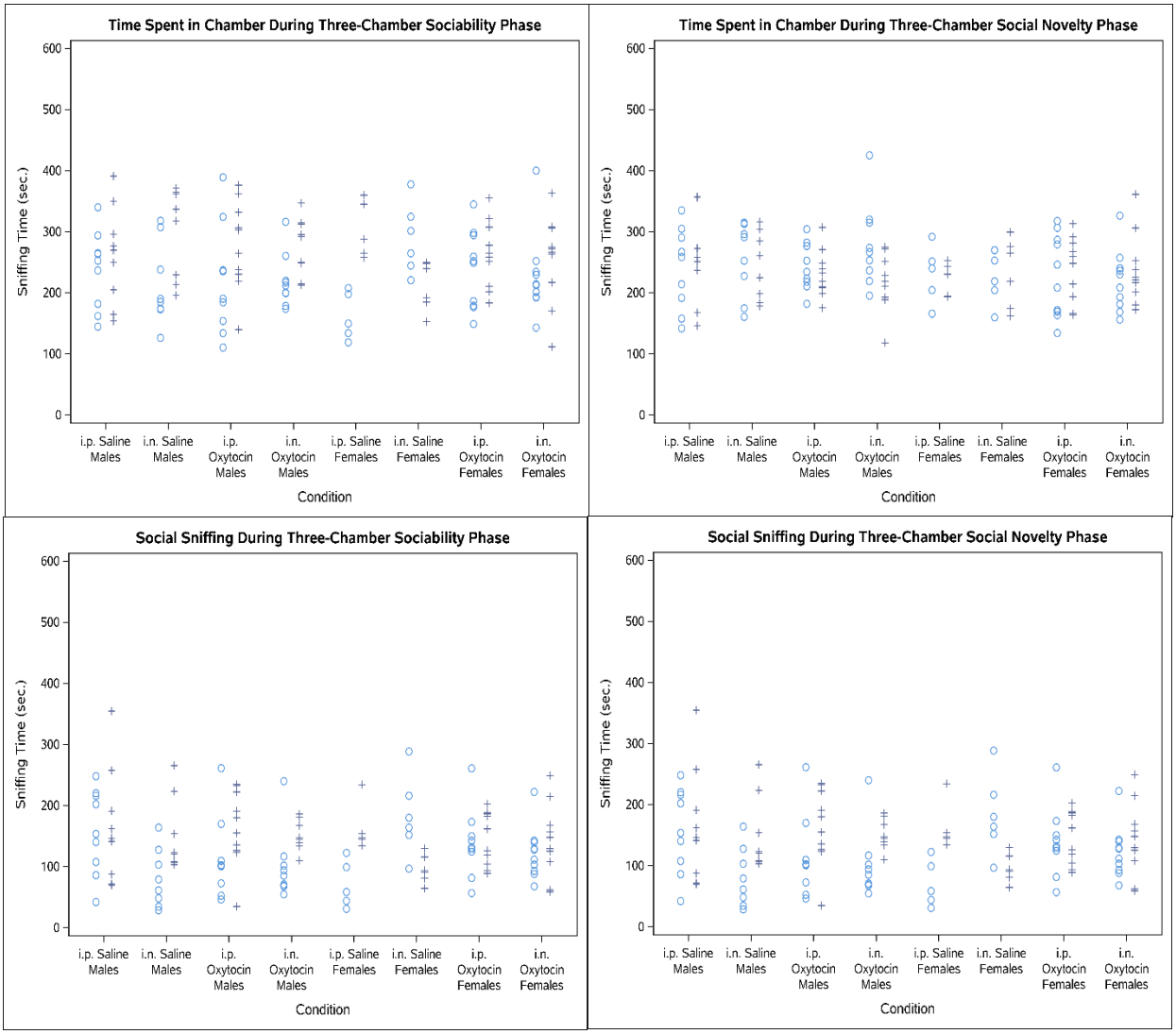
Comparison Split	Comparisons		Mean Difference	Std. Error	*p*-value
	Condition	Condition			
Oxytocin	i.n. Female	i.n. Male	11.48	20.35	0.942
	i.n. Female	i.p. Female	18.73	20.35	0.794
	i.n. Male	i.p. Male	-3.13	20.90	0.999
	i.p. Female	i.p. Male	-10.39	20.90	0.960
Saline	i.n. Female	i.n. Male	**-81.87**	**25.38**	**0.011***
	i.n. Female	i.p. Female	6.58	27.55	0.812
	i.n. Male	i.p. Male	**80.83**	**22.11**	**0.003***
	i.p. Female	i.p. Male	-7.62	24.57	0.990
i.n.	OT Female	OT Male	11.48	20.35	0.942
	OT Female	Saline Female	45.02	24.92	0.281
	OT Male	Saline Male	-48.33	20.90	0.107
	Saline Female	Saline Male	**-81.87**	**25.38**	**0.011***
i.p.	OT Female	OT Male	-10.39	20.90	0.960
	OT Female	Saline Female	32.86	23.49	0.505
	OT Male	Saline Male	35.63	22.11	0.380
	Saline Female	Saline Male	-7.62	24.57	0.990
Female	OT i.n.	OT i.p.	18.73	20.35	0.974
	OT i.n.	Saline i.n.	45.01	24.92	0.281
	OT i.p.	Saline i.p.	32.86	23.49	0.505
	Saline i.n.	Saline i.p.	6.58	27.55	0.995
Male	OT i.n.	OT i.p.	-3.13	20.90	0.999
	OT i.n.	Saline i.n.	-48.33	20.90	0.107
	OT i.p.	Saline i.p.	35.63	22.11	0.380
	Saline i.n.	Saline i.p.	**80.83**	**22.11**	**0.003***

*Significant based on a Tukey-Kramer adjusted alpha of 0.025.

Appendix O: Three-chamber sociability task graphs

Figure 7.

Graphs depicting time in seconds the test mouse spent: (a) in the social chamber during the sociability phase, (b) social sniffing of the conspecific mouse during the sociability phase, (c) in the novelty chamber during the social novelty task, and (d) social sniffing during the social novelty chamber. (a-b) Blue circles indicate social chamber and purple crosses indicate empty chamber. (c-d) Blue circles indicate familiar chamber and purple crosses indicate novel chamber.



Appendix P: Group means of social behaviors during free dyadic social interaction

Table 9.

Group Means for Social Approach, Social Avoidance, and Anxiety-like Behavior During Free Dyadic Social Interaction.

			Social Approach		*Social Avoidance*		*Grooming*	
Drug	Administration	Sex	*M*	*SD*	*M*	*SD*	*M*	*SD*
OT	i.p.	Female (*N* = 10)	25.70	5.50	25.60	5.93	3.90	1.66
		Male (*N* = 9)	42.22	12.93	38.78	15.15	4.56	2.07
	i.n.	Female (*N* = 9)	35.22	10.32	25.89	9.03	10.44	6.27
		Male (*N* = 10)	33.00	12.31	27.10	12.35	10.30	3.59
Saline	i.p.	Female (*N* = 7)	27.00	8.62	28.00	2.89	18.14	6.82
		Male (*N* = 8)	41.63	11.29	40.63	15.04	8.75	3.85
	i.n.	Female (*N* = 5)	28.00	4.36	17.60	5.50	10.20	3.11
		Male (*N* = 8)	12.88	5.00	10.63	3.62	18.88	3.56
Saline	i.p. (*N* = 15)		34.80	12.35	34.73	12.62	13.13	7.13
	i.n. (*N* = 13)		18.69	8.92	13.31	5.50	15.54	5.47
OT	i.p. (*N* = 19)		33.53	12.70	31.84	12.86	4.21	1.84
	i.n. (*N* = 19)		34.05	11.15	26.53	10.63	10.37	4.89
Saline		Female (*N* = 12)	27.42	6.91	23.67	6.65	14.83	6.75
		Male (*N* = 16)	27.25	17.07	25.63	18.75	13.81	6.34
OT		Female (*N* = 19)	30.21	9.29	25.74	7.34	7.00	5.49
		Male (*N* = 19)	37.37	13.13	32.63	14.63	7.58	4.13
	i.p.	Female (*N* = 17)	26.24	6.73	26.59	4.94	9.76	8.44
		Male (*N* = 17)	41.94	11.81	39.65	14.65	6.53	3.64
	i.n.	Female (*N* = 14)	32.64	9.18	22.93	8.75	10.36	5.21
		Male (*N* = 18)	24.06	14.01	19.78	12.53	14.11	5.59
OT (*N* = 38)			33.79	11.79	29.18	11.94	7.29	4.80
Saline (*N* = 28)			27.32	13.47	24.79	14.64	14.25	6.42
	i.p. (*N* = 34)		34.09	12.37	33.12	12.64	8.15	6.61
	i.n. (*N* = 32)		27.81	12.72	21.16	10.99	12.47	5.67
		Female (*N* = 31)	29.13	8.44	24.94	7.04	10.03	7.06
		Male (*N* = 35)	32.74	15.69	29.43	16.77	10.43	6.06

Appendix Q: Tukey-Kramer pairwise comparisons for social approach

Table 10.

Tukey-Kramer Pairwise Comparisons for Social Approach of the Test Mouse during Free Dyadic Social Interaction.

Comparisons				
Condition	Condition	Mean Difference	Std. Error	*p*-value
OT i.n.	OT i.p.	0.15	3.14	0.961
Saline i.n.	Saline i.p.	**-13.88**	**3.72**	**< 0.001***
i.n. OT	i.n. Saline	**13.67**	**3.54**	**< 0.001***
i.p. OT	i.p. Saline	-0.35	3.34	0.917
i.n. Female	i.p. Female	5.26	3.60	0.149
i.n. Male	i.p. Male	**-18.99**	**3.28**	**< 0.001***
i.n. Female	i.n. Male	**8.67**	**3.54**	**0.017***
i.p. Female	i.p. Male	**-15.57**	**3.34**	**< 0.001***

*Significant based on a Tukey-Kramer adjusted alpha of 0.025.

Appendix R: Tukey-Kramer pairwise comparisons for social avoidance

Table 11.

Tukey-Kramer Pairwise Comparisons for Social Avoidance of the Test Mouse during Free Dyadic Social Interaction.

Comparisons		Mean Difference	Std. Error	*p*-value
Condition	Condition			
OT i.n.	OT i.p.	-5.69	3.31	0.098
Saline i.n.	Saline i.p.	**-20.20**	**3.92**	**< 0.001***
i.n. OT	i.n. Saline	**12.38**	**3.73**	**0.002***
i.p. OT	i.p. Saline	-2.12	3.53	0.550
i.n. Female	i.p. Female	-5.06	3.79	0.188
i.n. Male	i.p. Male	**-20.84**	**3.46**	**< 0.001***
i.n. Female	i.n. Male	2.88	3.73	0.443
i.p. Female	i.p. Male	**-12.90**	**3.53**	**0.001***

*Significant based on a Tukey-Kramer adjusted alpha of 0.025.

Appendix S: Tukey-Kramer pairwise comparisons for anxiety-like behavior

Table 12.

Tukey-Kramer Pairwise Comparisons for Anxiety-like Behavior of the Test Mouse during Free Dyadic Social Interaction.

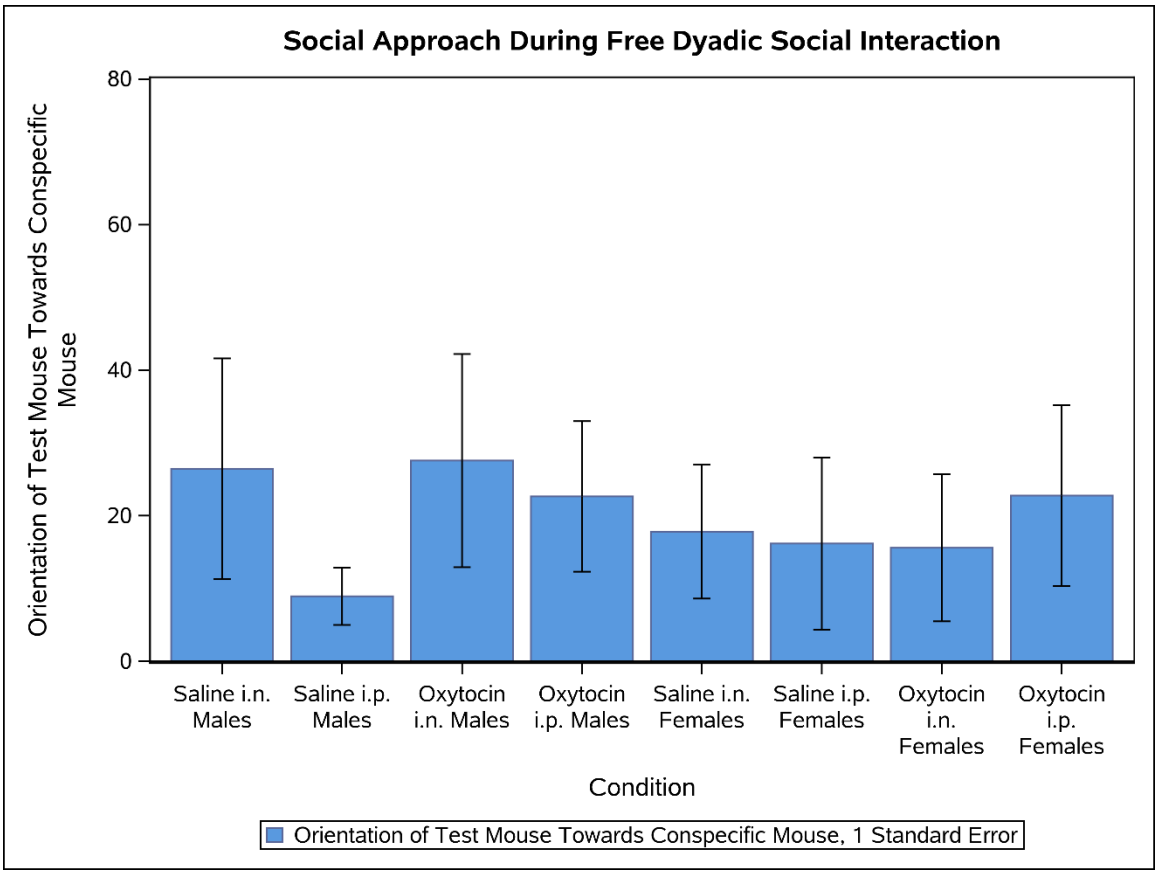
Comparison Split	Comparisons		Mean Difference	Std. Error	*p*-value
	Condition	Condition			
Oxytocin	i.n. Female	i.n. Male	0.14	1.91	1.000
	i.n. Female	i.p. Female	**6.54**	**1.91**	**0.006***
	i.n. Male	i.p. Male	**5.74**	**1.91**	**0.020***
	i.p. Female	i.p. Male	-0.66	1.91	0.986
Saline	i.n. Female	i.n. Male	**-8.68**	**2.37**	**0.003***
	i.n. Female	i.p. Female	**-7.94**	**2.43**	**0.010***
	i.n. Male	i.p. Male	**10.13**	**2.08**	**< 0.001***
	i.p. Female	i.p. Male	**9.39**	**2.15**	**< 0.001***
i.n.	OT Female	OT Male	0.14	1.91	1.000
	OT Female	Saline Female	0.24	2.32	1.000
	OT Male	Saline Male	**-8.58**	**1.97**	**< 0.001***
	Saline Female	Saline Male	**-8.68**	**2.37**	**0.003***
i.p.	OT Female	OT Male	-0.66	1.91	0.986
	OT Female	Saline Female	**-14.24**	**2.05**	**< 0.001***
	OT Male	Saline Male	-4.19	2.02	0.172
	Saline Female	Saline Male	**9.39**	**2.15**	**< 0.001***
Female	OT i.n.	OT i.p.	**6.54**	**1.91**	**0.006***
	OT i.n.	Saline i.n.	0.24	2.32	1.000
	OT i.p.	Saline i.p.	**-14.24**	**2.05**	**< 0.001***
	Saline i.n.	Saline i.p.	**-7.94**	**2.43**	**0.010***
Male	OT i.n.	OT i.p.	**5.74**	**1.91**	**0.020***
	OT i.n.	Saline i.n.	**-8.58**	**1.97**	**< 0.001***
	OT i.p.	Saline i.p.	-4.19	2.02	0.172
	Saline i.n.	Saline i.p.	**10.13**	**2.08**	**< 0.001***

*Significant based on a Tukey-Kramer adjusted alpha of 0.025.

Appendix T: Graph of social approach during free dyadic social interaction task.

Figure 8.

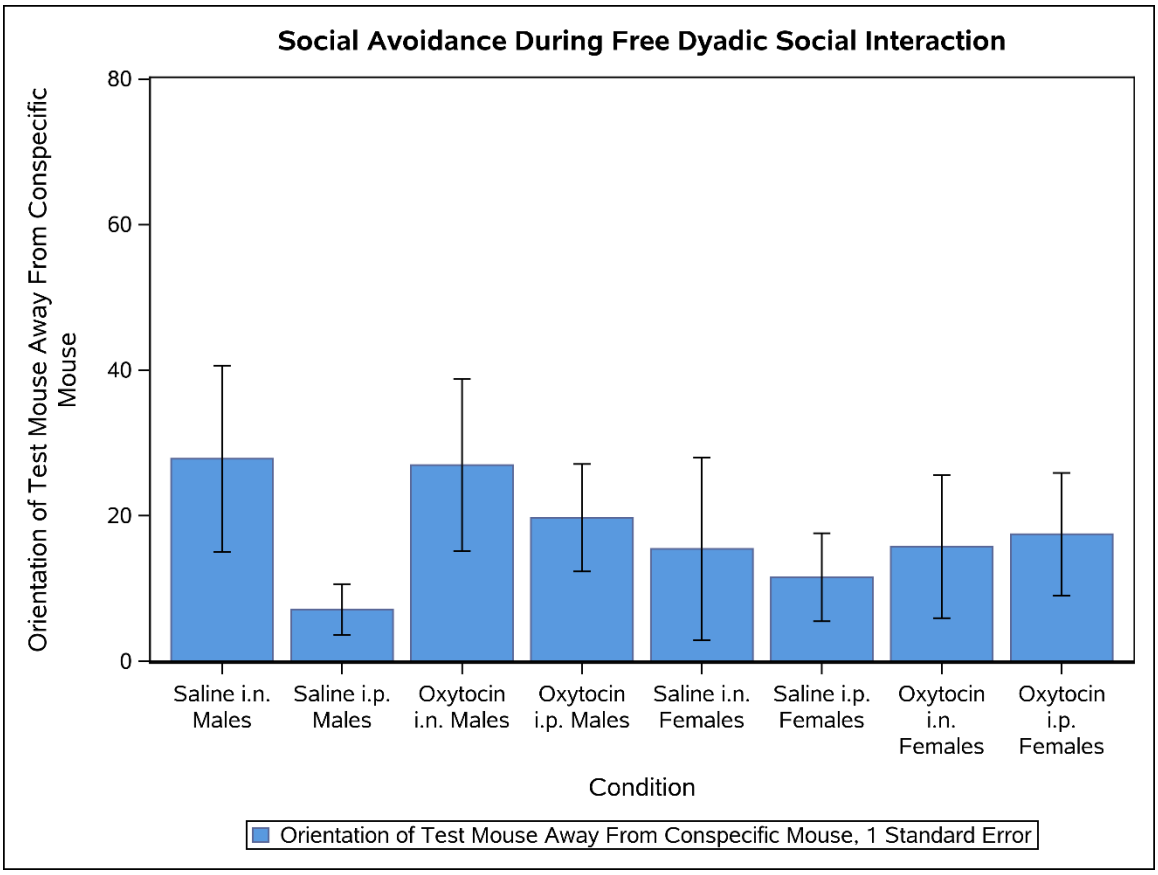
Graph depicting total frequency of social approach during free dyadic social interaction.



Appendix U: Graph of social avoidance during free dyadic social interaction task.

Figure 9.

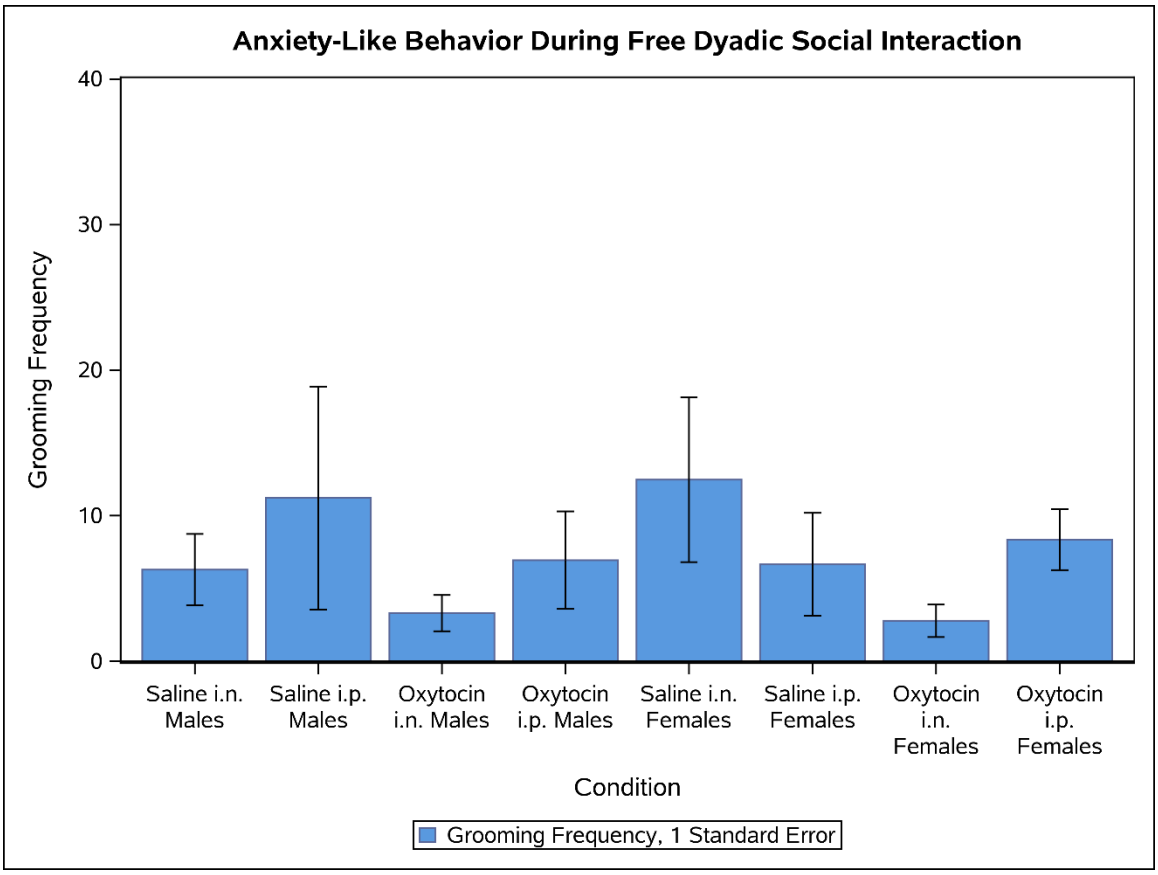
Graph depicting total frequency of social avoidance during free dyadic social interaction.



Appendix V: Graph of social anxiety-like behavior during free dyadic social interaction task.

Figure 10.

Graph depicting total frequency of test mouse grooming during free dyadic social interaction.



Appendix W: IACUC protocol approval

Figure 11.

IACUC Protocol Approval.

IACUC
INSTITUTIONAL ANIMAL CARE and USE COMMITEE
Office of Research Compliance,
010A Sam Ingram Building,
2269 Middle Tennessee Blvd
Murfreesboro, TN 37129

MIDDLE TENNESSEE STATE UNIVERSITY

IACUCN006: FCR PROTOCOL APPROVAL NOTICE

Thursday, May 14, 2020

Senior Investigator	**Tiffany Rogers** (ROLE: Principal Investigator)
Co-Investigators	NONE
Investigator Email(s)	*tiffany.rogers@mtsu.edu*
Department	Psychology
Protocol Title	***Neurochemical and Behavioral Analysis of Social Interaction in Mice***
Protocol ID	**20-3003**

Dear Investigator(s),

The MTSU Institutional Animal Care and Use Committee has reviewed the REVISED animal use proposal identified above under the ***Full Committee Review (FCR) mechanism***. The IACUC met on 5/12/2020 to determine if your proposal meets the requirements for approval. The Committee determined through a majority of vote that this REVISED protocol meets the guidelines for approval in accordance with PHS policy. In view of the current COVID-19 crisis, the IACUC also introduced a few restrictions. A summary of the IACUC action(s) and other particulars of this this protocol are tabulated below:

IACUC Action	**APPROVED for one year**	
Date of Expiration	**5/31/2021**	
Number of Animals	192 (ONE HUNDRED and NINETY TWO)	
Approved Species	**C57BL/6J Mice (Jackson Laboratories)**	
Category	☐ **Teaching**	☒ **Research**
Subclassifications	☐ Classroom ☐ Laboratory	☒ Laboratory ☐ Field Research ☐ Field Study ☒ Handling/Manipulation ☐ Observation
	Comment: NONE	
Approved Site(s)	MTSU Vivarium: Rooms SCI1170L (housing) and SCI 1170K (procedures)	
Restrictions	**1. Must comply with all FCR requirements;** **2. Mandatory compliance with CDC guidelines during COVID-19; Social distancing guidelines are made by the Dean of CBAS.** **3. The PI must make alternative plans to ensure proper animal care, including euthanasia if needed, in the event the research team is quarantined due to COVID19**	
Comments	NONE	

Appendix W: IACUC protocol approval

Figure 11 (cont.).

IACUC Protocol Approval.

IACUC Office of Compliance MTSU

This approval is effective for three (3) years from the date of this notice till 5/31/2023 The investigator(s) MUST file a Progress Report annually updating the status of this study. Refer to the schedule for Continuing Review shown below; NO REMINDERS WILL BE SENT. A continuation request (progress report) must be **approved** by the IACUC prior to 5/31/2021 for this protocol to be active for its full term. Once a protocol has expired, it cannot be continued and the investigators must request a fresh protocol.

Continuing Review Schedule:

Reporting Period	Requisition Deadline	IACUC Comments
First year report	4/30/2021	NONE
Second year report	4/30/2022	NONE
Final report	4/30/2023	NONE

Post-approval Amendments:

Date	*Amendment*	*IACUC Notes*
NONE	NONE	NONE

Post-approval Actions:

Date	*Amendment*	*IACUC Notes*
05/14/2020	Mekenzie Meadows, Psychology graduate student) is added as a co-investigator. CITI training and health screening are confirmed	Initial FCR approval

MTSU Policy defines an investigator as someone who has contact with live or dead animals for research or teaching purposes. Anyone meeting this definition must be listed on your protocol and must complete appropriate training through the CITI program. Addition of investigators requires submission of an Addendum request to the Office of Research Compliance.

The IACUC must be notified of any proposed protocol changes prior to their implementation. Unanticipated harms to subjects or adverse events must be reported within 48 hours to the Office of Compliance at (615) 494-8918 and by email – compliance@mtsu.edu.

All records pertaining to the animal care be retained by the MTSU faculty in charge for at least three (3) years AFTER the study is completed. In addition, refer to MTSU Policy 129: Records retention & Disposal (https://www.mtsu.edu/policies/general/129.php) for Tennessee State requirements for data retention. Please be advised that all IACUC approved protocols are subject to audit at any time and all animal facilities are subject to inspections at least biannually. Furthermore, IACUC reserves the right to change, revoke or modify this approval without prior notice.

Sincerely,

Compliance Office
(On behalf of IACUC)
Middle Tennessee State University
Tel: 615 494 8918
Email: iacuc_information@mtsu.edu (for questions) and
iacuc_submissions@mtsu.edu (for sending documents)

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